VOL. 308 JUNE 8, 1984
(Biomedical Applications, Vol. 33)
COMPLETE IN ONE ISSUE

JOURNAL OF CHROMATOGRAPHY BIOMEDICAL APPLICATIONS

ELSEVIER
ELSEVIER

JOURNAL OF CHROMATOGRAPHY

Scope. The *Journal of Chromatography* publishes papers on all aspects of chromatography, electrophoresis and related methods. Contributions consist mainly of research papers dealing with chromatographic theory, instrumental development and their applications. The section *Biomedical Applications,* which is under separate editorship, deals with the following aspects: developments in and applications of chromatographic and electrophoretic techniques related to clinical diagnosis (including the publication of normal values); screening and profiling procedures with special reference to metabolic disorders; results from basic medical research with direct consequences in clinical practice; combinations of chromatographic and electrophoretic methods with other physicochemical techniques such as mass spectrometry. In *Chromatographic Reviews,* reviews on all aspects of chromatography, electrophoresis and related methods are published.

Submission of Papers. Papers in English, French and German may be submitted, in three copies. Manuscripts should be submitted to: The Editor of *Journal of Chromatography,* P.O. Box 681, 1000 AR Amsterdam, The Netherlands, or to: The Editor of *Journal of Chromatography, Biomedical Applications,* P.O. Box 681, 1000 AR Amsterdam, The Netherlands. Review articles are invited or proposed by letter to the Editors and will appear in *Chromatographic Reviews* or *Biomedical Applications.* An outline of the proposed review should first be forwarded to the Editors for preliminary discussion prior to preparation. Submission of an article is understood to imply that the article is original and unpublished and is not being considered for publication elsewhere. For copyright regulations, see below.

Subscription Orders. Subscription orders should be sent to: Elsevier Science Publishers B.V., P.O. Box 211, 1000 AE Amsterdam, The Netherlands. The *Journal of Chromatography* and the *Biomedical Applications* section can be subscribed to separately.

Publication. The *Journal of Chromatography* (incl. *Biomedical Applications, Chromatographic Reviews* and *Cumulative Author and Subject Indexes, Vols. 101-150 and 276-300*) has 30 volumes in 1984. The subscription prices for 1984 are:

J. Chromatogr. (incl. *Chromatogr. Rev.* and *Cum. Indexes Vols. 101-150 and 276-300*) + *Biomed. Appl.* (Vols. 282-311):
Dfl. 4800.00 plus Dfl. 630.00 (postage) (total *ca.* US$ 2088.50)

J. Chromatogr. (incl. *Chromatogr. Rev.* and *Cum. Indexes Vols. 101-150 and 276-300*) only (Vols. 282-304):
Dfl. 4025.00 plus Dfl. 483.00 (postage) (total *ca.* US$ 1734.00)

Biomed. Appl. only (Vols. 305-311):
Dfl. 1225.00 plus Dfl. 147.00 (postage) (total *ca.* US$ 528.00).

Journals are automatically sent by airmail to the U.S.A. and Canada at no extra costs, and to Japan, Australia and New Zealand, with a small additional postal charge. Back volumes of the *Journal of Chromatography* (Vols. 1 through 281) are available at Dfl. 190.00 (plus postage). Claims for issues not received should be made within three months of publication of the issue. If not, they cannot be honoured free of charge. Customers in the U.S.A. and Canada wishing information on this and other Elsevier journals, please contact Journal Information Center, Elsevier Science Publishing Co. Inc., 52 Vanderbilt Avenue, New York, NY 10017. Tel. (212) 867-9040.

Abstracts/Contents Lists published in Analytical Abstracts, Biochemical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Current Contents/Physical, Chemical & Earth Sciences, Current Contents/Life Sciences, Deep-Sea Research/Part B: Oceanographic Literature Review, Index Medicus, Mass Spectrometry Bulletin, Referativnyi Zhurnal and Science Citation Index.

See page 3 of cover for Publication Schedule, Information for Authors and information on Advertisements.

0378-4347/84/$03.00

Printed in The Netherlands

(Biomedical Applications, Vol. 33)

CONTENTS

(Abstracts/Contents Lists published in Analytical Abstracts, Biochemical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Current Contents/Physical, Chemical & Earth Sciences, Current Contents/Life Sciences, Deep-Sea Research/Part B: Oceanographic Literature Review, Index Medicus, Mass Spectrometry Bulletin, Pharmaceutical Abstracts, Referativnyi Zhurnal, and Science Citation Index)

(Continued overleaf)

(Continued overleaf)

CHROMATOGRAPHY

Fundamentals and Applications of Chromatographic and Electrophoretic Methods

Part A: Fundamentals and Techniques
Part B: Applications

ERICH HEFTMANN, U.S. Department of Agriculture, Berkeley, CA, U.S.A. *(editor)*

Journal of Chromatography Library Vol. 22 A + B

This two-part handbook is an up-to-date treatment of the entire field of chromatography and electrophoresis, written by scientists who are internationally recognized as the foremost authorities in their specific fields.

Chromatography and electrophoresis are probably the most widely used analytical techniques today. Since these techniques were last presented in a comprehensive treatise in 1975, significant changes have taken place, both in the techniques themselves and in their applications to analytical problems.

The work is in two parts:
Part A – Fundamentals and Techniques – presents an introduction to the field, including the development and theory of chromatography and electrophoresis, and then gives the theoretical and instrumental basis of each technique.
Part B – Applications – is a critical review of the isolation and quantification methods in current use for various classes of substances.

Both parts of this work belong in all libraries serving chromatographers at technical colleges, research institutes, universities, pharmaceutical companies and instrument manufacturers. Practising chromatographers and analysts will refer to it often when faced with new or unusual analytical problems and situations.

CONTENTS

February 1983
Part A: xxii + 388 pp. ISBN 0-444-42043-6
US $83.00 (in USA & Canada)
Dfl. 195.00 (Rest of the World)

Part B: xviii + 564 pp. ISBN 0-444-42044-4
US $138.25 (in USA & Canada)
Dfl. 325.00 (Rest of the World)

Parts A & B: ISBN 0-444-42045-2
US $221.25 (in USA & Canada)
Dfl. 520.00 (Rest of the World)

ELSEVIER

P.O. Box 211,
1000 AE Amsterdam,
The Netherlands.

P.O. Box 1663,
Grand Central Station,
New York, NY 10163.

The Dutch guilder price is definitive US $ prices are subject to exchange rate fluctuations.

JOURNAL OF CHROMATOGRAPHY

VOL. 308 (1984)

(Biomedical Applications, Vol. 33)

JOURNAL
of
CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY,
ELECTROPHORESIS AND RELATED METHODS

BIOMEDICAL APPLICATIONS

Vol. 33 1984

ELSEVIER
AMSTERDAM — OXFORD — NEW YORK — TOKYO

0378-4347/84/$03.00

Printed in The Netherlands

Journal of Chromatography, 308 (1984) 1—9
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2068

EFFECTS OF STARVATION AND REFEEDING ON THE EXCRETION OF URINARY STEROID METABOLITES IN MICE WITH DIFFERENT GENETIC BACKGROUND

M. ALASANDRO, D. WIESLER and M. NOVOTNY*

Department of Chemistry, Indiana University, Bloomington, IN 47405 (U.S.A.)

(First received May 25th, 1983; revised manuscript received January 10th, 1984)

SUMMARY

Gas chromatographic steroid metabolic profiling procedures have been applied to investigations of the effects of starvation and refeeding in mice. Urinary steroid metabolites were quantitatively followed during the starvation—refeeding experiments for mice with different genetic backgrounds. Some quantitative alterations were noted for certain congenic strains of mice. The metabolites which exhibited such quantitative variations were tentatively identified by means of combined gas chromatography—mass spectrometry.

INTRODUCTION

Various physiological and biochemical changes induced by starvation and subsequent refeeding processes have been extensively studied [1, 2] in both humans and animals. Among numerous studies, some chronological alterations in the secretion of steroid hormones have also been investigated. In particular, the adrenal gland appears to respond to the state of starvation by both enhanced secretion and an altered frequency of hormonal release. The enhanced gluconeogenesis seen in starvation is expected to be related to an increase in corticosteroid metabolism. Additionally, some physiological parallels exist between the states of starvation and refeeding and certain diabetic conditions [3, 4].

Observations by Takahashi and co-workers [5, 6], that the circadian rhythm of adrenocortical activity in rats is strongly affected by the food intake, have been corroborated by others [7—9]. Food deprivation for five days completely disturbed the circadian rhythm [5], measured as the amount of corticosterone (the major corticosteroid hormone of rodents). Diurnal cycling and increase of plasma cortisol levels were also observed in humans during a three-day fasting

0378-4347/84/$03.00

period [10]. More recently, Quigley and Yen [11] observed a dramatic midday surge of plasma cortisol with an onset concomitant with food intake; food deprivation appears to attenuate markedly both the magnitude and time course of this cortisol surge.

While the hormone levels in plasma have been commonly measured under the conditions of food deprivation, Kley et al. [8] have suggested that steroid measurements in urine are more advantageous than the corresponding plasma measurements while working with small animals. The advantages are due to the larger volume of urine sample available by obtaining a 24-hour urine sample and the avoidance of diurnal hormone fluctuations. In their work on the adrenal evaluations in rats, Kley et al. [8] reported that the adrenal stimulations through starvation increased the urinary concentration of free corticosterone three to four times, while the aldosterone excretion was significantly suppressed. However, little is known about the effect of starvation—refeeding on the numerous other urinary steroids.

While the value of multicomponent steroid determinations under different metabolic circumstances has been demonstrated in humans [12—15], similar procedures could be even more useful in the assessment of metabolic alterations in animal models of human diseases. The significant advantage of using animal models is the elimination of several variables which can not be controlled in humans, such as genetic background, diet, environment, etc.

This report deals with the effects of acute starvation on the urinary excretion of steroidal metabolites in mice. Selected metabolites have been quantitated by capillary gas chromatography (GC), while combined gas chromatography—mass spectrometry (GC—MS) has provided the essential means of identification.

Since some genetic variations in the hormonal secretion and steroid metabolism in mice can be expected [16], the starvation—refeeding model experiment reported here involved male mice of two distinctly different genotypes (BALB/c and C57BL). In addition, the profiles of congenic C57BL animals were quantitatively compared for three haplotypes (B10, B10-RIII, and B10-AKM), differing only in a small section of the major histocompatibility gene complex (H-2) on the seventeenth chromosome [17].

EXPERIMENTAL

Test animals were housed in groups of five to a cage. The experimental animals used were all male mice (13—14 weeks of age) of the following strains: BALB/cAnNHap BR, and three different haplotypes B10, C57BL/10SnJ, (H-2^b); B10.RIII (71Ns/Sn), (H-2^r); B10.AKM/Sn, (H-2^m) of the C57BL strain. All C57BL mice were obtained from the Jackson Laboratory (Bar Harbor, ME, U.S.A.) while the BALB/c mice were obtained from Harlan Industries (Indianapolis, IN, U.S.A.). 24-hour urine samples were collected from different animal groups in standard metabolism cages with wire-mesh floors. The urine was directed into a collection cup placed on dry ice so that the samples were immediately preserved by freezing. The specimens of mouse urine were collected prior to the starvation—refeeding experiment (controls), during the following two days of starvation, and during two days of refeeding.

Water was provided ad libitum, while a rat chow (Purina, Indianapolis, IN, U.S.A.) was given prior to the starvation experiment and during refeeding.

Mouse urine aliquots (5 ml) were analyzed by a previously reported [15] procedure, except that C_{18} Sep-Pak cartridges (Waters Assoc., Milford, MA, U.S.A.) were used for the initial isolation of steroids instead of the XAD-2 resin. In the described procedure [15], subsequent sample treatment consists of enzymatic and solvolytic deconjugations, removal of interfering acids and bases through ion-exchange chromatography, and, finally, preparation of methoxime–trimethylsilyl derivatives for gas chromatography.

A modified Varian 1400 gas chromatograph, a preconcentration technique [18], and a 20 m × 0.25 mm I.D. glass capillary column (coated statically with SE-30 silicone gum) were employed to record the urinary profiles of steroids. A PEP-2 Perkin-Elmer data system performed the peak integrations. The glass capillary column was later directly coupled to the ion source of a Hewlett-Packard 5980A quadrupole mass spectrometer for identification work. All spectra were recorded at scan rates of 100 a.m.u./sec, ion source temperature 280° C, and 70 eV ionization energy.

RESULTS AND DISCUSSION

To our knowledge, no systematic multicomponent determination of mouse urinary steroids has been reported in the literature. Yet, the metabolic profiling approach seems highly attractive for various inquiries into physiology and genetics of the laboratory rodents that are used so often as various models of human conditions. A high-resolution chromatogram of mouse urinary steroid metabolites is shown in Fig. 1, while tentatively identified mixture constituents are listed in Table I.

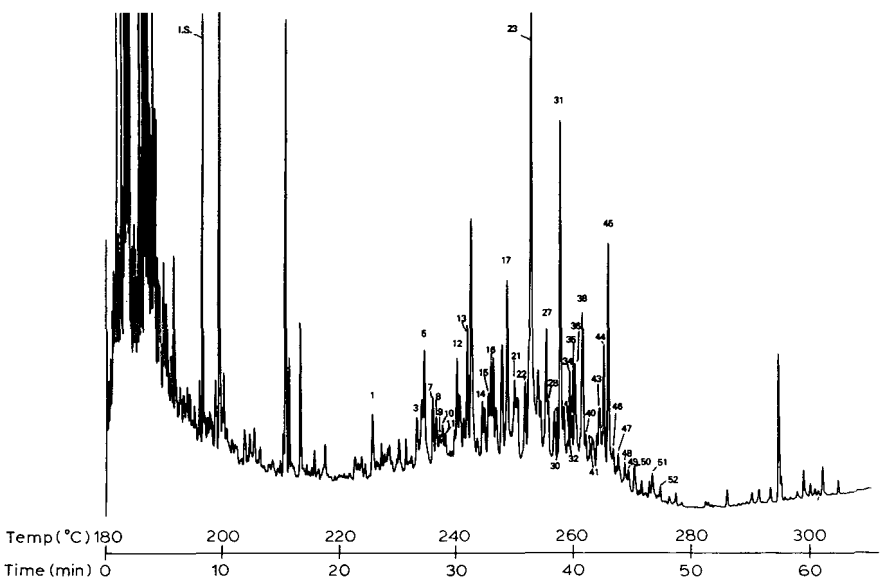


Fig. 1. Capillary gas chromatogram of methoxime–trimethylsilyl derivatives of mouse urinary steroids. Chromatographic conditions, see text. Tentative identifications are listed in Table I.

TABLE I

MOUSE URINARY STEROIDS

Peak No.	Derivatized MW	Identification	Principal *m/e*	Retention index (SE-30)
1	567	An androstanetriol-17-one	567, 536, 446, 356	2735
2	522	An androstenetriol	522, 507, 467, 432	
3	595	A pregnanetriol-20-one	564, 492, 474	2839
4	567	An androstanetriolone	536, 476, 446	2853
5	507	A pregnane-3,17-diol-20-one	507, 492, 476, 386	2863
6	567	An androstanetriol-16-one	536, 446, 356	
7	565	An androstenetriolone	534, 444, 354	2885
8	655	An androstanetetrolone	624, 565, 534, 475, 444	2895
9	550	A pregnanedioldione	550, 520, 417	2901
10	595	A pregnanetriolone	492, 402	2910
11	595	A pregnanetriolone	564, 474	2916
12	520	An androstadienetriol	520, 505, 431, 417	2949
13	595	A pregnanetriolone	580, 564, 474	2988
14	595	A pregnanetriolone	564, 492, 474, 384	3013
15	595	A pregnanetriolone	564, 474, 384	3034
16	683	A pregnanetetrolone	652, 562, 472	3041
17	655	An androstane-3,7,11,16-tetrol-17-one	624, 534, 444, 354	3072
18	683	A pregnanetetrolone	652, 562, 472	
19	595	A pregnanetriolone	580, 564, 474	
20	458	Cholesterol	368, 329	
21	624	An androstenetetrol-11-one	624, 534, 519, 444, 354	3106
22	609	A pregnanetriol-11,20-dione	609, 578, 506, 488	3107
23	683	A pregnanetetrol-20-one	652, 562, 472, 382	3141
24	683	A pregnanetetrolone	652, 562, 472, 382	
25	595	A pregnanetriolone	580, 564, 474	
26	683	A pregnanetetrol-20-one	652, 562, 472, 382	
27	683	A pregnanetetrol-20-one	652, 562, 472, 382	3180
28	654	A pregnanetetrol-11-one	461, 371	3186
29	566	A pregnanetriol-11-one	551, 461, 431, 371	
30	636	A pregnenetrioldione	605, 515, 425	
31	728	A pregnanepentol	535, 445, 355	3220
32	683	A pregnanetetrolone	494, 404	
33	683	A pregnanetetrolone	652, 562, 472	
34	683	A pregnanetetrolone	490, 400, 310	3244
35	636	A pregnenetrioldione	621, 605, 589, 469	
36	683	A pregnanetetrolone	490, 400, 310	3258
37	681	A pregnenetetrolone	488, 398, 308	
38	683	A pregnanetetrolone	652, 562, 472	3279
39	640	A pregnane-3,11,20,21-tetrol	486, 396, 381, 357	
40	728	A pregnanepentol	535, 445, 355	3293
41	683	A pregnanetetrolone	562, 472, 382	3306
42	683	A pregnanetetrolone	578, 488, 398, 308	
43	681	A pregnene-3,11,20,21-tetrol-16-one	488, 398, 308	3330
44	681	A pregnene-3,11,20,21-tetrol-16-one	578, 488, 398, 308	3340
45	728	A pregnane-3,11,16,20,21-pentol	535, 445, 355	3351
46	681	A pregnenetetrolone	578, 488, 398, 308	
47	728	A pregnanepentol	535, 445, 355	
48	593	A pregnenetriolone	593, 562, 472	
49	681	A pregnenetetrolone	488, 398, 308	
50	681	A pregnenetetrolone	488, 398, 308	3425
51	728	A pregnane-3,11,16,20,21-pentol	535, 445, 355	3482
52	728	A pregnane-3,11,16,20,21-pentol	535, 445, 355	3495

Although mass spectra of various steroid polyols were reported [19—26], positive identification of different isomers remains problematic. Mass spectrometry does not readily distinguish such isomers. There are two solutions to this problem: (1) acquisition of an extensive collection of various polyol samples, or (2) development of ancillary techniques that would be more powerful than mass spectrometry. Both approaches represent long-term goals. Until then, it is felt that qualitative information, as represented by Table I, is better

than none. In order to provide further documentation on these tentatively identified metabolites, retention indices relative to normal hydrocarbons are also included. Based on three determinations, variation in retention indices was found to be less than 0.4%.

Of particular interest are a number of late-eluting constituents: peaks 45, 51, and 52, apparently stereoisomers, whose mass spectra feature intensities at *m/e* 265, 355, 445, and 535; and peaks 43 and 44, also apparently stereoisomers, whose mass spectra feature *m/e* 308, 398, and 488. In none of these cases does there appear to be a molecular ion. Mass spectral information about steroid polyols provided by Gustafsson and co-workers [19—26], however, leads us to postulate that the first three substances are fully silylated pregnanepentols (MW 728), while the other two are silylated, methoximated pregnenetetrolones (MW 681).

From a biosynthetic point of view, it is reasonable to propose hydroxylation at positions 3, 11, 16, 20, and 21 for the supposed pregnanepentols. If so, an initial cleavage of the C_{20}—C_{21} bond would result in the loss of 103 mass units to give an ion of mass 625, from which the observed masses would be obtained by successive losses of trimethylsilanol (MW 90) units. Hydroxylation at C_{17} is unlikely, for a different set of mass peaks would thus be generated through C_{17}—C_{20} bond cleavage.

For the supposed pregnenetetrolones, a peak at *m/e* 276 suggests one oxo group and two hydroxyl groups at C_{16}, C_{20}, and C_{21}. Again, an initial loss of 103 mass units from an ion of mass 681, followed by successive losses of trimethylsilanol molecules, would yield the observed fragmentation pattern. The fact that these methoxime spectra are not dominated by the customary M—31, M—121, etc., peaks may indicate a structure significantly different from those of the common 20-oxosteroids. Tentatively, we propose 3,11,20,21-pregnenetetrol-16-one structures for constituents 43 and 44, with the position of the double bond unspecified.

As indicated above, multiple oxidations that occur at different sites of the basic steroidal skeleton present a challenge for identification studies. In this respect, it has become obvious that the steroid metabolites excreted into the urine of rodents are substantially different from those encountered in humans

TABLE II

EFFECTS OF STARVATION AND REFEEDING ON CONCENTRATION OF MOUSE URINARY STEROID CONSTITUENTS

S1 = first day of starvation; S2 = second day of starvation; R1 = first day of refeeding; R2 = second day of refeeding. Concentrations are reported as percent of control.

Steroid	BALB/c				B10.AKM				B10				B10.RIII			
	S1	S2	R1	R2	S1	S2	R1	R2	S1	S2	R1	R2	S1	S2	R1	R2
17	49	96	104	166	25	18	114	150	43	11	103	99	41	19	119	99
23	41	50	50	57	78	43	65	117	144	57	147	122	86	58	76	119
31	50	114	64	72	80	60	113	139	104	59	151	93	137	78	95	134
43	105	169	128	133	266	109	85	133	182	127	190	118	98	70	57	105
44	128	134	151	103	25	16	59	104	80	40	154	93	97	31	77	172
45	68	137	100	119	38	34	62	106	110	60	195	173	155	140	124	245
51	43	301	58	49	138	64	96	132	132	65	109	132	118	68	84	97
52	54	303	57	80	152	56	86	148	92	42	45	71	107	201	108	174

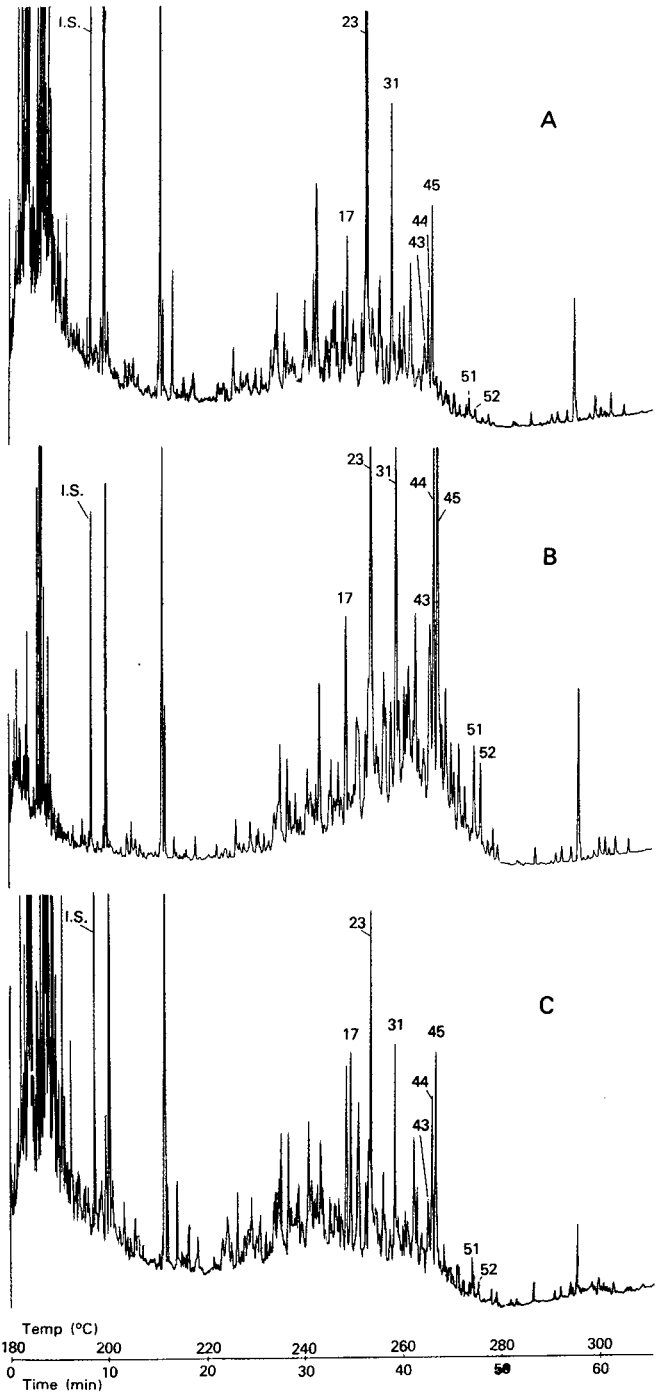


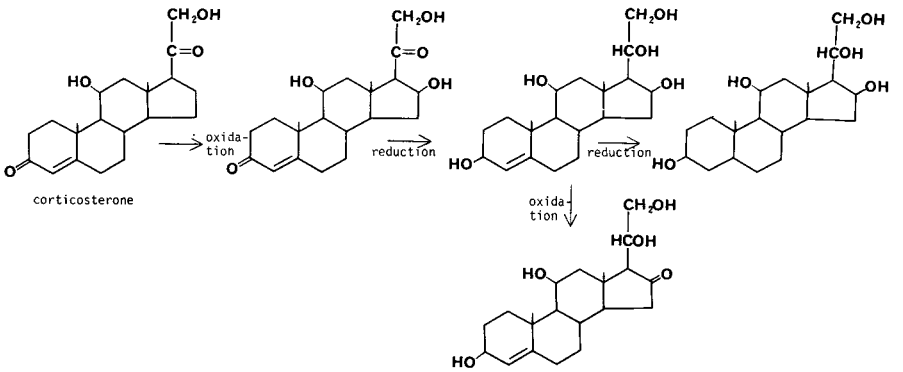
Fig. 2. Comparison of steroid metabolic profiles from the urine of BALB/c mice. (A) Control; (B) starvation; (C) refeeding. Chromatographic conditions, see text.

[15, 27]. Furthermore, the solvent extraction methodology commonly used in human studies [13, 27] fails to provide sufficient recoveries of very polar steroids from mouse urine. Fortunately, "solid extractions" [27] appear capable of extracting a sufficient amount of such steroids for reliable and reproducible analyses. While the absolute values of excreted mouse steroids are largely unknown (estimated at microgram amounts of corticosterone metabolites per 24 h), the coefficient of variation in our samples is estimated to be below 15%, enabling fairly reliable comparisons of mouse urinary steroid profiles. Individual variations of steroid excretion in different animals have been minimized through the urine collection from five animals per group.

The results of starvation–refeeding experiments are shown in Table II for those steroids that show significant alterations in urinary excretion and appear related to the physiological changes incurred during the experiment. A representative illustration of these effects is seen in Fig. 2, where the increases of certain profile components are evident for the starved animals (B) as compared to the controls (A); the profile of refed animals (C) is fairly comparable to the control. The values of Table II suggest, however, that a few profile components do not come back to their original value after the mice are refed. In some cases (e.g. peaks 23, 31, 51 and 52 in BALB/c mice) excretion rates below normal are observed.

Any results of metabolic profiling during starvation–refeeding experiments should be seen in view of various chronological alterations in the metabolism of various body fuels and the associated changes in the secretion of hormones. Of particular interest is that the starvation and refeeding processes evoke many of the same metabolic alterations that are observed in the mild forms of diabetes mellitus [3, 4]. It is known that the major known glucocorticoid in rodents, corticosterone, interacts with insulin and glucagon to effect the glycogenolytic and gluconeogenetic processes. Additionally, various glucocorticoids are known to participate in decreasing the responsiveness of muscle and adipose tissue to insulin-stimulated glucose uptake [29]. Starvation is expected to cause an elevation of corticosterone in rats with a return to normal levels during refeeding. However, if the starvation exceeds three or four days, the fat stores become depleted and the organism depends increasingly on the catabolism of body protein. Refeeding after such a prolonged fast may result in a period of carbohydrate intolerance and a less prompt return to the original hormonal conditions. Mlekusch et al. [30] reported that plasma concentrations of corticosterone in rats were increased at the initial period of starvation, but flattened off during the following two days of starvation, presumably due to an "adaptation" mechanism.

While the increases of corticosterone during the starvation of rodents are documented [5–9], virtually nothing is known about the numerous metabolites of corticosterone. The structures of steroids that show elevations in Table II are consistent with the general pathways of corticosteroid metabolism, i.e. biological oxidations taking place in the liver. Some increases in pregnenetetrolones (peaks 43 and 44) and pregnanepentols (peaks 45, 51 and 52) in BALB/c mice appear to be in agreement with the known corticosterone increases. It is conceivable that these compounds are biosynthesized as follows:



It must be pointed out, however, that the trends in metabolic excretions (Table II) are not uniform for all measured metabolites. While substantial increases of 200—300% of the original 24-h concentrations during starvation are observed in some instances (e.g. peaks 51 and 52 in BALB/c and peak 43 in B10.AKM mice), the levels of certain other steroids remain relatively less affected, while some show even decreases. It would be hard to explain such variations by a simple mechanism. The starvation process is known to induce a variety of liver enzymes [30]; some steroid oxidations could thus be viewed as non-specific metabolic interactions. Alternatively, some corticosterone products may have a metabolic role of their own; it has been reported [31] that similar compounds (e.g. synthetic glucocorticoids prednisolone and dexamethasone) may have contrasting effects on glucose uptake by rat adipocytes. Similarly, refeeding has shown variations that are not easily explained. It is, however, obvious that most metabolites show both positive and negative deviations from the control values.

Differences observed in steroid excretion among the different mouse strains indicate some genetic control over either the response of the adrenal gland to the starvation process, or alternatively, the liver metabolism of steroids. Variations observed with the H-2 congenic strains further support the notion that the major histocompatibility complex may exert a visible influence over the pathways of secondary metabolites [32—35]. While the biological reasons for this are presently unknown (the major biological role of H-2 is known to involve primarily the immune interactions), other "non-specific traits" of this gene complex have been observed [36, 37], including a linkage to the appearance of cortisone-induced cleft palate [38]. Although the liver is not generally considered in association with the immune response, an H-2 linkage with the liver metabolism was found for cAMP [33]. Moreover, glucagon binding to liver cell membranes appears also under some influence of the major histocompatibility complex [34].

ACKNOWLEDGEMENT

This project was supported in part by BRSG S07-RR07031 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institute of Health.

REFERENCES

1 P. Felig, in P. Felig, J.D. Baxter, A.E. Broadus and L.A. Frohman (Editors), Endocrinology and Metabolism, McGraw-Hill, New York, 1981, p. 761.
2 S. Seino, H. Sakurai, Y. Seino, K. Tsuda, K. Tanigawa, H. Kuzuya, Y. Goto and H. Imura, Diabetes, 29 (1980) 323.
3 R. Arky, in S.S. Fajans (Editor), Environmental Factors Influencing the Development of the Diabetic State, DHEW Publication No. (NIH) 76-854, Bethesda, MD, 1976, p. 89.
4 R.S. Bosboom, J. Zweens and P.R. Bouman, Diabetologia, 9 (1973) 243.
5 K. Inoue, K. Takahashi and Y. Takahashi, Nippon Naibumpi Gakkai Zasshi, 52 (1976) 898; C. A. 86 (1977) 15581 q.
6 K. Takahaski, K. Inoue and Y. Takahashi, Endocrinology (Philadelphia), 100 (1977) 1097.
7 Z. Maletova and I. Ahlers, Endocrinol. Exp., 11 (1977) 214.
8 H.K. Kley, W. Elsasser, H. Dehnen and H.L. Krueskemper, Steroids, 32 (1978) 223.
9 Y. Morimoto, T. Oishi, K. Arisue and Y. Yamamura, Neuroendocrinology, 29 (1979) 77.
10 C. Faiman and J.A. Moorhouse, Clin. Sci., 32 (1967) 111.
11 M. Quigley and S.S.C. Yen, J. Clin. Endocrinol. Metab., 49 (1979) 945.
12 C.D. Pfaffenberger and E.C. Horning, J. Chromatogr., 112 (1975) 581.
13 C.D. Pfaffenberger, L.R. Malinak and E.C. Horning, J. Chromatogr., 158 (1978) 313.
14 P. Pfeifer and G. Spiteller, J. Chromatogr., 223 (1981) 21.
15 M. Alasandro, D. Wiesler, G. Rhodes and M. Novotny, Clin. Chim. Acta, 126 (1982) 243.
16 A. Bartke, J. Endocrinol., 60 (1974) 145.
17 J. Klein, Biology of the Mouse Histocompatibility-2 Complex, Springer, Berlin, 1975.
18 M. Novotný and R. Farlow, J. Chromatogr., 103 (1975) 1.
19 B.E. Gustafsson, J.A. Gustafsson and J. Sjövall, Eur. J. Biochem., 4 (1968) 568.
20 H. Eriksson, J.A. Gustafsson and J. Sjövall, Eur. J. Biochem., 6 (1968) 219.
21 J.A. Gustafsson and J. Sjövall, Eur. J. Biochem., 6 (1968) 236.
22 J.A. Gustafsson, Eur. J. Biochem., 6 (1968) 248.
23 J.A. Gustafsson, B.P. Lisboa and J. Sjövall, Eur. J. Biochem., 6 (1968) 317.
24 J.A. Gustafsson, C.H.L. Shackleton and J. Sjövall, Eur. J. Biochem., 10 (1969) 302.
25 J.A. Gustafsson and B.P. Lisboa, Eur. J. Biochem., 16 (1970) 475.
26 J.A. Gustafsson, R. Ryhage, J. Sjövall and R.M. Moriarty, J. Amer. Chem. Soc., 91 (1969) 1234.
27 K.D.R. Setchell, B. Almé, M. Axelson and J. Sjövall, J. Steroid Biochem., 7 (1976) 615.
28 H. Eriksson, Eur. J. Biochem., 18 (1971) 86.
29 J.M. Olefsky, J. Clin. Invest., 56 (1975) 1499.
30 W. Mlekusch, B. Paletta, W. Truppe, E. Paschke and R. Grimus, Horm. Metab. Res., 13 (1981) 612.
31 R. DePirro, A. Green, M. Y.-C. Kao and J.M. Olefsky, Diabetologia, 21 (1981) 149.
32 P. Ivanyi, R. Hampl, L. Starka and M. Mickova, Nature (London), 238 (1972) 280.
33 D. Meruelo and M. Edidin, Proc. Nat. Acad. Sci. U.S., 72 (1975) 2644.
34 W. Lafuse and M. Edidin, Biochemistry, 19 (1980) 49.
35 F.J. Schwende, J.W. Jorgenson and M. Novotny, J. Chem. Ecol., in press.
36 J. Klein, Advan. Immunol., 26 (1978) 58.
37 J. Klein, F. Figueroa and D. Klein, Immunogenetics, 16 (1982) 285.
38 J.J. Bonner and H.C. Slavkin, Immunogenetics, 2 (1975) 213.

Journal of Chromatography, 308 (1984) 11—18
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2072

QUANTITATIVE METABOLIC PROFILING OF TESTICULAR STEROID SECRETIONS WITH BONDED-PHASE CAPILLARY GAS CHROMATOGRAPHY

CURTIS CHUBB* and CATHERINE NOLAN

Department of Cell Biology, Southwestern Medical School, The University of Texas Health Science Center at Dallas, 5323 Harry Hines Boulevard, Dallas, TX 75235 (U.S.A.)

(First received October 7th, 1983; revised manuscript received January 16th, 1984)

SUMMARY

A procedure is described for quantitative metabolic profiling of nine steroids secreted by mouse testes. The procedure incorporates Celite column chromatography and a gas chromatograph equipped with a bonded-phase capillary column. Validation tests demonstrated that the procedure is precise, efficient, sensitive, and capable of providing information about the spectrum of steroids secreted by testes perfused in vitro. The procedure was used to determine the effect of the hemimelic extra toes gene mutation on the steroidogenic potential of inbred mouse testes perfused in vitro.

INTRODUCTION

The evolution of capillary columns has dramatically increased the resolution power of gas chromatography (GC) [1]. The high resolution of capillary columns coupled with the universal detection ability of flame ionization detectors provide a valuable method for the metabolic profiling of steroids. Metabolic profiles are "multicomponent GC analyses that define or describe metabolic patterns for a group of metabolically or analytically related metabolites" [2, 3].

Previously, investigators have analyzed urinary steroids [4—6] and, to a lesser extent, plasma steroids [7, 8] with capillary GC. The current report describes an efficient and practical procedure utilizing capillary GC for the metabolic profiling of steroids secreted by mouse testes perfused in vitro. Although a spectrum of steroids occurs naturally [9], nine steroids were selected for metabolic profiling because they account for more than 85% of the total steroid secretion of mouse [10], rat [11], and rabbit [11] testes

0378-4347/84/$03.00

perfused in vitro. The selected steroids were profiled quantitatively by a combination of Celite column chromatography and capillary GC.

MATERIALS AND METHODS

Animals

B10.D2/nSn (F43) male mice with (Hx/+) or without (+/+) the hemimelic extra toes (Hx) gene mutation were obtained from the Jackson Laboratory (Bar Harbor, ME, U.S.A.) at six weeks of age and housed in sibling groups for two months before use. Lab-Blox (Wayne Feed Division, Chicago, IL, U.S.A.) and water were available at all times. The animal room was maintained at 23 ± 1°C with 14 h of light per 24 h.

Chemicals and steroids

Isooctane (Spectra-Analyzed, Fisher, Pittsburgh, PA, U.S.A.), benzene and chloroform (Omnisolv, MCB, Cincinatti, OH, U.S.A.), and hexane (Pesticide Grade, J.T. Baker, Phillipsburg, NJ, U.S.A.) were redistilled and stored in glass-stoppered bottles. Acetic anhydride (Pfaltz and Bauer, Stamford, CT, U.S.A.) and pyridine (Certified ACS, Fisher) were also redistilled and the pyridine stored in a desiccator. Ethylene glycol (Fisher) was used without modification. Celite Analytical Filter Aid (Fisher) was heated at 550°C for at least 18 h before use and allowed to cool to room temperature in a desiccator.

Reference samples of the steroids selected for analysis were obtained from Steraloids (Wilton, NH, U.S.A.) and recrystallized three times. Ethanolic solutions (2—4 mg/ml) of the following steroids were stored at —20°C: pregnenolone (PREG, 3β-hydroxy-5-pregnen-20-one); dehydroepiandrosterone (DHA, 3β-hydroxy-5-androsten-17-one); androstenediol (DIOL, 5-androstene-3β,17β-diol); progesterone (PROG, 4-pregnene-3,20-dione); androstenedione (DIONE, 4-androstene-3,17-dione); testosterone (T, 17β-hydroxy-4-androsten-3-one); dihydrotestosterone (DHT, 17β-hydroxy-5α-androstan-3-one); 3α-androstanediol (3α-DIOL, 5α-androstane-3α,17β-diol); 3β-androstanediol (3β-DIOL, 5α-androstane-3β,17β-diol); 5α-cholestane.

Two radio-labelled steroids were purchased from New England Nuclear (Boston, MA, U.S.A.): [1,2,6,7,16,17-^{3}H]testosterone (135 Ci/mmol) and [1,2-^{3}H]androstenediol (45 Ci/mmol). The ^{3}H-labelled steroids were purified by Sephadex LH-20 chromatography prior to use.

In vitro testis perfusion

Testes were perfused in vitro for 4 h at 32°C via the capsular artery with an artificial medium containing 0.2% glucose, 3% bovine serum albumin and 25% bovine erythrocytes [12]. Luteinizing hormone (LH; NIAMDD-oLH-24) was infused at 100 ng/ml in order to maximally stimulate steroidogenesis. After an initial 1-h equilibration period, three consecutive 60-min samples of venous effluent were collected. Supernatants of the venous effluent and saline washes of the erythrocytes were combined and stored at —20°C prior to steroid analysis by GC.

Steroid extraction

Two 60-min collections of supernatant plus wash from each testis were

combined and [^{3}H]testosterone (approximately 1000 dpm) and [^{3}H]-androstenediol (approximately 1000 dpm) were added for recovery determinations. For validation tests, aliquots of each reference steroid solution were dissolved in 3 ml of perfusion medium supernatant. Steroids were extracted from the analytical and validation samples by adding 6 ml benzene—hexane (2:1) and vortexing for 90 sec. After centrifugation (500 *g* for 10 min), aqueous phases were frozen in an acetone—dry ice bath. Organic phases were decanted into 13 × 100 mm disposable test tubes and evaporated under nitrogen in a water bath (47° C). Residues were then redissolved in 0.5 ml of isooctane (saturated with ethylene glycol) and applied to the bed of a Celite column.

Glassware was silanized and subsequently rinsed with methanol and chloroform before use. Re-usable glassware was chromic acid-washed.

Celite column chromatography

Celite column chromatography was performed as described previously [10, 13] with the exception that only two consecutive fractions of eluent were collected: fraction A = 3.5 ml of isooctane + 3.5 ml of 5% benzene in isooctane + 5 ml of 20% benzene in isooctane; fraction B = 6 ml of 20% benzene in isooctane + 10 ml of 35% benzene in isooctane.

The collected fractions were transferred with chloroform to 1-ml reaction vials, dried under nitrogen (60° C), and acetylated (0.2 ml pyridine—acetic anhydride (5:1) at 60° C for 1 h) [14]. Afterwards, the contents of the reaction vials were evaporated under nitrogen at 60° C and redissolved in 100 μl of chloroform. Aliquots (10 μl) of analytical samples were removed for recovery determination. Acetylated samples were stored at —20° C until GC analysis.

Gas chromatography

Acetylated samples were transferred to injection tubes (6 × 50 mm disposable test tubes) containing 100 ng of the internal standard, 5α-cholestane. The injection mixture was evaporated under nitrogen in a 60° C water bath and redissolved in 4 μl of chloroform. After vortexing for 5 sec, 2 μl of the contents were injected into a Spectra-Physics SP7100 gas chromatograph (Spectra-Physics, San Jose, CA, U.S.A.) equipped with a capillary inlet and dual flame ionization detectors. Steroids were separated on a WCOT bonded-phase vitreous silica capillary column (12 m × 0.2 mm, 12 QC2/BP1-0.25, Scientific Glass Engineering, Austin, TX, U.S.A.) coated with non-polar dimethyl silicone. The conditions selected for steroid analyses are presented in Table I.

TABLE I

GC CONDITIONS FOR SEPARATION OF NINE TESTICULAR STEROIDS

Injector temperature: 250° C	Carrier gas: helium
Detector temperature: 300° C	Capillary inlet: splitless mode
Oven temperature: 160° C initial, hold for 1 min;	Splitless injection time: 0.6 min
240° C final, hold for 5 min;	Linear gas velocity: 25 cm/sec
4° C/min ramp	Capillary column head pressure: 100 kPa
Electrometer: $1 \cdot 10^{12}$ A/mV	Septum purge: 1.3 ml/min
Attenuation: 32	Hydrogen flow-rate: 30 ml/min
	Air flow-rate: 400 ml/min

Peaks were integrated by a built-in programmable data system/printer plotter. Component peaks were identified by their relative retention times and quantified by comparison to the internal standard peak area.

RESULTS AND DISCUSSION

Steroid extraction and preliminary purification

Organic solvent extraction was selected because of the resulting quantitative recoveries as reported in an earlier study [10]. The extracted steroids were chromatographed on a Celite column to provide a preliminary purification step and to divide the steroids into two fractions: fraction A steroids are DIONE, DHA, DHT, T, PROG, PREG; fraction B steroids are 3α-DIOL, DIOL, 3β-DIOL.

Steroid derivatization

Acetylation stabilized the steroids, resulted in single peaks, and was simple to perform. Additionally, the residue remaining after evaporation of the reaction mixture did not significantly contribute to the background of the GC analyses. In contrast, the formation of methoxime—trimethylsilyl ether derivatives [15] of 3-oxo-4-ene steroids (PROG, DIONE, T) produced isomers [3] and required additional clean-up columns as reported by Axelson and Sjövall [8].

Two steroids, 17α-hydroxypregnenolone and 17α-hydroxyprogesterone, were not acetylated under the stated conditions. The sterically hindered hydroxyl at the 17-position has been reported to be difficult to derivatize [14].

Precision of retention times and peak identification

Because of the high resolution of capillary GC, retention times can be used to identify peaks if the retention times can be proven to be stable [16]. The

TABLE II

PRECISION OF RETENTION TIMES

Retention times (min) are of steroids analyzed by the described GC method on three different days. Steroid concentrations ranged from 10 to 200 ng.

Steroid	n	$\bar{x}$	Coefficient of variation ($\frac{\text{S.D.}}{\bar{x}} \times 100$)
Androstenedione	16	12.54	0.3
Dehydroepiandrosterone	16	13.50	0.3
Dihydrotestosterone	16	14.02	0.3
Testosterone	16	15.25	0.3
Progesterone	16	15.84	0.3
Pregnenolone	16	16.71	0.2
3α-Androstanediol	16	15.58	0.3
Androstenediol	16	16.16	0.3
3β-Androstanediol	16	16.30	0.3
5α-Cholestane	32	17.26	0.2

highly reproducible and stable retention times of steroids analyzed on our gas chromatograph (Table II) support our decision to use retention times for peak identification.

Sensitivity and range of detection

The sensitivity and range of detection were evaluated by recording the detector response to different amounts of injected steroids (Fig. 1). The sensitivity was 2 ng for each of the derivatized steroids. Detector response was linear from 0 to 200 ng as indicated by the high correlation coefficient (r = 0.98). However, the increased error for masses above 100 ng suggests that column overloading occurred.

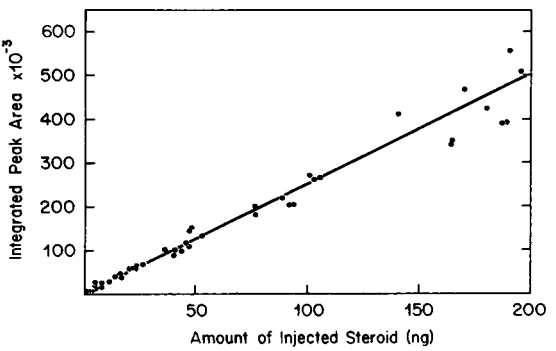


Fig. 1. Linear-regression analysis of the relationship between steroid mass injected and detector response. In total 44 data points were analyzed. The data points represent nine steroids and five mass ranges (5, 25, 50, 100, and 200 ng). The correlation coefficient (r = 0.982) was highly significant ($p < 0.001$) as determined by Student's t test; $y = 2089 + 1260x$.

Recovery and precision

The recovery and precision of the procedure were assessed by extracting known amounts of all nine steroids from 2 ml of water or perfusion medium supernatant. Examples of the chromatograms are presented in Figs. 2 and 3. The results (Table III) demonstrate that the recovery and precision of the method compare favorably with previously reported capillary GC analyses of plasma steroids [7, 8, 17]. The presence of a close-eluting background peak may explain the high recovery for androstenedione. The background peak has been subsequently diminished by a more defined distillation of isooctane.

Metabolic profile of nine steroids secreted by mouse testes perfused in vitro

The hemimelic extra toes (Hx) gene mutation causes infertility in male mice [18, 19]. Since successful spermatogenesis requires a milieu of steroids, we hypothesized that the Hx gene mutation may alter steroidogenesis. The hypothesis was tested by comparing the metabolic profile of steroids secreted by sibling inbred mice that were coisogenic for the Hx gene mutation. Testicular venous effluent was analyzed for steroids by the described GC procedure. The results (Fig. 4) suggest that the Hx gene mutation does not deleteriously affect steroidogenesis. Additionally, the steroid secretion profile presently

TABLE III

RESULTS OF ASSAYS TO DETERMINE RECOVERY AND PRECISION

Identical amounts of a solution containing the nine selected steroids were added to 2 ml water (n = 3), 2 ml perfusion medium supernatant (n = 3), and two reaction vials. The steroids in the reaction vials were used to determine recoveries of the steroids extracted from the aqueous solutions and analyzed by the described method.

Steroid	n	Mass ($\bar{x}$, ng)	Recovery percentage (%, $\bar{x}$ ± S.E.M.)	Precision coefficient of variation ($\frac{\text{S.D.}}{\bar{x}} \times 100$)
Androstenedione	5	61.7	102 ± 7	14.7
Dehydroepiandrosterone	6	100.5	82 ± 3	8.4
Dihydrotestosterone	6	68.1	83 ± 3	7.8
Testosterone	6	83.4	81 ± 2	7.7
Progesterone	6	49.4	84 ± 3	9.9
Pregnenolone	6	55.5	86 ± 3	8.4
3α-Androstanediol	5	86.3	93 ± 4	8.2
Androstenediol	6	74.2	93 ± 4	12.9
3β-Androstanediol	6	47.9	86 ± 3	7.7

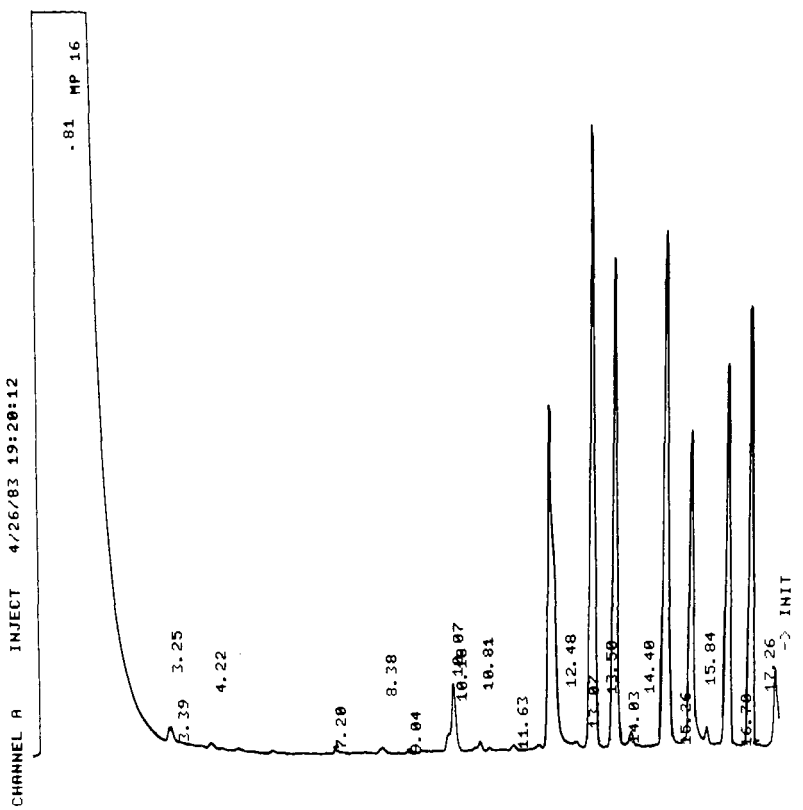


Fig. 2. A chromatogram of the six fraction A steroids extracted from 2 ml of perfusion medium supernatant containing the nine selected steroids and processed by the described procedure. Steroids were identified by retention times as specified on the chromatogram: androstenedione, 12.48 min; dehydroepiandrosterone, 13.50 min; dihydrotestosterone, 14.03 min; testosterone, 15.26 min; progesterone, 15.84 min; pregnenolone, 16.70 min; 5α-cholestane, 17.26 min. The 5α-cholestane peak represents 50 ng.

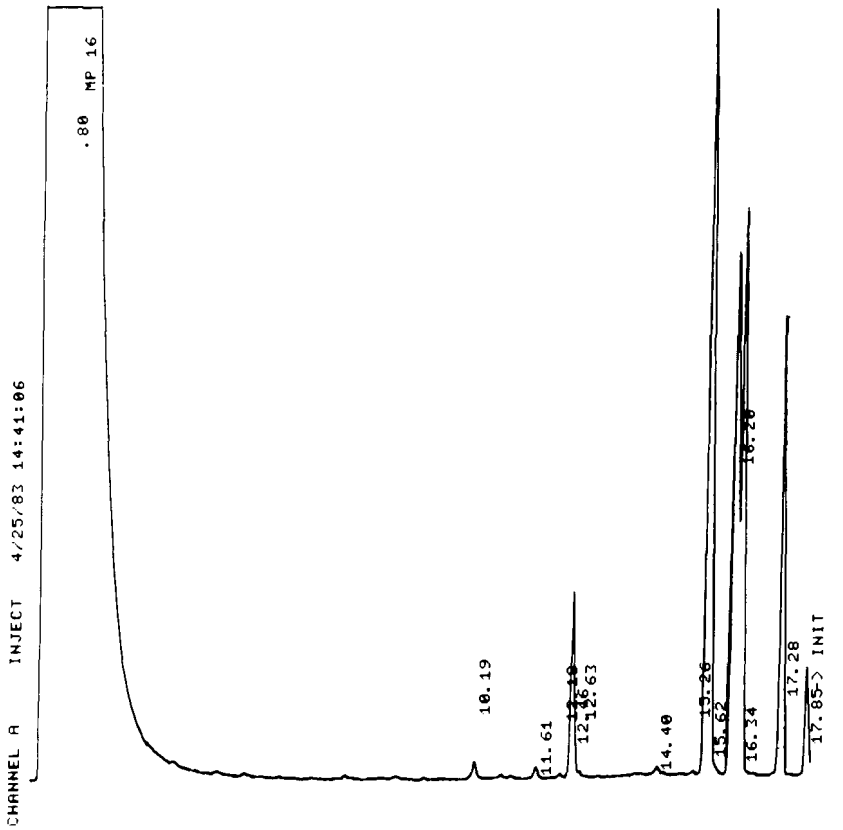


Fig. 3. A chromatogram of the three fraction B steroids extracted from 2 ml of perfusion medium supernatant containing the nine selected steroids and processed by the described procedure. The steroids were identified by retention times as specified on the chromatogram: 3α-androstanediol, 15.62 min; androstenediol, 16.20 min; 3β-androstanediol, 16.34 min; 5α-cholestane, 17.28 min. The 5α-cholestane peak represents 50 ng.

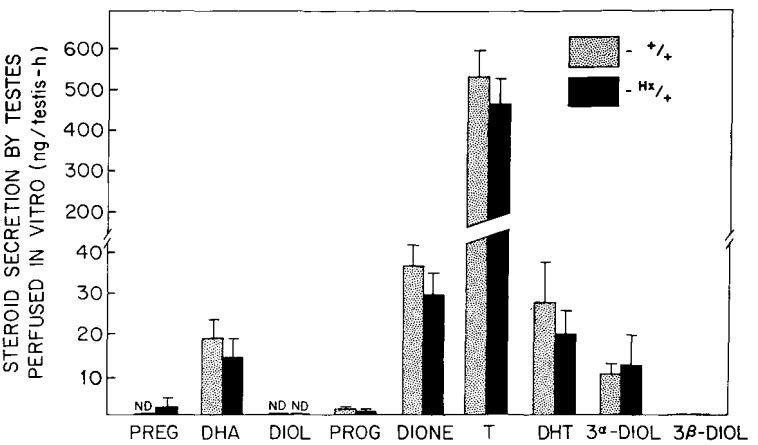


Fig. 4. Metabolic profile of steroids secreted by LH-stimulated testes of hemimelic extra toes mutant mice (Hx/+) and control siblings (+/+). Testes were perfused in vitro for 4 h and the venous effluent was analyzed for steroids by the described capillary GC procedure. The steroid abbreviations are defined in Materials and methods. Each value represents the mean ± standard error. $n = 6$; ND = non-detectable.

reported is comparable to an earlier report of steroid secretion by CBF_1 mouse testes perfused in vitro [10]. The previous study was accomplished with column chromatography and radioimmunoassay procedures. Differences in absolute secretion rates may be due to the different strains of mice used in the two studies.

CONCLUSION

Together, the data support the applicability of the described procedure employing capillary GC for the metabolic profiling of steroids secreted by testes. All of the "most desirable achievements of a GC analysis" as listed by Schomburg [20] are attained: adequate resolution, reasonable analysis time, sensitive detection, highly precise and accurately obtained data.

ACKNOWLEDGEMENTS

Luteinizing hormone, NIAMDD-oLH-24, was obtained from the National Hormone and Pituitary Program, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, MD, U.S.A.

The research was supported, in part, by Public Health Service Research Grant HD-15594 from the National Institute of Child Health and Human Development.

REFERENCES

1 A.L. German, C.D. Pfaffenberger, J.-P. Thenot, M.G. Horning and E.C. Horning, Anal. Chem., 45 (1973) 930.
2 E.C. Horning and M.G. Horning, Clin. Chem., 17 (1971) 802.
3 C.D. Pfaffenberger, in W.G. Jennings (Editor), Applications of Glass Capillary Gas Chromatography, Marcel Dekker, New York, 1981, p. 203.
4 M. Axelson, B.-L. Sahlberg and J. Sjövall, J. Chromatogr., 224 (1981) 355.
5 C.D. Pfaffenberger, L.R. Malinak and E.C. Horning, J. Chromatogr., 158 (1978) 313.
6 P. Pfeifer and G. Spiteller, J. Chromatogr., 223 (1981) 21.
7 M. Axelson and B.-L. Sahlberg, J. Steroid Biochem., 18 (1983) 313.
8 M. Axelson and J. Sjövall, J. Steroid Biochem., 8 (1977) 683.
9 R.I. Dorfman and F. Ungar, Metabolism of Steroid Hormones, Academic Press, New York, 1965.
10 C. Chubb and C. Desjardins, Amer. J. Physiol., 244 (1983) E575.
11 C. Chubb and L.L. Ewing, Amer. J. Physiol., 237 (1979) E231.
12 C. Chubb and C. Desjardins, Comp. Biochem. Physiol., 74A (1983) 231.
13 R.J. Moore, J.M. Gazak, J.F. Quebbeman and J.D. Wilson, J. Clin. Invest., 64 (1979) 1003.
14 O.V. Dominguez, J.R. Seely and J. Gorski, Anal. Chem., 35 (1963) 1243.
15 C.D. Pfaffenberger and E.C. Horning, Anal. Biochem., 80 (1977) 329.
16 F. Rowland, Amer. Lab., (March 1982) 110.
17 R.G. Frith and G. Phillipou, Ann. Clin. Biochem., 18 (1981) 84.
18 M.M. Dickie, Mouse News Lett., 38 (1968) 24.
19 M.C. Green, in M.C. Green (Editor), Genetic Variants and Strains of the Laboratory Mouse, Gustav Fischer Verlag, Stuttgart, 1981, p. 8.
20 G. Schomburg, J. Chromatogr. Sci., 21 (1983) 97.

Journal of Chromatography, 308 (1984) 19—29
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2066

DETERMINATION OF ACIDS IN WHOLE LIPOPOLYSACCHARIDE AND IN FREE LIPID A FROM *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* AND *HAEMOPHILUS APHROPHILUS*

ILIA BRONDZ*

Department of Chemistry, University of Oslo, Blindern, Oslo 3 (Norway)

and

INGAR OLSEN

Department of Microbiology, Dental Faculty, University of Oslo, Blindern, Oslo 3 (Norway)

(First received October 26th, 1983; revised manuscript received January 6th, 1984)

SUMMARY

Acids from whole lipopolysaccharide and free lipid A of the closely related bacteria *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* were determined by gas chromatography and gas chromatography—mass spectrometry. In whole lipopolysaccharide, 3-hydroxymyristic acid was most abundant, followed by myristic and 3-deoxy-D-manno-2-octulosonic acid. In the lipid A moiety, myristic acid dominated, followed by 3-hydroxymyristic acid. The acid composition of whole lipopolysaccharide and free lipid A from *A. actinomycetemcomitans* and *H. aphrophilus* was not so specific as to allow taxonomic differentiation between these bacteria. If fatty acids of lipopolysaccharide are essential for expression of endotoxicity, the present results suggested no marked difference in the endotoxic activities of *A. actinomycetemcomitans* and *H. aphrophilus*.

INTRODUCTION

Actinobacillus actinomycetemcomitans and *Haemophilus aphrophilus* are morphologically very similar Gram-negative rods [1] indigenous to dental plaque [2]. Taxonomic differentiation between these bacterial species is difficult and usually based only on a few physiological characters [3]. Both *A. actinomycetemcomitans* and *H. aphrophilus* have been associated with endocarditis, dental plaque often being the source of extraoral infections [4].

0378-4347/84/$03.00

A. actinomycetemcomitans has recently attracted much attention due to its suspected role as a major pathogen in juvenile periodontitis (periodontosis) [5]. *H. aphrophilus* has not been implicated to the same extent, haemophili traditionally being considered to have a low pathogenic potential in the periodontal pocket [6].

Lipopolysaccharide (LPS) located in the outer membrane of Gram-negative bacteria is considered an essential factor in chronic inflammatory periodontal disease where it may elicit damage by its ability to stimulate macrophages, activate complement, act as a thymus-independent antigen and B lymphocyte mitogen, stimulate bone resorption, and by its cytotoxicity (see ref. 7 for review). Chemically, LPS is made up of a polysaccharide portion, the O-antigenicity determining chains and the core, and a covalently bound lipid, lipid A [8]. The serological properties of LPS are related to the O-specific chains, the endotoxic activity to lipid A. The latter, which is a highly biologically active molecule with a great variety of endotoxic activities [9], also promotes the functional and structural integrity of the bacterial membrane. Knowledge on what specific components of the LPS molecule are responsible for the endotoxic activity is sparse. What is known though, is that chemical [10] or enzymatic [11] removal of fatty acids results in detoxification of endotoxins. Therefore, the presence of fatty acids seems to be essential for the expression of endotoxicity. If *A. actinomycetemcomitans* and *H. aphrophilus* really have a different periopathogenic potential, this might be reflected in the fatty acid composition of their endotoxins. In the present study, the nature and quantity of acids, mainly fatty acids, present in the entire LPS molecule or in free lipid A of *A. actinomycetemcomitans* and *H. aphrophilus* isolated from periodontitis, were compared by means of gas chromatography (GC) and gas chromatography—mass spectrometry (GC—MS). Analysis of the fatty acid composition of lipid A may provide important information as to the taxonomic position of a bacterial strain [12, 13]. It was therefore hoped that differences, if present, would help to distinguish between *A. actinomycetemcomitans* and *H. aphrophilus*.

Fatty acids of the LPS macromolecule are generally removed by acidic or alkaline hydrolysis and then derivatized before GC analysis. In this report, hydrolysis and derivatization were performed simultaneously by transesterification with hydrochloric acid in anhydrous methanol. This method also enabled direct GC determination of 3-deoxy-D-manno-2-octulosonic acid (KDO), to which lipid A is covalently linked.

MATERIAL AND METHOD

Bacteria

The strains of *A. actinomycetemcomitans* and *H. aphrophilus* investigated and the sources from which they were obtained, are shown in Table I. Strains ATCC 33389, 33384, and 19415 were obtained directly from the American Type Culture Collection, strain HK 435 from M. Kilian, Aarhus, Denmark, and the remaining strains through Forsyth Dental Center. The organisms were maintained and mass cultivated as described previously [14].

TABLE I

LIST OF BACTERIA INVESTIGATED

Organism	Strain	Source*	Site of origin
Actinobacillus actinomycetemcomitans	33384 (9710)	ATCC (NCTC)	Lung abscess
	29524	ATCC	Chest aspirate
	29522	ATCC	Mandibular abscess
	511	FDC	Periodontitis
	HK435	Kilian	Pus
	N27	FDC	Periodontosis
	Y4	FDC	Periodontosis
Haemophilus aphrophilus	33389 (5906)	ATCC (NCTC)	Endocarditis
	19415 (5886)	ATCC (NCTC)	Endocarditis
	655	FDC	Periodontitis
	654	FDC	Periodontitis
	626	FDC	Periodontitis
	621	FDC	Periodontitis

*ATCC = American Type Culture Collection (Rockville, MD, U.S.A.); NCTC = National Collection of Type Cultures (London, U.K.); FDC = Forsyth Dental Center (Boston, MA, U.S.A.).

Preparation of lipopolysaccharide

LPSs from *A. actinomycetemcomitans/H. aphrophilus* strain pairs were isolated by the phenol—water procedure [15]. Lyophilized bacterial cells (1 g) were suspended in 35 ml of deionized distilled water preheated to 68°C. The suspension was mixed vigorously for 20 min at 68°C with 35 ml of preheated 90% phenol (E. Merck, Darmstadt, F.R.G.). After cooling (—20°C, 1 h), the phases were separated by centrifugation (900 *g*, 20 min, 4°C). The aqueous phase was pipetted off and the phenol portion extracted with 30 ml of prewarmed deionized distilled water for another 20 min at 68°C. The combined aqueous phases were dialysed against tap water (24 h) and several changes of deionized distilled water at 4°C. They were concentrated to 6—8 ml and precipitated with 10 vols. of absolute ethanol in the presence of 2—3 drops of saturated sodium acetate. After cooling (—20°C, 1 h), the suspension was centrifuged (900 *g*, 20 min, 4°C) and the precipitate dried. It was then dissolved in deionized distilled water under sonication in the presence of ice and centrifuged (12,100 *g*, 15 min, 4°C). The supernatant was purified further by ultracentrifugation (100,000 *g*, 1 h, 4°C) twice. These procedures virtually eliminated the absorbance top at 260 nm. The resulting pellet was dissolved in deionized distilled water by sonication and lyophilized.

Preparation of free lipid A

Lipid A was prepared from *A. actinomycetemcomitans* strain ATCC 33384 and *H. aphrophilus* strain ATCC 33389 by treating LPS with 1% glacial acetic acid (Merck) at 100°C for 90 min [16]. Crude lipid A was first homogenized in an agate mortar and then sonicated slowly for 1—2 min under ice cooling in the presence of 3 ml of 1% glacial acetic acid. The suspension was heated to 70°C

for 25 min in a PTFE-sealed tube with screw cap, cooled to room temperature and centrifuged (900 *g*, 10 min, 4° C). The pellet was washed with warm (50° C) glacial acetic acid and then with warm (50° C) deionized distilled water, and lyophilized.

Methanolysis

Fatty acids were liberated from samples of whole LPS (0.5—1.5 mg) and lipid A (0.2—0.3 mg) by methanolysis (2 *M* hydrochloric acid in anhydrous methanol, 2 ml, 24 h, 85° C). After cooling, the methanolysate was concentrated, while kept on ice, to 0.1—0.2 ml by a stream of nitrogen. Chloroform (Fluka, Buchs, Switzerland) (2 ml) was added and the mixture transferred to a 20-ml separatory funnel, followed by two 1-ml batches of chloroform used to wash the methanolysis tube. Distilled water (4 ml) was added to the organic phase and the mixture shaken carefully. After separation of the organic phase from the water phase, the former was washed twice, each time with 4 ml of distilled water, and the water phase twice, with 4-ml batches of chloroform. The organic phases, like the water phases, were pooled. The water phases were lyophilized and their sugar content will be published later. Anhydrous magnesium sulphate (Merck) (400 mg) was added to the organic phases, which after 30 min were filtered through a paper filter. Filter and storage flasks for the organic phases were washed three times, each time with 3 ml of chloroform. The chloroform phase, kept on ice, was dried with nitrogen. It was dissolved in *n*-hexane (Merck) previously dried by filtration through alkaline aluminium oxide grade 1 (Woelm Pharmacia, F.R.G.) and analysed by GC and GC–MS.

Reference compounds

LPSs, prepared by the phenolic extraction procedure, from *Escherichia coli* serotype 055:B5, *Salmonella typhimurium* and *Serratia marcescens* were obtained from Sigma, St. Louis, MO, U.S.A. The methyl ester of KDO was provided by incubating KDO (Sigma) in a PTFE-sealed tube with screw cap in the presence of 2 *M* hydrochloric acid in anhydrous methanol at 85° C for 24 h. Methyl esters of lauric, tridecanoic, myristic, 2-hydroxymyristic, pentadecanoic, and palmitic acid were purchased from Supelco, Bellefonte, PA, U.S.A. 3-Hydroxymyristic acid was synthesised as described previously [17], and N-glucosaminemyristate was a gift from O. Lüderitz, Max-Planck-Institut für Immunbiologie, Freiburg, F.R.G. We obtained *n*-triacontane from Fluka, and *n*-triacontanol from Fluka and from *H. perforatum* L. [18].

Recovery of acids from LPS

To determine the recovery of acids from LPS, three separate methanolysis experiments were performed, using N-glucosaminemyristate as reference. The recovery was determined by weighing the product and by means of GC with two internal standards: methyltridecanoate and methylpentadecanoate. The product consisted of methyltetradecanoate, the recovery of which was 95%. Traces of tetradecanoic acid were not detected with methods previously described [19].

Gas chromatography

A Sigma 3 gas chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.) furnished with an electronic integrator (Sigma 10) was used. The Chrompack (Middelburg, The Netherlands) CP-Sil 5 CB (polydimethylsiloxane) capillary column used was 25 m × 0.22 mm I.D. with film thickness 0.14 μm and height equivalent of a theoretical plate (HETP) 0.25 mm. Helium served as carrier gas at a flow-rate of 2 ml/min. The pressure at the inlet of the column was 151.5 kPa. The temperature of the injector and flame ionization detector was 220° C. The gas chromatograph was programmed from 120° C to 260° C at 5° C or 10° C/min with the attenuator set at 8, and the attenuator of the Sigma 10 data system at −1. The paper speed was 10 mm/min.

The identity of the fatty acids (methyl esters) of whole LPS and free lipid A was established by direct cochromatography and by GC—MS. The acids were identified tentatively by comparing their retention times with those of authentic standards. KDO peaks were recognized tentatively by cochromatography of KDO-methyl derivatives and by chromatography of methanolysates of LPS from *E. coli*, *S. typhimurium* and *S. marcescens*. Their identity was verified by GC—MS.

Quantification of fatty acids (methyl esters) was made by correlating the percentual area on the chromatograms constituted by each acid derivative with it percentual content in three standard solutions composed of methyl esters of myristic, 3-hydroxymyristic and palmitic acid, using *n*-triacontane and *n*-triacontanol as internal standards. KDO and 2-hydroxymyristic acid were calculated in the same manner with *n*-triacontane and *n*-triacontanol as internal standards, and the $C_{17:cycl}$ substance as percentage of the total area on the chromatograms.

Irreversible retention of substances with free hydroxy groups by the capillary column was measured by comparing molar response rates between equimolar solutions of *n*-triacontane and *n*-triacontanol.

Gas chromatography—mass spectrometry

The instrument used for combined GC—MS consisted of a Carlo Erba 4200 gas chromatograph, a Micromass 7072F (Vg Micromass, Cheshire, U.K.) mass spectrometer, and a Vg data system 2200. The gas chromatograph was equipped with a glass capillary OV-1 methylsilicone column (20 m × 0.3 mm I.D.). Helium was used as carrier gas. The column temperature was programmed from 120° C to 250° C at 5° C/min. Electron impact ionizing spectra were recorded under the following conditions: ionizing energy 70 eV, ionizing current 200 μA, ion-source temperature 240° C, and accelerating voltage 4 kV. High-resolution mass spectra were obtained at 70 eV from an MS902 double-focus spectrometer connected to an AEI computer (Scientific Apparatus, Manchester, U.K.).

RESULTS

Gas chromatography

A typical gas chromatogram of the acids (methyl esters) recovered from whole LPS of *A. actinomycetemcomitans* and *H. aphrophilus* is given in Fig. 1.

TABLE II

ACIDS RECOVERED FROM WHOLE LIPOPOLYSACCHARIDE

Values (means) are expressed as relative percentage (w/w) of the total in the bacterial strain (S.D. = 5%).

Strain	KDO*	$C_{12:0}$	$C_{14:0}$	2-OH-$C_{14:0}$	3-OH-$C_{14:0}$	$C_{16:0}$	$C_{14:0}$/3-OH-$C_{14:0}$	$C_{17:cycl}$
Actinobacillus actinomycetemcomitans								
33384	9.8	tr**	37.7		52.0	0.2	0.725	0.3
29524	8.2	tr	34.6		57.5	0.1	0.602	0.3
29522	9.4	tr	39.9		50.3	0.1	0.793	0.3
511	6.7	tr	37.5		55.1	0.3	0.681	0.4
HK435	8.4	tr	25.0		66.3	0.1	0.377	0.2
N27	9.5	tr	30.3		59.9	0.1	0.506	0.2
Y4	9.7	tr	34.7		55.0	0.2	0.631	0.4
Haemophilus aphrophilus								
33389	7.7	tr	36.3		55.4	0.1	0.655	0.5
19415	6.5	tr	36.4		56.7	0.1	0.642	0.3
655	6.5	tr	37.7		55.1	0.3	0.684	0.4
654	6.8	tr	36.4		56.3	0.1	0.647	0.4
626	6.9	tr	36.9		54.7	0.7	0.675	0.8
621	6.8	tr	36.6		55.1	0.7	0.664	0.8
Escherichia coli								
055:B5	17.4	8.8	14.5		58.3	0.4	0.250	0.6
Salmonella typhimurium								
	8.9	7.3	15.1	4.8	52.3	8.6	0.290	3.0

*3-Deoxy-D-manno-2-octulosonic acid and/or its degradation products.

**tr = trace amount.

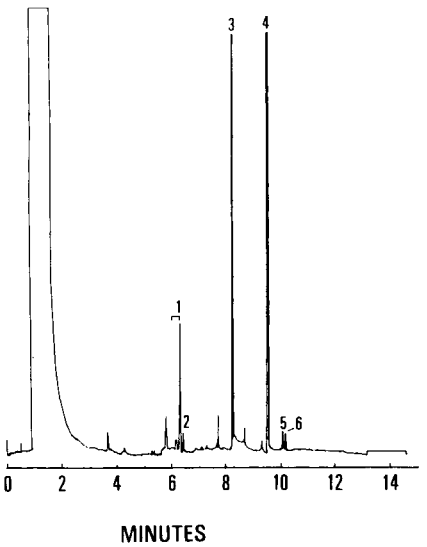


Fig. 1. Typical gas chromatogram of fatty acids (methyl esters) in whole LPS from *A. actinomycetemcomitans* and *H. aphrophilus* represented by *H. aphrophilus* strain ATCC 19415. Programme: 120°C to 260°C at 10°C/min. Temperature of the injector and flame ionization detector, 220°C. Attenuator of the Sigma 3 gas chromatograph, 8; of the Sigma 10 data system, —1. Paper speed, 10 mm/min. 1 = KDO, 2 = $C_{12:0}$, 3 = $C_{14:0}$, 4 = 3-OH-$C_{14:0}$, 5 = $C_{16:0}$, and 6 = $C_{17:cycl}$.

The composition of the major fatty acids recovered from all the LPS preparations investigated is presented in Table II. In *A. actinomycetemcomitans* and *H. aphrophilus*, the most abundant acid was 3-OH-$C_{14:0}$, followed by $C_{14:0}$. Contrary to what was observed among *A. actinomycetemcomitans* strains, the $C_{14:0}$/3-OH-$C_{14:0}$ ratio of *H. aphrophilus* strains varied little. The quantity of KDO and/or its degradation products tended to be higher in LPS from *A. actinomycetemcomitans* than from *H. aphrophilus*, and the amount of the $C_{17:cycl}$ substance higher in *H. aphrophilus* than in *A. actinomycetemcomitans*. The content of fatty acids in LPS from *E. coli* and *S. typhimurium* differed markedly from that of *A. actinomycetemcomitans* and *H. aphrophilus*, particularly with respect to $C_{12:0}$, $C_{14:0}$, and the $C_{14:0}$/3-OH-$C_{14:0}$ ratio. LPS of *S. typhimurium* also contained 2-OH-$C_{14:0}$ and was considerably higher in the $C_{17:cycl}$ substance than the other bacterial strains.

Only two major fatty acids were detected in free lipid A of *A. actinomycetemcomitans* and *H. aphrophilus*: $C_{14:0}$ and 3-OH-$C_{14:0}$ (Fig. 2), of which $C_{14:0}$ was the most abundant (Table III). It should be recognized that with flame ionization detection the detector response, expressed as molar response, was 0.33 for KDO, 1.00 for $C_{14:0}$ and 3-OH-$C_{14:0}$. By using a thermal conductivity detector, the molar response for all the detected components was 1.00.

Gas chromatography—mass spectrometry

The fragmentation pattern of the major fatty acids (methyl esters) of whole LPS and lipid A from all the strains of *A. actinomycetemcomitans* and *H. aphrophilus* investigated were in agreement with those described by Moss and

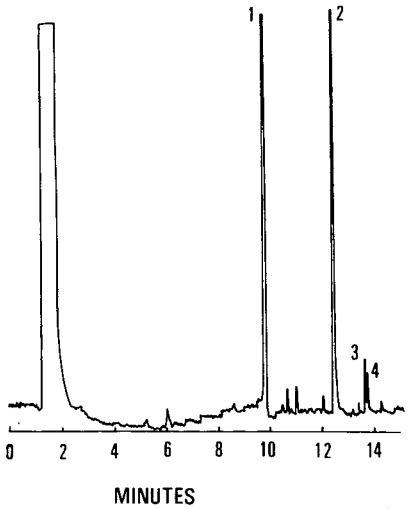


Fig. 2. Typical gas chromatogram of fatty acids (methyl esters) in free lipid A from *A. actinomycetemcomitans* and *H. aphrophilus*, represented by *A. actinomycetemcomitans* strain ATCC 33384. Programme and setting as in Fig. 1, except 5°C/min. 1 = $C_{14:0}$, 2 = 3-OH-$C_{14:0}$, 3 = $C_{16:0}$, and 4 = $C_{17:cycl}$.

TABLE III

ACIDS RECOVERED FROM FREE LIPID A

Values (means) are expressed as the relative percentage (w/w) of the total in the strain (S.D. = 5%).

Strain	$C_{14:0}$	3-OH-$C_{14:0}$	$C_{16:0}$	$C_{17:cycl}$
Actinobacillus actinomycetemcomitans 33384	57.5	38.8	2.0	1.9
Haemophilus aphrophilus 33389	58.8	36.6	2.3	2.3

Dees [20]. The fragmentation pattern of KDO (methyl ester) revealed characteristic fragments with M − 59 (loss of $COOCH_3$) and M − 61 (loss of HO—CH—CH_2—OH). The mass spectrum of the $C_{17:cycl}$ substance is presented in Fig. 3.

DISCUSSION

A number of extraction methods for the isolation of LPS from Gram-negative bacteria have been described, but the method of choice is usually the phenol—water procedure which yields a water-soluble extract subsequently purified by ultracentrifugation [21]. These LPS preparations usually have little contamination, mostly protein (about 1%). The LPSs prepared as presently described, were biologically active, as assessed from the ability to release the local Shwartzman reaction in rabbits and to stimulate bone resorption in cultured explants of mouse calvaria [22].

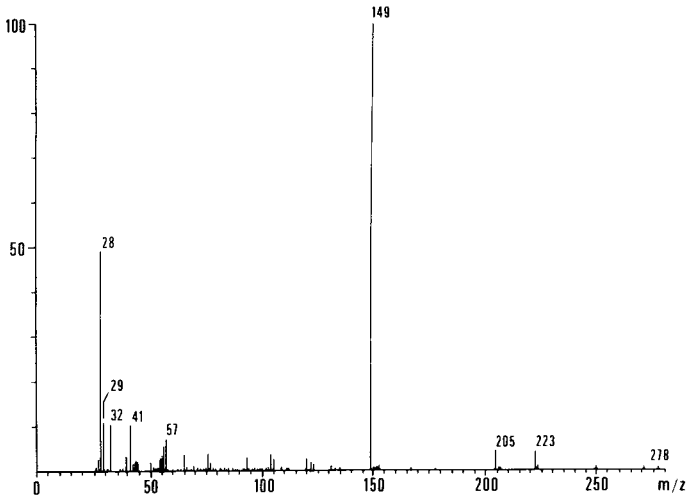


Fig. 3. Mass spectrum of unknown substance tentatively identified as $C_{17:cycl}$ acid.

Our estimates for fatty acids present in LPS from *E. coli* and *S. typhimurium*, which were used as controls, agreed well with those given by previous authors [23]. The acid composition of LPS from *A. actinomycetemcomitans* has not previously been studied systematically, and, as far as we know, the acid content of LPS from *H. aphrophilus* has not been examined at all. In this study, 3-hydroxymyristic acid was the dominant fatty acid in whole LPS from both *A. actinomycetemcomitans* and *H. aphrophilus*. Bryn and Jantzen [24], who examined one strain of *A. actinomycetemcomitans*, also found 3-hydroxymyristic acid most abundant. Since this acid is rarely found in other lipids of Gram-negative bacteria, its isolation from the gingival fluid may be taken as an indication that endotoxin or lipid A is present in the periodontal pocket. Hydroxy acids released down to picomolar amounts from lipid A of LPS sediments have been used successfully to provide an estimate of Gram-negative bacteria [25]. Our estimates for $C_{14:0}$, 3-OH-$C_{14:0}$, and $C_{16:0}$ in free lipid A from LPS of *A. actinomycetemcomitans* agreed with those of Kiley and Holt [26].

KDO appears to be a unqiue substance of Gram-negative bacteria. In LPS, KDO residues are situated at the reducing ends of the polysaccharide domains, linking them by ketosidic bonds to lipid A [27]. No entirely satisfactory method exists for the quantitative determination of KDO in polysaccharides of unknown structure and substitution pattern [27]. The thiobarbiturate assay is most frequently used, but it is subject to interference by materials in the LPS as well as other cellular components [28]. Determination of the KDO content by gas–liquid chromatography (GLC) has infrequently been reported in the literature, mostly due to lack of generally applicable, quantitative derivatization procedures. Like Kochetkov et al. [29], who studied GLC derivatives from whole LPS by methanolysis and pertrimethylsilylation, we found three peaks in the chromatograms which were due to KDO and/or its degradation products.

An unidentified substance of chain length C_{17} was detected in whole LPS

and lipid A from all the examined strains of *A. actinomycetemcomitans* and *H. aphrophilus* as well as from LPS of *E. coli*, *S. typhimurium*, and *S. marcescens*. We found a similar substance among the bound cellular acids of *A. actinomycetemcomitans* and *H. aphrophilus* [17]. The base peak of this substance at *m/e* 149 and its chemical brutto formula provided by high-resolution mass spectrometry, suggested the presence of a cyclopropane fatty acid [30]. Cyclopropane fatty acids are widely distributed among Gram-negative bacteria, including *E. coli* [31], *S. typhimurium* [32], and *S. marcescens* [33]. They have occasionally been detected in lipid A from various bacteria [34], but, to our knowledge, never in *A. actinomycetemcomitans* and *H. aphrophilus*. Most bacteria convert a major fraction of their membrane phospholipids to cyclopropane derivates (for review see ref. 35), particularly when they enter stationary phase. It should be noted that phospholipid is not present in phenol—water-extracted LPS [36]. Nitrogen limitation is another factor which may promote cyclopropane fatty acid formation [37].

The similarity in the qualitative distribution of fatty acids in whole LPS and free lipid A from *A. actinomycetemcomitans* and *H. aphrophilus* indicated that these parameters are of little value as additional tools for distinction between these bacteria. The quantitative variation in fatty acids of whole LPS was larger in the strains of *A. actinomycetemcomitans* than in those of *H. aphrophilus*. We observed a similar relationship when whole cells of *A. actinomycetemcomitans* and *H. aphrophilus* were examined for free fatty acids [14].

The great similarity in the acid composition of the LPS and lipid A preparations of *A. actinomycetemcomitans* and *H. aphrophilus* questioned the validity of considering *A. actinomycetemcomitans* as more periopathogenic than *H. aphrophilus*. If these parameters are valid measures for the relative potency of *A. actinomycetemcomitans* and *H. aphrophilus* as periopathogens, our results suggested that the ability of *H. aphrophilus* to cause periodontal diseases could be underestimated. It cannot be excluded that strains isolated from periodontal lesions may occasionally have been designated as *A. actinomycetemcomitans* when they in fact were *H. aphrophilus* due to problems with differentiation. It is also possible that different biological activities of LPS preparations cannot be predicted on the basis of their structural features. Differences in the serological behaviour of free lipid A and the parent LPS may thus indicate a masking of the determinants of the LPS bound lipid A by the O chains or by the polar head groups [21].

ACKNOWLEDGEMENTS

This work was supported in part by the Anders Jahres fond til vitenskapens fremme. The authors gratefully acknowledge the technical assistance of G. Isaksen, J. Vedde, and M.-B. Jørgensen. We are also indebted to Drs. O. Lüderitz, Max-Planck-Institut für Immunbiologie, Freiburg, F.R.G., for N-glucosaminemyristate, and M. Kilian, Royal Dental College, Aarhus, Denmark, for the *A. actinomycetemcomitans* strain HK 435.

REFERENCES

1 S.C. Holt, A.C.R. Tanner and S.S. Socransky, Infect. Immun., 30 (1980) 588.
2 M. Kilian and R.C. Schiött, Arch. Oral Biol., 20 (1975) 791.
3 J. Slots, Arch. Microbiol., 131 (1982) 60.
4 M.I. Page and E.O. King, N. Engl. J. Med., 275 (1966) 181.
5 S.S. Socransky, J. Clin. Periodontol., 6 (1979) 16.
6 W. Sims, Arch. Oral Biol., 17 (1972) 1029.
7 C.G. Daly, G.J. Seymour and J.B. Kieser, J. Oral Pathol., 9 (1980) 1.
8 O. Lüderitz, O. Westphal, A.M. Staub and H. Nikaido, in G. Weinbaum, S. Kadis and S.J. Ajl (Editors), Microbial Toxins, Vol. 4, Bacterial Endotoxins, Academic Press, New York, London, 1971, pp. 145—233.
9 S.G. Bradley, Annu. Rev. Microbiol., 33 (1979) 67.
10 E. Neter, O. Westphal, O. Lüderitz, E.A. Gorzynski and E. Eichenberger, J. Immunol., 76 (1956) 377.
11 D. Malchow, O. Lüderitz, B. Kickhöfen and O. Westphal, Eur. J. Biochem., 7 (1969) 239.
12 H. Nikaido, Int. J. System. Bacteriol., 20 (1970) 383.
13 E.T. Rietschel and O. Lüderitz, Forum Mikrobiol., 80 (1980) 12.
14 I. Brondz and I. Olsen, J. Chromatogr., 278 (1983) 13.
15 E.A. Kabat and M.M. Mayer (Editors), Experimental Immunochemistry. Revised and Enlarged, C.C. Thomas, Springfield, 2nd ed., 1971, pp. 833—835.
16 K. Sveen and N. Skaug, Scand. J. Dent. Res., 88 (1980) 535.
17 I. Brondz and I. Olsen, J. Chromatogr., 308 (1984) 282.
18 I. Brondz, T. Greibrokk and A.J. Aasen, J. Nat. Prod., 46 (1983) 940.
19 I. Brondz, I. Olsen and T. Greibrokk, J. Chromatogr., 274 (1983) 299.
20 C.W. Moss and S.B. Dees, J. Chromatogr., 112 (1975) 595.
21 O. Lüderitz, M.A. Freudenberg, C. Galanos, V. Lehmann, E.T. Rietschel and D.H. Shaw, Curr. Top. Membr. Transp.,17 (1982) 79.
22 I. Olsen, unpublished results.
23 U. Feige, Zentralbl. Bakteriol. Parasitenk. Infektionskr. Hyg., Abt. 1: Orig. Reihe A, 250 (1981) 52.
24 K. Bryn and E. Jantzen, J. Chromatogr., 240 (1982) 405.
25 J.H. Parker, G.A. Smith, H.L. Fredrickson, J.R. Vestal and D.C. White, Appl. Environ. Microbiol., 44 (1982) 1170.
26 P. Kiley and S.C. Holt, Infect. Immun., 30 (1980) 862.
27 F.M. Unger, Advan. Carbohyd. Chem. Biochem., 38 (1981) 323.
28 J.N. Saddler and A.C. Wardlaw, Antonie van Leeuwenhoek, J. Microbiol. Serol., 46 (1980) 27.
29 N.K. Kochetkov, B.A. Dmitriev and L.V. Backinowsky, Carbohydr. Res., 11 (1969) 193.
30 W.W. Christie and R.T. Holman, Lipids, 1 (1966) 176.
31 S. Dauchy and J. Asselineau, C.R. Acad. Sci. Ser. D, 250 (1960) 2635.
32 G.M. Gray, Biochim. Biophys. Acta, 65 (1962) 135.
33 D.G. Bishop and J.L. Still, J. Lipid Res., 4 (1963) 81.
34 E.T. Rietschel, S. Hase, M.-T. King, J. Redmond and V. Lehmann, in D. Shlesinger (Editor), Microbiology — 1977, American Society for Microbiology, Washington, D.C., 1977, pp. 262—268.
35 C.O. Rock and J.E. Cronan, Jr., Curr. Top. Membr. Transp., 17 (1982) 207.
36 O. Westphal, O. Lüderitz and F. Bister, Z. Naturforsch. Teil B, 7B (1952) 148.
37 A.G. Marr and J.L. Ingraham, J. Bacteriol., 84 (1962) 1260.

Journal of Chromatography, 308 (1984) 31—41
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2089

PICOGRAM DETECTION OF EICOSANOIDS BY ULTRAVIOLET ABSORBANCE AFTER NARROW-BORE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

COMPARISON WITH CONVENTIONAL-BORE COLUMN

RACHELLE RYDZIK

Department of Pharmacology and Toxicology, College of Pharmacy, University of Georgia, Athens, GA 30602 (U.S.A.)

ALICIA TERRAGNO

Instituto de Farmacologia Clinica y Experimental, Avenida Tellier 2160, 1440 Buenos Aires (Argentina)

and

RANDALL TACKETT*

Department of Pharmacology and Toxicology, College of Pharmacy, University of Georgia, Athens, GA 30602 (U.S.A.)

(First received November 11th, 1983; revised manuscript received January 25th, 1984)

SUMMARY

Mobile-phase variations were employed to achieve optimal separation by narrow-bore reversed-phase high-performance liquid chromatography of eleven eicosanoids. Separation and quantitation by ultraviolet absorbance at 190 nm using conventional-bore ODS columns were compared. Using the improved sensitivity obtained by means of the narrow-bore column, i.e. 250-pg detection limits of a standard solution, analysis of eicosanoids in kidney medulla was achieved. Parallel quantitation by radioactivity, using [1-^{14}C]arachidonic acid as substrate, was applied.

INTRODUCTION

The involvement of eicosanoids has been implicated in numerous physiological and pathological processes. Consequently, several attempts to quantitate eicosanoids directly after high-performance liquid chromatographic (HPLC) separation have been made. Most of these studies have involved derivative formation to achieve sensitivity in the nanogram range of the eicosanoids by elec-

0378-4347/84/$03.00

trochemical [1,2], ultraviolet (UV) [3—6], or fluorescence [6—9] detection. Although picogram detection with some fluorescent derivatives has been accomplished [6,7], the derivatization reaction is often not quantitative at the picogram level [7]. Others have used UV absorbance directly for detection in the nanogram or microgram range [10—17]. After HPLC separation, quantitation in biological samples was accomplished by subsequent radioimmunoassay [18,19], gas chromatography—mass spectrometry [20], or radioactivity of converted radiolabeled substrate [5,16,17,21—24]. Previously, we reported quantitation of 6-ketoprostaglandin $F_{1\alpha}$ ($6KPGF_{1\alpha}$), the stable hydrolysis product of prostacyclin (PGI_2), in plasma by direct UV absorbance [25]. However, detection sensitivity precluded quantiation of other eicosanoids.

With the advent of small-diameter columns, there have been reports of increased sensitivity of detection [26,27]. This increased sensitivity is due to the higher concentration of solute reaching the detector cell, since there is less dilution by the mobile phase. In this report, an HPLC separation using a narrow-bore C_{18} column was accomplished for eleven eicosanoids: $6KPGF_{1\alpha}$; 6-ketoprostaglandin E_1 ($6KPGE_1$); 6,15-diketoprostaglandin $F_{1\alpha}$ ($6,15DiKPGF_{1\alpha}$); thromboxane B_2 (TxB_2); prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$); prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$); prostaglandin E_2 (PGE_2); 15-ketoprostaglandin $F_{2\alpha}$ ($15KPGF_{2\alpha}$); prostaglandin E_1 (PGE_1); prostaglandin D_2 (PGD_2); and 15-ketoprostaglandin E_2 ($15\ KPGE_2$). In addition, the UV detection sensitivity of a narrow-bore column (2.0 mm) was compared with that of conventional-bore column (4.6 mm). Subsequently, narrow-bore HPLC separation, with direct absorbance quantitation at 190 nm, of the HPLC effluent was applied to detect several eicosanoids from the kidney medulla. Correlative radioactive quantitation of the HPLC effluent as well as the use of a cyclo-oxygenase inhibitor was used as a verification of the method.

EXPERIMENTAL

Reagents and materials

Methanol, chloroform, ethyl acetate, acetonitrile, water and orthophosphoric acid were HPLC grade (Fisher Scientific). Kreb's solution salts were all ACS grade (Baker Chemical). Prepacked, 1-g LH-20 columns (Isolabs) were used for sample purification. Meclofenamic acid (Warner-Lambert) and eicosanoid standards (Upjohn) were kind gifts of Dr. Roger Westland and Dr. John Pike, respectively. The specific activity of [1-^{14}C] arachidonic acid (AA) (New England Nuclear) was 51.6 mCi $mmol^{-1}$.

Chromatographic apparatus

Mobile phase, in the solvent reservoir, passed through a 2-μm, 1.5-mm solvent inlet filter (Alltech) by way of PTFE tubing (1.5 mm × 0.8 mm I.D.) to a six-position, 0.8-mm bore, PTFE low-pressure Rheodyne rotary valve. A Beckman Model 112 reciprocating pump delivered the selected solvent to a Rheodyne Model 7125 injector equipped with a 10-μl sample loop. Either a 250-mm conventional-bore (4.6 mm) or narrow-bore (2.0 mm) Altex Ultrasphere 5-μm particle size, C_{18} column, protected by a 2-μm precolumn filter (Upchurch), was used for sample separation. Absorbance detection at 190 nm of the HPLC effluent was accomplished using a Kratos Model 773 variable-

wavelength UV—Vis spectrophotometer equipped with an 8-μl flow cell having a 1-cm pathlength. Sensitivity on the detector was adjusted between 0.007 and 2.500 absorbance units full scale (a.u.f.s.). Absorbance changes, resulting in the chromatogram, were recorded on a single-pen recorder (Kipp-Zonen, Model BD 40/04) set at 1 mV full scale deflection (f.s.d.) and operated at a chart speed of 5 mm min^{-1}. Radioactive quantitation was done by collecting 1-min fractions of the detector effluent, mixing with 4 ml scintillation cocktail (Scintiverse II, Fisher Scientific) followed by ^{14}C-counting for 2 min in a scintillation counter (Beckman, Model LS 7500).

Mobile phase systems were prepared by mixing proportions of acetonitrile with 0.0025 *M* phosphoric acid. Eicosanoid standards (25 pg/μl—25 ng/μl) as well as purified biological extracts were dissolved in water—acetonitrile (70:30).

Biological sample preparation

New Zealand white rabbits (3—4 kg) were killed by cervical dislocation and their kidneys quickly removed. The kidney medulla was dissected free from the cortex, finely cut and incubated in 2.5 ml Kreb's solution per 500 mg tissue wet weight containing 0.5 μCi/ml [1-^{14}C] AA for 1 h at 37°C under an oxygen—carbon dioxide (95:5) atmosphere as previously described [17]. Meclofenamic acid was dissolved in Kreb's solution at a concentration of 1 mg/ml for inhibition studies [28].

After incubation, the medium was acidified to pH 3.5 with 0.8 *M* phosphoric acid and extracted once by vigorous shaking using 4 vol. ethyl acetate. The organic phase was dried under nitrogen, reconstituted in 0.5 ml chloroform—ethyl acetate (85:15) and applied onto a chloroform pre-equilibrated LH-20 column. After sequential washing of the column with an additional 3.5 ml chloroform—ethyl acetate (85:15), 10 ml chloroform, and 2 ml methanol, the eicosanoid fraction was collected with 1.5 ml methanol. The methanol fraction was dried under nitrogen and stored until the HPLC separation. Recoveries using this purification scheme were 51%, 81% and 86% for $6KPGF_{1\alpha}$, $PGF_{2\alpha}$, and PGE_2, respectively, as previously reported [25].

RESULTS AND DISCUSSION

Separation

By ionic suppression of the carboxyl group with acidification of the mobile phase by phosphoric acid, separation of eicosanoids by reversed-phase chromatography can be governed by mobile-phase polarity, i.e. water—acetonitrile ratios [11]. Fig. 1 shows the effect of changing the percentage from 27.5% to 35.0% of acetonitrile in 0.0025 *M* phosphoric acid on the capacity factors for the eleven eicosanoids using a 250 × 2.0 mm ODS column. A similar profile is seen by conventional-bore ODS columns [11], with the curves shifted slightly to the right in Fig. 1. That is, a higher percentage of acetonitrile was necessary to give the same k' value. Likewise, the optimal solvent strength for the narrow-bore column was a 69:31 ratio of 0.0025 *M* orthophosphoric acid—acetonitrile compared to a 67.2:32.8 ratio for a conventional bore column. At optimal solvent strengths for both columns, migration of the various eicosanoids was governed by their hydrophobicity. Therefore, the more polar two series,

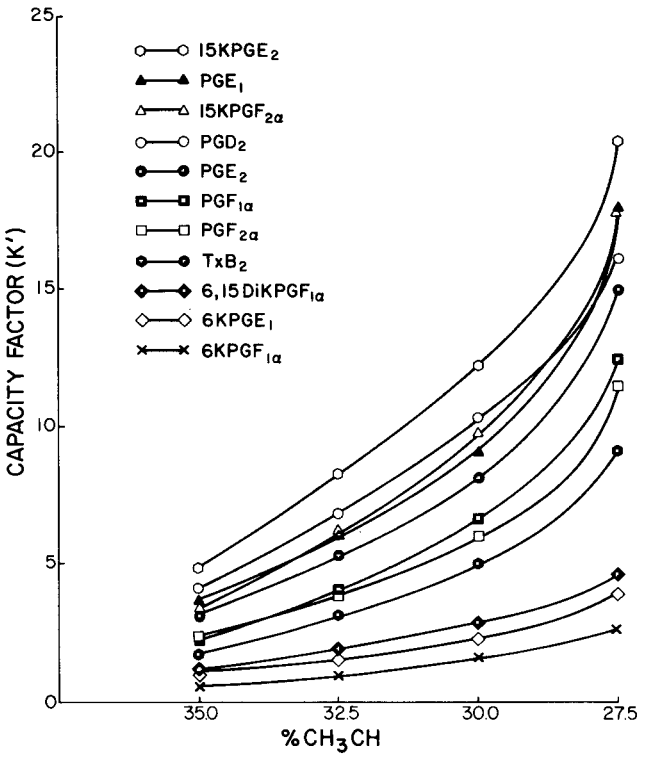


Fig. 1. Relationship between capacity factor (k') and amount of acetonitrile in the mobile phase. Column: 5 μm ODS Ultrasphere, 250 × 2.0 mm; mobile phase: various percentages of 0.0025 *M* orthophosphoric acid—acetonitrile; flow-rate: 0.3 ml/min.

eicosanoids derived from arachidonic acid, having two carbon—carbon double bonds eluted before the one series, products of eicosatrienoic acid which contain only a single alkene group, i.e., $PGF_{2\alpha}$ eluted before $PG_{1\alpha}$ and PGE_2 prior to PGE_1. Furthermore, the addition of either an hydrophilic hydroxyl or carbonyl group lowered the capacity factor, i.e. $6KPGF_{1\alpha}$ and $6KPGE_1$ eluted before $PGF_{1\alpha}$ and PGE_1, respectively. Substitution of an oxygen for the hydrogen atom and hydroxyl group at a given position, however, lengthened the retention time. This was illustrated by PGE_2 eluting after $PGF_{2\alpha}$, due to the substitution at C-9, and 6,15 $DiKPGF_{1\alpha}$ after $6KPGF_{1\alpha}$ by the oxygen substitution at position 15.

Quantitation

Table I gives the regression analysis, i.e. $y = mx + b$ where m is the slope and b the intercept, for both diameter columns using peak height in mm normalized at 0.01 a.u.f.s. on the detector. Analysis was done in triplicate for each point over a range of concentrations of 25 pg/μl—25 ng/μl for the 2.0-mm I.D. column and 0.1—25 ng/μl for the 4.6-mm I.D. column. By comparing the slopes of the regression lines, (m_1/m_2), the increase in sensitivity with the narrow-diameter column is seen to be 3—3.5 fold that of the conventional-diameter column. The higher concentration of eicosanoid reaching the detector cell after

TABLE I

REGRESSION ANALYSIS OF PEAK HEIGHT VERSUS CONCENTRATION OF STANDARD EICOSANOIDS USING NARROW-BORE AND NORMAL-BORE COLUMNS

Eicosanoid	Narrow-bore column			Normal-bore column			m_1/m_2
	b_1	m_1	r_1^2	b_2	m_2	r_2^2	
6KPGF$_{1\alpha}$	85.74	0.081	0.999	98.91	0.028	0.999	2.9
6KPGE$_1$	77.35	0.100	0.999	60.62	0.033	0.999	3.0
6,15DiKPGF$_{1\alpha}$	25.38	0.022	0.999	56.33	0.007	0.995	3.1
PGF$_{2\alpha}$	27.87	0.080	0.999	27.23	0.025	0.999	3.2
PGF$_{1\alpha}$	93.69	0.052	0.999	74.15	0.015	0.998	3.5
PGE$_{2\alpha}$	97.25	0.082	0.999	39.30	0.026	0.999	3.2
15KPGF$_2$	1.63	0.037	0.999	11.67	0.011	0.999	3.4
PGE$_1$	73.50	0.042	0.999	65.03	0.012	0.998	3.5
PGD$_2$	−65.61	0.049	0.999	−61.73	0.014	0.998	3.5
15KPGE$_2$	44.89	0.037	0.999	27.59	0.011	0.999	3.4
Conditions							
Column:	ODS 5 μm Ultrasphere, 250 × 2.0 mm			ODS 5 μm Ultrasphere, 250 × 4.6 mm			
Flow-rate:	0.3 ml/min			1.5 ml/min			
Mobile phase:	0.0025 *M* orthophosphoric acid—acetonitrile (69:31)			0.0025 *M* orthophosphoric acid—acetonitrile (67.2:32.8)			
Detection:	UV 190 nm, 5 sec rise time peak height (mm) normalized to 0.01 a.u.f.s.			UV 190 nm, 5 sec rise time peak height (mm) normalized to 0.01 a.u.f.s.			
Recorder:	1 mV f.s.d.			1 mV f.s.d.			
Sample loop:	10 μl			10 μl			
Concentration:	25 pg/μl—25 ng/μl			0.1—25 ng/μl			

the narrow-bore HPLC separation is illustrated by the chromatograms in Fig. 2. Only 2 ng of each eicosanoid were injected onto the narrow-bore column compared to 8 ng on the conventional-bore column. Both mobile phases were of optimal solvent strength, with all other conditions, except flow-rate, constant.

Biological application

Having realized the improvement in sensitivity using a narrow-bore column, the separation was applied to biological samples. Because of the end absorption of many substances at 190 nm, sample clean-up was necessitated. Purification by LH 20 chromatography, after acidic organic extraction, offers multiple separation mechanisms by solvent alterations [29]. Since impurities in the extract also show a high distribution coefficient for ethyl acetate, separation by partition chromatography is seen by using the chloroform—ethyl acetate mixture with a chloroform-rich gel as the stationary phase and ethyl acetate as the mobile phase. Using chloroform alone, the structural characteristics of eicosanoids are exploited by absorption of their hydroxyl and carboxyl groups, by hydrogen bonding, to the glucose hydroxyls within the LH-20 gel. Therefore, some of the coextracted impurities, having affinity for the chloroform, are removed. Gel permeation with methanol alone as the mobile phase, elutes the eicosanoids by their molecular weight.

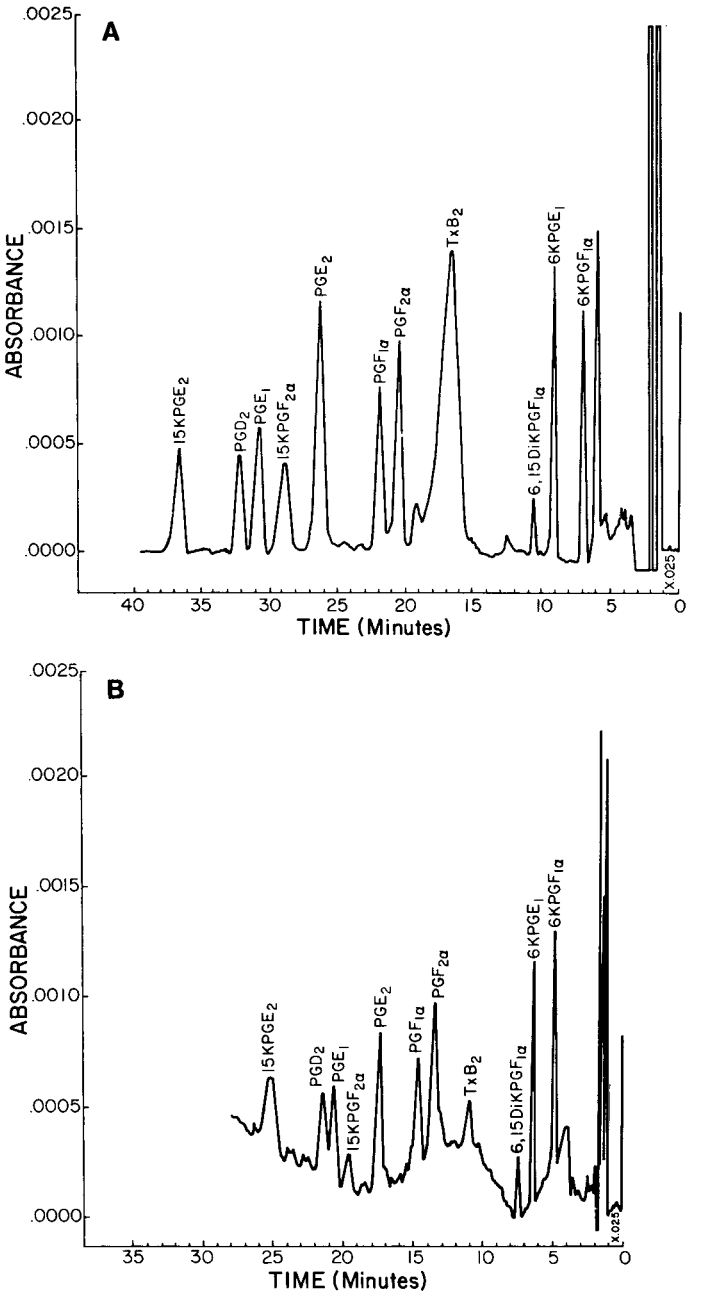


Fig. 2. Chromatograms of eicosanoid standards. (A) Column: 250 × 2.0 mm Ultrasphere ODS; flow-rate: 0.3 ml/min; mobile phase: 0.0025 *M* orthophosphoric acid—acetonitrile (69:31); amount: 2 ng of each eicosanoid. (B) Column: 250 × 4.6 mm Ultrasphere ODS; flow-rate: 1.5 ml/min; mobile phase: 0.0025 *M* orthophosphoric acid—acetonitrile (67.2:32.8); amount: 8 ng of each eicosanoid. Detection: UV at 190 nm, 0.025 a.u.f.s.; recorder: 1 mV.

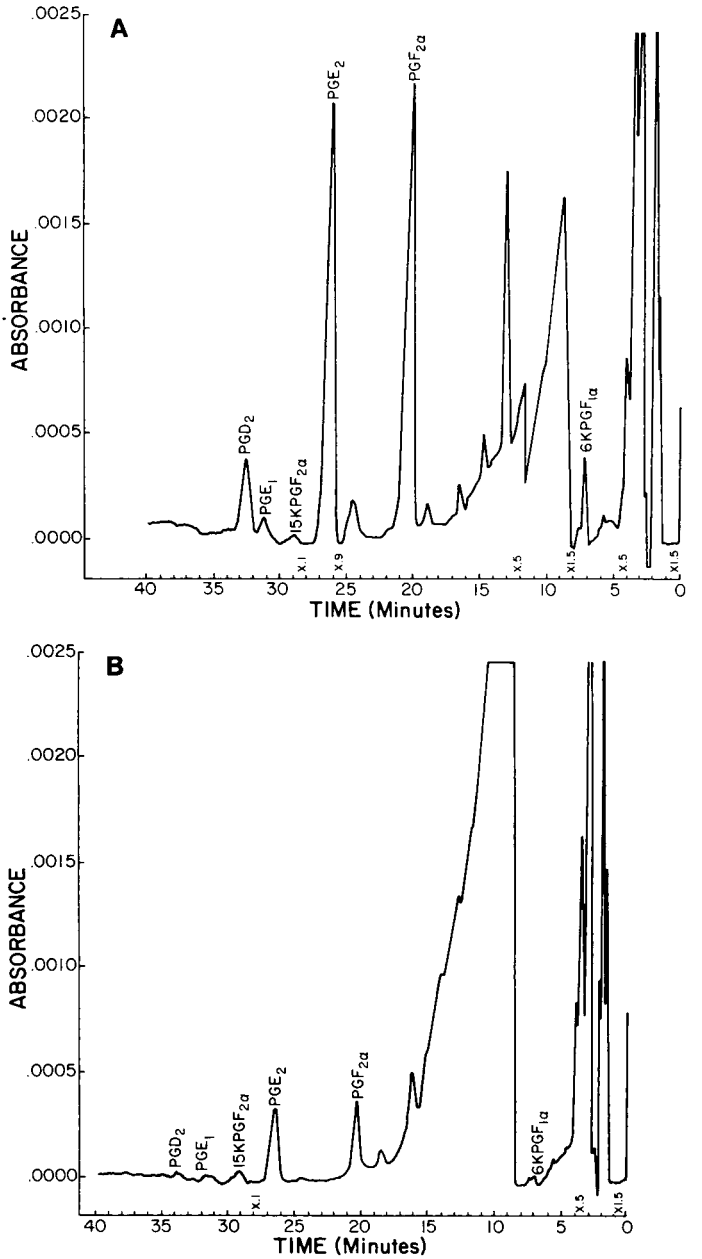


Fig. 3. Chromatogram of kidney medulla extract using narrow-bore HPLC. (A) Control: 56 mg tissue (wet wt.); (B) meclofenamic acid: 1 mg/ml, 67 mg tissue (wet wt.). Conditions: same as Fig. 2A.

Fig. 3. shows the UV absorbance of eicosanoids produced by approximately 60 mg of kidney medulla tissue. When the same sample incubate was injected at twice the concentration, the UV absorbance peaks corresponding to the eicosanoids, doubled. The kidney medulla was selected because of the wide variety

of eicosanoids produced, its pivotal role in maintaining physiological homeostasis, as well as its function in the elimination of potentially toxic substances. As illustrated in Fig. 3A, several eicosanoids were readily detected. Meclofenamic acid, a cyclo-oxygenase inhibitor, showed a marked reduction of eico-

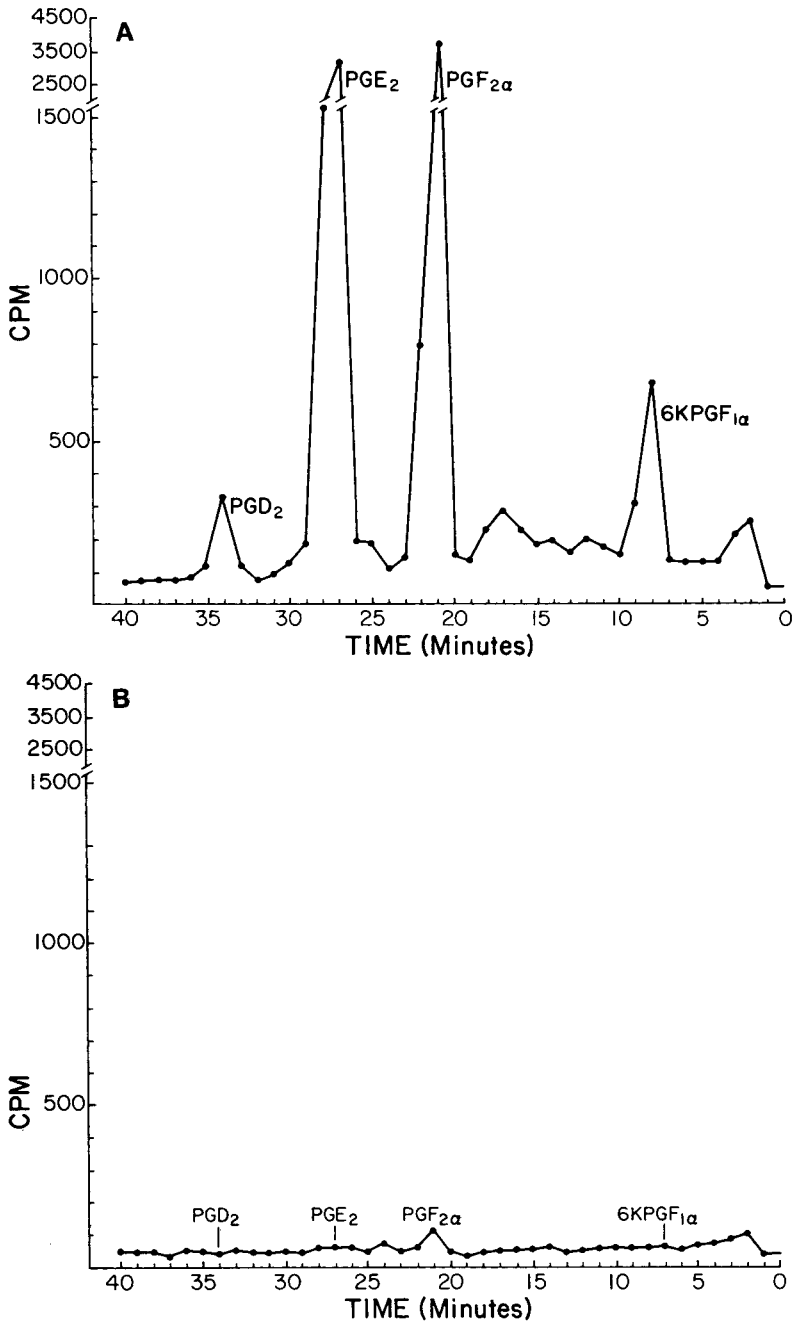


Fig. 4. Radioactivity patterns of HPLC effluent from Fig. 3. (A) Control; (B) meclofenamic acid.

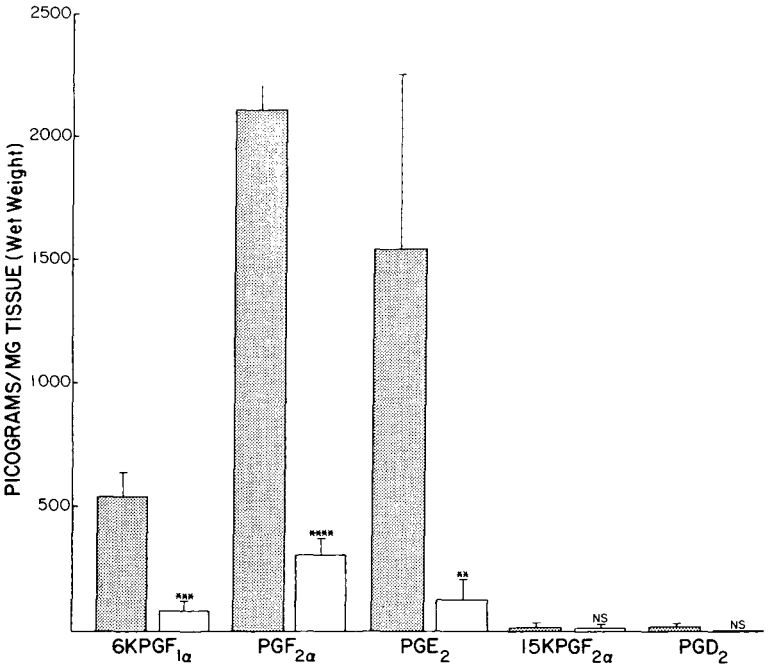


Fig. 5. Pattern of eicosanoids produced by kidney medulla, absorbance quantitation. Mean pg/mg tissue wet weight ± S.E.M. (▫) Control (n=5); (▪) meclofenamic acid (n=3). (****) $p<0.005$, (***) $p<0.010$, (**) $p<0.050$, (NS) $p>0.10$.

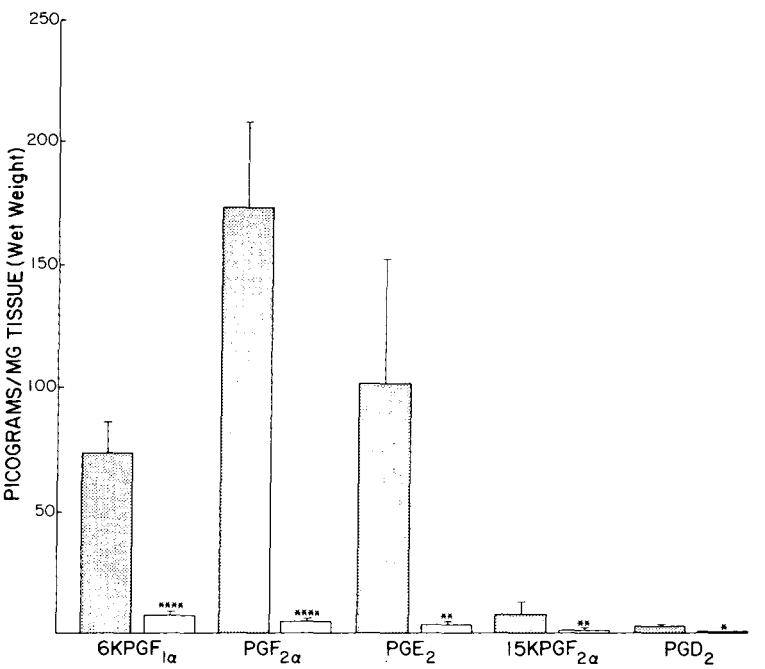


Fig. 6. Pattern of eicosanoids produced by kidney medulla as determined by radioconversion of [1-^{14}C]arachidonic acid. Mean pg/mg tissue wet weight ± S.E.M. (▫) Control (n=5), (▪) meclofenamic acid (n=3). (****) $p<0.005$, (**) $p<0.050$, (*) $p<0.10$.

sanoids produced (Fig. 3B). Correlation by conversion of [1-^{14}C] AA into radiolabeled products collected from the HPLC effluent and counted shows a similar production pattern, Fig. 4, with the radioactivity coincident with the absorbance peak.

A one-tailed t=statistic (n=8) showed a significant reduction in eicosanoid levels for both absorbance and radioactive quantitiation after meclofenamic acid, respectively (Figs. 5 and 6). The radioconversion pattern of eicosanoids produced by the kidney medulla is typical of that shown by others [30] as well as being similar to the absorbance detection profile. An approximate ten-fold increase in eicosanoid production is given by absorbance quantitation, reflecting the endogenous release of arachidonic acid by phospholipase A_2, the rate limiting step, as well as that produced by the addition of [1-^{14}C)AA.

CONCLUSIONS

This study demonstrates a method for detecting picogram quantities of eicosanoids using narrow-bore HPLC. Application of this method to kidney medulla tissue shows several chromatographic peaks, although not structurally elucidated by mass spectrometry, identified as eicosanoids by: (1) retention times identical to standard eicosanoids; (2) absorbance, i.e. peak height, proportional to concentration; (3) radioactivity in the HPLC effluent of the absorbance peak; (4) inhibition by a cyclo-oxygenase inhibitor, meclofenamic acid.

ACKNOWLEDGEMENTS

We are grateful to Mrs. Judy Bates for preparation of this manuscript. Our gratitude is also expressed to Drs. John Pike of Upjohn and Roger Westland of Warner-Lambert for their generous gifts of eicosanoids and meclofenamic acid, respectively. This work was supported by BRSG S07 RR-7025-17, awarded by the Biomedical Research Support Grant Program, Division of Research Resources, NIH.

REFERENCES

1 W.P. King and P.T. Kissinger, Clin. Chem., 26 (1980) 1484.
2 S. Ikenoya, O. Hiroshima, M. Ohmae and K. Kawabe, Chem. Pharm. Bull., 28 (1980) 2941.
3 W. Morozowich and S.L. Douglas, Prostaglandins, 10 (1975) 19.
4 M.V. Merritt and G.E. Bronson, Anal. Chem., 48 (1976) 1851.
5 M.V. Merritt and G.E. Bronson, Anal. Chem., 80 (1977) 392.
6 W.D. Watkins and M.B. Peterson, Anal. Biochem., 125 (1982) 30.
7 M. Hatsumi, S. Kimata and K. Hirosawa, J. Chromatogr., 253 (1982) 271.
8 K. Yamada, M. Onodera and Y. Aizawa, J. Pharm. Methods, 9 (1983) 93.
9 J. Turk, S.J. Weiss, J.E. Davis and P. Needleman, Prostaglandins, 16 (1978) 291.
10 D.M. Desiderio, M.D. Cunningham and J.A. Trimble, J. Liquid Chromatogr., 4 (1981) 1261.
11 A. Terragno, R. Rydzik and N.A. Terragno, Prostaglandins, 21 (1981) 101.
12 G.T. Hill, J. Chromatogr., 176 (1979) 407.
13 K. Nagayo and N. Mizuno, J. Chromatogr., 178 (1979) 347.

14 M.A. Wynalda, F.H. Lincoln and F.A. Fitzpatrick, J. Chromatogr., 176 (1979) 413.
15 M. VanRollins, S.H.K. Ho, J.E. Greenwald, M.S. Alexander, N.J. Dorman, L.K. Wong and L.A. Horrocks, Prostaglandins, 20 (1980) 571.
16 T. Eling, B. Tainer, A. Ally and R. Warnock, in W.M. Lands and W.L. Smith (Editors), Methods in Enzymology 86, Academic Press, New York, 1982, p. 511.
17 R. Rydzik, N.A. Terragno, R.L. Tackett and A. Terragno, submitted for publication.
18 L. Levine and I. Alam, Prostaglandin Med., 3 (1979) 295.
19 I. Alam, K. Ohuchi and L. Levine, Anal. Biochem., 93 (1979) 339.
20 A.R. Whorton, K. Carr, M. Smigel, L. Walker, K. Ellis and J.A. Oates, J. Chromatogr., 163 (1979) 64.
21 F.A. Russell and D. Deykin, Prostaglandins, 18 (1979) 11.
22 F.F. Sun and B.M. Taylor, Biochemistry, 17 (1978) 4096.
23 M. Ghias-Ud-Din, E.B. Olson and J. Rankin, J. Chromatogr., 192 (1980) 463.
24 K. Carr, B.J. Sweetman and J.C. Frolich, Prostaglandins, 11 (1976) 3.
25 R. Rydzik, N.A. Terragno, R.L. Tackett and A. Terragno, submitted for publication.
26 F.J. Yang, J. High Resolut. Chromatogr. Chromatogr. Commun., 6 (1983) 348.
27 J. Bowermaster and H.M. McNair, LC Magazine, 1 (1983) 362.
28 D.A. Terragno, K. Crowshaw, N.A. Terragno and J.C. McGiff, Circ. Res., 36 (1975) I76.
29 L. Fischer, An Introduction to Gel Chromatography, Elsevier-North Holland Biomedical Press, New York, 1969, p. 200.
30 N.A. Terragno, A. Terragno, J.A. Early, M.A. Roberts and J.C. McGiff, Clin. Sci. Mol. Med., 55 (1978) 199s.

Journal of Chromatography, 308 (1984) 43—53
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2081

AN INTEGRATED SCHEME FOR THE SIMULTANEOUS DETERMINATION OF BIOGENIC AMINES, PRECURSOR AMINO ACIDS, AND RELATED METABOLITES BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

KAZUHIRO OKA*, KOHICHI KOJIMA, AKIFUMI TOGARI and TOSHIHARU NAGATSU*

Laboratory of Cell Physiology, Department of Life Chemistry, Graduate School at Nagatsuta, Tokyo Institute of Technology, Yokohama 227 (Japan)

and

BELA KISS

Medical Division, Department of Biochemistry, Chemical Works of Gedeon Richter, 1475 Budapest 10 (Hungary)

(Received December 28th, 1983)

SUMMARY

A new method using high-performance liquid chromatography with electrochemical detection (HPLC—ED) for the simultaneous determination of monoamines, their precursor amino acids, and related major metabolites in small samples of brain tissue weighing from 0.5 to 50 mg is described. The method is based on the preliminary isolation of monoamines (dopamine, norepinephrine, epinephrine, and serotonin), their precursor amino acids (tyrosine, 3,4-dihydroxyphenylalanine, tryptophan and 5-hydroxytryptophan), and their major metabolites (3-methoxytyramine, normetanephrine, 3,4-dihydroxyphenylacetic acid, homovanillic acid, vanillylmandelic acid, 3-methoxy-4-hydroxyphenylethyleneglycol, and 5-hydroxyindoleacetic acid) by chromatography on small columns of Amberlite CG-50 and Dowex 50W, and by ethyl acetate extraction. All the compounds in the four isolated fractions were measured by HPLC—ED on a reversed-phase column under four different conditions. The sensitivity was from 0.1 to 40 pmol, depending on the substances analysed. This newly established method was applied to the study of the effects of an aromatic L-amino acid decarboxylase inhibitor (NSD-1015) and a monoamine oxidase inhibitor (pargyline) on the levels of monoamines, their precursor amino acids and their major metabolites in brain regions of mice.

*Present address: Roche Institute of Molecular Biology, Nutley, NJ 07110, U.S.A.

0378-4347/84/$03.00

INTRODUCTION

High-performance liquid chromatography (HPLC) with electrochemical detection (ED) [1—3] has proved to be a sensitive and inexpensive method for measuring catecholamines, indoleamines and their precursor amino acids and related metabolites in tissues and body fluids [4—21]. However, the simultaneous determination of all of the monoamines, their metabolites, and their amino acid precursors has not been reported.

Although HPLC—ED is highly sensitive, it is relatively non-specific and without prior isolation procedures gives rise to complex chromatograms. Therefore, the identification of each peak is sometimes difficult [10, 20, 21]. Most procedures for the HPLC—ED analysis of biogenic amines include a preliminary separation of compounds by alumina adsorption, ion-exchange or Sephadex G-10 columns [2, 6—8] to make the assay more specific.

In this report, we describe a new HPLC—ED assay for the simultaneous determination of all the monoamines, their metabolites and precursor amino acids [dopamine (DA), norepinephrine (NE), epinephrine (E), serotonin (5-HT), tyrosine (TYR), 3,4-dihydroxyphenylalanine (DOPA), tryptophan (TRP), 5-hydroxytryptophan (5-HTP), 3-methoxytyramine (3-MT), normetanephrine (NMN), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), vanillylmandelic acid (VMA), 5-hydroxyindoleacetic acid (5-HIAA), and 3-methoxy-4-hydroxyphenylethyleneglycol (MOPEG)] based on their prior isolation by Amberlite CG-50 and Dowex 50 columns and by ethyl acetate extraction, using six internal standards [N-methyldopamine (NMDA), α-methyl-DOPA (MDOPA), α-methyl-*p*-tyrosine (MTYR), α-methyltryptophan (MTP), α-methyl-*p*-hydroxytryptophan (MHTP), and 3,4-dihydroxycinnamic acid (DHCA)]. Application of the method to pharmacological studies is also described.

MATERIALS AND METHODS

Materials

The following drugs or chemicals were used: HVA (Calbiochem, La Jolla, CA, U.S.A.); L-TYR, L-DOPA, DL-MDOPA, L-MTYR, L-TRP, MTRP, L-5-HTP, L-NE, L-E bitartrate, DA · HCl, 5-HT creatinine sulphate, NMN · HCl, 3-MT · HCl, NMDA, DOPAC, 5-HIAA dicyclohexylammonium salt, MOPEG piperazine salt, and pargyline, *m*-hydroxybenzylhydrazine (NSD-1015) (Sigma, St. Louis, MO, U.S.A.); and VMA and DHCA (Tokyo Kasei, Tokyo, Japan). MHTP was a kind gift from Dr. J. Daly (NIH, Bethesda, MD, U.S.A.). Other chemicals were of analytical grade.

Animal experiments

All experiments were carried out on male DDY mice weighing about 20 g (six weeks of age). They were housed five per cage with food and water ad libitum and under a normal dark and light rhythm. Animals were sacrificed between 10:30 a.m. and 12:00 a.m. to avoid possible changes caused by circadian rhythm in the levels of biogenic amines, their metabolites and precursor amino acids, and in their biosynthesis rate. NSD-1015 and pargyline

were dissolved in saline, and all injections were given intraperitoneally (i.p.) in a volume of 1 ml per 100 g body weight.

Mice were killed by cervical dislocation; the brains were removed within 30 sec, and were immediately dropped into ice-cold saline. The brains were dissected on a glass plate over ice into ten regions by the method of Carlsson and Lindqvist [22] with modifications [23]. Tissue samples after dissection were frozen on dry ice and analysed immediately or stored at −80°C until analysis.

Tissue samples (0.5—50 mg) were weighed and homogenized by a tissue sonicator (Ohtake Works, Tokyo, Japan) with 1.0 ml of ice-cold 0.32 *M* sucrose. An aliquot of the homogenate can be used both for the analysis of monoamines, their metabolites and precursor amino acids and for the enzymatic analysis of monoamine-related enzymes. When the latter enzyme analysis was not necessary, homogenization was carried out with 0.8 ml of the perchloric acid solution described below.

Preparation of Amberlite CG-50 and Dowex 50 columns

Amberlite CG-50 (type II, 200—400 mesh) and Dowex 50W (200—400 mesh) were washed with water and then by cycling through sodium and acid forms with 2 *M* sodium hydroxide and 2 *M* hydrochloric acid and finally with distilled water. A column of Amberlite CG-50 (1 cm × 0.4 cm I.D.) was washed with 3 ml of 0.1 *M* sodium phosphate buffer (pH 6.5) containing 0.1% Na_2EDTA and 3 ml of water [24]. A column of Dowex 50 W (H^+) (1.8 cm × 0.4 cm I.D.) was also prepared.

Both Amberlite and Dowex columns can be regenerated after use by washing them with water, 3 ml of 2 *M* NaOH—1% Na_2EDTA, 5 ml of water, 3 ml of 2 *M* hydrochloric acid and 5 ml of water.

Isolation of monoamines, their precursor amino acids, and metabolites

The isolation of monoamines, their metabolites and precursor amino acids on Amberlite CG-50 and Dowex 50 columns was carried out according to the method of Kiss et al. as modified by Oka et al. [23]. Freshly prepared 1.0 ml of ice-cold 0.4 *M* perchloric acid containing 0.25% Na_2EDTA, 0.5% $Na_2S_2O_5$, and internal standards (NMDA, MDOPA, MTYR, MTRP, MHTP, and DHCA) were added to 0.5 ml of the homogenate. After standing for 15 min in an ice bath, samples were centrifuged at 20,000 *g* for 15 min at 4°C. Supernatants were transferred to test tubes containing 0.1 ml of 0.5 *M* sodium phosphate buffer (pH 6.5), and 0.2 ml of 1 *M* potassium carbonate was added to adjust the pH to 6.1—6.3; the mixtures were frozen at −80°C. After thawing, extracts were centrifuged at 1600 *g* for 10 min at 4°C and the supernatants were poured onto Amberlite CG-50 columns (1 cm × 0.4 cm I.D.). After the samples had passed through, the columns were washed successively with 1 ml of 0.01 *M* sodium phosphate buffer (pH 6.5) containing 0.1% Na_2EDTA, 1 ml of water and 0.1 ml of 0.5 *M* hydrochloric acid. Column effluents and washings were collected in tubes containing 0.1 ml of 1 *M* phosphoric acid and 0.05 ml of 2 *M* hydrochloric acid and carefully mixed. Monoamines (NE, E, DA, 3-MT, 5-HT, and NMDA) were eluted with 0.4 ml of 0.5 *M* hydrochloric acid from the Amberlite CG-50 columns, and their HPLC—ED analysis (System 1) was performed immediately. The effluents plus washings from the Amberlite

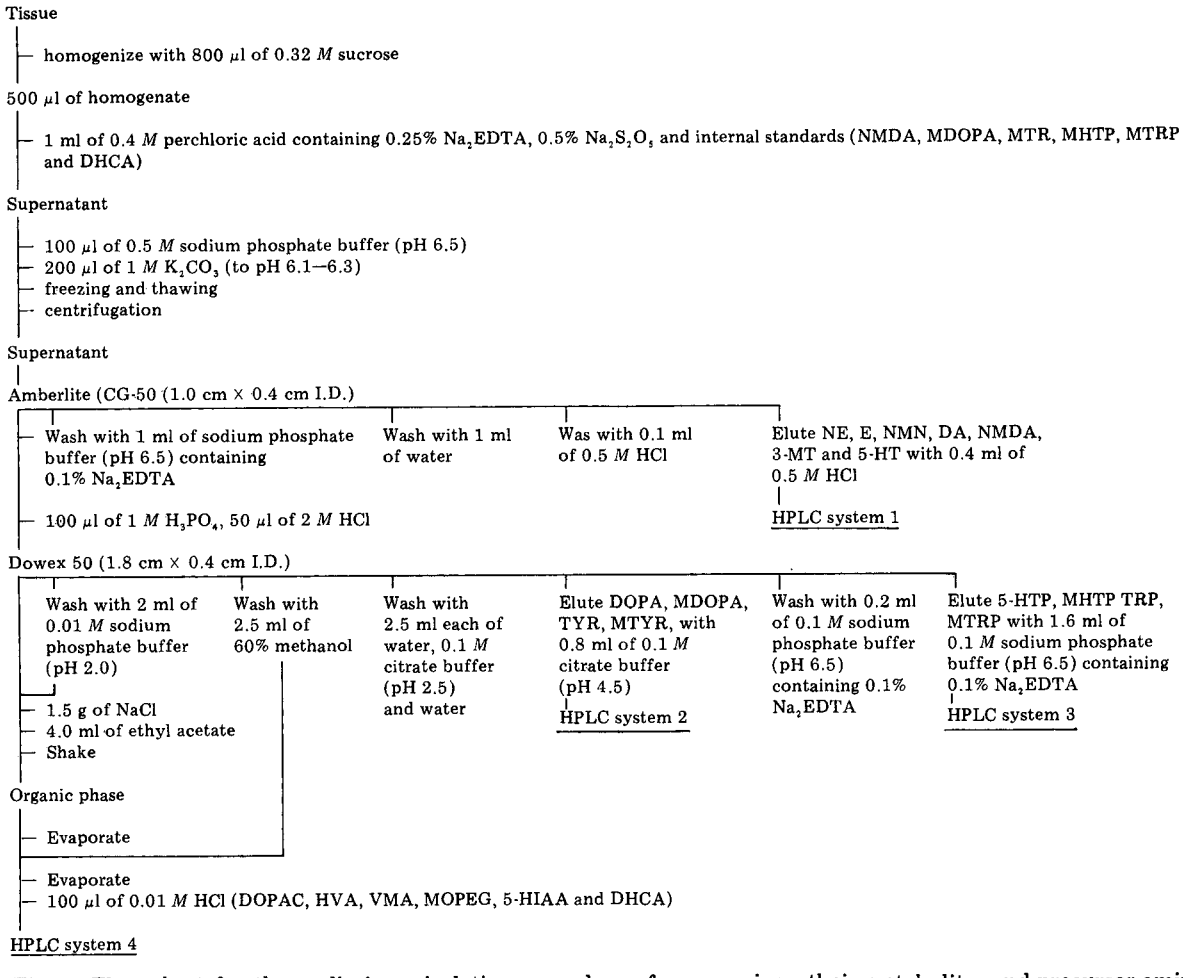


Fig. 1. Flow-chart for the preliminary isolation procedure of monoamines, their metabolites and precursor amino acids.

CG-50 columns were applied to Dowex 50 columns (1.8 cm × 0.4 cm I.D.). After the samples had passed through, the columns were washed with 1 ml of 0.01 *M* sodium phosphate buffer (pH 2.0), and with 2.5 ml of 60% methanol. The column effluents plus buffer washings, and 60% methanol washings were collected separately in different tubes. Then the columns were washed successively with 2.5 ml of water, 2.5 ml of 0.1 *M* citrate phosphate buffer (pH 2.5), and 2.5 ml of water. DOPA, MDOPA, and TYR were eluted with 0.8 ml of 0.1 *M* citrate—sodium hydroxide buffer (pH 4.5). The columns were washed with 0.2 ml of 0.1 *M* sodium phosphate buffer (pH 6.5) containing 0.1% Na_2EDTA, and then 5-HTP, MHTP, TRP and MTRP were eluted with 1.6 ml of the same buffer. The effluent plus buffer washing were saturated with 1.5 g of sodium chloride and extracted with 4.0 ml of ethyl acetate with vigorous shaking. The organic phase was collected and evaporated. Previously saved 2.5 ml of 60% methanol washing was added to the same test tube and then evaporated to dryness. This fraction, which contained VMA, DOPAC, MOPEG, 5-HIAA, HVA and DHCA, was dissolved with 0.1 ml of 0.01 *M* hydrochloric acid before HPLC—ED analysis. The isolation procedure is summarized in Fig. 1.

Apparatus

The chromatograph used was a Yanaco L-2000 with a Yanaco VMD-101 electrochemical detector, a two-pen recorder, and a column (25 cm × 0.4 cm I.D.) packed with Nucleosil 7 C_{18} (Macherey-Nagel, Düren, F.R.G.) using a slurry column packing apparatus Model 124 (Chemco Scientific, Osaka, Japan) and a column packer (Umetani Seisakusho, Osaka, Japan). For column packing, 2.5 g of reversed-phase packing material in 3.8 ml of methanol containing 0.1% ammonium acetate, 3.8 ml of acetonitrile, 17.3 ml of carbon tetrachloride and 5.3 ml of slurry solvent C (Macherey-Nagel) were pumped into an empty column, the back-pressure being maintained at 550 kg/cm^2 with methanol containing 0.1% ammonium acetate. The pumping continued with 100 ml of methanol, and then the pressure was reduced during 15 min [25]. The column was washed with 200 ml of 50% methanol, and with water prior to use in order to obtain a low background signal for electrochemical detection.

HPLC analysis

The following analytical systems were used. System 1 for monoamines: 0.1 *M* sodium phosphate buffer (pH 2.2) as the mobile phase with a flow-rate of 0.74 ml/min and the detector potential at 0.65 V against Ag/AgCl electrode. System 2 for DOPA and TYR: 0.1 *M* citrate—sodium hydroxide buffer (pH 3.0) as the mobile phase with a flow-rate of 0.74 ml/min at 0.73 V. System 3 for TRP and 5-HTP: 0.1 *M* sodium phosphate buffer (pH 6.5)—acetonitrile (96:4, v/v) as the mobile phase with a flow-rate of 0.74 ml/min at 0.55 V. System 4 for metabolites: 0.1 *M* sodium acetate buffer (pH 5.2) as the mobile phase with a flow-rate of 1.0 ml/min at 0.6 V.

All mobile phases were freshly prepared from 0.5 *M* stock solution and degassed before use.

Calculations

The concentration of each compound in brain tissue was calculated from the

ratio of peak height (peak height of each compound/peak height of internal standard). The calibration factor was determined from the peak height ratio of each known amount of the standard which was carried through the whole extraction procedure with internal standards.

RESULTS

Fig. 2A shows chromatograms of a mixture of standards of NE, E, NMN, DA, NMDA (internal standard), 3-MT and 5-HT. The separation of monoamine standards on a reversed-phase column was complete. Isolation of monoamines from the midbrain was also complete as shown in Fig. 2B (a saline-treated mouse) and in Fig. 2C (mouse treated with pargyline, a monoamine oxidase inhibitor). Marked increases in the peaks of NE, NMN, DA, 3-MT, and 5-HT were observed in the pargyline-treated mouse. No detectable interfering substances were observed in the chromatograms. The limit of sensitivity for standard monoamines was about 0.1 pmol, and that for NMN and 3-MT was about 0.2 pmol. Since a detector potential of 0.65 V is not maximal for NMN and 3-MT, the sensitivity for NMN and 3-MT depends on the applied potential for electrochemical detection. Linearity was satisfactory up to 5 nmol. NMDA was used as an internal standard for this fraction because its elution profile from an Amberlite CG-50 column was similar to that of each monoamine, and its separation by HPLC was complete.

The chromatographic separation of the fraction containing DOPA and TYR

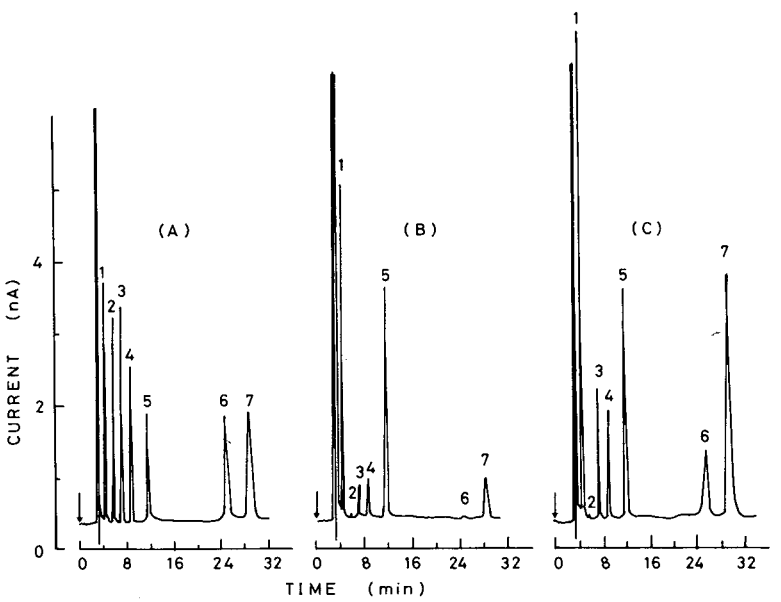


Fig. 2. Chromatograms of the monoamine fractions. (A) A mixture of standards: 5 pmol each of NE, E, DA and NMDA, and 10 pmol each of NMN, 3-MT and 5-HT. (B) The extract from 44 mg of midbrain from a saline-treated mouse (i.p., 60 min), and (C) the extract from 36 mg of midbrain from a pargyline-treated mouse (75 mg/kg, i.p., 60 min) were subjected to the whole procedure. Peaks: 1 = NE; 2 = E; 3 = NMN; 4 = DA; 5 = NMDA (internal standard); 6 = 3-MT; and 7 = 5-HT. HPLC conditions are described in Materials and methods.

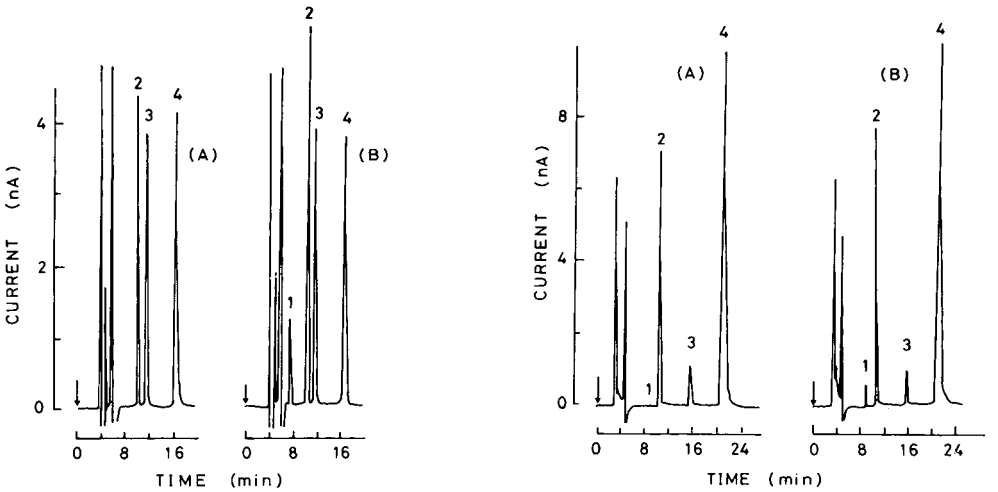


Fig. 3. Chromatograms of DOPA and TYR fractions. The extract from each 39 mg of midbrain of (A) a saline-treated mouse (i.p., 30 min), and of (B) an NSD-1015-treated mouse (100 mg/kg, i.p., 30 min), were subjected to the complete extraction procedure. HPLC conditions are described in Materials and methods. Peaks: 1 = DOPA; 2 = TYR; 3 = MDOPA (internal standard); and 4 = MTYR (internal standard).

Fig. 4. Chromatograms of 5-HTP and TRP fractions. Each 6 mg of hypothalamus from (A) a saline-treated mouse (i.p., 30 min) and from (B) an NSD-1015-treated mouse (100 mg/kg, i.p., 30 min) were carried through the whole extraction procedure. HPLC conditions are described in Materials and methods. Peaks: 1 = 5-HTP; 2 = MHTP (internal standard); 3 = TRP; and 4 = MTRP (internal standard).

from the midbrain is shown in Fig. 3. The fraction from saline-treated animals did not show a detectable peak of DOPA (Fig. 3A), but the extract from the midbrain of a mouse treated with an aromatic L-amino acid decarboxylase inhibitor (NSD-1015) showed a marked DOPA accumulation as indicated in Fig. 3B. We used 0.73 V as an applied oxidation potential. At higher oxidation potential the TYR peak was increased but interfered with the peak of MDOPA, because of high TYR concentration in tissues. At the oxidation potential of 0.73 V, the sensitivity of DOPA was 1 pmol, that of TYR 10 pmol.

Fig. 4 shows the chromatographic separation of the fraction containing 5-HTP and TRP. The chromatogram of the extract from the hypothalamus of a saline-treated animal (Fig. 4A) shows a trace peak of 5-HTP. Fig. 4B is the chromatogram of the extract from the hypothalamus of a mouse treated with a DOPA decarboxylase inhibitor (NSD-1015), and the peak of 5-HTP was observed. Both 5-HTP and TRP could be measured accurately based on two different internal standards, MHTP and MTRP, because MHTP and MTRP had similar chemical properties on a Dowex 50 column and similar oxidation potentials to 5-HTP and TRP, respectively. The sensitivities for 5-HTP and TRP were 1 pmol and 40 pmol, respectively.

Fig. 5A illustrates the separation of a mixture of standards of VMA, DOPAC, MOPEG, 5-HIAA, HVA and DHCA (internal standard). The sensitivity for DHCA was lower than that for other metabolites. However, DHCA proved to

be a suitable internal standard because the separation from the other compounds on a reversed-phase column was complete. Fig. 5B shows the chromatogram of the extract from the midbrain of saline-treated mouse. Clear peaks of DOPAC, MOPEG, 5-HIAA and HVA were observed, but the peak of VMA was not detected. The sensitivity of each metabolite was about 1 pmol.

In order to demonstrate the specificity and the utility of this assay, we investigated the levels of all monoamines and related compounds that can be measured by our newly established method, after pharmacological manipulations, i.e. administration of an aromatic L-amino acid decarboxylase inhibitor (NSD-1015) and of a monoamine oxidase inhibitor (pargyline). The effects on the levels of biogenic amines, their precursor amino acids and major metabolites in the striatum, hypothalamus, and pons—medulla oblongata are shown in Table I. Increases of NE, NMN, DA, 3-MT, 5-HT and decreases of DOPAC, MOPEG, 5-HIAA and HVA were observed in the tissues from monoamine oxidase inhibitor (pargyline) treated animals. As indicated in Table I, NSD-1015 inhibited aromatic L-amino acid decarboxylase and caused DOPA and 5-HTP accumulations in each brain region which give the in vivo activity of tyrosine and tryptophan hydroxylases. However, NSD-1015 also had an inhibitory effect on monoamine oxidase, and incrased monoamine levels and decreased their metabolite levels.

TABLE I

EFFECTS OF INHIBITORS OF AROMATIC L-AMINO ACID DECARBOXYLASE (NSD-1015) AND MONOAMINE OXIDASE (PARGYLINE) ON THE LEVELS OF BIOGENIC AMINES, PRECURSOR AMINO ACIDS AND THEIR METABOLITES IN DIFFERENT BRAIN REGIONS OF MICE

Values are expressed in nmol/g wet tissue (mean ± S.E.M. from five animals). Treatments: saline, i.p., 60 min; NSD-1015, 100 mg/kg, i.p., 30 min; and pargyline, 75 mg/kg, i.p., 60 min.

Compound	Striatum			Hypothalamus
	Saline	NSD-1015	Pargyline	Saline
NE	1.16 ± 0.13	0.96 ± 0.04	1.82 ± 0.23	5.47 ± 0.51
E	0.11 ± 0.01	0.11 ± 0.01	0.13 ± 0.01	0.42 ± 0.06
NMN	0.15 ± 0.03	0.22 ± 0.03	0.69 ± 0.04 §	0.58 ± 0.04
DA	29.4 ± 2.79	27.5 ± 2.21	36.5 ± 1.76 §	0.62 ± 0.05
3-MT	2.42 ± 0.21	3.45 ± 0.26**	6.55 ± 0.27 §	n.d. § §
5-HT	1.21 ± 0.12	0.94 ± 0.16	2.30 ± 0.49**	n.d.
DOPA	n.d.	4.03 ± 0.23 §	n.d.	n.d.
TYR	68.3 ± 4.0	103 ± 2.2 §	57.8 ± 1.7	76.7 ± 4.5
5-HTP	0.06 ± 0.00	0.61 ± 0.03 §	n.d.	n.d.
TRP	31.1 ± 2.8	28.0 ± 2.7	27.8 ± 2.0	27.3 ± 2.6
VMA	n.d.	n.d.	n.d.	n.d.
DOPAC	14.8 ± 1.68	7.54 ± 0.36***	2.59 ± 0.34 §	1.96 ± 0.15
MOPEG	0.23 ± 0.01	n.d.	n.d.	0.46 ± 0.06
5-HIAA	1.58 ± 0.18	0.91 ± 0.05**	0.54 ± 0.04*	1.27 ± 0.19
HVA	5.71 ± 0.83	4.52 ± 0.21	1.59 ± 0.26 §	1.04 ± 0.09

*$P < 0.05$.
**$P < 0.02$.
***$P < 0.01$.
§$P < 0.001$.
§ § n.d. = not detectable.

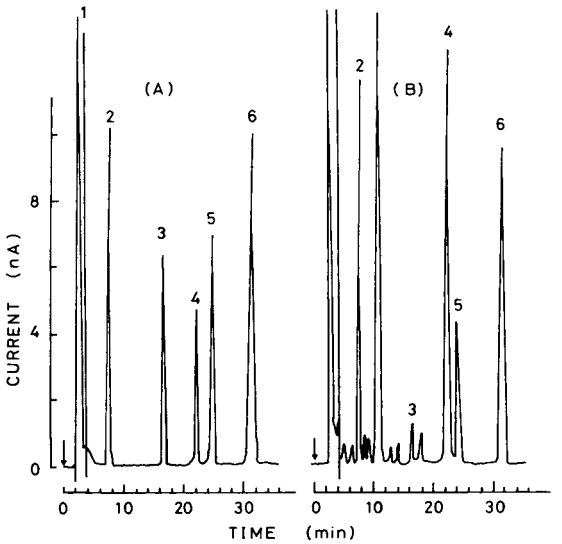


Fig. 5. Chromatograms of the fractions of monoamine metabolites. (A) A mixture of standards (20 pmol of VMA, 40 pmol each of DOPAC, MOPEG, and 5-HIAA, and 200 pmol of DHCA). (B) A tissue sample of 42 mg of midbrain from saline-treated mouse (i.p., 60 min) was subjected to the whole extraction procedure as described in Materials and methods. HPLC conditions are described in Materials and methods. Peaks: 1 = VMA; 2 = DOPAC; 3 = MOPEG; 4 = 5-HIAA; 5 = HVA; and 6 = DHCA (internal standard).

		Pons—medulla oblongata		
NSD-1015	Pargyline	Saline	NSD-1015	Pargyline
9.68 ± 0.35 §	13.2 ± 0.98 §	4.00 ± 0.14	4.24 ± 0.18	5.02 ± 0.34** §
0.41 ± 0.04	0.57 ± 0.05	0.18 ± 0.02	0.10 ± 0.01 §	0.10 ± 0.01 §
1.13 ± 0.14***	2.76 ± 0.15 §	0.80 ± 0.09	1.16 ± 0.16	1.60 ± 0.18***
0.86 ± 0.09*	2.10 ± 0.21 §	0.11 ± 0.01	0.06 ± 0.01**	0.21 ± 0.01 §
n.d.	0.82 ± 0.05	n.d.	n.d.	0.15 ± 0.04
n.d.	2.65 ± 0.47	n.d.	0.61 ± 0.08	0.80 ± 0.22
1.55 ± 0.20 §	n.d.	n.d.	0.56 ± 0.03 §	n.d.
124 ± 6.2 §	94.4 ± 7.1	58.7 ± 4.9	95.8 ± 4.9 §	44.1 ± 1.5*
2.89 ± 0.24 §	n.d.	n.d.	0.88 ± 0.03 §	n.d.
42.9 ± 2.6***	40.3 ± 3.5**	27.7 ± 4.0	38.7 ± 2.1*	26.7 ± 2.6
n.d.	n.d.	n.d.	n.d.	n.d.
2.48 ± 0.13***	1.28 ± 0.18*	0.91 ± 0.06	1.53 ± 0.14***	0.83 ± 0.10
0.49 ± 0.07	n.d.	0.32 ± 0.02	0.26 ± 0.03	n.d.
0.48 ± 0.15*	n.d.	0.83 ± 0.05	0.15 ± 0.02 §	n.d.
0.80 ± 0.04	n.d.	0.16 ± 0.02	0.12 ± 0.01	n.d.

DISCUSSION

This new assay for monoamines and their precursor amino acids and metabolites by HPLC—ED has several advantages.

First, the method permits the simultaneous assay of almost all the monoamines and related compounds. Many methods for the assay of monoamines and related compounds [4—21] have been reported. The method by Mayer and Shoup [19] measured eight compounds (DA, NE, 5-HT, HVA, DOPAC, MOPEG, 5-HIAA and TRP) without any prelimary isolation. The method of Ishikawa and McGaugh [15] uses primary butanol extraction before HPLC—ED and measured twelve compounds (DA, NE, 5-HT, MTYR, NMN, DOPAC, HVA, MOPEG, 5-HIAA, TYR, DOPA, and TRP). Kempf and Mandel [7] used alumina adsorption and diethyl ether extraction to measure nine compounds (DA, NE, 5-HT, MTYR, NMN, DOPAC, HVA, MOPEG, and 5-HIAA). However, our method can measure simultaneously all fourteen monoamines, and their metabolites and precursor amino acids from a single, small tissue sample.

Secondly, this method is highly specific. Wagner et al. [21] simultaneously determined DOPA, 5-HTP, DA, 3-MT, NE, DOPAC, HVA, 5-HT and 5-HIAA in brain tissues by direct injection of perchloric acid extract into the HPLC—ED system. Although direct injection without prior isolation is simple and rapid, the complex chromatogram causes difficulties in identification of compounds of interest and also low sensitivity. Since we used Amberlite CG-50 and Dowex 50 columns and ethyl acetate extraction as a preliminary isolation procedure before HPLC—ED, we obtained simple and clean chromatograms of each substance, and the identification of each compound is easy. The specificity of the method is further increased both by HPLC and by specific detection depending on the applied oxidation potential with ED.

Thirdly, this method is accurate, since proper internal standards are used. We corrected the recovery of each compound during the isolation by using six internal standards.

Fourthly, since the baseline is very stable due to preliminary cleaning of the tissue samples before HPLC—ED, this method is highly sensitive. Sensitivities for the monoamines, their precursor amino acids, and their derivatives are approximately 0.1 pmol, 10 pmol, and 1 pmol, respectively.

We also demonstrated that the present newly established HPLC—ED assay of monoamines and their related compounds is useful in pharmacological studies. We homogenized the tissues with sucrose or perchloric acid, and we could not observe significant differences between the two homogenizing media. A sucrose homogenate has advantages, because, as reported previously [23], the monoamines and their related compounds and the activities of monoamine-related enzymes can be measured simultaneously. In addition, it is also possible to compare in vivo enzyme activities in animals treated with an aromatic L-amino acid decarboxylase inhibitor, measured for example by the accumulation of DOPA (tyrosine hydroxylase activity) and 5-HTP (tryptophan hydroxylase activity), with the in vitro enzyme activities. This is important for the study of the regulation of tyrosine hydroxylase and tryptophan hydroxylase.

In conclusion, this new simultaneous HPLC—ED assay for biogenic mono-

amines and related compounds holds great promise for biochemical, physiological, and pharmacological studies.

REFERENCES

1 C. Refshauge, P.T. Kissinger, R. Dreiling, L. Blank, R. Freeman and R.N. Adams, Life Sci., 14 (1974) 311.
2 D.K. Koch and P.T. Kissinger, J. Chromatogr., 164 (1979) 441.
3 G.C. Davis, P.T. Kissinger and R.E. Shoup, Anal. Chem., 53 (1981) 156.
4 S. Sasa and C.L. Blank, Anal. Chem., 49 (1977) 354.
5 P. Hjemdahl, M. Daleskog and T. Kahan, Life Sci., 25 (1979) 131.
6 I.N. Mefford, M. Gilberg and J.D. Barchas, Anal. Biochem., 104 (1981) 469.
7 E. Kempf and P. Mandel, Anal. Biochem., 112 (1981) 223.
8 B.H.C. Westerink and T.B.A. Mulder, J. Neurochem., 36 (1981) 1449.
9 K. Fujita, K. Maruta, S. Ito and T. Nagatsu, Clin. Chem., 29 (1983) 876.
10 G.M. Anderson, J.G. Yong, D.K. Batter, S.N. Young, D.J. Cohen and B.A. Shaywitz, J. Chromatogr., 223 (1981) 315.
11 J.J. Warsh, A. Chiu and D.D. Godse, J. Chromatogr., 228 (1982) 131.
12 G. Sperk, J. Neurochem., 38 (1982) 840.
13 W.H. Lyness, Life Sci., 31 (1982) 1435.
14 A.M. Krstulovic, L. Bertani-Dziedzic, S. Bautista-Cerquerira and S.E. Gitlow, J. Chromatogr., 227 (1982) 379.
15 K. Ishikawa and J.L. McGaugh, J. Chromatogr., 229 (1982) 35.
16 M. Van Bockstaele, L. Dillen, M. Claeys and W.P. DePotter, J. Chromatogr., 275 (1983) 11.
17 W.E. Willson, S.W. Mietling and J.-S. Hong, J. Liquid Chromatogr., 6 (1983) 871.
18 W.A. Wolf and D.M. Kuhn, J. Chromatogr., 275 (1983) 1.
19 G.S. Mayer and R.E. Shoup, J. Chromatogr., 255 (1983) 533.
20 O. Magnusson, L.B. Nilsson and D. Westerlund, J. Chromatogr., 221 (1980) 237.
21 J.W. Wagner, P. Vitali, M.G. Palfreyman, M. Zraika and S. Huot, J. Neurochem., 38 (1982) 1241.
22 A. Carlsson and M. Lindqvist, J. Pharm. Pharmacol., 25 (1973) 437.
23 K. Oka, G. Ashiba, B. Kiss and T. Nagatsu, Neurochem. Int., 4 (1982) 375.
24 L. Atack and T. Magnusson, Acta Pharmacol. Toxicol., 42 (1978) 35.
25 T.J.N. Webber and E.H. McKerrell, J. Chromatogr., 122 (1976) 243.

24 L. Atack and T. Magnusson, Acta Pharmacol. Toxicol., 42 (1978) 35.
25 T.J.N. Webber and E.H. McKerrell, J. Chromatogr., 122 (1976) 243.

Journal of Chromatography, 308 (1984) 55—63
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2083

RAPID AND SIMPLE METHOD FOR THE SIMULTANEOUS DETERMINATION OF 3,4-DIHYDROXYPHENYLACETIC ACID, 5-HYDROXYINDOLE-3-ACETIC ACID AND 4-HYDROXY-3-METHOXYPHENYLACETIC ACID IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

A. MINEGISHI and T. ISHIZAKI*

Division of Clinical Pharmacology, Clinical Research Institute, National Medical Center, Toyama 1-21-2, Shinjuku-ku, Tokyo 162 (Japan)

(First received October 17th, 1983; revised manuscript received January 19th, 1984)

SUMMARY

A simple procedure for the simultaneous determination of major metabolites of dopamine and serotonin in plasma, i.e. 3,4-dihydroxyphenylacetic acid, 5-hydroxyindole-3-acetic acid, and 4-hydroxy-3-methoxyphenylacetic acid, was developed. The method is based on rapid isolation of the compounds by one-step clean-up on a small C_{18} column, followed by high-performance liquid chromatography with dual electrochemical detection. The system is readily used for clinical applications.

INTRODUCTION

Abnormalities of biogenic amine metabolism are implicated in various pathological states including psychiatric [1] and neurological disorders [2]. A number of investigations have concentrated on measurement of the metabolites of biogenic amines in biological fluids, particularly cerebrospinal fluid (CSF) and urine. This seems to be helpful for the diagnosis and interpretation of the disorders. Unexpectedly, information on plasma monoamines and their metabolites, except for catecholamines [3, 4], is scanty. The plasma concentrations of 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), major metabolites of dopamine [5], have been suggested to reflect changes in the brain dopaminergic system [6]. 5-Hydroxyindoleacetic acid (5-HIAA), another acidic compound, is a major metabolite of serotonin which is known as a neurotransmitter and vasoconstrictor. Interaction between the dopaminergic

0378-4347/84/$03.00

and serotonergic systems has been suggested [7]. Modifications of these plasma metabolites have been documented in hepatic encephalopathy [8] and psychoses [9]. However, to our knowledge, no method for the simultaneous determination of DOPAC, HVA and 5-HIAA in blood appears to have been available.

The development of high-performance liquid chromatography (HPLC) with electrochemical detection (ED) facilitated the establishment of highly sensitive and selective assay procedures for these metabolites. Under certain conditions, direct injection of the deproteinized brain tissue [10], CSF [11], or urine [12] into the HPLC system has been reported to permit a clear separation and detection of these compounds, which is not easily applicable to plasma metabolites owing to the much lower levels and various interfering substances relative to other matrices as described above. The difficulty in determining the plasma metabolites could be overcome by the use of Sephadex G-10 [13, 14] or organic solvent extraction [15, 16] prior to HPLC. However, our preliminary test with a small C_{18} column revealed that the purification or enrichment procedure became less time-consuming and simpler compared to the use of Sephadex G-10 or organic solvent extraction.

This paper describes a simple and rapid procedure for the simultaneous determination of DOPAC, 5-HIAA and HVA in plasma by one-step purification on a small C_{18} column followed by HPLC—ED.

EXPERIMENTAL

Reagents

3,4-Dihydroxyphenylacetic acid (DOPAC) and 5-hydroxy-2-indolecarboxylic acid (5-HICA) were obtained from Aldrich (Milwaukee, WI, U.S.A.); 5-hydroxyindole-3-acetic acid (5-HIAA), 4-hydroxy-3-methoxyphenylacetic acid (HVA), and other related compounds were from Sigma (St. Louis, MO, U.S.A.). Stock solutions of these compounds were prepared in 0.1 *M* hydrochloric acid in a concentration of 100 μg/ml. Standard solutions were diluted from their corresponding stock solutions on the day of experiment. All other reagents were of analytical reagent grade.

Chromatography

The LC—ED system was purchased from Yanagimoto Mfg. Co. (Kyoto, Japan), and consisted of a Yanako Model L-4000W pump, a 250 × 4.6 mm I.D. 7-μm Yanapak ODS-A reversed-phase column, and a Model VMD-501 dual electrochemical detector with series-adjacent twin glassy carbon electrodes. Applied electrode potentials were 0.6 V and 0.75 V vs. Ag/AgCl at the upstream (W_1) and downstream (W_2), respectively. The mobile phase consisted of 0.1 *M* potassium phosphate buffer (pH 3.2), containing EDTA · 2Na (10 μM) and methanol (18%). The flow-rate was 1.2 ml/min, and the column temperature was set at 40° C.

Extraction

Blood was collected in a tube containing 0.1% EDTA · 2Na and 0.1% sodium metabisulphite and put on ice. Plasma was separated by centrifugation at 600 *g* for 7 min at 4° C, and stored at −80° C until analysed.

Extractions were performed under vacuum using Bond-Elut columns prepacked with 100 mg of C_{18} bonded silica (40 μm) in a 1-ml capacity disposable syringe (Analytichem International, Harbor City, CA, U.S.A.). The columns, which were inserted into a vacuum chamber connected with water aspirator, were prepared by washing with 2 ml of methanol followed by 2 ml of water.

To 1 ml of plasma, 100 μl of a solution of the internal standard (equivalent to 10 ng), 5-hydroxy-2-indolecarboxylic acid (5-HICA), and 250 μl of 1 *M* hydrochloric acid were added. Samples were then applied to and passed through the columns, followed by a 1-ml water wash to rinse off residual samples and easily eluted hydrophilic compounds. The adsorbed materials were eluted with 200 μl of methanol and 5—20 μl of this solution were injected into the HPLC system.

Calibration curves were generated by processing authentic standard substances through the entire procedure of extraction and comparing the relative peak heights to the internal standard.

RESULTS AND DISCUSSION

The usefulness of electrochemical detection for endogenous monoamines including catecholamines and related compounds has been repeatedly emphasized in recent years [17]. Many aspects can be further improved with dual amperometric detection [18].

In the present chromatographic conditions, DOPAC and 5-HIAA are oxidized readily at potentials above 0.6 V (Fig. 1). Since higher selectivity and lower background noise can be obtained at lower potentials, we adopted 0.6 V for the determination of DOPAC and 5-HIAA. However, HVA showed a poor response at 0.6 V but gave a quantifiable response current at potentials above 0.7 V (Fig. 1). Thus, the upstream (W_1) and downstream (W_2) potentials were selected at 0.6 V for the detection of DOPAC, 5-HIAA and 5-HICA (internal standard) and 0.75 V for HVA, respectively.

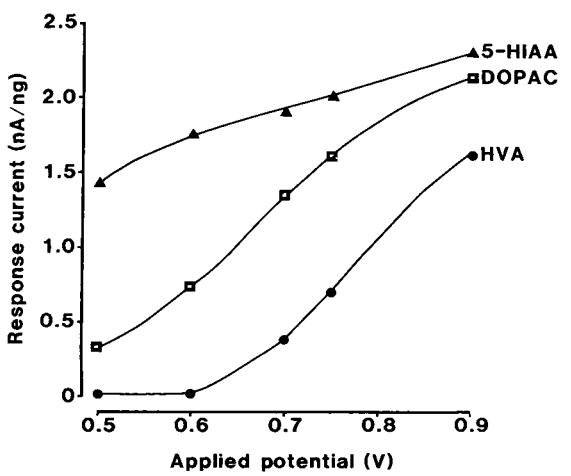


Fig. 1. Hydrodynamic voltammograms of DOPAC, 5-HIAA and HVA. The details of HPLC conditions are described in Experimental.

Chromatograms demonstrating the resolution of authentic DOPAC, 5-HIAA, 5-HICA and HVA are shown in Fig. 2. To achieve an optimal separation of the compounds from interfering substances, the pH of the mobile phase was critical. The retention times of monoamine-related compounds examined using the system described above are listed in Table I. All the compounds determined herein, which are acidic metabolites of biogenic amines, had much longer retention times than any other amines and neutral compounds examined, except for tryptophan which was eluted immediately after DOPAC using this system but did not interfere with the detection of DOPAC because of little or no response at 0.6 V. The retention times of these acidic compounds were shortened at higher pH values and substantial overlap of peaks occurred.

Representative chromatograms of an extract from normal human plasma are shown in Fig. 3. DOPAC, 5-HIAA and 5-HICA are clearly separated from other biological materials (which could not be identified) and selectively detected at 0.6 V (W_1). Only HVA needed to be detected at 0.75 V (W_2), while the peak of DOPAC was overlapped by tryptophan (whose recovery was not

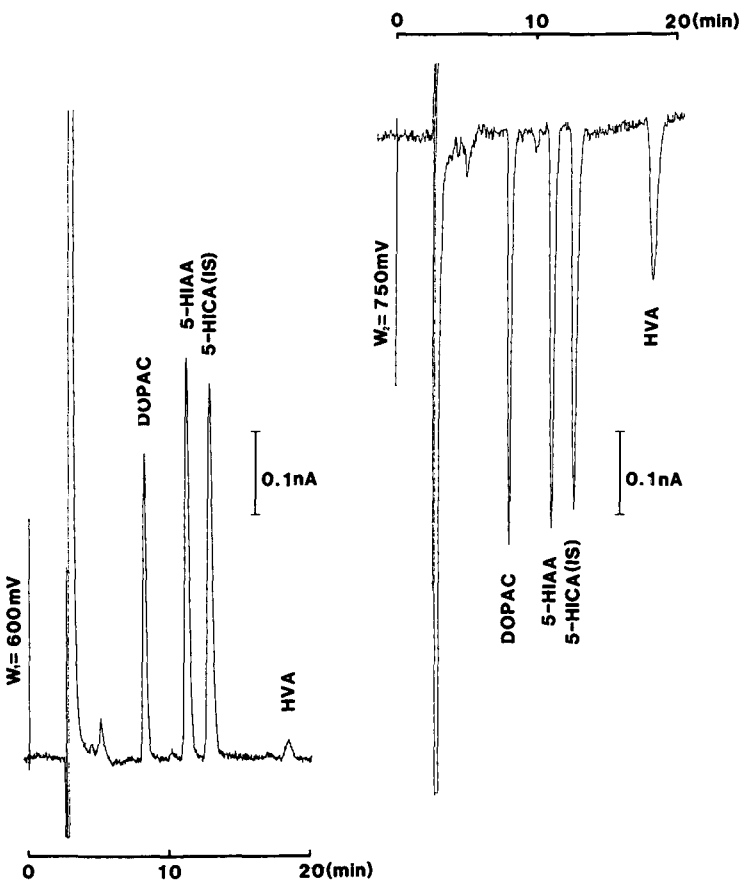


Fig. 2. Chromatogram demonstrating the separation of DOPAC, 5-HIAA, HVA and 5-HICA (internal standard) in the standard solution (0.3 ng each) using a dual electrochemical detector.

TABLE I

RETENTION TIMES OF SOME MONOAMINE-RELATED COMPOUNDS

Compound	Retention time (min)
Norepinephrine Epinephrine	2.79
l-DOPA	3.02
Dopamine	3.08
Tyrosine	3.37
3,4-Dihydroxyphenylglycol	3.38
Serotonin	3.87
Vanillylmandelic acid	4.12
5-Hydroxytryptophan	4.22
4-Hydroxy-3-methoxyphenylglycol	4.80
DOPAC	8.06
l-Tryptophan	8.18
5-HIAA	11.14
5-HICA	12.70
HVA	18.20

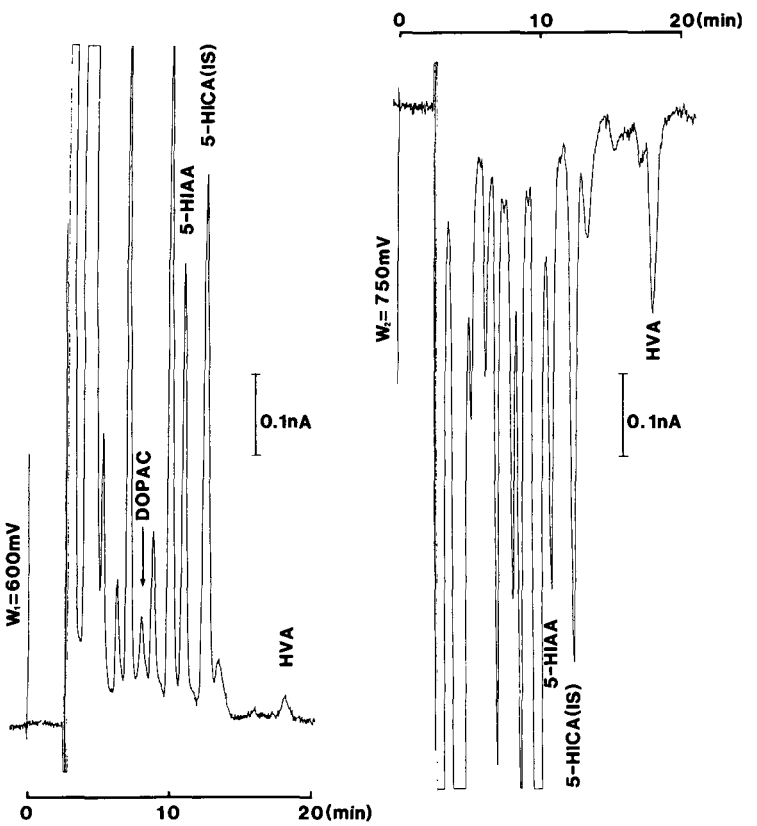


Fig. 3. Typical chromatograms demonstrating the separation of DOPAC, 5-HIAA and HVA in an extract from human plasma using a dual electrochemical detector. 5-HICA was used as an internal standard.

determined) at this potential. Identities of the peaks were confirmed by chromatographic behaviour using several solvent systems differing in pH and percentage of organic solvent and electrochemical characteristics. The dual ED system revealed another advantage that the peak identities were confirmed by comparing the polarities and relative magnitudes of current responses of standards and unknowns [18].

To establish the most effective extraction procedure from plasma various adsorbents for the extraction columns were tested in preliminary experiments. When the authentic standard solution was used, C_{18}, phenyl and cyclohexyl columns showed a high recovery to a similar extent for all three compounds, i.e. 5-HIAA, DOPAC and HVA, compared with cyanopropyl, aminopropyl, quarternary amine, and C_8 columns. When using blood samples, the C_{18} column proved to be better than or not different from phenyl or cyclohexyl columns in terms of selectivity against possible interfering endogenous substance(s) and recoveries. We chose a C_{18} column on the basis of this finding and also because of its general distribution and ready availability. The recovery rates of substances when using the extraction columns were further influenced by the differences in extraction conditions (Table II). The recoveries of the substances were all around 90% when the plasma samples were treated according to the standard method. Deproteinization of plasma samples with perchloric acid prior to application on to the extraction columns (Method 1) markedly reduced those recoveries, although the acidic condition was indispensable. Differences in the composition of the eluent also seriously influenced recovery. Extraction with a much more polar eluent containing more than 50% of aqueous solution in place of methanol (Method 2) reduced the recoveries of less hydrophilic compounds. Although fairly good recoveries were obained with another eluent containing less than 50% of aqueous solution (Method 3), this eluent extracted interfering substances simultaneously, which interfered with the determination of DOPAC. We can prepare a sample for HPLC injection through the entire extraction procedure within a few minutes.

TABLE II

COMPARISON OF RECOVERIES OF SUBSTANCES (10 ng/ml) ADDED TO NORMAL PLASMA UNDER VARYING EXTRACTION CONDITIONS

Procedures are similar to the standard method except for the following: in Method 1, plasma was deproteinized with perchloric acid (40 μg/ml) before applying to the Bond-Elut column; in Method 2, adsorbed materials were eluted from the Bond-Elut column with 0.1 *M* phosphate buffer (pH 3.2)—methanol mixture (60:40); and in Method 3, adsorbed materials were eluted from the Bond-Elut column with 0.05 *M* hydrochloric acid—methanol mixture (40:60).

	Recovery (%)			
	Standard method (mean ± S.D., n = 5)	Method 1	Method 2	Method 3
DOPAC	87.7 ± 4.1	57.0	87.7	95.6
5-HIAA	92.2 ± 4.1	52.4	80.7	93.1
5-HICA	87.1 ± 1.8	33.1	65.3	82.0
HVA	91.1 ± 3.8	75.6	55.6	85.6

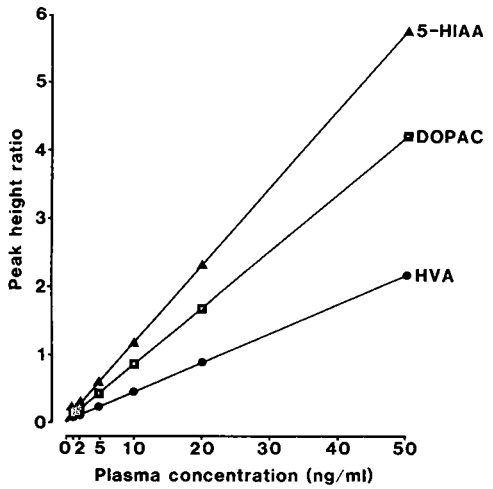


Fig. 4. Calibration curves of the peak height ratio of DOPAC, 5-HIAA and HVA vs. 5-HICA (10 ng/ml) in plasma. A three- or four-point standard curve was prepared by plotting the ratio of each compound's peak height to that of the internal standard for each concentration. Linear regression analysis of calibration data indicated no significant deviation from linearity (r = 0.9985—0.9990). In addition, the intercept did not differ significantly from zero.

The standard curves were run on each occasion with plasma standards carried through the entire procedure. Linearity was demonstrated by plotting the peak height ratio of compounds vs. 5-HICA over the range 1—50 ng/ml (Fig. 4). Standard curves had r values of 0.9985—0.9990 and passed through the origin. The sensitivity of the present method permitted the assay of at least 200 pg/ml for DOPAC and 5-HIAA and 800 pg/ml for HVA in plasma. The precision of the assay is given in Table III. High reproducibility was verified by the coefficients of variation which ranged from 2.1% to 4.3%.

The analytical results of human plasma from five healthy subjects are given in Table IV. The concentrations of DOPAC, 5-HIAA and HVA are compatible with the values recently reported with HPLC [3, 19, 20] and gas chromatography—mass spectrometry [9, 21—23].

The present method offers a means for the rapid and simultaneous determination of three monoamine metabolites, i.e. DOPAC, 5-HIAA and HVA, in human plasma. The readiness of the procedure may augment the utilization of these values as indices for evaluating the activities of dopaminergic and

TABLE III

PRECISION OF THE ASSAY FOR DOPAC, 5-HIAA AND HVA IN POOLED PLASMA

	Concentration ± S.D. (ng/ml)	n	C.V. (%)
DOPAC	1.87 ± 0.04	5	2.1
5-HIAA	11.56 ± 0.33	5	2.9
HVA	15.88 ± 0.68	5	4.3

TABLE IV

DETERMINATION OF MONOAMINE METABOLITES IN PLASMA FROM HEALTHY HUMAN SUBJECTS

Subject No.	DOPAC (ng/ml)	5-HIAA (ng/ml)	HVA (ng/ml)
1	1.70	6.8	15.6
2	2.44	6.8	16.9
3	2.10	8.2	11.7
4	1.90	11.4	10.0
5	1.95	12.9	10.3
Mean	2.02	9.22	12.9
± S.D.	±0.28	±2.79	±3.2

serotonergic systems in diverse clinical conditions. The assay method presented here is also of sufficient sensitivity, precision, and accuracy for clinical application. In addition, the procedure may be applicable to other biological fluids or tissues with minor modifications.

ACKNOWLEDGEMENTS

The authors wish to thank Miss Kaori Narita for her secretarial assistance. This work was supported in part by a Grant-in-Aid from the Ministry of Human Health and Welfare, Japan.

REFERENCES

1 D.L. Murphy, I. Campbell and J.L. Costa, in M.A. Lipton, A. DiMascio and K.F. Killam (Editors), Psychopharmacology: A Generation of Progress, Raven Press, New York, 1978, p. 1235.
2 G. Curzon, in W.B. Essman (Editor), Serotonin in Health and Disease, Vol. 3, The Central Nervous System, Spectrum Publications, New York, 1978, p. 403.
3 I.N. Mefford, M.M. Ward, L. Miles, B. Taylor, M.A. Chesney, D.L. Keegan and J.D. Barchas, Life Sci., 28 (1981) 477.
4 F. Smedes, J.C. Kraak and H. Poppe, J. Chromatogr., 231 (1982) 25.
5 B.H.C. Westerink and J. Korf, Eur. J. Pharmacol., 37 (1976) 249.
6 N.G. Bacopoulos, S.E. Hattox and R.H. Roth, Eur. J. Pharmacol., 56 (1979) 225.
7 G. Roccatagliata, C. Albano, L. Cocito and M. Maffini, J. Neurol. Neurosurg. Psychiat., 42 (1979) 1159.
8 J. Borg, J.M. Warter, J.L. Schlienger, M. Imler, C. Marescaux and G. Mack, J. Neurol. Sci., 57 (1982) 343.
9 M.B. Bowers, M.E. Swigar and P.I. Jatlow, N. Engl. J. Med., 308 (1983) 845.
10 G. Sperk, J. Neurochem., 38 (1982) 840.
11 K.H. Le Quan-Bui, J.-L. Elghozi, M.-A. Devynck and P. Meyer, Eur. J. Pharmacol., 81 (1982) 315.
12 K. Fujita, K. Maruta, S. Ito and T. Nagatsu, Clin. Chem., 29 (1983) 876.
13 B.H.C. Westerink and T.B.A. Mulder, J. Neurochem., 36 (1981) 1449.
14 B.H.C. Westerink, F.J. Bosker and J.F. O'Hanlon, Clin. Chem., 28 (1982) 1745.
15 F. Hefti, Life Sci., 25 (1979) 775.
16 T. Shibuya, K. Sato and B. Salafsky, Int. J. Clin. Pharmacol. Ther. Toxicol., 20 (1982) 297.
17 K. Ishikawa and J.L. McGaugh, J. Chromatogr., 229 (1982) 35.

18 D.A. Roston, R.E. Shoup and P.T. Kissinger, Anal. Chem., 54 (1982) 1417A.
19 E. Martinez, F. Artigas, C. Sunol, J.M. Tusell and E. Gelpi, Clin. Chem., 29 (1983) 1354.
20 B. Petruccelli, G. Bakris, T. Miller, E.R. Korpi and M. Linnoila, Acta Pharmacol. Toxicol., 51 (1982) 421.
21 F.A.J. Muskiet, G.T. Nagel and B.G. Wolthers, Anal. Biochem., 109 (1980) 130.
22 F.A.J. Muskiet, H.J. Jeuring, G.T. Nagel, H.W.A. de Bruyn and B.G. Wolthers, Clin. Chem., 24 (1978) 1899.
23 E. Ånggård, B. Sjöquist, B. Fyrö and G. Sedvall, Eur. J. Pharmacol., 24 (1973) 37.

Journal of Chromatography, 308 (1984) 65—73
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2077

MEASUREMENT OF HYDROXYLYSINE GLYCOSIDES IN URINE AND ITS APPLICATION TO SPINAL CORD INJURY

GLADYS P. RODRIGUEZ* and JACQUELINE CLAUS-WALKER

Department of Rehabilitation, Baylor College of Medicine, Neuroendocrine Laboratory, The Institute for Rehabilitation and Research, 1333 Moursund, Houston, TX 77030 (U.S.A.)

(Received December 14th, 1983)

SUMMARY

A new technique to evaluate the degradation of skin or bone collagen by measuring glucosylgalactosyl hydroxylysine and galactosyl hydroxylysine is presented. The method utilizes an automated amino acid analyzer. Eluents used are lithium buffers, and the color reagent is ninhydrin. Both glycosides elute in 3.5 h. Samples require minimum preparation. Urinary concentrations of both glycosides in ten patients with cervical spinal cord injuries of less than six months duration were higher than in five healthy controls. Proportional increases were different for each of the two glycosides. Variations in the proportional increase of each glycoside indicate different rates of degradation of skin and bone collagen. Repeated evaluations of the two urinary glycosides may help to predict whether patients are likely to develop skin- or bone-related clinical complications.

INTRODUCTION

Cervical spinal cord injury (SCI) produces an immediate alteration of the collagen metabolism of the affected patients [1]. The specific mechanism whereby the cord injury alters the collagen metabolism is not known. The consequences of bone collagen losses are well documented: greatly increased bone loss leading frequently to hypercalcemia, hypercalciuria, bladder stones, and osteoporosis. In contrast, the consequences of skin collagen loss are not widely documented, although SCI patients present an increased susceptibility to pressure ulcers, suggesting a deterioration of the skin quality. The changes in the collagen metabolism of SCI patients may be monitored by measuring the urinary excretion of collagen metabolites. Two collagen metabolites are especially informative: glucosylgalactosyl hydroxylysine (glu-gal Hyl) and galactosyl hydroxylysine (gal Hyl) (Fig. 1). Glu-gal Hyl is predominant in skin

0378-4347/84/$03.00

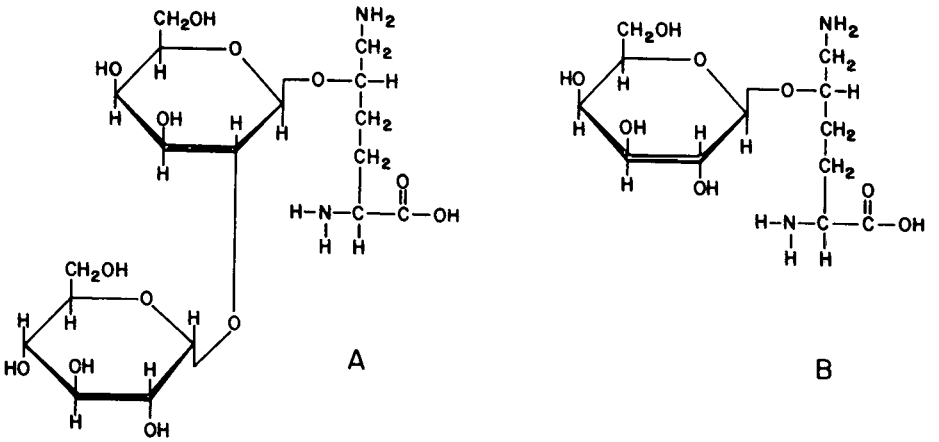


Fig. 1. Structural formulae of (A) 2-O-α-D-glucopyranosyl-O-β-D-galactosylpyranosyl hydroxylysine and (B) O-β-D-galactosylpyranosyl hydroxylysine.

collagen, and its urinary excretion is increased in patients with skin abnormalities such as erythema multiforme or burns [2—4]. Gal Hyl is predominant in bone collagen, and its urinary excretion is increased in patients with bone disease such as osteomalacia or Paget's disease [2—4]. The relative concentration of the glycosides in the urine of SCI patients would give some indication of the origin of the collagen being degraded and might be predictive of particular complications likely to occur in a specific patient.

The methods previously described to measure urine hydroxylysine glycosides have several disadvantages: (1) multiple columns in the analyzer [5]; (2) incomplete resolution of several amino acids [6]; (3) very lengthy chromatography [7, 8]; (4) mechanical alterations to analyzer [9]; (5) extensive preparation of sample prior to chromatography [4, 10—12].

The method we have developed to measure the urinary concentration of each hydroxylysine glycoside has overcome all these disadvantages. It utilizes an automated single-column amino acid analyzer and completely resolves glu-gal Hyl and gal Hyl in 3.5 h, providing a full amino acid profile in 5 h. It uses a standard commercial amino acid analyzer without any mechanical modification. The alterations to the elution buffer system which make the separation possible are straightforward and preparation of the sample prior to chromatography is minimal.

EXPERIMENTAL

Glu-gal Hyl and gal Hyl are not available commercially. They were extracted from the anterior lens capsule of dog lenses by a modification of Askenasi's method [6]. The lens capsules were homogenized in 0.15 *M* sodium chloride and lyophilized. Portions of the dried homogenate were hydrolyzed in 2 *M* lithium hydroxide at 110°C for 18 h. The hydrolysate was adjusted to pH 6 with perchloric acid and centrifuged at 60,000 *g* for 15 min at 4°C. Portions of the supernatant were placed on a 124 × 0.9 cm column filled with Bio-Gel P-2, 400 mesh, resin and eluted with 0.1 *M* pyridine—acetate buffer, pH 4.8.

Fractions of 3.7 ml were collected (LKB Model 2070 fraction collector) and tested for the presence of hexoses by the method of Dubois et al. [13], and for the presence of amino acids by the method of Moore and Stein [14]. Fractions positive for both were subjected to acid hydrolysis in 2 *M* hydrochloric acid for 3 h at 109°C; an increase in free Hyl content is an indication of the presence of either or both glycosides.

The amino acid analyzer used was Beckman's Model 119CL provided with a data processor Model 126 capable of integrating the peaks in the chromatogram produced by the attached Bristol recorder. The analyzer column was 460 × 6 mm, filled with a cross-linked sulfonated sytrene copolymer resin (Beckman W3P) to a height of 22 cm. The resin was changed to the lithium form by treatment with lithium hydroxide. The eluting buffers were made from Beckman concentrates diluted according to directions but altered in the following way: (a) Beckman 0.2 *M* lithium citrate buffer, pH 2.83 was changed to pH 2.70 (buffer A); (b) to Beckman 0.2 *M* lithium citrate buffer, pH 3.70, 2 g of lithium chloride per l of buffer was added and pH changed to 3.21 (buffer B); (c) to Beckman 1 *M* lithium citrate buffer, pH 3.75, 6% 2-propanol was added and pH changed to 3.68 (buffer C). The color reagent was a standard ninhydrin solution using stannous chloride as a reducing agent. Starting temperature was 40°C, raised to 65°C after 40 min. Buffer flow-rate was 44 ml/h. Ninhydrin flow-rate was 22 ml/h. Buffer A ran for 66 min, buffer B for 101 min, and buffer C for 98 min (Table I).

TABLE I

ELUTION PROGRAM FOR THE AMINO ACID ANALYZER

Buffer (lithium)	Additive	pH (25°C)	Pumping time (min)	Temperature
A 0.2 *M*	—	2.70	66	40°C for 40 min then 65°C
B 0.2 *M*	2 g lithium chloride per l	3.21	101	65°C
C 1.0 *M*	6% 2-propanol	3.68	98	65°C

Urine samples from five healthy controls and ten spinal cord injury patients were assayed for their glu-gal Hyl and gal Hyl concentrations using the present method. The controls were males between 30 and 52 years of age. The patients were males, injured less than six months, between 14 and 50 years of age, with no previous history of chronic diseases. Both controls and patients gave informed consent to participate in the project and provided 24-h urine samples. The urines were kept cold during the collections and frozen if not assayed immediately. Ammonia was removed from the sample aliquots by raising the pH between 11.5 and 12.0 with 4 *M* lithium hydroxide and placing them in a dessicator containing concentrated sulfuric acid under reduced pressure for 6 h. The pH of the urine was then adjusted to pH 2.2 with 6 *M* hydrochloric acid,

filtered through a Millipore 0.22-μm filter and brought back up to its original volume with 0.2 *M* lithium citrate buffer, pH 2.2. An aliquot of 100 μl was applied to the amino analyzer column. In accordance with the findings of Lou and Hamilton in 1971 [7], the molar ninhydrin color equivalent for Hyl was used to calculate the glycoside concentrations. Results of the assay of the control and patient urine samples were compared by analysis of variance by means of a taped program of the Wang table top computer.

RESULTS

Fractions 15 through 24 of the Bio-Gel P2 eluate of the lens hydrolysate tested positive for both hexoses and amino acid (Fig. 2). When each of these fractions was subjected to acid hydrolysis, only fraction 15 exhibited an increase in free Hyl content (Figs. 3 and 4). This fraction was used to adjust the elution protocol of the amino acid analyzer to elicit the desired resolution of glu-gal Hyl and gal Hyl in the chromatogram. Since only 5% of the glycosylated Hyl in lens capusule is the monosaccharide [15], fraction 15 was subjected to a mild acid hydrolysis in 0.2 *M* hydrochloric acid for 4 h at 109° C. This releases only the terminal glucose residue in some of the molecules of glu-gal Hyl and makes it possible for the analyzer to detect the augmented gal Hyl [6, 7, 9, 16]. With the present protocol, the retention time for glu-gal Hyl was approximately 115 min and for gal Hyl 185 min (Fig. 5).

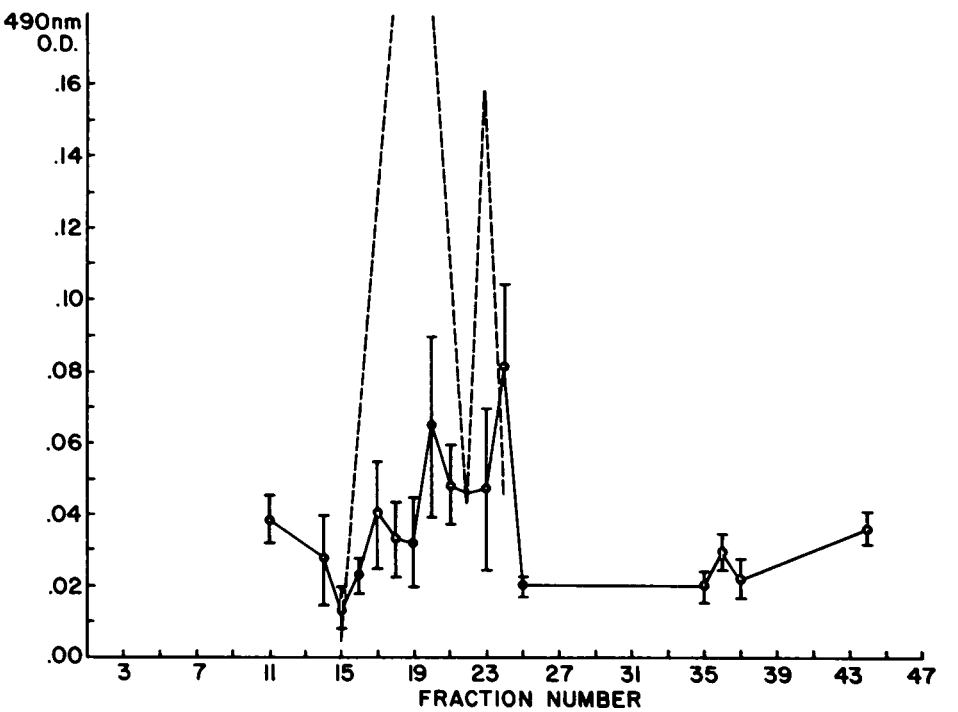


Fig. 2. Hexose determinations in Bio-Gel P2 filtrate fractions. Average of determinations in four separate filtrations. Bars indicate maximum variation. Abscissa: fractions numbers; ordinate: optical density at 490-nm wavelength; (- - -), largest amino acid peaks.

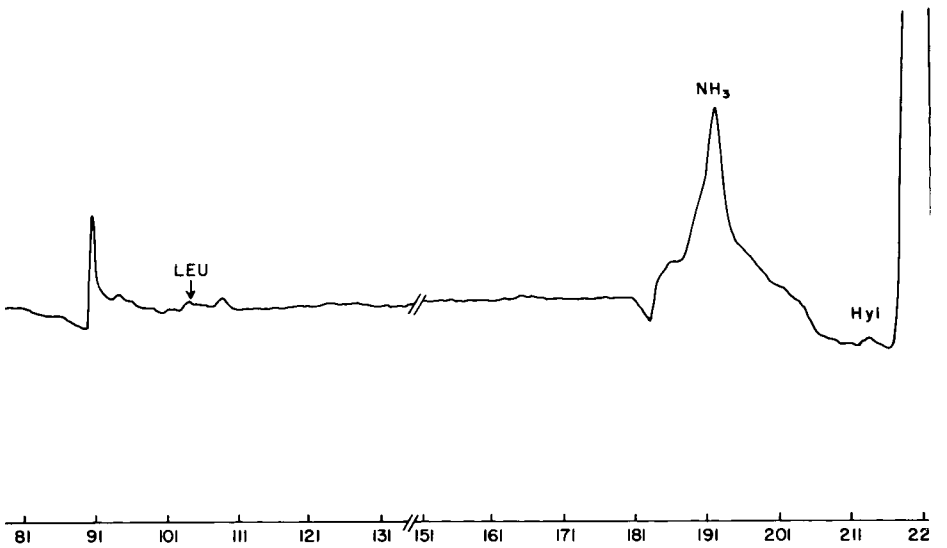


Fig. 3. Amino acid analyzer chromatogram of Bio-Gel P2 filtrate fraction No. 15. Curve represents the sum of the absorptions at 440 and 570 nm. Only sections of interest of the chromatogram are shown. Abscissa: retention times in min; ordinate: absolute units proportional to optical density. Size of sample: 100 μl; recorder set at highest sensitivity (0.1 absorption).

Fig. 4. Amino acid analyzer chromatogram of the same fraction as in Fig. 3 after it had been subjected to acid hydrolysis in 2 *M* hydrochloric acid for 4 h at 109°C and brought to pH 3 with 4 *M* lithium hydroxide. Curve represents the sum of the absorptions at 440 and 570 nm. Only sections of interest of the chromatogram are shown. Abscissa: retention times in min; ordinate: absolute units proportional to optical density. Size of sample: 200 μl; recorder set at highest sensitivity.

The 24-h urine samples from five healthy controls and ten spinal cord injury patients were assayed using the protocol outlined in Table I. The mean concentration of glu-gal Hyl in the healthy controls was 18.7 ± 2.4 (S.E.M.) μmol per g creatinine. The mean gal Hyl in the healthy controls was 12.2 ± 3.8 (S.E.M.) μmol per g creatinine. In spite of the large differences between the means of

the two groups [31.8 (S.E.M.) μmol per g creatine for glu-gal Hyl and 20.5 (S.E.M.) μmol per g creatinine for gal Hyl], these differences were not statistically significant unless we discard the results on patient 9 (Table II). This patient's hydroxyproline was also extremely high, 266 mg per g creatinine.

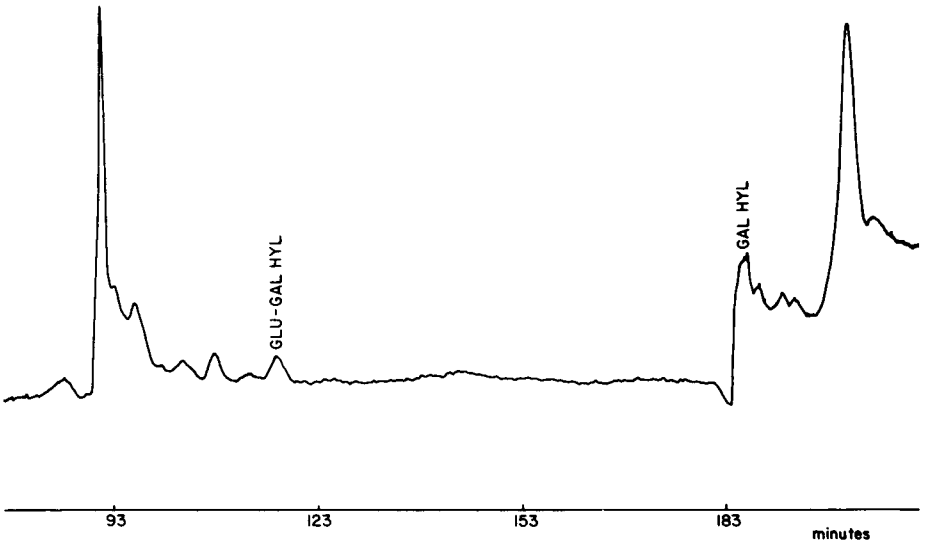


Fig. 5. Amino acid analyzer chromatogram of the same sample as in Fig. 3 but done under the final parameters chosen for this method. Only the section of interest of the chromatogram is shown. Curve represents the sum of the absorptions at 440 and 570 nm. Abscissa: retention times in min; ordinate: absolute units proportional to optical density; size of sample: 625 μl; recorder set at highest sensitivity (0.1 absorption).

TABLE II

CONCENTRATIONS OF GALACTOSYL HYDROXYLYSINE AND GLUCOSYLGALACTOSYL HYDROXYLYSINE IN CONTROLS AND PATIENTS

Sample	Controls			Patients		
	glu-gal (μmol per g creatinine)	gal (μmol per g creatinine)	glu-gal/ gal	glu-gal (μmol per g creatinine)	gal (μmol per g creatinine)	glu-gal/ gal
1	16.7	9.6	1.7	20.4	15.5	1.3
2	13.9	9.3	1.5	44.5	30.2	1.5
3	21.2	14.0	1.5	30.0	14.9	2.0
4	26.8	18.2	1.5	42.9	23.5	1.8
5	14.7	10.1	1.5	84.3	25.4	3.3
6				27.0	24.8	1.1
7				35.2	24.6	1.4
8				28.5	21.8	1.3
9				142.7	106.0	1.3
10				49.7	40.3	1.2
Mean	18.7	12.2		50.5	32.7	
S.D.	5.4	3.8		37.1	26.7	
S.E.	2.4	1.7		11.8	8.5	
p				>0.05	>0.1	
If sample 9 is eliminated:			Mean	40.3	24.6	
			S.D.	19.0	7.6	
			S.E.	6.3	2.5	
			p	0.05	0.01	

To analyze the inter-assay variation, a random urine sample was analyzed eight times. The determinations were carried out over a period of three weeks, utilizing several different batches of the eluting buffers and color reagent. The mean of the eight glu-gal Hyl determinations was 17.2 ± 0.2 (S.E.M.) μmol per g creatinine.

To analyze the intra-assay variation, a different random urine sample was analyzed ten consecutive times. The determinations were carried out over a period of three days, utilizing the same batches of eluting buffers and color reagent. The mean of the ten glu-gal Hyl determinations was 11.0 ± 0.3 (S.E.M.) μmol per g creatinine.

DISCUSSION

The method described herein is a straightforward procedure adaptable to many uses. It has five main positive features: (1) the chromatography is carried out in a single-column Beckman 119CL amino acid analyzer with the resin in the lithium form, and only three buffers are needed; (2) in the urine chromatograms, glu-gal Hyl and gal Hyl peaks are clearly resolved from the other peaks and accurately read and quantitated by the data analyzer; the magnitude of the inter- and intra-assay variations attest to the reproducibility of the method; (3) only 100 μl of urine are required for the chromatography; (4) the preparation of the urine sample requires little handling or chemical alteration; (5) both glycosides elute within 3.5 h.

The concentration of glu-gal Hyl and gal Hyl found in the control group had a narrow range of values which are similar to those obtained by previous authors (Table III). In contrast, the concentration of the glucosides in the patient group exhibited a very wide range of values. The mean values in both groups were very different, but, because of the large variation in the patient group, these differences were not statistically significant unless we disregard the results for patient 9. Nevertheless, a trend can be seen (Fig. 6). Of the patients 90% had glu-gal Hyl values above the control mean and all patients had gal Hyl values above the control mean. The glu-gal Hyl/gal Hyl ratio in the patient

TABLE III

COMPARISON OF NORMAL VALUES IN URINE FOR HYDROXYLYSINE GLYCOSIDES CITED IN THE LITERATURE WITH THOSE OBTAINED BY PRESENT METHOD

glu-gal Hyl (μmol per g creatinine)	gal Hyl (μmol per g creatinine)	glu-gal Hyl (μmol per 24 h)	gal Hyl (μmol per 24 h)	glu-gal Hyl/ gal Hyl	Reference
16.5	11.0	26.3	17.7	1.5	3
17.8	11.6	21.5	13.7	1.6	18
—	—	31.9	28.3	1.1	11
20.0	12.0	—	—	1.7	10
30.0	20.0	—	—	1.6	19
14.0	8.0	—	—	1.7	15
18.7	12.0	28.9	18.7	1.5	present paper

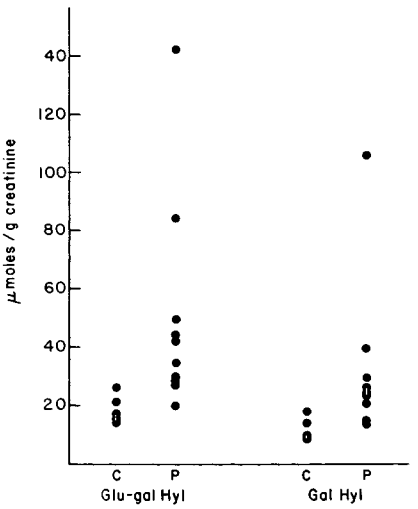


Fig. 6. Distribution of values of hydroxylysine glycosides in the urine of controls (C) and patients (P).

group had a wide range of values. A longitudinal study in a large population will be needed to determine whether a high ratio will increase the probability that the patient will develop skin complications and a low ratio will be associated with bone-related complications.

This method was developed to explore the metabolism of collagen in spinal cord injury. Previous data indicated that trauma of the spinal cord causes an increased bone mineral and collagen metabolism. The collagen loss occurs first in all the bones, later only in the bones of the paralyzed body areas [1]. In addition, there is a loss of collagen from the skin [17]. The urinary concentrations of glu-gal Hyl and gal Hyl will be an indication not only of total collagen loss after spinal cord injury, but also of which tissue is most at risk.

The content of hydroxylysine varies with the type of collagen. In human skin collagen, glu-gal Hyl represents 61% of the total glycosides, while in bone collagen it represents only 13—40% [4, 18]. The relative and absolute concentrations of the hydroxylysine glycosides excreted in urine indicate the tissue origin of the collagen metabolites and the rate of the degradation of the collagen [3, 11, 15, 19]. Measuring the excretion of these glycosides could be a means of monitoring the effect of the therapies used to minimize the sequelae of spinal cord injury (osteoporosis, skin ulcers, etc.), as well as evaluating the progress and the efficacy of the therapy in many diseases that affect collagen (e.g., Ehler-Danlos syndrome, hyperthyroidism, chronic uremia).

ACKNOWLEDGEMENTS

This work was supported in part by grant from the National Institute for Handicapped Research, No. RSA 16P 56813/6. The invaluable assistance of Joseph Eichberg, Ph. D., Department of Biochemical and Biophysical Sciences, University of Houston, Houston, TX, U.S.A., is gratefully acknowledged.

REFERENCES

1 J. Claus-Walker, Int. J. Rehab. Res., 3 (1980) 540.
2 R. Askenasi, Clin. Chim. Acta, 59 (1975) 87.
3 R. Askenasi, J. Lab. Clin. Med., 83 (1979) 673.
4 S.R. Pinnell, R. Fox and S.M. Krane, Biochim. Biophys Acta, 229 (1971) 119.
5 D.T. DiFerrante, N.Y. Wilson and C.S. Leach, J. Chromatogr., 187 (1980) 271.
6 R. Askenasi, Biochim. Biophys. Acta, 304 (1973) 782.
7 M.F. Lou and P.B. Hamilton, Clin. Chem., 17 (1971) 782.
8 R.G. Spiro, in S.P. Colowick and N.O. Kaplan (Editors), Methods in Enzymology, Vol XXVII, Academic Press, New York and London, 1972, p. 3.
9 V. Odell, L. Wegener, B. Peczon and B.G. Hudson, J. Chromatogr., 88 (1974) 245.
10 Y. Kakimoto and S. Akagawa, J. Biol. Chem., 245 (1970) 5751.
11 J.P. Segrest and L.W. Cunningham, J. Clin. Invest., 49 (1970) 1497.
12 R.G. Spiro, J. Biol. Chem., 244 (1969) 606.
13 M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith, Anal. Chem., 28 (1956) 350.
14 S. Moore and W. Stein, J. Biol. Chem., 211 (1954) 907.
15 H.J. Bremer, M. Duran, J.P. Kamerling, H. Przyrembel and S.K. Wadman, Disturbances of Amino Acid Metabolism: Clinical Chemistry and Diagnosis, Urban and Schwarzenberg, Baltimore and Munich, 1981, p. 428.
16 N.A. Kefalides, in E.A. Balaz (Editor), Chemistry and Molecular Biology of the Intracellular Matrix, Academic Press, London and New York, 1970, p. 535.
17 J. Claus-Walker, J. Singh, C.S. Leach, D.V. Hatton, C.W. Hubert and N. DiFerrante, J. Bone Joint Surg., 59 (A) (1977) 209.
18 R. Askenasi, in D.A. Hall (Editor), The Methodology of Connective Tissue Research, Joynson-Bruvvers, Oxford, 1976, p. 251.
19 W.C. Bisbee and P.C. Kelleher, Clin. Chim. Acta, 90 (1978) 29.

Journal of Chromatography, 308 (1984) 75—82
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2079

CYSTEINYLGLYCINE IN URINE DETERMINED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

BERTIL KÅGEDAL* and MAGNUS KÄLLBERG

Department of Clinical Chemistry, University Hospital, S-581 85 Linköping (Sweden)

(Received December 5th, 1983)

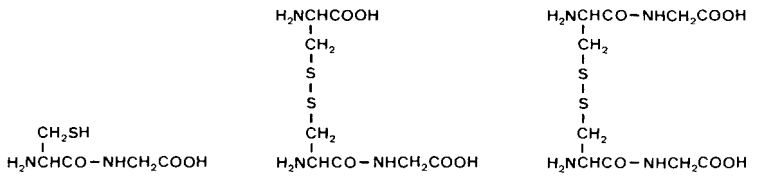
SUMMARY

Using N-(1-pyrene)maleimide as a reagent for thiol compounds and high-performance liquid chromatography with fluorometric detection, we have identified cysteinylglycine as an endogenous compound in dithiothreitol-reduced urine. In a quantitative method developed for cysteinylglycine, reduction of urinary disulphides was effected by dithiothreitol at pH 6. The pH was then brought to 1.5 and excess dithiothreitol together with acid thiols was extracted with water-saturated ethyl acetate. After derivatization the concentration was determined by reversed-phase liquid chromatography. Precision of the method (C.V. = 6.5%) and analytical recovery (86 ± 6.4%) were satisfactory. The urinary excretion of cysteinylglycine was 7.4 ± 2.3 μmol/l (mean ± S.D.) in eight healthy subjects.

INTRODUCTION

A number of thiol compounds are present in animal cells and take an active part in metabolism. One of the best known of these thiols is glutathione which is present in high amounts in, for example, liver cells and erythrocytes, but is present in much lower concentrations extracellularly [1]. According to the γ-glutamyl cycle the first step in glutathione breakdown is catalysed by γ-glutamyltranspeptidase, which removes the γ-glutamyl moiety of glutathione and transfers it to an acceptor amino acid [2]. The remaining cysteinylglycine is then split to cysteine and glycine in a reaction which may be very rapid. Thus, after injection of radioactively labelled glutathione to rats [3], the radioactivity within the tissue was essentially recovered as labelled glycine, and no radioactive cysteinylglycine was observed in the blood or tissues examined. However, when isolated renal tubules were incubated with radioactive glutathione disulphide, a third radioactive compound in addition to glutathione disulphide and glycine appeared, which was identified as cystinyldiglycine, the symmetrical disulphide of cysteinylglycine (Fig. 1).

0378-4347/84/$03.00



A. Cysteinylglycine B. Cystinylglycine C. Cystinyldiglycine

Fig. 1. Structure of (A) cysteinylglycine, (B) cystinylglycine (mixed disulphide of cysteine and cysteinylglycine), and (C) cystinyldiglycine (symmetric disulphide of cysteinylglycine).

The compound cystinylglycine (Fig. 1), which probably also is a metabolite of glutathione [4], occurs regularly in blood plasma, but has not been observed in urine [5]. Both cystinyldiglycine and cystinylglycine give the thiol cysteinylglycine after reduction of their disulphide bridges.

We have previously developed a highly sensitive method for the determination of the acid thiols mercaptoacetate and N-acetylcysteine in urine [6]. After reduction of disulphide bridges and clean-up of the liberated acid thiols from the urine, the thiols were reacted with a non-fluorescent maleimide compound [7] thereby forming fluorescent derivatives which could be separated by high-performance liquid chromatography (HPLC) and quantified by fluorimetry. Using similar techniques we have now identified the compound cysteinylglycine in reduced urine and developed an HPLC method for its determination.

EXPERIMENTAL

Materials

3-Mercaptopropionic acid was obtained from E. Merck (Darmstadt, F.R.G.) and cystinyldiglycine $(Cys\text{-}Gly)_2$ was from Serva (Heidelberg, F.R.G.). Dithiothreitol was obtained from Sigma (St. Louis, MO, U.S.A.) and the anion exchanger AG1-X4 (100—200 mesh, Cl^-) was from Bio-Rad Labs. (Richmond, CA, U.S.A.). The organomercurial adsorbent *p*-acetoxymercurianiline—Sepharose 4B (PAMAS) was prepared according to the procedure of Sluyterman and Wijdenes [8]. N-(1-Pyrene)maleimide was a product of Fluka (Buchs, Switzerland). We purified the substance by chromatography on a Lobar LiChroprep Si-60 column, size B (Merck). For this procedure we used an Eldex E-120-S-2 (Eldex Labs., Menlo Park, CA, U.S.A.) pump and a mobile phase of toluene—acetone (9:1, v/v). After equilibration of the column, 5 mg of N-(1-pyrene)maleimide, dissolved in 3 ml of the mobile phase were transferred to the column and eluted at a flow-rate of 1.0 ml/min. Detection was by ultraviolet (UV) absorption at 339 nm (Spectromonitor III, LDC, Riviera Beach, FL, U.S.A.). Fractions (5 ml) were collected and the relevant fractions evaporated; the N-(1-pyrene)maleimide was dissolved in ethanol—acetone (1:1, v/v) and its concentration determined from its UV absorption at 339 nm. A stock solution, 1.0 mmol/l, was prepared in ethanol—acetone (1:1, v/v) and stored in the refrigerator.

Apparatus

We used a Constametric III pump from LDC, a Rheodyne (Cotati, CA,

U.S.A.) Model 7125 sample injector with a 100-μl sample loop, and a Perkin-Elmer (Beaconsfield, U.K.) Model 3000 spectrofluorometer with an LC-cell accessory. Excitation wavelength was 342 nm (band width 15 nm) and emission wavelength was 396 nm (band width 20 nm).

The analytical column was a Supelcosil LC-8 column (250 × 4.6 mm), particle size 5 μm, from Supelco (Bellefonte, PA, U.S.A.). In order to protect the analytical column, a precolumn packed with Solvecon Silica, particle size 37—53 μm (Pierce, Rockford, IL, U.S.A.), was put in line between the pump and the injector. The columns were jacketed and held at constant temperature (25 or 35°C) by connection to a circulating water-bath in order to obtain reproducible retention times. The mobile phase was prepared from phosphoric acid (85%, Merck) diluted with distilled water to a concentration of 50 mmol/l and mixed with methanol in the proportions 11:9 (v/v).

Urine collection

Urine was collected for 24 h in plastic bottles containing 5 ml of thymol, 0.7 mol/l, in isopropanol as a preservative [9].

Reduction of urinary disulphides with thiopropyl-Sepharose 6B

We followed the procedure described by Hannestad and Sörbo [10] for reduction of urinary disulphides. Thus we added 0.2 ml of Na_2EDTA, 0.13 mol/l, to a 5-ml aliquot of urine, adjusted the pH to 9.8—10.0 with ammonia, 5 mol/l, and made up the volume to 6 ml with water. Then 1 ml of thiopropyl-Sepharose 6B suspension containing 20 μmol of SH-groups per ml gel was added and reduction was effected during 30 min mixing. Acidification was then done to pH 3.5—4.0 by the addition of 1 ml of acetic acid, 4 mol/l, and the sample was centrifuged. From this solution samples were taken either for derivatization or further clean-up. For derivatization 500 μl were transferred to new tubes; 5 ml of carbonate buffer, 50 mmol/l, pH 9.0, containing Na_2EDTA, 10 mmol/l, were added followed by 250 μl of sodium hydroxide, 1 mol/l, and 500 μl of N-(1-pyrene)maleimide, 1 mmol/l.

Clean-up of reduced urine with PAMAS and anion exchanger (AG1-X4)

From the urine reduced by thiopropyl-Sepharose, an amount (usually 5 ml) containing less than 2 μmol of thiols as measured by the method of Rootwelt [11] was adsorbed on a PAMAS column (bed dimension 1.3 × 0.7 cm) and after washing with 2 ml of water the adsorbed thiol compounds were eluted with 3 ml of 3-mercaptopropionic acid, 10 mmol/l, into tubes containing 0.2 ml of Na_2EDTA, 0.18 mol/l. We then adjusted the pH to about 7, and applied the sample to a 2.6 × 0.5 cm AG1-X4 (Cl^-) column (equilibrated with imidazole solution, 50 mmol/l, adjusted to pH 7.0 with hydrochloric acid), which retains the 3-mercaptopropionic acid. The effluent was collected in a tube containing 0.2 ml of Na_2EDTA, 0.18 mol/l, together with a further 1 ml of imidazole buffer applied to the column. A 500-μl aliquot of the combined effluents was mixed with 5 ml of carbonate buffer, 50 mmol/l, pH 9.0, containing Na_2EDTA, 10 mmol/l, followed by 500 μl of N-(1-pyrene)-maleimide, 1 mmol/l.

Quantitative analysis of cysteinylglycine

To a 5-ml aliquot of urine were added 200 μl of Na_2EDTA, 0.13 mol/l, and 500 μl of dithiothreitol, 1 mol/l. Standard was prepared daily as an aqueous solution of cystinyldiglycine, 10 μmol/l, corresponding to a cysteinylglycine concentration of 20 μmol/l after reduction. A 5-ml volume of this solution was mixed with Na_2EDTA and dithiothreitol in the same way as urine. We then adjusted the pH of the sample to 6.0 with sodium hydroxide, 1 mol/l, or hydrochloric acid, 1 mol/l, and made up to a final volume of 6.0 ml with water. After mixing, the tubes were left at room temperature for 1.5 h. With hydrochloric acid, 6 mol/l, the pH was then adjusted to 1.5 ± 0.1 and water was added to a final volume of 6.2 ml. From this solution 1 ml was shaken horizontally for 5 min with 5 ml of ethyl acetate saturated with water. The phases were separated by centrifugation and the organic phase was discarded. After four-fold extraction the water phase was evaporated at 40° C in a Vortex-Evaporator (Buchler, Fort Lee, NJ, U.S.A.). Then 10 ml of carbonate buffer, 50 mmol/l, pH 9.0, containing Na_2EDTA, 10 mmol/l, were added, the pH was adjusted to 9.0 ± 0.1 with sodium hydroxide, 1 mol/l, and the volume made up to 10.2 ml with water. From this solution 5 ml were derivatized with 500 μl of N-(1-pyrene)maleimide, 1 mmol/l, and HPLC was performed as described below.

Derivatization and high-performance liquid chromatography

After mixing of sample and standard solutions with derivatization reagent at pH 9.0 in tubes with PTFE-lined screw-caps, the tubes were placed in a water-bath at 37° C for approximately 20 h before five-fold dilution with the mobile phase and loop injection on the HPLC column. In all experiments we used a flow-rate of 1 ml/min. In initial studies we used a column temperature of 35° C, which gave satisfactory separation. However, at this temperature a peak from remaining dithiothreitol, used in the final method, interfered with the cysteinylglycine peak. Separation of these peaks was accomplished by lowering the temperature to 25° C.

RESULTS

Chromatography of reduced urine

Fig. 2 shows a chromatogram of thiopropyl-Sepharose 6B-reduced urine. From earlier work we knew the identification of 3-mercaptolactate, N-acetylcysteine, mercaptoacetate and cysteine. Among the unknown peaks there was one peak eluting between N-acetylcysteine and mercaptoacetate with the same retention time as cysteinylglycine. Additional peaks were seen in the void volume and with retention times around 20 min.

Anion-exchange experiments

Cysteinylglycine is a dipeptide with amphoteric character, and thus its properties differ markedly from the properties of acid thiols. In contrast to these it is retained by a cation exchanger, and we found that it disappeared completely when reduced urine was purified for determination of acid thiols [6]. To purify cysteinylglycine in urine from acid thiols, thiopropyl-Sepharose-

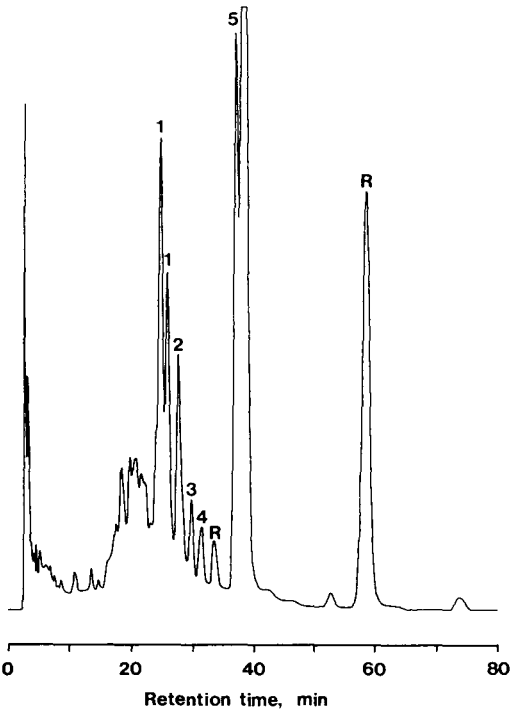


Fig. 2. Chromatogram of a urine sample after reduction with thiopropyl-Sepharose 6B. No prepurification was done before derivatization. Peak identification: 1 = 3-mercaptolactate as a double peak, 2 = N-acetylcysteine, 3 = cysteinylglycine, 4 = mercaptoacetate, 5 = cysteine as a double peak, and R = reagent peaks. Column temperature 35° C.

reduced urine was transferred to a PAMAS column, the column was washed with water, and elution was then effected by 3-mercaptopropionic acid. The sample adjusted to pH 7.0 was then transferred to an AG1-X4 column equilibrated with an imidazole buffer, 50 mmol/l, pH 7.0, a pH at which acid thiols like 3-mercaptopropionate should be retained. The first effluent together with 1 ml of washing with imidazole buffer was derivatized. On HPLC we found a chromatogram (Fig. 3) with only a few peaks, among them cysteinylglycine and cysteine. However, although cysteinylglycine was highly purified from acid thiols by this procedure the recoveries of cysteinylglycine were not reproducible. This may be due to incomplete recovery from the ion exchanger or to instability of cysteinylglycine at the high pH used in the reduction step. From other studies it has been claimed [12] that cysteinylglycine is subject to non-enzymatic oxidation at pH values above 7, and we therefore abandoned this procedure as a quantitative method for cysteinylglycine in urine.

Method for determination of cysteinylglycine in urine

Since cysteinylglycine is unstable at alkaline pH we finally chose to reduce urine with dithiothreitol at pH 6.0. Before the ensuing extraction of dithiothreitol with ethyl actate the pH was adjusted to 1.5, a pH at which cysteinylglycine remains in the water phase in contrast to weakly acid thiols like 3-mercaptolactate. In spite of four extractions, traces of dithiothreitol

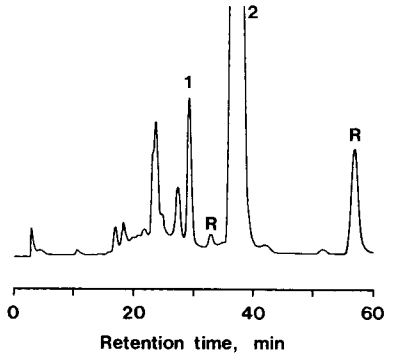


Fig. 3. Chromatogram of thiopropyl-Sepharose 6B-reduced urine purified on PAMAS and AG1-X4 columns. Peak identification: 1 = cysteinylglycine, 2 = cysteine, and R = reagent peaks. Column temperature 35° C.

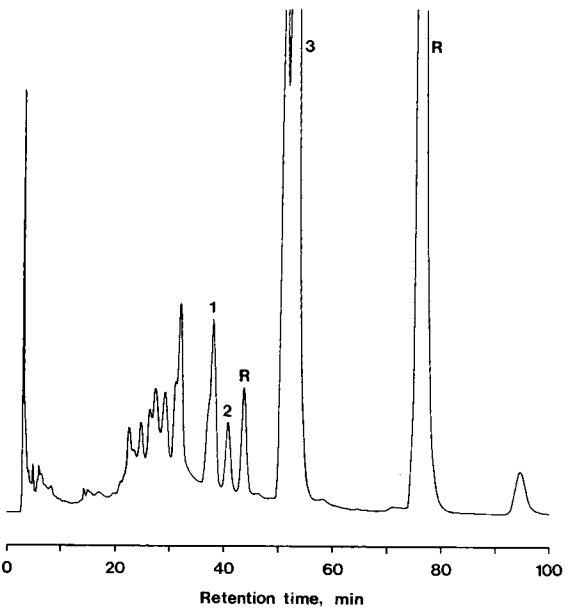


Fig. 4. Chromatogram of dithiothreitol-reduced urine. Peak identification: 1 = dithiothreitol, 2 = cysteinylglycine, 3 = cysteine, and R = reagent peaks. Column temperature 25° C.

appeared in the chromatogram close to cysteinylglycine by the standard HPLC procedure. This peak, however, was well separated from the cysteinylglycine peak when the chromatography was performed at 25° C instead of 35° C. The resulting chromatogram is shown in Fig. 4. Well resolved peaks for cysteinylglycine and cysteine were seen. This procedure was adopted for quantitative determination of cysteinylglycine in urine.

Precision and recovery of the quantitative method

Duplicate determinations were performed on seven urine samples. They had a mean cysteinylglycine concentration of 6.3 μmol/l. From the duplicate determinations the S.D. was calculated as 0.41 μmol/l, which gave a C.V. of

6.5%. Recovery was determined from urinary samples to which cystinyldiglycine was added to increase the cysteinylglycine concentration by 20 μmol/l. The recovery was determined as 86 ± 6.4%.

Normal values

Urine samples were collected from eight healthy members of staff. They had a mean urinary excretion of 7.4 ± 2.3 μmol per 24 h. The range was 5.0 to 10.8 μmol per 24 h.

DISCUSSION

By the experiments presented here we have identified cysteinylglycine as an endogenous compound in reduced urine. It may be of interest to summarize the criteria we have used to make this statement. Thus, with the HPLC we found that the retention time of authentic cysteinylglycine corresponded perfectly with the peak in question from urine. This was also confirmed as an increase of the peak when cysteinylglycine was added to the urine. The retention time also changed in a similar way for standard and the urinary compound when the temperature of the chromatography procedure was changed. Further, the compound was adsorbed by the organomercurial adsorbent used in the clean-up of other thiol compounds [6, 10] from urine, and was competitively eluted by 3-mercaptopropionate. Its reaction with N-(1-pyrene)maleimide is a similar sign of thiol property. The behaviour of the endogenous compound in cation- and anion-exchange experiments was as expected and similar to that of standard solution. Also pH-stability experiments performed (data not shown) showed identical properties for the endogenous compound and the standard.

With the present method the amount of cysteinylglycine is quantified as free thiol after reduction of disulphides in urine. The method thus determines the sum of free cysteinylglycine, cystinyldiglycine $(Cys\text{-}Gly)_2$ and cystinylglycine (mixed disulphide of cysteine and cysteinylglycine). Also other mixed disulphides of cysteinylglycine may be found in urine, and still other non-disulphide compounds which can be reduced to cysteinylglycine may exist in human urine.

Recently a scheme for the renal metabolism of glutathione was suggested by Jones et al. [4]. Glutathione is first oxidized to glutathione disulphide and thereafter a first γ-glutamyl residue is removed. This is effected by the enzyme γ-glutamyltransferase. Then either the remaining γ-glutamyl residue is removed, which gives cystinyldiglycine, or a glycine moiety is removed, leaving the disulphide of cysteine and glutathione (preferred pathway). Subsequent removal of a γ-glutamyl residue from the latter disulphide or removal of glycine from cystinyldiglycine gives cystinylglycine. Cleavage of the disulphide bridge of the latter compound or of cystinyldiglycine gives cysteinylglycine.

Hahn et al. [3] incubated radioactively labelled glutathione disulphide with renal tubules from rats. They clearly detected cystinyldiglycine, but they could not show the presence of cysteinylglycine in renal tissue after injection of glutathione. These findings are at variance with the findings of a cysteinylglycine dipeptidase [13], which may degrade cyst(e)inylglycine and cystinyldiglycine rapidly.

It has been shown that cystinylglycine is present in human serum at a concentration of about 10 μmol/l [5], but the compound was not demonstrated in urine. The findings in plasma were confirmed by Perry and Hansen [14], and they further showed that it was absent in plasma of a patient with pyroglutamic acidaemia, and its concentration was greatly reduced in homocystinuria. Small amounts (0.23 μmol/l) of cysteinylglycine have been reported to occur in human serum [15]. The present work shows the presence of cysteinylglycine or compounds that can be reduced to this compound in urine. From the foregoing discussion it is probable that it comes from degradation products of glutathione. Further studies will show the exact nature of the compound in urine.

ACKNOWLEDGEMENT

This work was supported by Grant No. B84-13X-05644-05A from the Swedish Medical Research Council.

REFERENCES

1 M.E. Anderson and A. Meister, J. Biol. Chem., 25 (1980) 9530—9533.
2 A. Meister, in H. Sies and A. Wendel (Editors), Functions of Glutathione in Liver and Kidney, Springer, New York, 1978, pp. 43—59.
3 R. Hahn, A. Wendel and L. Flohé, Biochim. Biophys. Acta, 539 (1978) 324—337.
4 D.P. Jones, P. Moldéus, A.H. Stead, K. Ormstad, H. Jörnvall and S. Orrenius, J. Biol. Chem., 254 (1979) 2787—2792.
5 M.D. Armstrong, Biochim. Biophys. Acta, 584 (1979) 542—544.
6 B. Kågedal and M. Källberg, J. Chromatogr., 229 (1982) 409—415.
7 M. Machida, N. Ushijima, M.I. Machida and Y. Kanaoka, Chem. Pharm. Bull., 23 (1975) 1385—1386.
8 L.A.AE. Sluyterman and J. Wijdenes, Biochim. Biophys. Acta, 200 (1970) 593—595.
9 L. Naftalin and L.R. Mitchell, Clin. Chim. Acta, 3 (1958) 197—199.
10 U. Hannestad and B. Sörbo, Clin. Chim. Acta, 95 (1979) 189—200.
11 K. Rootwelt, Scand. J. Clin. Lab. Invest., 19 (1967) 325—330.
12 S.S. Tate, in W.B. Jacoby (Editor), Enzymatic Basis of Detoxication, Vol. II, Academic Press, London, 1980, pp. 95—120.
13 A. Meister and S.S. Tate, Annu. Rev. Biochem., 45 (1976) 559—604.
14 T.L. Perry and S. Hansen, Clin. Chim. Acta, 117 (1981) 7—12.
15 R. Thachuk, Can. J. Biochem., 48 (1970) 1029—1036.

Journal of Chromatography, 308 (1984) 83—91
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2067

ANALYSIS OF HEPATIC REDUCED GLUTATHIONE, CYSTEINE AND HOMOCYSTEINE BY CATION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

EUGENE G. DEMASTER*, FRANCES N. SHIROTA, BETH REDFERN, DAVID J.W. GOON and HERBERT T. NAGASAWA

Medical Research Laboratories, Veterans Administration Medical Center, and the Department of Medicinal Chemistry, University of Minnesota, Minneapolis, MN 55417 (U.S.A.)

(First received October 26th, 1983; revised manuscript received December 28th, 1983)

SUMMARY

A high-performance liquid chromatographic method employing a mercury-based electrochemical detector and a cation-exchange column is described for the simultaneous measurement of reduced glutathione, cysteine, and homocysteine in liver homogenates. Sample preparation involves precipitation of protein with perchloric acid, removal of perchlorate by precipitation as its potassium salt and dilution with mobile phase. Mercaptoethylglycine is used as the internal standard. Using this procedure, the sum of the individual hepatic thiols agreed well with the total thiols determined with Ellman's reagent. Comparisons were made with (a) control rats, (b) rats depleted of hepatic thiols by pargyline pretreatment, and (c) rats administered L-cysteine.

INTRODUCTION

Interest in the role of reduced glutathione (GSH) in drug-induced hepatotoxicity [1] and the possible sparing effect of sulfhydryl-containing therapeutic agents and prodrug forms thereof on hepatic GSH stores [2—4], prompted us to evaluate and modify the high-performance liquid chromatographic—electrochemical detection method (HPLC—ED) for the simultaneous measurements of GSH, cysteine and homocysteine in liver homogenates. The application of HPLC—ED to the quantitation of biologically relevant sulfhydryl substances was first described by Rabenstein and Saetre [5] using a mercury-based detector. Although their initial report was limited to standard solutions, they [6—8] and others [9, 10] have subsequently used HPLC—ED

0378-4347/84/$03.00

for the determination of cysteine and homocysteine in plasma; D-penicillamine in whole blood, red blood cells, plasma and urine; and GSH in whole blood.

The HPLC—ED procedure was adapted for measurement of GSH and cysteine in brain and liver homogenates by Mefford and Adams [11]. They used graphite rather than mercury for the working electrode. Although graphite or glassy carbon can be used as an electrode for oxidizing thiols, mercury is the preferred electrode material for thiol detection. The operating potential of the latter is significantly lower [12]. Differences in the operating potential are based on the half-reactions which occur at their respective electrode surfaces, viz., $2\ RSH \rightarrow RSSR + 2H^+ + 2e^-$ (carbon), and $2\ RSH + Hg \rightarrow Hg(SR)_2 + 2H^+ + 2e^-$ (mercury). Consequently, electrochemically active substances such as uric acid and ascorbate are detected and can interfere with thiol analysis using graphite but not mercury electrodes [12].

In our initial attempts to use cation-exchange HPLC—ED for quantitation of thiols in protein-free supernatants prepared from rat liver with perchloric acid and other protein precipitants, a rapid and apparent irreversible loss of column affinity for thiols was observed with these biological samples, but not with identically prepared standards. In this report, we describe a modified HPLC—ED method for simultaneous separation and quantitation of the individual sulfhydryl compounds in liver homogenate using the mercury-based detector. Chromatographic anomalies with liver-derived samples have been eliminated by changing the sample preparation procedure. The method requires minimal sample preparation, and utilizes mercaptoethylglycine (MEG) as an internal standard.

MATERIALS AND METHODS

Chemicals

Reduced glutathione, L-cysteine, pargyline · HCl all from Sigma (St. Louis, MO, U.S.A.), and DL-homocysteine from ICN Pharmaceuticals (Cleveland, OH, U.S.A.) were used as received. Mercaptoethylglycine was prepared as previously described [13].

Animals

Male, random-bred rats of Sprague—Dawley descent from BioLab, (St. Paul, MN, U.S.A.) weighing 200—260 g were used. Food was withdrawn 16 h before sacrifice by decapitation. The livers were perfused in situ through the portal vein with 20 ml of isotonic saline, then excised and weighed.

Sample preparation

A 0.5-g section of the lower median lobe was removed from the liver and homogenized in 2.5 ml of a cold 0.3 *M* perchloric acid—5 m*M* EDTA solution. The homogenate was centrifuged at 12,000 *g* for 15 min resulting in a clear supernatant. Of the protein-free supernatant 1 ml was mixed with an equal volume of a solution containing 0.15 *M* dipotassium hydrogen phosphate, 0.01 *M* tripotassium citrate and 5 m*M* tetrasodium EDTA. The mixture was maintained at 0°C for 30 min and then centrifuged for 15 min at 12,000 *g* to remove the potassium perchlorate precipitate. For analysis, 0.1—0.4 ml of the

perchlorate-free supernatant depending on the anticipated levels of thiols, 0.1 ml of 1 m*M* mercaptoethylglycine (internal standard in 5 m*M* EDTA, and 0.1 ml of 0.2 m*M* N-acetylcysteine were added to 1.4—1.7 ml of mobile phase for a final volume of 2.0 ml. After bubbling with nitrogen to remove dissolved oxygen, 20 μl of the final solution were injected onto the column.

HPLC system

Separation of the sulfhydryl compounds was carried out using a 50 × 0.2 cm glass column dry packed with Zipax® SCX (Dupont, Wilmington, DE, U.S.A.). A precolumn (5 × 0.2 cm) with the same packing was used. The precolumn was changed after every 500 biological samples. The mobile phase composed of 0.01 *M* citric acid and 0.01 *M* disodium hydrogen phosphate was adjusted to pH 2.1 with metaphosphoric acid (ionic strength equalled approximately 0.038 *M*), then filtered through a 0.45-μm Millipore® filter (Bedford, MA, U.S.A.) and degassed under vacuum for 10 min before use. An oxygen-free eluent was maintained by keeping the mobile phase reservoir at 50—55°C and by continuously flushing the head space of the reservoir with nitrogen, deoxygenated by bubbling through a chromous chloride solution (Fisher Scientific, Fairlawn, NJ, U.S.A.).

The samples were injected onto the column using a 20-μl loop sample injector (Rheodyne, Model 7125, Berkeley, CA, U.S.A.). A flow-rate of 0.7 ml/min was maintained using a Milton Roy Model 396 minipump (Riviera Beach, FL, U.S.A.). The electrochemical detection system consisted of an LC-4 amperometric detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) and a thin-layer transducer (Model TL-6A, Bioanalytical Systems) composed of a Au/Hg working electrode and a glassy carbon auxillary electrode. The potential of the working electrode was 0.0 V versus an Ag/AgCl reference electrode [5]. Between injections a 10-sec cleansing voltage (—0.20 V) was applied to the working electrode [5]. Failure to apply the cleansing voltage between samples resulted in a gradual decline in detector response. The peak areas were integrated using a Hewlett-Packard Model 3390A integrator (Palo Alto, CA, U.S.A.).

Spectrophotometric method

Total sulfhydryls were determined using Ellman's reagent according to the method of Buttar et al. [14]. The same homogenization procedure was used as described under Sample preparation.

Calculations and statistical analysis

Standards containing GSH, cysteine and homocysteine prepared in 0.3 *M* perchloric acid—5 m*M* EDTA were carried through the sample preparation procedure. The following equation was used for calculating the results:

$$\text{Concentration of unknown} = \frac{(\text{Peak area ratio of unknown/MEG}) \times 6.0\ (= \text{dilution factor}) \times \text{concentration of external standard}}{\text{Peak area ratio of external standard/MEG}}$$

The results were expressed as mean ± S.E.M. of triplicate samples unless indicated otherwise. Statistical analyses were carried out using the Student's

t test, paired or unpaired, as appropriate. Differences were considered significant for p values < 0.05.

RESULTS

Homogenization media and chromatography

A typical chromatogram for a combined standard containing GSH, cysteine and homocysteine is shown in Fig. 1A. The citrate—phosphate mobile phase, pH 2.1, is a modification of that used by others [5, 9, 11] with a Zipax SCX column for separation of sulfhydryl compounds. N-Acetylcysteine was included to mask a negative peak [5] which interfered with the electronic integration of the GSH peak area. MEG was present as the internal standard. The retention times for GSH, cysteine, homocysteine and MEG were 1.7, 2.6, 3.8 and 6.8 min, respectively.

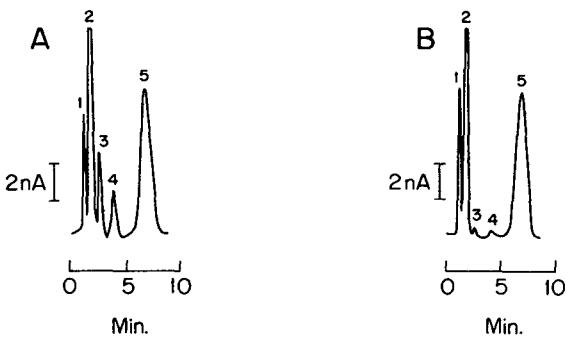


Fig. 1. HPLC—ED chromatograms of biologically significant sulfhydryl compounds in (A) a standard solution and (B) a sample prepared from rat liver. Peaks: (1) N-acetylcysteine; (2) GSH; (3) cysteine; (4) homocysteine; and (5) mercaptoethylglycine. N-Acetylcysteine (1) and mercaptoethylglycine (5) were added for analytical purposes (see text). The amounts of standard thiol injected were: GSH and mercaptoethylglycine, 1.0 nmol and cysteine and homocysteine, 0.05 nmol.

A number of deproteinizing agents including trichloroacetic acid (TCA), metaphosphoric acid, perchloric acid, sulfosalicylic acid and phosphotungstic acid were evaluated for their compatibility with the chromatography of standards. Of these, metaphosphoric acid (1.5%), TCA (5%), and perchloric acid (10%) appeared compatible. Standards in metaphosphoric acid could be injected directly onto the column, whereas standards in TCA and perchloric acid required prior diethyl ether extraction and perchlorate precipitation with tripotassium citrate, respectively, for unaltered chromatographic characteristics.

The chromatograms of the sulfhydryl compounds in protein-free supernatants from rat liver homogenates prepared with metaphosphoric acid, TCA and perchloric acid were compared. Before chromatography, the TCA samples were extracted with diethyl ether and perchlorate was precipitated from the perchloric acid samples by the addition of a potassium phosphate—citrate solution. The biological samples prepared in metaphosphoric acid and TCA caused a rapid deterioration of the cation-exchange column. One to five 20-μl

injections caused a pronounced decrease in retention times of all thiols. The loss of column affinity for the thiols in metaphosphoric acid and in diethyl ether-extracted TCA biological samples was not observed with standards. The original retention times could not be restored by washing the column with water, 1 *M* trisodium phosphate or 1 *M* orthophosphoric acid or a combination thereof. No column deterioration was observed with biological samples prepared with perchloric acid as described. Fig. 1B shows a typical chromatogram of the sulfhydryl compounds from liver.

Standard curves

The standard curves for GSH, cysteine, homocysteine and MEG were linear over a range of 0—1.0 nmol injected. Using MEG as the internal standard, the peak area ratios of individual standards prepared over a concentration range of 0—2 m*M* (and carried through the sample preparation procedure described in Materials and methods) versus the theoretical amount of standard injected, viz., 0 to 1.0 nmol, also gave linear plots.

Recovery and sample storage

The recoveries of GSH, cysteine and homocysteine standards carried through the sample preparation procedure were 100%. The calculated recoveries for externally added GSH, cysteine and homocysteine from a pooled biological sample were 96, 94 and 77%, respectively (Table I).

The effect of storage at −70°C on the GSH content of protein-free and perchlorate-free supernatants prepared from rat liver homogenates is shown in Fig. 2. The relative GSH content of the biological samples and of the identically prepared GSH standards were similar up to eleven days of storage. A minimum recovery of 89% was observed. Although the presence of perchlorate did not significantly affect the recovery of GSH in either the stored biological samples or GSH standard solutions, it is prudent to store biological samples perchlorate-free.

TABLE I

RECOVERY OF GSH, CYSTEINE AND HOMOCYSTEINE FROM PROTEIN-FREE SUPERNATANTS OF RAT LIVER HOMOGENATES

Protein-free supernatants were prepared from liver homogenates from two rats. To 5-ml aliquots of the supernatants (n = 4) were added 3.0 nmol GSH, 1.5 nmol cysteine or 1.5 nmol homocysteine. The protein-free supernatants with and without additions were analyzed for sulfhydryl content by HPLC—ED. The results were compared against data obtained using a combined standard solution of GSH, cysteine and homocysteine. Other experimental details are given under Materials and methods.

Sulfhydryl	Concentration (μmol/g wet weight liver)		Found after addition	Percent recovery (%)
	Initial	Calculated after addition		
GSH	3.72 ± 0.07	7.32	7.19 ± 0.05	96
Cysteine	0.059 ± 0.008	1.86	1.75 ± 0.03	94
Homocysteine	0.109 ± 0.005	1.91	1.50 ± 0.04	77

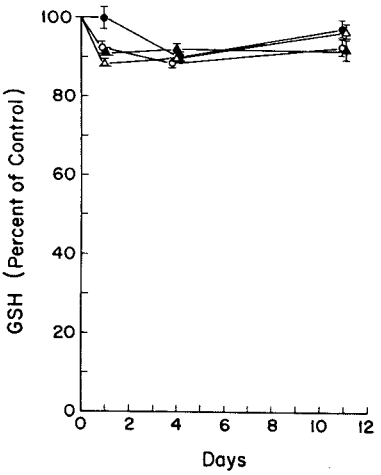


Fig. 2. Effect of storage at −70°C on GSH levels in protein-free and potassium perchlorate-free supernatants prepared from liver homogenates. The livers from two rats were individually homogenized in 0.3 *M* perchloric acid—5 m*M* EDTA, pooled and processed as described in Materials and methods. A GSH standard (3.0 m*M*) was treated identically to the biological samples. The protein-free and perchlorate-free supernatants prepared from the pooled liver homogenate and the GSH standard were stored in 2.0-ml aliquots, deaerated by bubbling with nitrogen and sealed for storage. The data represent mean ± S.E.M. of triplicate samples compared to freshly prepared GSH standards. The initial hepatic GSH concentration was 3.26 ± 0.03 μmol per g wet weight tissue. Protein-free supernatants from liver homogenate (○) and corresponding GSH standard (△); perchlorate-free supernatants from liver homogenate (●) and corresponding GSH standard (▲).

Comparison of hepatic sulfhydryls by HPLC—ED and spectrophotometric methods in control and drug-treated rats

Liver homogenates were prepared from isotonic saline (control), pargyline and L-cysteine pretreated rats. The hepatic GSH, cysteine and homocysteine levels were determined in these animals using the described HPLC—ED method (Table II). Pargyline is known to decrease hepatic GSH levels [15] and L-

TABLE II

HEPATIC GSH, CYSTEINE AND HOMOCYSTEINE LEVELS IN RATS PRETREATED WITH SALINE, PARGYLINE OR L-CYSTEINE

The drugs were administered, intraperitoneally, 1 h before sacrifice. Levels are given in μmol per g wet weight liver.

Drug	*n*	HPLC—ED method				Chemical method	*p***
		GSH	Cysteine	Homocysteine	Total thiols*	Total thiols	
Saline control	6	4.07 ± 0.21	0.050 ± 0.032	0.087 ± 0.006	4.20 ± 0.21	4.67 ± 0.23	N.S.
Pargyline (1.0 mmol/kg)	6	0.53 ± 0.14 (<0.001)***	0.023 ± 0.007 (N.S.)	0.099 ± 0.008 (N.S.)	0.66 ± 0.14 (<0.001)	0.83 ± 0.14 (<0.001)	N.S.
L-Cysteine (1.25 mmol/kg)	5	5.04 ± 0.33 (<0.05)	0.183 ± 0.025 (<0.01)	0.075 ± 0.010 (N.S.)	5.30 ± 0.31 (<0.05)	5.61 ± 0.29 (<0.05)	N.S.

*Equals sum of the individual thiols. None of the data are corrected for recovery.
**Total thiols for the two methods are compared using the paired Student's *t* test. N.S. = non-significant.
***The *p* values in parentheses represent statistical comparisons against the control within each column.

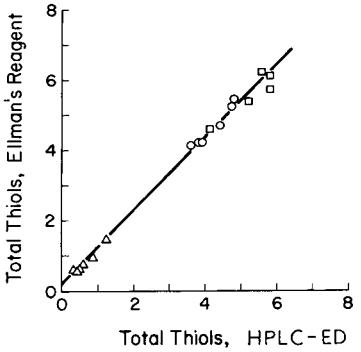


Fig. 3. Comparison of the total hepatic thiols measured by the spectrophotometric versus HPLC—ED method. The data are from Table II. Control (○), pargyline (△) and cysteine (□) treated animals.

cysteine is a biochemical precursor of GSH. In the control animals, GSH constituted nearly 97% of the total hepatic sulfhydryl content, whereas GSH was reduced to approximately 80% of the total in the pargyline-treated animals. Administration of L-cysteine caused a 20% increase in hepatic GSH and 266% increase in cysteine levels, over controls.

In these same groups of animals, the total hepatic sulfhydryl content measured by HPLC—ED, i.e., the sum of GSH, cysteine and homocysteine, was compared with the total sulfhydryl content measured by a spectrophotometric method using Ellman's reagent (Table II). For each group of animals, the total thiols measured by these two methods were not statistically different. Furthermore, the correlation curve comparing these two sets of data was linear with a slope of 1.04 and a correlation coefficient of 0.996 (Fig. 3).

DISCUSSION

Methods development

The attractive features of HPLC—ED and the mercury-based electrode for the measurement of hepatic sulfhydryl substances include (a) facile sample preparation, (b) high detector sensitivity for thiols, and (c) detector insensitivity toward electrochemically inactive substances present in biological samples. The lower operating potential of the mercury compared to the carbon electrode provides additional detector selectivity [12]. Other HPLC procedures utilizing spectrophotometric detectors contain one or more derivatization steps and are characterized by long elution times [16, 17].

Our application of HPLC—ED to the analysis of hepatic thiols is based on the earlier work of others [5, 8, 9, 11]. The resolution of GSH, cysteine and homocysteine was optimized by altering the pH and ionic strength of citrate—phosphate eluents while retaining short elution times. MEG which has a sulfhydryl functional group was selected as the internal standard based on its optimal elution time.

A major problem encountered was finding a protein-precipitating agent which did not alter the chromatographic characteristics. Of those evaluated, metaphosphoric acid, TCA, and perchloric acid appeared compatible when

used with standard thiol solutions. However, the removal of excess reagents, TCA by diethyl ether extraction and perchlorate by precipitation as its potassium salt, was required before analysis. Analysis of protein-free supernatants of biological samples prepared with metaphosphoric acid or TCA — even after extraction with diethyl ether — caused a nearly complete loss of column affinity. The cation-exchange column could not be regenerated with either acid or base. Protein-free supernatant prepared with perchloric acid followed by removal of perchlorate could be injected directly onto the column without column deterioration or changes in the chromatographic characteristics of the thiols when compared with chromatograms of standard thiol solutions.

Hepatic thiol levels and application

The sum of the individual hepatic thiols determined by HPLC—ED, viz., the sum of GSH, cysteine and homocysteine levels, is in good agreement with the total thiols determined spectrophotometrically using Ellman's reagent (Table II, Fig. 3). However, the HPLC—ED values are consistently slightly lower suggesting that the total thiols measured using Ellman's reagent may include an additional sulfhydryl component.

GSH constituted more than 95% of the total thiols in the liver of control rats, whereas GSH still represented about 80% of total thiols in the GSH-depleted livers of pargyline-treated animals (Table II). These results suggest that the measurement of total hepatic thiols using Ellman's reagent represents only an approximation of the actual GSH levels.

The application of this HPLC—ED method may be particularly relevant to studies on the biochemical mechanisms of GSH restoration in experimental animals following GSH-depletion by highly reactive metabolites derived from xenobiotic substances such as acetaminophen [18] and after therapeutic intervention with L-cysteine and/or its prodrug derivatives [2—4, 19].

ACKNOWLEDGEMENT

This research was supported by the Veterans Administration.

REFERENCES

1 L.F. Chasseaud, in C. Klein and R. Weinhouse (Editors), Advances in Cancer Research, Vol. 29, Academic Press, New York, 1979, p. 175.
2 H.T. Nagasawa, D.J.W. Goon, R.T. Zera and D.L. Yuzon, J. Med. Chem., 25 (1982) 489.
3 J.M. Williamson and A. Meister, Proc. Nat. Acad. Sci. U.S., 78 (1981) 936.
4 B.H. Lauterburg, G.B. Corcoran and J.R. Mitchell, J. Clin. Invest., 71 (1983) 980.
5 D.L. Rabenstein and R. Saetre, Anal. Chem., 49 (1977) 1036.
6 R. Saetre and D.L. Rabenstein, Anal. Biochem., 90 (1978) 684.
7 R. Saetre and D.L. Rabenstein, Anal. Chem., 50 (1978) 276.
8 D.L. Rabenstein and R. Saetre, Clin. Chem., 24 (1978) 1140.
9 R.F. Bergstrom, D.R. Kay and J.G. Wagner, J. Chromatogr., 222 (1981) 445.
10 F. Kreuzig and J. Frank, J. Chromatogr., 218 (1981) 615.
11 I. Mefford and R.N. Adams, Life Sci., 23 (1978) 1167.
12 L.A. Allison and R.E. Shoup, Anal. Chem., 55 (1983) 8.
13 H.T. Nagasawa, J.A. Elberling and E.G. DeMaster, J. Med. Chem., 23 (1980) 140.

14 H.S. Buttar, A.Y.K. Chow and R.H. Downie, Clin. Exp. Pharmacol. Physiol., 4 (1977) 1.
15 E.G. DeMaster, H.W. Sumner, E. Kaplan, F.N. Shirota and H.T. Nagasawa, Toxicol. Appl. Pharmacol., 65 (1982) 390.
16 D.J. Reed, J.R. Babson, P.W. Beatty, A.E. Brodie, W.W. Ellis and D.W. Potter, Anal. Biochem., 106 (1980) 55.
17 J. Reeve, J. Kuhlenkamp and N. Kaplowitz, J. Chromatogr., 194 (1980) 424.
18 J.R. Mitchell, S.S. Thoregeirsson, W.Z. Potter, D.J. Jallow and H. Keiser, Clin. Pharmacol. Ther., 16 (1974) 676.
19 E.C. deFerreyra, O.M. DeFenos, A.S. Bernacchi, C.R. DeCastro and J.A. Castro, Toxicol. Appl. Pharmacol., 48 (1979) 221.

Journal of Chromatography, 308 (1984) 93—100
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2061

ASSAY FOR CATECHOL-O-METHYLTRANSFERASE IN ERYTHROCYTES USING A NEW FLUOROGENIC SUBSTRATE, 2-(3,4-DIHYDROXYPHENYL)NAPHTHO[1,2-*d*]THIAZOLE

HITOSHI NOHTA, SHUNYA NOMA and YOSUKE OHKURA*

Faculty of Pharmaceutical Sciences, Kyushu University 62, Maidashi, Higashi-ku, Fukuoka 812 (Japan)

and

BEONG-TAE YOO

Department of Pharmacy, College of Science, Chungnam National University, Gung-dong, Chung-ku, Daejeon, Chungnam 300-31 (Korea)

(First received October 3rd, 1983; revised manuscript received December 21st, 1983)

SUMMARY

A highly sensitive method for the assay of catechol-O-methyltransferase in erythrocytes is described, which employs high-performance liquid chromatography with fluorescence detection. A newly synthesized catechol compound, 2-(3,4-dihydroxyphenyl)naptho[1,2-*d*]-thiazole is used as a highly fluorogenic substrate for catechol-O-methyltransferase; the *m*- and *p*-methylated products formed enzymatically from the substrate under the optimum conditions, after extraction with *n*-hexane—chloroform, are separated by normal-phase chromatography on LiChrosorb Si 100. The limits of detection for *m*- and *p*-methylated products are 3 pmol per assay tube (60 fmol per injection volume of 20 μl) in each case. The ratio of *m*- and *p*-methylated products was 0.54. This method requires as little as 50 μl of human erythrocytes.

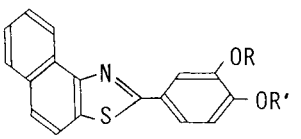
INTRODUCTION

Catechol-O-methyltransferase (COMT, EC 2.1.1.6) occurs in many tissues of mammals and catalyzes the transfer of the methyl group of S-adenosyl-L-methionine (SAM) to one of the phenolic groups of a catechol compound [1]. COMT plays an important role in the metabolism of catecholamines, and the

0378-4347/84/$03.00

relationship between its activity in human erythrocytes and some diseases has therefore been investigated [2].

Many assay methods for COMT in biological materials have been reported: spectrophotometry [3—6], fluorimetry [1, 7—10], high-performance liquid chromatography (HPLC) with electrochemical [11] and ultraviolet [12] detection, and radioisotopic methods [13—18]. COMT activity in human erythrocytes is very low and can be assayed only by the radioisotopic methods. The methods, however, require radioisotopic substrate or SAM and do not permit the determination of the ratios of two O-methylated products. In the previous paper, we reported an assay method for COMT which allowed the determination of the product ratios [20]. This is based on the quantification of vanillin and isovanillin (*m*- and *p*-O-methylated products, respectively) formed enzymatically from the substrate 3,4-dihydroxybenzaldehyde by derivatizing these products with 2,2′-dithiobis(1-aminonaphthalene) (DTAN, a selective fluorogenic reagent for aromatic aldehydes [19]) to highly fluorescent 2-(3-methoxy-4-hydroxyphenyl)naphtho[1,2-*d*]thiazole (*m*-MNT; Fig. 1) and 2-(3-hydroxy-4-methoxyphenyl)naphtho[1,2-*d*]thiazole (*p*-MNT; Fig. 1), respectively, extracting the compounds into *n*-hexane—chloroform and separating the extract by normal-phase HPLC on LiChrosorb Si 100 [20]. The sensitivity of the method permits the assay of COMT in various tissues, but is not sufficient for assay of the enzyme in erythrocytes.



R=R′=H : DNT

R=CH_3, R′=H : m-MNT

R=H, R′=CH_3 : p-MNT

Fig. 1. Chemical structures of DNT, *m*-MNT and *p*-MNT.

We have synthesized 2-(3,4-dihydroxyphenyl)naphtho-[1,2-*d*]thiazole (DNT; Fig. 1) as a highly fluorogenic substrate for COMT by the reaction of 3,4-dihydroxybenzaldehyde with DTAN, and thus developed an HPLC method for the assay of the enzyme in erythrocytes. *m*- and *p*-MNT (*m*- and *p*-O-methylated products) formed enzymatically from DNT under the optimum conditions are determined as in the previous method. A COMT preparation from human erythrocytes was employed to establish the assay procedure.

EXPERIMENTAL

Material and reagents

All chemicals were of reagent grade, unless otherwise noted. Deionized and distilled water was used. 3,4-Dihydroxybenzaldehyde was recrystallized from toluene. DTAN was purchased from Dojindo Laboratory (Kumamoto, Japan). *m*-MNT and *p*-MNT were prepared as previously described [20].

Preparation of DNT

To 100 mg of 3,4-dihydroxybenzaldehyde dissolved in 100 ml of water was added DTAN solution (200 mg of DTAN, 30 ml of methanol, 0.4 ml of tri-*n*-butylphosphine and 50 ml of 6 *M* sulphuric acid). The mixture was stirred at room temperature for 3 h. Yellow resinous substance was extracted with ethyl acetate, and the solvent was removed in vacuo. The residue was crystallized from *n*-hexane—ethyl acetate (about 9:1, v/v) to give yellowish needles (m.p. 116—117°C) which seemed to have crystal solvent. The crystals were further purified by sublimation in vacuo at 100—110°C, and yielded colourless needles (m.p. 212°C; yield 20 mg). Analytical data were as follows. Calc. for $C_{17}H_{11}O_2NS$: 69.61% C, 3.78% H, 4.78% N. Found: 69.50% C, 3.73% H, 4.79% N. Mass spectrum: *m/z* 293 (M^+, base peak), 247 ($M^+ - H_2O - CO$), 246 ($M^+ - H_2O - CHO$). ^{1}H-NMR spectrum δ (ppm): 6.48—7.92 (9 H, complex multiplet, aromatic protons), 8.93 (2H, broad doublet, hydroxyl protons, disappeared on adding a small amount of heavy water). IR spectrum: ν_{max} 3440 (OH), 1595 (aromatic C=C and/or C=N).

COMT preparation from human erythrocytes

Venous blood (5 ml) obtained from a normal volunteer was collected into a 10-ml glass tube containing 100 μl of 0.1 *M* EDTA · 2Na. The blood was centrifuged for 10 min at 1500 *g* and the plasma removed. The erythrocytes were washed twice with isotonic phosphate buffer (pH 7.4) and stored at −75°C until used. A 1-ml volume of the erythrocytes was lysed with 9.0 ml of ice-cold water and centrifuged at 1500 *g* for 10 min. The supernatant was used as the COMT preparation.

Apparatus

The column for HPLC was prepared by packing LiChrosorb Si 100 (particle size 5 μm; Japan Merck, Tokyo, Japan) into a stainless-steel tube (150 × 4 mm I.D.) by the slurry technique using tetrabromoethane—tetrachloroethylene (3:2, v/v) as solvent.

A Hitachi 635A liquid chromatograph was used, equipped with a Rheodyne 7125 syringe-loading sample injection valve (20-μl loop) and a Hitachi 650-10S spectrofluorimeter fitted with a 20-μl flow-cell operating at an emission wavelength of 390 nm and an excitation wavelength of 348 nm; spectral bandwidths of 10 nm and 5 nm, respectively, were used. Uncorrected fluorescence excitation and emission spectra were measured with a Hitachi MPF-4 spectrofluorimeter in quartz cells (optical pathlength, 10 × 10 mm); spectral bandwidths of 5 nm were used in both the excitation and emission monochromators. The mass spectrum was measured with a JEOL JMS-01-SG mass spectrometer. The ^{1}H-NMR spectrum was obtained with JEOL JNM-PS-110 spectrometer at 100 MHz using an approximately 15% (w/v) solution in dimethylsulfoxide-d_6 containing tetramethylsilane as internal standard. The infrared (IR) spectrum was taken with a Nihonbunko DS 701G spectrometer using a potassium bromide pellet. The melting points are uncorrected.

Procedure for COMT assay (procedure A)

To 0.5 ml of the enzyme preparation (corresponding to 50 μl of

erythrocytes) were added 200 μl each of 0.1 *M* phosphate buffer (pH 6.5) and 0.5 m*M* SAM and 50 μl of 40 m*M* magnesium chloride. The mixure was preincubated at 37° C for 5—15 min, then incubated again at 37° C for 30 min after the addition of 50 μl of 1 m*M* DNT in isopropanol—water (1:1, v/v). The reaction was stopped by adding 1.0 ml of 0.2 *M* hydrochloric acid. To the mixture, 1.0 ml of *n*-hexane—chloroform (4:1, v/v) was added and the resulting *m*- and *p*-MNT were extracted with mechanical shaking (approximately 300 rpm) for 10 min. A 20-μl volume of the upper organic layer was injected into the chromatograph. The organic layer could be used for more than a week when stored at room temperature (approximately 20° C).

The mobile phase comprised 25 m*M* acetic acid in *n*-hexane—chloroform (4:1, v/v), and the flow-rate was 2.0 ml/min. Column temperature was ambient (20—25° C).

For the blank, the same procedure was carried out except that DNT solution was added after the addition of 0.2 *M* hydrochloric acid. To prepare the calibration curves, 50 μl of DNT solution were replaced with 50 μl of DNT solution containing *m*- and *p*-MNT (each 0.05—1.0 nmol per 50 μl) and the same procedure as for the blank was carried out.

The peak heights in the chromatogram were used for the quantification of *m*- and *p*-MNT.

Procedure for COMT assay in the presence of pyrogallol (procedure B)

To 0.5 ml of the enzyme preparation were added 200 μl of 0.1 *M* phosphate buffer (pH 6.5), 100 μl of $5 \cdot 10^{-5}$ *M* pyrogallol and 50 μl each of DNT solution and 40 m*M* magnesium chloride. The mixture was preincubated at 37° C for 5 min, then incubated again at 37° C for 30 min after the addition of 100 μl of 1 m*M* SAM, and then treated in the same way as in procedure A.

RESULTS AND DISCUSSION

The optimum pH values for the enzymatic *m*- and *p*-methylation reactions were 6.5 and 6.5—8.0, respectively (Fig. 2A). The pH affects the product ratio of *m*- and *p*-MNT and the ratio increases with increasing pH, particularly in the pH range 6.0—8.5 (Fig. 2B); pH 6.5 was used in the standard procedure.

Maximum and constant velocities of the *m*- and *p*-methylation reactions were attained in the presence of 20—70 μ*M* DNT in the incubation mixture; DNT at a concentration greater than 70 μ*M* caused inhibition of the enzyme (Fig. 3). The Michaelis constant (K_M) values for DNT were 2.1 ± 0.5 μ*M* and 3.2 ± 0.6 μ*M* (mean ± S.D.) when measured on the basis of the formation of *m*- and *p*-MNT, respectively (n = 7 in each case). This indicates that the affinity of COMT is fairly high for DNT. Therefore, 50 μ*M* DNT was used as saturating concentration in the procedure. DNT is hardly soluble in water, so it had to be dissolved in isopropanol. The alcohol did not affect COMT activity at a concentration range of 0.5—5% (v/v) in the incubation mixture; aqueous isopropanol (1:1, v/v) was used for the preparation of DNT solution (the concentration in the incubation mixture was 2.5%).

Almost maximum and constant velocities of the *m*- and *p*-methylation reactions were achieved over SAM concentrations of 50—200 μ*M*, with the K_M

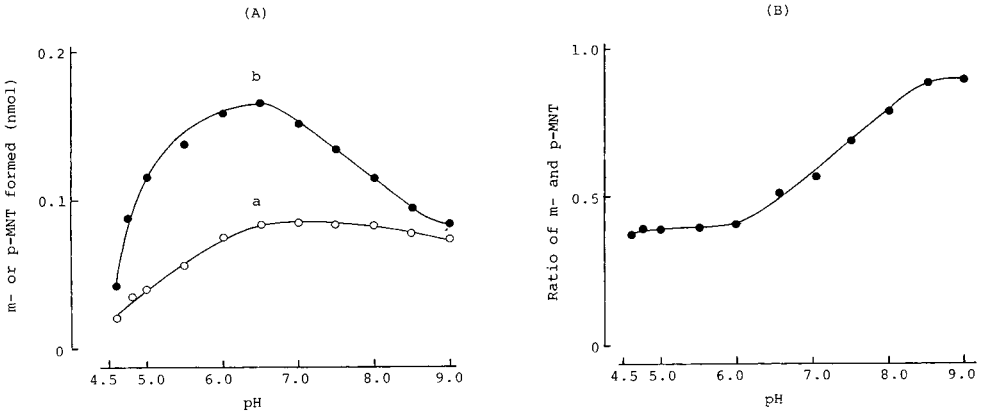


Fig. 2. Effect of pH on (A) the amounts of *m*- and *p*-MNT formed and (B) the ratio of *m*- and *p*-MNT. Curves: a = *m*-MNT; b = *p*-MNT. Buffers: pH 4.6—5.0, 0.1 *M* acetate buffer; pH 5.0—8.0, 0.1 *M* phosphate buffer; pH 8.0—9.0, 0.1 *M* Tris—hydrochloric acid buffer.

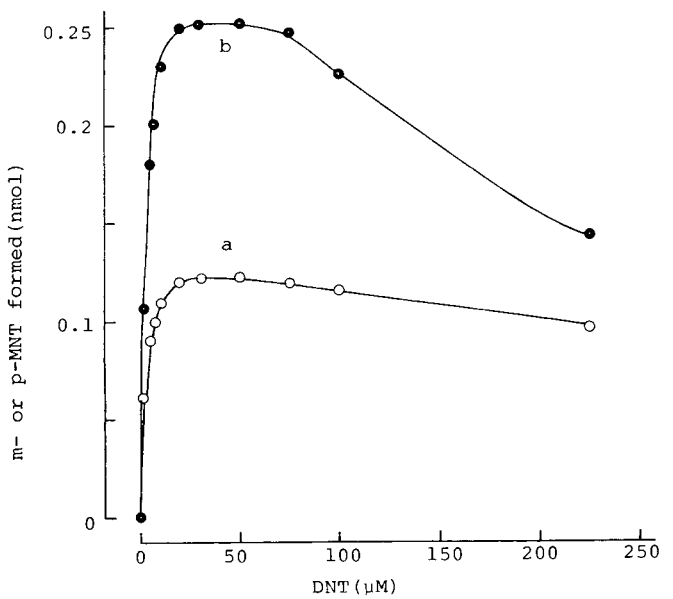


Fig. 3. Effect of DNT concentration on the amounts of (a) *m*-MNT and (b) *p*-MNT formed.

value at 3.0 ± 0.2 μM (mean ± S.D.) in both the methylation reactions; 100 μM SAM was used for convenience. Magnesium chloride gave maximum activity at 2 m*M* in the incubation mixture. Calcium ion has been described to inactivate human erythrocyte [17] and rat liver [21] COMT. In our study, however, the removal of the ion from the enzyme preparation by adding Chelex-100 (particulate chelating agent of divalent cations; Bio-Rad, Richmond, CA, U.S.A.) according to the method of Raymond and Weinshilboum [17] had no effect on the enzyme activity.

The amounts of *m*- and *p*-MNT formed enzymatically were proportional to the volume of erythrocytes in the enzyme preparation up to at least 200 μl

when the enzyme reaction mixture was incubated at 37° C for 30 min (Fig. 4), and to the incubation time up to at least 120 min; 50 μl of erythrocytes and 30-min incubation were used in the procedure.

Hydrochloric acid (0.2 *M*) was used for the termination of the enzyme reaction, otherwise *m*- and *p*-MNT could not be reproducibly extracted with *n*-hexane—chloroform mixture. When the incubation mixture was deproteinized with perchloric acid or trichloroacetic acid, the recovery of *m*- and *p*-MNT in the organic layer was almost nil, probably due to the coprecipitation of the products with the denatured protein.

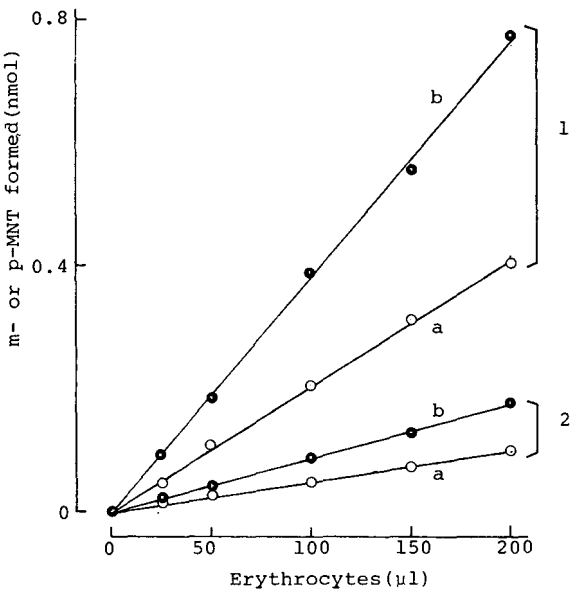
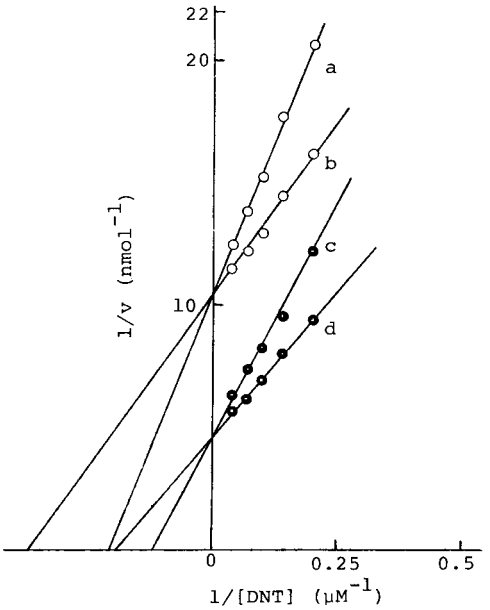


Fig. 4. Effect of the volume of erythrocytes obtained from two healthy men (1 and 2) on the amounts of (a) *m*-MNT and (b) *p*-MNT formed.

Pyrogallol was reported to be a potent inhibitor of COMT [22—24], and thus its inhibitory action was examined according to procedure B. Pyrogallol inhibited COMT in a competitive mode against DNT (Fig. 5) and the observed inhibitory constant value calculated according to the method of Dixon [25] was 7 m*M* in both the *m*-and *p*-O-methylation reactions. This value is almost identical to that obtained with rat liver COMT using a natural substrate, norepinephrine [24].

The HPLC conditions in this method were the same as those in the previous method [20]. Fig. 6 shows a typical chromatogram obtained using procedure A. No peak was observed in the chromatogram of the blank. The eluates from peaks 1 and 2 in Fig. 6 had fluorescence excitation (maximum, 349 and 347 nm, respectively) and emission (maximum, 393 and 389 nm, respectively) spectra that were identical with those of authentic *m*- and *p*-MNT, respectively, dissolved in the mobile phase.

DNT dissolved in the mobile phase had only a weak fluorescence with an excitation maximum at 350 nm and an emission maximum at 404 nm; the



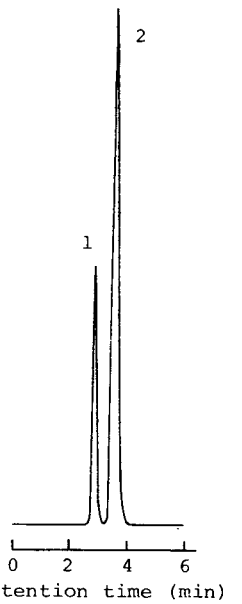


Fig. 5. Inhibition of human erythrocyte COMT by pyrogallol, examined according to procedure B. The data were plotted by linear regression analysis. Lines: a and b, *m*-MNT; c and d, *p*-MNT. Pyrogallol in the incubation mixture: a and c, 5 m*M*; b and d, absent.

Fig. 6. Chromatogram obtained with COMT preparation (50 μl of human erythrocytes per 0.5 ml) according to procedure A. Peaks: 1 = *m*-MNT; 2 = *p*-MNT. COMT activity: 6.2 nmol *m*-MNT and 11.6 nmol *p*-MNT per ml erythrocytes per h.

intensity was less than 1% of that of *m*- or *p*-MNT. DNT was strongly retained on the silica gel column for HPLC and did not elute with the mobile phase. However, the column was deactivated when the sample solution was repeatedly applied on to it for a long time. This was probably caused by small amounts of water contained in the sample solutions. In this case, DNT eluted and interfered with the quantification of *m*- and *p*-MNT. Therefore, the column should be washed every 300—500 injections of the sample by passing approximately 100 ml of chloroform containing a small amount of acetic acid (0.15%, v/v) through it. The retention times for *m*- and *p*-MNT were reproducible even in the deactivated column.

The recoveries of *m*- and *p*-MNT added to the incubated mixture of the blank in amounts of 0.25 nmol were 67.3 ± 2.1% and 71.2 ± 1.6% (mean ± S.D., n = 12 in each case), respectively. The lower limits of detection for *m*- and *p*-MNT formed enzymatically were both 3 pmol per assay tube (60 fmol per injection volume of 20 μl). The precision was established with respect to repeatability. The coefficients of variation were 2.0% and 1.9% for mean activity of 2.7 nmol *m*-MNT and 5.2 nmol *p*-MNT per ml erythrocytes per h, respectively (n = 14).

COMT activities in normal human erythrocytes assayed by the present method were 5.6 ± 1.6 nmol *m*-MNT and 10.5 ± 3.2 nmol *p*-MNT per ml erythrocytes per h (mean ± S.D., n = 12); the product ratio of *m*- and *p*-MNT was 0.54 ± 0.02.

This study provides the first method for the assay of COMT in erythrocytes that does not use radioisotopic substrate or cofactor. This method may also permit the assay of COMT in preparations obtained from other tissues such as liver, kidney, brain and heart muscle; the results will be published elsewhere. This method is simple and precise, and should be useful for biological and biomedical investigations.

REFERENCES

1 J. Axelrod and R. Tomchick, J. Biol. Chem., 233 (1958) 702.
2 H.C. Guldberg and C.A. Marsden, Pharmacol. Rev., 27 (1975) 135.
3 P.J. Anderson and A. D'Iorio, Can. J. Biochem., 44 (1966) 347.
4 J.K. Coward and F. Y-Hsiueh Wu, Anal. Biochem., 55 (1973) 406.
5 W.F. Herblin, Anal. Biochem., 51 (1973) 19.
6 R.T. Borchardt, Anal. Biochem., 58 (1974) 382.
7 M. Assicot and C. Bohuon, Life Sci., 8 (1969) 93.
8 O.J. Broch, Jr. and H.C. Guldberg, Acta Pharmacol. Toxicol., 30 (1971) 266.
9 H. Thomas, J. Veser and D. Müller-Enoch, Hoppe-Seyer's Z. Physiol. Chem., 357 (1976) 1347.
10 Y. Okada, K. Zaitsu, K. Ohtsubo, H. Nohta and Y. Ohkura, Chem. Pharm. Bull., 29 (1981) 1670.
11 R.T. Borchardt, M.F. Hegazi and R.L. Schowen, J. Chromatogr., 152 (1978) 255.
12 E.J.M. Pennings and G.M.J. Van Kempen, Anal. Biochem., 452 (1979) 98.
13 J. Axelrod, W. Albers and C.D. Clemente, J. Neurochem., 5 (1959) 68.
14 R.E. McCaman, Life Sci., 4 (1965) 2353.
15 M. Assicot and C. Bohuon, Nature (London), 213 (1966) 861.
16 G.W. Bates, C.D. Edman, J.C. Porter, J.M. Johnston and P.C. Macdonald, Clin. Chim. Acta, 94 (1979) 63.
17 F.A. Raymond and R.M. Weinshilboum, Clin. Chim. Acta, 58 (1975) 185.
18 H. Masayasu, M. Yoshioka and Z. Tamura, Clin. Chim. Acta, 27 (1979) 633.
19 Y. Ohkura, K. Ohtsubo, K. Zaitsu and K. Kohashi, Anal. Chim. Acta, 99 (1978) 317.
20 K. Zaitsu, Y. Okada, H. Nohta, K. Kohashi and Y. Ohkura, J. Chromatogr., 211 (1981) 129.
21 R.M. Weinshilboum and F.A. Raymond, Biochem. Pharmacol., 25 (1976) 573.
22 Z.M. Bacq, L. Gosselin, A. Dresse and J. Renson, Science, 130 (1959) 453.
23 J. Axelrod and M.J. LaRoche, Science, 130 (1959) 800.
24 J.R. Crout, Biochem. Pharmacol., 6 (1961) 47.
25 M. Dixon, Biochem. J., 55 (1953) 170.

Journal of Chromatography, 308 (1984) 101—109
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2065

DETECTION OF AN ABNORMAL LIPOPROTEIN IN A LARGE COLONY OF PEDIGREED BABOONS USING HIGH-PERFORMANCE GEL EXCLUSION CHROMATOGRAPHY

MARY CARR WILLIAMS

Southwest Foundation for Biomedical Research, San Antonio, TX 78284 (U.S.A.)

JIM LEE KELLEY

The University of Texas Health Science Center, San Antonio, TX 78284 (U.S.A.)

and

RAMPRATAP SINGH KUSHWAHA*

Southwest Foundation for Biomedical Research, P.O. Box 28147, San Antonio, TX 78284 (U.S.A.)

(First received October 20th, 1983; revised manuscript received January 3rd, 1984)

SUMMARY

High-performance gel exclusion chromatography was used to detect an abnormal lipoprotein in a large colony of baboons. Serum obtained from fasting baboons was adjusted to density 1.21 g/ml and ultracentrifuged to obtain lipoproteins. A small fraction (equivalent to 50 μl serum) was separated using a gel filtration column (TSK 4000 SW) or a combination of TSK 4000 PW and TSK 3000 PW columns. The unusual lipoprotein was detected either as a distinct peak between low- and high-density lipoproteins or as a distinct shoulder to the high-density lipoprotein peak. In some baboons the unusual lipoprotein was present on both chow and atherogenic diet, but in most cases it was induced by feeding an atherogenic diet.

INTRODUCTION

The composition and levels of plasma lipoproteins in humans and animals are affected by a number of genetic and environmental factors. Recently a special class of lipoproteins has been detected in some baboons upon feeding a diet rich in cholesterol and saturated fats. These abnormal lipoproteins accumulate in the density region between low-density (LDL) and high-density (HDL) lipo-

0378-4347/84/$03.00

proteins corresponding to a flotation rate of $F^{\circ}_{1.20}$ 9—28 and, therefore, have been designated as F° 9—28 lipoproteins by Nichols et al. [1]. The lipid and apolipoprotein compositions of these abnormal lipoproteins are not well characterized; however, they seem to be rich in apolipoprotein A-I and E. The F° 9—28 phenotype appears to be genetically transmitted.

Plasma lipoproteins differ in their hydrated densities [2], electrophoretic mobilities [3,4], and particle size [5]. Based on these characteristics a number of methods have been used for the separation and characterization of the plasma lipoprotein profile of an individual. Among these methods are the preparative ultracentrifuge technique using sequential density centrifugation [6], the vertical rotor [7], or density gradient ultracentrifugation [8,9], low-pressure gel permeation chromatography using agarose as the separation medium [10,11], and electrophoretic techniques [12—14]. Although the preparative ultracentrifuge, column chromatographic, and electrophoretic techniques are very reliable for a limited number of samples, they cannot be used to screen a large number of animals. Since high-performance liquid chromatography (HPLC) using gel permeation columns of the TSK-Gel type has been shown to be effective for rapid profiling of human [15—20] and monkey [21] plasma lipoproteins, we applied and further developed this technique for this study.

MATERIALS AND METHODS

Animals and diets

A group of pedigreed baboons consisting of 400—500 animals was used for these studies. These animals varied from 1 to 10 years of age. Animals were first screened while they were on the chow diet (Ralston Purina, St. Louis, MO, U.S.A.). They were again screened on an atherogenic diet, rich in cholesterol and saturated fat, at the seventh and eighth week. The composition of these diets is given in Table I.

TABLE I

COMPOSITION OF CHOW AND ATHEROGENIC DIETS

Nutrients	Chow diet*	Atherogenic diet**
Carbohydrates (% kcal)	62	39
Protein (% kcal)	28	21
Fat (% kcal)	10	40
Energy (kcal per 100 g diet)	329	377
Cholesterol (mg/kcal)	0.03	1.7

* Baboon chow manufactured by Ralston Purina.

** Atherogenic diet was prepared by mixing 79.1% (dry weight basis) of Purina monkey meal 25-5045-6 (a special mix with no added fat, dehydrated alfalfa, sodium chloride, ascorbic acid or retinyl acetate) with dried egg yolk (4.8%), lard (14.3%), sodium chloride (1.1%), retinyl acetate (0.001%), ascorbic acetate (0.1%), and cholesterol (0.5%).

Isolation and characterization of standard lipoproteins

For isolation of standard lipoproteins, blood was drawn from fasting animals in tubes containing EDTA (1 mg/ml). The plasma was separated using low-speed centrifugation. Lipoproteins were isolated by sequential ultracentrifugation (Beckman L5-50) as described by Kushwaha and Hazzard [22] using densities for very-low-density lipoproteins (VLDL), LDL, and HDL as 1.006, 1.019—1.063, 1.063—1.21 g/ml, respectively, using a 50.3 Ti rotor. The densities were adjusted using solid potassium bromide. The lipoproteins (upper 2.5-ml layer) were obtained by slicing the tube. To detect if the lipoprotein fractions isolated by the sequential ultracentrifugation were pure fractions, their electrophoretic mobility and apoproteins were characterized. The lipoproteins had single bands corresponding to their characteristic electrophoretic mobility [4] on agarose gel electrophoresis. The apoproteins were characterized by separating delipidated lipoproteins [23] using 3.5% and 10% polyacrylamide gel electrophoresis (PAGE) containing 0.1% sodium dodecyl sulfate (SDS) [24]. The VLDL and LDL fractions had apo-B as their major protein, while HDL had apo-A-I and apo-A-II as its major proteins. Albumin was present in HDL in minor amounts even after washing once.

Preparation of lipoproteins for HPLC separation

Fasted animals (16—20 h) were bled to obtain 3 ml of blood in a tube containing EDTA (1 mg/ml). The blood was centrifuged in a low-speed refrigerated centrifuge to separate plasma. A 0.5-ml aliquot of plasma was adjusted to a density of 1.21 g/ml and ultracentrifuged (Beckman L5-50 and L3-50) at a speed of 125,000 *g* for 24 h at 6° C. The lipoproteins were harvested by carefully pipetting the upper 1.5-ml fraction.

Chromatographic (HPLC) separations of lipoproteins

The apparatus consisted of a Waters Assoc. Model 204 liquid chromatograph, a Model 6000A pump, with a high-sensitivity noise filter in line, a Model 440 UV detector, and a WISP autosampler. This was coupled with a Perkin-Elmer Model Sigma 15 integrator for plotting and integration.

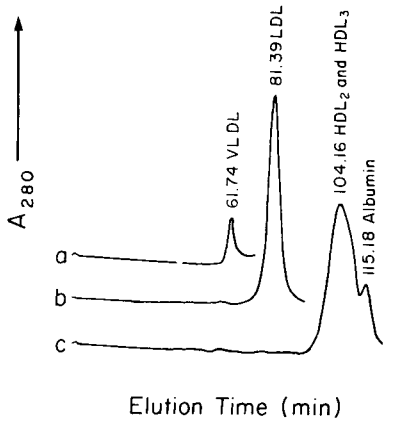
The 0.1 *M* sodium phosphate buffer, pH 7.0, containing 0.1 *M* sodium sulfate and 0.05% sodium azide was filtered through 0.45-μm Millipore filters and degassed. The 0.2 *M* Tris—acetate buffer, pH 7.0, containing 0.05% sodium azide was filtered and degassed prior to use. Initial separation of lipoproteins was performed using a gel permeation column TSK 4000 SW (Kratos, 600 × 7.5 mm). Later studies were conducted by using a TSK 4000 PW (600 × 7.5 mm) followed by a TSK 3000 PW (600 × 7.5 mm). Both column set-ups were preceded by the appropriate guard column TSK GSWP or GPWP (Kratos, 100 × 7.5 mm). Using the phosphate or Tris—acetate buffer at pH 7.0 with a flow-rate of 0.2 ml/min, proteins were detected by monitoring absorbance at 280 nm (attenuation of 3 using Perkin-Elmer Sigma 15 recorder—integrator) and a chart speed of 0.1 cm/min. Samples were applied automatically in a total volume of 150 μl equivalent to 50 μl serum or plasma. The autosampler was used for a 24-h operation.

RESULTS

HPLC separation of ultracentrifugally isolated standard lipoprotein fractions

Lipoprotein standards were isolated by sequential ultracentrifugation and were washed once by diluting with a potassium bromide solution of equivalent density and ultracentrifuging under similar conditions as described above for isolation of standard lipoproteins. These fractions gave a single peak when separated by HPLC (Fig. 1). VLDL had a retention time of 61.74 min (corresponding to an elution volume of 12.35 ml). LDL and HDL had retention times of 81.39 and 104.16 min, respectively. The HDL fraction had mainly HDL_2 and therefore, the retention time of 104.16 min corresponds to the HDL_2 fraction. The HDL fraction was contaminated with albumin (even after washing) as judged by SDS-PAGE [24] and, therefore, gave another HPLC peak corresponding to albumin (retention time of 115.18 min).

A number of other standards with different molecular weights were sep-



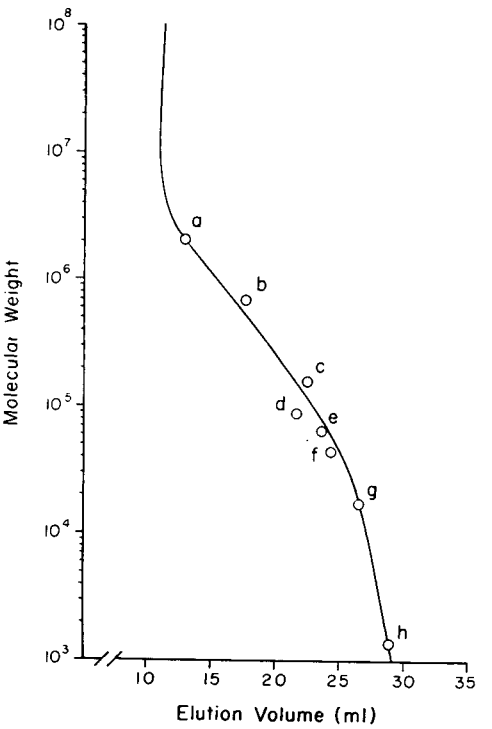


Fig. 1. HPLC elution patterns of the A_{280} for baboon lipoproteins isolated by sequential ultracentrifugation. Column, TSK 4000 SW (600 × 7.5 mm) + GSWP guard column (100 × 7.5 mm); eluent, 0.2 *M* Tris—acetate buffer, pH 7.0; flow-rate, 0.2 ml/min. (a) VLDL (density <1.006 g/ml); (b) LDL (density = 1.019—1.063 g/ml); and (c) HDL_2, HDL_3, and albumin (density = 1.063—1.21 g/ml).

Fig. 2. Relationship between molecular weight and elution volume (× 5 elution time). HPLC conditions were the same as Fig. 1. Reference compounds: (a) blue dextrin (2,000,000); (b) thyroglobulin (670,000); (c) gamma globulin (158,000); (d) transferrin (83,000); (e) baboon albumin (60,000); (f) ovalbumin (44,000); (g) myoglobulin (17,000); and (h) vitamin B_{12} (1350).

arated to determine the linear range of the TSK 4000 SW column. As shown in Fig. 2, the column gave a linear response between molecular weights $2 \cdot 10$ and $3.6 \cdot 10^4$ daltons corresponding to elution volumes between 12 and 25 ml. Elution volumes for plasma lipoproteins fell within the linear range.

Effect of phosphate buffer and Tris—acetate buffer on HPLC separation of lipoproteins

Initial studies were conducted using phosphate buffer. Under these conditions only 80—90 lipoprotein samples could be successfully separated. Afterwards, the column would retain most of the lipoproteins. Washing with organic solvents such as methanol, tetrahydrofuran, dimethylsulfoxide, and tetramethylurea did not regenerate the column. Smaller proteins (MW < 60,000), however, were still separated by this column.

To determine if a change in buffer would prolong the column life, we used the Tris—acetate buffer to separate the lipoproteins by HPLC. Use of the Tris—acetate buffer improved the resolution of lipoproteins and increased the column life span. Using the Tris—acetate buffer more than 300 samples were processed by a single column during the initial phases of this study. After the initial six months of this study (using twelve columns), the second group of columns (six) were not of the same quality as the earlier TSK 4000 SW columns. The absorption of lipoproteins occurred within the first 50—80 samples resulting in a very high cost per sample. At this point it was necessary to change to PW type columns as discussed by Carroll and Rudel [21]. A combination of TSK 4000 PW plus TSK 3000 PW gave adequate detection of the F° 9—28 lipoproteins. Again we were able to run at least 300 samples per column.

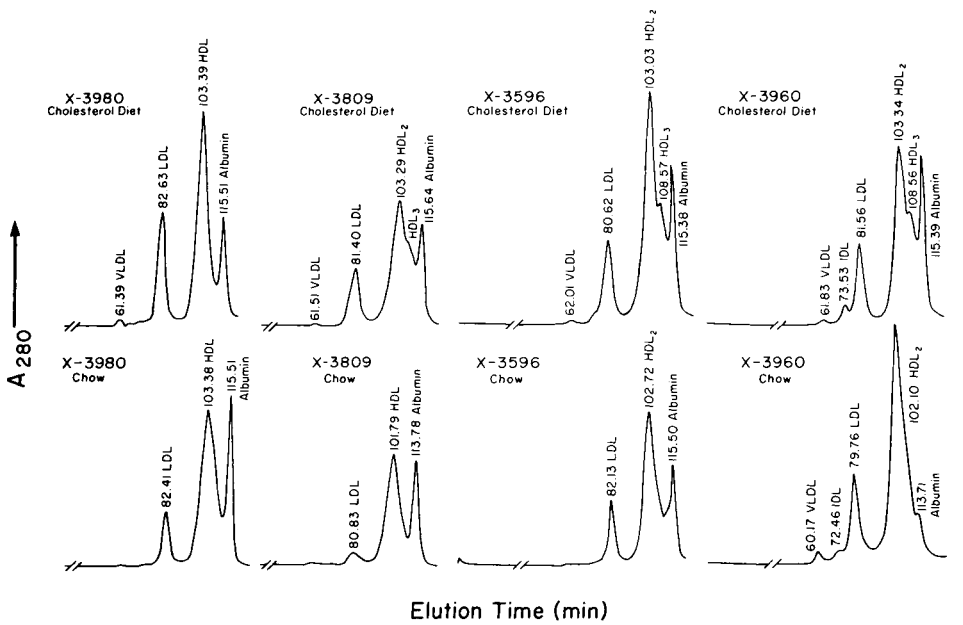


Fig. 3. Lipoprotein patterns from four representative animals, which do not show F° 9—28 lipoproteins on the chow (bottom) or atherogenic diet (top). Atherogenic diet has raised LDL and HDL levels.

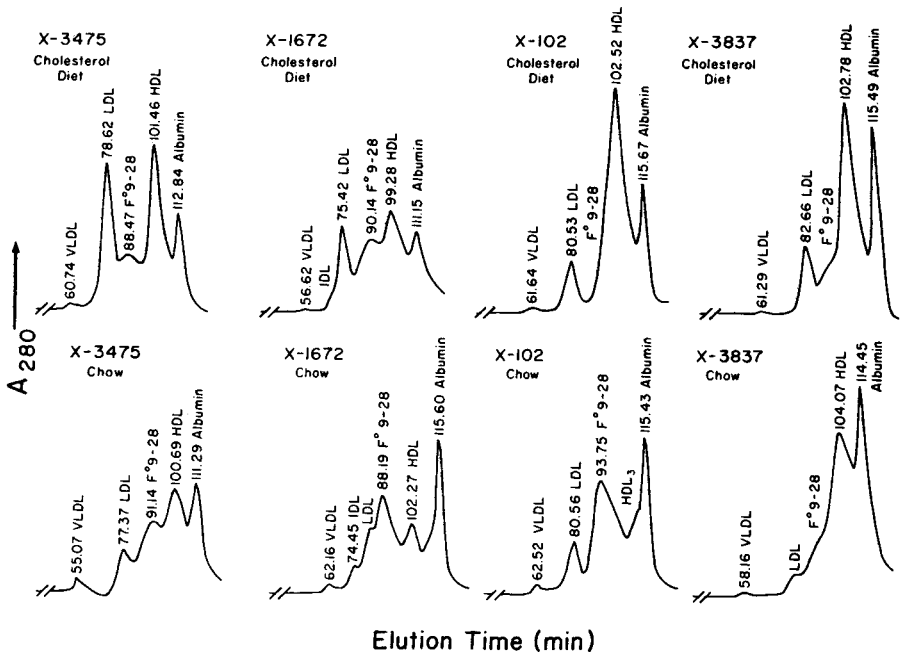


Fig. 4. Lipoprotein patterns from four representative animals, which show F° 9—28 lipoproteins on chow diet (bottom panel). F° 9—28 lipoproteins (elution time 88—91 min) appear either as a peak or as a shoulder to HDL towards LDL. Upon feeding atherogenic diet the F° 9—28 lipoproteins do not seem to be affected to a considerable extent (upper panel). LDL and HDL have been raised by feeding atherogenic diet as in case of animals without F° 9—28 lipoproteins.

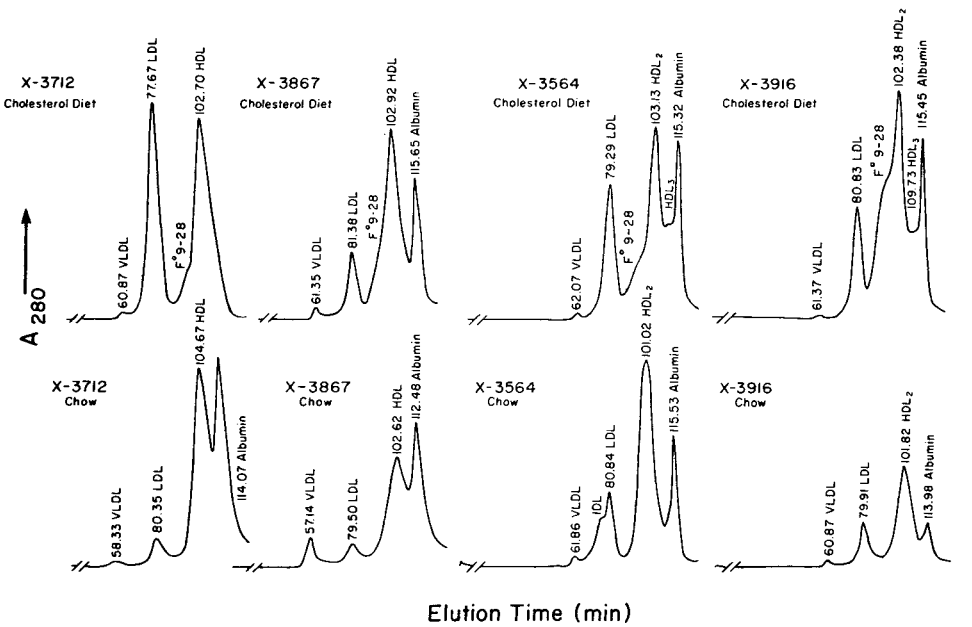


Fig. 5. Lipoprotein patterns from four animals that did not show high levels of F° 9—28 lipoproteins on chow diet (lower panel) but seem to induce these lipoproteins upon feeding atherogenic diet (upper panel).

Lipoprotein profiles of chow and atherogenic diet-fed animals

The majority of the animals on chow diet had lipoprotein profiles similar to those exhibited in Fig. 3. The major peak (A_{280}) corresponded to HDL_2. Varying amounts of LDL were present. VLDL was present in minute quantities, but was detectable in most animals. Since the lipoproteins (density <1.21 g/ml) were not washed, albumin was detectable in variable amounts. Upon cholesterol and saturated fat feeding their lipoprotein profiles changed. There was an increase in LDL and HDL with a distinct appearance of an HDL_3 fraction (elution time 108.19 min) in many animals. The VLDL concentration did not seem to change (Fig. 3).

As shown in Fig. 4, a distinct peak between LDL and HDL was detected in some animals which would correspond to the lipoprotein fraction between LDL and HDL_2 with a hydrated density F°_{i20} between 9 and 28. These F° 9—28 lipoproteins were present in variable amounts on both chow and the atherogenic diet. Some animals on the other hand did not exhibit these F° 9—28 lipoproteins on the chow diet, but they seem to appear upon feeding an atherogenic diet (Fig. 5).

DISCUSSION

F° 9—28 lipoproteins are abnormal lipoproteins and were previously detected by analytical ultracentrifugation in some of the baboons fed a diet high in saturated fats and cholesterol [1]. The results of the present study demonstrate that gel permeation HPLC using either TSK 4000 SW or a combination of TSK 4000 PW and TSK 3000 PW columns can detect this lipoprotein and is suitable for screening a large population of pedigreed baboons. This technique is simple and rapid and therefore could be used three times (once on a chow diet and twice (seventh and eighth weeks) on an atherogenic diet) for 400—500 baboons. The use of a Tris—acetate buffer, pH 7.0, improved the resolution. The TSK 4000 SW columns, however, cannot be used for large numbers of samples, because they start absorbing lipoproteins. Carroll and Rudel [21] have similarly reported that these columns absorb lipoproteins. A combination of TSK 4000 PW and TSK 3000 PW, however, gave similar results and would, therefore, be of choice for similar studies. Albumin was seen as a contaminant in lipoproteins and gave a separate peak with a retention time of 115.8 min. The presence of an albumin peak, however, enabled us to calculate the relative retention of various lipoproteins for the new or other sets of columns. This relative retention time for lipoproteins did not change in the various columns used.

The comparison of plasma lipoprotein profiles of baboons indicated that F° 9—28 lipoproteins were not only present in some baboons on an atherogenic diet as observed earlier [1], but they were also present in some baboons on a low-cholesterol chow diet. This special class of lipoproteins was detected either as a distinct peak between LDL and HDL or as a distinct shoulder to the HDL_2 peak. The F° 9—28 lipoproteins were present in some baboons on both the chow and the atherogenic diet, but in certain cases it seems to be induced by feeding the atherogenic diet.

HDL_C, a lipoprotein induced upon cholesterol feeding [25] and Lp(a) lipo-

protein or sinking prebeta lipoprotein [26] in humans have been shown to occur between the LDL and HDL density region. Since Lp(a) has an apo-B-like protein as its major protein, it would be different from F° 9—28 lipoproteins which have apo-A-I and E as their major apoproteins. The F° 9—28 lipoproteins may, however, be similar to HDL_C. Further characterization of these abnormal lipoproteins in baboons will determine if this is the case. One major difference between HDL_C and F° 9—28 lipoproteins, however, is that HDL_C is induced upon cholesterol feeding, whereas F° 9—28 lipoproteins have been seen on a low-cholesterol, low-fat chow diet. The application of this technique has provided the means of phenotyping for the F° 9—28 lipoproteins. The collection of these fractions from preparative samples would provide material for lipid and apoprotein characterization of this lipoprotein. The metabolic basis and genetic inheritance of this lipoprotein are presently being investigated.

ACKNOWLEDGEMENTS

Supported by NIH Grants HL-28972 and HL-25874, and NHLBI Contract No. NV-53030. The authors thank Cynthia Stenoien for technical help and Linda Clayton for typing the manuscript.

This paper was presented at the Second International Symposium on HPLC of Proteins, Peptides, and Polynucleotides, December 6—8, 1982, at Baltimore, MD, Abstract No. 210.

REFERENCES

1 A.V. Nichols, T.J. Kuehl, E.L. Gong, H.C. McGill, Jr., P. Blanche and T.M. Forte, Arteriosclerosis, 1 (1981) 89.
2 J.W. Gofman, F.T. Lindgren and H. Elliot, J. Biol. Chem., 179 (1949) 973.
3 G. Blix, A. Tiselius and H. Svensson, J. Biol. Chem., 137 (1941) 485.
4 R.L. Jackson, J.D. Morrisett and A.M. Gotto, Jr., Physiol. Rev., 56 (1976) 259.
5 P.N. Herbert, A.M. Gotto and D.S. Fredrickson, in J.B. Stanbury, J.S. Wyngaarden and D.S. Frederickson (Editors), The Metabolic Basis of Inherited Diseases, McGraw Hill Book Co., New York, 1978, p. 544.
6 R.J. Havel, H.A. Eder and J.H. Bragdon, J. Clin. Invest., 34 (1955) 1345.
7 B.H. Chung, T. Wilkinson, J.C. Geer and J.P. Segrest, J. Lipid Res., 21 (1980) 284.
8 D.W. Anderson, A.V. Nichols, T.M. Forte and F.T. Lindgren, Biochim. Biophys. Acta, 493 (1977) 55.
9 M.J. Chapman, S. Goldstein, D. Lagrange and P.M. Laplaud, J. Lipid Res., 22 (1981) 339.
10 L.L. Rudel, J.A. Lee, M.D. Morris and J.M. Felts, Biochem. J., 139 (1974) 89.
11 L.L. Rudel, D.G. Greene and R. Shah, J. Lipid Res., 18 (1977) 734.
12 R.W. Mahley and K.H. Weisgraber, Biochemistry, 13 (1974) 1964.
13 W. Neubeck, H. Wieland, A. Habenicht, P. Muller, G. Baggio and D. Seidel, Clin. Chem., 23 (1977) 1296.
14 G.R. Warnick, T. Nguyen, R.O. Bergelin, P.W. Wahl, and J.J. Albers, Clin. Chem., 28 (1982) 2116.
15 M. Okazaki, Y. Ohno and I. Hara, J. Chromatogr., 221 (1980) 257.
16 M. Okazaki, Y. Ohno and I. Hara, J. Biochem., 89 (1981) 879.
17 Y. Ohno, M. Okazaki and I. Hara, J. Biochem., 89 (1981) 1675.
18 D.L. Busbee, D.M. Payne, D.W. Jasheway, S. Carlisle and A.G. Lacko, Clin. Chem., 27 (1981) 2052.
19 I. Hara, K. Shiraishi and M. Okazaki, J. Chromatogr., 239 (1982) 549.

20 R. Vercaemst, M. Rossenue and J.P. Van Biervliet, J. Chromatogr., 276 (1983) 174.
21 R.M. Carroll and L.L. Rudel, J. Lipid Res., 24 (1983) 200.
22 R.S. Kushwaha and W.R. Hazzard, Biochim. Biophys. Acta, 619 (1980) 142.
23 B.E. Cham and B.R. Knowles, J. Lipid Res., 17 (1976) 176.
24 K. Weber and M. Osborn, J. Biol. Chem., 244 (1969) 4406.
25 R.W. Mahley., in J.M. Deitschy, A.M. Gotto, Jr. and J.A. Ontko (Editors), Disturbances in Lipid and Lipoprotein Metabolism, American Physiological Society, Bethesda, MD, 1978, p. 251.
26 J.A. Albers, V.G. Cabana, G.R. Warnick and W.R. Hazzard, Metabolism, 24 (1975) 1047.

Journal of Chromatography, 308 (1984) 111—119
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2102

THE SEPARATION OF COLLAGEN α-CHAINS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

COMPARISON OF COLUMN ALKYL STATIONARY PHASES AND TEMPERATURE EFFECTS

S.J.M. SKINNER*

Postgraduate School of Obstetrics and Gynaecology, National Women's Hospital, Claude Road, Epsom, Auckland 3 (New Zealand)

B. GREGO and M.T.W. HEARN

St. Vincent's School of Medical Research, Fitzroy, Melbourne 3065 (Australia)

and

G.C. LIGGINS

Postgraduate School of Obstetrics and Gynaecology, National Women's Hospital, Claude Road, Epsom, Auckland 3 (New Zealand)

(First received November 29th, 1983; revised manuscript received February 2nd, 1984)

SUMMARY

Procedures for the separation of α_1(I), α_2(I), α_1(II) and α_1(III) chains of human collagen by reversed-phase high-performance liquid chromatography are described. The influence of several different chromatographic parameters (stationary phase, mobile phase and temperature) has been examined and procedures to optimise resolution presented. These reversed-phase high-performance liquid chromatographic conditions also permit the separation of collagen α_1(I), α_2(I), α_1(II) and α_1(III) monomers from their corresponding dimeric β- and γ-components.

INTRODUCTION

The separation and quantitation of collagen α chains by polyacrylamide gel electrophoresis (PAGE) has remained the most convenient and practical

0378-4347/84/$03.00

method for many years [1—3]. Recent reports have suggested that high-performance liquid chromatography (HPLC) may provide a suitable alternative [4—6] with the added attraction of easy recovery prior to characterisation of the purified materials. The speed of separation and the preparative options available with reversed-phase HPLC for polypeptide fractionation are also attractive when compared with classical chromatographic methods [7, 8]. We report here a comparison of the performance of several different reversed-phase supports with water—acetonitrile eluents at both room temperature and at 62° C, for the separation of collagen α-chains. The identity and purity of the separated collagen chains were confirmed by PAGE and by amino acid analyses.

MATERIALS AND METHODS

Reagents

Acetonitrile was obtained from Waters Assoc. (Milford, MA, U.S.A.). Trifluoroacetic acid was obtained form Pierce (Rockford, IL, U.S.A.). Human collagen standards (Types I, II and III) were donated by Professor A.L. Bailey (Bristol, U.K.). Samples of ^{3}H-labelled Type I collagen were donated by Dr. J. Bateman (Melbourne, Australia). Other collagen samples were prepared in this laboratory by established techniques

Chromatographic equipment

Two gradient elution HPLC systems were used. The HPLC system from Waters Assoc. consisted of two Model M6000A pumps, a gradient programmer Model M660, a Model U6K injector, and a Model M450 variable-wavelength detector. The second HPLC system was a DuPont Model 850 liquid chromatograph (Wilmington, DE, U.S.A.) equipped with a heated column compartment. Detection was performed with a Micromeritics Model 786 variable-wavelength detector (Norcross, GA, U.S.A.). Chromatograms from both HPLC systems were displayed on either an Omniscribe dual-pen recorder (Houston Instruments, Austin, TX, U.S.A.) or a Hewlett-Packard Model 3390A integrator (Waldbronn, F.R.G.). Samples were injected with SGE syringes (SGE, Melbourne, Australia). The *n*-alkyl silica reversed-phase packing material consisted of LiChrospher silica, 10-μm particle diameter with a nominal 50-nm pore diameter, bonded with *n*-butyldimethylchlorosilane or *n*-octyldimethylchorosilane to a ligand density of approximately 2.8 μmol/m^2 followed by maximal end-capping (with hexamethyldisilazane) and slurry packed into stainless-steel columns (15 × 0.40 cm) using procedures described elsewhere [9, 10]. Bakerbond wide-pore diphenyl columns (25 × 0.46 cm) were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.).

The solvents used to chromatograph the collagen samples were water and water—acetonitrile (1:1), each containing 0.1% trifluoroacetic acid. New columns were equilibrated overnight in the aqueous solvent and prepared for chromatography by running 60-min gradients from 0 to 50% acetonitrile at 1 ml/min until a stable baseline was achieved. After each gradient run with different collagen samples the columns were equilibrated at initial conditions for 10 min.

Sample and chromatographic fraction treatment

The samples of Types I, II and III collagens were dissolved in acetic acid (0.5 *M*, 2 mg/ml) and diluted with an equal volume of 0.1% trifluoroacetic acid. The sample was heated to 50°C for 15 min and allowed to cool to room temperature. The sample was injected onto the column within 20 min of heating at 50°C.

The collagen fractions separated by HPLC were collected in glass tubes and evaporated under nitrogen to remove most of the acetonitrile. The aqueous material was then frozen and lyophilized.

Sodium dodecyl sulphate—polyacrylamide gel electrophoresis

Sodium dodecyl sulphate (SDS)—PAGE separation of collagens was carried out according to the method of Sykes et al. [1] as modified by Sodek and Limeback [3] with a 4% stacking gel and a 7.5% separating gel. The gels were stained according to the method of Fairbanks et al. [11] with Coomassie Blue R 250.

Amino acid analysis

Aliquots of fractions identified as α_1(I) and α_2(I) by SDS—PAGE were hydrolysed with 6 *M* hydrochloric acid for 24 h at 110°C. The hydrolysates were dried, dissolved in the starting buffer and analysed with a Durrum amino acid analyser, Model D502 (Palo Alto, CA, U.S.A.).

Quantitation of tritium

Fractions (1 ml) containing ^{3}H-labelled collagens were mixed with 10 ml Soluene (Packard Instruments, Downers Grove, IL, U.S.A.) and counted for a minimum of 4 min using a β-counter (Packard Tricarb).

RESULTS

Type I collagen

In preliminary experiments designed to optimise resolution and recovery, samples of human Type I collagen (20—500 μg) were chromatographed on the LiChrospher *n*-butyl and Bakerbond diphenyl columns at both 20°C and 62°C. In these experiments a 90-min linear gradient from 0 to 50% acetonitrile in 0.1% trifluoroacetic acid with a flow-rate of 1 ml/min at 20°C was used. Using these conditions the human Type I collagen samples eluted from the LiChrospher *n*-butyl column as two peaks (with retention times 46.4 ± 0.3 min and 51.5 ± 0.1 min over the sample loading range 20—500 μg) which contained the collagen chains α_1(I) and α_2(I) respectively (Fig. 1a). There was, however, contamination as assessed by SDS—PAGE of these main peaks by dimeric β- and trimeric γ-components. With the Bakerbond diphenyl column, using the same elution conditions, similar selectivity was observed (Fig. 1b). The presence of collagen chain dimers and trimers in many collagen preparations has been recognised previously [12]. The fact that these aggregates exhibit similar hydrophobicities as isolated collagen chains under the above reversed-phase conditions is thus not unexpected. However, by employing similar criteria for the optimisation of gradient elution conditions which have been

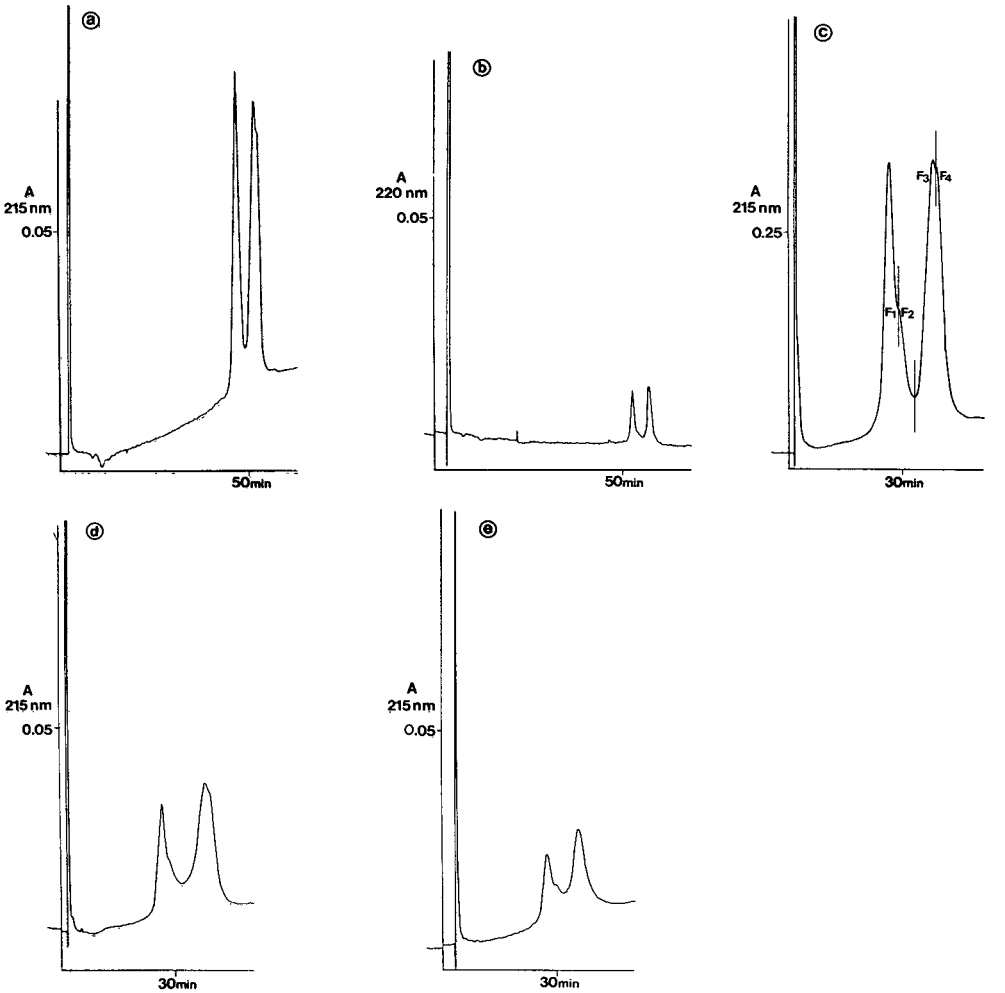


Fig. 1. The separation of type I collagen by HPLC using the *n*-butyl LiChrospher and the Bakerbond diphenyl columns at 20°C and at 62°C. The samples of Type I collagen were heated in 0.5 *M* acetic acid—0.1% trifluoroacetic acid (1:1) at 50°C for 15 min, cooled to 20°C and injected onto the column within 20 min of cooling. Solvent A was 0.1% trifluoroacetic acid in water. Solvent B was 0.1% trifluoroacetic acid in water—acetonitrile (1:1). The traces represent the chromatograms of: (a) 60 μg Type I collagen, linear gradient from 0 to 100% B in 90 min at 1 ml/min, butyl column, 20°C; (b) 60 μg Type I collagen, linear gradient from 0 to 100% B in 90 min at 1 ml/min, diphenyl column, 20°C; (c) 500 μg Type I collagen, linear gradient from 35 to 70% B in 70 min at 1.2 ml/min, butyl column, 62°C; (d) 60 μg Type I collagen, linear gradient from 35 to 70% B in 70 min at 1.2 ml/min, butyl column, 20°C; (e) 40 μg Type I collagen, linear gradient from 40 to 70% B in 50 min at 1 ml/min, diphenyl column, 20°C.

applied [13] to the separation of other high-molecular-weight proteins, improved resolution of Type I collagen chains could be obtained using the LiChrospher *n*-butyl column at 62°C. The elution conditions which were finally selected to take advantage of the large *s*-values [10] of collagen chains involved a shallower gradient, namely a 70-min linear gradient from 17.5 to 35% aceto-

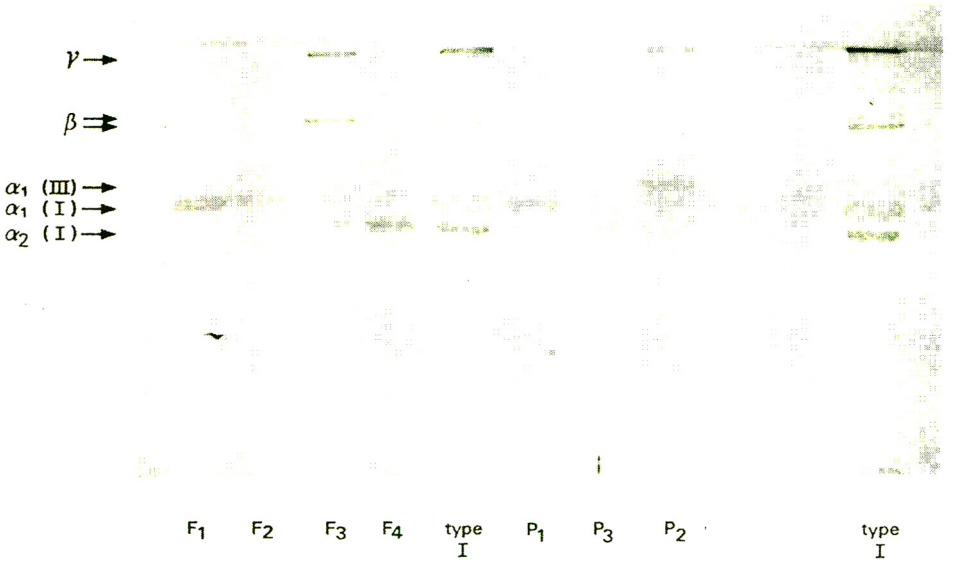


Fig. 2. SDS—PAGE of HPLC fractions of Type I and Type III collagens. SDS—PAGE was performed as described in Materials and methods. The fractions F_1—F_4 were collected from the separation of Type I collagen shown in Fig. 1c. The fractions P_1, P_2 and P_3 (not in sequence) were collected from the separation of Types I and III collagen shown in Fig. 3a.

TABLE I

AMINO ACID ANALYSIS OF α_1(I) AND α_2(I) ISOLATED BY HPLC (RESIDUES/1000 TOTAL RESIDUES)

	α_1(I)		α_2(I)	
	Found	Reported*	Found	Reported*
Aspartic acid	46.2	42	49.4	44
Threonine	17.2	16	19.5	19
Serine	37.2	34	39.3	30
Glutamic acid	73.4	73	73.7	68
Proline	114.9	124	104.1	113
Hydroxyproline	110.3	109	93.6	94
Glycine	332.6	333	321.0	338
Alanine	114.3	115	97.1	102
Half-cystine	—	0	—	0
Valine	21.1	21	34.6	35
Methionine	3.0	7	7.0	5
Isoleucine	10.3	6	20.5	14
Leucine	20.1	19	31.9	30
Tyrosine	—	1	—	4
Phenylalanine	11.6	12	9.6	12
Histidine	4.2	3	12.8	12
Lysine	26.0	26	20.2	18
Arginine	52.5	50	52.3	50
Hydroxylysines	5.1	9	13.6	12

*As reported by Miller and Gay [12].

nitrile in 0.1% trifluoroacetic acid at a flow-rate of 1.2 ml/min. With these conditions human Type I collagen was separated into several component peaks F_1—F_4 (Fig. 1c). The separated fractions were identified by SDS—PAGE (Fig. 2) and by amino acid composition (Table I) as α_1(I) (F_1), the dimeric β_{11}-component (F_2), the dimeric β_{12}- and trimeric γ-components (F_3) while F_4 was essentially pure α_2(I). The same separation carried out at 20°C gave a similar result (Fig. 1d). Using analogous optimisation procedures with the Bakerbond diphenyl column a comparable separation between the α_1(I) monomer and β_{11} dimer was obtained with a 50-min linear gradient from 20 to 35% acetonitrile in 0.1% trifluoroacetic acid at a flow-rate of 1 ml/min at 20°C. With these conditions the α_1(I) and α_2(I) peaks eluted at 28.0 ± 0.1 min and 36.6 ± 0.2 min, respectively. Isolation of the β_{11} dimer from the α_1(I) monomer could be achieved by careful peak shaving of the second eluting peak (Fig. 1e).

The recovery of the Type I collagen from the column was determined by running a sample of [^{3}H]Type I collagen. The majority of the radioactivity (74%) was eluted with the α_1(I) and α_2(I) peaks in a ratio of 2.3:1 which corresponds well to the expected ratio α_1(I):α_2(I) of 2:1.

Type III collagen

Type III collagen is composed of a trimer of α_1(III) chains which, unlike Type I and II collagens, is not dissociated into its monomeric components unless a sulfhydryl-reducing agent, such as β-mercaptoethanol, is present. The unreduced Type III trimer eluted between the α_1(I) and α_2(I) peaks of the type I collagen (Fig. 3a) on the LiChrospher *n*-butyl and *n*-octyl columns as well as on the Bakerbond diphenyl columns at both 20°C and 62°C. After reduction of Type III collagen with β-mercaptoethanol the α_1(III) monomer

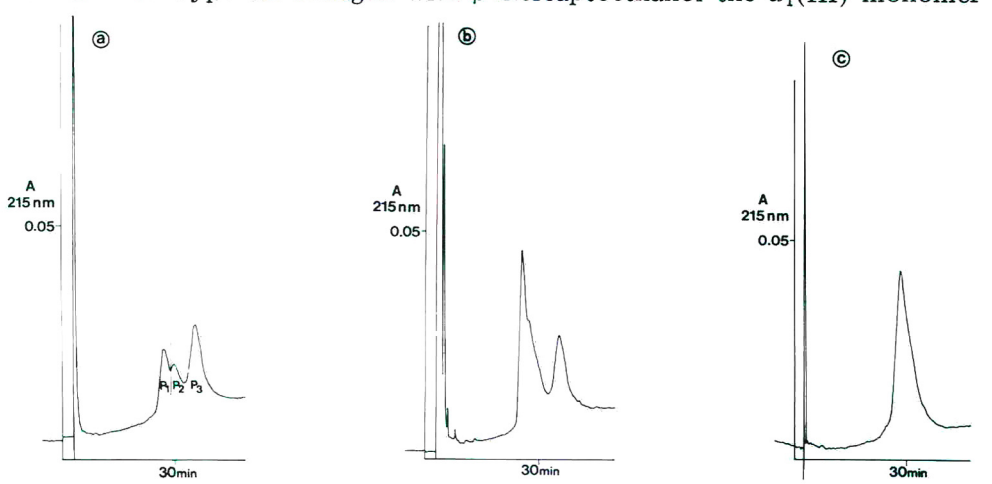


Fig. 3. The separation of types I, II and III (monomer and trimer) collagens. The samples were prepared as in Fig. 1 and were separated on the Bakerbond diphenyl column at 20°C with a linear gradient from 40 to 70% B in 50 min at 1 ml/min (as in Fig. 1e). Detection was at 215 nm. The chromatograms represent: (a) Types I and III collagens (40 μg of each), the fractions P_1, P_2 and P_3 were collected manually; (b) types I and III collagens (reduced with β-mercaptoethanol, 40 μg of each); (c) type II collagen (40 μg).

elutes as a sharply defined peak immediately before the α_1(I) peak (Fig. 3b). The identity of the fractions obtained from the run shown in Fig. 3a was confirmed by SDS—PAGE (Fig. 2).

Type II collagen

Type II collagen is composed of three α_1(II) chains which are dissociated into the monomeric form on heating. Using either the LiChrospher *n*-butyl packing material or the commercial Bakerbond diphenyl columns at both 20° C and 62° C the α_1(II) monomer was found to elute immediately after the α_1(I) peak (Fig. 3c).

DISCUSSION

Recent reports on the separation of collagen chains by reversed-phase HPLC have suggested a need for high column temperatures [4] or have advocated [5] the use of the UV opaque pyridine—formate elution conditions to maintain adequate chromatographic selectivity. However, these elution conditions require sophisticated post-column derivatization procedures for sample detection. Since these requirements are not easily accommodated in many laboratories, a simplified elution system for collagen chain separation by HPLC was sought and a comparison of widely differing column stationary phases made.

As has been noted in previous studies [10, 13] on the separation of proteins on large-pore alkyl silicas, the *n*-alkyl chain length did not have a significant influence on the resolution of collagen chains under gradient elution conditions with low pH water—acetonitrile combinations. In addition, there appeared to be very little effect of temperature on the separation of α_1(I), α_2(I), α_1(II) and α_1(III) chains provided the samples were initially heated to 50° C to dissociate the collagen chains before injection. One practical caveat in this approach, however, is that if pepsin (a likely preparation contaminant) is present in the collagen sample, heating to 50° C will lead to rapid proteolysis of the dissociated collagen triple helix. Under the chromatographic conditions employed in this study there appeared to be no discernible improvement in resolution if the column temperature is raised to 62° C.

In the comparison of the *n*-butyl and diphenyl stationary phases no major selectivity differences were evident. The slightly higher efficiency of the diphenyl silica support was in accord with the smaller particle size of the parent silica matrix than was used for the preparation of the *n*-butyl silica support. Despite the slightly sharper peaks obtained with the Bakerbond diphenyl silica column, the *n*-butyl LiChrospher column gave better overall resolution, i.e. higher selectivity for the β_{12}- and γ-components from α_2(I). Preliminary attempts to separate collagens on standard octadecyl silica columns which were neither macroporous nor fully capped were without success.

As part of the evaluation of elution conditions several solvent systems and gradients were tried in this investigation. Increasing the mobile phase pH to 6.0 at constant low ionic strength resulted in no eluted components and we assume the collagens precipitated or adhered to the column matrix. Similar results were obtained when a 60 m*M* phosphate buffer gradient from pH 2.5 to 7.0 was

run in conjunction with an acetonitrile gradient (0 to 50%). A primary mobile phase based on 0.1% phosphoric acid also appears to be a suitable eluent for the reversed-phase HPLC of collagens but in contrast to 0.1% trifluoroacetic acid this ionic modifier lacks volatility and cannot be removed by lyophilisation.

Although it appears from this and other investigations [4—6] that collagens may be chromatographed with a variety of reversed-phase columns and solvent systems, the procedures do not yet allow the complete resolution of all differing collagen types. The greatest separation is between α_1(I) and α_2(I) while α_1(II) and the α_1(III) trimer elute just after α_1(I) and the α_1(III) monomer elutes just before α_1(I). These differences are sufficient to allow micropreparative separations of individual chains which can be recovered in high purity. However, reliable quantitative analytical separations will only be obtained when the contamination from β- and γ-components is adequately controlled, possibly by preliminary salt precipitation [14] or by gel permeation chromatography [8, 15]. The relatively minor contribution which the molecular size differences between the collagen species (100,000—300,000) make on the separation with reversed-phase columns is noteworthy. As has been extensively documented [16] retention to *n*-alkyl silicas by macromolecules is dominated by the hydrophobic contact area with molecular weight playing a minor role on resolution, mainly through its influence on column efficiencies.

The advantages of HPLC of proteins are well recognized and may apply particularly in collagen biochemistry. Newly discovered collagen types and genetic variants have recently resulted in a three-fold increase in known collagenous proteins all of which may potentially be purified by rapid reversed-phase HPLC techniques. The high resolution and rapidity of HPLC should find ready acceptance in the clinical diagnosis of genetic defects in collagen synthesis and post-translational processing. There is also the evidence from this investigation that the relatively neglected β- and γ-components may also be separated by reversed-phase HPLC. Availability of these components should lead to new insights into α-chain cross-linking patterns and hence the structural complexity which results in the unique architecture of the collagenous tissue framework.

ACKNOWLEDGEMENTS

This investigation has been supported by grants from the Medical Research Council of New Zealand and The National Health and Medical Research Council of Australia. The excellent technical assistance of F. Lambrou with the amino acid analyses is acknowledged. We are grateful to Dr. G. Gibson, Department of Surgery, University of Auckland for his advice and discussion.

REFERENCES

1 B. Sykes, B. Puddle, M. Frances and R. Smith, Biochem. Biophys. Res. Commun., 72 (1976) 1472.
2 H. Sage and P. Bornstein, Methods Enzymol., 82 (1982) 96.
3 J. Sodek and H.F. Limeback, J. Biol. Chem., 254 (1979) 10496.
4 M. van der Rest and P. Fietzek, Eur. J. Biochem., 125 (1982) 491.

5 A. Fallon, R.V. Lewis and K.D. Gibson, Anal. Biochem., 110 (1981) 318.
6 K.A. Smolenski, A. Fallon, N.D. Light and A.J. Bailey, Biosci. Rep. 3 (1983) 93.
7 T.F. Kresina and E.J. Miller, Biochemistry, 18 (1979) 3089.
8 K.A. Piez, E.A. Eigner and M.S. Lewis, Biochemistry, 2 (1963) 58.
9 B.L. Karger, K. Benedek, N. Miller, B. Feibush and S.A. Cohen, in I. Chaiken, M. Wilchek and I. Parikh (Editors), Affinity Chromatography and Biological Recognition, Academic Press, New York, 1983, p. 479.
10 M.T.W. Hearn and B. Grego, J. Chromatogr., 296 (1984) in press.
11 G. Fairbanks, T.L. Steck and D.S.H. Wallach, Biochemistry, 10 (1971) 2606.
12 E.J. Miller and S. Gay, Methods Enzymol., 82 (1982) 3.
13 M.T.W. Hearn and B. Grego, J. Chromatogr., 255 (1983) 125.
14 G. Chandrakasan, D.A. Torchia and K.A. Piez, J. Biol. Chem., 251 (1976) 6062.
15 Z. Deyl, K. Macek, M. Adam and M. Horáková, J. Chromatogr., 230 (1982) 409.
16 M.T.W. Hearn, Adv. Chromatogr., 20 (1982) 1.

Journal of Chromatography, 308 (1984) 121—131
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2080

RAPID ISOLATION, HYDROLYSIS AND CHROMATOGRAPHY OF FORMALDEHYDE-MODIFIED DNA

FREDERICK A. BELAND*, NANCY F. FULLERTON and ROBERT H. HEFLICH

National Center for Toxicological Research, Jefferson, AR 72079 (U.S.A.)

(First received September 6th, 1983; revised manuscript received January 19th, 1984)

SUMMARY

Deoxyadenosine, deoxyguanosine, deoxycytidine and thymidine were reacted with formaldehyde. High-performance liquid chromatographic (HPLC) analysis indicated that each deoxynucleoside had formed one major product. With the exception of the thymidine product, these adducts were analyzed by nuclear magnetic resonance spectroscopy and identified as hydroxymethyl derivatives at the exocyclic amines. Calf thymus DNA was incubated with [^{3}H]formaldehyde and, after purification, enzymatically hydrolyzed to nucleosides. HPLC analysis indicated the presence of a substantial proportion of non-covalently bound formaldehyde and the following hydroxymethyl adducts, listed in order of decreasing concentration: N^6-hydroxymethyldeoxyadenosine $\gg$ N^4-hydroxymethyldeoxycytidine $>$ N^2-hydroxymethyldeoxyguanosine. Incubation of Chinese hamster ovary (CHO) cells with [^{3}H]formaldehyde resulted in metabolic incorporation of the formaldehyde into purines and pyrimidines plus an appreciable concentration of formaldehyde noncovalently associated with the DNA. However, HPLC analysis clearly indicated the presence of N^6-hydroxymethyldeoxyadenosine in the CHO cell genome.

INTRODUCTION

The reaction of formaldehyde with nucleic acids to form hydroxymethyl derivatives was first described by Fraenkel-Conrat in 1954 [1]. This interaction, which can occur with both exocyclic and endocyclic amino groups, has been extensively reviewed by Feldman [2] and Auerbach et al. [3]. More recently, McGhee and Von Hippel, in an elegant series of studies [4—7], demonstrated that formaldehyde reacted preferentially with A · T rich regions in DNA and that the interaction was preceded by an unstacking of the polynucleotide. In addition, they found that although thymidine reacted much faster with formaldehyde, the adduct formed from adenosine was quantitatively more important. This is due to differences in the stabilities of the adducts;

endocyclic hydroxymethyl derivatives, such as N3-hydroxymethylthymidine, have very short lifetimes ($t_{1/2}$ ca. 0.1 sec at 40°C), whereas exocyclic N^6-hydroxymethyladenosine is a comparatively more stable adduct with a $t_{1/2}$ of 100 min at 40°C.

Given sufficient time, the hydroxymethyl derivatives can react with another amine function to yield methylene diadducts [2, 3]. These diadducts are much more stable than the hydroxymethyl monomers and Chaw et al. have developed techniques for their analysis in DNA [8]. It should be noted, however, that in vitro formation of formaldehyde diadducts requires prolonged reaction times (e.g., 40 days) and rather high formaldehyde concentrations.

Another type of diadduct involving DNA is a methylene protein—DNA crosslink [2, 3]. These products are thought to form through an initial interaction of formaldehyde with an amine function on proteins followed by reaction with the polynucleotide. This type of adduct may be important in vivo because the interaction of formaldehyde with amino acids has been reported to increase the rate of reaction of formaldehyde with DNA [9, 10]. The structures of these protein—DNA crosslinks have not been elucidated. Furthermore, although the interactions of formaldehyde with isolated macromolecules have been studied extensively, the identity of the products obtained in intact cells has been inferred only through indirect evidence [11—17].

An additional DNA lesion that results from formaldehyde treatment is single-strand breaks. This type of damage, which appears to be dependent upon DNA repair processes, has been observed in yeast [18] and mammalian cells [14—17].

Of the products formed from the interaction of formaldehyde, one class, the hydroxymethyl adducts, has not been investigated in cells. The relative instability of these hydroxymethyl products has probably hindered their analysis and yet these adducts could be responsible for some of the biological effects produced by formadehyde. We have, therefore, developed methods for the analysis of these derivatives in mammalian cells.

MATERIALS AND METHODS

Chemicals

Formaldehyde was obtained from Fisher Scientific (Fair Lawn, NJ, U.S.A.) as a 37% aqueous solution which contained 10—15% methanol as a preservative. [^{3}H] Formaldehyde (specific activity, 85 mCi/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.) as 1% aqueous solution. Deoxyguanosine, deoxyadenosine, deoxycytidine, calf thymus DNA (Type I), RNase A (Type XII-A), RNase T_1 (grade IV), proteinase K (Type XI), lysozyme (grade 1), bis(2-hydroxyethyl)amino-tris(hydroxymethyl)methane (Bis-Tris), sodium dodecyl sulfate, and alkaline phosphatase (Type III-S) were acquired from Sigma (St. Louis, MO, U.S.A.). Thymidine and nuclease P_1 were obtained from Calbiochem-Behring (LaJolla, CA, U.S.A.). 2,4-Dinitrophenylhydrazine was purchased from MCB Manufacturing Chemists (Gibbstown, NJ, U.S.A.).

Instrumentation

High-performance liquid chromatography (HPLC) was conducted with a Waters Assoc. system consisting of two 6000A pumps, a U6K injector, a 440 UV detector and an automated gradient controller. UV spectra were recorded with either a Cary 210 or a Gilford 2400-2 spectrometer. Radioactivity was measured in Scintisol (Isolabs, Akron, OH, U.S.A.) using a Searle Mark III liquid scintillation counter. ^{1}H-Nuclear magnetic resonance (NMR) spectra were recorded with a Bruker WM 500 spectrometer.

Formaldehyde analysis

Formaldehyde was analyzed by HPLC as its 2,4-dinitrophenylhydrazone derivative [19]. The formaldehyde (ca. 10 μg) was added to a solution of 12 mg 2,4-dinitrophenylhydrazine in 20 ml 0.2 *M* hydrochloric acid. The mixture was incubated 30 min at 37°C and then extracted three times with 10-ml aliquots of chloroform. The combined chloroform extracts were washed twice with 2 *M* hydrochloric acid, twice with water and then evaporated under reduced pressure. The residue was dissolved in ethanol and analyzed by reversed-phase HPLC using a 10 μm μBondapak C_{18} column (300 × 3.9 mm) and a 30-min linear gradient of 20—100% methanol at 2 ml/min. The absorbance was monitored at 340 nm and the amount of 2,4-dinitrophenylhydrazone was quantified by comparison to an authentic standard [20] which eluted at 18.5—19.0 min. When [^{3}H]formaldehyde was assayed, 30-sec fractions were collected for measurement of radioactivity.

Preparation of hydroxymethyldeoxynucleoside standards

Solutions of 10 m*M* deoxynucleoside and 670 m*M* formaldehyde in 100 m*M* potassium phosphate buffer, pH 7.0, were incubated for 24 h at 37°C. HPLC analysis of the solutions, using the conditions outlined below, indicated the presence of two major UV-absorbing peaks from each incubation. In each case, the early eluting peak corresponded to the starting material while the second peak contained the modified deoxynucleoside. The product from each incubation was collected for spectral characterization.

Reaction of calf thymus DNA with [^{3}H]formaldehyde

A 400-μl solution consisting of 650 μg DNA and 2 μg [^{3}H]formaldehyde in 40 m*M* sodium acetate buffer, pH 4.5, was incubated 20 h at 37°C. The mixture was then cooled on ice, and the DNA was precipitated by addition of 30 μl of 1 *M* sodium chloride followed by 800 μl ice-cold ethanol. The DNA was recovered by centrifugation and dissolved in 400 μl of 5 m*M* Bis-Tris—0.1 m*M* EDTA buffer, pH 7.1. After determination of the specific activity, the DNA was again precipitated, collected by centrifugation and dissolved in the Bis-Tris buffer.

Enzymatic hydrolysis of calf thymus DNA and HPLC separation of products

The DNA solutions (400 μl) were hydrolyzed by addition of 20 μl 20 m*M* zinc sulfate, 20 U nuclease P_1 and 0.5 U alkaline phosphatase [21]. After incubating 1 h at 37°C, 75 μl 1 *M* Tris—HCl, pH 8.0, was added and the hydrolysis was continued one additional hour. The solution was centrifuged

with a Beckman microfuge at 22,000 *g* for 1 min and aliquots of the supernatant were analyzed directly by HPLC. Individual adducts were separated with a 10 μm μBondapak C_{18} reversed-phase analytical column (300 × 3.9 mm) with a linear gradient of 10 m*M* ammonium acetate, pH 5.3, to 20% methanol in 10 m*M* ammonium acetate, pH 5.3, over 30 min at a flow-rate of 2 ml/min [21]. The absorbance was monitored at 254 nm and 30-sec fractions were collected for analysis of radioactivity.

Incubation of Chinese hamster ovary (CHO) cells with [^{3}H]formaldehyde, isolation of DNA and analysis of adducts

Suspension cultures of CHO-K_1-BH_4 cells (obtained from A.W. Hsie, Oak Ridge, TN, U.S.A.) were centrifuged and the cell pellet was resuspended at a concentration of approximately 1 × 10^7 cells/ml in Ham's medium F12, containing 25 m*M* N-2-hydroxyethylpiperazine-N′-ethanesulfonic acid buffer, pH 7.3 [22]. Samples were treated with 1 m*M* [^{3}H]formaldehyde or 2.5 m*M* and 5 m*M* formaldehyde for 2 h at 37°C. Aliquots were used to determine cell survival by relative cloning ability and the remaining cells from the [^{3}H]formaldehyde exposure were isolated by centrifugation for adduct analysis.

DNA was isolated using a modification of the technique of Gupta [23]. Cell pellets were suspended in 10 ml 1% sodium dodecyl sulfate—1 m*M* EDTA and homogenized for 30 sec with a Polytron (Brinkmann Instruments, Westbury, NY, U.S.A.) at speed setting 4—5. After centrifugation for 5 min at 3400 *g* to break the emulsion, the solution was treated with 5 mg proteinase K for 30 min at 37°C. After addition of 500 μl 1 *M* Tris—HCl, pH 8.0, the solution was gently extracted for 5 min with 10 ml phenol and then centrifuged at 3400 *g* for 5 min. (The phenol and all other organic solvents used in the DNA isolation and purification were previously saturated with 50 m*M* Tris—HCl, pH 8.) The aqueous phase was then sequentially extracted with 10 ml phenol—chlorform—isoamyl alcohol (25:24:1), followed by 10 ml chloroform—isoamyl alcohol (24:1). After adding 1.1 ml 5 *M* sodium chloride to the aqueous fraction, the DNA was precipitated with 11 ml ice-cold ethanol, washed twice with 5 ml 70% ethanol, and redissolved in 2 ml 1.5 m*M* sodium chloride—0.15 m*M* trisodium citrate—1 m*M* EDTA buffer. The solution was treated with 40 μl 1 *M* Tris—HCl, pH 7.4, 600 μg heat-treated RNase A, and 300 U RNase T_1, and incubated for 15 min at 37°C. Following the addition of 250 μl 1 *M* Tris—HCl, pH 8, the mixture was extracted with 6 ml phenol—chloroform—isoamyl alcohol (25:24:1) and then with 6 ml chloroform—isoamyl alcohol (24:1). The DNA was precipitated by sequential addition of 600 μl 5 *M* sodium chloride and 6 ml ice-cold ethanol, washed twice with 70% ethanol, and dissolved in 4 ml 5 m*M* Bis-Tris—0.1 m*M* EDTA, pH 7.1, for analysis. The DNA was quantified by UV spectroscopy (48 μg/ml = 1.0 $A_{260\,nm}$) and its specific activity was determined by liquid scintillation spectrometry. Aliquots were enzymatically digested and analyzed for adducts by the technique used for calf thymus DNA.

RESULTS

Formaldehyde analysis

The purity of the formaldehyde was estimated by formation of its 2,4-dinitrophenylhydrazone derivative and analysis of this product by reversed-phase HPLC using a modification of the procedure of Mansfield et al. [19]. Based upon comparisons to an authentic standard, the recovery of formaldehyde, using this technique, was 103 ± 3%. When the [^{3}H] formaldehyde was analyzed in a similar manner, 93% of the radioactivity partitioned into the organic phase, and 90% of the radioactivity which migrated on the HPLC coeluted with the standard. The identity of the impurities is not known.

Preparation of formaldehyde—deoxyribonucleoside standards

HPLC analysis of the deoxyribonucleoside—formaldehyde reaction mixtures indicated the presence of one major product from incubation with each of the deoxynucleosides. Each adduct, with the exception of the thymidine derivative, was isolated for characterization by UV and NMR spectroscopy. The spectral parameters are given in Table I. The UV spectra are consistent with the previously reported values for the formaldehyde adducts [2]. In each case, the NMR spectra confirmed the hydroxymethyl substitution at the exocyclic amines. Although a product was observed from the reaction of thymidine with formaldehyde, it was formed in insufficient quantity and was too labile for characterization.

Reaction of [^{3}H] formaldehyde with calf thymus DNA

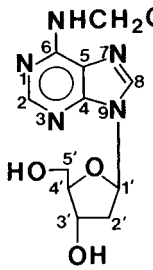
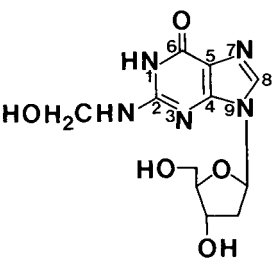
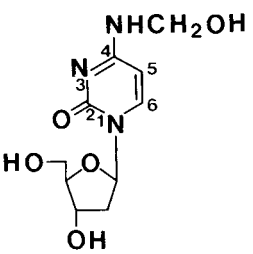
Calf thymus DNA was incubated with [^{3}H] formaldehyde for 20 h. The DNA was precipitated and then reprecipitated until constant specific activity was obtained. The enzymatically digested DNA was analyzed by HPLC which indicated two major bands of radioactivity (Fig. 1). The first migrated with the void volume and accounted for 85% of the radioactivity applied to the column; the second band coeluted with N^6-hydroxymethyldeoxyadenosine and contained 7% of the activity. In some experiments, small amounts of radioactivity coeluted with N^4-hydroxymethyldeoxycytidine (0.6%) and with N^2-hydroxymethyldeoxyguanosine (0.2%).

The HPLC profile indicated that the enzymatic hydrolysis of the DNA was complete because UV absorbance was associated only with the deoxynucleoside monomers and the hydroxymethyldeoxynucleoside markers. The absence of UV absorbance in the vicinity of the void volume suggested that the radioactivity eluting in this region might be due to nonreacted [^{3}H] formaldehyde. To examine this possibility, the modified DNA was incubated with 2,4-dinitrophenylhydrazine and the products formed were analyzed by HPLC. Of the radioactivity applied to the column, more than 85% migrated with the 2,4-dinitrophenylhydrazone standard, while the remaining activity eluted as a single peak slightly later.

McGhee and Von Hippel have reported rapid removal of residual noncovalently bound formaldehyde from polynucleotides by use of a Bio-Rad P2 column [6]. Repeated attempts to use this technique were not successful; in each case all the radioactivity applied to the column eluted with the DNA.

TABLE I

UV AND ^{1}H-NMR SPECTRAL PARAMETERS OF HYDROXYMETHYL DEOXYRIBONUCLEOSIDE ADDUCTS

Compound	UV*	NMR**	
		Chemical shift (δ)	Assignment
N^6-Hydroxymethyldeoxyadenosine 	263	2.29	H2'a
		2.72	H2'b
		3.57	H5'ab
		3.87	H4'
		4.41	H3'
		4.93***	-CH_2-
		6.35	H1'
		8.26***	H8
		8.41***	H2, -$NHCH_2$-
N^2-Hydroxymethyldeoxyguanosine 	253 (shoulder, 275)	2.20	H2'a
		2.55	H2'b
		3.50, 3.54	H5'a,b
		3.80	H4'
		4.30	-CH_2OH
		4.35	H3'
		4.76	-CH_2-
		4.85	3'OH
		5.25	5'OH
		6.18	H1'
		6.99	-$NHCH_2$-
		7.92	H8
N^4-Hydroxymethyldeoxycytidine 	271	1.90	H2'a
		2.09	H2'b
		3.57	H5'ab
		3.76	H4'
		4.19	H3'
		4.72	-CH_2-
		5.75	H5
		6.15	H1'
		7.81	H6
		8.15	-$NHCH_2$-

*UV spectra were recorded in water and are reported as the maximum absorbance in nm.

**^{1}H-NMR spectra were recorded at 500 mHz in dimethylsulfoxide-d_6 and are referenced to internal tetramethylsilane. The adducts formed from reaction of formaldehyde with the deoxynucleosides were isolated by HPLC. Due to the adducts' lability, some decomposition occurred during the preparation of the samples for NMR analysis. Therefore, the products were analyzed as mixtures of the adducts and their nonmodified nucleosides. Resonance assignments were made through comparisons to nonmodified deoxynucleosides.

***These signals were unusually broad when spectra were obtained at 300°K. When measured at 325°K, the resonances were dramatically sharpened. A similar broadening has been reported for H2 of an N^6-substituted deoxyadenosine [24].

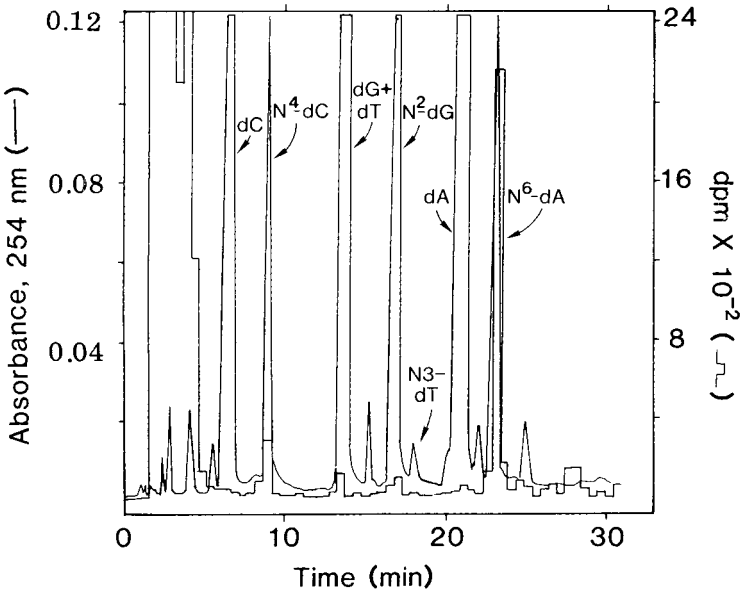


Fig. 1. Reversed-phase HPLC of adducts obtained from reacting [^{3}H]formaldehyde with calf thymus DNA. Following enzymatic hydrolysis, the solution was centrifuged and aliquots of the supernatant were analyzed directly. Synthetic hydroxymethyldeoxynucleosides were added to serve as UV markers. N^6-dA = N^6-hydroxymethyldeoxyadenosine; N^2-dG = N^2-hydroxymethyldeoxyguanosine; N^4-dC = N^4-hydroxymethyldeoxycytidine; N3-dT = N^3-hydroxymethylthymidine. The structure of the thymidine adduct has not been confirmed by spectral analysis. The adducts were separated by running a linear gradient of 10 m*M* ammonium acetate, pH 5.3, to 20% methanol in 10 m*M* ammonium acetate, pH 5.3, over 30 min at a flow-rate of 2 ml/min. (—), Absorbance at 254 nm of the hydrolyzed DNA plus the hydroxymethyldeoxynucleoside markers; (⌐¬), radioactivity associated with the DNA.

In control experiments where [^{3}H]formaldehyde was applied in the absence of DNA, all the radioactivity was retained. When the enzymatic hydrolysate was applied to a P2 column, all of the radioactivity remained on the column as did all of the nucleosides. Prolonged drying of either the enzymatic hydrolysate or the DNA in vacuo also did not remove the noncovalently bound radioactivity.

Treatment of CHO cells with [^{3}H]formaldehyde

CHO cells were treated in suspension with either 1 m*M* [^{3}H]formaldehyde or 2.5 and 5 m*M* formaldehyde for 2 h at 37°C. After aliquots were removed to measure cell survival, the remainder of the [^{3}H]formaldehyde-treated cells was recovered by centrifugation to be used for adduct analysis.

The cell survival of the treated CHO cells, compared to control cultures, was 103, 9, and < 0.04% for 1, 2.5, and 5 m*M* formaldehyde, respectively.

The yield of DNA from 3.85×10^8 cells was 3.3 mg with a specific activity of 1.90×10^5 dpm per mg DNA. Aliquots (340 μg) were enzymatically hydrolyzed and following centrifugation, the supernatant was analyzed by HPLC. Enzymatic hydrolysis released 91% of the radioactivity associated with the DNA and four radioactive bands were observed in the HPLC profile (Fig. 2). The first peak, containing 70% of the radioactivity, eluted with the void volume and based upon previous results was probably nonreacted formaldehyde. The second band (25%) coeluted with deoxyguanosine and/or

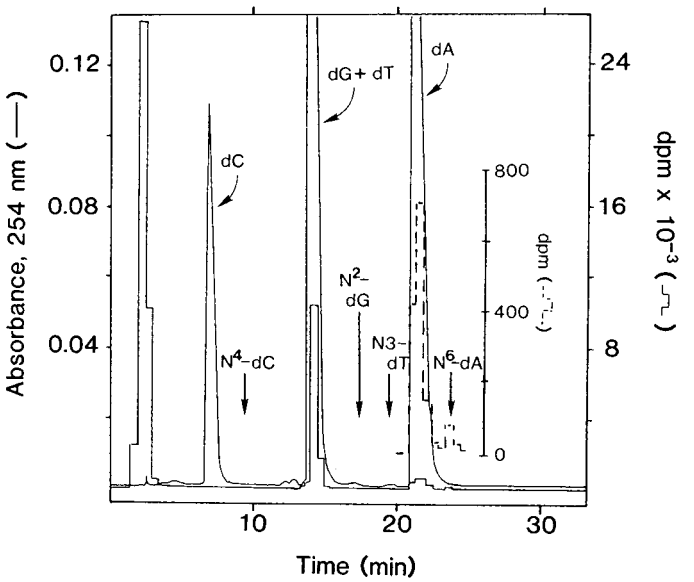


Fig. 2. Reversed-phase HPLC of supernatant from enzymatic hydrolysis of DNA isolated from CHO cells treated with [^{3}H]formaldehyde. Chromatographic conditions are identical to those used in Fig. 1. Under these conditions the hydroxymethyl adducts elute as follows: N^4-hydroxymethyldeoxycytidine, 9.0 min; N^2-hydroxymethyldeoxyguanosine, 17.2 min; N3-hydroxymethylthymidine, 19.2 min; N^6-hydroxymethyldeoxyadenosine, 23.8 min. (—), Absorbance at 254 nm from the hydrolyzed CHO cell DNA; (⎍⎍), radioactivity associated with the CHO cell DNA; (⎍⎍), enlargement of the solid histogram ((included to emphasize the contributon from N^6-hydroxymethyldeoxyadenosine). The arrows indicate the elution volume of the hydroxymethyldeoxynucleoside standards.

thymidine. Subsequent analysis, using a program which would separate these two nucleosides, indicated that all of the activity was due to thymidine. The third peak (2%) coeluted with deoxyadenosine while the final band of radioactivity (0.2%) comigrated with N^6-hydroxymethyldeoxyadenosine. HPLC separation of twice the amount of DNA hydrolysate gave a concentration-dependent increase in all peaks. Assuming that the deoxyadenosine adduct had the same specific activity as the [^{3}H]formaldehyde, the level of binding in the CHO cells was 65 N^6-hydroxymethyldeoxyadenosines per 10^8 nucleotides. In a control experiment in which [^{3}H]formaldehyde-modified calf thymus DNA was subjected to the same DNA isolation procedure used for the CHO cells, the specific activity of the DNA decreased by 70% and the concentration of N^6-hydroxymethyldeoxyadenosine decreased by 60%. Thus, the concentration of N^6-hydroxymethyldeoxyadenosine in the CHO cell genome was probably at least twofold higher than that detected by the HPLC analysis.

DISCUSSION

The reaction of formaldehyde with deoxynucleosides has been reported to yield hydroxymethyl derivatives (see refs. 2 and 3 for pertinent references). Since these products are unstable, techniques had to be developed for the rapid isolation, hydrolysis and chromatography of formaldehyde-modified DNA.

In addition, since formaldehyde adducts have a polarity similar to nonmodified nucleosides and because metabolic incorporation of the formaldehyde into purines and pyrimidines could occur, the chromatographic system had to be able to resolve both modified and nonmodified deoxynucleosides.

When formaldehyde was reacted with deoxyadenosine, deoxycytidine and deoxyguanosine, one major adduct was detected in each instance. Although it was assumed that these were hydroxymethyl derivatives formed by reacting with the exocyclic nitrogens, with the exception of adenosine [4], this had not been unambiguously proven. Therefore, the major product from each reaction was isolated for spectral characterization. The UV spectra of the compounds were consistent with previously reported spectra [2] and NMR spectra contained a characteristic methylene doublet (ca. δ 4.7) which collapsed to a singlet upon irradiation of the adjacent amine proton. (It should be noted that NMR spectra have recently been reported on N-ethoxymethyl derivatives of ribonucleosides [25].) These data taken together indicate that the adducts are, indeed, exocyclic hydroxymethyl derivatives.

Incubation of [^{3}H] formaldehyde with calf thymus DNA yielded an apparent binding of seven formyl residues per 10^4 nucleotides. However, it became apparent that the great majority of this radioactivity was due to nonreacted formaldehyde. Attempts to remove this noncovalently bound material were unsuccessful. McGhee and Von Hippel [6] reported that a Bio-Rad P2 column could be used to lower the formaldehyde concentration to less than 1—2 μM, which was the limit of detection in their fluorescent analysis. In the absence of DNA, we found that the P2 column would remove more than 98% of the applied formaldehyde. With DNA present, however, the concentration of [^{3}H]-formaldehyde (ca. 3 μM) associated with the DNA did not change. Nevertheless, the HPLC procedure reported herein provided adequate resolution between the nonreacted formaldehyde and the adducts which were formed.

Initial attempts to separate the adducts were based upon acid hydrolysis and cation-exchange HPLC [26]. Although this did provide adequate resolution, we were concerned that the acidic conditions might destroy the relatively unstable hydroxymethyl derivatives. The procedure adopted was a modification of the enzymatic hydrolysis and reversed-phase HPLC developed by Kuo et al. [21]. By using enzymatic hydrolysis, neutral and mild conditions were maintained. When combined with the DNA isolation technique of Gupta [23], the entire analysis could be conducted within 6 h and complete resolution was obtained between the adducts, the nonmodified nucleosides, and formaldehyde.

Analysis of calf thymus DNA modified with [^{3}H] formaldehyde indicated that the majority of the activity was due to noncovalently bound formaldehyde. However, radioactivity was observed to comigrate with the synthetic hydroxymethyl derivatives of deoxyadenosine, deoxyguanosine, and deoxycytidine. Furthermore, the relative proportion of the adducts was N^6-hydroxymethyldeoxyadenosine $\gg$ N^4-hydroxymethyldeoxycytidine $>$ N^2-hydroxymethyldeoxyguanosine which is the anticipated order based upon the ease of formation of the adducts and their relative stability [4—7].

Incubation of CHO cells with 1 mM [^{3}H] formaldehyde gave an apparent binding to DNA of 3.2 formyl residues per 10^4 nucleotides. Upon hydrolysis,

more than 90% of the radioactivity was released and could be chromatographed. However, the majority of the radioactivity coeluted with formaldehyde, while a substantial proportion of the remainder was incorporated metabolically into nonmodified nucleosides, primarily thymidine. Nevertheless, N^6-hydroxymethyldeoxyadenosine was clearly present at a level of 65 adducts per 10^8 nucleotides. Other adducts may have been present in the CHO cell genome; however, if their relative proportion was similar to that found in the calf thymus DNA, the concentration of these adducts would have been below our limit of detection.

In conclusion, we have confirmed that the major adducts obtained from reacting formaldehyde with deoxynucleosides are hydroxymethyl derivatives at the exocyclic amines. Since these products are quite labile, we developed techniques for the rapid isolation, hydrolysis and chromatography of formaldehyde-modified DNA. We were able to demonstrate that the reaction of formaldehyde with isolated DNA yielded three adducts plus a substantial proportion of noncovalently bound formaldehyde. When CHO cells were incubated with formaldehyde, there was an appreciable amount of noncovalently bound formaldehyde associated with the DNA and, in addition, metabolic incorporation into the nucleoside bases had also occurred. However, we were also able to demonstrate clearly the formation of a hydroxymethyldeoxyadenosine adduct at a level similar to that reported for protein—DNA crosslinks [11] and single-stranded breaks [14]. The biological consequences which result from the formation of this adduct remain to be elucidated.

ACKNOWLEDGEMENTS

We thank Robert Levine for obtaining the NMR spectra and Ruth York for helping prepare this manuscript. This work was supported, in part, by Interagency Agreement 224-82-007 with the U.S. Consumer Product Safety Commission and by Interagency Agreement 224-84-002 with the Veteran's Administration Hospital, Little Rock, AR, U.S.A.

REFERENCES

1 H. Fraenkel-Conrat, Biochim. Biophys. Acta, 15 (1954) 307—309.
2 M.Ya. Feldman, Prog. Nucleic Acid Res. Mol. Biol., 13 (1973) 1—49.
3 C. Auerbach, M. Moutschen-Dahmen and J. Moutschen, Mutat. Res., 39 (1977) 317—362.
4 J.D. McGhee and P.H. von Hippel, Biochemistry, 14 (1975) 1281—1296.
5 J.D. McGhee and P.H. von Hippel, Biochemistry, 14 (1975) 1297—1303.
6 J.D. McGhee and P.H. von Hippel, Biochemistry, 16 (1977) 3267—3276.
7 J.D. McGhee and P.H. von Hippel, Biochemistry, 16 (1977) 3276—3293.
8 Y.F.M. Chaw, L.E. Crane, P. Lange and R. Shapiro, Biochemistry, 19 (1980) 5525—5531.
9 Yu.A. Siomin, V.V. Simonov and A.M. Poverenny, Biochim. Biophys. Acta, 331 (1973) 27—32.
10 Yu.A. Siomin, E.N. Kolomyitseva and A.M. Poverenny, Mol. Biol. (Engl. Transl.), 8 (1974) 276—285.
11 R.J. Wilkins and H.D. MacLeod, Mutat. Res., 36 (1976) 11—16.
12 N. Magaña-Schwencke and B. Ekert, Mutat. Res., 51 (1978) 11—19.

13 N. Magaña-Schwencke and E. Moustacchi, Mutat. Res., 70 (1980) 29—35.
14 A.J. Fornace, Jr., Cancer Res., 42 (1982) 145—149.
15 A.J. Fornace, Jr., J.F. Lechner, R.C. Grafstrom and C.C. Harris, Carcinogenesis, 3 (1982) 1373—1377.
16 R.C. Grafstrom, A.J. Fornace, Jr., H. Autrup, J.R. Lechner and C.C. Harris, Science, 220 (1983) 216—218.
17 W.E. Ross and N. Shipley, Mutat. Res., 79 (1980) 277—283.
18 N. Magaña-Schwencke, B. Ekert and E. Moustacchi, Mutat. Res., 50 (1978) 181—193.
19 C.T. Mansfield, B.T. Hodge, R.B. Hege, Jr. and W.C. Hamlin, J. Chromatogr. Sci., 15 (1977) 301—302.
20 R.L. Shriner, R.C. Fuson and D.Y. Curtin, The Systematic Identification of Organic Compounds, Wiley, New York, 4th ed., 1956, pp. 111, 112, 283.
21 K.C. Kuo, R.A. McCune, C.W. Gehrke, R. Midgett and M. Ehrlich, Nucleic Acids Res., 8 (1980) 4763—4776.
22 R.H. Heflich, D.T. Beranek, R.L. Kodell and S.M. Morris, Mutat. Res., 106 (1982) 147—161.
23 R.C. Gupta, submitted for publication.
24 D.L. Tullis, K.M. Straub and F.F. Kadlubar, Chem.-Biol. Interact., 38 (1981) 15—27.
25 P.K. Bridson, J. Jiricny, O. Kemal and C.B. Reese, J. Chem. Soc., Chem. Commun., (1980) 208—209.
26 D.T. Beranek, C.C. Weis and D.H. Swenson, Carcinogenesis, 1 (1980) 595—606.

Journal of Chromatography, 308 (1984) 133—141
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2062

DETERMINATION OF CYANIDE AND THIOCYANATE IN BLOOD PLASMA AND RED CELLS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUOROMETRIC DETECTION

TOSHIHIKO TOIDA, TADAYASU TOGAWA, SHINZO TANABE and TOSHIO IMANARI*

Faculty of Pharmaceutical Sciences, Chiba University, 1-33, Yayoi-cho, Chiba-shi, Chiba, 260 (Japan)

(Received September 30th, 1983)

SUMMARY

A method for the determination of cyanide and thiocyanate in blood plasma and red cells of humans was established. It involved high-performance liquid chromatography and fluorometric detection by the Kõnig reaction. Calibration curves for cyanide and thiocyanate were linear in the range 1—200 pmol and 2—300 pmol, respectively. Clean-up methods for the determination of cyanide and thiocyanate in red cells were also developed. These methods were applied for the determination of cyanide and thiocyanate in the blood of smokers and non-smokers.

INTRODUCTION

Much is now known about the metabolism of cyanide in normal humans. Cyanide is converted to thiocyanate by an irreversible reaction catalyzed by rhodanese (EC 2.8.1.1), which is a mitochondrial enzyme in liver and kidney [1, 2]. On the other hand, several workers have demonstrated in vivo conversion of thiocyanate to cyanide by an erythrocytic enzyme, "thiocyanate oxidase", and suggested the physiological effects of its conversion [3—7]. Recently, Vesey and Wilson [8] have reported that thiocyanate oxidase activity was not observed in erythrocytes, and have pointed out that the earlier reported cyanide assays in erythrocytes were not suitable for the determination of cyanide because haemoglobin in erythrocytes oxidized thiocyanate chemically to give cyanide. However, the presence of thiocyanate oxidase in body fluids cannot be ruled out clinically, since a large dose of thiocyanate as a hypotensive agent caused cyanide poisoning [9]. Therefore, an accurate and reliable method for the determination of cyanide and thiocyanate in biological samples is desired.

0378-4347/84/$03.00

A large number of reports have been published for the determination of cyanide and thiocyanate in biological fluids. A usual method for the determination of cyanide in blood plasma and erythrocytes is the pyridine—pyrazolone method developed by Epstein [10] coupled with a microdiffusion method using a Conway cell [11] or Cavett flask [12]. For the determination of thiocyanate in blood plasma, the method of Boxer and Rickards [13] has been widely used. In the assay, thiocyanate is first oxidized to cyanide under mild acidic conditions and the hydrogen cyanide liberated is then absorbed to alkali by aeration; the cyanide is determined colorimetrically using the pyridine—pyrazolone method.

We have already reported a method for the simultaneous determination of cyanide and thiocyanate using high-performance liquid chromatography (HPLC) and colorimetric detection by the König reaction [14]. Recently, we found that the reaction products formed from cyanide and thiocyanate by the König reaction have an intense fluorescence [15]. A few picomoles of both anions can be determined by this reaction.

In the present work, we devised a highly sensitive method for the determination of cyanide and thiocyanate by HPLC employing this fluorescence reaction, as well as the development of a pretreatment method for the determination of both anions in human plasma and red cells.

EXPERIMENTAL

Reagents

A standard solution of cyanide (0.1 *M*) was prepared by dissolving potassium cyanide (Wako Pure Chemicals, Osaka, Japan) in 0.1 *M* sodium hydroxide; the concentration of cyanide was calibrated by titration with silver nitrate according to the Liebig-Dénigés method [16]. A standard solution of thiocyanate (Wako) was prepared with redistilled water. Other chemicals were of analytical reagent grade.

Chromatographic conditions

Fig. 1 is a flow diagram of the HPLC system, which consists of reciprocating pumps (PSU-2.5, Seishin Seiyaku Co., Tokyo, Japan), a variable-wavelength fluoromonitor (RF-530, Shimadzu Seisakusho Co., Kyoto, Japan) with a xenon lamp and 12-μl flow cell, a variable-input recorder (SS-250F, Sekonic Co., Tokyo, Japan) and a sample injector (VMW-6, Seishin Seiyaku Co., Tokyo, Japan). HPLC conditions were as follows: column, a strong base anion-exchange resin, TSK Gel LS-222 (6 μm, 100 mm × 3 mm I.D., Toyo Soda Co., Tokyo, Japan); eluent, 0.1 *M* sodium acetate buffer (pH 5.0) containing 0.2 *M* sodium perchlorate (flow-rate, 0.5 ml/min); chlorination reagent, 0.1% chloramine T aqueous solution (flow-rate, 0.1 ml/min); pyridine—barbituric acid reagent, a mixture of barbituric acid (1.5 g), pyridine (15 ml), concentrated hydrochloric acid (3 ml) and redistilled water (82 ml) (flow-rate, 0.1 ml/min); excitation and emission wavelengths of the detector were set at 583 and 607 nm, respectively.

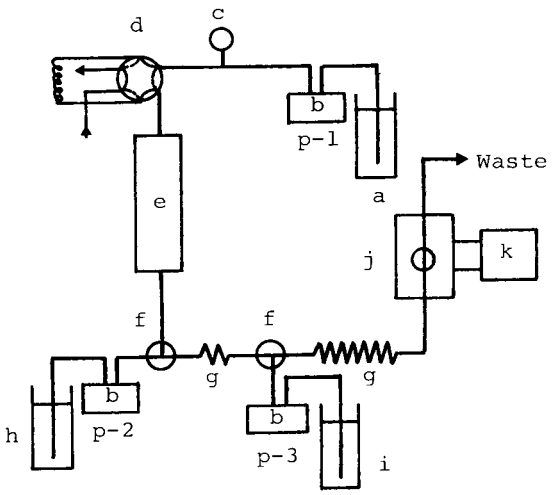


Fig. 1. Schematic diagram of HPLC: a = eluent (p-1), b = pump, c = pressure gauge, d = sample injector, e = analytical column, f = mixing joint, g = mixing coil, h = chloramine T reagent (p-2), i = pyridine—barbituric acid reagent (p-3), j = fluorescence spectromonitor, k = recorder.

Determination of cyanide in red cells

Isolation of red cells. Fresh heparinized blood, obtained by the standard techniques, was used. For the determination of total cyanide in red cells, the heparinized blood was centrifuged at 1500 *g* for 20 min and the plasma and buffy coat were removed with a micropipette. The packed red cell volume (haematocrit) was determined using a haemocytometer. Red cells were washed four times with 0.9% sodium chloride and centrifuged at 1500 *g* for 20 min.

Determination of cyanide. Portions of red cells (0.2 or 0.4 ml) were placed in the outer well of the Conway cell and 1.0 ml of 0.1 *M* sodium hydroxide was placed in the center chamber. The Conway cell and ground-glass cover were coated with silicone grease, and a glass cover was placed on top of the microdiffusion cell, leaving a small space for the addition of a hemolyzing agent. To the samples described above for the determination of total cyanide 1.0 ml of 20% ascorbic acid was added, and to those for the determination of stable cyanide 1.0 ml of redistilled water was added. After about 1 h, 1.5 ml of 10% sulphuric acid were added to the outer chamber. Subsequently, the ground-glass cover was moved to seal the microdiffusion cell. These cells were rotated carefully to mix the solutions in the outer chamber. The cells were rotated every 30 min. Cyanide in the sample was allowed to diffuse for 4 h at room temperature and the hydrogen cyanide liberated was absorbed into the sodium hydroxide solution in the center chamber. An aliquot in the center chamber solution was applied to HPLC.

Determination of thiocyanate in red cells

An appropriate volume of red cells prepared for total cyanide assay was added to 0.3 *M* phosphate buffer (pH 8.0) containing 0.2 *M* sodium perchlorate. The mixture was agitated once and maintained for 30 min at room temperature. Then the red cell suspension was filtered with an Amicon

Centriflo ultrafiltration membrane cone (Amicon, Lexington, MA, U.S.A.) by centrifuging at 1000 *g* for 20 min. The filtrate was submitted to HPLC.

Determination of thiocyanate in blood plasma

A 100 μl volume of blood plasma was mixed with 500 μl of 10% perchloric acid in a microtube. After 10 min, the suspension was centrifuged at 8000 *g* for 5 min. The supernatant was injected for HPLC.

RESULTS AND DISCUSSION

Detection and HPLC separation of cyanide and thiocyanate

In order to find the optimum detection conditions, we examined the reaction time (reaction coil length) and the concentration of chloramine T and pyridine–barbituric acid reagents by using a flow injection system consisting of the HPLC apparatus (Fig. 1) with the separation column removed. In this series of studies, 2.5 μ*M* cyanide and thiocyanate aqueous solutions were submitted to the test sample and the pH of the eluent was adjusted to 5.0 with acetate buffer as reported previously [14, 15].

The effect of the concentration of chloramine T reagent on response for cyanide and thiocyanate is shown in Fig. 2. The peak heights remained constant between the concentrations of 0.08% and 0.3%. In this range, the time required for chlorination was within about 1 min. The concentration of chloramine T reagent, therefore, was fixed at 0.1%.

Various concentrations of pyridine–barbituric acid reagent containing 3 ml of concentrated hydrochloric acid in 100 ml of reagent solution were prepared and their effects on response for cyanide were examined (Fig. 3). The optimum

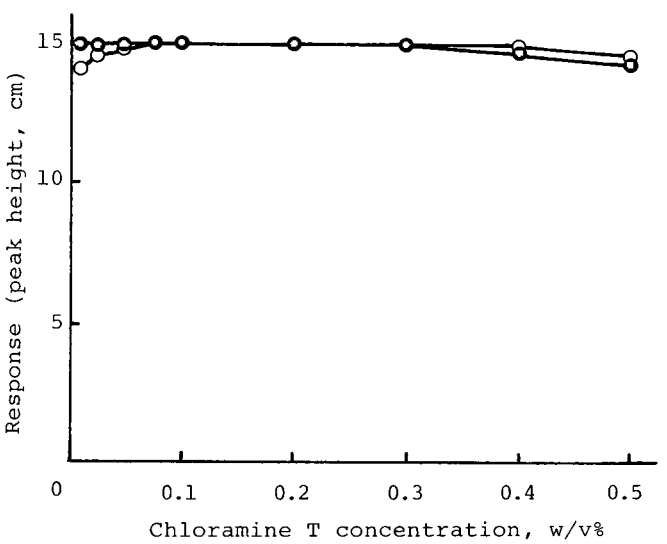


Fig. 2. Effect of chloramine T concentrations on fluorescence intensity. Conditions of flow injection system (FIA): p-1, 0.1 *M* acetate buffer (pH 5.0); p-2, chloramine T; p-3, 15% (v/v) pyridine—1.5% (w/v) barbituric acid containing 3 ml of concentrated hydrochloric acid in 100 ml; sample volume, 20 μl. Flow-rates and detection wavelength are given under Experimental. (○), Thiocyanate (50 pmol); (●), cyanide (50 pmol).

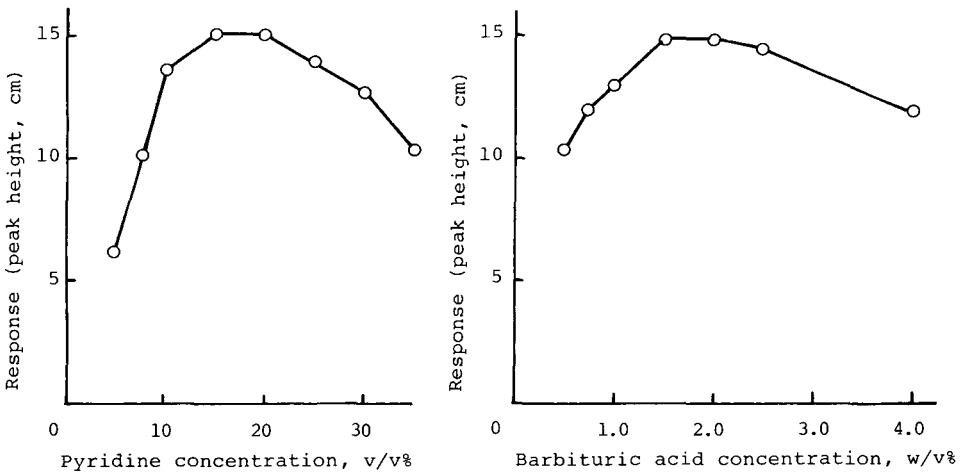


Fig. 3. Effect of pyridine and barbituric acid concentrations on fluorescence intensity. FIA conditions: p-2, 0.1% (w/v) chloramine T; p-3, pyridine—1.5% (w/v) barbituric acid (left) and 15% (v/v) pyridine—barbituric acid (right) containing 3 ml of concentrated hydrochloric acid in 100 ml. Other conditions as in Fig. 2. (○), Cyanide (50 pmol).

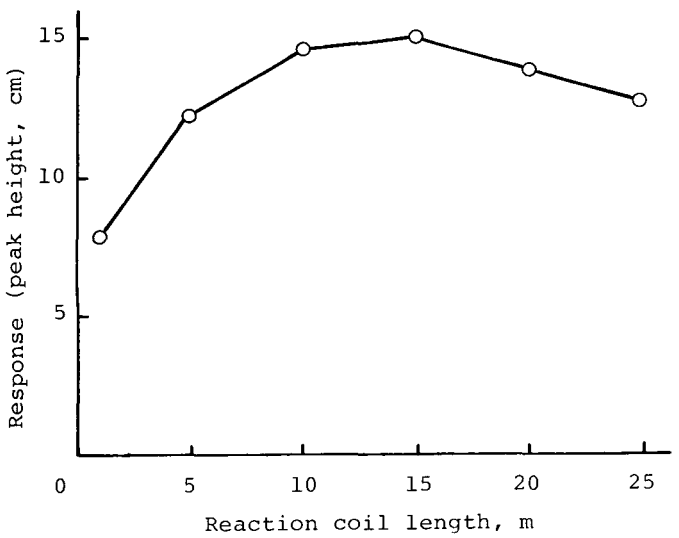


Fig. 4. Effect of reaction coil length on fluorescence intensity. FIA conditions: p-3, 15% (v/v) pyridine—1.5% (w/v) barbituric acid containing 3 ml of concentrated hydrochloric acid in 100 ml. Other conditions as in Fig. 2. (○), Cyanide (50 pmol).

concentrations of the components of pyridine—barbituric acid reagent were estimated at 15% (v/v) and 1.5% (w/v), respectively, as described in Experimental.

The effect of the reaction time on response for cyanide is shown in Fig. 4. Reaction time means the period for the reaction with barbituric acid and glutaconic aldehyde formed from cyanogen chloride and pyridine in the reaction coil. It is well known that the reaction product decomposes rapidly

[17]. In the flow system, however, reproducible peak heights proportional to the concentrations of cyanide and thiocyanate were obtained with a reaction coil of 15 m × 0.5 mm I.D.

The above data were almost the same as those obtained using HPLC with colorimetric detection [14].

For the separation of cyanide and thiocyanate, we used the same conditions as those described in the earlier report [14], except that the separation column length was changed from 15 to 10 cm. Under these chromatographic conditions, cyanide and thiocyanate were separated at the respective retention times of 6 and 9 min (Fig. 5), and were determined in the range 1—200 pmol and 2—300 pmol, respectively, in a sample size of 20 μl. The limit of detection for each anion was 0.4 pmol.

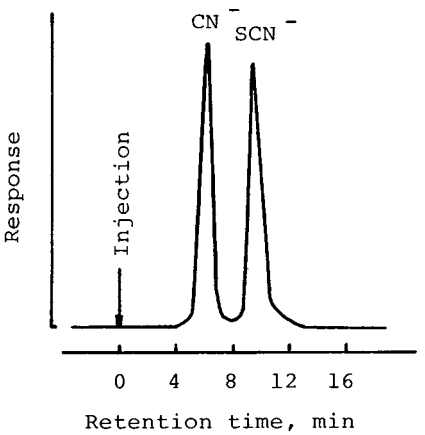


Fig. 5. Typical chromatogram of cyanide and thiocyanate (10 pmol of each). HPLC conditions are given under Experimental.

Regeneration of the separation column

When a sample containing heavy metal ions was submitted to the column, the chromatograms of cyanide and thiocyanate were not reproducible because the heavy metal ions were adsorbed on the column. In this case, the column was regenerated with 40 ml of 0.1 *M* sodium hydroxide solution containing 1% EDTA, followed by about 40 ml of eluent.

Cyanide in blood plasma and red cells

The normal molar ratio of thiocyanate to cyanide in blood plasma is more than 50:1. Furthermore, when cyanide is added to blood plasma in vitro, and left at room temperature, it survives only briefly (Fig. 6). Since a concentration step was involved for the determination of cyanide in blood plasma reported earlier [3—7], this step must be treated with some caution as suggested by Pettigrew and Fell [18]. On the other hand, Vesey and Wilson [8] suggested that the amount of cyanide released on acidifying the whole blood was much greater than the total amount from the blood plasma and erythrocytes assayed separately. The value also varied with blood plasma thiocyanate concentrations. This suggests that this artifactual release of cyanide from the whole blood was

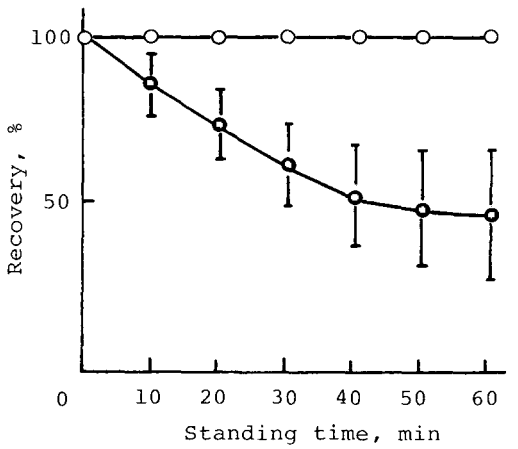


Fig. 6. Disappearance of cyanide added to plasma. Conditions: 1.0 μM cyanide is added to plasma (•) and saline (○). The mixtures are left to stand at room temperature in the outer wells of the Conway cells; then sulphuric acid is added to each sample. Other conditions are given under Experimental.

due to the oxidation of thiocyanate by methaemoglobin under acidic conditions.

Table I indicates the effect of ascorbic acid as the reducing reagent for the determination of cyanide in red cells containing thiocyanate by the microdiffusion method. Ascorbic acid effectively prevented artifactual formation of cyanide. These data were obtained from the determination of the total cyanide in red cells, where thiocyanate was not washed out by saline. The same result was obtained for the determination of stable cyanide in red cells, where thiocyanate and the free cyanide were washed out by saline as suggested by Vesey and Wilson [8]. The procedure for the determination of the total cyanide was as described in Experimental.

TABLE I

EFFECT OF ASCORBIC ACID ON CYANIDE* FORMATION IN THE PRESENCE OF RED CELLS AND THIOCYANATE

Agent	Thiocyanate added (μM)	Cyanide added (μM)	Cyanide recovery (%)
None	0	1.0	98.9
	200	1.0	398.0
Ascorbic acid**	0	1.0	100.6
	200	1.0	102.8

*Measured as total cyanide (see Experimental).
**20% Aqueous solution.

Thiocyanate in blood plasma and red cells

Methods for the determination of thiocyanate in red cells have not been reported, except Lang's [1] method. He has obtained approximately

quantitative recoveries of thiocyanate from whole blood using trichloroacetic acid as the deproteinizing reagent. But it was difficult to obtain quantitative and reproducible recoveries for thiocyanate by the method of Lang. Consequently, the new pretreatment method of red cells for the determination of thiocyanate was developed.

Blood plasma (100 μl) was deproteinized with 10% perchloric acid (400 μl) and centrifuged. Portions (20 μl) of the supernatant were then analyzed for thiocyanate by HPLC. The specificity of this deproteinization procedure was checked by a series of recovery tests, by adding various concentrations of potassium thiocyanate standard solution to blood plasma.

It was noted that thiocyanate in red cells was completely washed out by saline. So, the effective extraction of thiocyanate from red cells was accomplished by the use of phosphate buffer, pH 8.0, containing 0.3 *M* sodium perchlorate. Table II shows the results of the recovery tests for the determination of thiocyanate in blood plasma and red cells.

TABLE II

RECOVERY TESTS OF THIOCYANATE FROM BLOOD PLASMA AND RED CELLS

	Amount added (μM)	Recovery (%) (mean ± S.D.)*
Plasma	5.0	98.3 ± 2.1
	50.0	97.6 ± 3.3
Red cells	2.5	101.5 ± 1.4
	10.0	100.2 ± 0.7

*Mean of six analyses.

TABLE III

DETERMINATION OF CYANIDE AND THIOCYANATE IN HUMAN BLOOD SAMPLES

Subjects	Red cells			Plasma
	Total cyanide (μM)	Stable cyanide (μM)	Thiocyanate (μM)	Thiocyanate (μM)
1	0.15	0.15	10.6	13.3
2*	0.42	0.40	80.8	82.8
3*	0.44	0.42	84.7	90.0
4*	0.27	0.27	8.0	12.0
5	0.15	0.15	12.4	17.5
6	0.13	0.13	19.3	28.6
7*	0.27	0.25	34.7	44.4

*Cigarette smoker.

Application

The results for the determination of cyanide and thiocyanate in blood plasma and red cells are shown in Table III. Blood plasma thiocyanate concentrations in smokers were much higher than in non-smokers, and cyanide

concentrations in red cells of smokers tended to be higher but the difference was not significant. A marked difference between the total cyanide and the stable cyanide in red cells was neither observed. From these data, it is suggested that most cyanide in red cells exists as a "stable cyanide" and may have no toxic effects in vivo.

CONCLUSION

A new fluorometric method for the specific and sensitive determination of cyanide and thiocyanate based on the König reaction was developed using HPLC. The sensitivity of this sytem was more than one order of magnitude better than that obtained by the earlier colorimetric method using HPLC [15]. Anions present in environmental and biological materials mostly did not affect the chromatogram of cyanide and thiocyanate. It seems that our pretreatment methods are effective for the accurate determination of cyanide in red cells in the presence of large amounts of thiocyanate.

REFERENCES

1 K. Lang, Biochem. Z., 259 (1933) 243.
2 R.G. Baumeister, H. Schievelvein and G. Zickgraf-Rüdel, Arzneim.-Forsch., 25 (1975) 4056.
3 F. Goldstein and F. Reiders, Amer. J. Physiol., 167 (1951) 47.
4 J. Chung and J.L. Wood, J. Biol. Chem., 246 (1971) 555.
5 J.D. Oram and B. Reister, Biochem. J., 100 (1966) 373.
6 B. Sörbo and J.G. Llungren, Acta Chem. Scand., 12 (1958) 470.
7 F. Maloof and M.J. Soodak, J. Biol. Chem., 239 (1964) 1955.
8 C.J. Vesey and J. Wilson, J. Pharm. Pharmacol., 20 (1978) 20.
9 K.L. Pines and M.M. Crymble, Proc. Soc. Exp. Biol. Med., 81 (1952) 160.
10 J. Epstein, Anal. Chem., 19 (1947) 272.
11 R.L. Moran, G.E. Isom and J.L. Way, Toxicol. Appl. Pharmacol., 50 (1979) 323.
12 S. Baar, Analyst, 91 (1966) 268.
13 G.E. Boxer and J.C. Rickards, Arch. Biochem. Biophys., 39 (1952) 292.
14 T. Imanari, S. Tanabe and T. Toida, Chem. Pharm. Bull., 30 (1982) 3800.
15 T. Toida, S. Tanabe and T. Imanari, Chem. Pharm. Bull., 29 (1981) 3763.
16 W.J. Williams, Handbook of Anion Determination, Butterworths, London, 1979, p. 73.
17 G.V.L.N. Murty and T.S. Viswanathan, Anal. Chim. Acta, 25 (1961) 293.
18 A.R. Pettigrew and G.S. Fell, Clin. Chem., 18 (1972) 996.

Journal of Chromatography, 308 (1984) 143—151
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2097

QUANTITATIVE CAPILLARY COLUMN GAS CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF GLYCOPYRRONIUM IN HUMAN PLASMA

G.R. MURRAY

School of Pharmacy, Liverpool Polytechnic, Liverpool L3 3AF (U.K.) and Pharmacy Department, Whiston Hospital, Prescot, Merseyside L35 5DR (U.K.)

T.N. CALVEY* and N.E. WILLIAMS

Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool L69 3BX (U.K.) and Whiston Hospital, Prescot, Merseyside L35 5DR (U.K.)

and

K. CHAN*

School of Pharmacy, Liverpool Polytechnic, Liverpool L3 3AF (U.K.)

(First received September 30th, 1983; revised manuscript received February 2nd, 1984)

SUMMARY

A new sensitive and selective capillary column gas chromatographic method for the anticholinergic agent glycopyrronium bromide in human plasma is described. The procedure involves preliminary ion-pair extraction of the drug into dichloromethane, followed by concentration and analysis of the ion-pair complex by capillary column gas chromatography using a nitrogen-sensitive detector. The method depends on the thermal dequaternisation of the quaternary ammonium compound and can be used to detect 5 ng/ml in a 3-ml plasma sample. The assay procedure has been applied to the determination of the plasma concentration of glycopyrronium after intravenous administration to an anaesthetised patient.

*Present address: Department of Pharmacology, Chinese University of Hong Kong, Shatin, New Territories, Hong Kong.

0378-4347/84/$03.00

INTRODUCTION

Glycopyrronium bromide (glycopyrrolate; Robinul) is a quaternary amine with antimuscarinic properties, which may be used as an alternative to atropine, during the antagonism of non-depolarising neuromuscular blockade. In these conditions, it may have several important advantages. It does not enter the central nervous system to a significant extent, or produce central effects [1, 2]; it does not cause tachycardia or cardiac arrhythmias [3, 4]; and its duration of action is 4—7 h [5]. Nevertheless, little is known of the clearance, distribution or metabolism of the drug in man.

This paper presents a sensitive and selective analytical method for the determination of glycopyrronium in human plasma based on capillary column gas chromatography (GC) with nitrogen-sensitive detection. The procedure used is a modification of the method developed to measure the concentration of neostigmine and pyridostigmine in plasma [6] and is based on ion-pair extraction of the drug.

EXPERIMENTAL

Materials

The following materials were used: dichloromethane, diethyl ether and methanol, all of AnalaR grade and freshly redistilled; AnalaR anhydrous sodium sulphate; 0.1 mol/l and 5 mol/l sodium hydroxide solutions; 3 mol/l hydrochloric acid solution; 0.1 mol/l glycine buffer (AnalaR glycine, 7.505 g; AnalaR sodium chloride, 5.185 g and distilled water to 1 l); potassium iodide—glycine buffer (AnalaR potassium iodide, 12.8 g; 0.1 mol/l sodium hydroxide solution, 4 ml and 0.1 mol/l glycine buffer, 6 ml); potassium triiodide solution; iodine—potassium iodide—water (1:2:20, w/w/v); glycopyrronium bromide (A.H. Robins, Horsham, U.K.); [^{14}C]glycopyrronium bromide (A.H. Robins, Richmond, VA, U.S.A.); mepenzolate bromide (M.C.P. Pharmaceuticals, Wembley, U.K.); neostigmine bromide and pyridostigmine bromide (Roche, Welwyn Garden City, U.K.).

Apparatus

The following apparatus was used: a Sigma 3 gas chromatograph fitted with a phosphorus—nitrogen detector and linked to a Hitachi chart recorder Model 56 (Perkin-Elmer, Beaconsfield, U.K.). This system was operated with quartz capillary columns (25 m × 0.21 mm I.D.) and with coiled glass columns (1.5 m × 4 mm O.D.) silanized with hexamethyldisilazane (HMDS, Chromatography Services, Merseyside, U.K.) before use. These columns were packed with various stationary phases. The operating temperature for the injector and detector was 320° C and a suitable one for the column. Gas flow-rates were helium (carrier gas) 1 ml/min for capillary columns and 30 ml/min for glass columns, hydrogen 4 ml/min and air 100 ml/min. The setting of the rubidium bead in the phosphorus—nitrogen detector was adjusted to optimum condition before use. Other apparatuses used were: 15-ml capacity centrifuge tubes with well fitting screw caps (Sovirel, Levallois-Perret, France); 15-ml capacity stoppered evaporation tubes with finely tapered bases. All glassware was

cleaned by soaking overnight in a 2% solution of RBS 25 [Chemical Concentrates (RBS), London, U.K.] in water, then rinsing thoroughly with hot tap water followed by methanol and distilled water. These tubes were subsequently silanized by rinsing with a 3% (v/v) solution of HMDS in redistilled chloroform, and dried at 250°C overnight. This treatment of glassware was found to be necessary to eliminate possible loss of drug due to adsorption on the glass wall [7].

Gas chromatography

Methanolic solutions of glycopyrronium bromide and possible internal standards were injected on to the following chromatographic systems at various oven temperatures. System 1: Chromosorb W-AW (100—120 mesh) packed with 3% OV-17, in a coiled glass column (1 m × 4 mm O.D.). System 2: fused-silica capillary column coated with OV-101 (25 m × 0.21 mm I.D.). System 3: fused-silica capillary column coated with OV-1 (25 m × 0.21 mm I.D.).

All columns were conditioned at 20°C below that of the maximum recommended temperature of the relevant stationary phase for 24 h. Retention times, resolution and symmetry factors of the chromatographic peaks for these systems were then established. Compounds which were investigated as possible internal standards were: mepenzolate bromide, neostigmine bromide and pyridostigmine bromide.

Gas chromatography—mass spectrometry

Mass spectra of the GC resolved compounds were acquired using a Finnigan 1020 Series automated gas chromatograph—mass spectrometer operated in the electron-impact mode of ionisation. An electron energy of 24 eV and an ion source temperature of 80°C were used. Helium was used as carrier gas at a flow-rate of 1 ml/min.

General procedure for the determination of glycopyrronium in plasma

Blood samples were obtained by venous puncture after intravenous administration and collected in heparinised polythene tubes. The red blood cells were separated from the plasma by centrifugation (2500 *g* for 10 min). A sample of plasma (3.0 ml) in a 15-ml glass centrifuge tube was made alkaline (pH 10–12) with 20 μl of 5 mol/l sodium hydroxide solution and the internal standard, mepenzolate bromide (60 μl of a standard solution equivalent to a concentration of 2 μg/ml in water), was added. The alkaline solution was extracted with diethyl ether (10 ml) using an automatic shaker at a speed of 40 rpm for 10 min. The organic layer was then separated from the aqueous layer by centrifugation (2500 *g* for 10 min) and the ethereal extract was discarded. The remaining traces of diethyl ether were removed by purging nitrogen gas over the aqueous phase. Potassium iodide—glycine buffer (1 ml) was added to the ether-washed plasma and the resultant iodide—glycine drug complexes were extracted into dichloromethane (10 ml).

After mixing and centrifugation at 3500 *g* for 15 min, the plasma (upper) layer was discarded and the dichloromethane extract was dried by shaking with a quantity of anhydrous sodium sulphate (approximately 2 g). The water-free

extract was transferred carefully into an evaporation tube and evaporated to dryness by a gentle stream of nitrogen gas. The stoppered evaporation tube was then stored at −20°C before GC analysis. The residue was reconstituted in redistilled methanol (30 μl), by vortexing for 5 sec, immediately before GC analysis. An aliquot (2—5 μl) of the final concentrate was injected on to the GC system. The concentration of glycopyrronium present in the plasma sample was determined from the ratio of the peak height of glycopyrronium to that of the internal standard.

Calibration graphs

A standard solution of glycopyrronium bromide was prepared by dissolving the salt in distilled water. This was diluted to give a series of solutions in plasma (3.0 ml) covering the concentration range 5—100 ng/ml. The solutions were then analysed as described in the general procedure and the peak height ratios were plotted against the corresponding concentrations.

Recovery

The recovery of [^{14}C]glycopyrronium bromide from plasma was investigated using potassium iodide- glycine buffer at pH 10 and also using potassium triiodide as counter ions at pH 5—6. The recoveries at three different drug concentrations were studied (20, 50, and 100 ng/ml).

Samples of fresh plasma (3.0 ml) were spiked with [^{14}C]glycopyrronium bromide (specific activity = 11.7 μCi/mg) in water to give drug concentrations of 20, 50 and 100 ng/ml. The total radioactivity before extraction was expressed as 100%. The percentage of radioactivity recovered after extraction indicated the efficiency of the extraction procedure.

Each plasma sample was assayed as described in the general procedure. Where potassium triiodide, instead of potassium iodide, was used as counter ion, the ether-washed plasma was acidified with 100 μl of 3 mol/l hydrochloric acid solution (pH 5) and 100 μl potassium triiodide solution were added. The resultant drug complexes were extracted into 10 ml dichloromethane as in the general procedure. In all samples the water-free dichloromethane extracts were transferred into plastic scintillation vial inserts and evaporated to dryness under nitrogen gas. Redistilled methanol (200 μl) was added followed by 7 ml scintillation fluid (Triton-X-100/toluene cocktail). The radioactivity in each vial was counted for 100 min and the number of counts recorded (cpm) and converted to dpm.

The radioactivity before extraction (i.e. 100%) was determined by making up concentrations equivalent to 20, 50 and 100 ng/ml [^{14}C]glycopyrronium in 200 μl methanol in vial inserts and adding 7 ml scintillation fluid and counting radioactivity as above.

Selectivity

Samples of plasma obtained from volunteers were analysed to determine if they produced peaks after chromatography which interfered with that of glycopyrronium. In some experiments, basic drugs (e.g. pethidine and lignocaine) were added to plasma samples and assayed in order to detect if these drugs interfered with the chromatogram of glycopyrronium.

Reproducibility

Eight replicate samples of glycopyrronium (40 ng/ml) in plasma were assayed as in the general procedure and the peak height ratio of the drug to internal standard (mepenzolate) was calculated for each sample.

Storage

Samples of freshly collected plasma were analysed immediately and after storage at −20° C for 24 h and 7 days. Concentrates of the extracts containing drugs were assayed immediately and after storage at −20° C for 24 h and 7 days.

RESULTS AND DISCUSSION

Choice of GC system

All three systems investigated resolved the dequaternised analogue of glycopyrronium (see Mass spectrometry for explanation). System 3 (OV-1 capillary column), however, offered little selectivity and substances in plasma such as cholesterol and fatty acids interfered with the drug peak. Systems 1 (3% OV-17 glass column) and 2 (OV-101 capillary column) were both suitable for the determination of glycopyrronium. System 2, using splitless injection but with the split vent opened after 30 sec, gave superior peak shape and much less interference from the components present in the solvent front. System 2 was therefore used for routine analysis. With the column at 240° C, a vent time of 30 sec and a split ratio of 50:1, a methanolic solution of glycopyrronium bromide and a methanolic solution of its potassium iodide–glycine complex following extraction both gave one peak at a retention time of 4.8 min.

Mepenzolate bromide was the most suitable compound for use as an internal standard. A methanolic solution of mepenzolate bromide and of its potassium iodide–glycine complex gave only one peak at a retention time of 7.0 min.

TABLE I

PERFORMANCE OF SYSTEM 2 (OV-101 QUARTZ CAPILLARY COLUMN)

Temperatures: injection, 320° C; oven, 240° C; detector, 320° C. Gas flow-rates: helium (carrier), 1 ml/min, split ratio 50:1 and vent time 30 sec; hydrogen 4 ml/min and air 100 ml/min; bead setting 400.

Compound	Retention time (min)	Symmetry factor* (0.95–1.05)	Resolution between marker* (≥ 1.0)
Glycopyrronium bromide	4.8	1.0	2.75
Glycopyrronium– potassium iodide complex			
Mepenzolate bromide	7.0	0.96	
Mepenzolate–potassium iodide complex			

*British Pharmacopoeia specification for gas–liquid chromatographic analysis [8].

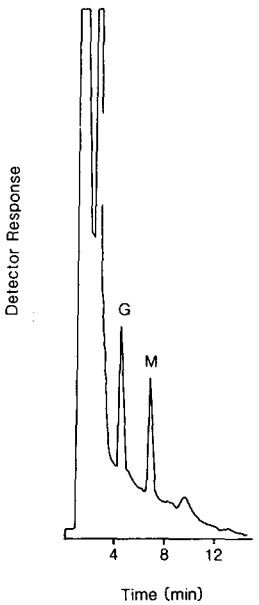


Fig. 1. Chromatogram of glycopyrronium (G) and the internal standard mepenzolate (M) after ion-pair extraction from human plasma.

Both neostigmine and pyridostigmine bromide produced peaks at less than 3 min and could not be separated from other components in the solvent front.

The respective retention times, symmetry factors and resolution of glycopyrronium bromide and its potassium iodide—glycine complex and of mepenzolate bromide and its potassium iodide—glycine complex are summarised in Table I and Fig. 1.

Gas chromatography—mass spectrometry

It was demonstrated that both glycopyrronium and mepenzolate (either as the bromide salt or as the potassium iodide—glycine complex) were thermally

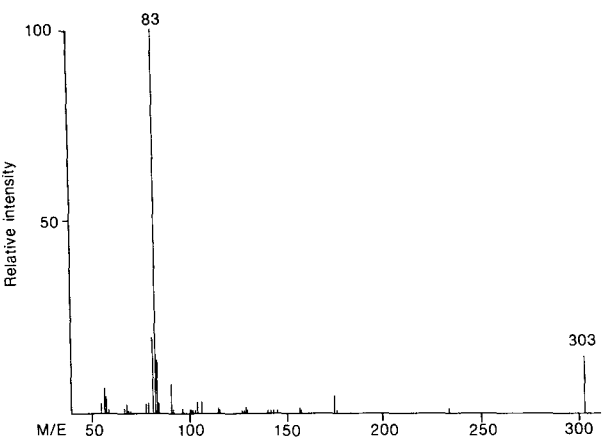


Fig. 2. Electron-impact mass spectrum of glycopyrronium bromide.

dequaternised to their corresponding tertiary analogues which were then resolved by the GC system, by coupling System 2 with a mass spectrometer and recording the mass spectrum of each compound. The mass spectra of glycopyrronium bromide and of its potassium iodide—glycine complex following ion-pair extraction were found to be identical. Both spectra had a molecular ion at *m/e* 303, and a base peak at *m/e* 83 resulting from breakdown of the ester grouping and subsequent release of an N-methylpyrrolidinyl fragment (Figs. 2 and 3). The mass spectra of mepenzolate bromide and of its ion pair complex were identical, both having a molecular ion at *m/e* 325 and a base peak at *m/e* 97, resulting from the breakdown of the ester grouping and subsequent release of the N-methylpiperidinyl fragment (Figs. 3 and 4).

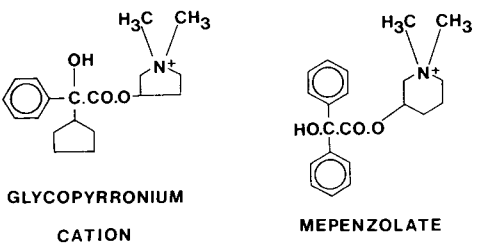


Fig. 3. Structure of glycopyrronium and mepenzolate.

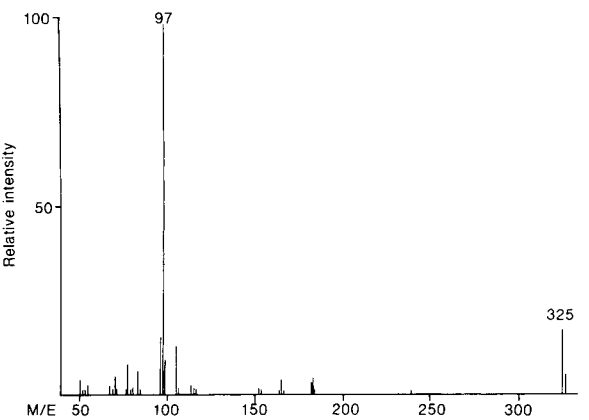


Fig. 4. Electron-impact mass spectrum of mepenzolate bromide (internal standard).

TABLE II

RECOVERY STUDIES

Each value is mean ± S.E.M. of four experiments.

Concentration (ng/ml)	Percent recovery	
	Potassium iodide—glycine buffer	Potassium triiodide
20	52.29 ± 1.06	48.59 ± 1.73
50	51.41 ± 1.61	47.46 ± 2.27
100	49.62 ± 1.46	47.99 ± 1.14

Recovery

The recovery of [^{14}C] glycopyrronium from plasma using potassium iodide—glycine buffer ranged from 49.6 to 52.3% at concentrations between 20 and 100 ng/ml (Table II). Using potassium triiodide as pairing ion, the recovery of [^{14}C] glycopyrronium ranged from 47.5 to 48.6% (Table II). Therefore as no substantial difference was found in drug recovery using a stronger pairing ion, potassium iodide—glycine buffer was used in the general procedure.

Selectivity

The preliminary ether wash eliminated commonly used basic drugs (for instance pethidine and lignocaine) which might be extracted simultaneously from plasma and be analyzed by the GC system. In the present procedure, there were no chromatographic peaks from a normal plasma extract which interfered with the measurement of peaks corresponding to glycopyrronium and mepenzolate.

Reproducibility

When eight replicate samples of glycopyrronium (40 ng/ml) in plasma were assayed using mepenzolate (2 μg/ml) as internal standard, the reproducibility of the peak height ratio was 100 ± 9%. The calibration graphs of glycopyrronium using mepenzolate as internal standard were found to be reproducible when repeated five times during the studies. There was invariably a linear relationship between the concentration of glycopyrronium in plasma (in the range from 5—100 ng/ml) and the peak height ratio of glycopyrronium: mepenzolate.

Storage

There was no difference in peak height ratios to internal standard in extracts assayed immediately and after storage at —20° C for 24 h and 7 days.

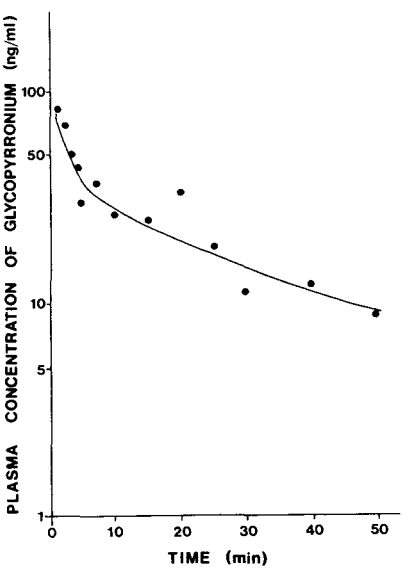


Fig. 5. Semi-logarithmic plot of the plasma concentration of glycopyrronium after intravenous administration to an anaesthetised patient. Dose of glycopyrronium = 0.3 mg.

Application

The procedure has been used to measure the concentration of glycopyrronium in human plasma after intravenous injection of the quaternary amine. Glycopyrronium was used in anaesthetised patients to prevent undesirable muscarinic effects during the antagonism of non-depolarising neuromuscular blockade.

After intravenous injection of glycopyrronium bromide (0.3 mg), samples of blood were collected at 1, 2, 3, 4, 5, 7, 10, 15, 20, 25, 30, 40 and 50 min; plasma was separated as soon as possible by centrifugation. In the one patient studied, the concentration of glycopyrronium fell rapidly from 80.8 ng/ml at 1 min to 36.5 ng/ml at 7 min. Subsequently the concentration of the drug declined more slowly to 8.6 ng/ml after 50 min (Fig. 5). It should be emphasised that these results are only based on a single study. A more detailed investigation of the plasma concentration of glycopyrronium after intravenous administration is in progress.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. C.J. Jones for his help and encouragement. They are also indebted to the A.H. Robins Co. and the Peel Medical Research Trust for financial assistance.

REFERENCES

1 A.G. Proakis and G.B. Harris, Anaesthesiology, 48 (1978) 339.
2 S. Ramamurthy, M.H. Shaker and A.P. Winnie, Can. Anaesth. Soc. J., 19 (1972) 399.
3 L. Gyermek, Curr. Ther. Res., 18 (1975) 377.
4 R.R. Mirakhur, R.S.J. Clarke, J. Elliott and J.W. Dundee, 33 (1978) 906.
5 G.M. Wyant and E. Kao, Can. Anaesth. Soc. J., 21 (1974) 230.
6 K. Chan, N.E. Williams, J.D. Baty and T.N. Calvey, J. Chromatogr., 120 (1976) 349.
7 K. Chan and A. Dehghan, J. Pharmacol. Methods, 1 (1978) 311.
8 British Pharmacopoeia, Her Majesty's Stationary Office London, 1980, p. A63.

Journal of Chromatography, 308 (1984) 153—164
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2085

DETERMINATION OF THE PARTITIONING, STABILITY, AND METABOLITE FORMATION OF ISOSORBIDE DINITRATE IN HUMAN AND RAT BLOOD USING AN IMPROVED GAS—LIQUID CHROMATOGRAPHIC ASSAY

R.A. MORRISON and H.-L. FUNG*

Department of Pharmaceutics, School of Pharmacy, Cooke Hall, State University of New York at Buffalo, Buffalo, NY 14260 (U.S.A.)

(First received August 8th, 1983; revised manuscript received January 23rd, 1984)

SUMMARY

A simple and highly sensitive gas—liquid chromatographic method using electron-capture detection has been developed for the simultaneous determination of isosorbide dinitrate (ISDN) and its mononitrate metabolites in rat and human plasma. This method has a limit of quantitation of about 5 ng/ml for the mononitrates and of 1 ng/ml for ISDN using 0.1 ml of plasma, and is thus useful for pharmacokinetic studies of these compounds in small animals, and in humans when the available volume of blood is limited. Using this method, we found the apparent in vitro partitioning ratio of ISDN between erythrocyte and plasma in rat and human blood at 37°C to be 0.22 and 0.13, respectively. In spite of this poor affinity for red blood cells, ISDN degradation in whole blood was mediated primarily via this blood fraction. Loss of ISDN in blood appeared to proceed exclusively through its mononitrate metabolites, resulting in a 6:1 product ratio of the 5-mononitrate to its 2-isomer. These data suggest that although blood degradation of ISDN and erythrocyte partitioning occur per se, these phenomena do not contribute significantly to the very rapid in vivo clearance of ISDN observed in man and in the rat.

INTRODUCTION

Isosorbide dinitrate (ISDN) has been shown to be an effective drug for the management of angina pectoris [1,2], congestive heart failure [3], and acute pulmonary edema [4]. Recently, reliable gas chromatographic techniques for the determination of this drug in plasma have become available and have been used in clinical pharmacology studies of ISDN [5,6]. The denitrated, primary metabolites of ISDN, isosorbide-2-mononitrate (2-ISMN) and isosorbide-5-mononitrate (5-ISMN), have been shown to be pharmacologically active vasodilators in dogs [7,8] and in man [9].

0378-4347/84/$03.00

Assay procedures for ISDN only [5,10—12], mononitrates only [13], or for all three compounds [14—16] in plasma have been described. The simultaneous methods [14—16] are superior when throughput is considered since sample splitting for separate determinations is not required. However, these procedures employed relatively large plasma volumes (2—4 ml) to attain the requisite sensitivity. In pharmacokinetic studies with small laboratory animals, such a sample size is not feasible. In studies with selected patient populations, the availability of blood may also be limited. Thus, it appears desirable to develop a more sensitive procedure for the simultaneous determination of ISDN and its mononitrate metabolites.

Several reports have indicated that organic nitrates, particularly nitroglycerin, are highly unstable in whole blood and in plasma [17—22]. Johson et al. [17] examined the in vitro disappearance rate of ISDN in rat whole blood at 37°C and reported a degradation half-life ($t_{½}$) of 15—20 min. Their study, however, used a relatively high initial ISDN concentration (ca. 2.5 μg/ml) and therefore these results may not apply to the lower drug concentrations typically observed in vivo. ISDN was found to be considerably more stable in human plasma, $t_{½}$ = 55 h at 37°C [5], but its stability and partitioning characteristics in human whole blood has not been investigated. Knowledge of these parameters is necessary for the proper planning and interpretation of ISDN pharmacokinetic studies. In addition, the contribution of in vivo blood metabolism to the total body clearance might also be estimated from this information. This estimation may be important in understanding the reason(s) for the very high in vivo total body clearance observed for ISDN [23].

EXPERIMENTAL

Standard solutions

ISDN was extracted with diethyl ether from a 20% (w/w) mannitol adsorbate supplied by Stuart Pharmaceuticals (Wilmington, DE, U.S.A.), recrystallized with an aqueous ethanol solution, and checked for purity with the United States Pharmacopoeia (USP) [24] method modified to effect separation of metabolites using a celite column with isooctane elution. The isomeric mononitrates, obtained from Ayerst Pharmaceuticals (Rouses Point, NY, U.S.A.), as well as heptachlorepoxide (HCE) and dichlorodiphenyltrichloroethane (DDT), both from Supelco (Bellefonte, PA, U.S.A.), were received as pure solids (> 99%) and were used without further purification.

Cleaning and silanization procedure

All glassware used was acid-washed and consecutively rinsed with distilled water and pesticide-grade methanol and then dried. A 10% (v/v) dimethyldichlorosilane solution in toluene was used to silanize tubes. Tubes were immediately rinsed with toluene to remove excess reagent, and then successively rinsed with toluene—methanol (1:1) and pesticide-grade methanol before being dried.

Effect of gas—liquid chromatographic injection solvent

Four solutions were prepared in which the isoamyl alcohol concentration in

hexane varied from 5 to 62.5% (v/v). In these co-solvents, as well as neat hexane, the concentrations of the three organic nitrates were held constant in ratios similar to those found for in vivo plasma samples (ISDN = 201, 2-ISMN = 107, 5-ISMN = 674 pg/μl). The effect of injection co-solvent composition on the apparent electron-capture detector response was assessed after triplicate 1-μl injections.

Gas—liquid chromatographic assay

Organic nitrates in 0.1 ml of plasma were first stabilized with 0.01 ml of 1 *M* silver nitrate [18] and extracted once with 3 ml of pesticide-grade chloroform containing an appropriate amount of HCE or DDT as internal standard. This tube was then sealed with a PTFE stopper and shaken at slow speed for 30 min. The upper, aqueous phase was aspirated, and a suitable aliquot (0.05—2.0 ml) of the chloroform was transferred to a 3-ml centrifuge tube for evaporation to dryness under pre-purified nitrogen in an ice bath (4°C). As soon as the tube was dry, care was taken to stop the nitrogen flow quickly. The compounds were reconstituted with an appropriate volume of 25% (v/v) isoamyl alcohol in hexane, depending on the anticipated concentration calculated from reported pharmacokinetic parameters [9,23]. These solutions were then vortexed, covered with parafilm, and stored in crushed ice prior to injection of 0.5—3.0 μl on the chromatograph. The glass column used (1.85 m × 4 mm) was custom-made with an extension to the septum so as to facilitate on-column injection. The column was packed with 3% SP-2401 and 100—120 mesh Chromosorb 750 purchased from Supelco. On a daily basis, the column was primed and stabilized for response via multiple injections of the highest and lowest standards. The nitrogen carrier gas flow-rate was approximately 80 ml/min with operating temperatures of 200°C, 160°C and 200°C for the inlet, column, and detector, respectively. The gas—liquid chromatographic (GLC) instruments (Packard Models 7500 and 428, Downers Grove, IL, U.S.A.), equipped with a ^{63}Ni electron-capture detector, maintained a linear response for these compounds over the range of 25—1500 pg injected on-column. The GLC—electron-capture detector (ECD) response for the internal standards was linear over the range of 5—500 pg injected on-column. Calibration curves were constructed daily using standards prepared in 25% (v/v) isoamyl alcohol in hexane containing either HCE or DDT as internal standard.

Recovery from spiked plasma

For recovery from rat plasma, six male Sprague-Dawley rats supplied by Blue Spruce Farms (Altamont, NY, U.S.A.) were fasted overnight and blood was collected via cardiac puncture into heparinized tubes. The separated plasma was then pooled and spiked using aqueous stock solutions of organic nitrates. For recovery from human plasma, blood was withdrawn from a cubital vein, heparinized, and the separated plasma was similarly spiked. Recovery was evaluated over wide ranges of plasma concentrations for rat plasma in order to encompass expected levels in future pharmacokinetic studies. Recovery studies from human plasma covered smaller ranges of the three nitrates but these were sufficient to include concentrations typically seen after therapeutic doses.

Stability studies

Stability of ISDN was studied in heparinized (50 U/ml) rat and human whole blood, and rat plasma incubated at 37°C. Rat fluids were obtained from three rats via cardiac puncture and pooled. Human whole blood was collected from a peripheral arm vein from three healthy volunteers. Incubation trials were conducted in triplicate. Each incubation (ca. 8 ml) was covered and gently stirred to prevent sedimentation of erythrocytes. Serial aliquots (0.25 ml) were collected at 0, 2, 5, 10, 30, 60, 90, 120, 180 and 240 min for whole blood experiments, and plasma (0.1 ml) was separated for analysis. For the study of ISDN stability in rat plasma, an additional sample at 300 min was obtained.

The procedure developed for the assay of plasma concentrations of these organic nitrates was not suitable for determining the concentrations of these compounds in whole blood, since both specificity and precision were unacceptably impaired. Thus, the in vitro whole blood stability of ISDN was determined by monitoring the concentrations of organic nitrates in plasma which was separated from whole blood after serial sampling. This being the case, the disappearance of ISDN from plasma obtained from spiked whole blood samples might be contributed, at least initially, by distribution of drug into the erythrocytes, as well as by degradation. This distribution phase might be avoided if ISDN stability was studied with blood in which erythrocyte-to-plasma equilibrium has been achieved. Thus, ISDN stability in rat whole blood was carried out with spiked samples and with samples obtained from animals which have been infused in vivo to apparent steady state with ISDN.

The spiked blood was prepared by adding 0.5 ml of a normal saline solution of ISDN (5.1 μg/ml) to 25 ml of whole blood. Plasma incubation samples were prepared similarly. Rat whole blood was also prepared to contain ISDN by using a constant-rate in vivo infusion (7 μg/min) into a right jugular vein catheter implanted [25] in the animal. A 2-h infusion duration was sufficient to attain apparent steady state [26] and resulted in plasma concentrations similar to those obtained from in vitro spiking (about 200 ng/ml).

RESULTS AND DISCUSSION

Standardization

Because of its explosive nature, ISDN cannot be handled in its pure form. Primary standards of ISDN have to be prepared via extraction from a mannitol adsorbate mixture. Quantitation of primary standards of ISDN, on which all subsequent determinations are based, is therefore extremely important. When this work was initiated, the USP XIX method [24] quantified ISDN via hydrolytic liberation of inorganic nitrate and nitrate ions, and subsequent reaction with phenoldisulfonic acid to form a stable chromophore. There was no provision for a celite column purification step to remove the possible degradation or contaminant products; viz., the mononitrates, inorganic nitrate and/or nitrite ions. This type of interference has been shown to be present when nitroglycerin is assayed by this method [27]: The USP XIX method [24] was therefore modified in this study by adding a celite column elution step to overcome this deficiency. Under certain circumstances (e.g. equipment or expertise availability), this method may be a useful alternative to the polarographic assay recommended in USP [28].

Choice of internal standard

Nitroglycerin (NTG) can be used as an internal standard for this assay. However, in some therapeutic situations, the concomitant use of NTG and ISDN cannot be avoided, and the use of NTG as an internal standard for assay is obviously inappropriate. Isoidide mononitrate, which has been used for this purpose [16], was shown to be a urinary metabolite of ISDN in the rat [29]. Other organic nitrates used, viz., isoidide dinitrate [11,12] and isomannide dinitrate [16] are not available commercially.

The pesticides used here, HCE and DDT, are useful alternatives to NTG as internal standards. They are available commercially in pure form, are highly sensitive to ECD, and chromatograph at locations which were usually free from interference from peaks which appeared with blank plasma samples. When interference does occur with either one of the internal standards (as we found occasionally with HCE in some human plasma samples), the other internal standard may be employed. Although DDT has a long retention time, we seldom found any interference at its location in patient plasma samples.

Ideally, the internal standard should be added to plasma directly. However, in this case, HCE and DDT are so poorly soluble in water that an alcoholic co-solvent would have been needed; this would then have decreased the extraction efficiency of the polar mononitrates into chloroform. Thus, in this assay, the internal standards were added to plasma via the extraction solvent.

Choice of injection solvent

Mononitrates were found to adsorb to the injection syringe if a non-polar solvent such as hexane was used. Smith and Besic [13] attempted to decrease such adsorptive losses via *tert.*-butylsilyl derivatization of the mononitrate's polar hydroxy groups. This method, which required two back-extractions as well as the derivatization step, was unsuccessful in decreasing the detection limit of 5-ISMN in plasma below 250 ng/ml. LeBel and Williams [30] demonstrated that syringe adsorption in the GLC analysis of polar insecticides could be overcome by using acetone instead of the relatively non-polar hexane as injection solvent. Since acetone was not compatible with the trifluoropropyl liquid phase of our packing material, isoamyl alcohol was added to hexane in order to increase injection solvent polarity, and hence, mononitrate solubility.

The effect of isoamyl alcohol concentration in the injection co-solvent on the assay sensitivity of the three organic nitrates was examined. Dramatic improvements in the absolute detector response and in the coefficient of variation (C.V.) were observed for all three organic nitrates when isoamyl alcohol concentration in hexane was increased (Fig. 1). When compared with neat hexane, an injection co-solvent composition of 63% (v/v) isoamyl alcohol in hexane caused 6-, 15-, and 25-fold increases in apparent detector response for ISDN, 2-ISMN, and 5-ISMN respectively. Additionally, this co-solvent mixture produced an approximate 50% reduction in the C.V. value with triplicate injections. Unfortunately, injections containing high concentrations of isoamyl alcohol produced a more prolonged solvent front, and possibly lessened column stability. The co-solvent composition of 25% (v/v) of isoamyl alcohol in hexane appeared to provide an optimal balance between the need to increase detector response and the desirability to minimize the amount of isoamyl alcohol in-

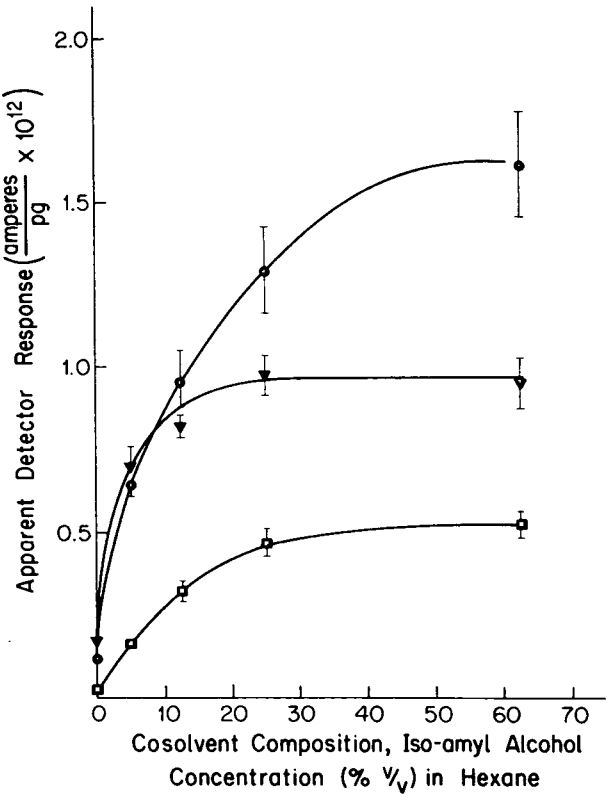


Fig. 1. Effect of co-solvent composition on the mean (± S.D.) GLC—ECD response of ISDN (▾), 2-ISMN (•), and 5-ISMN (▪). Each point represents three determinations.

jected; this particular composition was therefore chosen for all subsequent work.

Fig. 2 shows the chromatogram of a standard solution, and those of extracts of spiked and blank rat plasma. The key determinant of limit of quantitation for these compounds was the degree of possible interference from other substances in blank plasma or in the system. Typically, blank values for ISDN, 2-ISMN, and 5-ISMN were at or below 0.5, 1, and 2 ng/ml, respectively. The higher blank values for the mononitrates reduced their limits of quantitation relative to ISDN. Compared to previously published assays, however, this method still represents considerable increase in sensitivity for the mononitrate metabolites.

Choice of extraction solvent

Although ethyl acetate and diethyl ether were both satisfactory in extracting the three organic nitrates, chromatograms of blank rat plasma using these solvents were found to contain numerous interference peaks which limited the sensitivity for mononitrate detection. This problem was absent with chloroform as the extraction solvent. However, high-density polyethylene could not be used with this solvent since a contaminant occurred which co-chromatographed with 5-ISMN. Use of PTFE in place of this plastic removed this

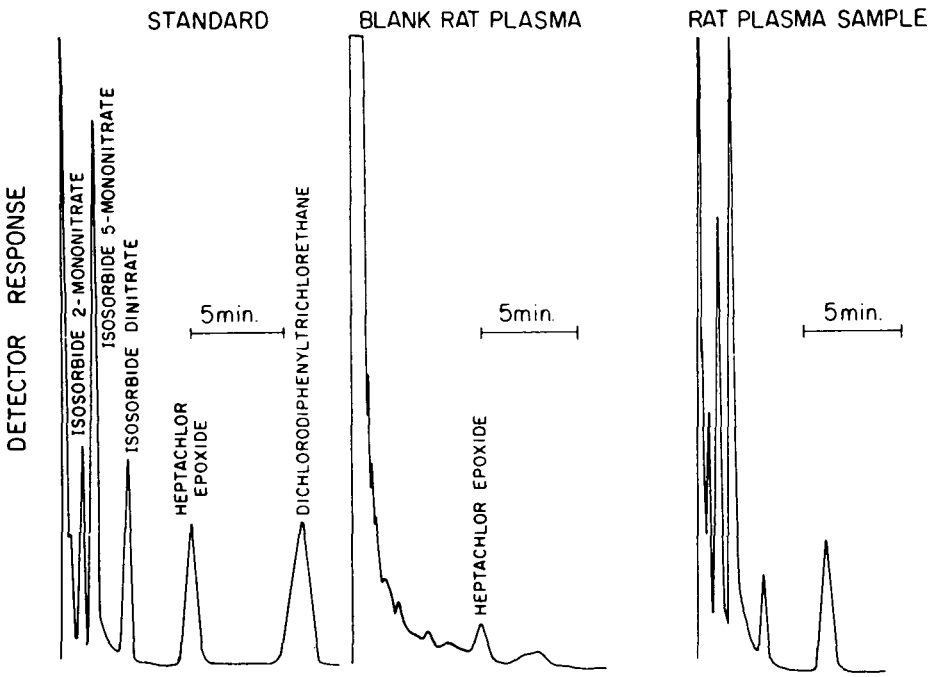


Fig. 2. Chromatograms showing simultaneous determination of ISDN, 2-ISMN, and 5-ISMN in rat plasma. The approximate on-column amounts injected for the standard shown were 2-ISMN (0.1 ng), 5-ISMN (0.5 ng), ISDN (0.2 ng), HCE (0.2 ng) and DDT (0.5 ng).

problem. Chloroform is highly electron capturing and therefore must be evaporated completely before reconstitution with 25% (v/v) isoamyl alcohol—hexane. Recovery was reduced by 80% and 10% for 2-ISMN and 5-ISMN, respectively, if evaporation to dryness was carried out at ambient temperatures rather than in an ice bath (4°C).

Recovery and reproducibility

The extraction efficiency and overall reproducibility of this procedure were examined following multiple determinations on spiked rat and human plasma. For the mononitrates, mean recovery was about 80% and did not appear to depend on the added concentration over a wide range of concentrations studied (Table I). ISDN, however, had significantly higher recovery (analysis of variance, $p < 0.001$, 35 pairs) with increasing spiked rat plasma ISDN concentration. A significant correlation ($r = 0.66$, $p < 0.001$, 35 pairs) was observed between percent recovery and spiked concentration (Fig. 3). Although the absolute difference in percent recovery over this concentration range was not large (ca. 20%), the differences were statistically significant. This concentration dependency in recovery was found not to arise from artifactual factors such as the percentage of water in diluted plasma, aliquot size and the presence of different concentrations of the mononitrate metabolites.

Since the concentration range covered almost four orders of magnitude, a ln—ln plot was used to examine the relationship between the experimentally determined concentration [not corrected for recovery, i.e., $C_{p(\text{assayed})}$] and the

TABLE I

RECOVERY OF ISDN AND ITS MONONITRATE METABOLITES FROM RAT AND HUMAN PLASMA AT DIFFERENT ADDED CONCENTRATIONS

	ISDN			2-ISMN			5-ISMN		
	Concentration* (ng/ml)	Percent recovery (mean)	C.V. (%)	Concentration (ng/ml)	Percent recovery (mean)	C.V. (%)	Concentration (ng/ml)	Percent recovery	C.V. (%)
Rat plasma	1.49 (11)	69	13	1.89	78	9	7.71	81	10
	6.88	75	15	3.92 (5)	73	7	18.4	81	8
	68.8	78	10	196	78	8	920	84	12
	1560	87	13	1980	75	11	8080	100	12
	6880	88	7	19,600	83	6	92,000	88	9
Grand mean		**			78	8		87	10
Human plasma	1.56	80	22	2.13	79	12	8.69	80	9
	3.12	79	22	4.26	76	11	17.4	75	11
	31.2	90	11	42.6	79	16	174	81	12
Grand mean		83	18		78	12		79	10

*n = 6 determinations for rat plasma unless otherwise indicated in brackets, n = 4 for human plasma.

**Recovery was concentration-dependent and was therefore not averaged; see Fig. 3 and text.

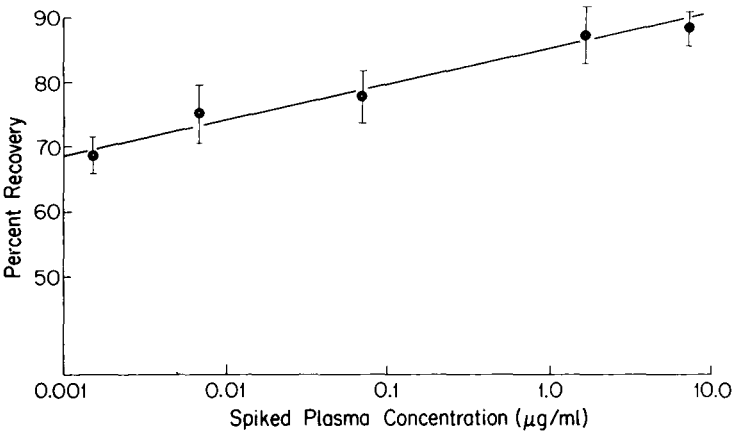


Fig. 3. Percent recovery of ISDN (± S.E.M.) as a function of a spiked rat plasma concentration.

theoretical, spiked concentration, $C_{p(\text{spiked})}$. Regression constants which were generated from individual recoveries (n = 35 pairs) are shown in eqn. 1.

$$\ln C_{p\ (\text{spiked})} = \frac{\ln C_{p\ (\text{assayed})} + 0.366}{1.029} \quad (1)$$

This relationship allows for correction of the concentration dependency of ISDN recovery over the wide range of rat plasma concentrations studied.

The recovery of ISDN from human plasma was studied over a much smaller range (1.5—31 ng/ml) and no significant effect of concentration on recovery was observed (Table I). Over this range of concentrations the mean percent recovery was 83%, 78%, and 79% for ISDN, 2-ISMN, and 5-ISMN, respectively.

Stability and partitioning studies

Fig. 4 shows that ISDN was metabolized rapidly in whole blood from rat and human ($t_{½}$ = 100 min) but was more stable in isolated rat plasma ($t_{½}$ = 8.7 h). A previous report showed ISDN to have a half-life of 55 h in human plasma [20]. Ideally, the stability of ISDN in whole blood should be directly monitored in blood and/or erythrocytes in addition to measurements of drug and metabolite concentrations in plasma. However, the present assay technique did not allow for these direct determinations in either blood or erythrocytes. Thus, the observed decline in plasma ISDN concentration after spiking of intact drug into whole blood may have been contributed, in part, by drug distribution into erythrocytes. Relatively slow uptake of drug into red blood cells has been reported for acetazolamide [31] and lithium [32]. To examine this possibility for ISDN, its stability in whole blood was assessed after incorporation of this drug into rat blood by both in vivo steady-state infusion as well as by in vitro spiking. A previous study [26] indicated that steady-state plasma ISDN levels were attained (ca. 150—200 ng/ml) with a 2-h constant-rate infusion at 7 μg/min. Therefore, it is reasonable to assume that after this infusion regimen in the rat, distributional equilibrium between plasma and erythrocytes has been achieved. No difference in the in vitro stability of ISDN was found between

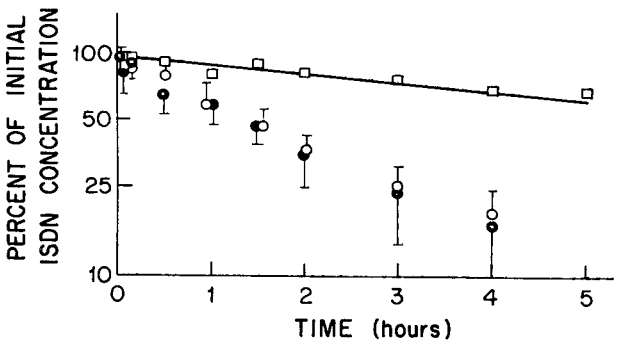


Fig. 4. Stability of ISDN following incubation (37°C) in whole blood (mean ± S.D.); (•) human (n = 3); (○) rat (n = 6) and (□) in rat plasma (mean, n = 2).

blood prepared to contain drug by in vitro spiking or in vivo infusion. This finding suggested that (1) erythrocyte partitioning of ISDN was essentially instantaneous and (2) loss of ISDN in incubated whole blood was due to physical and/or biotransformation processes rather than time-dependent accumulation by erythrocytes. The data suggested that degradation was linear, at least over the concentration range monitored (ca. 200 down to 25 ng/ml).

If it is assumed that plasma-to-erythrocyte partitioning of ISDN is instantaneous, the apparent partition coefficient can be quantitated without determination of actual corpuscle concentrations. Using the measured initial plasma concentration, the theoretical initial whole blood concentration, and the hematocrit, the erythrocyte-to-plasma apparent partition coefficient (K_p) was estimated at 0.22 and 0.13 for rat and human blood, respectively. The reason(s) for this relatively low erythrocyte partitioning of ISDN is (are) not clear at present. Using radiolabelled ISDN, Sisenwine and Ruelius [33] reported negligible radioactivity in dog erythrocytes for 2 h after in vivo ISDN administration. Their finding is consistent with the present results which indicated minimal erythrocyte partitioning of ISDN in blood obtained from the rat and human species.

Wu et al. [20] and Sokoloski et al. [22] recently concluded that the mechanism responsible for the loss of nitroglycerin after incubation in human blood was physical in nature, since they could not detect the presence of any denitrated metabolites concomitant with nitroglycerin loss. On the other hand, Johnson et al. [17] and Noonan and Benet [21] reported the formation of denitrated metabolites following whole blood incubation of organic nitrates. In the present study, significant concentrations of mononitrate metabolites were observed when ISDN was incubated in blood (Fig. 5). During ISDN degradation in human whole blood, plasma mononitrate metabolite levels increased during the first 2 h and then remained relatively constant thereafter (Fig. 5). The 5-ISMN isomer was the major metabolite formed (approximately 6:1, 5-ISMN:2-ISMN). This ratio of mononitrates formed from ISDN in vitro was similar to the ratio observed after in vivo dosing of ISDN in man [34] and animals [26, 33,35].

Assuming that the calculated partition coefficient of ISDN is constant throughout the 4-h period studied, the total amount of ISDN lost in whole

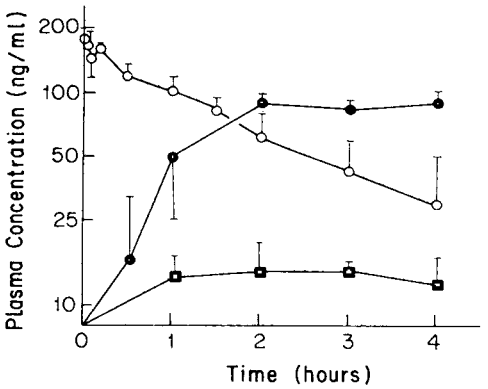


Fig. 5. Plasma concentration (mean ± S.D.) of ISDN (○) and its mononitrate metabolites 2-ISMN (■) and 5-ISMN (●) observed after incubation of ISDN in human whole blood (37°C).

blood can be quantitated and compared with the amount of denitrated metabolites formed in plasma. For the first 2 h, 96% of the total molar amount of ISDN that was lost (0.28 nmol/ml) was accounted for by summation of the mononitrate amounts produced in the plasma (2-ISMN = 0.04 nmol/ml; 5-ISMN = 0.23 nmol/ml). This metabolite mass balance steadily decreased to about 50% at 4 h of incubation, presumably due to further denitration of the mononitrates themselves or slow accumulation of these mononitrates into erythrocytes. The quantitative production of mononitrate metabolites in the present study indicated that biotransformation via denitration, rather than physical loss, was the operative mechanism responsible for the loss of ISDN in whole blood. In addition, the fact that 96% of ISDN lost from whole blood was accounted for by the amounts of mononitrates formed in plasma suggested that, within the first 2 h at least, erythrocyte partitioning of these metabolites was also minimal.

Results of these in vitro stability experiments can also be used in furthering our understanding of in vivo pharmacokinetic data of ISDN. The contribution of blood degradation itself to the total body clearance of ISDN can be estimated using the product of the in vitro disappearance rate constant (ca. 0.007 min^{-1}) and the physiologic blood volume in these species (rat, 65 ml/kg [36] and man, 70 ml/kg [37]). This estimation produces a blood clearance value of 0.46 ml/min/kg in the rat and 0.49 ml/min/kg in man, respectively. This value represents less than 1% of the systemic clearance for ISDN in either species. Thus, denitration by whole blood itself does not appear to contribute significantly to the high systemic clearance and the extensive extrahepatic metabolism of ISDN suggested in rats [26] and cardiac patients [23].

ACKNOWLEDGEMENTS

Supported in part by NIH Grant HL22273. We thank J. Dolce and J. Rogan for their excellent technical assistance.

REFERENCES

1 T. Sweatman, G. Strauss, A. Selzer and K.E. Cohn, Amer. J. Cardiol., 29 (1972) 475.
2 D.T. Danahy and W.S. Aronow, Circulation, 56 (1977) 205.
3 J.A. Franciosa, E. Mikulic, J.N. Cohn, E. Jose and A. Fabie, Circulation, 50 (1974) 1020.
4 P. Chiche and S. Baligadoo, Amer. J. Cardiol., 44 (1979) 392.
5 H.-L. Fung, E.F. McNiff, D. Ruggirello, A. Darke, U. Thadani and J.O. Parker, Brit. J. Clin. Pharmacol., 11 (1981) 579.
6 S. Sporl-Radun, G. Betzien, B. Kaufmann, V. Liede and U. Abshagen, Eur. J. Clin. Pharmacol., 18 (1980) 237.
7 R.L. Wendt, J. Pharmacol. Exp. Ther., 180 (1972) 732.
8 M.G. Bogaert and M.T. Rosseel, Naunyn-Schmiedeberg's Arch. Pharmacol., 275 (1972) 339.
9 M. Kaltenbach, W.D. Bussmann and A. Schrey (Editors), Mononitrat, Universitätsdruckerei und Verlag Dr. C. Wolf und Sohn, Munich, 1981.
10 H. Laufen, F. Scharpf and G. Bartsch, J. Chromatogr., 146 (1978) 457.
11 M.T. Rosseel and M.G. Bogaert, J. Pharm. Sci., 62 (1973) 755.
12 J.O. Malbica, K. Monson, K. Neilson and R. Sprissler, J. Pharm. Sci., 66 (1977) 384.
13 R.V. Smith and J. Besic, Microchem. J., 23 (1976) 185.
14 L. Richard, G. Klein and J.M. Orr, Clin. Chem., 22 (1976) 2060.
15 D.A. Chin, D.G. Prue, J. Michelucci, B.T. Kho and C.R. Warner, J. Pharm. Sci., 68 (1977) 1143.
16 M.T. Rosseel and M.G. Bogaert, J. Pharm. Sci., 68 (1979) 659.
17 E.M. Johnson, A.B. Harkey, D.J. Blehm and P. Needleman, J. Pharmacol. Exp. Ther., 182 (1972) 56.
18 G.A. Maier, A. Poliszczuk and H.-L. Fung, Int. J. Pharm., 4 (1979) 75.
19 J.A. Armstrong, S.E. Slaughter, G.S. Marks and P.A. Armstrong, Can. J. Physiol. Pharmacol., 58 (1980) 459.
20 C.C. Wu, T.D. Sokoloski, M.F. Blanford and A.M. Burkman, Int. J. Pharm., 8 (1981) 323.
21 P.K. Noonan and L.Z. Benet, Int. J. Pharm., 12 (1982) 334.
22 T.D. Sokoloski, C.C. Wu, L.S. Wu and A.M. Burkman, J. Pharm. Sci., 72 (1983) 335.
23 R.A. Morrison, U.-W. Wiegand, E. Jahnchen, D. Hohmann, H. Bechtold, T. Meinertz and H.-L. Fung, Clin. Pharmacol. Ther., 33 (1983) 747.
24 The United States Pharmacopeia, 19th rev., Mack Publishing Co., Easton, PA, 1975.
25 J. Weeks and J. Davis, J. Appl. Physiol., 19 (1964) 541.
26 R.A. Morrison, Ph.D. Thesis, State University of New York at Buffalo, 1983.
27 R.A. Morrison and H.-L. Fung, J. Pharm. Sci., 68 (1979) 1197.
28 The United States Pharmacopeia, 20th rev., Mack Publishing Co., Easton, PA, 1980.
29 M.T. Rosseel and M.G. Bogaert, Biochem. Pharmacol., 22 (1973) 67.
30 G.L. LeBel and D.T. Williams, J. Assoc. Offic. Anal. Chem., 62 (1979) 1353.
31 S.M. Wallace and S. Riegelman, J. Pharm. Sci., 66 (1977) 729.
32 G.N. Pandey, J. Baker, S. Chang and J.M. Davis, Clin. Pharmacol. Ther., 24 (1978) 343.
33 S.F. Sisenwine and H.W. Ruelius, J. Pharmacol. Exp. Ther., 175 (1971) 296.
34 T. Taylor, D.A. O'Kelly, R.M. Major, A. Darragh and L.F. Chasseaud, Arzneim.-Forsch., Drug Res. , 28 (1978) 1426.
35 D.E. Reed, J.M. Akester, J.F. Prather, J.R. Tuckosh, D.H. McCurdy and C. Yeh, J. Pharmacol. Exp. Ther., 202 (1977) 32.
36 Technical Bulletin, ARS/Sprague—Dawley, P.O. Box 4220, Madison, Wisconsin, 53711.
37 K. Diem and C. Lentrer (Editors), Documenta Geigy, Ciba-Geigy Corporation, Ardsley, NY, 1970.

Journal of Chromatography, 308 (1984) 165—179
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2104

AMITRIPTYLINE AND ITS BASIC METABOLITES DETERMINED IN PLASMA BY GAS CHROMATOGRAPHY

J.E. BURCH*

Department of Biochemistry, University of Leeds, Leeds LS2 9JT (U.K.)

and

S.G. ROBERTS and M.A. RADDATS

Regional Metabolic Research Unit, High Royds Hospital, Menston, Ilkley LS29 6AQ (U.K.)

(Received December 22nd, 1983)

SUMMARY

Gas chromatography was used to determine plasma levels of amitriptyline, nortriptyline and their 10-hydroxy derivatives after conversion to the dehydro compounds by heating with acid. The primary amine 10-hydroxydesmethylnortriptyline is also dehydrated and the dehydro compound coincides on the chromatogram with dehydronortriptyline. Treatment of the extract with salicylaldehyde selectively removed the primary amine, which was determined by difference. *Cis*- and *trans*-hydroxydesmethylnortriptyline were isolated from urine by thin-layer chromatography and used to standardize the estimation.

The stability of all the metabolites in plasma was investigated. Results are given for hydroxydesmethylnortriptyline levels in the plasma of 41 patients treated with amitriptyline.

INTRODUCTION

The hydroxy metabolites of amitriptyline (AT) and nortriptyline (NT) have recently been shown to be pharmacologically active [1—3], suggesting that where plasma levels of the parent drugs are monitored the 10-hydroxy derivatives should also be determined. We have modified our routine method for estimating AT and NT [4] to include the assay of these metabolites.

Gas chromatograpy (GC) has previously been used to determine 10-hydroxyamitriptyline (OHAT) and/or 10-hydroxynortriptyline (OHNT) in plasma, with electron-capture detection [5] or mass spectrometry [6—9]. In

0378-4347/84/$03.00

these methods *cis* and *trans* isomers were estimated together after dehydration to dehydroamitriptyline (DHAT) and dehydronortriptyline (DHNT), respectively. The methods for determining OHAT [8, 9] require mass spectrometry and are therefore unavailable to many laboratories.

Bertilsson and Alexanderson [10] separated and quantified the *cis* and *trans* isomers of OHNT in urine, using thin-layer chromatography (TLC). In plasma extracts, Breyer and Villumsen [11] demonstrated the partial separation of these isomers by TLC but without quantification. Breyer-Pfaff et al. [12] assayed *trans*-OHNT in plasma by TLC with reflectance spectrophotometry but the method was insufficiently sensitive to determine the other hydroxy metabolites.

High-performance liquid chromatography (HPLC) offers the possibility of determining AT and all its basic metabolites in plasma with separation of *cis* and *trans* isomers. Early work [13, 14] demonstrated the separation of the OHAT and OHNT isomers but provided no validated estimation procedures. In some later studies [15—18] sensitivity was inadequate and one or both pairs of isomers were unseparated. More recently, using methods with sufficient sensitivity, Edelbroek et al. [19] determined *cis*- and *trans*-OHAT together while Suckow and Cooper [20] and Bock et al. [21] separated both pairs of geometrical isomers.

Hydroxydesmethylnortriptyline (OHDMNT) has not previously been determined in plasma. It was identified in urine by Borgå and Garle [5] and shown by Alexanderson and Borgå [22] to constitute about 16% of the total metabolites recovered from the urine of patients treated with NT. These determinations were unreliable, however, as no reference OHDMNT isomers were available for standardization. Biggs et al. [23] used HPLC to determine AT, NT, desmethylnortriptyline (DMNT) and all their 10-hydroxy metabolites after dehydration, in urine. Standardizing with synthetic OHDMNT they found that conjugated and unconjugated OHDMNT together constituted about 13% of the metabolites recovered from the urine after single doses of AT. The sensitivity of the method was much too low for use with plasma.

We showed previously [24] that DMNT does not separate from NT on OV-17 but can be selectively removed by treatment of the plasma extract with salicylaldehyde (SA). Patients' plasma extracts, prepared by the present method and hence containing AT, NT, DHAT and DHNT, were treated with SA. Reductions occurred not only in the peak at the NT position but also in that in the position of DHNT, suggesting the presence of dehydrodesmethylnortriptyline (DHDMNT) formed from OHDMNT in the plasma. Pure samples of the OHDMNT isomers were not available to us. However, during the isolation of *cis*- and *trans*-OHNT from patients' urine by TLC, some of the fractions showed very large reductions in the peaks in the DHNT position on SA treatment. TLC with a different solvent produced two clear spots from such preparations. When material eluted from these spots was assayed by our usual procedure, SA treatment completely eliminated the dehydro compound peak from the front spots and had no effect on the corresponding peak from the rear spots. By successive TLC using three different solvents (Table II), two preparations were obtained from patients' urine, containing about 100 μg of *cis*- and *trans*-OHDMNT, respectively. These solutions were standardized on

the basis of their ultraviolet (UV) spectra and used to validate the determination of OHDMNT in plasma samples by the SA method.

MATERIALS AND METHODS

Drugs and metabolites

These were kindly donated as follows: amitriptyline hydrochloride by Merck Sharp & Dohme (Hoddesdon, U.K.). nortriptyline hydrochloride by E.R. Squibb and Sons (Twickenham, U.K.), desmethylnortriptyline and the *cis* and *trans* isomers of 10-hydroxyamitriptyline and 10-hydroxynortriptyline by Dr. A. Jørgensen of H. Lundbeck & Co. (Copenhagen, Denmark), maprotiline and desmethylclomipramine by Ciba-Geigy Pharmaceuticals (Horsham, U.K.).

Solvents

These were obtained from BDH (Poole, U.K.). *n*-Heptane, *n*-pentane, toluene (low in sulphur), all general purpose reagent grade, and diethylamine (analytical reagent grade) were distilled before being used for plasma extractions. Analytical reagent grade amyl alcohol was used without purification. For TLC, solvents were used without purification and consisted of general purpose reagent grade heptane and analytical reagent grade diethylamine, chloroform, *n*-butan-1-ol and glacial acetic acid.

Preparation of standard solutions

Stock solutions of drug compounds and metabolites in 0.01 *M* hydrochloric acid contained 250 μg/ml, expressed as base. Dilutions, containing 10 μg/ml or 1 μg/ml, were used when making additions to plasma. Standard solutions of the bases of AT, NT, maprotiline and desmethylclomipramine were prepared by extraction from 0.01 *M* hydrochloric acid into an equal volume of heptane containing 0.5% diethylamine [4]. A standard solution of DHAT and DHNT bases in the same solvent was prepared by mixing 5 ml stock OHAT, 5 ml stock OHNT and 10 ml of 1 *M* hydrochloric acid. After heating for 1 h in a boiling water bath, 3 ml of 4 *M* sodium hydroxide and 300 μl diethylamine were added and the solution was extracted with 20 ml heptane containing 0.5% diethylamine. The organic layer thus contained 625 ng/ml of DHAT and of DHNT, expressed in terms of OHAT and OHNT base, respectively. Mixtures of bases in the same solvent were prepared for calibrating the chromatograph. A 10-μl volume of each mixture contained 50 ng tetracosane, 150 ng maprotiline, 150 ng desmethylclomipramine and equal amounts (either 150 or 7.5 ng) of AT, NT, DHAT and DHNT.

Extraction from plasma

The three stoppered tubes required for each extraction were treated as previously described [4]. A 1.0-ml volume of a solution, prepared by diluting 125 ml of 4 *M* sodium hydroxide and 10 ml of diethylamine to 200 ml with water, was pipetted into a 35-ml tube, which was stoppered and rotated in the hand until the whole surface had been wetted with alkali. To the plasma sample (1—8 ml) in a polystyrene tube was added 1.0 ml of 0.01 *M* hydrochloric acid containing 1 μg of maprotiline as internal standard. After mixing, the plasma

was washed quantitatively into the 35-ml tube with water. Then 10 ml toluene and 0.1 ml amyl alcohol were added and water run in until the 35-ml tube was almost full. After mixing for 30 min on a rotary tumbler at 30 rpm, the tube was centrifuged for 15 min at 2000 *g*. The toluene layer was transferred to a 15-ml tube, using a Pasteur pipette that had been rinsed first with diethylamine and then with toluene. (Between successive samples the pipette was rinsed with toluene only.) After adding 2 ml of 0.05 *M* sulphuric acid the tube was tumbled at 30 rpm for 15 min and centrifuged briefly with stopper in place. Most of the toluene was aspirated, using a filter-pump. Pentane (10 ml) was added to the aqueous layer and the tube was stoppered, inverted several times and briefly left for the layers to separate. The pentane was removed quantitatively with the pump. Then 0.1 ml of 1 *M* hydrochloric acid was added and the tube, without stopper, placed in an ordinary domestic pressure cooker. The rack of tubes was covered with aluminium foil and heated at 1 bar excess pressure for 70 min. After cooling to room temperature, 1 μg of desmethylclomipramine in 0.1 ml of 0.01 *M* hydrochloric acid was added. Into a conical tube was pipetted 0.3 ml of a solution prepared by diluting 100 ml of 4 *M* sodium hydroxide and 45 ml diethylamine to 300 ml with water. The whole surface was wetted with the alkali as before. The acid solution from the 15-ml tube was poured into the conical tube and the final extraction of the compounds into 50 μl of heptane containing diethylamine and 250 ng tetracosane was carried out as previously described [4].

Treatment of the extract with salicylaldehyde was performed as described before [24].

Gas chromatography

Chromatography on OV-17 packed columns with flame ionization detectors was carried out as before [4]. The system was calibrated daily by injecting the standard mixtures of bases to obtain peak area ratios and corrections for losses in chromatography [4]. Results for plasma samples were then calculated assuming that the relative amounts of maprotiline and all the compounds to be estimated were the same in the plasma extract as in the original plasma sample. Desmethylclomipramine was not used in the calculation, having been introduced half-way through the extraction in order to minimize adsorptive losses of other compounds.

Table I shows that the retention times of NT and DHAT differ by a factor of only 1.08. Resolution of these peaks was incomplete (Figs. 1 and 2) and the integrator was used to measure only their combined area. This was allocated between the two individual peaks in proportion to their measured heights, using a weighting factor of 1.08 for DHAT.

With patients' plasma samples, the peak area of NT may be up to fifteen times greater than that of DHAT. On injecting a series of base mixtures, each containing 150 ng maprotiline and 15 ng DHAT per 10 μl, but with amounts of NT ranging from 15 to 300 ng, it was found that the measured DHAT peak area rose with increasing NT content. Presumably the NT peak tails slightly under the DHAT. When 2% of the measured NT area was subtracted from the measured area of DHAT, the resulting corrected value for DHAT was independent of the amount of NT present. DHAT peak areas corrected in this way were used when estimating plasma OHAT concentrations.

TABLE I

RELATIVE RETENTION TIMES

OV-17 packed columns. Compounds extracted from aqueous alkali into heptane.

Compound	RR_{AT}
Tetracosane	0.68
Amitriptyline (AT)	1.00
Nortriptyline (NT)	1.16(5)
Desmethylnortriptyline (DMNT)	1.16(5)*
Dehydroamitriptyline (DHAT)	1.26
Dehydrodesmethylnortriptyline (DHDMNT)	1.41(5)**
Dehydronortriptyline (DHNT)	1.43
Maprotiline	1.71(5)
trans-10-Hydroxyamitriptyline (OHAT)	1.88
cis-10-Hydroxyamitriptyline (OHAT)	1.97
trans-10-Hydroxynortriptyline (OHNT)	2.22
trans-10-Hydroxydesmethylnortriptyline (OHDMNT)	2.25
cis-10-Hydroxynortriptyline (OHNT)	2.36
Desmethylclomipramine	2.40
cis-10-Hydroxydesmethylnortriptyline (OHDMNT)	2.44
Cyclizine	0.59
Benzhexol	1.09
Zimelidine	1.16
Nomifensine	1.20
Didesmethylclomipramine (primary amine)	2.47
Flurazepam	7.32
Temazepam	no peak found
Lorazepam	no peak found
Atenolol	no peak found
Chlordiazepoxide	no peak found
Salbutamol	no peak found

*Also a minor peak at 1.30.
**Also a minor peak at 1.63.

Isolation of cis- and trans-hydroxydesmethylnortriptyline from urine

Glass plates (Merck), 20 × 20 cm with silica gel 60 containing fluorescent indicator were used in a tank lined with filter paper.

Morning samples of urine from patients being treated with AT were made alkaline with sodium hydroxide (0.1 *M* final concentration) and extracted with 0.33 vol toluene. The toluene was extracted with about 0.2 of its volume of 0.01 *M* hydrochloric acid and the acid layer was washed with pentane. After aspiration of the pentane and addition of sodium hydroxide to 0.1 *M* final concentration, the bases were reextracted from the aqueous layer into about 0.1 of its volume of toluene. This solution was evaporated to small volume under a stream of nitrogen and applied to the plate with a 5-μl capillary. After development the spots or bands seen under UV light were scraped off and eluted with 0.01 *M* hydrochloric acid. For further TLC this solution was made alkaline and the bases were extracted into toluene and concentrated as before.

Initial chromatography in solvent A (Table II) was used to isolate the OHAT isomers for other investigations. This solvent did not separate the primary from the secondary amines, and because the plates were grossly overloaded with

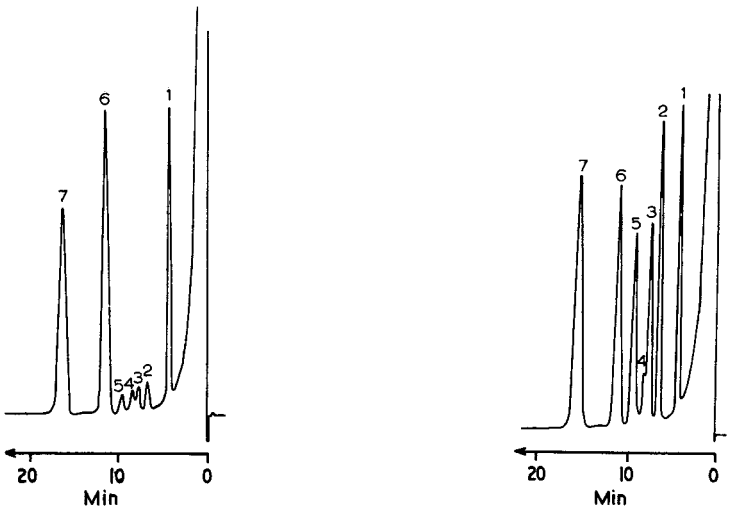


Fig. 1. Chromatogram from 10 μl of standard base mixture containing (1) 50 ng tetracosane; (2) 7.5 ng AT; (3) 7.5 ng NT; (4) DHAT from 7.5 ng OHAT; (5) DHNT from 7.5 ng OHNT; (6) 150 ng maprotiline, internal standard; and (7) 150 ng desmethyl clomipramine.

Fig. 2. Chromatogram from 10 μl of extract from 5 ml of a patient's plasma. Peaks numbered as in Fig. 1. Calculated concentrations: 130 ng/ml AT; 104 ng/ml NT; 26 ng/ml OHAT; 132 ng/ml OHNT.

TABLE II

THIN-LAYER CHROMATOGRAPHY

Compound	Distance above baseline (cm)		
	Solvent A*	Solvent B*	Solvent C*
cis-OHNT	1.6	7.2	3.0
cis-OHDMNT	**	8.5	**
trans-OHNT	2.1	7.2	3.8
trans-OHDMNT	**	8.5	**
cis-OHAT	6.0	5.5	9.0
DHNT	6.4	8.2	—
trans-OHAT	6.9	5.5	9.0
NT	8.0	8.3	9.0
DMNT	**	9.2	**
DHAT	13.6	6.4	—
AT	15.1	6.5	14

*Solvent A: heptane—diethylamine (7:1, v/v), run to top of 20-cm plate four times. Solvent B: butanol—glacial acetic acid—water (4:1:1, v/v/v), run on 20-cm plate once, for 4 h. Solvent C: heptane—chloroform—diethylamine (7:2:1, v/v/v), run to top of 20-cm plate three times.

**In solvents A and C the primary amines gave elongated spots in approximately the same positions as the corresponding secondary amines.

cis- and *trans*-OHNT these did not separate from each other. However, they were freed from the other bases listed in Table II as well as from various unidentified UV-absorbing materials.

Solvent B (Table II) was then used to separate OHDMNT from OHNT, followed by solvent C to separate the two isomers of OHDMNT. The final eluates were made alkaline and extracted with toluene. These bases were back-extracted into 0.01 *M* hydrochloric acid and traces of toluene removed by two successive extractions with pentane, mixing for 10 min each time. This procedure, applied to eluates of blank plate areas after TLC, gave solutions with zero UV absorbance.

The UV spectra of the preparations of OHDMNT from urine, together with those of AT, NT and the five metabolites of which pure samples were available, were observed in 0.01 *M* hydrochloric acid in 1-cm cuvettes in a Cecil spectrophotometer, Model CE 292.

RESULTS

Figs. 1 and 2 show chromatograms from a standard mixture of bases and from a patient's plasma extract.

Heparinized plasma obtained from drug-free human subjects gave no peaks in the region of interest. However, plasma prepared from out-dated blood-bank blood usually produced a small peak interfering with NT. Ox serum gave no peaks and was used for experiments except where heparinized plasma from volunteer human subjects is specified.

Compounds were added to ox serum, and extracts were prepared and injected before and after SA treatment. AT, NT, *cis*- and *trans*-OHAT and OHNT and the internal standards, tested separately and in various appropriate combinations, gave no interfering peaks. DMNT showed only the expected peak coinciding with NT, together with the minor peak of retention time 1.12 times that of the major peak [24]. *cis*- or *trans*-OHDMNT isolated from urine gave only a major peak of retention time 0.99 times that of DHNT, together with a minor peak of retention time 1.15 times that of the main peak. As with DMNT the ratio of minor to major peak areas was about 0.04 and both peaks disappeared completely on treatment with SA.

Determination of tertiary and secondary amines

Table III gives the results of assays of equal amounts of AT, NT and either *cis*- or *trans*-OHAT and OHNT, added to ox serum. The determinations were carried out on many different days, usually as single specimens in batches of patients' samples. Results for the *cis* and *trans* isomers were indistinguishable and have been combined.

Interactions between the different compounds were investigated by analysing samples containing 20 ng/ml of one substance, either alone or together with 200 ng/ml of the other three compounds. AT, NT and OHNT assays were unaffected by the presence of the other substances, while OHAT assays based on uncorrected peak areas were increased, as expected, by the presence of NT. When DHAT peak areas were corrected for NT tailing as already described, the OHAT assays were no longer influenced by the presence of NT. This was also tested using other concentrations of OHAT, with amounts of NT ranging from 1 to 20 times the OHAT present. A series of determinations of 20 ng/ml OHAT with 200 ng/ml NT gave a mean OHAT value of 19.2

TABLE III

SECONDARY AND TERTIARY AMINES DETERMINED IN SERUM SAMPLES CONTAINING EQUAL AMOUNTS OF ADDED COMPOUNDS

Amount added (ng/ml in 5 ml)	*n*	AT		NT		OHAT		OHNT	
		Mean	C.V. (%)	Mean	C.V. (%)	Mean	C.V. (%)	Mean	C.V. (%)
200	27	197.3	3.0	191.1	2.3	205.7	2.8	201.0	2.9
100	14	97.6	2.3	93.2	2.4	102.3	2.7	100.1	3.0
40	17	38.4	3.7	36.3	3.2	41.2	4.0	39.5	2.9
20	19	19.2	3.3	18.2	3.0	20.1	4.3	19.7	3.4
10	22	9.55	6.4	9.77	11.4	9.70	9.1	9.54	9.4

ng/ml with a coefficient of variation (C.V.) of 3.5% (n = 11), which is no greater than the value obtained for this concentration of OHAT with an equal amount of NT (Table III).

The quantity of serum or plasma present was shown not to affect the assay results for added compounds. Human plasma (1—8 ml) and ox serum (3—12 ml) all gave indistinguishable results for AT, NT and their *cis* and *trans* hydroxy metabolites.

These six compounds were found not to be adsorbed from human plasma (either fresh or previously frozen and thawed) on to glass pipettes or polystyrene blood tubes. Plasma was sucked up and down in five pipettes in series and poured from one tube to another, using five in series, without loss of compounds.

Determination of primary amines

Absorption spectra of the *cis*- and *trans*-OHDMNT preparations from urine are shown in Fig. 3, together with those of DMNT, NT, AT and *cis*-OHAT. The curves for *trans*-OHAT and both isomers of OHNT were extremely similar to those illustrated and have been omitted for clarity. All compounds showed maxima at 237 nm and minima at 228 nm. The close resemblance between the spectra of the preparations from urine and those of the pure compounds suggests that no other absorbing material contributed appreciably to the absorbance of the urine preparations. The molar extinction coefficients at 237 nm (Table IV) for all seven pure compounds were similar, the ratio of highest (*trans*-OHNT) to lowest (DMNT) being only 1.07. As neither the degree of methylation of the nitrogen atom, nor ring hydroxylation appreciably affected the maximum molar extinction coefficient, the mean value of 13,500 was taken to give a reasonably accurate measure of the concentration of OHDMNT in each of the preparations from urine.

To each of five serum samples 1.1 μg of *cis*-OHDMNT (based on this spectrophotometric standardization) were added and 1.1 μg of the *trans*-isomer to five more. After extraction with internal standards in the usual way, the peak areas of DHDMNT produced were found not to differ significantly between the two isomers. The mean value was 0.75 ± 0.03 (S.D.) times the peak area of DHNT produced by 1.1 μg of OHNT (*cis* or *trans*). The ratio is close to the value of 0.767 found previously [24] for the ratio of DMNT and NT peak areas

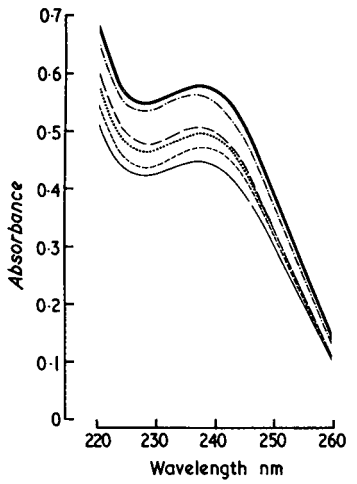


Fig. 3. Absorption spectra of compounds in 0.01 *M* hydrochloric acid. Traces: (—), *trans*-OHDMNT; (— • — • —), *cis*-OHDMNT; (— —), DMNT; (• • • • •), NT; (- - -), AT; (—), *cis*-OHAT.

TABLE IV

MOLAR EXTINCTION COEFFICIENTS AT PEAK WAVELENGTH (237 nm)

Measurements on 10 μg/ml solutions in 0.01 *M* hydrochloric acid.

Compound	$\epsilon \cdot 10^{-3}$
AT	13.4
NT	13.4
DMNT	12.9
cis-OHAT	13.4
trans-OHAT	13.8
cis-OHNT	13.6
trans-OHNT	13.8
Mean	13.5

after extraction. This agreement supports the accuracy of the spectrophotometric standardization of the OHDMNT concentrations.

When serum extracts are treated with SA the peak areas of all the secondary amines decrease by a few percent relative to the tertiary amines and tetracosane [24]. Of the extracts prepared for the assays in Table III, six at each concentration from 20 to 200 ng/ml were treated with SA and rechromatographed. The peak area ratio of DHNT to maprotiline (which is a secondary amine) was found to be almost unaffected by SA treatment. It increased by 0.6% on average, with a standard deviation of 1.4% (n = 24).

Using symbols as before [24], F is defined as the ratio of apparent OHNT concentrations before and after SA treatment, using maprotiline as internal standard for the calculations. Let the ratio of the weight of OHDMNT to that of OHNT in the sample be r, and the ratio of the peak area of DHDMNT to that of DHNT (for equal weights of the two compounds in the sample) be a.

Then before SA treatment the apparent amount of OHNT in the sample is $(1 + ar)$ times the true amount. After SA treatment the apparent amount is 1.006 times the true amount. Hence $F = (1 + ar)/1.006$. Substituting the value of 0.75 found above for a, we have

$$F = 0.994 + 0.746r \quad (1)$$

This equation was verified using twenty determinations with *cis*-OHDMNT and twenty with the *trans* isomer. In each case the ratio r of OHDMNT to OHNT was held constant at 0.137, which is close to the mean value found in patients' plasma (see below). Five determinations were carried out at each of four OHNT concentrations, i.e. 20, 40, 100 and 200 ng/ml. F was found to be the same for *cis* and *trans* isomers and independent of concentration. Its mean value was 1.0943 ± 0.0166 (n = 40, S.E.M. = 0.0026), in good agreement with the value of 1.0962 calculated from eqn. (1) with r = 0.137. Thus, as in the determination of DMNT with NT [24], the relative amounts of primary and secondary amines extracted by the chosen procedure are independent of their absolute concentrations. The effect of SA treatment of extracts of mixtures of the two compounds agrees with predictions based on the relative peak areas of the compounds extracted separately. Hence, in patients' plasma samples, the ratio of OHDMNT to OHNT may be calculated as: $r = (F - 0.994)/0.746$.

Altogether 47 plasma samples were analysed, from 41 different patients being treated with AT at a wide range of daily dosage. OHNT concentrations ranged from 15 to 266 ng/ml. The mean value of F was 1.102 ± 0.0541 (S.D.) and no relationship between F and dose or OHNT concentration was apparent. The mean OHDMNT concentration was therefore 14.5 ± 7.25% of the OHNT level present, and before SA treatment the apparent OHNT concentration averaged 110.9 ± 5.4% of the true value.

Stability of the drugs and metabolites

Solutions in 0.01 *M* hydrochloric acid of AT, NT, DMNT and all six of their 10-hydroxy metabolites were stable in the refrigerator for many months and no detectable dehydration occurred. The standard base solutions in heptane containing 0.5% diethylamine, with AT, NT, DHAT, DHNT, maprotiline, desmethylclomipramine and tetracosane were stable at room temperature for at least one year. Both isomers of OHAT and of OHNT were stable as bases in toluene at room temperature for at least eight months. However, the OHDMNT isomers were unstable in tolune at room temperature. After two weeks only 80% of the *trans* and 60% of the *cis* compound remained.

In human plasma freshly separated from heparinized blood, AT, NT and their *cis* and *trans* hydroxy metabolites were reasonably stable at room temperature. When the compounds were added to drug-free plasma, NT declined slowly, losing 12% of its concentration in ten days at room temperature, while the other five compounds were unchanged. However, in plasma that had previously been frozen and thawed the compounds were much less stable, for unknown reasons. Perhaps the increase in pH caused by loss of carbon dioxide in freezing was responsible. Compounds were added to drug-free plasma that had been stored frozen. In eight days at room temperature, NT decreased on average by 22%, AT by 20% and the four hydroxy compounds by about 5%. Patients' plasma samples were also tested at room temperature and

gave similar results, both for freshly separated and for previously frozen and thawed samples. Thus there was no evidence of bases being liberated from conjugated forms in patients' plasma. In some samples that had been frozen for many months, much larger losses occurred after thawing, e.g. 55% of NT lost during ten days at room temperature.

The primary amines were less stable in plasma than the secondary and tertiary amines. When added to fresh plasma and kept at room temperature for seven days, DMNT declined by 38%, *cis*-OHDMNT by 25% and *trans*-OHDMNT by 16%. However, in 17 h these compounds showed negligible losses, so that estimations carried out promptly would be unaffected.

All the compounds were stable in plasma during freezing and thawing. AT, NT, DMNT and all six of their hydroxy metabolites, added to drug-free human plasma, showed no significant losses when frozen and thawed eleven times in succession and estimated directly after the final thawing. Patients' plasma samples treated similarly gave no evidence either of loss of bases or their liberation from conjugates.

The compounds were stable for long periods in frozen plasma. AT, NT, their four hydroxy metabolites and DMNT, when added to drug-free human plasma and frozen, were not detectably changed after six months storage, while *cis*- and *trans*-OHDMNT declined by about 10%. Patients' plasma samples stored frozen for periods up to one year showed no change in the tertiary and secondary amines. (Primary amines were not estimated in these samples, so that the stability of their conjugates was not tested.)

The compounds were not adsorbed appreciably by the precipitates that develop in frozen heparinized plasma on storage. A pool of stored plasma samples from patients treated with AT was thawed and centrifuged. Determinations of AT, NT, OHAT and OHNT before and after centrifugation showed no differences and assay of the precipitate showed negligible amounts of these compounds. Similarly, blank human plasma with added *cis*- and *trans*-OHDMNT, stored frozen for several months, showed negligible amounts of these compounds adhering to the precipitates.

All blood samples were equilibrated and centrifuged at 37°C as previously described [25], in order to prevent the redistribution of compounds between cells and plasma. Blood samples from six patients were divided into two. One aliquot was equilibrated and centrifuged at once, and the other after 24 h at room temperature. No significant differences in plasma levels of AT, NT, OHAT or OHNT were found.

Other drugs

Retention times relative to AT (RR_{AT}) were given previously [4] for 29 other drugs and some of their metabolites. In Table I, eleven further compounds are listed. The estimation of additional metabolites necessarily increases the likelihood of interference by other drugs. In the current method, peaks with RR_{AT} values between about 0.9 and 1.9 will interfere. The principal potential offenders are other tricyclics and related antidepressants, propranolol, benzhexol, possible metabolites of procyclidine, and the smaller, less polar benzodiazepines and phenothiazines. Most benzodiazepines and neuroleptics, however, have RR_{AT} values greater than 1.9.

The extraction, like the procedure [4] for AT and NT only, excludes small

polar bases as well as all non-basic compounds. Many large bases appear equally in extracts prepared by either technique, but in other cases the heating step causes complications. Some drugs (cyclizine, benztropine) are destroyed and give no detectable peak. Other drugs produce new peaks during heating. Chlordiazepoxide (RR_{AT} = 15.3), which does not itself extract into heptane, gives a peak at RR_{AT} = 0.81 during heating. Diazepam (RR_{AT} = 3.16) is largely excluded by the present extraction method with or without heating, and gives no other peak. However, in the plasma of patients taking diazepam there is apparently a metabolite which produces a peak at RR_{AT} = 0.81 during the heating step. Fortunately, peaks at this retention time do not interfere. Flurazepam (RR_{AT} = 7.32) gives an additional peak at RR_{AT} = 3.0 on heating.

DISCUSSION

Development of the method

Toluene was chosen for the extraction of compounds from plasma because heptane proved to be a poor solvent for the OHNT isomers. Partition coefficients between heptane and 0.1 *M* sodium hydroxide (with about 0.5% diethylamine in the heptane layer and 1.5% in the aqueous phase) were only 6.0 for *cis*-OHNT and 3.5 for the *trans* isomer.

The amount of diethylamine added to the plasma was adjusted so that the quantity passing from the toluene layer into the aqueous acid was about 0.065 mmol, neutralising about one-third of the available acid.

If hydrochloric acid was used instead of sulphuric acid for extracting the compounds from toluene, significant amounts of AT and NT were left in the organic phase. The higher the hydrochloric acid concentration, the more drug was left behind, suggesting ion pair formation with Cl^-. Sulphuric acid extracted all the compounds well, but in catalysing the dehydration of the hydroxy metabolites sulphuric acid was found to be no more effective than an equal molarity of hydrochloric acid. This is presumably because at pH $\leqslant$ 1 the HSO_4^- ion is largely undissociated and sulphuric acid acts as a monobasic acid. In order to minimise the sodium hydroxide required for final neutralisation, the compounds were extracted into sulphuric acid and hydrochloric acid was added before heating. In an earlier version of the method, 0.4 *M* hydrochloric acid was used and the mixture heated at 100°C for 1 h. However, results were less reproducible than expected and recoveries of DHNT were low; the high concentration of sodium chloride present in the final extraction was thought to be responsible. Use of dilute acid at a higher temperature greatly improved the results.

In the dehydration of hydroxy metabolites the *cis* isomers were found to react more slowly than the *trans*. However, the difference was neither large nor reproducible enough to be used for determining the ratio of *cis* to *trans* isomers in an extract. If either clomipramine or desmethylclomipramine was present during the heating step, about 30% of its peak area was lost and an interfering peak at RR_{AT} = 1.08 was produced from either compound.

Performance and results

The accuracy and precision of this method for the determination of the

tertiary and secondary amines are as good as those of other published methods [8, 17—21]. The measurement of low concentrations (e.g. 10—20 ng/ml) requires several millilitres of plasma, while volumes up to at least 8 ml can be used (if available) to make possible the determination of lower concentrations. Many other methods specify 1 ml plasma only, implying greater absolute sensitivity. However, in many cases their effective sensitivity is unclear as results with spiked samples at low concentrations have not been reported.

As in other GC methods, *cis* and *trans* isomers are determined together. Levels of total OHAT in plasma are so low [8, 9, 19—21] and the proportion of the *cis* form is so small [20, 21] that the determination of *cis*-OHAT by currently available methods [20, 21] is often impossible. Joint measurement of the isomers may therefore be preferable. Total OHNT levels in plasma are comparable with NT levels and the ratio of the isomers present differs considerably between individuals [19—21] so that separate determination would seem both desirable and feasible. However, the two available HPLC studies of plasma levels of *cis*- and *trans*-OHNT in patients treated with AT differ remarkably. Suckow and Cooper [20] found a mean *cis/trans* ratio of 0.30 (S.E.M. = 0.034, n = 11) while Bock et al. [21] obtained a mean of 0.14 (S.E.M. = 0.013, n = 27).

Relative retentions given in Table I show that resolution of all six hydroxy metabolites, as such, on OV-17 packed columns is impracticable. Whether or not the *cis* and *trans* isomers differ in antidepressive effect is unknown; pharmacological studies have given different results. While Bertilsson et al. [1] found *cis* and *trans* forms to be equally effective, Hyttel et al. [2] found differences in certain tests, while Potter et al. [3] obtained much greater activity from *cis*-OHNT (the minor form in plasma) than from *trans*-OHNT, the more abundant form. The test systems were different and their relevance to therapeutic action remains doubtful. Racemic mixtures of all metabolites were used although both the *cis*- and *trans*-OHNT formed in the body are known to be optically active [10]. It may perhaps be doubted whether the separate contributions to clinical effects of all the individual isomers of the various metabolites are likely to be evaluated in sufficient detail to justify the separate monitoring of all the compounds in patients' plasma.

The present study is the first to report plasma levels of OHDMNT. The average amount present was found to be 14.5 ± 7.25% of the OHNT. Thus, there is often at least as much OHDMNT as OHAT in the plasma and it seems surprising that the primary amines have not been identified on HPLC chromatograms. If they should happen to interfere with OHAT peaks, large errors could result. In the present method, the minor peak formed from DMNT during extraction will interfere with DHAT, but as its area is only about 4% of the DMNT area which is about 6% of the NT area [24] the errors will be unimportant.

Results with spiked serum samples showed that the standard deviation of OHDMNT determinations by the salicylaldehyde method was 0.0166 in F, corresponding to 0.022 in r, the ratio of OHDMNT to OHNT present. Expressed in relation to the mean level in patients' plasma, this gives a coefficient of variation of 15%. Most of the variation arises in the process of salicylaldehyde treatment and rechromatography, since when only NT was

present the S.D. observed for F was 0.014. Experimental error contributes only a small proportion of the total variation observed in the population of patients. Subtraction of variances leaves a standard deviation of 0.069 in r as the true inter-individual variation. This represents a coefficient of variation of 48% for inter-individual differences in the ratio of OHDMNT to OHNT.

Compared to DMNT estimations by a similar procedure [24], the OHDMNT method has a slightly smaller standard deviation. The coefficient of variation is considerably better because the mean amount of OHDMNT in plasma (in relation to OHNT) is 1.7 times as great as the mean amount of DMNT (in relation to NT). The present extraction method was not, however, found suitable for determining DMNT. Although DMNT peaks were of the expected area and disappeared completely on salicylaldehyde treatment, experiments with spiked serum samples containing a DMNT/NT ratio of 0.1 gave unsatisfactory results. For unknown reasons, the peak area reductions on SA treatment were too small. DMNT is therefore better determined by the original extraction procedure [24].

The stability of the secondary and tertiary amines in fresh plasma is such that samples can be sent by post without significant losses. The compounds are stable for several months in frozen plasma but, once thawed, samples should be extracted within a few hours or refrozen for further storage.

For the determination of primary amines, fresh plasma cannot be left at room temperature for more than a few hours before extraction. It may be stored frozen, if extracted immediately after thawing.

The methods described in this paper have been in routine clinical use for more than two years, with regular analysis of spiked plasma samples. Trouble from interfering peaks has been rare and has always been from drugs known to interfere on OV-17 [4], notably other tricyclics and procyclidine. Benztropine is a useful non-interfering drug that can be used in place of procyclidine or benzhexol. Benzodiazepines, most of which have long retention times and/or fail to extract, have not given trouble in practice; nor have interfering phenothiazines been encountered. The method is suitable for the many laboratories currently estimating AT and NT by GC, making possible the routine determination of the hydroxy metabolites that are believed to be pharmacologically active.

REFERENCES

1 L. Bertilsson, B. Mellström and F. Sjöqvist, Life Sci., 25 (1979) 1285.
2 J. Hyttel, A.V. Christensen and B. Fjalland, Acta Pharmacol. Toxicol., 47 (1980) 53.
3 W.Z. Potter, H.M. Calil, A.A. Manian, A.P. Zavadil and F.K. Goodwin, Biol. Psychiat., 14 (1979) 601.
4 J.E. Burch, M.A. Raddats and S.G. Thompson, J. Chromatogr., 162 (1979) 351.
5 O. Borgå and M. Garle, J. Chromatogr., 68 (1972) 77.
6 G. Alván, O. Borgå, M. Lind, L. Palmér and B. Siwers, Eur. J. Clin. Pharmacol., 11 (1977) 219.
7 V.E. Ziegler, T.A. Fuller and J.T. Biggs, J. Pharm. Pharmacol., 28 (1976) 849.
8 W.A. Garland, R.R. Muccino, B.H. Min, J. Cupano and W.E. Fann, Clin. Pharmacol. Ther., 25 (1979) 844.
9 B. Vandel, M. Sandoz, S. Vandel, G. Allers and R. Volmat, Eur. J. Clin. Pharmacol., 22 (1982) 239.

10 L. Bertilsson and B. Alexanderson, Eur. J. Clin. Pharmacol., 4 (1972) 201.
11 U. Breyer and K. Villumsen, Eur. J. Clin. Pharmacol., 9 (1976) 457.
12 U. Breyer-Pfaff, H.J. Gaertner, F. Kreuter, G. Scharek, M. Brinkschulte and R. Wiatr, Psychopharmacology, 76 (1982) 240.
13 J.C. Kraak and P. Bijster, J. Chromatogr., 143 (1977) 499.
14 B. Mellström and R. Braithwaite, J. Chromatogr., 157 (1978) 379.
15 S.H. Preskorn, K. Leonard and C. Hignite, J. Chromatogr., 197 (1980) 246.
16 R. Dixon and D. Martin, Res. Commun. Chem. Path. Pharmacol., 33 (1981) 537.
17 G.A. Smith, P. Schulz, K.M. Giacomini and T.F. Blaschke, J. Pharm. Sci., 71 (1982) 581.
18 S.M. Johnson, C. Chan, S. Cheng, J.L. Shimek, G. Nygard and S.K. Wahber Khalil, J. Pharm. Sci., 71 (1982) 1027.
19 P.M. Edelbroek, E.J.M. de Haas and F.A. de Wolff, Clin. Chem., 28 (1982) 2143.
20 R.F. Suckow and T.B. Cooper, J. Chromatogr., 230 (1982) 391.
21 J.L. Bock, E. Giller, S. Gray and P. Jatlow, Clin. Pharmacol. Ther., 31 (1982) 609.
22 B. Alexanderson and O. Borgå, Eur. J. Clin. Pharmacol., 5 (1973) 174.
23 S.R. Biggs, L.F. Chasseaud, D.R. Hawkins and I. Midgley, Drug Metab. Dispos., 7 (1979) 233.
24 J.E. Burch, M.A. Raddats and S.G. Roberts, J. Chromatogr., 274 (1983) 350.
25 J.E. Burch, S.G. Roberts and M.A. Raddats, Psychopharmacology, 75 (1981) 262.

Journal of Chromatography, 308 (1984) 181—187
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2105

MONITORING OF *S*- AND *R*-TOCAINIDE IN HUMAN PLASMA AFTER HEPTAFLUOROBUTYRYLATION, SEPARATION ON CHIRASIL-VAL® AND ELECTRON-CAPTURE DETECTION*

ANN-MARIE ANTONSSON, OLLE GYLLENHAAL*, KERSTIN KYLBERG-HANSSEN, LARS JOHANSSON and JÖRGEN VESSMAN

Analytical Chemistry, AB Hässle, S-431 83 Mölndal (Sweden)

(First received December 28th, 1983; revised manuscript received January 31st, 1984)

SUMMARY

The conditions for the heptafluorobutyrylation of tocainide have been studied. An almost instantaneous reaction was obtained with 0.01% of heptafluorobutyric anhydride in toluene at 40°C. Higher anhydride concentration caused degradation of the initially formed derivative, mainly by the loss of water, as shown by mass spectral analysis.

Tocainide was isolated from plasma by extraction into dichloromethane at alkaline pH. Gas chromatographic separation was performed with a fused-silica capillary column coated with a methyl silicone gum. The enantiomers were separated on a glass capillary column coated with Chirasil-Val®.

Upon analysing 0.1 ml of plasma eight times the precision was 4.7% at the 10 μmol/l level for the *S*-form of tocainide.

INTRODUCTION

Tocainide [2-amino-N-(2,6-xylyl)propanoic acid amide] is an orally active antiarrhythmic drug (Fig. 1). Its determination in biological samples has been performed by liquid chromatographic [1—6] and gas chromatographic [7—13]

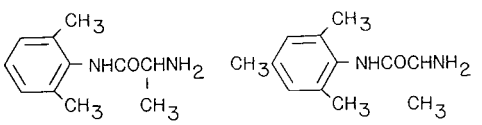


Fig. 1. Chemical structures of tocainide (left) and the internal standard (H 155/73, right)

*Presented at the Annual Meeting of The Swedish Academy of Pharmaceutical Sciences, 1982, and The 1st International Symposium on Drug Analysis, Brussels, 1983.

0378-4347/84/$03.00

methods. Under certain conditions the steady-state concentration of tocainide in plasma is high enough to allow the use of gas chromatography with flame-ionization detection of the free base [10]. Normally, the primary amino group is blocked. This can be accomplished by perfluoroacylation [7, 9, 11, 12] or by Schiff base formation [13]. Flame-ionization [9, 12], electron-capture [8, 11] and nitrogen-selective [12, 13] detection have all been used.

As an alternative to the liquid chromatographic [2] and Schiff base [13] methods developed in our laboratories we have now investigated the possibility of using a method based on the heptafluorobutyryl derivative. The aim was also to reduce the sample volume required and to lower the detection limit. Moreover, it was of interest to obtain a derivative suitable for the chiral resolution of the enantiomers instead of using an optically active derivatizing agent [12].

We report here conditions for the acylation of tocainide with heptafluorobutyric anhydride and a complete method for the determination of the individual enantiomers by electron-capture gas chromatography after separation on Chirasil-Val®. Detailed studies of the acylation reaction and the degradation when an excess of anhydride is used will be published elsewhere [14].

EXPERIMENTAL

Chromatography

A Varian 3700 gas chromatograph equipped with a nitrogen-selective detector was used with a glass column (120 × 0.2 cm I.D.) filled with 3% OV-17 on Gas-Chrom Q, 100—120 mesh. The nitrogen carrier gas flow-rate was 30 ml/min. Hydrogen and air flow-rates were 5 and 175 ml/min, respectively. The temperatures of the injector, oven and detector were 250° C, 200° C and 300° C, respectively.

Electron-capture gas chromatography was performed with a Hewlett-Packard 5730 instrument with a glass column (200 × 0.2 cm I.D.) filled with the same packing as above. Argon—methane (95:5) was used as carrier gas with a flow-rate of 20 ml/min. The temperatures were as above.

The tocainide racemate was also chromatographed on a fused-silica capillary column (20 m × 0.32 mm I.D., siloxane-deactivated) coated with a methyl silicone gum, in the Hewlett-Packard instrument with an electron-capture detector. The inlet pressure was 80 kPa (helium). The split flow-rate was 10 ml/min, and that of the argon—methane used for the electron-capture detector make-up gas was 20 ml/min. The column was maintained isothermally at 170° C. Samples were injected automatically (Hewlett-Packard Model 7671) every fifth minute. Peaks were evaluated by a Spectra Physics integrator Model 4270.

The enantiomers were separated on a Chirasil-Val (Applied Science Labs., PA, U.S.A.; No. 13815) glass capillary column (25 m × 0.3 mm I.D.). The column was connected to a Varian 3700 gas chromatograph by a Dani PC-IN 68/156 injector (Monza, Italy) and to the detector by a coupling from Gerstel (Mülheim an der Ruhr, F.R.G.). The all-glass injectors were high-temperature silanized and filled with 1-cm long silanized glass wool plugs. The inlet pressure was 140 kPa and the split flow-rate 10 ml/min. The injector and the detector were maintained at 250° C. The column was kept at 110° C for 1 min after the

injection and then taken to 210°C by increasing the oven temperature by 25°C/min. Samples were introduced every 18 min by a Varian 8000 autosampler unit.

Reagents and chemicals

Tocainide hydrochloride, internal standard as the hydrochloride [H 155/73, 2-amino-N-(2,4,6-mesityl)propanoic acid amide, Fig. 1], *S*- and *R*-tocainide hydrochloride and the heptafluorobutyric derivative of tocainide (H 191/12) were from the department of Organic Chemistry, AB Hässle. The following metabolites were also from the same source: the lactic xylidide (H 170/82), the pyruvic xylidide (H 170/83), the oxime (H 170/84) and the 3-(2,6-xylyl)-5-methyl hydantoin.

p-Bromobenzophenone was from BDH (Poole, U.K.). The methyl carbamate of dibenzylamine was prepared as previously described [15].

Heptafluorobutyric anhydride (Regis, Morton Grove, IL, U.S.A.) was stored in small glass bottles with PTFE-lined screw caps. A 0.5% solution in toluene was prepared and used the same day. Care should be taken that the solution is homogeneous. The corresponding imidazol was from Pierce (Rockford, IL, U.S.A.).

HPLC grade dichloromethane and glass-distilled toluene were obtained from Rathburn (Walkerburn, U.K.) and ethyl acetate p.a. from Merck (Darmstadt, F.R.G.).

A 1.25% solution of hydroxylamine hydrochloride (Merck p.a.) was prepared with deionized water.

Methods

Studies on the acylation of tocainide. Acylation studies of tocainide were performed with 10 μg of tocainide (5.2 nmol) and a marker (4-bromobenzophenone for electron-capture detection, and methyl carbamate of dibenzylamine for nitrogen selective detection) in 2 ml of toluene. After addition of the acylating agents aliquots were withdrawn and washed with buffer pH 7.4. Analysis with electron-capture detection necessitated dilution of the solution, and analysis by nitrogen-selective detection required the elimination of toluene as solvent. This was accomplished by evaporation and reconstitution in ethyl acetate.

Determination of tocainide in human plasma. A 0.1-ml plasma sample was mixed with 50 μl of the internal standard solution (H 155/73, 25 μmol/l) and 50 μl of 1.25% aqueous hydroxylamine hydrochloride solution. After 30 min at room temperature the mixture was made alkaline by the addition of 0.1 ml of 1 *M* sodium hydroxide and extracted with 5 ml of dichloromethane for 10 min. After centrifugation and aspiration of the aqueous phase, the organic phase was decanted into a new tube and evaporated to dryness at 30°C under a stream of dry nitrogen. The residue was dissolved in 0.5 ml of toluene, and 10 μl of the heptafluorobutyric solution, 0.5% in toluene (or 50 μl of 0.1% solution), were added. The reaction was allowed to proceed at 40°C for 10 min or at room temperature for 30 min, and was taken to dryness as above. The remainder was dissolved in 0.2—1 ml of toluene. A 3-μl volume was injected into the gas chromatograph.

RESULTS AND DISCUSSION

Heptafluorobutyrylation of tocainide

Several authors have gas chromatographed tocainide after heptafluorobutyrylation [8, 9, 11, 12]. Both the anhydride [8, 11, 12] and the imidazol [9] have been used. In our hands acylation with the imidazol was slower than with the anhydride. By increasing the concentration of the imidazol the reaction time could be reduced to 10 min (0.05%). However, interfering peaks appeared in the electron-capture gas chromatograms [16] and the yield was 10% lower than with the anhydride. Also, the reagent itself was labile once the ampoule had been opened although the contents were protected against moisture. For these reasons heptafluorobutyric anhydride was preferred as acylating reagent.

The absolute yield with toluene as solvent was found to be 92% as determined with the synthetic derivative as reference. The actual concentration of the reagent was only 0.01% since higher concentrations of the anhydride resulted in degradation of the derivative (Fig. 2). Upon temperature programming of the column it was revealed that at least three new compounds with shorter retention times than the heptafluorobutyryl tocainide derivative were present. Preliminary results indicate that two of the compounds have lost water. The instability of the derivative with excess anhydride was also apparent with other solvents such as hexane, diethyl ether, ethyl acetate, dichloromethane and acetonitrile. Other losses of the derivative were also observed with hexane.

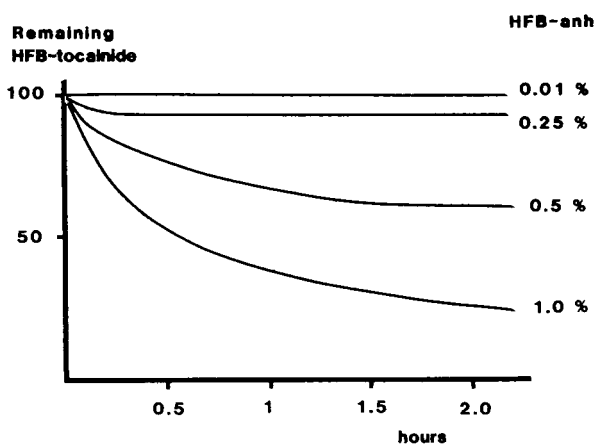


Fig. 2. Stability of the heptafluorobutyryl (HFB) derivative of tocainide in toluene in the presence of excess anhydride (anh) at 40°C.

The heptafluorobutyrylation was also studied after isolation of tocainide and the internal standard from plasma to ensure that co-extracted components did not reduce the yield. A pooled plasma sample (60 μmol/l) was studied. The concentration of the anhydride could be reduced from 0.01% to 0.003% before the yield of tocainide and the standard became irregular.

Due to varying quality of the anhydride from batch to batch, we recommend a check of its potency when a new batch is used or when the method is started

up anew. A slight increase in the anhydride will only have a minor influence on the yield, which will be compensated for by the internal standard. At present we use 0.03%.

Isolation of tocainide from plasma

Tocainide was extracted from alkaline human plasma into dichloromethane. The extraction coefficient is 8 [2, 17] and the high organic-to-aqueous-phase ratio (> 15) will give a near complete extraction. The addition of hydroxylamine prior to extraction improves the yield and precision [13] of the method.

Purification of the reaction mixture

Although a low concentration of heptafluorobutyric anhydride is used in this method, it was not possible to inject aliquots of the reaction solution directly without any disturbance to the electron-capture gas chromatographic system. The racemate of tocainide eluted on the tail of the solvent front. The excess of anhydride was thus always removed and this is also a prerequisite for the use of the Chirasil-Val column. Evaporation of the anhydride was preferred to the buffer wash as it is less time-consuming. The yields were equivalent with both methods and no sign of degradation was observed either by hydrolysis or by dehydration.

The mild heptafluorobutyrylation conditions, i.e. low concentration of the reagent, short reaction time and low temperature, have a positive effect on the chromatographic background noise caused by endogenous compounds (Fig. 3) [18, 19]. If the anhydride concentration was raised to 10% the baseline increased considerably, but few new peaks were observed.

Gas chromatography of heptafluorobutyric tocainide

Heptafluorobutyric tocainide can be gas chromatographed on a Carbowax 20M fused-silica capillary column [11]. When using a methyl silicone as stationary phase we found adequate separation from the methyl homologue used as internal standard. The peaks were symmetrical and the standard devia-

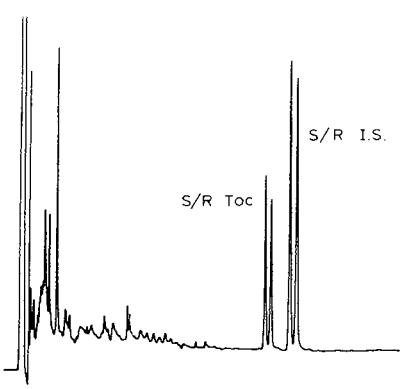


Fig. 3. Separation of the enantiomers of tocainide (Toc) and the internal standard (I.S.) as heptafluorobutyryl derivatives. Sample: 0.1 ml of plasma from a male volunteer taken 0.5 h after a 400-mg oral dose of tocainide hydrochloride, with 1.25 nmol of the internal standard added. Found levels: 2.7 and 2.3 μmol/l of *S*- and *R*-tocainide, respectively. Column: Chirasil-Val. Temperature: 110° C for 1 min and then taken to 210° C at 25° C/min. Retention time of *R*-internal standard: 8 min.

tion upon repeated injection was < 5%. No signs of adsorption or degradation compared with an inert marker could be observed [20]. As methyl silicone capillary columns are used routinely for many other drug determinations in our laboratories there was no need to investigate other stationary phases.

In order to separate the enantiomers of tocainide, capillary columns coated with Chirasil-Val were investigated. A column from Applied Science Labs. gave adequate separation of the enantiomers (Fig. 3). The resolution of the enantiomers as their heptafluorobutyryl derivatives was marginally inferior to that of the pentafluoropropionyl derivative (Table I) and superior to that obtained with the trifluoroacetyl derivatives. The responses of the pentafluoropropionyl and heptafluorobutyryl derivatives were about eight times greater than that of the trifluoroacetyl derivative. Recently a method was published describing the enantiomeric separation of heptafluorobutyric tocainide on Chirasil-Val [21]. Optically active derivatizing agents such as *S*-α-methoxy-α-trifluoromethylphenylacetyl chloride [12] can also be used but such methods demand a high optical purity of the reagent.

TABLE I

RESOLUTION OF PERFLUOROACYL DERIVATIVES OF TOCAINIDE ON CHIRASIL-VAL

Conditions: 190°C, split flow-rate 20 ml/min, inlet pressure 100 kPa.

Derivative	k'_S*	k'_R	α
Trifluoroacetyl	3.49	3.68	1.055
Pentafluoropropionyl	3.09	3.31	1.071
Heptafluorobutyryl	3.46	3.70	1.070

*k' = capacity factor.

Selectivity of the present method towards metabolites

Possible interferences from four metabolites [22] were investigated. Plasma was spiked to a concentration of 200 μmol/l of the pyruvic and lactic xylidides, the oxime and the hydantoin. Only the hydantoin, which is formed as an artefact at alkaline pH from the N-carboxytocainide glucuronide, was a potential interference. It elutes just after *R*-tocainide, but was not observed in any of the experimental plasma samples analysed.

Application to plasma samples

Standard curves were prepared by analysing plasma spiked with tocainide in the range 0.3—20 μmol/l. The precision upon repeated analysis of plasma samples is presented in Table II. For routine quantitative determinations five

TABLE II

PRECISION DATA FROM THE REPEATED ANALYSIS OF TOCAINIDE IN PLASMA

Conditions: 0.1 ml of plasma, $n = 8$.

	μmol/l	R.S.D. (%)
Tocainide (racemate)	10	3.3
	0.3	7.7
R-Tocainide	10	4.5
	0.3	9.5
S-Tocainide	10	4.7
	0.3	6.0

reference samples were prepared by adding 50 μl of a stock solution to 0.1 ml of plasma (1.3 μmol/l). The minimum determinable concentration is about 0.3 μmol/l.

Pharmacokinetic data of *R*- and *S*-tocainide in healthy subjects obtained by this method will be published elsewhere [23].

CONCLUSIONS

We have developed an electron-capture gas chromatographic method for the simultaneous determination of *S*- and *R*-tocainide in minute plasma samples. By careful control of the heptafluorobutyrylation conditions a low concentration of the reagent can be used. A methyl analogue is used as internal standard and the detection limit is 0.3 μmol/l (58 ng/ml) with a precision < 10%. A Chirasil-Val capillary column is used for separation of the enantiomers and a methyl silicone capillary column for the racemate. No interference was observed from four metabolites. The present method offers significant improvements over other methods [13, 21].

ACKNOWLEDGEMENTS

We are greatly indebted to Ms. Gunilla Edvardsson and Mr. Torsten Eklund for skilful technical assistance.

REFERENCES

1 P.J. Meffin, S.R. Harapat and D.C. Harrison, J. Pharm. Sci., 66 (1977) 583.
2 P.-O. Lagerström and B.-A. Persson, J. Chromatogr., 149 (1978) 331.
3 E.M. Wolshin, M.H. Cavanaugh, C.V. Marion, M.B. Meyer, E. Milano, C.R. Reardon and S.M. Wolshin, J. Pharm. Sci., 67 (1978) 1692.
4 P.A. Reece and P.E. Stanley, J. Chromatogr., 183 (1980) 109.
5 A.J. Sedman and J. Gal, J. Chromatogr., 232 (1982) 315.
6 R.A. Ronfeld, E.M. Wolshin and A.J. Block, Clin. Pharmacol. Ther., 31 (1982) 384.
7 D.G. McDevitt, A.S, Nies, G.R. Wilkinson, R.F. Smith, R.L. Woosley and J.A. Oates, Clin. Pharmacol. Ther., 19 (1976) 396.
8 R. Venkataramanan and J.E. Axelson, J. Pharm. Sci., 67 (1978) 201.
9 S. Supradist, Thesis, University of Bath, U.K., 1980.
10 S.D. Gettings, R.J. Flanagan and D.W. Holt, J. Chromatogr., 225 (1981) 469.
11 G.K. Pillai, J.E. Axelson and K.M. McErlane, J. Chromatogr., 229 (1982) 103.
12 J. Gal, T.A. French, T. Zysset and P.E. Haroldsen, Drug Metab. Dipos., 10 (1982) 399.
13 L. Johansson and J. Vessman, J. Chromatogr., 239 (1982) 323.
14 O. Gyllenhaal, K.-J. Hoffmann, B. Lamm, R. Simonsson and J. Vessman, in preparation.
15 O. Gyllenhaal, L. Johansson and J. Vessman, J. Chromatogr., 190 (1980) 347.
16 R. Venkataramanan, Thesis, University of British Columbia, Vancouver, Canada, 1978.
17 P.-A. Johansson, Acta Pharm. Suecica, 19 (1982) 137.
18 J. Vessman, in E. Reid (Editor), Methodological Surveys in Biochemistry, Vol. 7, Ellis Horwood, Chichester, 1978, p. 124.
19 M. Ervik, K. Kylberg-Hanssen and P.-O. Lagerström, J. Chromatogr., 182 (1980) 341.
20 M. Ahnoff, L. Johansson and M. Ervik, in R.E. Kaiser (Editor), Fourth International Symposium, Capillary Chromatography, Hindelang, Hüthig, Heidelberg, 1981, p. 487.
21 K.M. McErlane and G.K. Pillai, J. Chromatogr., 274 (1983) 129.
22 R. Venkataramanan, F.S. Abbot and J.E. Axelson, J. Pharm. Sci., 71 (1982) 491.
23 B. Edgar, A. Heggelund, L. Johansson, G. Nyberg and C.-G. Regårdh, Brit. J. Clin. Pharmacol., 17 (1984) 216.

Journal of Chromatography, 308 (1984) 189—197
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2106

SENSITIVE GAS—LIQUID CHROMATOGRAPHIC—ELECTRON-CAPTURE DETECTION METHOD FOR DETERMINATION OF SOBREROL IN BIOLOGICAL FLUIDS

UMESH A. SHUKLA

College of Pharmacy, and Upjohn Center for Clinical Pharmacology, The University of Michigan, Ann Arbor, MI 48109 (U.S.A.)

PHILIP L. STETSON

Upjohn Center for Clinical Pharmacology, The University of Michigan, Ann Arbor, MI 48109 (U.S.A.)

and

JOHN G. WAGNER*

College of Pharmacy, The University of Michigan, Ann Arbor, MI 48109 (U.S.A.)

(First received December 8th, 1983; revised manuscript received February 10th, 1984)

SUMMARY

A sensitive gas—liquid chromatographic assay for sobrerol in biological fluids has been developed. The assay procedure involves esterification of both hydroxyl groups of sobrerol with pentafluoropropionic anhydride to form the diester which can be quantitated using gas—liquid chromatography—electron-capture detection. The detection limit of plasma sobrerol under the conditions described is 5 ng/ml. The assay procedure permits the measurement of unchanged drug in plasma, whole blood or urine in the presence of its metabolites.

INTRODUCTION

DL-*trans*-Sobrerol (5-hydroxy-α,α,4-trimethyl-3-cyclohexene-1-methanol) is a terpenoid-like compound which has been shown in animal and clinical studies to have bronchial mucokinetic activity [1—4]. The drug is commercially available in European countries [5].

The structure and physico-chemical properties of sobrerol necessitate the use

0378-4347/84/$03.00

of gas—liquid chromatography (GLC) for measuring the plasma or blood levels achieved after administration of the drug by various routes. The available analytical methods for measuring sobrerol in biological fluids utilize GLC with flame ionization detection [6] and GLC—mass spectrometry (MS) [7]. The GLC method with flame ionization detection lacks specificity and the sensitivity is limited. The newer GLC—MS procedure is both sensitive and specific but the cost of the instrumentation is prohibitive and the training required for routine use of the instrument is demanding. This report describes a rapid and sensitive GLC—electron-capture detection (ECD) method for analysis of sobrerol in biological fluids.

MATERIALS AND METHODS

Reagents

Benzene, spectrophotometric grade (J.T. Baker, Phillipsburg, NJ, U.S.A.), chloroform for gas chromatography (GC) (without ethanol; Burdick & Jackson Labs., Muskegon, MI, U.S.A.), pentafluoropropionic anhydride, PFAA (Pierce, Rockford, IL, U.S.A.), sodium hydroxide (Fisher Scientific, Pittsburgh, NJ, U.S.A.), ammonium hydroxide reagent (DuPont), 4′-aminoacetophenone (Eastman Organic Chemicals, Rochester, NY, U.S.A.), and DL-*trans*-sobrerol (Key Pharmaceuticals, Miami, FL, U.S.A.) were used.

Stock solutions

Sobrerol solution (1 mg/ml in methanol) and 4′-aminoacetophenone solution (1 mg/ml in methanol) were prepared and stored at 4° C. Ammonium hydroxide reagent was diluted 1:20 (final concentration of 1.5%) and stored at room temperature.

Standards

Sobrerol stock solution was diluted with water to yield 20 μg/ml sobrerol solution; 4′-aminoacetophenone stock solution (internal standard) was also diluted to yield 20 μg/ml internal standard solution. Quality control samples were prepared by spiking blank plasma to a final sobrerol concentration of 1 μg/ml.

Chromatographic conditions

The gas chromatograph (Varian Model 3700) was equipped with a ^{63}Ni electron-capture detector, an autosampler (Varian Model 8000) and a deactivated silylated 2-m glass column (6.35 mm O.D. and 1.8 mm I.D.) packed with 10% SE-30 on 100—120 mesh Gas-Chrom Q (Applied Science Labs.). The column was conditioned at 190° C for 24 h. The GC operating conditions were: injector temperature, 200° C, column temperature, 150° C, detector temperature, 240° C, and the electrometer attenuation at 32-128 and range 10. The carrier gas was prepurified nitrogen set to a flow-rate of 40 ml/min. Of each sample 4 μl were injected on-column and the resulting signal monitored on a Linear strip-chart recorder. The retention times of sobrerol and internal standard were 4.5 and 8 min, respectively.

Sample preparation

The 1-ml plasma samples in 15-ml screw-capped test tubes were spiked with 50 μl of the internal standard solution (4'-aminoacetophenone, 20 μg/ml solution) and 250 μl of 10 *M* sodium hydroxide solution. Chloroform (10 ml) was then added. The tubes were capped and shaken for 15 min. Samples were then centrifuged at 1000 *g* for 10 min to ensure complete separation. The upper aqueous layer was removed and the organic layer transferred to another tube and evaporated at 40° C under a stream of air until the sample volume was reduced to approximately 3 ml. These samples were then transferred to 3-ml Reacti vials, evaporated to dryness, and immediately cooled to room temperature.

The sample preparation procedure for sobrerol analysis in whole blood and urine was essentially the same, except for the following modifications: in the case of blood, a 1-ml sample was deproteinized with 1 ml trichloroacetic acid (8%) before subjecting it to the sample preparation procedure, and in the case of urine, a 10-μl sample was diluted to 1 ml with water and subjected to the sample preparation procedure.

Derivatization procedure

Benzene (1 ml) and PFAA (20 μl) were added to each Reacti vial. The vials were then tightly capped with PTFE-lined screw caps and vortexed for 30 sec to ensure complete mixing of the reagent in benzene. Derivatization was accomplished at 50° C for 3 h. The vials were then cooled and excess derivatization reagent was removed by washing with 1 ml water followed by 1 ml aqueous ammonia (1.5%) solution. The samples were centrifuged at 1000 *g* for 5 min. Approximately 0.7 ml of the benzene phase was then transferred to autosampler vials. Samples were injected on-column and peak heights of sobrerol and the internal standard were measured for quantitation.

Standard calibration curve

Blank plasma samples were spiked in duplicate with sobrerol to concentrations ranging from 0.1 to 2.0 μg/ml and subjected to the sample preparation procedure described above. Peak heights of sobrerol and the internal standard were measured and a standard calibration curve was constructed by plotting peak height ratio versus concentration. The drug concentrations of unknown samples were calculated from the least-squares regression line of the standard curve.

GLC—MS of derivatives

Electron-impact mass spectra of the derivatives were obtained with a Finnigan Mat Model 4021 gas—liquid chromatograph—mass spectrometer equipped with a fused silica column (30 m × 0.25 mm I.D.) coated with DB-5 (film thickness 0.25 μm). The instrument was operated under the following conditions: energy of the ionization beam 70 eV, 0.3 mA, the injector temperature at 250° C, the column temperature was held at 40° C for 2 min and then increased to 275° C at 10° C/min. The ion source temperature was 250° C. The sample was introduced via a Grob injector. Helium was the carrier gas at 1.36 bar.

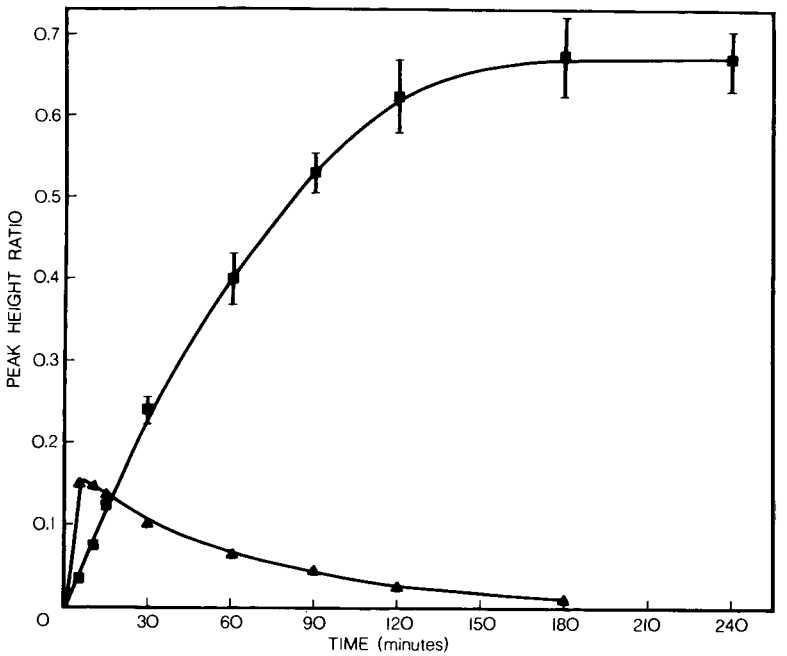


Fig. 1. Kinetics of derivatization of sobrerol at 50°C. Plot of ratio of peak heights of the derivatives to *m*-dinitrobenzene as a function of time. (■), diester; (▲), monoester.

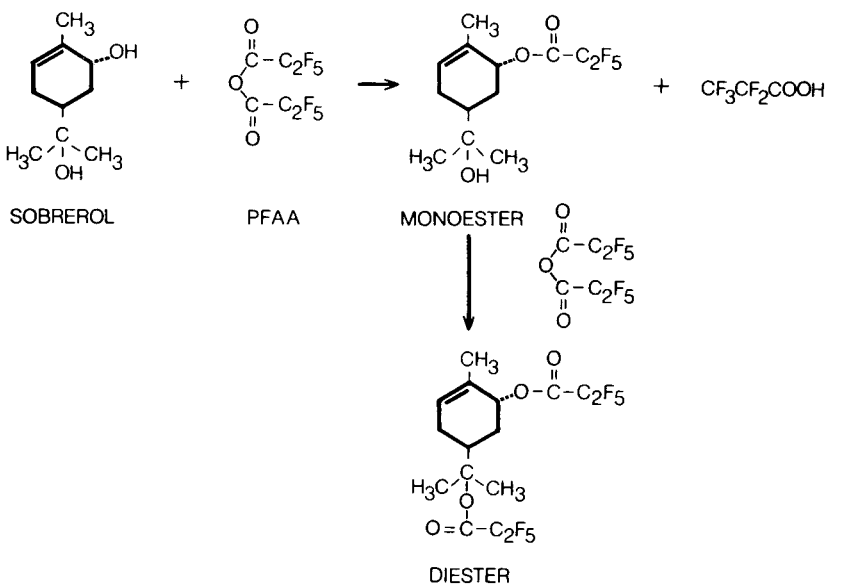


Fig. 2. Reaction between sobrerol and pentafluoropropionic anhydride (PFAA) resulting in formation of monopentafluoropropionyl derivative (monoester) which further reacts with PFAA to form dipentafluoropropionyl derivative (diester).

RESULTS AND DISCUSSION

Kinetics of derivatization reaction

Since sobrerol has two reactive hydroxyl groups, conditions of the esterification reaction were found which would yield a single product to be used for quantitation. The kinetics of derivatization were studied by transferring 1-ml aliquots of sobrerol solution in benzene (1 μg/ml) to Reacti vials followed by addition of 20 μl of PFAA and 50 μl of *m*-dinitrobenzene (20 μg/ml solution in benzene). The assay internal standard (4'-aminoacetophenone) could not be used for studying reaction kinetics because it also reacts with PFAA. Therefore, *m*-dinitrobenzene was used as internal standard in studying the derivatization kinetics. The reaction was carried out at 50° C and samples were withdrawn at regular intervals, quickly cooled to room temperature, and the reaction stopped by washing with water followed by ammonium hydroxide reagent 1.5%) as previously described. Of the benzene phase 2—4 μl were injected on-column for GLC analysis. The mono- and diesters were separated under the following conditions; injector, 200° C, column, 130° C, detector, 240° C, carrier gas flow-rate, 25 ml/min. The peak height ratios of monoester and diester to *m*-dinitrobenzene versus time were plotted as shown in Fig. 1. The plot shows that monoester is formed instantaneously (less than 5 min) and its concentration decreases as the reaction time increases. On the other hand, diester is formed slowly and reaches a plateau at 3 h suggesting completion of the reaction. It should also be noted that the electron-capture detector response to diester is much greater than its response to monoester. The reaction is presumed to be as shown in Fig. 2.

Mass spectrometry of derivatives

The existence and structure of mono- and diester was confirmed by GLC—MS. The mass spectrum of parent compound sobrerol [8] shows a molecular ion ($-H_2O$) peak at *m/e* 152 and the base peak at *m/e* 59 corresponding to $C_3H_7O^+$ (tertiary alcohol moiety). Mass spectra of mono- and diester are shown in Fig. 3A and B, respectively. In Fig. 3A the peak at *m/e* 298 corresponds to the molecular ion ($-H_2O$) for monoester, and the base peak at *m/e* 59 corresponds to $C_3H_7O^+$ as in the case of the parent compound's mass spectrum [8] and therefore suggests the presence of the unreacted tertiary alcohol group. The peak at *m/e* 119 corresponds to $C_2F_5^+$ and confirms derivatization of the secondary hydroxyl group. In the mass spectrum of diester (Fig. 3B), the heaviest *m/e* peak is at 298 which is the same as that observed for monoester. The diester molecular ion peak is not observed because the diester fragments easily give a greatly increased base peak at *m/e* 119 (corresponding to $C_2F_5^+$). The base peak at *m/e* 59 observed for monoester disappeared in the diester spectrum suggesting the hydroxyl group at the tertiary alcohol is derivatized. As would be expected the diester also has longer GLC retention time as compared to monoester.

Stability of sobrerol and the derivative

It has been reported that sobrerol has a tendency to sublime [5]. This observation was confirmed by allowing the stream of air to continue for various time intervals after the last drop of chloroform phase was evaporated. The

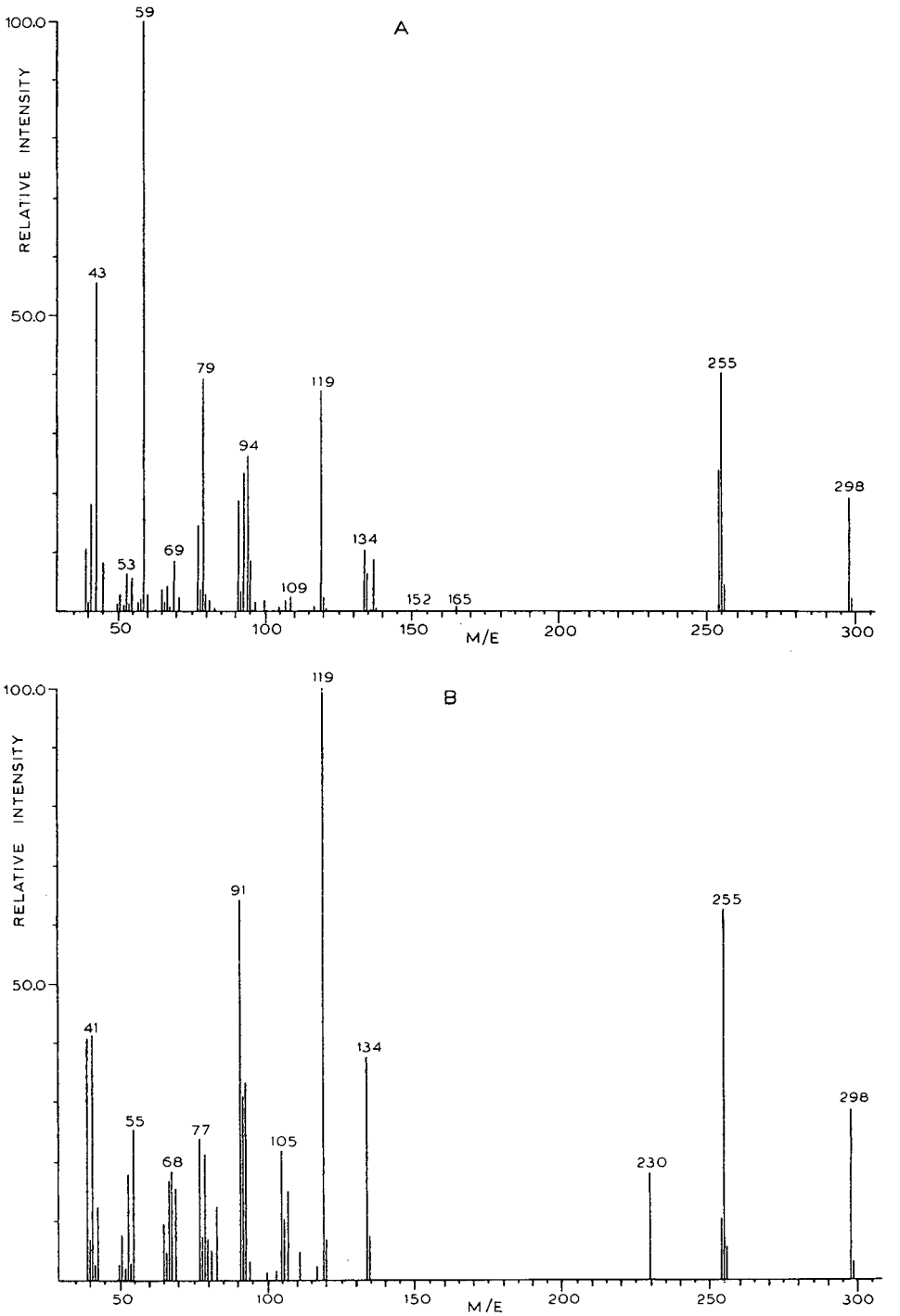


Fig. 3. Mass spectra obtained by GLC—MS of (A) the monoester and (B) the diester.

recovery of sobrerol decreased as the time of evaporation increased. Therefore, caution must be exercised in the evaporation to dryness of the initial chloroform extract.

The sobrerol derivative has also been found to be volatile at room temperature and evaporation of the reaction mixture to remove excess reagent resulted in loss of the derivative. Therefore removal of excess reagent was accomplished by addition of water to hydrolyze the anhydride, followed by the addition of ammonium hydroxide to extract the acid into the aqueous layer. The derivative under anhydrous conditions was found to be stable at room temperature for several days.

Extraction of sobrerol

Sobrerol is a neutral and polar molecule. The extraction efficiency of sobrerol from plasma samples was tested in chloroform, methylene chloride and ethyl acetate. Chloroform and methylene chloride gave similar results, but ethyl acetate showed poor extraction efficiency at alkaline pH. Use of sodium chloride did not result in significant improvement in extraction efficiency. The background increased when extraction was attempted at neutral or acidic pH.

Reproducibility, accuracy and precision

A typical chromatogram for plasma assay is shown in Fig. 4. No interfering peaks were observed when blank plasma, urine or whole blood were subjected

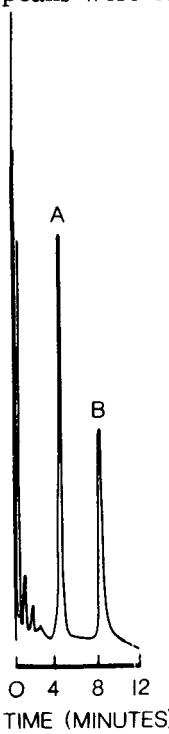


Fig. 4. A typical chromatogram of sobrerol (1 μg/ml, measured as diester) and the internal standard, 4′-aminoacetophenone (1 μg/ml) extracted from plasma. Peaks: A = sobrerol and B = internal standard.

TABLE I

INTRA-DAY REPRODUCIBILITY OF THE ASSAY

Each peak height ratio was obtained from an independently prepared calibration sample; all samples were assayed on the same day.

	Concentration (μg/ml)				
	0.10	0.20	0.50	1.50	2.00
Peak height ratio	0.18	0.33	1.04	3.83	4.50
	0.17	0.37	0.94	3.65	4.44
	0.19	0.33	1.14	3.75	4.81
Mean (μg/ml)	0.18	0.34	1.04	3.74	4.58
S.D. (μg/ml)	0.01	0.02	0.10	0.09	0.20
C.V. (%)	5.55	6.70	9.60	2.40	4.33

to the assay. The validity of the assay procedure was established through a study of linearity of response, reproducibility, accuracy and precision.

The calibration curve was found to be linear over a range of 0.10—2.0 μg/ml sobrerol concentration. The best fit least-squares line was obtained using linear-regression analysis. The correlation coefficients for inter-day standard calibration curves (in triplicate and duplicate) ranged from 0.995 to 0.998 for the plasma assay, from 0.991 to 0.993 for the whole blood assay and from 0.995 to 0.999 for the urine assay. The coefficients of variation (C.V., %) calculated

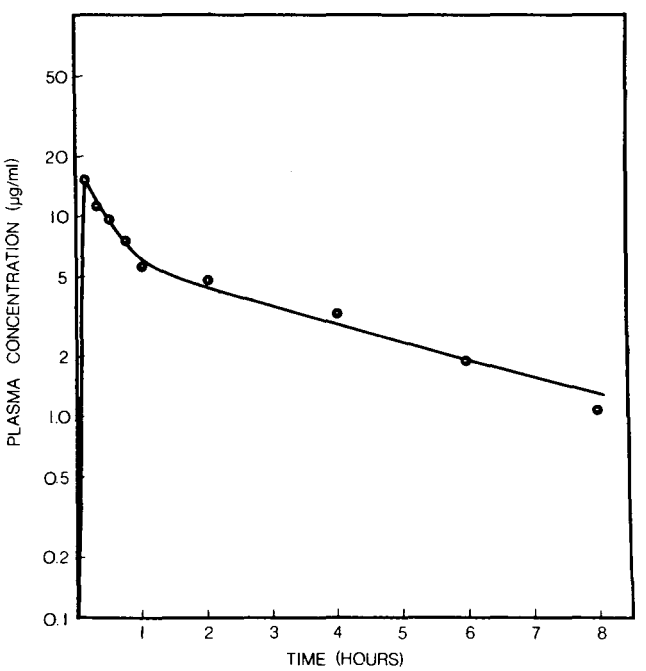


Fig. 5. A demonstration of assay applicability to pharmacokinetic studies. Plasma concentration—time curve of sobrerol after oral administration of a 500-mg oral solution to a normal human volunteer.

from inversely estimated concentrations for inter-day standard calibration curves ranged from 6.36 to 10.6 for the plasma assay, from 7.41 to 8.21 for the whole blood assay and from 3.8 to 7.5 for the urine assay. The intra-day reproducibility of the standard curve (samples analyzed in triplicate) had C.V. values ranging from 2.40 to 9.60 as shown in Table I.

The accuracy of the method was assessed by analyzing quality control samples on each assay day. The quality control sample variability was found to have a C.V. of 7.57% and a mean concentration of 1.03 μg/ml (n = 5) with the theoretical concentration being 1.0 μg/ml.

Measurement of sobrerol in plasma

Application of the method developed was demonstrated by measuring plasma levels of sobrerol in a normal human volunteer after oral administration of 500 mg as an oral solution. Peripheral venous blood samples were withdrawn at regular intervals and the plasma obtained was used for drug analysis. Plasma levels obtained are shown in Fig. 5. The data best fit the triexponential equation:

$$C = 6.9\ e^{-0.213t} + 14.01\ e^{-3.03t} - 20.92\ e^{-81.69t}$$

Interference by other compounds

A systematic investigation of potential interferences by other compounds has not been carried out. The intended use of this assay in our laboratory is measurement of sobrerol levels in controlled clinical trials on normal human volunteers. However, ephedrine, which proved to be a good internal standard for spiked plasma samples, was found to have the same retention time as one of the metabolites of sobrerol (possibly hydrated carvone). A systematic investigation of interference by commonly coadministered drugs is anticipated.

ACKNOWLEDGEMENT

This research was supported by Key Pharmaceuticals, Miami, FL, U.S.A.

REFERENCES

1 R. Scuri, C. Cellari and V. Dalla Valle, Boll. Chim. Farm., 115 (1976) 581.
2 R. Scuri, C. Frova, P.L. Fantini, G. Mondani and C. Alfieri, Farmaco Ed. Prat., 36 (1981) 36.
3 L. Allegra, R. Bossi and P.C. Braga, Respiration, 42 (1981) 105.
4 C. Marchioni, R. Scuri, C. Moretti, E.V. Baldini and A. Bisetti, VIth Asia-Pacific Congress on Diseases of the Chest, Bombay, November 18—22, 1979, p. 72.
5 Pharmacokinetic Evaluation of a New Mucous Regulating Agent, Sobrerol; Protocol No. 83-10-S01, Key Pharmaceuticals, Miami, FL, August 11, 1983.
6 Key Pharmaceuticals, Miami, FL, personal communication.
7 P.C. Braga, L. De Angelis, R. Bossi, F. Scaglione, G. Scarpazza, L. Allegra and F. Fraschini, Eur. J. Clin. Pharmacol, 24 (1983) 209.
8 P. Ventura, M. Schiavi and S. Serafini, Xenobiotica, 13 (1983) 139.

Journal of Chromatography, 308 (1984) 199—208
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2069

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CITALOPRAM AND FOUR OF ITS METABOLITES IN PLASMA AND URINE SAMPLES FROM PSYCHIATRIC PATIENTS

E. ØYEHAUG* and E.T. ØSTENSEN

Agder College, 4600 Kristiansand S (Norway)

and

B. SALVESEN

Institute of Pharmacy, University of Oslo, Blindern, Oslo 3 (Norway)

(First received September 30th, 1983; revised manuscript received January 12th, 1984)

SUMMARY

A high-performance liquid chromatographic method is used for the determination of citalopram [1-(3-dimethylaminopropyl)-1-(4-fluorophenyl)-5-phthalancarbonitrile] and four of its metabolites (the methylamino, amino, propionic acid and N-oxide derivatives) in plasma and urine. The plasma samples were extracted with diethyl ether at pH 10 and pH 4. Filtered urine samples could be injected directly on to the column. Steady-state drug and metabolite levels were investigated in fifteen psychiatric patients. In urine, 12 ± 5% (mean ± S.D.) of a given dose of citalopram was excreted in unchanged form. The propionic acid derivative was further conjugated, possibly to glucuronic acid. Mean steady-state plasma levels and metabolites in 24-h urine are given as percentages of the dose.

INTRODUCTION

Citalopram (I, Fig. 1), an antidepressant, is a potent and selective inhibitor of serotonin re-uptake [1]. From previous investigations it appears that the kinetics in man is characterized by approximately complete systemic availability and slow elimination [2]. Compared to the tricyclic antidepressants, which are eliminated mainly by hepatic metabolism [3], citalopram seems to be less extensively metabolized. Thus roughly 13% of a given dose of citalopram has been recovered unchanged in urine, suggesting elimination by renal as well as hepatic processes [2].

0378-4347/84/$03.00

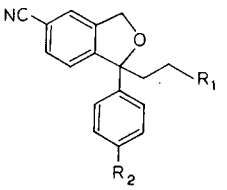


Fig. 1. Formulae of the compounds investigated

	R_1	R_2
I: Citalopram	$-CH_2N(CH_3)_2$	F
II: Methylamine metabolite	$-CH_2NHCH_3$	F
III: Amino metabolite	$-CH_2NH_2$	F
IV: Propionic acid metabolite	$-COOH$	F
V: N-Oxide	$-CH_2\overset{\uparrow}{N}(CH_3)_2$ (O)	F
VI: Internal standard	$-CH_2N(CH_3)_2$	Cl

Citalopram is metabolized by demethylation (II and III, Fig. 1). These metabolites are less potent than citalopram, but retain its specific effect [1]. The N-oxide of citalopram and a deaminated metabolite (IV and V, Fig. 1) have been observed in human urine [4]. We have therefore extended our previously described high-performance liquid chromatographic (HPLC) method [5] to include determination of IV (the propionic acid metabolite) in plasma. This paper also describes a method for the determination of unconjugated and conjugated compounds of I—V (Fig. 1) in urine.

EXPERIMENTAL

Standards and reagents

Lu-10-177 HBr (I), Lu-11-109 HCl (II), Lu-11-161 oxalate (III), Lu-16-073 (IV), Lu-11-305 HCl (V) and the internal standard Lu-10-202 HBr (VI) were supplied by Lundbeck (Copenhagen, Denmark). Methanolic stock solutions (5 mg/ml) of compounds I—VI were stable at room temperature for at least two months.

All reagents were analytical-reagent grade and aqueous solutions were prepared using glass-distilled water; 1 *M* sodium hydroxide was ether-washed; 0.6% (w/v) potassium dihydrogen phosphate was adjusted to pH 3 with concentrated orthophosphoric acid; 0.2 *M* sodium acetate was adjusted to pH 5 with concentrated acetic acid; β-glucuronidase—arylsulphatase from *Helix pomatia* was supplied by Boehringer Mannheim (Mannheim, F.R.G.); acetonitrile (HPLC grade) and diethyl ether were supplied by Rathburn Chemicals (Walkerburn, U.K.).

Chromatography

HPLC analyses were performed as described earlier [5] on a Perkin-Elmer Series 2/2 liquid chromatograph with a Perkin-Elmer 3000 fluorescence spectrometer (Perkin-Elmer, Norwalk, CT, U.S.A.) operating at an excitation wavelength of 240 nm, slit 15 nm, and an emission wavelength of 300 nm, slit

20 nm. The column was Spherisorb ODS 5 μm (25 cm × 3 mm I.D.) with an MPLC^{TM} guard column of RP-18 (3 cm × 4.6 mm I.D.). The mobile phase used for determination of I, II and III was 55% (v/v) acetonitrile in potassium dihydrogen phosphate buffer at a constant flow-rate of 1.3 ml/min. The mobile phase used for determination of IV was 45% (v/v) acetonitrile in potassium dihydrogen phosphate buffer at a flow-rate of 1 ml/min.

The mobile phase used for urine analysis was 50% (v/v) acetonitrile in potassium dihydrogen phosphate buffer at a flow-rate of 1.3 ml/min.

Extraction from plasma

Citalopram and the amino metabolites (I, II and III) were extracted according to the reported method [5] with minor modifications. To 1 ml of plasma (either patient plasma or spiked plasma blank) were added 75 ng of internal standard (VI) followed by 50 μl of 1 *M* sodium hydroxide to bring the solution to a pH of about 10. The mixture was extracted twice with 3-ml portions of diethyl ether by mechanical shaking for 15 min. After centrifuging for 10 min at 3200 *g*, the combined ether layers were transferred to 10-ml evaporation tubes containing 100 μl of potassium dihydrogen phosphate buffer pH 3. The diethyl ether was evaporated under a stream of nitrogen in a 40°C water-bath. The residual extract was purified by whirlmixing with 0.5 ml of diethyl ether for 10 sec and centrifuged at 625 *g*. The ether layer was then removed and discarded; 15—20 μl of the remaining extract were injected on to the column.

The propionic acid metabolite (IV) was extracted from plasma as follows. To the residual plasma sample after basic extraction were added 150 μl of 1 *M* hydrochloric acid and 1 ml of 0.6% phosphate buffer pH 3, and extracted twice with 3-ml portions of diethyl ether by mechanical shaking for 15 min. After centrifuging for 10 min at 3200 *g*, the combined ether layers were transferred to 10-ml evaporation glass tubes. The diethyl ether was evaporated under a stream of nitrogen in a 40°C water-bath. The residue was dissolved in 100 μl of mobile phase and 20 μl of the extract were injected on to the column.

Four to five standards of blank plasma spiked with I, II, III and IV, and patient plasma samples were run simultaneously. Amounts of I, II and III were determined by peak height ratios of compound to internal standard. The propionic acid metabolite (IV) was determined by peak height measurement.

Pretreatment of urine samples

To 200 μl of urine (either patient urine or spiked blank urine) were added 200 μl of 0.2 *M* sodium acetate pH 5 containing 5 μg/ml internal standard (VI). The solution was sterile filtered through a MF-7 centrifugal Microfilter with an OE 0.2-μm filter (Bioanalytical Systems, West Lafayette, U.S.A.) and 20 μl were injected on to the column.

For determination of conjugated metabolites, the patient urine samples were also incubated with β-glucuronidase. To 1 ml of patient urine were added 1 ml of 0.2 *M* acetate buffer pH 5 containing internal standard 5 μg/ml and 2 μl of β-glucuronidase reagent. The mixture was incubated for 15 h at 37°C and sterile filtered; 20 μl were injected on to the column. Standards and patient

urine samples were run simultaneously. Calibration curves of spiked blank urines were constructed and patient urine levels of compounds I—V were determined using peak height ratios of compound to internal standard.

Plasma samples

Plasma samples were obtained from thirteen psychiatric patients, eight men and five women, aged 22—79 years, who had been treated with citalopram 40—60 mg/day for at least fourteen days. The drug was given as a single morning dose. No other medication, except for nitrazepam, was given.

Citalopram and metabolites were measured in plasma at minimum steady-state levels; i.e. before the morning dose, 10—15 ml of venous blood were drawn into heparinized glass tubes and centrifuged. The plasma samples were stored frozen at −20° C for a maximum of four months before analysis.

Urine samples

Twenty-four-hour urine samples were collected from fifteen psychiatric patients, nine men and six women, aged 29—79 years, who had been treated with citalopram 40—60 mg/day for at least fourteen days. In addition the patients had been given nitrazepam, alimemazine or levomepromazine. The samples were stored frozen at −20° C for a maximum of four months before analysis.

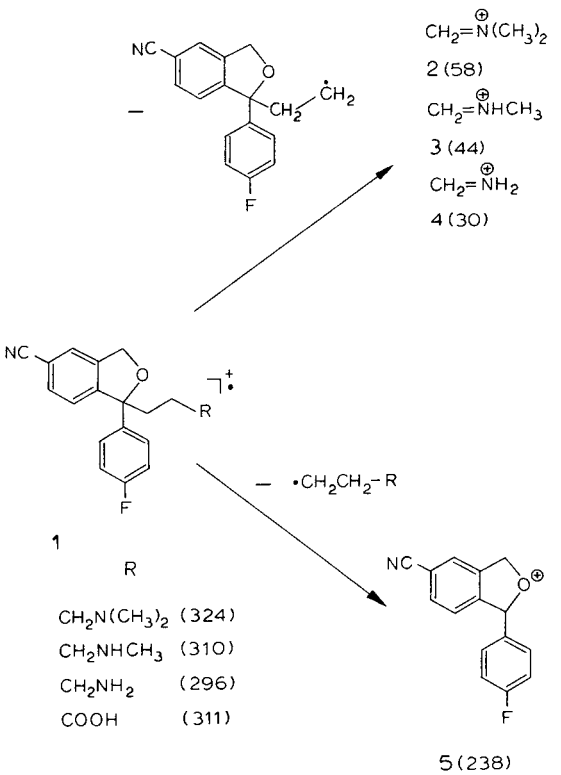


Fig. 2. Fragmentation of citalopram derivatives.

Identification of compounds by fluorescence ratios

Fluorescence ratios of the pure compounds were determined by stop flow and fluorescence measurements at three different wavelengths as described by Yost et al. [6]. When measuring a possible citalopram derivative in a patient urine sample, the fluorescence ratios of the compound were determined by the same technique. Compounds that eluted at the retention times of citalopram (I), the methylamino metabolite (II) and the amino metabolite (III) were identified by this method.

Mass spectrometric identification

Citalopram derivatives eluted from a patient urine by repeated injection of the sample on to the analytical column were collected separately. The acetonitrile was evaporated and the concentrated isolates of the basic and acid compounds were extracted with diethyl ether at pH 10 and 3, respectively. The diethyl ether extracts were evaporated and analysed by electron-impact mass spectrometry (MS) at 20 eV.

A probable fragmentation pattern of citalopram derivatives is shown in Fig. 2. In the mass spectra of isolates with retention times identical to those of citalopram (I), the methylamino metabolite (II), the amino metabolite (III)

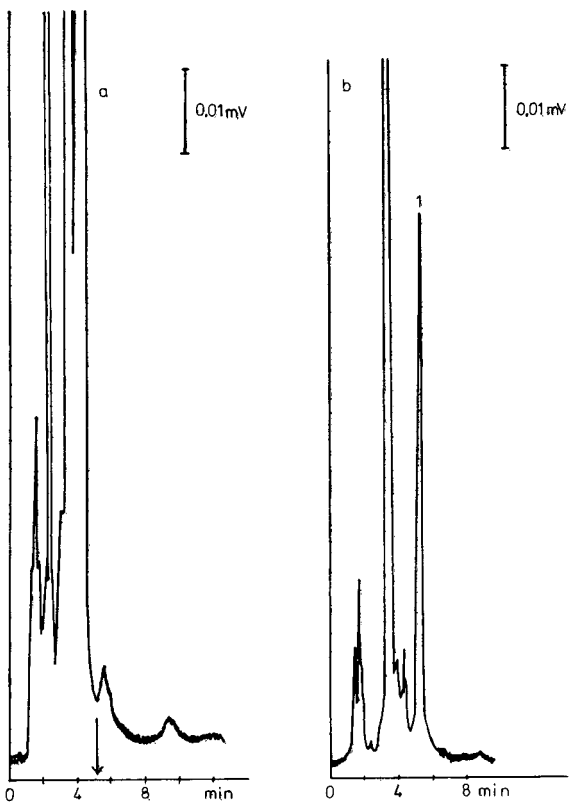


Fig. 3. Chromatograms of extracts from plasma at pH 4. (a) Plasma blank. (b) Patient plasma containing 75 nmol/l propionic acid metabolite (1). Daily dose of citalopram was 40 mg. Mobile phase: acetonitrile—phosphate buffer 0.6%, pH 3 (45:55) at a flow-rate of 1 ml/min.

and the propionic acid metabolite (IV), major ions of *m/e* 238 were found, corresponding to fragment 5 (Fig. 2). In addition, ions (*m/e* 44 and 58) corresponding to fragments 3 and 2 were evident in the mass spectra of citalopram and the methylamino metabolite isolates.

RESULTS

Determination in plasma

Chromatograms of an acidic extract of blank plasma from a healthy person and patient plasma containing 75 nmol/l propionic acid metabolite (IV) are shown in Fig. 3a and b. Inter-individual variations in the amounts of early eluted plasma constituents were observed. Equations for the calibration curves were as follows:

I: $X = 2.73Y - 6.3$; $n = 5$; $r^2 = 1.00$; range 60—375 nmol/l.

II: $X = 1.95Y - 14.0$; $n = 5$; $r^2 = 1.00$; range 60—375 nmol/l.

III: $X = 1.86Y - 3.2$; $n = 5$; $r^2 = 1.00$; range 13—90 nmol/l.

IV: $X = 1.28Y + 12.6$; $n = 5$; $r^2 = 0.99$; range 37.5—225 nmol/l.

Day-to-day variation in the calibration curve of IV was observed; relative standard deviation was 9% and the detection limit 15 nmol/l. The extraction procedure yielded a recovery of IV better than 80% from plasma (Table I). The N-oxide could not be determined by the given method for plasma analysis.

TABLE I

ANALYSIS OF PLASMA

$n = 10$.

	Within-run analysis of spiked blank plasma		Recovery (%) (mean ± S.D.)
	Mean conc. (nmol/l)	Relative S.D. (%)	
Citalopram	250	2	84 ± 4
Methylamino metabolite	260	3	88 ± 5
Amino metabolite	26	5	87 ± 5
Propionic acid metabolite	60	9	87 ± 7
Internal standard			84 ± 4

Determination in urine

Chromatogams of blank urine and of patient urine are shown in Fig. 4a and b.

Equations for the calibration curves were as follows:

I: $X = 6.7Y - 0.2$; $n = 5$; $r^2 = 1.00$; range 2.5—12.5 μmol/l.

II: $X = 4.4Y - 0.3$; $n = 5$; $r^2 = 1.00$; range 2.5—12.5 μmol/l.

III: $X = 3.8Y - 0.2$; $n = 5$; $r^2 = 1.00$; range 1.0—7.0 μmol/l.

IV: $X = 3.2Y - 0.1$; $n = 5$; $r^2 = 1.00$; range 1.0—7.0 μmol/l.

V: $X = 4.5Y - 0.02$; $n = 5$; $r^2 = 1.00$; range 0.15—0.7 μmol/l.

No day-to-day variation was observed. Relative standard deviations were < 5% (n = 10) in the upper and lower concentration ranges for compounds I—IV. The relative standard deviation of the N-oxide (V) was < 11% in the lower concentration range (Table II). The detection limits were in the range 0.03—0.06 μmol/l.

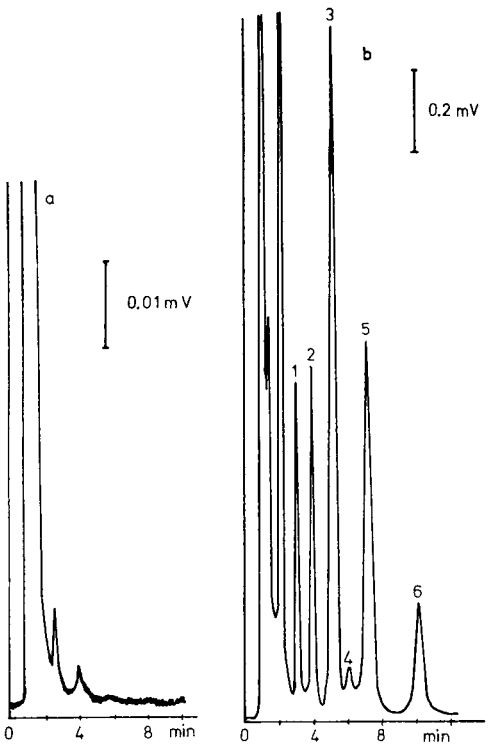


Fig. 4. Chromatograms of urine samples. (a) Urine blank. (b) Patient urine containing propionic acid metabolite 7 μmol/l (1), amino metabolite (10 μmol/l (2), methylamino metabolite 23 μmol/l (3), N-oxide 1.8 μmol/l (4) and citalopram 22 μmol/l (5); internal standard (6). Mobile phase: acetonitrile—phosphate buffer 0.6%, pH 3.2 (50:50), at a flow-rate of 1.3 ml/min.

Plasma samples

Minimum steady-state plasma levels of citalopram and metabolites are shown in Table III. Citalopram (I) plasma levels were in the range 108—334 nmol/l. The methylamino metabolite (II) levels were on average 50% of the citalopram levels and were in the range 70—167 nmol/l. The amino metabolite (III) plasma levels were approximately 10% of the parent drug (I) levels and were in the range 7—37 nmol/l.

In one patient, the propionic acid metabolite (IV) could not be detected. In twelve patients, the plasma levels of IV ranged from 33 to 117 nmol/l and were approximately 30% of the citalopram plasma levels (Table III).

The N-oxide could not be detected in plasma.

TABLE II

ANALYSIS OF URINE

$n = 10$.

	Within-run analysis of spiked blank urine	
	Mean conc. (μmol/l)	Relative S.D. (%)
Citalopram	1.25	3
	12.50	5
Methylamino metabolite	1.30	3
	13.00	4
Amino metabolite	0.65	4
	6.50	4
N-Oxide	0.67	6
	0.13	11
Propionic acid metabolite	0.75	5
	7.50	5

TABLE III

MINIMUM STEADY-STATE PLASMA LEVELS

$n = 13$. Citalopram dose was 40 mg/day.

	Plasma concentration (nmol/l)	
	Range	Mean ± S.D.
Citalopram	108—334	206 ± 69
Methylamino metabolite	70—167	105 ± 27
Amino metabolite	7—37	23 ± 9
Propionic acid metabolite*	<15—117	66 ± 27

*$n = 12$.

TABLE IV

PERCENTAGES OF A GIVEN DOSE IN 24-h URINE AT STEADY-STATE

$n = 15$.

	Range (%)	Mean ± S.D. (%)
Citalopram	6.3—21.0	12.2 ± 5.1
Methylamino metabolite	6.4—22.2	12.3 ± 4.0
Amino metabolite	0.3— 3.5	1.5 ± 1.2
Amino metabolite, conjugated	0.0— 1.9	
Propionic acid metabolite	0.1— 1.4	0.6 ± 0.4
Propionic acid metabolite, conjugated	2.1— 7.1	4.3 ± 1.5
N-Oxide	0.0— 1.0	
Total of drug and known metabolites	18.7—44.8	31.8 ± 5.1

Urine samples

Table IV shows amounts of citalopram and metabolites in 24-h urine samples as percentage of a given dose. Volume and pH of excreted urine were in the range 0.65—2.15 l per 24 h and 5.4—6.4, respectively. Ranges of excreted unchanged drug (I) and the methylamino metabolite (II) were 6.3—21.0% and 6.4—22.2%, respectively, with averages of 12% of a given dose.

The amino and propionic acid metabolites (III and IV) appeared to be conjugated in urine. The ranges of unconjugated and conjugated amino metabolite were 0.3—3.5% and 0—1.9%, respectively, of a given dose. Excreted unconjugated and conjugated propionic acid metabolite were in the range 0.06—1.44% and 2.1—7.1%, respectively.

The N-oxide (V) could not be detected in seven patients. The range of excreted amounts of V was 0—1.0% of a given dose.

DISCUSSION

In our clinical study, either nitrazepam, alimemazine or levomepromazine was given in addition to citalopram. However, because of the selectivity of the fluorescence detection these compounds do not give interfering peaks [5]. The 9% standard deviation in the determination of the propionic acid metabolite (IV) in plasma reflects the absence of internal standard and the presence of co-extracted endogenous plasma constituents (Fig. 3a). The presence of the propionic acid metabolite (IV) in plasma and urine seems to verify that citalopram is deaminated in the human body, possibly by α-oxidation as described by Beckett et al. [7].

This side-chain degradation is identical to the degradation of the corresponding phthalanes Lu-3-010 [8] and Lu-5-003 [9] and is different from degradation of the tricyclic antidepressants [3].

The propionic acid metabolite (IV) appears further to be conjugated to glucuronic acid. Conjugation of the amino metabolite (III) was also observed in some of the patient urine samples, while citalopram and the methylamino metabolite (I and II) did not seem to be excreted in conjugated form.

Observed amounts of citalopram N-oxide in urine were close to the detection limit of the analytical method and it seems likely that N-oxidation only plays a minor role in citalopram metabolism.

The average amount of citalopram excreted unmetabolized was 12% of a given dose. This result is in agreement with observations in an earlier investigation [2] and confirms that citalopram is eliminated by renal as well as hepatic clearance. The mean amount of excreted drug and metabolites in 24-h urine was 31.8% of the dose (Table IV). This result may in addition indicate faecal elimination or degradation pathways of citalopram other than demethylation and deamination.

In this study the steady-state citalopram and methylamino metabolite plasma levels were in the same range as reported earlier [1]. The corresponding amino metabolite and propionic acid metabolite levels were about one-tenth and one-third, respectively, of the citalopram levels.

Determination of plasma and urine levels of drug and metabolites may be useful in investigating drug interactions caused by induction or inhibition of

hepatic metabolism. We have been investigating the effect of phenothiazines on steady-state plasma levels of citalopram and metabolites. The results will be published elsewhere [10].

REFERENCES

1 J. Hyttel, Progr. Neuro-Psychopharm. Biol. Psychiat., 6 (1982) 275.
2 P. Kragh-Sørensen, K. Fredricson Overø, O. Lindegaard-Pedersen, K. Jensen and W. Parnas, Acta Pharmacol. Toxicol., 48 (1981) 53.
3 L.F. Gram, Dan. Med. Bull., 21 (1974) 218.
4 K. Fredricson Overø, personal communication.
5 E. Øyehaug, E.T. Østensen and B. Salvesen, J. Chromatogr., 227 (1982) 129.
6 R. Yost, J. Stoveken and W. Maclean, J. Chromatogr., 134 (1977) 73.
7 A.H. Beckett, J.M. van Dyk, H.H. Chissick and J.W. Gorrod, J. Pharm. Pharmacol., 23 (1971) 809.
8 G. Plym Forshell, P. Schauman, V. Hansen, U. Dahl Larsen, A. Jørgensen and K. Fredricson Overø, Acta Pharmacol. Toxicol., 26 (1968) 507.
9 K. Fredricson Overø, A. Jørgensen and V. Hansen, Acta Pharmacol. Toxicol., 28 (1970) 81.
10 E. Øyehaug, G. Eide and B. Salvesen, in preparation.

Journal of Chromatography, 308 (1984) 209—216
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2073

LIQUID CHROMATOGRAPHIC DETERMINATION OF NIFEDIPINE IN PLASMA AND OF ITS MAIN METABOLITE IN URINE

C.H. KLEINBLOESEM* and J. VAN HARTEN

Department of Pharmacology, Subfaculty of Pharmacy, State University of Leiden, Leiden (The Netherlands)

P. VAN BRUMMELEN

Department of Nephrology, University Hospital, Leiden (The Netherlands)

and

D.D. BREIMER

Department of Pharmacology, Subfaculty of Pharmacy, State University of Leiden, Leiden (The Netherlands)

(First received November 29th, 1983; revised manuscript received January 16th, 1984)

SUMMARY

A high-performance liquid chromatographic method was developed for the assay of nifedipine in plasma and its main metabolite (M-I) in urine. After liquid—liquid extraction nifedipine was chromatographed in a reversed-phase system with ultraviolet detection at 238 nm. The method was sensitive to 2 ng nifedipine per ml plasma and the standard curve was linear to at least 400 ng/ml. Standard deviations did not exceed 8.5%. There was no interference with photodecomposition products or metabolites. M-I was determined in urine after liquid—liquid extraction by ion-pair chromatography with ultraviolet detection at 290 nm. The method was sensitive to 0.02 μg M-I per ml urine and the standard curve was linear to at least 5 μg/ml. Standard deviations did not exceed 5.0%. The methods were used to study nifedipine disposition in healthy volunteers.

INTRODUCTION

Nifedipine, dimethyl-1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridine carboxylate, is a calcium-channel blocker, which selectively dilates arteries

0378-4347/84/$03.00

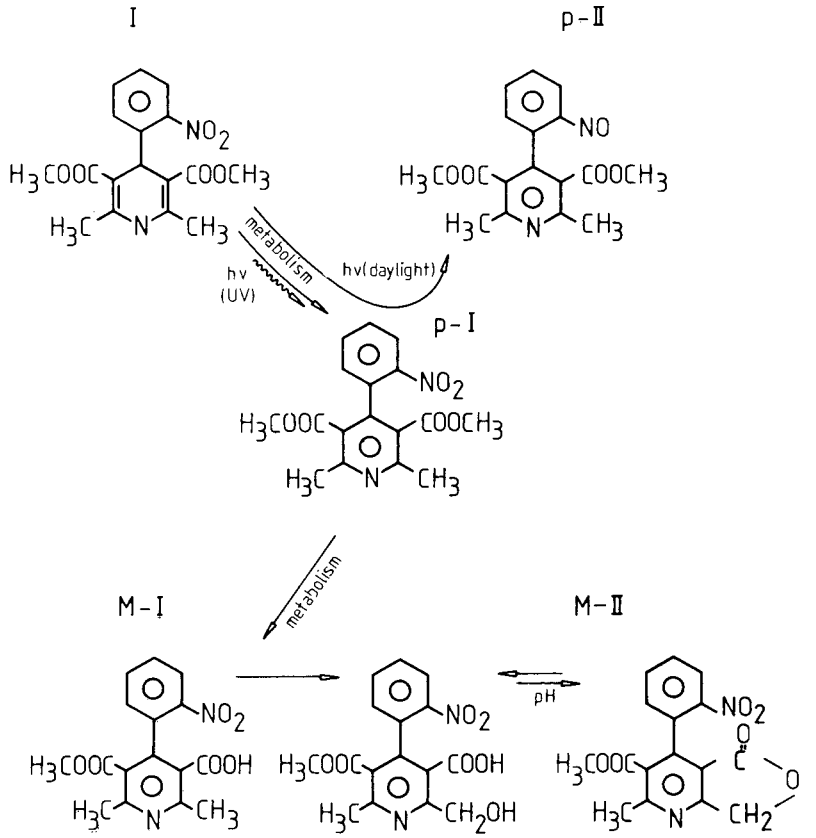


Fig. 1. Photodecomposition and biotransformation scheme. I is nifedipine; p-I and p-II are photodegradation products, and M-I and M-II are metabolites.

with little or no effect on other blood vessels. Therefore nifedipine is used in the treatment of angina pectoris and arterial hypertension [1, 2]. The compound is photolabile; a scheme of its photodecomposition and biotransformation is shown in Fig. 1. In the human body nifedipine is rapidly oxidized enzymatically into its pyridine metabolite (p—I) [3—5]. This product is also formed in ultraviolet (UV) light, whereas the 2-nitroso derivative (p-II) is formed in normal daylight. In the biotransformation process the ester moiety of the side chain can be hydrolysed (M-I) and further oxidation can occur in the 2-methyl position (M-II) [3—5]. M-I is excreted in urine for about 60% of the dose, whereas M-II is excreted for only 3—5% of the dose [6]. It is assumed that these metabolites have no pharmacological activity [3].

For the determination of nifedipine in biological fluids several gas chromatographic methods have been described, with either electron-capture detection or selective ion monitoring, providing a satisfactory detection limit of approximately 1—5 ng/ml [4—10]. However, the thermostability of nifedipine under the chromatographic conditions employed (230—250° C) represents a serious problem, since p-I is formed in non-reproducible amounts. Therefore Kondo et al. [6] and Higuchi and Shiobara [9] oxidized nifedipine prior to

analysis, despite the loss of selectivity. Pietta et al. [11] reported a selective high-performance liquid chromatographic (HPLC) method for nifedipine and metabolites in rat plasma with a detection limit of 10 ng of nifedipine, which is not low enough for pharmacokinetic studies in man. Recently, Bach [12] reported a sensitive liquid chromatographic method for the determination of nifedipine in serum, but some interference with the serum matrix occurred and a lengthy sample clean-up procedure was used.

In the present study a sensitive and selective reversed-phase HPLC method has been developed for the determination of nifedipine in plasma taking photodecomposition and metabolite formation into account. Also an ion-pair HPLC method is reported for the main metabolite of nifedipine in urine (M-I). Preliminary disposition data in healthy volunteers are included in this paper. In addition, the photostability of nifedipine in organic solvents, as well as in plasma, was investigated.

EXPERIMENTAL

Chemicals

Nifedipine, metabolites (M-I, M-II), photodegradation products (p-I, p-II), nitrendipine (internal standard) and Bay a-4160 [2,6-diethyl-4-(2-nitrophenyl)-5-methoxycarbonylpyridine-3-carboxylic acid, internal standard] were kindly supplied by Bayer (Wuppertal, F.R.G.). In all experiments double-distilled water and freshly distilled organic solvents (Baker, Deventer, The Netherlands) were used.

Apparatus and chromatographic system

Nifedipine. The HPLC system consisted of a solvent pump (Waters Assoc., U.S.A., Model M 45), an automatic sampler (Waters Assoc., Model Wisp 710B), a column (100 mm long), constructed from precison bore stainless-steel tubing (I.D. = 2.8 mm, O.D. = 6.35 mm), filled by a slurry technique with MOS-Hypersil® (dimethyloctyl silica), particle size 5 μm (Shandom, Astmoor, U.K.), and a UV detector (Waters Assoc., Model Lambda Max 480) set at 238 nm. Concentrations were calculated by using an integrator (Hewlett-Packard, U.S.A., Model 3390 A). The mobile phase consisted of 0.05 *M* acetate buffer, pH 4.0—acetonitrile (7:5). It was degassed ultrasonically and used at a flow-rate of 1.0 ml/min.

Metabolite I. M-I was chromatographed on a Rad-Pak C_8 cartridge (100 mm long, I.D. 5 mm, particle size 10 μm; Waters Assoc.). The mobile phase consisted of acetonitrile—water + 0.009 *M* cetrimide (1:3) and was degassed ultrasonically before use; the flow-rate was set at 4.0 ml/min. Detection took place at 290 nm.

Preparation of samples

Nifedipine. Plasma (1.0 ml) was transferred to a 15-ml centrifuge tube containing 0.5 ml of 1 *M* sodium hydroxide and 150 ng of nitrendipine (internal standard). The mixture was extracted with 5 ml of dichloromethane—pentane (3:7) on a vortex whirl mixer for 20 sec and centrifuged for 5 min at 2000 *g*. The organic layer was transferred to a 15-ml centrifuge tube using Pasteur dis-

posable pipettes and evaporated to dryness on a vortex vacuum evaporator (Buchler, Fort Lee, U.S.A.) at 40° C. The sample was reconstituted with 200 μl of the mobile phase before injection. All steps were carried out in sodium light, without influence of daylight.

Metabolite I. Urine (1.0 ml) was transferred to a 15-ml centrifuge tube containing 0.5 ml of 1 *M* acetate buffer, pH 4.0 and 3 μg of Bay a-4160 (internal standard). The mixture was extracted with 7 ml of chloroform on a vortex whirl mixer for 30 sec and further processed as described above.

Preparation of calibration graphs

Control plasma samples (1.0 ml) were spiked with 0, 5, 10, 25, 50, 100, 200 and 400 ng nifedipine. Urine samples were spiked with 0, 0.25, 0.5, 1.0, 2.5 and 5 μg/ml M-I. Samples were processed as described and the ratios of peak area of compound to be assayed to peak area of internal standard were calculated. Calibration curves were constructed by linear-regression analysis. Extraction yields were also determined using the same procedure, except that the internal standard was added now after extraction as external standard. The ratios obtained were compared with the ratio of standard amounts of nifedipine and M-I to the internal standard. The average recovery was determined by the slope ratio of the two calibration graphs as obtained after internal and external standardization.

Photostability of nifedipine

To 10 ml-centrifuge tubes 150 ng/ml nifedipine dissolved in dichloromethane—pentane—methanol (3:7:1) (total volume 5 ml) was added. The tubes were exposed to normal laboratory light on a normal summer day for the following periods: 0, 5, 10, 20, 30, 40, 50 and 60 min. After exposure to light, internal standard was added and the samples were analysed after evaporation of the organic solvents. Detection took place at 254 nm. The same procedure was performed after spiking plasma samples with nifedipine and exposing these to light. These samples, however, were also extracted prior to analysis as described above for nifedipine. The identity of the photodegradation product was confirmed by mass spectrometry as the nitroso derivative, by comparing the spectrum obtained with the mass spectrum of the reference compound.

Human experiments

In this preliminary study five healthy volunteers (four males, one female) received 4.5 mg of nifedipine as an intravenous infusion over a period of 50 min. Infusion lines were carefully protected from light by aluminum foil. Blood samples were taken from a forearm vein using a flexible cannula. Samples were drawn in heparinized tubes at 0, 10, 20, 30, and 45 min, and 1, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0 and 8.0 h after starting the infusion. Plasma was separated by centrifugation and samples were stored (protected from light) at −20° C until analysed. Urine samples were collected at regular intervals until 24 h after drug administration and these were stored at −20° C until analysed.

RESULTS AND DISCUSSION

Assay of nifedipine in plasma

In Fig. 2 typical chromatograms are shown which were obtained after extraction of blank plasma to which only internal standard had been added and plasma containing 25 ng of nifedipine and internal standard. No interfering substances seem to be coextracted. There was also a good separation with possible photodegradation products, because the retention times of p-I and p-II were 0.96 and 2.41 min, respectively. These compounds could not be detected in samples of volunteers who received nifedipine, indicating that protection from light had been adequate. Calibration curves were linear with concentration, as shown by the correlation coefficient of 0.999. The method was reproducible and had satisfactory precision; standard deviations did not exceed 8.5%. Extraction yields appeared to be linear in the concentration range 5—400 ng/ml and almost complete (mean 97.6 ± 3.0%; $n = 4$). The lowest measurable concentration was about 2 ng/ml. It can be concluded that the present HPLC method is highly selective, sensitive and rapid. As compared to previously reported GC methods [4—10] thermostability is no problem and the lowest measurable concentration is in the same order of magnitude (1—5 ng/ml); precision, however, is somewhat less: 8.5% vs. 2.8% [6].

Sensitivity is in the same order as that of the HPLC method reported by Bach [12], whereas sample clean-up is less lengthy in the present method.

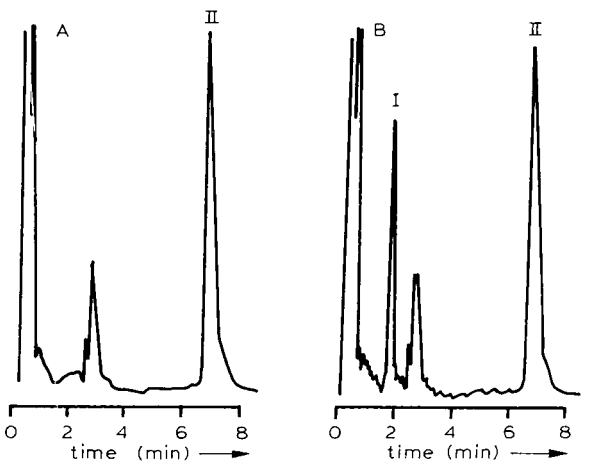


Fig. 2. Chromatograms of (A) control plasma with internal standard (peak II) and (B) plasma spiked with 25 ng/ml nifedipine (peak I) and internal standard (peak II).

Assay of M-I in urine

In Fig. 3 typical chromatograms are shown which were obtained after extraction of blank urine to which only internal standard had been added, and of urine containing 2.5 μg of M-I and internal standard. Interfering peaks were not observed. Calibration curves were linear with concentration, as shown by the correlation coefficient of 0.999. The method was reproducible and standard deviations were below 5%. Extraction yields determined in the same concentration range appeared to be constant and linear with concentration

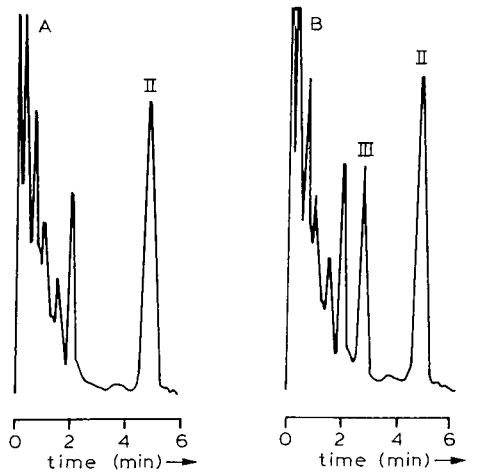


Fig. 3. Chromatograms of (A) control urine with internal standard (peak II) and (B) urine spiked with 3 μg/ml M-I (peak III) and internal standard (peak II).

with a mean value of 85.1 ± 4.2% (n = 4). The lowest measurable concentration in urine is 0.02 μg/ml. In comparison to the method of Kondo et al. [6] for this metabolite it can be concluded that the present ion-pair HPLC method is highly selective, and no derivatization step is required. However, the detection limit is somewhat higher (20 ng/ml urine vs. 5 ng/ml) and precision less (5.0% vs. 1.9%).

Photostability of nifedipine

In Fig. 4 the decomposition rate of nifedipine is shown in organic solvents, as well as in plasma. The half-life of nifedipine decompositon was 15 min in organic solvents and 44 min in plasma, which indicates that also in plasma oxidation of nifedipine takes place relatively rapidly in light, so that the whole assay procedure has to be carried out while excluding the light.

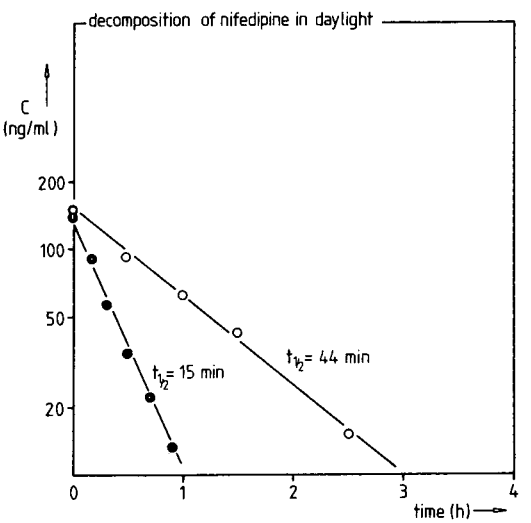


Fig. 4. Decomposition of nifedipine exposed to daylight in organic solvent (•) and plasma (○).

Human experiments

A representative plasma concentration—time curve is shown in Fig. 5. The concentrations of nifedipine could be fitted to an open two-compartment model, using weighted non-linear-regression analysis. Pharmacokinetic data are summarized in Table I. After stopping the infusion a rapid distribution phase ($t_{½}$, λ_1 = 10 ± 6 min) was followed by a first-order process of elimination ($t_{½}$, λ_2 = 106 ± 24 min). The volume of distribution at steady-state (V_{ss}) was 0.8 ± 0.2 l/kg, which indicates extensive tissue distribution, when considering the high extent of plasma protein binding of about 95—98% [14]. Mean systemic clearance was 0.55 ± 0.12 l/min, which implies that the rate of systemic drug elimination is not only dependent on drug metabolizing enzyme activity, but

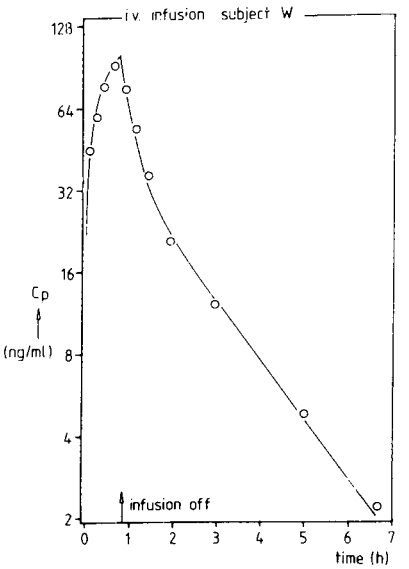


Fig. 5. Plasma concentration versus time profile of nifedipine in a healthy subject (W) following intravenous infusion for 50 min (dose 4.5 mg).

TABLE I

PHARMACOKINETIC DATA* OF NIFEDIPINE FOLLOWING INTRAVENOUS INFUSION

Subject	Age (years)	$t_{½,\lambda_1}$ (min)	$t_{½,\lambda_2}$ (min)	Cl (l/min)	V_{ss} (l/kg)	M-I (percentage dose per 24 h)
L	27	6	126	0.61	0.99	46.2
S	35	20	120	0.35	0.55	58.3
W	46	11	82	0.54	0.58	79.2
T	50	4	77	0.69	0.75	62.5
B	54	10	125	0.55	1.05	64.3
Mean	42	10	106	0.55	0.78	62.1
S.D.	11	6	24	0.12	0.23	11.9

*$t_{½,\lambda_1}$ = half-life of distribution; $t_{½,\lambda_2}$ = half-life of elimination; Cl = total plasma cleareance; V_{ss} = volume of distribution at steady-state.

also on hepatic blood flow. These results are in close agreement with the data reported by Rämsch [13].

The 24-h urinary excretion of M-I amounted to approximately 60% of the dose; Horster et al. [3] recovered about 65% of the dose with ^{14}C-labelled nifedipine after a bolus injection; the other part had been excreted in bile. This indicates that relatively small amounts are excreted in urine as other metabolites, since it has been shown by Kondo et al. [6] that unchanged nifedipine and/or p-I are excreted in urine in trace amounts (0.1% of an oral dose). Urinary excretion of M-I is also in agreement with the findings of Kondo et al. [6].

CONCLUSIONS

The phase systems described in this paper for the determination of nifedipine in plasma and its main metabolite (M-I) in urine allows their rapid, sensitive and selective quantitative analysis without interference from endogenous compounds, metabolites or photodegradation products. The described chromatographic procedure can be performed automatically, which permits the relatively rapid assay of many samples of nifedipine. The method is suitable for pharmacokinetic studies in man; drug elimination from plasma can be followed for 3—4 times its half-life following therapeutic dosages of nifedipine. It was also clearly shown in the present study that nifedipine preparations and plasma samples need to be handled without exposure to daylight.

ACKNOWLEDGEMENTS

The authors wish to thank H.C.N. Kappelle-de Vries for typing the manuscript and Drs. W. Onkenhout for producing the mass spectra. The study was supported in part by a grant from Bayer (Wuppertal, F.R.G.).

REFERENCES

1 M. Guazzi, M.T. Olivari, A. Polese, C. Fiorentini, F. Magrini and P. Moruzzi, Clin. Pharmacol. Ther., 22 (1977) 528.
2 K. Hashimoto, T. Kobayashi and E. Kimura (Editors), Nifedipine "Adalat", 1st International Symposium Tokyo, 1973, Bayer Yakuhin Ltd., Osaka, 1975.
3 F.A. Horster, B. Duhm, W. Maul, H. Medenwald, K. Patschke and L.A. Wegner, Arzneim.-Forsch., 22 (1972) 330.
4 R. Testa, E. Dolfini, C. Reschiotto, C. Secchi and P. Biondi, Farm. Ed. Pract., 34 (1979) 463.
5 H. Medenwald, K. Schlossmann and C. Wünsche, Arzneim.-Forsch., 22 (1972) 242.
6 S. Kondo, A. Kuchiki, K. Yamamoto, K. Akimoto, K. Takahashi, N. Awata and J. Sugimoto, Chem. Pharm. Bull., 28 (1980) 1.
7 P. Jakobsen, O. Lederballe Pedersen and E. Mikkelsen, J. Chromatogr., 162 (1979) 81.
8 J. Dokladalova, J.A. Tykal, S.J. Coco, P.E. Durkee, G.T. Quercia and J.J. Korst, J. Chromatogr., 231 (1982) 451.
9 S. Higuchi and J. Shiobara, Biomed. Mass Spectrom., 5 (1978) 220.
10 K.D. Rämsch, personal communication.
11 P. Pietta, A. Rava and P. Biondi, J. Chromatogr., 210 (1981) 516.
12 P.R. Bach, Clin. Chem., 29 (1983) 1344.
13 K.D. Rämsch, Schwerpunkt Med., 47 (1981) 55.
14 H. Rosenkranz, K. Schlossmann and W. Scholtan, Arzneim.-Forsch., 24 (1974) 455.

Journal of Chromatography, 308 (1984) 217—227
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2084

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF PYRIMETHAMINE, SULFADOXINE AND ITS N^4-ACETYL METABOLITE IN SERUM AND URINE AFTER INGESTION OF SULDOX

CARSTEN MIDSKOV

Biological Department, A/S Dumex (Dumex Ltd.), 37 Prags Boulevard, DK 2300 Copenhagen S (Denmark)

(First received October 24th, 1983; revised manuscript received January 19th, 1984)

SUMMARY

A sensitive and selective reversed-phase high-performance liquid chromatographic assay has been developed to determine the concentration of pyrimethamine, sulfadoxine and N^4-acetylsulfadoxine in serum and urine after oral administration of the antimalarial remedy Suldox®. Hitherto the literature describes no method being able to quantitate all three compounds in these fluids.

The compounds are extracted successively from the same sample and subjected to liquid chromatography followed by ultraviolet detection (280 nm). Calibration curves were linear ($\overline{r^2}$ = 0.999; S.E.M. less than 3%; n = 10) in the range 0—300 μg/ml (sulfadoxine) and 0—1000 ng/ml (N^4-acetylsulfadoxine and pyrimethamine). The limits of quantitation for the latter compounds were as low as about 5 ng/ml and 1 ng/ml, respectively. At therapeutic serum concentrations of 30 μg/ml (sulfadoxine), 350 ng/ml (N^4-acetylsulfadoxine) and 120 ng/ml (pyrimethamine) an interassay reproducibility below 8% (relative standard deviation) was found for all three compounds.

The assay was evaluated in a pilot study and proved convenient for pharmacokinetic studies in man following oral co-administration of pyrimethamine and sulfadoxine.

INTRODUCTION

Sulfadoxine (SDP) and pyrimethamine (DCE) are both long-acting drugs inhibiting sequential steps in the biosynthesis and utilization of folic acid [1]; used together they act synergistically [1, 2]. Given as a single drug SDP has been used in the treatment of various infections [3—6]. DCE monotherapy has been used in the treatment of meningeal leukaemia [7, 8], malaria due to *Plasmodium falciparum* [9, 10] and against coccidiosis [11, 12]. The fact that the concurrent use of DCE and SDP causes a double blockade in the folate pathway has been beneficial for the treatment and prophylaxis of protozoal

0378-4347/84/$03.00

diseases such as malaria tropica when caused by chloroquine- and/or pyrimethamine-resistant strains of *Plasmodium falciparum* [13—15].

Spectrophotometric methods were used for the analysis of both DCE [16, 17] and SDP [18, 19] until the late sixties. The first gas chromatographic method for DCE [20] dealt with its concentration in chicken tissue. Later, gas—liquid chromatographic (GLC) methods for DCE analysis in plasma were published [21–23]. The minimum concentration measurable in all cases was about 5 ng/ml. Disadvantageous to these techniques were a cumbersome procedure, a long time of chromatography [21] and the occurrence of ghost plasma peaks [22]. Two liquid chromatographic methods for DCE in plasma have been published [24, 25] and both signified simplifications of the procedure. A GLC method feasible for SDP in urine and whole blood was published in 1981 [23]; however, the chromatography involved a time-consuming temperature programming of the column oven. Some high-performance liquid chromatographic (HPLC) methods for sulphonamides in biological fluids have appeared [26—28], but to the authors knowledge none on SDP.

This publication describes a reversed-phase HPLC method to determine therapeutic concentrations of DCE, SDP and its N^4-acetyl metabolite (MAS) in plasma and urine after intake of Suldox® (Dumex). This antimalarial product contains DCE and SDP in the ratio of 1:20.

EXPERIMENTAL

Chemicals and reagents

SDP, MAS, DCE and the internal standards monobutyrylsulfadoxine (MBS) and *p*-aminopropionphenone (PAP) were supplied by the Synthesis Laboratory, Dumex. Acetonitrile was of HPLC grade and all other chemicals were of analytical grade.

Standard solution I: 10 mg of DCE and 10 mg of MAS were dissolved in 100 ml of methanol.

Standard solution II: 20 mg of SDP and 1 ml of standard solution I were added to 100 ml of methanol giving the concentrations 200 ng/μl SDP, 1 ng/μl MAS, 1 ng/μl DCE.

Internal standard solution I: 10 mg of MBS were dissolved in 100 ml of methanol.

Internal standard solution II: 10 mg PAP were dissolved in 100 ml of methanol.

Borate buffer (0.0125 *M*) resulted from the dissolution of 4.8 g of $Na_2B_4O_7 \cdot 10H_2O$ in 1 l of glass-distilled water, addition of 1.5 g of sodium hydroxide and final pH adjustment with 2 *M* sodium hydroxide to pH 10.0.

TBA reagent (0.25 *M*): 8.5 g of tetrabutylammonium hydrogen sulphate were dissolved in 100 ml of glass-distilled water and the pH was adjusted to 10.0 with 2 *M* sodium hydroxide

Sodium hydroxide (2 *M*): 8 g of sodium hydroxide were dissolved in 1 l of glass-distilled water.

Sodium phosphate buffer (0.1 *M*): 18.0 g of $Na_2HPO_4 \cdot 2H_2O$ were dissolved in 1 l of glass-distilled water and concentrated orthophosphoric acid was added to pH 4.0 or 5.4.

For hydrolysis of urine samples β-glucuronidase (EC 3.2.1.31) from Sigma was used.

Mobile phase for chromatography of SDP and DCE in serum and urine (mobile phase A): a mixture of equal volumes of acetonitrile and sodium phosphate buffer (0.1 *M*, pH 4.0)

Mobile phase for the chromatography of MAS in serum (mobile phase B): acetonitrile and sodium phosphate buffer (0.1 *M*, pH 5.4) were mixed (30:70, v/v).

Mobile phase for the chromatography of MAS in urine (mobile phase C): acetonitrile and sodium phosphate buffer (0.1 *M*, pH 5.4) were mixed (20:80, v/v).

The mobile phases were degassed before use by ultrasonication.

Chromatographic instrumentation

Liquid chromatography was performed on a Waters Model 6000A constant-flow solvent delivery system, a Waters U6K injection unit and a Waters Model 440 ultraviolet (UV) detector equipped with a 280-nm filter. The following 5 μm column packing materials were used: spherisorb S5 Phenyl, Spherisorb S5 Octyl and Spherisorb S5 ODS (Phase Separations, U.K.). The stainless-steel columns (Herbert Knauer, F.R.G.), 25 cm × 4 mm I.D., were packed using a downward slurry technique employing isopropanol and methanol. Detector response was monitored with a Hewlett-Packard Model 3380 integrator. A water-bath was used to maintain column temperatures of 20°C and 30°C.

Extraction from human serum and urine

The extractions of the three compounds (Fig. 1) were performed on the same sample. When internal standards for SDP and/or DCE were used, 80 μg of MBS (i.e. 800 μl of internal standard solution I) and/or 1500 ng of PAP (i.e. 15 μl of internal standard solution II) were added to 1 ml of the intact sample.

Pyrimethamine (DCE). A 1-ml sample was added to 3 ml of borate buffer (pH 10) and mixed. Then 5 ml of diethyl ether were added and after shaking for about 10 sec and centrifugation, 4 ml of the supernatant were transferred to a conical tube and evaporated to dryness under nitrogen. The residue was dissolved in 100 μl of mobile phase A and 40 μl were injected on to the column.

Sulfadoxine (SDP) and N^4-acetyl sulfadoxine (MAS). The aqueous phase remaining from the ether extraction of DCE was subjected to a nitrogen jet stream to evaporate residual diethyl ether. Then 1 ml of TBA reagent was admixed, thus using the tetrabutylammonium ion as an ion-pairing agent. After the addition of 5 ml of dichloromethane the tube was rotated for 3 min and centrifuged at 10°C. The aqueous supernatant was then removed using a pasteur pipette. At this point the procedure divided into separate routes for MAS and SDP. (1) A 3-ml portion of the remaining organic phase was transferred to a conical tube and evaporated with a nitrogen jet stream. The residue was dissolved in 100 μl of mobile phase B (serum) or mobile phase C (urine); 40 μl were injected on to the column for analysis of MAS. (2) From the remaining organic phase 20 μl (serum) or 200 μl (urine) were transferred to conical tubes. After evaporation with nitrogen the residue was redissolved in 200 μl of mobile phase A and 40 μl were injected on to the column for analysis of SDP.

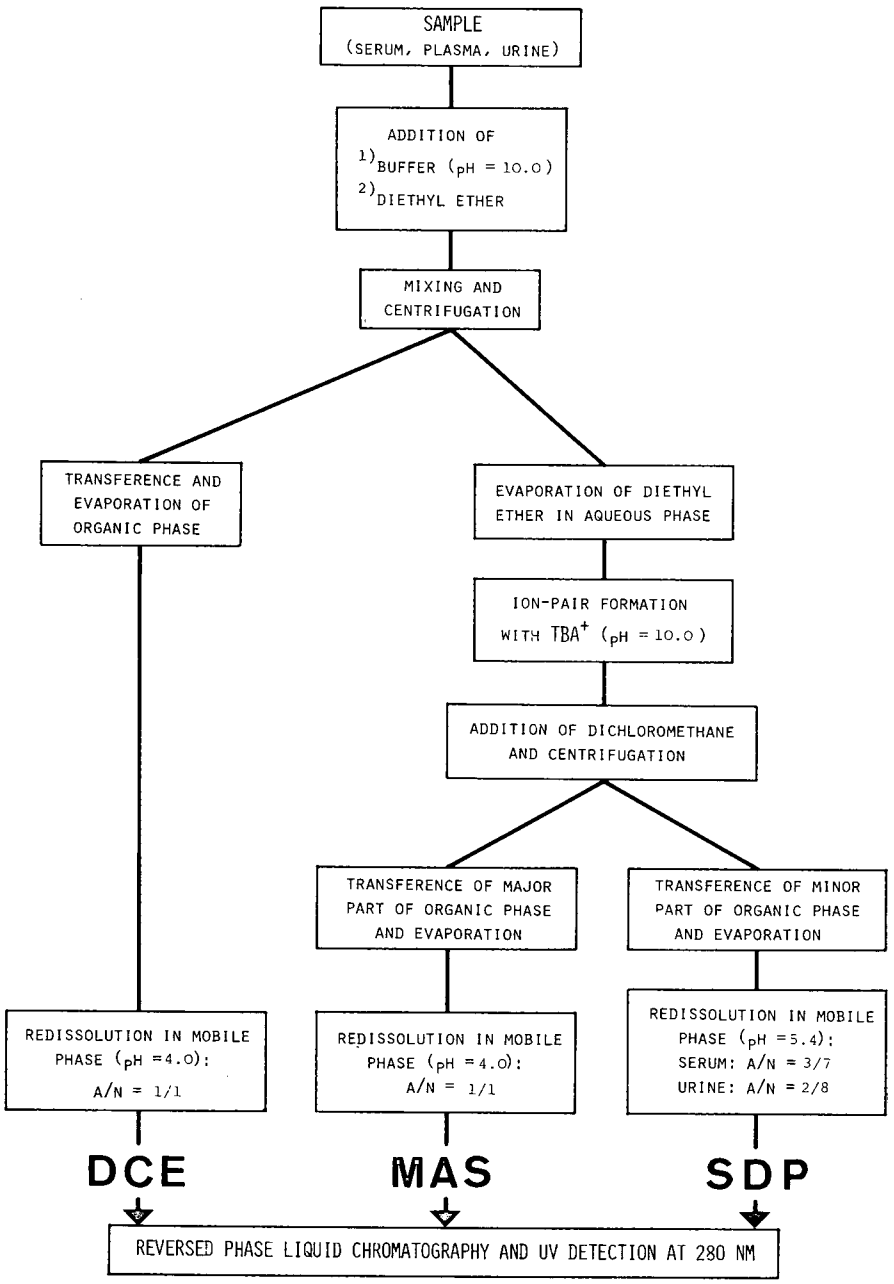


Fig. 1. Flow-chart of analytical procedures to quantitate sulfadoxine (SDP), N^4-acetylsulfadoxine (MAS) and pyrimethamine (DCE) in biological fluids. TBA^+ = tetrabutylammonium ion; A/N = volume ratio of acetonitrile and sodium phosphate buffer.

Liquid chromatography

The different mobile phases used were delivered through Spherisorb S5 ODS at a flow-rate of 1 ml/min giving in all cases a pressure drop of about 100 bar per 25 cm. The temperature of the column and of the solvent was thermo-

statically maintained at 20°C, except for chromatography of MAS in urine for which the temperature was 30°C.

Calibration curves

Standard samples were prepared by evaporating 0—1500 μl of standard solution II and volumes of the internal standard solutions I and II as mentioned above. To these residues were added 1 ml of drug-free urine or serum and these samples were then subjected to the procedure described above. The calibration curves were obtained by plotting the peak area ratios for DCE/PAP and SDP/MBS against concentration; for MAS the peak area was used. The curves were calculated according to linear regression by means of the method of least squares.

Quality control

To estimate the precision and accuracy of the analyses, quality control samples were prepared and analysed along with the biological samples. The pools were prepared by addition of the analytes to human blank serum and following gentle shaking the mixture was dispensed into tubes in 1-ml aliquots and stored at −20°C.

RESULTS AND DISCUSSION

Chromatographic conditions

Reversed-phase mode was preferred because a low retention of MAS relative to SDP was desired. This was considered beneficial for the detection sensitivity in that the plasma concentration of MAS was several orders of magnitude lower than that of SDP. C_8 and C_{18} bonded phases proved to be equally useful for the chromatography and an acceptable column performance was observed for about 600 succesive injections.

The components of the mobile phase were finally settled as acetonitrile and phosphate buffer. During the development work ion-pair chromatography for SDP and MAS was also tried with tetrabutylammonium hydrogen as the ion-pairing agent, but this was unsatisfactory regarding peak shapes. For the chromatography of MAS in urine the temperature of the mobile phase was increased from 20°C to 30°C to provide the required lower retention of endogenous urinary constituents relative to MAS.

The analysis of DCE in both serum and urine was selective and no peaks suspected of being metabolite peaks appeared. However, during the chromatography of about 600 serum samples including standards a small peak cluster was sometimes observed shortly after the peak of DCE. Occasionally it partly overlapped the peak of DCE (Fig. 2); thus peak heights were used rather than areas in the calculation of concentration.

Examples of typical serum and urine tracings are shown in Figs. 2 and 3. The capacity ratios (and theoretical plate numbers) were as follows: SDP, 3.5 (6000); MBS, 5.0 (5000); MAS (serum), 5.1 (6100); MAS (urine), 8.8 (5900); DCE, 7.0 (4300); PAP, 5.4 (4500). The peak resolution of SDP and MBS could be calculated as 1.8 from the serum chromatograms (Fig. 2).

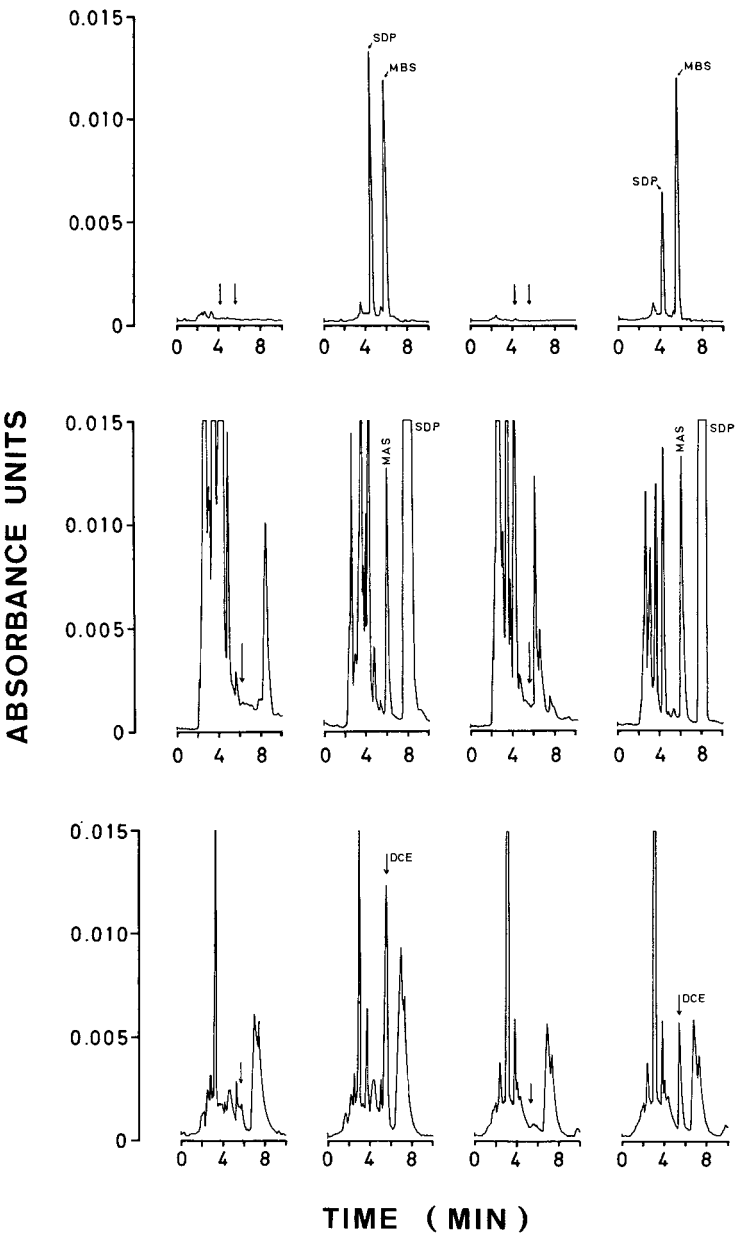


Fig. 2. Chromatograms of human serum extracts of 1-ml samples. UV detection at 280 nm. The arrows indicate retention times of the analytes. Each panel shows the following tracings (from left to right): blank serum standard, serum standard, volunteer serum prior to dosing, volunteer serum collected after a single oral dose of Suldox (25 mg DCE and 500 mg SDP). Peaks in the non-blank standard and volunteer serum tracings represent in the upper panel: 40 μg SDP and 55 μg MBS (standard), 18 μg SDP and 55 μg MBS (volunteer serum); central panel: 80 ng MAS (standard) and 85 ng MAS (volunteer serum); lower panel: 80 ng DCE (standard) and 30 ng DCE (volunteer serum).

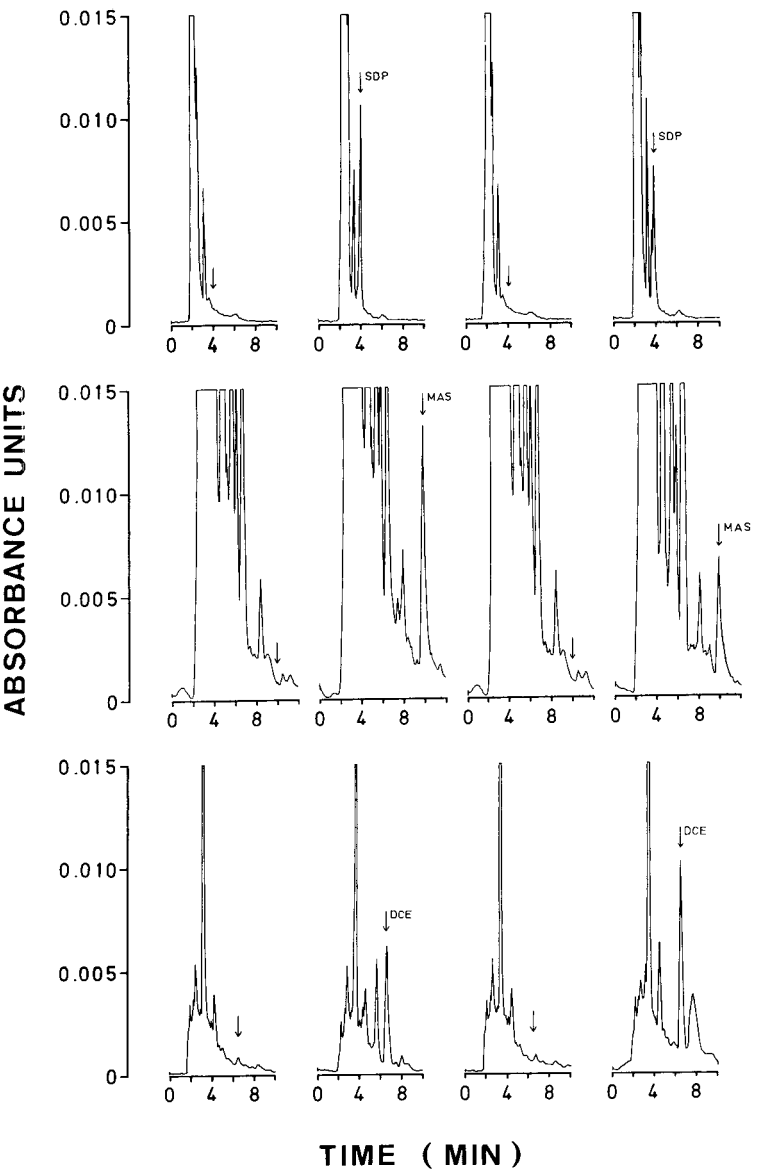


Fig. 3. Chromatograms of human urine extracts of 1-ml samples. UV detection at 280 nm. The arrows indicate retention times of the analytes. Each panel shows the following tracings (from left to right): blank urine standard, urine standard, volunteer urine collected after a single oral dose of Suldox (25 mg DCE and 500 mg SDP). Peaks in the non-blank standard and volunteer urine tracings represent in the upper panel: 40 μg and 28 μg SDP; central panel: 80 ng and 35 ng MAS; lower panel: 40 ng and 60 ng DCE.

Choice of internal standards

Internal standards for SDP and DCE were chosen as mentioned above. If a smaller amount of MBS was added it could apply for MAS instead of SDP. The internal standard for DCE (PAP) was sometimes not satisfactory because

of interfering constituents in serum and urine (Figs. 2 and 3). This interference amounted to about 5% of about 600 samples from twenty subjects. As a consequence PAP was excluded from routine work. The internal standard for SDP (MBS) was used throughout for analysis of serum. For SDP in urine, however, the applicability of MBS was verified but not used routinely.

Choice of extraction procedures

The final extraction procedure (Fig. 1) utilized a priori diethyl ether extraction of DCE from the alkalinized samples and ion-pair extraction of SDP and MAS from the remainder. It was found that addition of 85 mg of TBA dissolved in 1 ml of water was sufficient for optimum ion-pair formation as calculated per 1 ml sample and in the presence of the internal standard MBS as specified above (Experimental).

Extraction yields were assessed by injection of known amounts of the analytes on to the column and similarly for processed samples. The ratio of the slopes of these two linear correlations was used as an estimate of the extraction recovery for each analyte. It appeared that the extraction yields from urine were fully quantitative for MAS, MBS, PAP and DCE. Urine SDP was extractable to 83%. A lowering of the extraction yields resulted when serum concentrations were examined: 67% for SDP, 94% for MAS and MBS, 96% for PAP, and 90% for DCE. All extraction yield experiments were processed in quadruplicate and the S.E.M. was in all cases less than 3%.

Quantitation and reliability of the analytical results

Linearity of the calibration curves were established as described above in the concentration range 0—300 μg/ml (SDP) and 0—1000 ng/ml (MAS, DCE). For all three compounds a determination coefficient was found $\bar{r}^2 = 0.999$ ($n = 10$) with S.E.M. less than 3%. The ordinate intercepts did not deviate significantly from zero. The observed S.D. for the three analytes in the serum pools was in all instances within 8% of the mean ($n = 10$). The mean concentrations were 30 μg/ml (SDP), 350 ng/ml (MAS) and 120 ng/ml (DCE), i.e. within the therapeutic concentration range found (see below). An increase of the inter-assay reproducibility was seen when the internal standards were excluded from the calculations.

Limits of quantitation

The methods described for MAS and DCE had lower limits of quantitation of about 5 ng/ml (MAS) and 1 ng/ml (DCE). These limits resulted from optimization of the volume taken in the extraction as well as the volume injected on to the column. The reproducibilities of the assays seemed unaffected by such adjustments. A signal-to-noise ratio of about 2:1 was observed for these minimum quantifiable concentrations. Such a limit for the analysis of SDP was not relevant because the therapeutic range was several orders of magnitude above this limit.

Storage of urine and serum samples

Samples from the pilot study (see below), drug-free serum for standards and quality control pools were stored for about six months at −20° C and prior to

analysis 1—2 h at ambient temperature. This mode of storage was observed to be without significant detriment to the stability of the drugs. In addition no deterioration was noticeable for serum and urine samples undergoing two freeze—thaw cycles.

Analytical interferences

None of the following drugs interfered with the determination of SDP, MAS and DCE in serum or urine: acetylsalicylic acid, diazepam, quinine, chloroquine.

Application of the methods

Serum and urine samples from a 39-year-old female volunteer were successfully applied to demonstrate the validity of the methods. The samples were

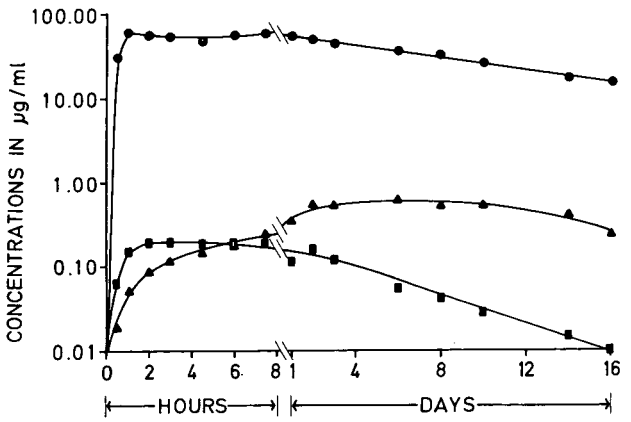


Fig. 4. Serum concentration profiles of the three analytes studied obtained following a single oral administration of one tablet of Suldox (25 mg DCE and 500 mg SDP) to a 39-year-old female volunteer. (•), SDP; (▲), MAS; (■), DCE.

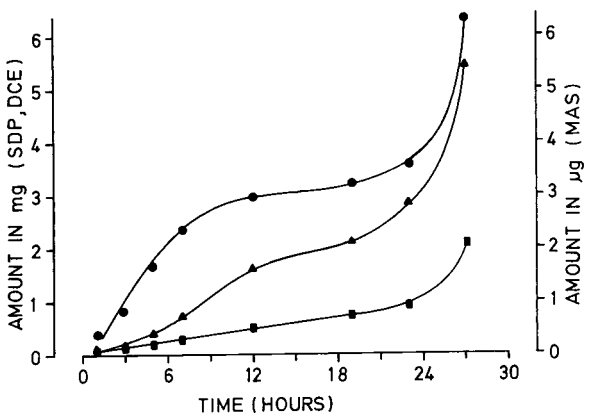


Fig. 5. Cumulative urinary excretion profiles of the three analytes studied obtained following a single oral administration of one tablet of Suldox (25 mg DCE and 500 mg SDP) to a 39-year-old female volunteer. The times of the data points are the midpoints of the collection time periods. (•), SDP; (▲), MAS; (■), DCE.

taken after oral administration, in a fasting state, of one tablet of Suldox containing 25 mg of DCE and 500 mg of SDP. The serum results for DCE, SDP and MAS (Fig. 4) show elimination half-lives from the circulation of about 100 h (DCE) and 190 h (SDP). The findings agree with the sparse pharmacokinetic data available in the literature on DCE [24, 29] and SDP [30]. Hitherto, lack of an analytical method has precluded knowledge about plasma levels of MAS.

Fig. 5 shows the accumulated urinary excretions. The data points are plotted in the midpoints of the collection time periods. It is evident that the excretion rates still increase at the time of the last data points. Within the collection period of 31 h about 1% of SDP and about 8% of DCE was recovered in the urine. The latter results compare well to a previous finding in these laboratories using a gas chromatographic method [22]. The urine samples were subjected to enzymatic hydrolysis with β-glucuronidase. Analysis of the hydrolysed samples showed the presence of at most 5% SDP and MAS glucuronides in the urine.

No comparisons to other estimations are feasible via the literature as regards the urinary excretion of SDP and MAS.

ACKNOWLEDGEMENTS

Mr. Thommy K. Nielsen is acknowledged for his unfailing efforts at all phases of the experimental and drawing work. Ms. Benthe Kold is thanked for her excellent secretarial work.

REFERENCES

1 M.G.D. Hurly, Trans. Roy. Soc. Trop. Med. Hyg., 53 (1959) 412.
2 G.B. Elion, S. Singer and G.H. Hitching, J. Biol. Chem., 208 (1954) 1977.
3 L. Weinstein, M.A. Madoff and C.A. Samet, N. Engl. J. Med., 263 (1960) 793.
4 J.C. Mihindukulasuriya, N. Velauthapillai and N.D. Lionel, Ceylon Med. J., 21 (1976) 201.
5 P. Saint-Andre, C. Ferracci, G. Baquillon and M. Travi Piacenti, Acta Leprol. (Switzerland), 76 (1979) 315.
6 O. Bedir, O. Ilter and S.B. Tumay, Monatsschr. Kinderheilkd., 122 (1974) 407.
7 J. Armata, R. Cyklis and W. Borkowski, Cancer, 42 (1978) 1216.
8 C. Smyth and P.H. Wiernik, Clin. Pharmacol. Ther., 19 (1976) 240.
9 L.H. Schmidt, Amer. J. Trop. Med. Hyg., 27 (1978) 703.
10 L.H. Schmidt, R. Crosby, J. Rasco and D. Vaughan, Antimicrob. Agents Chemother., 14 (1978) 292.
11 H.D. Chapman, J. Comp. Pathol., 92 (1982) 213.
12 J.S. Trier, P.C. Moxey, E.M. Schimmel and E. Robles, Gastroenterology, 66 (1974) 923.
13 I. Ebisawa, T. Muto, S. Kameko and G. Mitsu, Jap. J. Exp. Med., 40 (1970) 151.
14 E.J. Pearlman, R.M. Lampe, W. Theimanun and R.S. Kennedy, Amer. J. Trop. Med. Hyg., 26 (1977) 1108.
15 B. Simpson, W.S. Jamieson and A.H. Dimond, Trans. Roy. Soc. Trop. Med. Hyg., 66 (1972) 222.
16 R.L. DeAngelis, W.S. Simmons and C.A. Nichol, J. Chromatogr., 106 (1975) 41.
17 C.R. Jones and L.A. King, Biochem. Med., 2 (1968) 251.
18 A.C. Bratton and E.K. Marshall, J. Biol. Chem., 128 (1939) 537.
19 J. Rieder, Chemotherapy, 17 (1972) 1.
20 P.C. Cala, N.R. Trenner, R.P. Buhs, G.V. Downing, Jr., J.L. Smith and W.J.A. VandenHeuvel, J. Agr. Food Chem., 20 (1972) 337.

21 C.R. Jones, P.R. Ryle and B.C. Weatherby, J. Chromatogr., 224 (1981) 492.
22 C. Midskov, J. Chromatogr., 306 (1984) 388.
23 M. Bonini, F. Mokofio and S. Barazi, J. Chromatogr., 224 (1981) 332.
24 C.R. Jones and S.M. Ovenell, J. Chromatogr., 163 (1979) 179.
25 U. Timm and E. Weidekamm, J. Chromatogr., 230 (1982) 107.
26 K. Harzer, J. Chromatogr., 155 (1978) 399.
27 V. Ascalone, J. Chromatogr., 224 (1981) 59.
28 O. Spreux-Varoquax, J.P. Chapalain, P. Cordonnier, C. Advenier, M. Pays and L. Lamine, J. Chromatogr., 274 (1983) 187.
29 R.A. Ahmad and J.J. Rogers, Brit. J. Clin. Pharmacol., 10 (1980) 519.
30 W.A. Ritschel, G.V. Hammer and G.A. Thompson, Int. J. Clin. Pharmacol., 16 (1978) 395.

Journal of Chromatography, 308 (1984) 229—239
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2086

DETERMINATION OF PROGABIDE AND ITS MAIN ACID METABOLITE IN BIOLOGICAL FLUIDS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ELECTROCHEMICAL DETECTION

APPLICATION TO THE MEASUREMENT OF BLOOD/PLASMA PARTITION RATIO

P. PADOVANI, C. DEVES, G. BIANCHETTI, J.P. THENOT*,* and P.L. MORSELLI

LERS-Synthélabo, 58 rue de la Glacière, 75103 Paris (France)

(First received August 31st, 1983; revised manuscript received January 23rd, 1984)

SUMMARY

A method for the measurement in plasma, blood and urine of progabide, its main acid metabolite, and the corresponding benzophenone is described. This assay allows the determination of progabide and its acid metabolite for therapeutic drug monitoring, and with a minimum detectable concentration of 1—10 ng/ml for progabide and its acid metabolite, it is sensitive enough for pharmacokinetic studies. Progabide and its metabolites are extracted from biological samples with toluene at pH 4.5. Following reduction of the imine bond with sodium borohydride, the reduced drugs are back-extracted into an aqueous phase at acid pH and reextracted by diethyl ether at alkaline pH.

Progabide, its acid metabolite and the benzophenone are separated by high-performance liquid chromatography using a 3-μm ODS column with a quaternary solvent mixture of methanol—acetonitrile—phosphate buffer (0.033 *M*, pH 5.5)—sodium chloride (1.5 *M*) (30:30:40:9, v/v), and detected electrochemically at a potential of +850 mV vs. an Ag/AgCl electrode.

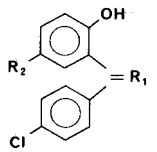
Antiepileptic drugs like carbamazepine, carbamazepine epoxide, phenytoin, valproic acid and ethosuximide do not interfere with the assay. Blood/plasma partition ratios of 0.69 and 0.55 for progabide and its acid metabolite, respectively, indicate that the former but not the latter is present in red blood cells.

INTRODUCTION

Progabide (Fig. 1, I), a new γ-aminobutyric acid (GABA) mimetic compound, which has been shown to possess a large spectrum of antiepileptic activity in

*Correspondence address: LERS-Synthélabo, 23—25 av. Morane Saulnier, 92360 Meudon la Foret, France.

0378-4347/84/$03.00



	R_1	R_2	
I	$N(CH_2)_3CONH_2$	F	PROGABIDE
II	$N(CH_2)_3$-COOH	F	PGA
III	$N(CH_2)_3CONH_2$	Cl	INTERNAL STANDARD 1
IV	O	F	BENZOPHENONE
V	O	Cl	INTERNAL STANDARD 2

Fig. 1. Structure of progabide, its metabolites and the internal standards.

resistant epileptic patients [1—3], is metabolized to other GABA agonists: progabide acid metabolite (PGA), gabamide and GABA (Fig. 1). PGA exerts the same pharmacological activity as the parent compound.

Progabide undergoes rapid hydrolysis into benzophenone in aqueous solution at acidic pH and in methanolic solution. The instability of the imine bond presents the main difficulty in the quantification of the drug. Hence, great care must be taken in the collection of the blood or plasma samples, and it is necessary to prevent the hydrolysis of the imine bond occurring naturally during the extraction and work-up procedure. This is made possible by reducing the double bond with sodium borohydride, as described by Yonekawa et al. [4]. The stable reduced compounds can then be purified by back-extraction in acidic conditions. However, Yonekawa's procedure, developed for plasma samples, required modifications for the determination of progabide in blood.

The present paper describes a method for the quantification of progabide, its acid metabolite (PGA) and, if needed, the benzophenone, in plasma, blood and urine with an optimized chromatographic separation under reversed-phase conditions. It also describes the determination of the blood/plasma partition ratio of progabide and of its acid metabolite PGA.

EXPERIMENTAL

Chemicals

Sodium borohydride, sodium chloride, sodium acetate and sodium citrate, p.a. grade, were purchased from Merck (Darmstadt, F.R.G.). Methanol (UV grade) and diethyl ether (p.a. grade) were obtained from Carlo Erba (Milan, Italy). The latter solvent should be freshly distilled. Acetonitrile (HPLC grade) and toluene (p.a. grade) were purchased from Baker (Deventer, The Netherlands).

Progabide, 4-{[(4-chlorophenyl)(5-fluoro-2-hydroxyphenyl)methylene]-amino}butanamide, SL-75102, 4-{[(4-chlorophenyl)(5-fluoro-2-hydroxyphenyl)-methylene]amino}butyric acid and their internal standard, SL-78050, 4-{[(4-chlorophenyl)(5-chloro-2-hydroxyphenyl)methylene]amino}butan- amide, were provided by the LERS Chemistry Department.

Standard solutions

Progabide and its internal standard were dissolved in ethyl acetate at a concentration of 10 ng/μl, whilst the solution of PGA at the same concentration was prepared in methanol. These solutions were stable for one month when kept at −20° C.

Apparatus and conditions

The liquid chromatograph consisted of a Model 750 Micromeritics pump (Norcross, GA, U.S.A.), a Waters Wisp automatic injector (Milford, MA, U.S.A.) and a Kipp Analytica Model 9205 (Emmen, The Netherlands) coulometric detector.

Separations were carried out on a Hypersil ODS 3-μm (150 × 4.6 mm) reversed-phase column packed according to the technique described by Broquaire [5]. Chromatograms were recorded using a linear stripchart recorder; peak areas were measured by a Perkin-Elmer Model Sigma 10 integrator.

The mobile phase was prepared by diluting 400 ml of 0.033 *M* phosphate buffer pH 5.05 with 300 ml of methanol and 300 ml of acetonitrile. The ionic strength was adjusted by adding 90 ml of 1.5 *M* sodium chloride. The mobile phase was adjusted to a flow-rate of 1.00 ml/min through the column, which was maintained at a constant temperature of 54° C using a water bath.

The column effluent was monitored electrochemically at an oxidation potential of +850 mV vs. an Ag/AgCl reference electrode.

Assay procedure

In the extraction procedure (Fig. 2), 1 ml of plasma, blood, or urine was added to a conical tapered tube containing 50 μl of a methanolic solution of internal standard (Fig. 1, III) (10 ng/μl) and 0.5 ml of 2 *M* acetate buffer pH 4.5. The mixture was extracted with toluene (8 ml) on a rotary shaker for 20 min. The two phases were then separated by centrifugation at 4° C (1000 *g* for 10 min).

A 0.5-ml volume of 0.5% sodium borohydride in ethanol was added to the organic phase. The mixture was vigorously shaken on a vortex mixer and the reduction proceeded during 20 min at room temperature. The reduced drugs were back-extracted for 20 min with 2 ml of 0.25 *M* citrate buffer pH 1.8. The organic layer contained the reduced benzophenone.

The pH of the aqueous phase was adjusted in the range 6.5—7.7 by adding 200 μl of 5 *M* sodium hydroxide and 500 μl of 1 *M* citrate buffer pH 7.7. This mixture was then extracted for 20 min with 7 ml of freshly distilled diethyl ether, and the organic phase was evaporated to dryness at 37° C under a gentle stream of nitrogen.

Following dissolution with 200 μl of methanol—0.015 *M* phosphate buffer pH 7.1 (4:6, v/v), the solution was transferred to an injection vial, and 100 μl were injected into the liquid chromatograph.

In order to quantify the benzophenone with its precursors (progabide, PGA) in one single chromatographic run, the organic extracts containing the reduced compounds were pooled prior to the HPLC separation (Fig. 2). The accuracy of the determination was improved by adding a second internal standard (Fig. 1, V) to blood samples prior to extraction.

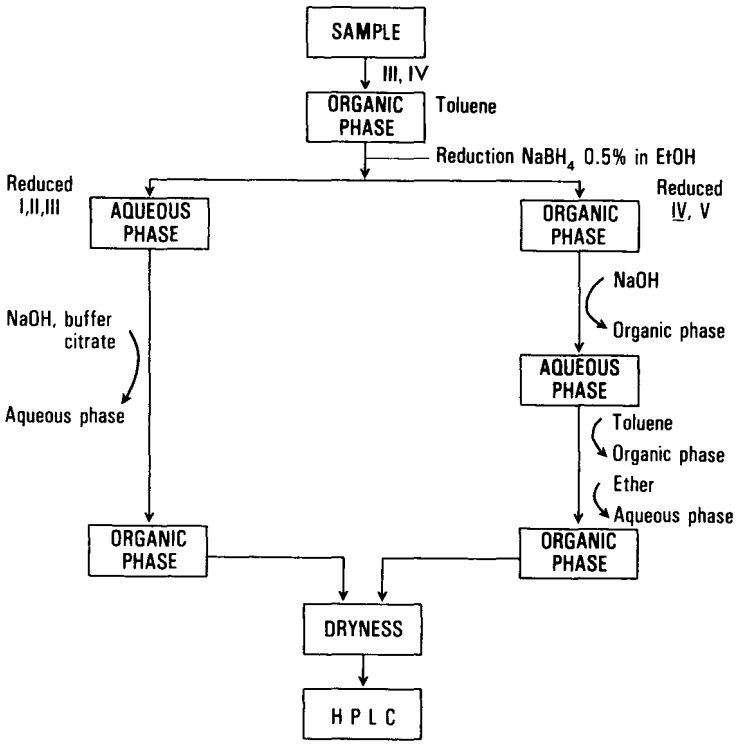


Fig. 2. Flow-chart of the extraction and reduction of progabide (I), its acid metabolite (II), and their corresponding benzophenone (IV). Compounds III and V are internal standards (see Fig. 1).

Peak area ratios progabide/internal standard, and PGA/internal standard were used to construct standard curves. The slope, intercept and correlation coefficient were determined by linear least-squares regression analysis.

Blood/plasma partition ratio

In order to determine the distribution ratio of progabide and PGA between blood and plasma, 600 mg of progabide were administered orally to three healthy volunteers (mean age 35 ± 8.7 years, mean body weight 60.3 ± 6.2 kg). Blood (15 ml) was collected at 0, 1, 2, 4, 6, and 8 h following drug administration. Half of the blood sample was immediately frozen, the second half was centrifuged and the plasma collected. Progabide and PGA were determined in blood and plasma, and the concentration ratio experimentally measured.

The relationship between blood and plasma concentrations is $C_b = C_p(1 - H) + C_eH$, where C_b, C_p and C_e are respectively blood, plasma and erythrocytes concentrations, and H is the haematocrit. For a haematocrit mean value of 0.45, C_b/C_p is superior or equal to 0.55. Only when $C_e = 0$ does C_b/C_p equal 0.55.

RESULTS

Stability

The stability of progabide and PGA was studied at 37° C. As shown in Fig. 3, the half-lives of progabide and PGA were 3.8 and 14.4 h, respectively. This indicates that it would be preferable to determine progabide in blood rather than in plasma samples in order to eliminate possible artefacts during the centrifugation step. From a clinical viewpoint it is also more practical to freeze blood immediately following sampling.

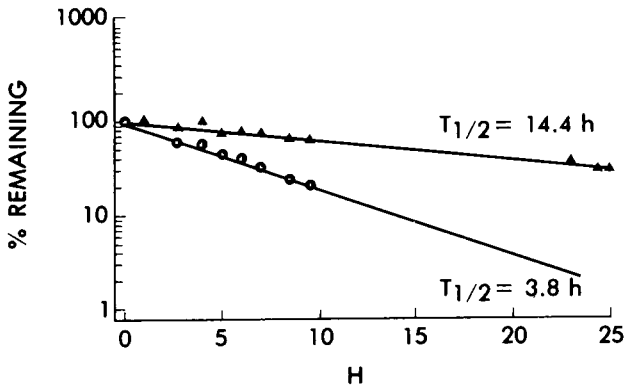


Fig. 3. Stability of progabide (•) and PGA (▲) in human blood at 37° C.

Extraction and reduction

Extraction curves for non-reduced compounds are shown in Fig. 4A and those of the reduced compounds in Fig. 4B. The pH value chosen for the initial extraction was in the pH range in which progabide is the most stable.

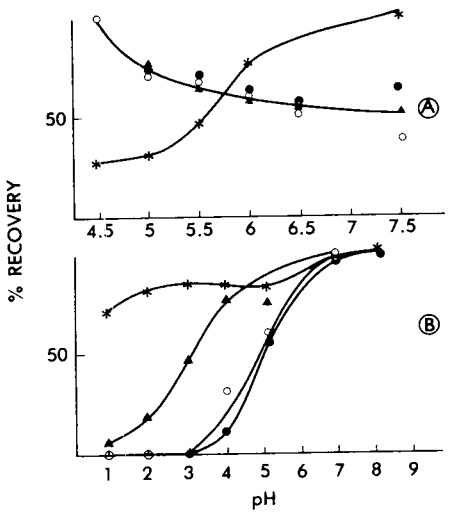


Fig. 4. Plots of percentage recovery vs. pH of (A) non-reduced compounds, and (B) reduced compounds. Recovery of progabide (•), PGA (▲), the internal standard 1 (○) and benzophenone, IV (*) as a function of pH (A) before and (B) after reduction with sodium borohydride.

Many solvents were tested for the extraction process, and diethyl ether proved to be the best for the non-reduced compounds; unfortunately it also extracted too many endogenous compounds to be useful, and the best compromise was provided by toluene. For the reduced compounds a more polar solvent was necessasry and diethyl ether gave the best recovery.

The influence of sodium borohydride reduction time was investigated and it was observed that yields, expressed as peak heights, were optimal for a 10—20 min reduction time, then decreased in a parabolic manner. However, peak height ratios between progabide, PGA and the benzophenone remained constant with time.

Chromatographic separation

The chromatogram obtained by high-performance liquid chromatography (HPLC) of unreduced progabide showed a large tailing peak. A marked improvement of the chromatographic properties was observed after reduction of the imine bond, but a simple binary mobile phase was not suitable to obtain a good separation of all the compounds. Thus each chromatographic parameter was investigated, and the solvent composition was optimized by the chromatographic resolution function introduced by Kaiser [6] using a two-factorial analysis.

TABLE I

OPTIMIZATION OF SOLVENT COMPOSITION — CHROMATOGRAPHIC RESOLUTION FUNCTION VALUES

KH_2PO_4 molarity			pH	NaCl molarity				CH_3CN (%)
0.01	0.033	0.1		0	0.041	0.082	0.1239	
—0.019	—100.0	—100.0	4.5	—0.233	—0.729	—0.1023	—0.0173	0
—0.116	—0.183	—0.105	5.2	—0.8493	—100	—0.9038	—0.0830	15
—100.0	—2.470	—100.0	5.9	—100.0	—0.771	—0.1431	—0.0841	30

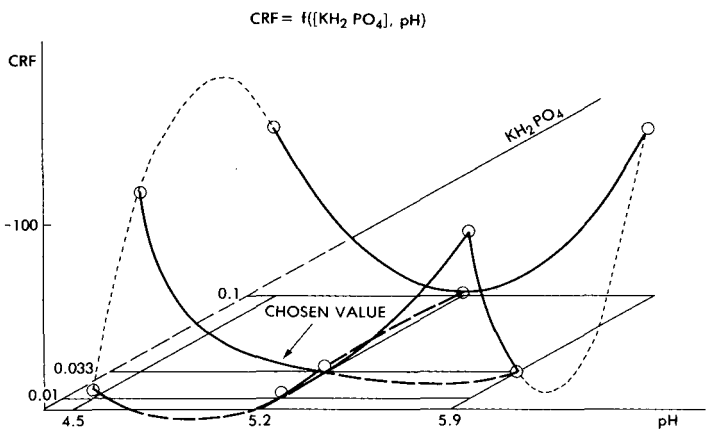


Fig. 5. Chromatographic resolution function (CRF) as a function of pH and molarity of phosphate buffer in the mobile phase.

The first step was optimization of the pH of the solution and the molarity of phosphate buffer; the second was to optimize the acetonitrile—methanol and the sodium chloride molarity. No mathematical development was used and the surface equation near the optimum was not calculated; thus, only qualitative results could be obtained. However, using the results from Table I, parabolic and cubic regressions were determined to draw Figs. 5 and 6. The chosen values were the experimental values near the surface minimum. It can be seen (Figs. 5 and 6) that the two minima were close to pH 5.1, 0.033 *M* KH_2PO_4, 30% acetonitrile, and 0.083 *M* sodium chloride.

Temperature had little effect on retention times of progabide and PGA, but benzophenone was greatly influenced by small variations of temperature. The results, illustrated in Fig. 7, indicate that the best resolution observed in the shortest analysis time was obtained at 54° C.

Fig. 8 shows a chromatogram of blood from a subject following administration of 900 mg of progabide. The retention times were, respectively, 3.1 min, 4.1 min and 5.9 min for PGA, progabide and their internal marker, and 5.0 and 7.0 min for benzophenone and its internal marker. The time interval between two injections was set at 8 min.

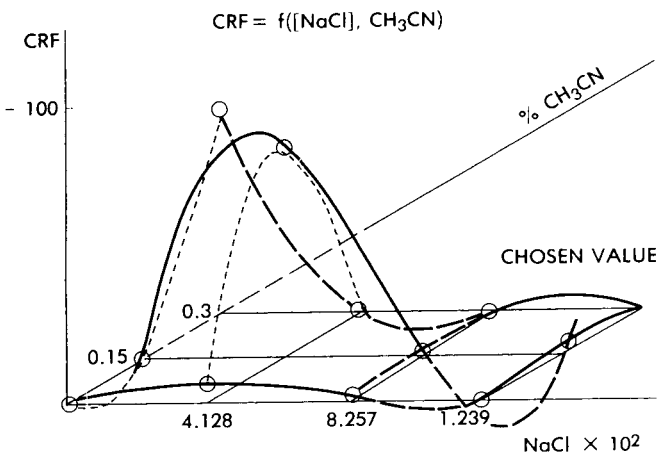


Fig. 6. Chromatographic resolution function as a function of sodium chloride molarity and percentage of acetonitrile in the mobile phase.

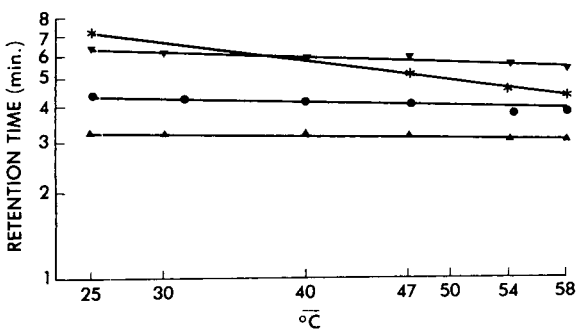


Fig. 7. Relationship between retention times of progabide (•), PGA (▲), their internal standard (▼) and benzophenone, IV (*), and the temperature of the mobile phase.

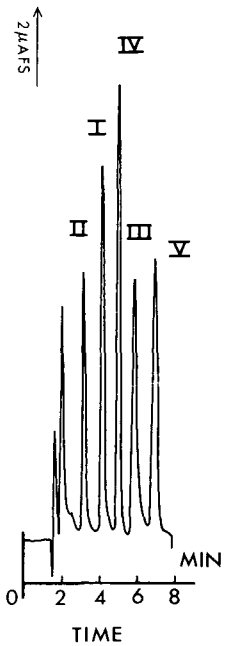


Fig. 8. Chromatogram obtained after extraction of blood following administration of 900 mg of progabide in one subject. Peaks: I = PG (559 ng); II = PGA (632 ng); III = internal standard 1 (500 ng); IV = benzophenone (500 ng); V = internal standard 2 (500 ng).

Assay linearity and precision

Calibration curves were constructed by analysing a series of blood samples of known progabide and PGA concentrations. Each data point was the mean of five measurements. The response was linear over the range 10—1500 ng/ml. Although the minimum detectable concentration was 1 ng/ml (three times the baseline noise), the practical limit was around 10 ng/ml.

TABLE II

REPRODUCIBILITY OF THE METHOD FOR THE MEASUREMENT OF PROGABIDE AND PGA AT DIFFERENT CONCENTRATIONS

	Spiked concentration (ng/ml)	No. of observations	Concentration observed (ng/ml)	Standard deviation (ng/ml)	C.V. (%)
Progabide	50	10	48	1.6	10
	250	5	259	2.5	2.2
	500	10	504	3.0	1.9
	750	5	745	4.0	1.2
	1000	10	999	2.9	0.9
PGA	50	10	51	1.3	8.0
	250	5	248	2.3	2.1
	500	10	499	2.6	1.7
	750	5	737	1.2	0.4
	1000	10	1008	2.2	0.7

The reproducibility and accuracy of the method are shown in Table II. The coefficient of variation (C.V.) ranged between 0.9% and 10% for concentrations of progabide of 1000 and 50 ng/ml, and between 0.7% and 8.0% for concentrations of PGA of 1000 and 50 ng/ml, respectively. Day-to-day variation of the slopes of the calibration curves was less than 5%.

Oxidation potentials

In order to obtain the best sensitivity, the resulting peak height was recorded for each compound as a function of the electrode potential. In the potential range explored (600—1000 mV) no limiting current was clearly visible except for the benzophenone. Moreover, two oxidative processes were visible for progabide, PGA and their internal marker (Fig. 9).

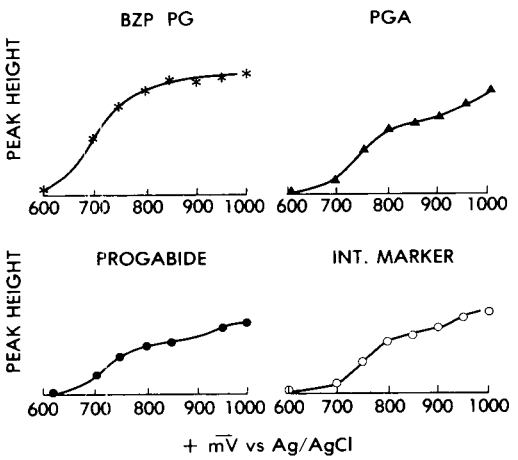


Fig. 9. Detector response, expressed as chromatographic peak height, for progabide (•), PGA (▲), benzophenone, IV (*) and the internal standard 1 (○) as a function of the electrode potential at pH 5.05.

Interferences

Progabide may be administered in combination with other antiepileptic drugs. For that reason 1-ml blood samples containing progabide and PGA (500 ng) were spiked separately with the most widely prescribed antiepileptic drugs (2 μg each of carbamazepine, carbamazepine epoxide, phenobarbital, phenytoin, ethosuximide and 5 μg of valproic acid), and were then processed according to the described method. No chromatographic interference could be observed.

HPLC—GLC correlation

This HPLC method was compared with a gas—liquid chromatographic (GLC) procedure previously used in our laboratory for the quantification of progabide alone [7]. The values obtained with 30 samples analysed with both methods were compared using Student's t test for paired data. This statistical analysis indicated a good correlation between the values calculated with both methods. The observed differences were not statistically significant. However, Fig. 10 shows that at concentrations higher than 1500 ng/ml experimental data tend to scatter.

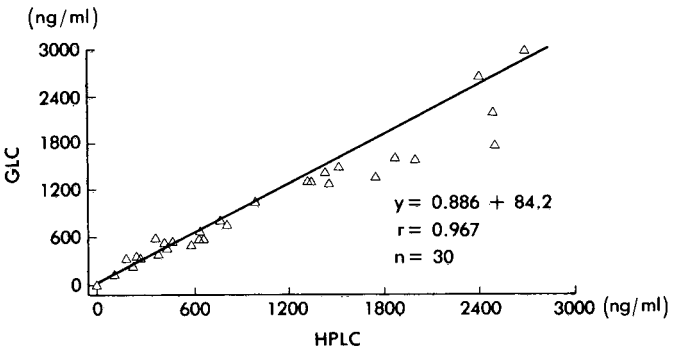


Fig. 10. Correlation of plasma concentrations of progabide obtained with a GLC method [7] and with the described HPLC method.

Application to blood/plasma partition ratio

The blood/plasma concentration ratio at 1, 2, 4, 6, and 8 h following administration of 600 mg of progabide was 0.68 for progabide and 0.55 for PGA (Table III). Two-way analysis of variance (subject, times) indicated that the experimental differences were not statistically significant. It is interesting to point out that the blood/plasma partition ratio of PGA of 0.55 is the theoretical value of a compound restricted to plasma. This was confirmed in a subsequent metabolic study in which progabide labelled with ^{14}C was administered to one volunteer. Progabide but not PGA was then detected in packed erythrocytes.

TABLE III

BLOOD/PLASMA CONCENTRATION RATIOS OF PROGABIDE AND PGA AFTER ORAL ADMINISTRATION OF 600 mg OF PROGABIDE

	Subjects	Time (h)				
		1	2	4	6	8
Progabide	1	0.964	0.736	0.803	0.689	0.737
	2	0.532	0.639	0.634	0.623	0.672
	3	0.707	0.662	0.733	0.552	0.611
	$\overline{M}$	0.734	0.679	0.723	0.621	0.673
	S.E.M.	0.125	0.029	0.049	0.040	0.036
	C.V. (%)	29.6	7.5	11.7	11.0	9.4
PGA	1	0.579	0.553	0.606	0.533	0.520
	2	0.400	0.538	0.474	0.521	0.500
	3	0.688	0.533	0.513	0.651	0.594
	$\overline{M}$	0.556	0.541	0.530	0.568	0.538
	S.E.M.	0.084	0.006	0.039	0.041	0.029
	C.V. (%)	26.2	1.92	12.8	12.6	9.6

DISCUSSION

Reduction of progabide, PGA and benzophenone yields stable products that can be back-extracted in acidic aqueous solutions and kept at room tempera-

ture for several hours during automatic injection of a large number of samples. It should be noted that PGA cyclizes during the reduction step to form a lactam; this is fortunate for it loses its amphoteric property. The decrease of peak height observed with reduction times longer than 20 min is puzzling; it may be due to oxidation of the phenol group at alkaline pH.

The chromatographic separation function developed by Kaiser [6] allows optimization of the separation without extensive calculation. In the present case more experimental data points would be needed to obtain a good resolution of the function. Nevertheless, this empirical determination of the function allowed an estimation of the optimal separation.

Blood/plasma partition coefficients indicate that progabide but not PGA diffuses into red cells. This does not seem to be due to insufficient time for equilibrium; 8 h should be more than necessary to reach equilibrium between plasma and red cells and there is no visible trend in the partition ratio as a function of time. However, it should be pointed out that outlying values were observed 1 h following oral administration.

The method described herein is time-consuming but its main advantage lies in the possibility of quantifying with precision and accuracy progabide, its acid metabolite and benzophenone in blood, plasma and urine. Progabide and PGA are normally not excreted in urine so the reduction step is necessary only for plasma and blood samples.

With automatic injection 30—40 samples and the corresponding calibration curves can be processed daily if the electrodes are rejuvenated every week by polishing them with alumina.

This method has been routinely used in our laboratory for over a year.

REFERENCES

1 P. Loiseau, B. Cenraud, L. Bossi and P.L. Morselli, in M. Dam, L. Gram and J.R. Penry (Editors), Advances in Epileptology, Proc. 12th Epilepsy Int. Symp., Copenhagen, September 6—10, 1980, Raven Press, New York, 1981, pp. 135—140.
2 G.J. Van Der Linden, H. Meinardi, W.A. Meijer and L. Bossi, in M. Dam, L. Gram and J.R. Penry (Editors), Advances in Epileptology, Proc. 12th Epilepsy Int. Symp., Copenhaven, September 6—10, 1980, Raven Press, New York, 1981, pp. 149—152.
3 M. Martinez, L. Bossi, G. Morales, E. Martinez Vila and H. Polanos, Epilepsia, in press.
4 W. Yonekawa, H.J. Kupferberg and T. Lambert, J. Chromatogr., 276 (1983) 103—110.
5 M. Broquaire, J. Chromatogr., 170 (1979) 43—52.
6 R. Kaiser, Gas Chromatographie, Akad. Verlagsges., Leipzig, 1959, p. 33.
7 G. Gillet, J. Fraisse-André, C.R. Lee, L.G. Dring and P.L. Morselli, J. Chromatogr., 230 (1982) 154—161.

Journal of Chromatography, 308 (1984) 241—249
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2091

QUANTITATIVE DETERMINATION OF FUROSEMIDE IN PLASMA, PLASMA WATER, URINE AND ASCITES FLUID BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

KATSUYOSHI UCHINO*, SADAO ISOZAKI*, YUKIYA SAITOH, FUJIO NAKAGAWA and ZENZO TAMURA**

Hospital Pharmacy, Faculty of Medicine, University of Tokyo, 3-1, Hongo 7-chome, Bunkyo-ku, Tokyo 113 (Japan)

and

NAOMI TANAKA

The First Department of Medicine, Faculty of Medicine, University of Tokyo, Tokyo (Japan)

(First received October 4th, 1983; revised manuscript received February 3rd, 1984)

SUMMARY

A high-performance liquid chromatographic method using spectrofluorometric detection is described for the determination of furosemide in plasma, plasma water, urine and ascites fluid. The extraction procedure decreases interference from endogenous substances. The detection limit of furosemide is 10 ng in 0.5 ml of biological sample. The method is sufficiently sensitive for pharmacokinetic study of furosemide with normal subjects and patients with liver cirrhosis and/or renal disease after oral administration of furosemide in a retard capsule, and for study of protein binding of furosemide in patients with various diseases.

INTRODUCTION

Furosemide (FD) is a potent diuretic agent which is widely used for the treatment of oedema and ascites. Measurements of FD concentration in both

*Present address: Hospital Pharmacy, Tokyo Teishin Hospital, 14-23, Fujimi 2-chome, Chiyoda-ku, Tokyo 102, Japan.
**Present address: Keio University Hospital Pharmacy, 35, Shinanomachi, Shinjuku-ku, Tokyo 160, Japan.

0378-4347/84/$03.00

plasma and urine are required for detailed studies on the pharmacokinetics and pharmacodynamics of FD in man.

Several analytical methods for the determination of FD in plasma and urine have been developed. Although an earlier colorimetric method [1] and a fluorometric method [2] are simple and rapid, they suffer from a lack of sensitivity and selectivity. Mikkelsen and Andreasen [3] reported an analytical method using thin-layer chromatographic (TLC) separation followed by fluorescence analysis. This method allows the determination of FD in plasma and urine in concentrations as low as 100 ng/ml, but the required sample volume is 2 ml. Although another TLC method [4] has a high sensitivity, it extends the complexity of FD assay. Two gas chromatographic (GC) methods [5, 6] based on derivatization of FD to its methyl ester are time-consuming since the incubation time for the derivation is about 1 h.

Recently high-performance liquid chromatographic (HPLC) methods [7—13] for the determination of FD in plasma and urine have been developed. In these methods, however, the complete separation of FD from endogenous substances in urine is not accomplished with a minimum detectable FD concentration of less than 100 ng/ml. Therefore, these HPLC methods can not be applied to the measurement of low FD concentrations (approximately 20 ng/ml) in plasma after its administration in retard capsules, or to the study of binding of FD to plasma protein at therapeutic plasma levels.

The present paper describes an HPLC method which has a high sensitivity, specificity and precision for the determination of FD in human biological fluids, and which would be suitable for a pharmacokinetic study following a low dose of FD.

EXPERIMENTAL

Drugs and reagents

Furosemide, 4-chloro-5-sulfamoyl anthranilic acid (CSA) and piretanide [4-phenoxy-3-(1-pyrrolidinyl)-5-sulfamoyl benzoic acid], which was used as internal standard (I.S.), were kindly supplied by Hoechst Japan (Tokyo, Japan). CSA has been reported as a major breakdown product of FD [3, 9], and its pharmacological activity was weak. In this study, therefore, the interference of CSA with the peak of FD was checked. The chemical structures of these drugs are shown in Fig. 1.

Retard capsules of FD (Eutensin®) in a sustained-release dosage form were obtained from Hoechst Japan, and contained 40 mg of FD. Lyophilized serum (Con Sera® "Nissui") was obtained from Nissui Pharmaceutical Co. (Tokyo, Japan). All the other solvents and reagents used were of reagent grade, and were obtained from Wako (Tokyo, Japan).

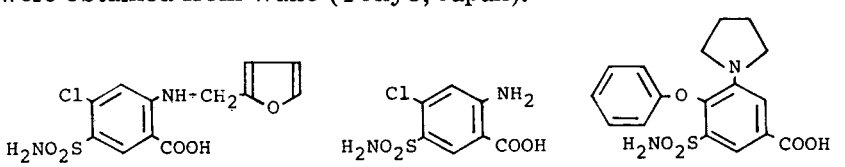


Fig. 1. Chemical structures of furosemide, 4-chloro-5-sulfamoyl anthranilic acid (CSA) and piretanide.

Extraction of FD from biological samples

To 0.5 ml of plasma, plasma water, 50 times-diluted urine or ascites fluid in a 50-ml glass-stoppered centrifuge tube were added 6 μg of internal standard in 100 μl of ethanol, 2 ml of H_3PO_4—KH_2PO_4 solution (1 *M*, pH 1.5) and 11 ml of methylene chloride. The mixture was vigorously shaken with a mechanical shaker for 15 min, then centrifuged in a KN-70 centrifuge (Kubota Seisakusho, Tokyo, Japan) at 1630 *g* for 5 min. Nine millilitres of lower organic phase were transferred to another tube, and were added with 2.5 ml of 0.001 *M* sodium hydroxide. The mixture was vigorously shaken for 15 min, then separated by centrifugation. Two millilitres of upper aqueous phase were transferred to another tube, mixed with 2 ml of H_3PO_4—KH_2PO_4 solution (1 *M*, pH 1.5), and extracted with 11 ml of methylene chloride once again. Ten millilitres of lower organic phase were transferred to another tube and evaporated to dryness using a rotary vacuum evaporator at room temperature. The dried residue was dissolved in 100 μl of ethanol (Super Special Grade, Wako) and 20 μl of this solution were injected into the HPLC column.

Light-protecting containers were used throughout this extraction procedure.

Chromatography

The HPLC apparatus used in this study was composed of a Shimadzu liquid chromatograph LC-3A and a Shimadzu spectrofluorophotometer RF-500 (Shimadzu, Kyoto, Japan). An excitation wavelength of 268 nm and an emission wavelength of 410 nm were used for the detection of FD. The mobile phase consisted of 65 vols. of ethanol and 35 vols. of $HClO_4$—$NaClO_4$ solution (0.02 *M*, pH 2.0), and was degassed before use. The flow-rate of the mobile phase was 0.2 ml/min (pressure approximately 70 kg/cm^2). The column was packed with 10-μm spherical porous particles of styrene—divinylbenzene (Hitachi Gel® 3011, Hitachi Seisakusho, Hitachi, Japan) in a stainless-steel column (50 cm × 2 mm I.D.). The column temperature was maintained by a column jacket connected to a water bath at 30° C.

Ascertainment of chromatographic peak of FD by mass spectrometry

Eight or nine tubes each containing 1 ml of FD solution 20 μg/ml in 0.01 *M* sodium hydroxide were extracted using the method described above. The dried residue was dissolved in 100 μl of ethanol, and 25 μl of this solution were injected into the HPLC column. One millilitre of mobile phase corresponding to the FD peak was collected in a 50-ml centrifuge tube just after passage through the cell of the detector, and this manipulation was repeated about thirty times. Thirty millilitres of the solution thus obtained were added to 1 ml of 1 *M* sodium hydroxide solution to prevent hydrolysis of FD, and the mixture was evaporated to dryness using a rotary vacuum evaporator at about 30° C. The dried residue was dissolved in 2 ml of 0.01 *M* sodium hydroxide, mixed with 2 ml of H_3PO_4—KH_2PO_4 solution (1 *M*, pH 1.5) and extracted with 11 ml of methylene chloride. The extracted residue was dissolved in 0.5 ml of ethanol, and this solution was subjected to mass spectrometry.

A JMS-300 mass spectrometer equipped with a multiple-ion detector and JMS-2000 data processing system including a JEC-980B computer (Jeol, Tokyo, Japan) were used in this study. Mass spectra were obtained by direct

inlet probe in electron-impact mode. The scanning mode for measurement of mass spectra was as follows: ionization energy, 70 eV; ionization current, 0.3 mA; scan speed, *m/z* 0 to 800 in 5 sec. The ion-source temperature was maintained at 210° C. The obtained data were stored directly on a disc and displayed on a cathode ray tube or printed out on a graphic printer.

Calibration curves

To prepare the plasma sample, 25 mg of FD were dissolved in 0.1 *M* sodium hydroxide in a 25-ml volumetric flask. An aliquot of this solution was diluted with reconstituted lyophilized serum in distilled water to produce a standard solution of the desired concentration (0, 10, 25, 50, 100, 250, 500 or 1000 ng per 0.5 ml). The other biological samples were prepared for calibration curves in the same manner. Thus, the 1 mg/ml FD solution in sodium hydroxide was diluted with diluted urine (× 50) or FD-free ascites fluid to produce standard solutions of the desired concentrations. Distilled water was used instead of plasma water, because a large volume of FD-free plasma water was difficult to obtain from normal volunteers. Furthermore, the extraction recovery of FD from plasma water was equal to that from distilled water in preliminary tests. These samples were analysed by the method described above. The calibration curve for each biological sample was obtained by plotting the peak height ratio (FD/I.S.) against the concentration of FD in each spiked sample.

Check for interference by coadministered drugs

The present method was checked for interference by drugs frequently used together with FD in the treatment of patients with ascites and/or oedema. Their prescriptions were used to search for these drugs, which were allopurinol, spironolactone, canrenone, diazepam, digitoxin, digoxin, dipyridamole, hydralazine, indomethacin, α-methyldopa, prednisolone, propranolol, reserpine, aspirin (salicylic acid), compound preparations of vitamins of the B group (Vitaneurin®-50, Takeda Chemical Industries, Osaka, Japan), and warfarin.

Sample collection

Three normal subjects were orally administered 40 mg of FD in a retard capsule at 09:00 a.m. once a day. All subjects were shown by physical and laboratory examinations to be in good physical condition. They consented to participate in this study after the aim and protocol were explained. Blood samples were drawn through an indwelling cannula at the times indicated in Fig. 4, until 12 h after administration; the plasma was immediately separated from whole blood by centrifugation. Urine samples were collected at the times shown in Fig. 4 until 24 h after the administration of FD.

Ascites fluid was collected by abdominal paracentesis from a decompensated cirrhotic patient with intractable ascites. The patient was intravenously administered 20—60 mg of FD for the treatment of ascites, but the ascites failed to decrease.

Plasma samples for separation of plasma water were collected from a normal volunteer after intravenous administration of FD, and from patients with liver

cirrhosis and/or renal disease who received FD for the treatment of ascites or oedema. The plasma water was separated from plasma by ultracentrifugation. According to the methods of Tanimura et al. [14] and Nishihara et al. [15], two 3-ml aliquots of plasma were pipetted into a length of curved seamless cellulose acetate tubing (Type 20/32, 130 mm long; Visking, U.S.A.) with both open ends held tightly by a silicone rubber stopper against the inner wall of a glass vessel. The glass vessel holding the tubing inside was centrifuged (Hitachi 20 PR-5) at 1630 *g* at 37 ± 0.5° C for about 40 min. Five hundred microlitres of ultrafiltrate (i.e. plasma water) were used for the determination of FD.

All biological samples were stored at −20° C until analysis.

RESULTS AND DISCUSSION

The extraction and HPLC procedures for the determination of FD in biological samples were set following the most desirable chromatographic and detection conditions. Methylene chloride for extraction of FD was contaminated with smaller amounts of endogenous substances present in biological fluids than are other organic solvents such as acetonitrile, choroform, diethyl ether and ethyl acetate. Furthermore, the basic extraction step reduced the quantities of endogenous substances extracted with methylene chloride from urine. As the fluorescence intensity of FD was most stable in ethanol and became greater with increasing acidity of the solution, the mobile phase was composed of 65 vols. of ethanol and 35 vols. of $HClO_4$—$NaClO_4$ solution (0.02 *M*, pH 2.0). The column was prepared with Hitachi Gel® 3011

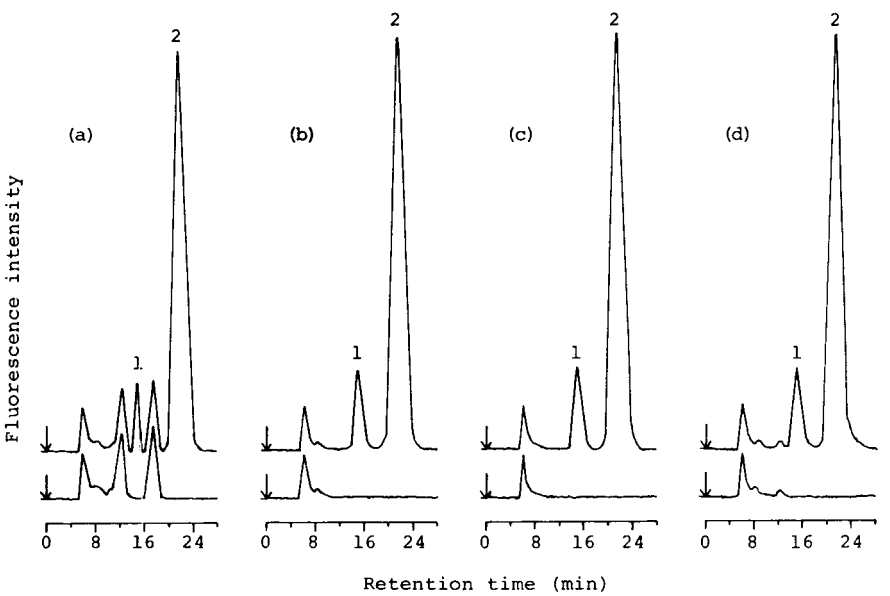


Fig. 2. Upper chromatograms obtained from 0.5 ml of (a) plasma, (b) plasma water, (c) urine and (d) ascites fluid from normal subjects or patients, spiked with 50 ng of furosemide and 6 μg of piretanide as the internal standard. Lower chromatograms obtained from drug-free (a) plasma, (b) plasma water, (c) urine and (d) ascites fluid. Peaks: 1 = furosemide, 2 = piretanide. Chromatographic conditions were as described in Experimental.

using the mobile phase because the degree of swelling of the gel was considerably influenced by the composition of the mobile phase. The column used in this study seems to be remarkably stable, since no changes in chromatographic properties were noticed after about 2000 injections of biological samples.

Typical chromatograms of FD and each blank obtained from biological samples are shown in Fig. 2. The retention times of FD and internal standard were 15.4 and 21.4 min, respectively. Since the retention time of CSA was 7 min, it did not interfere with the analysis of FD. Further, the blank chromatograms showed that no interference would occur with endogenous substances. The purity of the chromatographic peak of FD was ascertained by mass spectrometry. The results are shown in Fig. 3. The sample obtained from HPLC separation showed a mass spectrum identical to that of authentic FD.

The extraction recoveries of FD from biological fluids were determined at four different concentrations. As shown in Table I, the mean extraction recovery was about 95% for plasma and about 100% for plasma water, urine and ascites fluid. The coefficients of variation were less than 5%.

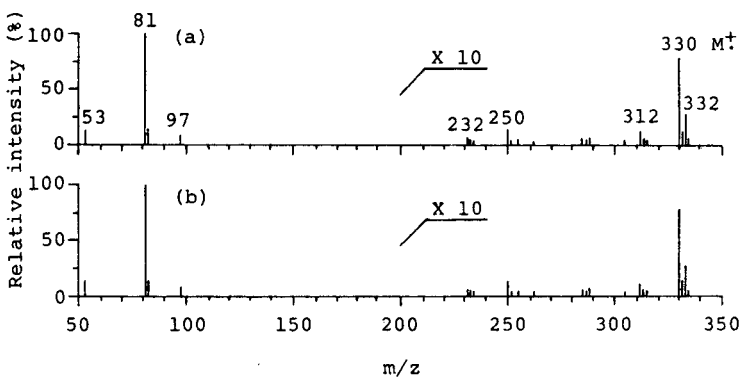


Fig. 3. Electron-impact (70 eV) mass spectra of (a) authentic furosemide and (b) sample from the present HPLC method obtained using a direct inlet system. Mass spectrometric conditions were as described in Experimental.

TABLE I

MEAN EXTRACTION RECOVERIES OF FUROSEMIDE FROM SEVERAL BIOLOGICAL SAMPLES

Each value represents mean ± S.D. of five experiments.

Added (ng)	Extraction recoveries (%)			
	Plasma	Urine	Plasma water	Ascites fluid
10	95.3 ± 3.9	100.4 ± 4.7	100.8 ± 3.2	102.1 ± 2.5
50	94.6 ± 4.1	105.3 ± 1.4	99.1 ± 2.8	99.2 ± 2.9
250	96.4 ± 1.6	100.9 ± 1.3	99.5 ± 2.8	103.5 ± 4.2
500	96.7 ± 2.7	101.3 ± 2.1	102.8 ± 3.3	100.6 ± 2.5
Mean	95.6	102.0	100.6	101.4

The calibration curves of FD for plasma, plasma water, urine and ascites fluid were linear over the FD concentration range 10—1000 ng per 0.5 ml, and the lines passed through the origin. The detection limit of FD in each biological fluid was 10 ng per 0.5 ml with a signal-to-noise ratio of about 10:1.

To examine the reproducibility of this method, a single dose of 40 mg of FD in a retard capsule was orally administered to a normal subject, and five 0.5-ml plasma samples at each sampling time were analysed. The results were satisfactory, as shown in Table II.

The interference by coadministered drugs is shown in Table III. Most drugs were completely removed by the first extraction step with methylene chloride under acidic conditions and/or by chromatographic separation, and only reserpine interfered. This, however, was removed by additional extraction with the basic aqueous solution.

TABLE II

REPRODUCIBILITY OF FUROSEMIDE (FD) MEASUREMENT AT EACH SAMPLING TIME AFTER ORAL ADMINISTRATION OF 40 mg OF FD IN A RETARD CAPSULE TO A NORMAL SUBJECT

Sampling time (h)	Plasma concentration of FD (ng/ml)	
	Mean ± S.D. (n = 5)	C.V. (%)
3	67.6 ± 2.8	4.1
6	205.2 ± 3.3	1.6
12	51.8 ± 2.0	3.9

TABLE III

INTERFERENCE BY COADMINISTERED DRUGS

Concomitant drug	Interference*		
	Acidic extraction	Chromatographic separation	Acidic + basic extraction
Allopurinol	—		
Spironolactone	—		
Canrenone	—		
Diazepam	—		
Digitoxin	—		
Digoxin	—		
Dipyridamole	+	—	
Hydralazine	+	—	
Indomethacin	—		
α-Methyldopa	—		
Prednisolone	—		
Propranolol	—		
Reserpine	+	+	—
Aspirin (salicylic acid)	+	—	
Vitaneurin®-50	—		
Warfarin	—		

*— indictes lack of interference.

The present method was applied to the determination of FD in several clinical studies. The time courses of mean plasma concentration and cumulative urinary excretion of FD after oral administration of 40 mg of FD in a retard capsule to three normal subjects are shown in Fig. 4. The plasma concentration of FD increased slowly to reach a maximum value (about 200 ng/ml) at about 6 h, and decreased gradually to approach about 30 ng/ml at 12 h. Furosemide was excreted in urine at an approximately constant rate for 10 h; the urinary excretion of FD during 24 h was about 8 mg, corresponding to 20% of the given dose.

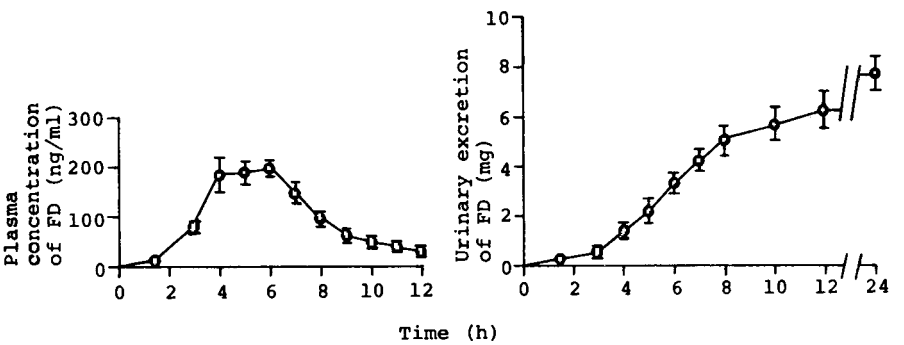


Fig. 4. Time courses of plasma concentration (left) and cumulative urinary excretion (right) of furosemide (FD) after oral administration of 40 mg of FD in retard capsules to three normal volunteers. Each value indicates the mean ± S.E. obtained from three samples.

TABLE IV

BINDING OF FUROSEMIDE (FD) TO PLASMA PROTEIN IN A NORMAL SUBJECT AND PATIENTS

The binding of FD to plasma protein is calculated using the equation

$$\text{Binding of FD to plasma protein (\%)} = \frac{\text{plasma concentration of FD} - \text{plasma water concentration of FD}}{\text{plasma concentration of FD}} \times 100.$$

	Concentration of FD (ng/ml)		Binding of FD to plasma protein (%)
	Plasma	Plasma water	
Normal subject	830.5	20.1	97.6
Liver cirrhosis	351.4	29.4	91.6
Renal disease	5702.0	391.0	93.1

In studies of the disposition of FD in patients with liver cirrhosis, the measurement of FD concentration in ascites fluid was particularly interesting. We applied this technique to ascites fluid from a patient with liver cirrhosis. The concentration of FD in the ascites fluid was 84.6 ng/ml and that in plasma collected simultaneously was 351.4 ng/ml.

The measurement of FD in plasma water at usual therapeutic plasma levels was required [16] since previous studies [17—21] for the binding of FD to plasma protein have been carried out by adding FD to plasma in vitro. Therefore, we applied this technique to plasma water from a normal subject, a

patient with liver cirrhosis and another patient with renal disease after the administration of FD; the results are shown in Table IV. The percentage of FD bound to plasma protein in the normal subject was higher than in the patients.

In conclusion, a sensitive, specific and precise method for the determination of FD in human biological fluids by HPLC was established. The method would be particularly useful for pharmacokinetic study following low doses of FD in retard capsule in normal subjects, as well as in patients with a variety of diseases. Such applications will be reported elsewhere.

ACKNOWLEDGEMENT

The authors wish to thank Dr. Hajime Kotaki for his excellent technical assistance and valuable comments on the mass spectrometric measurements.

REFERENCES

1 P. Hajdů and A. Häussler, Arzneim.-Forsch., 14 (1964) 709.
2 A. Häussler and P. Hajdů, Arzneim.-Forsch., 14 (1964) 710.
3 E. Mikkelsen and F. Andreasen, Acta Pharmacol. Toxicol., 41 (1977) 254.
4 B. Wesley-Hadzija and A.M. Mattocks, J. Chromatogr., 229 (1982) 425.
5 B. Lindström and M. Molander, J. Chromatogr., 101 (1974) 219.
6 J. Perez, D.S. Sitar and R.I. Ogilvie, Drug Metab. Dispos., 7 (1979) 383.
7 A.D. Blair, A.W. Forrey, B.T. Meijsen and R.E. Cutler, J. Pharm. Sci., 64 (1975) 1334.
8 K. Carr, A. Rane and J.C. Frölich, J. Chromatogr., 145 (1978) 421.
9 R.L. Nation, G.W. Peng and W.L. Chiou, J. Chromatogr., 162 (1979) 88.
10 E.T. Lin, D.E. Smith, L.Z. Benet and B.-A. Hoener, J. Chromatogr., 163 (1979) 315.
11 S.E. Swezey, P.J. Meffin and T.F. Blaschke, J. Chromatogr., 174 (1979) 469.
12 R.S. Rapaka, J.R.CT. Viswanathan, T.J. Goehl, V. Prasad and B.E. Cabana, J. Chromatogr., 227 (1982) 463.
13 A.L.M. Kerremans, Y. Tan, C.A.M. Van Ginneken and F.W.J. Gribnau, J. Chromatogr., 229 (1982) 129.
14 Y. Tanimura, M. Kimura, M. Ohkawa, Y. Saitoh, F. Nakagawa and T. Suzuki, in S. Yoshina (Editor), Abstracts of Papers (IV), The 96th Annual Meeting of Pharmaceutical Society of Japan, Nagoya, Japan, April 4—7, 1976, Pharmaceutical Society of Japan, Tokyo, p. 217.
15 K. Nishihara, K. Uchino, Y. Saitoh, Y. Honda, F. Nakagawa and Z. Tamura, Epilepsia, 20 (1979) 37.
16 D.E. Smith and L.Z. Benet, J. Pharmacokin. Biopharm., 10 (1982) 663.
17 F. Andreasen and P. Jakobsen, Acta Pharmacol. Toxicol., 35 (1974) 49.
18 J. Prandota and A.W. Pruitt, Clin. Pharmacol. Ther., 17 (1975) 159.
19 F. Andreasen, H.E. Hansen and E. Mikkelsen, Eur. J. Clin. Pharmacol., 13 (1978) 41.
20 A. Rane, J.P. Villeneuve, W.J. Stone, A.S. Nies, G.R. Wilkinson and R.A. Branch, Clin. Pharmacol. Ther., 24 (1978) 199.
21 S. Goto, H. Yoshitomi, A. Miyamoto, K. Inoue and M. Nakano, J. Pharm. Dyn., 3 (1980) 667.

Journal of Chromatography, 308 (1984) 251–259
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2090

SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY USING ELECTROCHEMICAL DETECTION FOR A NOVEL PRODRUG ESTER OF METHYLDOPA, PIVALOYLOXYETHYL 3-(3,4-DIHYDROXYPHENYL)-2-METHYLALANINATE, IN PLASMA AND URINE

D.G. MUSSON*, W.C. VINCEK, M.R. DOBRINSKA, S. VICKERS and H.L. LEIDY

Merck Sharp & Dohme Research Laboratories, West Point, PA 19486 (U.S.A.)

(First received November 8th, 1983; revised manuscript received January 27th, 1984)

SUMMARY

The pivaloyloxyethyl ester of methyldopa is an antihypertensive prodrug possessing improved bioavailability properties over methyldopa. A sensitive cation-exchange, high-performance liquid chromatographic assay using electrochemical detection has been developed for the ester in plasma and urine in order to determine the extent of its hydrolysis after oral administration. The chromatographic conditions involve two Altex Partisil 10 SCX columns (25 cm × 4.6 mm) in series; a mobile phase consisting of methanol, potassium phosphate buffer, pH 3.0, and EDTA disodium dihydrate; and an electrochemical detector set at 0.5 V. The pivaloyloxyethyl ester in plasma or urine is extracted into ethyl acetate, back-extracted into 0.1 *M* sulfuric acid, and analyzed directly by high-performance liquid chromatography. For urine, the ethyl acetate extract is washed with a buffer (pH 8.0) prior to the back-extraction step. The assay gives a linear response over the concentration range of 10—160 ng/ml in plasma and 20—400 ng/ml in urine.

INTRODUCTION

The pivaloyloxyethyl (POE) ester of methyldopa [Fig. 1, I, (*S*)- or (*R*)-pivaloyloxyethyl (*S*)-3-(3,4-dihydroxyphenyl)-2-methylalaninate hydrochloride dihydrate] is a new antihypertensive prodrug of methyldopa (Fig. 1, II). The ester possesses better oral absorption and antihypertensive properties than methyldopa [1—3]. In animals treated orally with a radioactive dose of the ester, the metabolites that have been isolated from urine are pivalic acid (Fig. 1, III) and methyldopa [3].

The POE ester of methyldopa has been developed as a prodrug that would be better absorbed than methyldopa and would readily hydrolyze to methyl-

0378-4347/84/$03.00

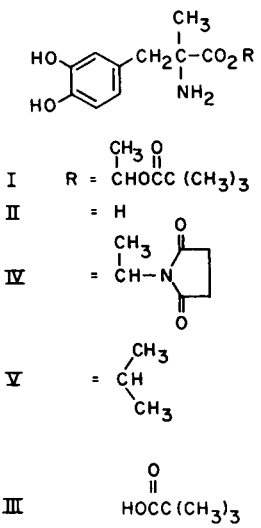


Fig. 1. Chemical structures of the POE ester of methyldopa (I), methyldopa (II), pivalic acid (III), internal standard for the plasma assay (IV) and internal standard for the urine assay (V).

dopa [1]. A sensitive and selective assay for the POE ester was needed to quantify the presence of the intact ester in plasma and urine following oral administration.

Methods available for the analysis of methyldopa include a non-specific fluorescent assay [4], a mass spectrometric assay [5], gas—liquid chromatography [6], and high-performance liquid chromatography (HPLC) using ultraviolet [7,8] and electrochemical detection (ED) [9—13]. The latter technique is the most sensitive for methyldopa and has been developed for the POE ester.

A cation-exchange, HPLC assay using ED is described for the determination of the POE ester of methyldopa. The plasma and urine assays are selective for the ester and are sensitive to 10 ng/ml and 20 ng/ml, respectively. Animal studies and preliminary human studies with prodrug have revealed trace levels in the urines and negligible levels in plasma.

EXPERIMENTAL

Reagents

HPLC solvents were obtained from Burdick & Jackson Labs.; water was purified using a Milli-Q system (Millipore). All chemicals were used as they were received.

Pivaloyloxyethyl 3-(3,4-dihydroxyphenyl)-2-methylalaninate·HCl·$2H_2O$ (*S,S*-diastereomer and an *R,S*- and *S,S*-diastereomeric combination, 50:50), (*S*)-succinimidoethyl (*S*)-(3,4-dihydroxyphenyl)-2-methylalaninate·HCl·$2H_2O$ (Fig. 1, IV, internal standard for the plasma assay), isopropyl (*S*)-2-amino-2-(3,4-dihydroxybenzyl)proprionate (Fig. 1, V, internal standard for the urine assay) were synthesized at Merck Sharp & Dohme Labs. [1].

High-performance liquid chromatography

The liquid chromatographic system consists of a solvent delivery system (Model M6000A) and an autosampler (WISP 710B) from Waters Assoc., a Spectra Physics computing integrator (Model SP4100) and an amperometric controller (LC-2A)/KEL F thin-layer detector cell (TL-8A) from Bioanalytical Systems. The mobile phase flows through the electrochemical detector cell (0.5 V) at 2 ml/min. Two cation-exchange columns from Altex (Partisil 10 SCX, 10-μm particles) are employed in series for the plasma and urine assays. The detector cell and the solvent waste container are enclosed within a copper mesh, Faraday cage (grounded to a water pipe).

The mobile phase for the plasma assay consists of 1.74 g of monobasic potassium phosphate, 850 ml of water, 150 ml of methanol, 50 mg of ethylenediaminetetraacetic acid, disodium salt, dihydrate (EDTA). The pH of the solution is adjusted to 3.0 with phosphoric acid (85%). The mobile phase for the urine assay is prepared similarly except 100 ml of methanol is mixed with 900 ml water. Both mobile phases are filtered (cellulosic membrane filter, 0.45 -μm) and kept under a helium atmosphere.

Extraction

A 1-ml sample of plasma is prepared for HPLC analysis by adding to it 100 μl of sodium fluoride (20 mg/ml), 200 μl of $Na_2S_2O_5$ (0.25 *M*) and 100 μl of (*S*)-succinimidoethyl (*S*)-(3,4-dihydroxyphenyl)-2-methylalanine hydrochloride dihydrate (1 μg/ml) in a 13-ml tube. The sample is then extracted with 5 ml of ethyl acetate (vortex for 1 min, centrifuge for 5 min at 653 *g*). The ethyl acetate layer is transferred to a 13-ml tapered centrifuge tube and back-extracted into 0.5 ml of 0.1 *M* sulfuric acid. The sulfuric acid layer is analyzed by HPLC.

A 2-ml urine sample is prepared by mixing with it isopropyl 2-amino-2-(3,4-dihydroxybenzyl)propionate (100 μl, 2 μg/ml), 100 μl of $Na_2S_2O_5$ and 0.5 ml of borate buffer, pH 9.75. The sample is then extracted with 6 ml of ethyl acetate (vortex for 1 min, centrifuge for 5 min at 653 *g*). The ethyl acetate layer is transferred to a new tube and is washed with 1 ml of phosphate buffer, pH 8.0—$Na_2S_2O_5$ solution (13.4 g Na_2HPO_4, 0.731 g NaH_2PO_4 and 100 ml of 10.25 *M* $Na_2S_2O_5$ in 1 l of water). The ethyl acetate is again transferred to a tapered centrifuge tube and extracted with 0.5 ml of dilute sulfuric acid. The aqueous layer is analyzed by HPLC.

Standard calibration curves

A 10 μg/ml stock solution of the POE ester is serially diluted to concentrations of 100, 200, 400, 800, 1200, 1600, 2000, 2200 and 2500 ng/ml. Standards are prepared by transferring 100 μl of each solution to 1 ml of plasma or 2 ml of urine. The standards are processed as described above.

Standard curves are drawn with peak height ratios plotted against concentrations. Sample concentrations are quantified from these curves which are generated with each set of samples (daily).

Plasma stability studies

The plasma stability of the POE ester was investigated with $Na_2S_2O_4$ and

with sodium fluoride. Aliquots from duplicate test standards were removed every 15 min, treated with internal standard and extracted. The test standards during the studies were mixed at room temperature. The percent of ester remaining overtime was determined from peak height ratios.

Animal and human studies

Animal and human studies were conducted with the *S,S*-diastereomer and/or the diastereomeric mixture to determine the systemic levels of the intact ester and/or bioavailability of methyldopa from the ester. In beagle dogs and rhesus monkeys, the drug was administered orally, 500 mg free base equivalents. Plasma samples were collected in time intervals up to 24 h in heparinized tubes containing 6 mg of sodium fluoride. The blood samples were centrifuged and the plasma frozen until analysis. Urine was collected over dry ice over 0—24 h and 24—48 h after dose. Samples were kept frozen until analysis.

Portal circulation levels of the prodrug were investigated in rhesus and cynomolgus monkeys after oral administration of the ester, 803 mg of the ester's free base. Portal blood samples were collected via a subcutaneously implanted catheter, in heparinized tubes containing 8 mg of sodium fluoride. Blood was collected in time intervals up to 8 h. Urine samples were collected as discussed above.

In human studies, male volunteers received 1000 mg of ester, per os. Blood and urine samples were collected in time intervals up to seven days. The samples were handled and stored as described above.

RESULTS AND DISCUSSION

The HPLC—ED assay is sensitive and specific for the POE ester of methyldopa in human and animal biological fluids. Pivaloyloxyethyl ester of methyldopa selectively elutes on two cation-exchange columns from methyldopa and its major metabolites: α-methyldopamine, α-methylnorepinephrine, 3-O-methyl-2-methyldopa, 3-O-methyl-α-methyldopamine and 3-methoxy-4-hydroxyphenylacetone. There is, however, no chromatographic distinction between the *R,S*- and the *S,S*-diastereomers of the POE ester. Organic extraction of the ester from plasma and urine by ethyl acetate and back-extraction into dilute sulfuric acid increases the selectivity of the method by isolating it from endogenous materials and conjugates that might interfere chromatographically (Figs. 2 and 3).

Investigations into isolation techniques for the POE ester from biological fluids for HPLC analysis were mainly centered on the urine matrix. There were no chromatographic interferences for the POE ester from plasma background using either deproteination (e.g. by acetonitrile precipitation or by ultrafiltration) or extraction methods (e.g. tributyl phosphate (TBP)—hexane extraction, aluminum oxide extraction) [9—13].

Reported isolation methods [9—13] for catecholamines and amino acids involving TBP—hexane and/or alumina extraction were tested on urine with minor success: TBP—hexane extraction—acid back-extraction resulted in interfering peaks; further isolation with aluminum oxide gave a clear chromatogram

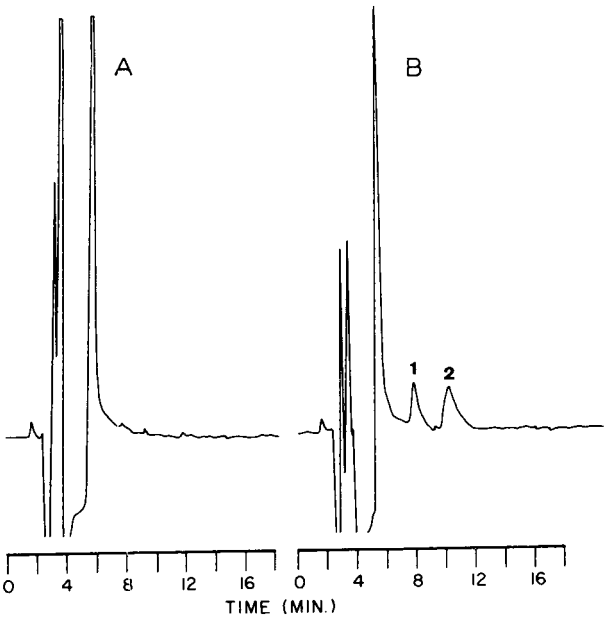


Fig. 2. Chromatograms of (A) a human plasma blank; (B) spiked plasma standard containing internal standard (1) and the POE ester (2) at 20 ng/ml. Electrochemical detector set at 50 nA full scale, offset 2—4.

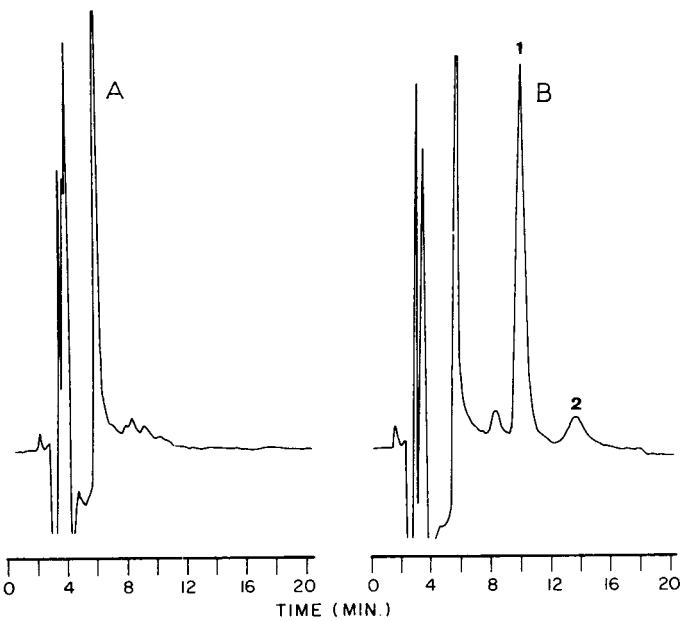


Fig. 3. Chromatograms of (A) a human urine blank; (B) a spiked urine containing the internal standard (1) and the POE ester (2) at 40 ng/ml. Electrochemical detector set at 50 nA full scale, offset 2—4.

for the POE ester, but recovery was less than 10%. Urine clean-up using $ZnSO_4 \cdot 7H_2O$—$Ba(OH)_2$ [14] was not any better than the TBP—hexane extraction—back-extraction technique.

POE ester extraction from urine with ethyl acetate [3] followed by back-extraction into dilute sulfuric acid was chromatographically successful with 70—80% recovery. An additional washing step in the isolation sequence was necessary to further clear the urine chromatogram for a suitable internal standard, isopropyl ester of methyldopa.

The POE ester's structure characteristically suggests chemical sensitivity to oxidation of its catechol and hydrolysis of its ester. The enzymatic hydrolysis of this ester in human plasma has been reported [3]. The enzymatic hydrolysis of esters in serum and their protection with sodium fluoride is known [15,16]. Therefore, the stability of the POE ester was tested at ambient temperature in human plasma, in plasma with $Na_2S_2O_5$ and in plasma with sodium fluoride. The results (Table I) indicate protection for the ester with sodium fluoride rather than with the reducing agent $Na_2S_2O_5$. Thus, sodium fluoride was added to the samples during collection to prevent enzymatic hydrolysis. The reducing agent was added later to the samples to prevent oxidation during preparation for analysis. Similar enzymatic losses for the POE ester of methyldopa in urine do not occur. The chemical stability of the ester in dilute hydrosulfuric acid was also tested, revealing no losses with time.

Intra-day coefficients of variation of replicates for the human plasma and the human urine assays are presented in Table II. These values were measured at concentrations over the linear range. The recovery yield for the POE ester extracted from plasma and back-extracted into dilute sulfuric acid is 79.7 ± 8.3%; for urine, 74.7 ± 5.9%. The plasma assay for the POE ester gives a linear plot (r^2 =0.9982) of peak height ratios versus concentration described by the equation y=0.0069+0.0043x. The concentration range is 10—160 ng/ml. The urine assay gives a linear plot (r^2 =0.9999) described by the equation y=0.0030+ 0.0019x over the concentration range of 20—400 ng/ml. The assay is sensitive for the ester to 10 ng/ml in plasma and to 20 ng/ml in urine. Lower concentration levels of the ester (approximately 1 ng/ml) can be obtained by adjusting

TABLE I

STABILITY OF POE ESTER (*S,S*-DIASTEREOMER) IN HUMAN PLASMA AND IN PLASMA CONTAINING $Na_2S_2O_5$ OR NaF AT AMBIENT TEMPERATURE

Time (min)	Percent remaining		
	Plasma*	Plasma with $Na_2S_2O_5$**	Plasma with NaF***
15	96.9	83.0	100
30	64.6	56.5	103
45	63.1	46.1	87.6
60	63.1	38.1	87.0

*POE ester in plasma at 5.8 μg/ml.
**POE ester (5.8 μg/ml) in plasma with 3 mg/ml $Na_2S_2O_5$.
***POE ester (5.8 μg/ml) in plasma with 2 mg/ml NaF.

TABLE II

REPRODUCIBILITY (n = 5—6) OF THE URINE AND THE PLASMA ASSAY OF THE *S,S*-DIASTEREOMER

Concentration (ng/ml)	Coefficients of variation (%)
Plasma assay	
10	18.3
20	7.5
40	5.0
80	6.1
160	2.2
Urine assay	
20	8.3
40	8.1
80	8.4
125	8.8
200	10.4
400	6.2

a lower attenuation or by extracting from a larger volume of fluid (in the urine assay, a different internal standard would be required). However, at lower attenuation baseline stability and assay reproducibility would be compromised.

The POE ester of methyldopa was developed with the objective of obtaining a prodrug which would be more efficiently absorbed from the gastrointestinal tract compared to oral methyldopa but which would also be readily hydrolyzed in the body to deliver the active moiety to the general circulation. When small oral doses (200 mg) of the *S,S*-diastereomer, labeled with ^{3}H, were administered to man, about 70% of the label was recovered in urine within 36 h and methyldopa was the major urinary metabolite [3]. In comparison, about 40% of a ^{14}C-labeled oral dose of methyldopa is recovered in urine [17]. With larger oral doses of the prodrug, equivalent to 500 and 1000 mg of methyldopa, the availability of methyldopa to the general circulation of man averaged 64% of the dose compared to 27% after oral methyldopa [2]. In vivo hydrolysis of the prodrug (*S,S*-diastereomer) was initially studied in rats, dogs, and rhesus monkeys after oral administration of the drug labeled with ^{3}H in the methyldopa moiety or ^{14}C in the pivalic acid moiety [3]. The differing temporal pattern of the two labels in the portal circulation and their selective solvent extraction profile from portal plasma was indicative that hydrolysis of the prodrug occurred pre-systematically and was rapid and extensive. In vitro experiments also demonstrated that plasma esterases are capable of hydrolyzing the ester at an appreciable rate in rat, dog, and human plasma [3] (see also Table I).

These data indicate that after oral administration of the POE ester, concentrations of the intact ester in biological fluids would be extremely low relative to the active hydrolysis product, methyldopa. The present HPLC method is highly sensitive and selective for the ester and was developed and applied in several animal and human studies to assess the degree to which the body is

exposed to the prodrug relative to the active therapeutic moiety, methyldopa. In these studies, blood was collected in the presence of sodium fluoride to limit any hydrolysis of the prodrug during collection and processing of the plasma samples. The assay was also used to determine if there is any stereoselectivity in absorption and/or hydrolysis of the ester by comparing results after administration of the *S,S*-diastereomer and an equimolar mixture of the *S,S*- and *R,S*-diastereomers of the drug.

In beagle dogs (n=6) given 500-mg oral doses of the single diastereomer and the mixture of diastereomers, the intact ester was not detected in plasma (<10 ng/ml); only a third of the 0—24 h urine samples contained detectable levels of the ester (<0.0003% of the dose), all below the minimum detection limit. In contrast, the urinary recovery of free methyldopa averaged 34.8% of the dose in either treatment. At the same dose level in rhesus monkeys (n=6), plasma samples contained trace levels of the intact ester varying between 8.4 and 32.8 ng/ml. The drug was present in the urine of three monkeys and accounted for less than 0.034% of the dose. No differences were observed in the bioavailability of methyldopa between the two treatments. The intact drug was detected in the portal circulation of rhesus monkeys (n=3) for 8 h after an oral 803-mg dose of the diastereomeric mixture. The area under the curve in portal plasma averaged 2.09 μg·h/ml compared to 135 μg·h/ml for methyldopa.

In a disposition study in man (n=4), intact ester was not detected in plasma after a 1000-mg oral dose of the *S,S*-diastereomer. The drug was detected in the initial 0—2 h urine collection of one subject and accounted for less than 0.0013% of the dose. Its identity was also verified by gas—liquid chromatographic—mass spectrometric analysis [18]. The single and mixed diastereomers were also compared in man (n=6) at oral doses of 1000 mg. The intact ester was detected in both treatments but did not exceed 7 ng/ml in plasma or 11.8 μg/ml (0.0012% of the dose) in urine. Methyldopa bioavailability in the two treatments was equivalent with about 40% of the dose recovered in urine as free methyldopa.

These results indicate that the prodrug is well absorbed and efficiently hydrolyzed to the active drug prior to reaching the general circulation. No stereoselectivity in absorption and hydrolysis is observed between the *S,S*- and *S,R*-diastereomers of the POE ester of methyldopa.

REFERENCES

1 W.S. Saari, M.D. Freedman, R.D. Hartman, S.W. King, A.W. Raab, W.C. Randall, E.L. Englehardt and R.F. Hirschmann, J. Med. Chem., 21 (1978) 746.
2 M.L. Dobrinska, W. Kukovetz, E. Beubler, H.L. Leidy, H.J. Gomez, J.L. Demetriades and J.A. Bolognese, J. Pharmacokin. Biopharm., 10 (1982) 587.
3 S. Vickers, C.A. Duncan, S.D. White, G.O. Breault, R.B. Royds, P.J. DeSchepper and K.F. Tempero, Drug Metab. Dispos., 6 (1978) 640.
4 B.K. Kim and R.T. Koda, J. Pharm. Sci., 66 (1977) 1632.
5 C.R. Freed, R.J. Weinkam, K.L. Melmon and N. Castagnoli, Anal. Biochem., 78 (1977) 319.
6 J.R. Whatson and R.C. Lawrence, J. Chromatogr., 103 (1975) 63.
7 P.D. Walson, K.S. Marshall, R.P. Forsyth, R. Rapport, K.L. Melmon and N. Castagnoli, Jr., J. Pharmacol. Exp. Ther., 195 (1975) 151.

8 L.D. Mell and A.B. Gustafson, Clin. Chem., 24 (1978) 23.
9 H.J.L. Janssen, Thesis, State University of Leiden, The Netherlands, 1981, p. 69.
10 G.M. Kochak and W.D. Mason, J. Pharm. Sci., 69 (1980) 897.
11 M.J. Cooper, R.F. O'Dea and B.L. Mirkin, J. Chromatogr., 162 (1979) 601.
12 H. Ong, S. Sved and N. Beaudoin, J. Chromatogr., 229 (1982) 433.
13 J.A. Hoskins and S.B. Holliday, J. Chromatogr., 230 (1982) 162.
14 L.L. Ng, J. Chromatogr., 257 (1983) 345.
15 M.J. Peach, in L.S. Goodman and A. Gilman (Editors), The Pharmacological Basis of Therapeutics, Macmillan, New York, 5th ed., 1975, p. 804.
16 L. Sharyl and S.A. Dorrbecker, Drug Metab. Dispos., 4 (1976) 72.
17 Stenback, E. Myhra, H.E. Rugstad, E. Arnold and T. Hansen, Eur. J. Clin. Pharmacol., 12 (1977) 117.
18 S. Vickers, C.A.H. Duncan, H.G. Ramjit, C.T. Dollery, H.L. Leidy, D.G. Musson and W.C. Vincek, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 42 (1983) 914.

Journal of Chromatography, 308 (1984) 261—271
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2094

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR THE DETERMINATION OF CEFPIMIZOLE IN PLASMA AND URINE

D.B. LAKINGS* and J.M. WOZNIAK

Drug Metabolism Research, Pharmaceutical Research and Development, The Upjohn Company, Kalamazoo, MI 49001 (U.S.A.)

(First received July 12th, 1983; revised manuscript received January 25th, 1984)

SUMMARY

Quantitative analytical methods have been defined for the determination of cefpimizole, a new broad-spectrum cephalosporin antibiotic, in plasma and urine specimens. The methods employ ion-pair reversed-phase high-performace liquid chromatography with both ethylenediaminetetraacetic acid (EDTA) and tetrabutylammonium hydroxide as pairing agents for separation and ultraviolet detection at 254 nm. Sample preparation for plasma aliquots consisted of acetonitrile protein precipitation followed by phase separation; the aqueous phase was filtered and assayed. For urine, sample preparation consisted of diluting an aliquot with chromatographic eluent, filtering, and assaying. The methods had a linear range of 17—0.3 μg/ml for plasma and 800—15 μg/ml for urine and had sufficient precision and accuracy to provide quantitative data. Stability studies in plasma and urine indicated that cefpimizole degraded rapidly at room temperature. Addition of EDTA to the physiological fluid substantially increased the stability at room temperature, and little or no degradation was observed in plasma or urine stored at −30°C for over 100 days. Utility of the methods was demonstrated by assaying plasma and urine specimens obtained from a human volunteer receiving three dose levels. Estimates of various pharmacokinetic parameters are presented.

INTRODUCTION

Cefpimizole (I), 7-β-D-(−)-α-[4(5)-carboxyimidazole-5(4)-carboxiamido]-phenylacetamido-3-(4-β-sulfoethylpyridinium)-methyl-3-cephem-4-carboxylate, sodium salt (Fig. 1), is a third generation cephalosporin analogue and is being evaluated as a broad-spectrum antibiotic. This compound has reported potent activity against both Gram-positive and Gram-negative bacterial species including *Proteus vulgaris*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, *Streptococcus mitis*, and *Staphylococcus aureus* [1, 2].

0378-4347/84/$03.00

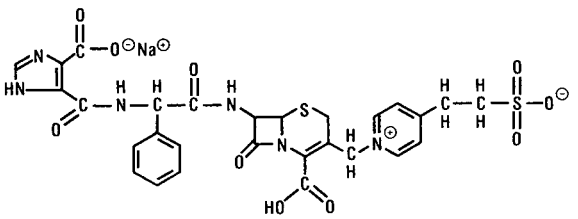


Fig. 1. Chemical structure of cefpimizole.

Pharmacokinetic and metabolism studies on I require a selective, quantitative assay. Previous studies on other cephalosporin analogues have employed high-performance liquid chromatography (HPLC) as the analytical technique for the determination of plasma and urine levels [3—7]. These methods employed reversed-phase HPLC or ion-pair HPLC to separate the cephalosporins from potentially interfering physiological fluid components. Previously reported pharmacokinetic evaluations on I in animals [8] and man [9] utilized microbiological techniques to determine serum and urinary levels of biological activity attributed to I. The results from the human studies indicated that the biological activity was rapidly excreted in the urine (80—90% of administered dose) and had a terminal serum half-life of 1.5—1.7 h. No definable metabolites of the antibiotic were observed and only minimal side effects were encountered in over 200 human patients. A bacteriological efficacy of 81.9% for a variety of Gram-negative strains was reported. While these data provide information of the disposition and efficacy of the antibiotic in humans, the methodology employed did not have adequate specificity to detect the parent compound or the precision and reproducibility for quantitative results.

This report describes quantitative HPLC methods for the determination of I in plasma and urine specimens. Applications of the methods are demonstrated by assaying for I in specimens obtained from a human volunteer and estimating various pharmacokinetic parameters from the data.

EXPERIMENTAL

Apparatus

A Laboratory Data Control (Riviera Beach, FL, U.S.A.) isocratic HPLC unit consisting of a Constametric III pump, UV Monitor III at 254 nm, and a Rheodyne (Cotati, CA, U.S.A.) Model 7125 variable-loop injector was employed. The HPLC column utilized was a Supelcosil LC-18, 5-μm particle size, 250 × 4.6 mm I.D. (Supelco, Bellefonte, PA, U.S.A.) with a Co:Pell ODS, 35-μm particle size, 50 × 2.1 mm I.D. guard column (Whatman, Clifton, NJ, U.S.A.). A Hamilton (Reno, NV, U.S.A.) Series 810RN 100-μl syringe was used to make injections.

Chemicals and reagents

Cefpimizole was supplied by the Pharmaceutical Research and Development Laboratories (Upjohn, Kalamazoo, MI, U.S.A.) and was used without further purification. Acetophenone, selected as the internal standard, and ethylenediaminetetraacetic acid (EDTA) were obtained from Aldrich (Milwaukee, WI,

U.S.A.). Tetrabutylammonium hydroxide (TBA) (Eastman-Kodak, Rochester, NY, U.S.A.) was 0.4 *M* titrant grade. Methanol and acetonitrile were UV, distilled-in-glass solvents (Burdick and Jackson Labs., Muskegon, MI, U.S.A.). All other chemicals used during these evaluations were of the highest purity available.

The final HPLC eluent was prepared by adding 40 ml of 0.1 *M* EDTA and 50 ml of 0.4 *M* TBA to 2510 ml distilled, deionized water. While stirring, 1400 ml methanol were added, and then the pH was adjusted to 6.0 with the dropwise addition of concentrated acetic acid. The prepared eluent was filtered through a 0.2-μm Nylon-66 (Rainin Instrument, Woburn, MA, U.S.A.) filter and helium-degassed for 45 min. The prepared eluent was stable for a minimum of five days; after that time, refiltration and degassing were performed to protect the analytical column. Prior to using the 0.1 *M* EDTA and 0.4 *M* titrant TBA solutions, they were filtered three times through 0.2-μm filters to remove particulate matter.

Sample preparation procedures

Plasma samples were prepared by adding 1.0 ml plasma to 1.0 ml of a solution containing 0.01 *M* EDTA and 0.05 *M* TBA, pH 5.0, and precipitating the plasma protein with the addition of 4.0 ml acetonitrile. After mixing, the plasma—acetonitrile mixture was allowed to stand a minimum of 2 h at 4° C to ensure complete protein precipitation. The sample was centrifuged and the supernatant transferred to a clean tube. The precipitate was washed with 2.0 ml (75:25, v/v) acetonitrile—0.01 *M* EDTA, 0.05 *M* TBA, pH 5.0; and after centrifugation, the wash was added to the original supernatant. Methylene chloride (200 μl) was added, and the solution was allowed to phase separate at —20° C. The upper organic layer was aspirated and discarded. The calculation internal standard (IS) (50 μl of a 200 μg/ml acetophenone in methanol solution) was added to the aqueous phase; the sample thoroughly mixed and filtered through a 0.45-μm filter (Acrodisc®-CR, Gelman, Ann Arbor, MI, U.S.A.). The prepared plasma sample was stored at 4° C until analysis by HPLC—UV (254 nm).

Urine samples were prepared for analysis by quantitatively pipetting a 100-μl urine aliquot into 4.0 ml HPLC eluent containing 10 μg/ml IS. The solution was filtered through a 0.45-μm filter and stored at 4° C until assay.

Plasma and urine specimens

Plasma and urine specimens were obtained from a normal volunteer who was administered three doses of I (1000, 2000, and 4000 mg) with a one-week wash-out period between doses. The drug was administered by intravenous infusion over a 20-min period. Blood specimens were obtained at 0 (predose), 0.33, 0.67, 1.0, 2.0, 4.0, 6.0, 8.0, 12.0, and 24.0 h using Vacutainers containing EDTA as anticoagulant and preservative. The blood specimens were centrifuged immediately after collection, and the plasma frozen and maintained at —30° C or lower until analysis. Urine specimens were obtained during the following collection periods: 0 (predose), 0—0.5, 0.5—0.75, 0.75—1.0, 1.0—2.0, 2.0—6.0, 6.0—12.0, 12—24, and 24—48 h after administration of the drug. During each collection period, urine was placed in a fask containing EDTA

as preservative and maintained at 4°C. After the collection period, the specimen was mixed, the urine volume measured, and an aliquot frozen and maintained at −30°C or lower until analysis.

Calculations

The level of I in plasma and urine specimens was calculated using the relative weight response (RWR, eqn. 1). Reference solutions of I in water for plasma and HPLC eluent for urine over a concentration range were analyzed with each sample set and the level of I in the sample aliquot determined by eqn. 2. Since some loss of I during the sample preparation was expected, fortified blank plasmas or urines over the concentration range were prepared with the sample set and the absolute recovery of I determined by eqn. 3. The level of I in the plasma and urine samples was corrected for sample preparation loss by eqn. 4. The method of calculation provided reference to a known quantity of I and served as a check on variations in the recovery of I between sample sets.

$$\text{RWR} = \frac{\text{Peak height I (standard)}}{\text{Peak height IS}} \times \frac{\mu\text{g IS}}{\mu\text{g I (standard)}} \tag{1}$$

$$\mu\text{g I/aliquot} = \frac{\text{Peak height I (sample)}}{\text{Peak height IS}} \times \frac{\mu\text{g IS}}{\text{average RWR}} \tag{2}$$

$$\text{Recovery I} = \frac{\mu\text{g I/aliquot found (fortified)}}{\mu\text{g I added}} \tag{3}$$

$$\text{Corrected } \mu\text{g/aliquot} = \frac{\mu\text{g I/aliquot}}{\text{average recovery}} \tag{4}$$

RESULTS AND DISCUSSION

HPLC parameter definition

Initial evaluations indicated that simple reversed-phase HPLC techniques employed for other cephalosporins did not provide acceptable chromatographic peak shape or retention for I. Likewise, single ion-pairing HPLC techniques reported for other cephalosporins either resulted in no retention (hexanesulfonic acid) or complete retention (TBA) of I. A combination of acidic and basic ion-pairing reagents in a methanol—water eluent gave some retention of the compound, and further evaluations indicated that a combination of EDTA and TBA gave acceptable peak shape. Experiments were conducted to optimize the pH, EDTA, TBA, and methanol content of the eluent and the following characteristics were observed: (a) at EDTA and TBA levels below 0.0005 *M* and 0.0025 *M*, respectively, the retention volume of I decreased substantially, but the retention volume was not affected by higher concentrations; (b) the retention volume was fairly consistent between pH values of 5.5 to 6.8, however, below pH 5.5 the retention volume decreased as the pH was lowered; (c) a 32—38% methanol level gave a retention volume between 8 and 16 ml and a sharp, well defined peak; and (d) increased column temperatures did not improve the chromatographic characteristics.

After establishing the HPLC eluent, an IS was selected. Since a homologue of I was not available and the HPLC eluent was different from the eluents employed for other cephalosporins, acetophenone, which had good chromatographic characteristics in the defined analytical system, was selected as IS.

Linearity and precision of the analytical technique

The HPLC analytical technique was evaluated for linearity and reproducibility by preparing and analyzing concentration series of I from 0.5 to 20 μg/ml. Each of the six reference solution levels was prepared and assayed in duplicate on four separate days. Excellent precision was obtained at each concentration with a relative standard deviation (R.S.D.) range for the eight determinations of 2.9—5.5%. A least-squares linear regression evaluation of the peak height ratio versus concentration gave $Y = (0.197 \pm 0.012)X - (0.010 \pm 0.011)$ with a correlation coefficient of 0.999. The Y-intercept was statistically insignificant ($p > 0.05$ with 95% confidence). The slope of the linear-regression equation was directly proportional to the relative weight response of I since the intercept was statistically equal to zero.

Sample preparation procedure for plasma/serum samples

Techniques commonly employed to isolate a compound from plasma/serum protein, i.e. liquid—liquid extraction and protein precipitation, were evaluated for I. The ionic nature of the compound prevented extraction from aqueous solution even when the ionic groups of the antibiotic were ion-paired with EDTA and TBA. Protein precipitation with acetonitrile, perchloric acid, or trichloroacetic acid resulted in co-precipitation of I. When EDTA and TBA were added to the physiological fluid prior to protein precipitation, recovery of the compound in the supernatant was obtained with acetonitrile but not with the acids as the precipitating reagent. However, two volumes of acetonitrile for each aqueous volume were required for complete protein precipitation, and the increased volume of the final solution prevented quantification at the 1 μg/ml level. Increasing the injection volume resulted in poor chromatography of I, thus preventing this technique from providing the necessary sensitivity. Concentration of the supernatant at approximately 35° C under a stream of nitrogen gave acceptable chromatography and met the sensitivity requirement. This sample preparation technique was employed to optimize the conditions, i.e. EDTA, TBA level; pH; sample handling, which gave the highest recovery of the compound with minimal interference from plasma components. The addition of 1.0 ml of a 0.05 *M* TBA—0.01 *M* EDTA, pH 5.0 solution to 1.0 ml serum/plasma and protein precipitation with 4 ml acetonitrile gave approximately 70% recovery of I. At lower TBA, EDTA levels, lower pH values, or less than 1.0 ml added aqueous solution, the recovery was decreased. To improve the recovery, the protein precipitate was washed with a (75:25, v/v) acetonitrile—0.05 *M* TBA, 0.01 *M* EDTA, pH 5.0 solution and the wash added to the original supernatant prior to concentration. This procedure gave 75—85% recovery of the compound; however, the concentration step was time-

consuming. Earlier experiments [10] had shown that I had limited stability in aqueous solution at room temperature. Because of this limited stability, the concentration step was a potential source of error as well as requiring substantial time. Two reported techniques, ultrafiltration [6] and acetonitrile precipitation followed by methylene chloride extraction [7] were evaluated. Ultrafiltration of plasma or serum gave HPLC chromatograms which had interfering plasma components at the elution position of I and the IS. The plasma components prevented quantitation below the 2 μg/ml level. The precipitation—extraction technique was modified because a partial phase separation had been observed earlier (attributed to the high salt content and 4°C employed to ensure complete protein precipitation). To ensure maximum phase separation, 200 μl methylene chloride were added and the sample frozen. After thawing, the upper organic layer was aspired. The aqueous layer contained 75—85% of I, and the chromatogram was relatively free of plasma components. The acetonitrile—methylene chloride layer had no detectable I. Representative HPLC chromatograms of a plasma blank and sample are shown in Fig. 2.

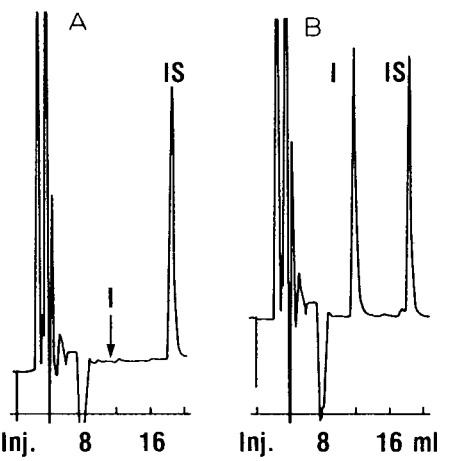


Fig. 2. HPLC—UV chromatograms of cefpimizole isolated from plasma; subject 1 given a 2000-mg dose. (A) Predose plasma; (B) 6-h plasma.

Sample preparation procedure for urine samples

The microbiology results [4] indicated that a relatively high urinary concentration of the parent compound (high μg/ml to mg/ml) could be expected. A simple procedure where a 100-μl aliquot of urine was diluted with 4.0 ml of HPLC eluent containing the IS and the solution filtered through a 0.45-μm filter provided an acceptable chromatogram with a quantifiable sensitivity of 15 μg/ml urine. Representative HPLC chromatograms of a urine blank and sample are shown in Fig. 3.

Linearity, precision and reproducibility of the sample preparation procedures

The procedures for the isolation of I in plasma and urine were validated for linearity, precision, and reproducibility by fortifying blank plasma and urine samples. For plasma samples, the concentration range was 17—0.33 μg/ml; and for urine was 800—17 μg/ml. The results of these validation studies are

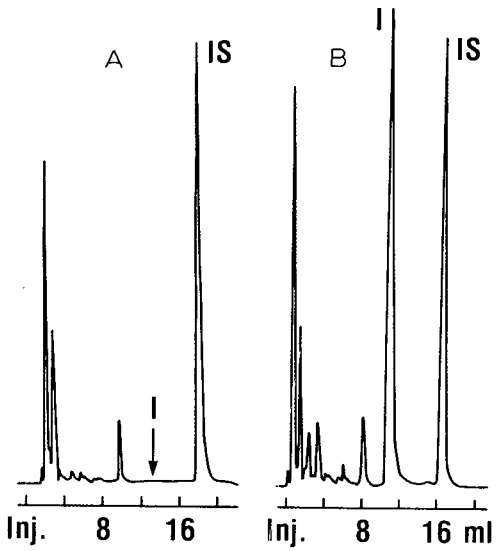


Fig. 3. HPLC—UV chromatograms of cefpimizole in urine; subject 1 given a 2000-mg dose. (A) Predose urine; (B) 6-h urine.

TABLE I

LINEARITY AND REPRODUCIBILITY OF PLASMA AND URINE DETERMINATION OF I

Plasma, 1.0-ml aliquots, n = 5, average recovery = 78.7 ± 1.3%. Urine, 100-μl aliquots, n = 4, average recovery = 99.3 ± 2.4%.

Plasma			Urine		
Concentration (μg/ml)	Average found (μg/ml)	Relative standard deviation	Concentration (μg/ml)	Average found (μg/ml)	Relative standard deviation
16.78	13.51	3.8	839	864	2.8
13.42	10.79	1.9	671	686	2.3
10.07	7.91	3.6	503	508	2.8
6.71	5.34	2.6	420	422	1.6
5.03	3.90	3.6	335	336	2.3
3.36	2.65	2.6	252	252	2.0
2.52	1.97	6.3	210	213	1.6
1.68	1.29	5.6	168	168	1.0
1.34	1.04	5.6	126	125	1.2
1.01	0.78	9.3	83.9	81.6	3.4
0.67	0.52	10.5	67.1	64.6	2.0
0.33	0.27	11.9	50.3	50.0	2.7
0	<0.05	—	33.6	32.7	2.8
			16.8	15.9	7.7
			0	<1.0	—

summarized in Table I. The least-squares linear-regression equations and correlation coefficients were $Y = (0.804 \pm 0.004)X - (0.044 \pm 0.031)$, r = 0.999 for plasma, and $Y = (1.028 \pm 0.005)X - (4.6 \pm 1.7)$, r = 0.999 for urine. The Y-intercept for the plasma and urine specimens was statistically

insignificant ($p > 0.05$). The minimal quantifiable level (defined as the lowest level assayed with an R.S.D. of less than 15% for multiple prepared samples on separate days) for plasma was 0.33 μg/ml and for urine was 16.8 μg/ml. The detection limit (based on signal-to-noise ratio of 5:1) was 0.05 μg/ml for plasma and 1.0 μg/ml for urine.

Stability in physiological fluids

The limited stability of I in aqueous solution indicated that stability studies in physiological fluids were necessary. The compound was added to plasma, serum, and urine and maintained at room temperature. At timed intervals, aliquots were taken and analyzed by the procedure described earlier. The results are shown graphically in Fig. 4 where the percent of the compound remaining (% T_0) is plotted versus time. Very rapid loss of I was observed with 80% of the original level being detected at 1 h for plasma, 4.5 h for serum, and 27 h for urine. The least-square linear-regression equations given in Fig. 4 showed that the loss of I in each matrix was linear ($r^2 > 0.96$) and the slope significant ($p < 0.05$). These data indicated that pharmacokinetic evaluations of I would be subject to substantial error due to the rapid loss of the compound in physiological samples.

When EDTA was added to plasma or urine containing I, a substantial increase in the room temperature stability was observed (Fig. 5). The 80% T_0 level was reached at 48 h for plasma containing EDTA and at 240 h for urine containing EDTA. Additional studies at −30° C for plasma—EDTA and urine—EDTA fortified with I indicated that collected specimens would be stable (greater than 95% of original level) for over 100 days when stored at −30° C.

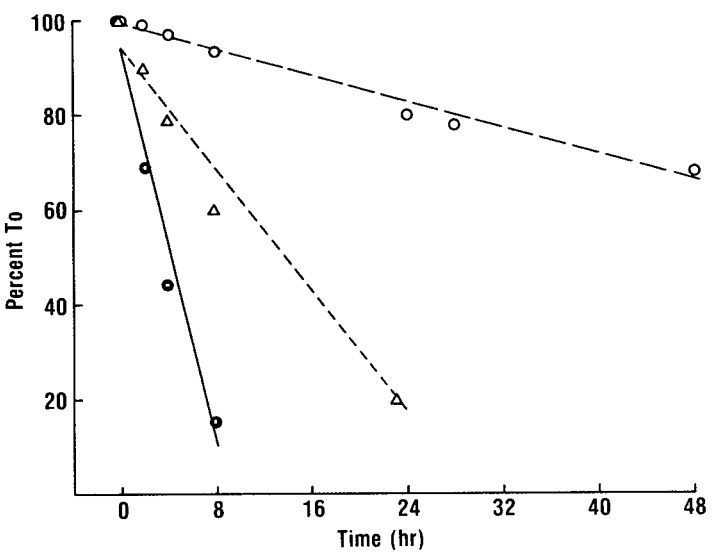


Fig. 4. Stability of cefpimizole in plasma, serum and urine samples maintained at room temperature. (•—•) Plasma; (△— —△) serum; (○— — ○) urine. Linear regression: plasma, $Y = (-10.4 \pm 0.8X) + (93.2 \pm 3.8)$, $r^2 = 0.96$; serum, $Y = (-3.17 \pm 0.22X) + (93.5 \pm 2.5)$, $r^2 = 0.96$; urine, $Y = (-0.68 \pm 0.04X) + (99.1 \pm 0.9)$, $r^2 = 0.96$.

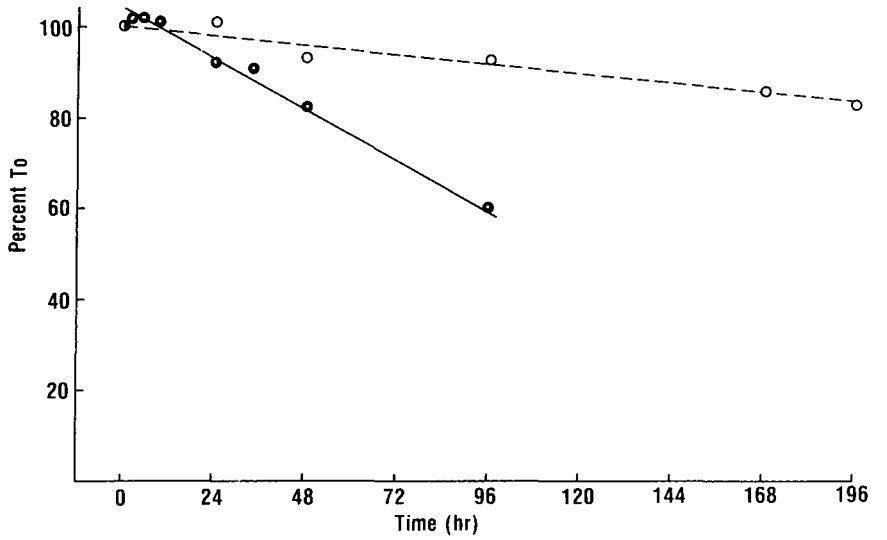


Fig. 5. Stability of cefpimizole in plasma—EDTA and urine—EDTA samples maintained at room temperature. (•—•) Plasma; (○— —○) urine. Linear regression: plasma, $Y = (-0.471 \pm 0.020X) + (104.1 \pm 0.8)$, $r^2 = 0.98$; urine, $Y = (-0.085 \pm 0.005X) + (100.0 \pm 1.0)$, $r^2 = 0.96$.

Plasma and urine specimen analysis

The utility of the developed HPLC methods for I in plasma and urine specimens was demonstrated by preparing and assaying samples obtained from a human volunteer receiving three intravenous doses of the drug.

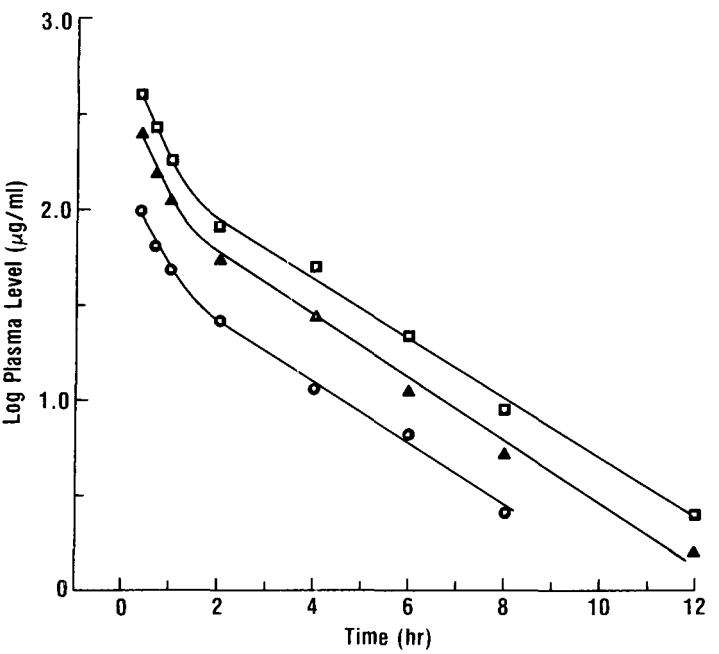


Fig. 6. Plasma concentration—time plot of cefpimizole from a human volunteer. Intravenous administration of three dose levels: (▪—▪) 4000-mg dose; (▲—▲) 2000-mg dose; (•—•) 1000-mg dose.

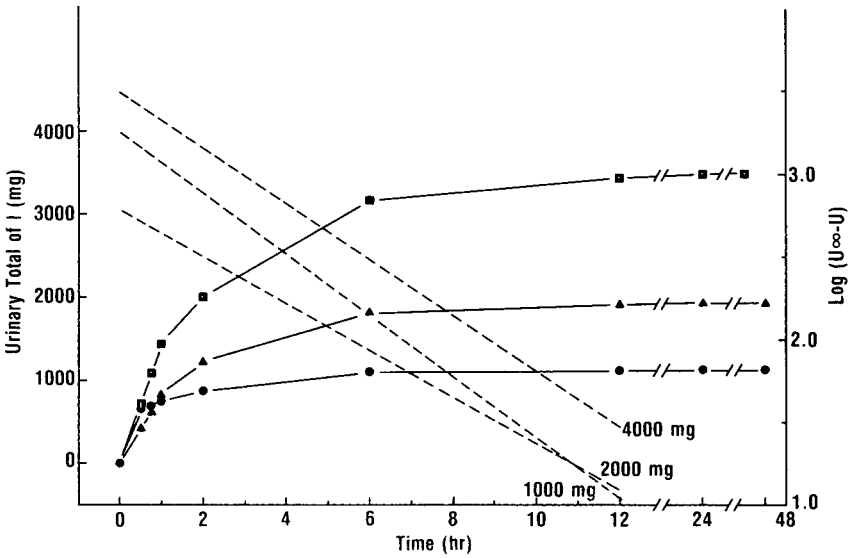


Fig. 7. Cumulative urinary excretion—time plot of cefpimizole from a human volunteer. Dashed lines represent log $(U_T - U)$—time plots for each dose level. (▪—▪) 4000-mg dose; (▲—▲) 2000 mg dose; (●—●) 1000-mg dose.

The results for the plasma assays of I are shown graphically in Fig. 6 where the log plasma concentration versus time is plotted for the three dose levels. The data indicate a two-compartment body model. Since the dose was administered by constant-rate infusion over a 20-min period and no samples were obtained during this time, the plasma level of the drug during the infusion process is not included. The urine assay results are shown in Fig. 7 where the cumulative amount excreted is plotted against time. The dashed lines represent least-squares linear-regression plots of the log $(U_T - U)$, i.e., total amount excreted − amount excreted to time t, versus time and indicate a first-order elimination process for I. The average amount of the unchanged drug excreted in the urine was over 75% during the first 6 h after administration.

Estimates of various pharmacokinetic parameters were made by subjecting the plasma and urine data to computer fitting using a NONLIN [11] program for a two-compartment constant-rate infusion model. The estimated plasma pharmacokinetic parameters (Table II) were fairly uniform at the three dose levels indicating that the drug was behaving similarly independent of dose, i.e. dose-independent pharmacokinetics. The urine data was evaluated graphically

TABLE II

PHARMACOKINETIC PARAMETER ESTIMATES FOR I FROM A HUMAN VOLUNTEER

Dose (mg)	Distribution rate constant α (h^{-1})	Terminal rate constant β (h^{-1})	Volume of distribution V_d (l)	Area under curve AUC (μg h/ml)	Total body clearance Cl_T (ml/min)	Elimination rate constant K_e (h^{-1})	Percent dose excreted (%)
1000	2.31	0.366	16.1	169.8	98.2	0.322	114.4
2000	1.98	0.349	15.0	391.1	85.2	0.424	97.2
3000	2.32	0.358	17.2	648.9	102.7	0.352	87.5

by plotting the log ($U_T - U$) versus time to estimate the urinary elimination rate constant (K_e) (Table II). As with the plasma pharmacokinetic parameters, the K_e and percent dose excreted were similar at the three dose levels indicating that the excretion of I was independent of dose. Additional pharmacokinetic evaluations of I are planned to more fully define the physiological distribution and elimination process for this drug.

ACKNOWLEDGEMENTS

The authors wish to express their appreciation to H.R. Allen who provided the clinical samples and to D.J. Weber, W.J. Adams, Gary Elfring and H. Ko for their helpful suggestions in defining the pharmacokinetic parameter estimates.

REFERENCES

1 G.E. Zurenko, G.P. Li, R.J. Yancey, M.L. Kinney, K.F. Stern and C.W. Ford, Twenty-Second Interscience Conference on Antimicrobial Agents and Chemotherapy, Miami Beach, FL, October 4—6, 1982, Amer. Soc. Microbiol., Washington, DC, paper 206.
2 H.C. Neu and P. Labthavidul, Twenty-Second Interscience Conference on Antimicrobial Agents and Chemotherapy, Miami Beach, FL, October 4—6, 1982, Amer. Soc. Microbiol., Washington, DC, paper 107.
3 M.C. Nahata, J. Chromatogr., 228 (1982) 429—433.
4 J.B. Lecaillon, M.C. Rouan, C. Souppart, N. Febvre and F. Juge, J. Chromatogr., 228 (1982) 257—267.
5 A.M. Birsson, J.B. Fourtillan and G. Berthon, J. Chromatogr. 233 (1983) 386—391.
6 G.R. Granneman and L.T. Sennello, J. Chromatogr., 229 (1982) 149—157.
7 R.E. Bawdon, D.L. Hemsell, and S.P. Guss, Antimicrob. Ag. Chemother., 22 (1982) 999—1003.
8 N. Kato, T. Matsuzawa, Y. Yugari and A. Saito, Twenty-Second Interscience Conference on Antimicrobial Agents and Chemotherapy, Miami Beach, FL, October 4—6, 1982, Amer. Soc. Microbiol., Washington, DC, paper 108.
9 J. Shimado, Y. Ueda, K. Hara, A. Saito, T. Nishiura, T. Yamamoto and K. Nakagawa, Twenty-Second Interscience Conference on Antimicrobial Agents and Chemotherapy, Miami Beach, FL, October 4—6, 1982, Amer. Soc. Microbiol., Washington, DC, paper 209.
10 D.B. Lakings, unpublished results.
11 C.M. Metzler, G.L. Elfring and A.J. McEwen, Biometrics, September (1974) 562.

Journal of Chromatography, 308 (1984) 273—281
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2100

DETERMINATION OF GLAUCINE IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

JEAN-PIERRE FELS*, PATRICK LECHAT, RAYMOND RISPE and WERNER CAUTREELS

Centre de Recherches Clin-Midy, Rue du Professeur J. Blayac, 34082 Montpellier (France)

(First received December 22nd, 1983; revised manuscript received February 8th, 1984)

SUMMARY

A sensitive method is described for the measurement of *d*-glaucine in pharmacokinetic studies using only 100-μl plasma samples or 200-μl urine samples. It requires a simple extraction clean-up on kieselguhr micro-columns and straight-phase high-performance liquid chromatography with fluorescence detection. Data on selectivity, sensitivity and precision demonstrate the reliability of this method. Its applicability is revealed by single and repeated oral administration pharmacokinetic studies in human subjects.

INTRODUCTION

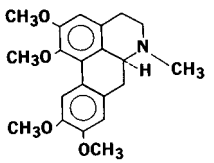
d-Glaucine (Fig. 1) is an aporphine alkaloid first isolated from *Glaucium flavum* Crantz (Papaveraceae) [1]. The drug is used widely in East Europe as a cough suppressant. The optical isomer, *l*-glaucine, and the racemic *dl*-glaucine seem to have a similar therapeutic effect [2].

Spectrophotometric and high-performance liquid chromatographic (HPLC) methods have been described for quantitative determination of *d*-glaucine [3—5]. None of these methods is sufficiently sensitive and specific for the determination of the drug after administration in the therapeutic range.

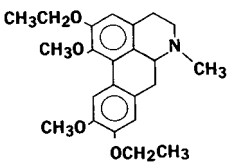
The fluorescent property of boldine, another aporphine, was previously described [6]. Glaucine is structurally related and shows a fluorescence spectrum close to that of boldine.

The aim of this paper was to develop a selective and highly sensitive method, based on the fluorescence behaviour of glaucine, in order to obtain pharmacokinetic data.

0378-4347/84/$03.00



[I]



[II]

Fig. 1. Chemical structures of *d*-glaucine [I] and diethyl boldine (internal standard) [II].

MATERIALS AND METHODS

Chemicals and reagents

d-Glaucine hydrochloride and the internal standard diethyl boldine hydrochloride dihydrate were supplied by Clin-Midy (Montpellier, France). *d*-[6a-^{14}C] Glaucine base (21 mCi/mmol) was obtained from CEA (Saclay, France). Analytical reagent grade *n*-hexane, tetrahydrofuran and diethylamine were purchased from Merck (Darmstadt, F.R.G.); methanol was from Farmitalia Carlo Erba (Milan, Italy), sodium hydroxide, ammonium chloride and ammonia 20% were from Prolabo (Paris, France).

Preparation of micro-columns

Kieselguhr extraction micro-columns were prepared in disposable polypropylene blue tips for Eppendorf pipettes (Hamburg, F.R.G.) They were filled with 0.1 g of kieselguhr (Extrelut Art. 11738, Merck). Glass-wool plugs were inserted to serve as bed support and avoid dispersion of the porous matrix.

Apparatus

The HPLC system consisted of a Model 5000 (Varian, Palo Alto, CA, U.S.A.) solvent delivery pump, a Model 710 B WISP automatic sample injector (Waters Assoc., Milford, MA, U.S.A.) and a Model JY3D spectrofluorimetric detector (Jobin Yvon Instruments, Paris, France) equipped with a 20-μl flow cell. The output signal was fed to a Model SP 4100 (Spectra-Physics, Santa Clara, CA, U.S.A.) integrator for recording and calculation of peak area.

The separation units consisted of a prepacked column of LiChrosorb Si 60 Merck (5-μm irregularly shaped particles), 125 mm $\times$ 4 mm I.D. Chromatographic conditions are given in Fig. 2. Other equipment included a vortex type mixer and an ultrasonic bath for degassing solvents.

Preparation of standards

Standards were prepared by dissolving compounds as salt forms in methanol. Two stock standard solutions were made each month, one containing 1 mg/ml

of *d*-glaucine base and the other containing 1 mg/ml diethyl boldine base. Working standards of 100 μg/ml to 10 ng/ml were prepared weekly by appropriate dilution of the stock solutions.

Procedure for plasma and urine extraction

Before addition of biological fluid each sample tube received an adjusted amount of internal standard as a methanolic solution evaporated to dryness under nitrogen at 50°C.

Plasma. A 100-μl aliquot of plasma was introduced into a sample tube and 200 μl of 0.05 *M* sodium hydroxide aqueous solution were added. The stoppered tube was vortexed for 30 sec, then the sample was applied on a kieselguhr micro-column. After equilibration for 15 min, 5 ml of *n*-hexane were added to the micro-column. The eluate was evaporated at 50°C in a conical tube under a gentle nitrogen steam; the sample residue was reconstituted with 100 μl of mobile phase and a 20-μl aliquot was injected into the HPLC column.

Urine. The procedure for urine was slightly different. To a kieselguhr micro-column were added 250 μl of a mixture of urine—0.1 *M* NH_4Cl—NH_3 buffer pH 8.1 (2:1). Other operations remained unchanged.

Quantitation

The concentration of *d*-glaucine in plasma and urine was determined from eight-point calibration curves of peak area ratios (*d*-glaucine and diethyl boldine) versus *d*-glaucine concentration expressed as free base in plasma and urine carried through the described procedures. A new calibration curve was made with each sample set.

RESULTS AND DISCUSSION

Method

Fig. 2 illustrates the chromatographic separation of *d*-glaucine in biological samples. Diethyl boldine was selected as an internal standard on the basis of the resolution achieved and the similarity of its fluorescence spectrum to that of *d*-glaucine.

The final composition of the mobile phase was the result of systematic work on silica thin-layer chromatography then extrapolation to HPLC plain silica column until adequate resolution was obtained. Diethylamine was added as a mobile phase modifier to avoid peak tailing.

A simple way of extracting glaucine is to operate in a slightly basic aqueous medium (pH 8) with *n*-hexane as extracting solvent. Unfortunately an emulsion often formed in biological samples when this procedure was used. To obtain cleaner extracts of small sample volumes extraction using kieselguhr micro-columns appeared to be suitable. The dilution and the volume of biological samples were adjusted as a function of porous matrix weight. The analytical recovery of extraction was determined by radiotracer techniques. A trace amount of *d*-[^{14}C]glaucine was added to the biological sample before the extraction clean-up step. The recovery measured by liquid scintillation counting prior to HPLC was 79.5% (n = 10, S.D. = 4) in plasma and 84.4% (n = 12, S.D. = 2) in urine.

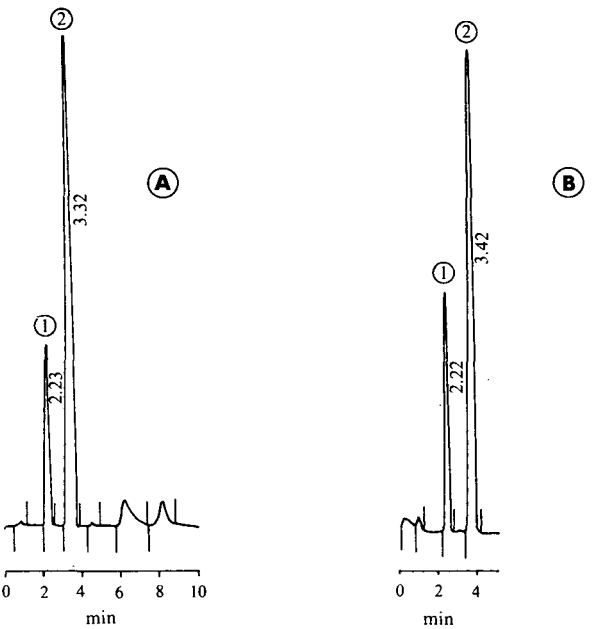


Fig. 2. (A) Typical HPLC chromatogram obtained from urine sample of a treated subject. (B) Typical HPLC chromatogram obtained from plasma sample of a treated subject. Operating conditions: column 125 mm × 4 mm I.D. LiChrosorb Si 60 (5 μm). Mobile phase: *n*-hexane—methanol—tetrahydrofuran—diethylamine (88.5:7.5:4:0.15). Flow-rate: 1.5 ml/min. Injection volume: 20 μl. Fluorescence detection: excitation wavelength 310 nm, emission 340 nm. Peaks: 1 = diethyl boldine (internal standard); 2 = *d*-glaucine.

Calibration curves of peak area ratio versus concentration were obtained by analysing plasma and urine standards containing *d*-glaucine in concentrations usually ranging from 10 to 500 ng/ml, but the curves were rectilinear for at least 0—5 μg/ml. The equations of the curves (10—500 ng/ml) were calculated by least-squares linear regression. A good linear relationship was obtained in the concentration range studied: correlation coefficients for 25 standard curves averaged 0.999 ± 0.0010 (± S.D.), with intercepts not significantly different from zero.

The peaks representing *d*-glaucine and the internal standard were symmetrical and well removed from the injection front and interfering peaks encountered in biological material. Attempts to quantitate *d*-glaucine by either gas chromato-

TABLE I

COMPARISON BETWEEN *d*-GLAUCINE CONCENTRATIONS OBTAINED BY MASS SPECTROMETRY WITH FIELD IONIZATION AND BY HPLC

Concentrations are expressed as ng ml^{-1}.

MS—field ionization	HPLC
97	90
190	175
17	20
205	200

graphy (GC) or gas chromatography—mass spectrometry (GC—MS) were unsuccessful. Adsorption on GC packing materials and thermal decomposition were the basis of the difficulties encountered. Hence, MS with field ionization — a mild ionization technique — was used with favourable results as reference method. The agreement between HPLC and MS methods was acceptable with the sample set checked (Table I).

The HPLC assay proved to be quite sensitive with a lower limit of 5 ng/ml for plasma and 2 ng/ml for urine, and a blank value equal to zero.

Duplicate measurements were performed on plasma and urine from treated subjects. The values were classified into three groups according to the concentration of *d*-glaucine. The differences d_i between the number (N) of duplicate measurements enables the estimation of the standard deviation (S.D.) which characterizes the reproducibility:

$$\text{S.D.} = \sqrt{\frac{\Sigma d_i^2}{2N}}$$

This estimation has been made for concentration ranges where the standard deviation is supposed to remain constant. The results for plasma in Table II indicate, as would be expected, a decrease of precision with decreasing *d*-glaucine concentration. The estimated precision in urine remains equal to 5% (Table III). This result is consistent with a better sensitivity in urine samples than in plasma samples.

TABLE II

PRECISION OF *d*-GLAUCINE DETERMINATION IN PLASMA

Plasma concentration (C_p) range (ng/ml)	Number of duplicate measurements	$\bar{x}_p$*	S.D.	C.V. (%)**
$5 < C_p \leq 20$	10	13.24	2.35	17.8
$20 < C_p \leq 50$	10	34.52	3.00	8.7
$C_p > 50$	10	147.34	4.74	3.2

*Mean value of C_p.

**C.V. (%) = $\frac{100 \text{ S.D.}}{\bar{x}_p}$.

TABLE III

PRECISION OF *d*-GLAUCINE DETERMINATION IN URINE

Urine concentration (C_u) range (ng/ml)	Number of duplicate measurements	$\bar{x}_u$*	S.D.	C.V. (%)**
$15 < C_u \leq 50$	5	23.44	1.30	5.6
$50 < C_u \leq 150$	6	91.5	5.41	5.9
$C_u > 150$	5	329.1	17.40	5.3

*Mean value of C_u.

**C.V. (%) = $\frac{100 \text{ S.D.}}{\bar{x}_{..}}$.

Application to biological samples

The assay was applied to the determination of *d*-glaucine in plasma and urine after single oral administration and repeated oral administration of the drug to

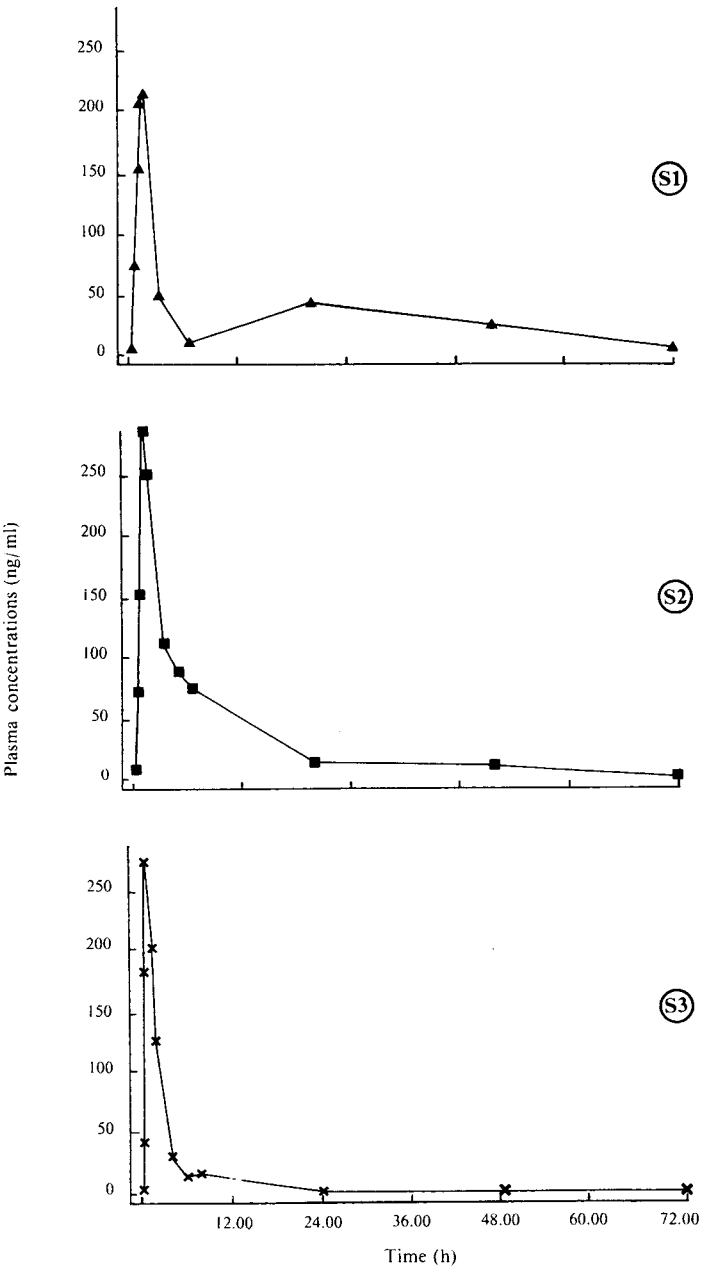


Fig. 3. Plasma concentration—time curves of *d*-glaucine from three healthy volunteers after an oral administration at a dose of 60 mg. Blood samples were collected at the following times: 0.00, 0.17, 0.33, 0.50, 0.75, 1.00, 1.50, 2.00, 4.00, 6.00, 8.00, 24.00, 48.00, 72.00 h after administration. Each concentration value is the mean of duplicate determinations.

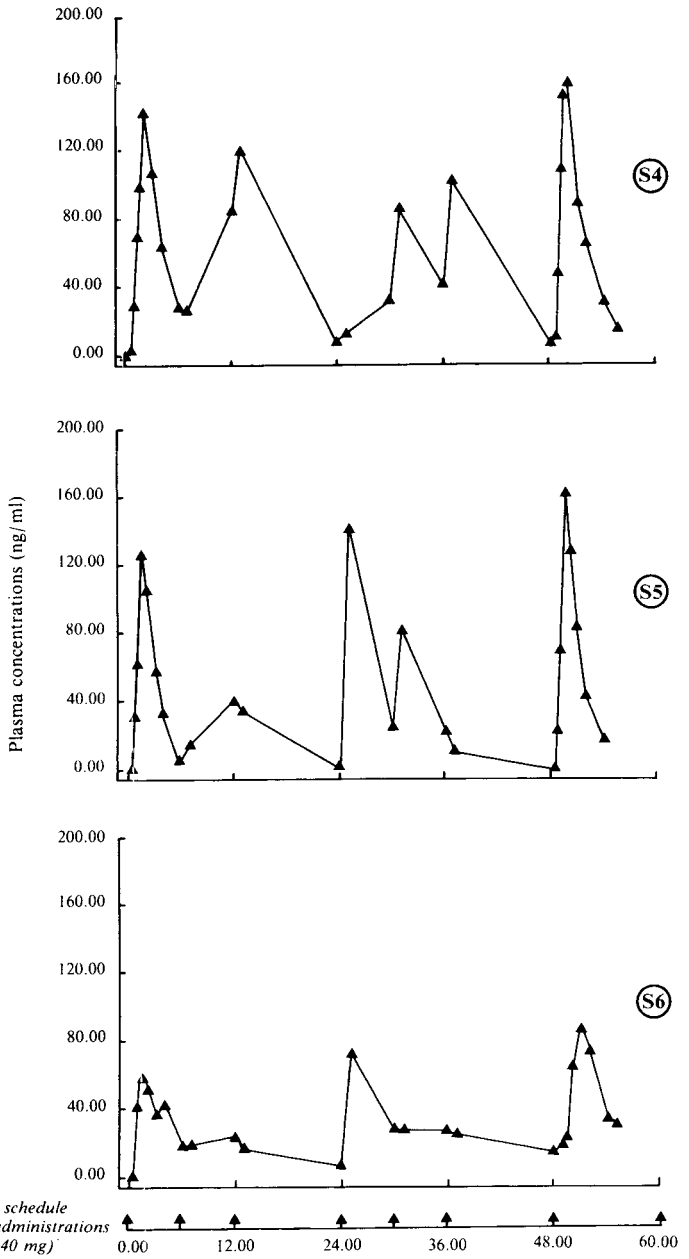


Fig. 4. Plasma concentration—time curves of *d*-glaucine from three healthy volunteers after repeated oral administration. Doses of 40 mg were given at following times: 0, 6, 12, 24, 30, 36, 48 h. Blood samples were collected at: 0.00, 0.17, 0.50, 0.75, 1.00, 1.50, 2.00, 3.00, 4.00, 6.00, 7.00, 12.00, 13.00, 24.00, 25.00, 31.00, 32.00, 36.00, 37.00, 48.00, 48.17, 48.50, 48.75, 49.00, 49.50, 50.00, 51.00, 52.00, 54.00, 55.00, 72.00, 96.00 h after the first administration. Each concentration value is the mean of duplicate determinations.

TABLE IV

URINARY EXCRETION OF *d*-GLAUCINE IN THREE SUBJECTS AFTER A SINGLE ORAL ADMINISTRATION OF 60 mg

Period of time (h)	*d*-Glaucine (μg)		
	S1	S2	S3
0— 8	53.2	140.8	68.5
8—24	6.7	16.9	10.5
24—48	2.0	2.2	0
48—72	0	1.0	0

TABLE V

SOME PHARMACOKINETIC PARAMETERS OF *d*-GLAUCINE OBTAINED AFTER A SINGLE ORAL ADMINISTRATION OF 60 mg

Parameter	S1	S2	S3
Weight (kg)	88	68	89
AUC (0—72 h)* (ng ml^{-1} h)	2076	2088	685
T_{max}** (h)	1.5	2	0.75
C_{max}*** (ng ml^{-1})	200	285	255
Urinary excretion (0—72 h) (percentage of dose)	0.10	0.27	0.13

*Area under the plasma drug concentration—time curve from 0 to 72 h.
**Time to reach maximum plasma drug concentration.
***Maximum plasma drug concentration.

healthy volunteers. Three subjects received a single oral dose of 60 mg and three others received seven doses of 40 mg each, given over a period of three days. The plasma concentration curves are shown in Figs. 3 and 4. Urinary excretion and some pharmacokinetic parameters were calculated after a single administration of the drug (Tables IV and V).

CONCLUSION

An HPLC assay has been developed for the analysis of *d*-glaucine in plasma and urine. This method, which involves rapid sample clean-up on micro-columns, is simple, selective and sensitive and requires only 100 μl of plasma or 200 μl of urine. Our procedure has been used for over one year with human and animal samples and has shown to be reliable and very reproducible in the long run.

The method could be a valuable tool for the further elucidation of human pharmacokinetics and animal toxicokinetics which are of great importance for establishing a relation between, on the one hand, glaucine plasma levels and, on the other hand, clinical and toxic effects [7]. In addition, the method may be suitable for *l*-glaucine and *dl*-glaucine assays.

ACKNOWLEDGEMENT

The authors are indebted to Mrs J.C. Prome (CNRS Toulouse) for mass spectrometric determination using field ionization.

REFERENCES

1 F.M. Probst, Justus Liebigs Ann. Chem., 31 (1839) 241.
2 P. Dierckx, G. Leblanc, A. Decoster and D. Criscuolo, Int. J. Clin. Pharmacol. Ther. Toxicol., 19 (1981) 396.
3 P.J. Davis, J. Chromatogr., 193 (1980) 170.
4 L.V. Benzel and L.Y. Ladnaya, Farm. Zh., 38 (1983) 48.
5 L.V. Benzel, L.Y. Ladnaya and D.Y. Rogovsky, Farmatsiya, 32 (1983) 49.
6 T. Gurkan, Mikrochim. Acta, 1 (1976) 173.
7 W. Cautreels, H. Davi and J.P. Jeanniot, in Proceedings of Eighth European Workshop on Drug Metabolism, Liège, September 5—9, 1982, University of Liège, Sart Tilman, 1983, p. 67.

Journal of Chromatography, 308 (1984) 282—288
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2070

Note

Determination of bound cellular fatty acids in *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* by gas chromatography and gas chromatography—mass spectrometry

ILIA BRONDZ*

Department of Chemistry, University of Oslo, Blindern, Oslo 3 (Norway)

and

INGAR OLSEN

Department of Microbiology, Dental Faculty, University of Oslo, Blindern, Oslo 3 (Norway)

(First received October 4th, 1983; revised manuscript received January 12th, 1984)

In some bacterial cells, fatty acids occur as free acids. Most bacterial acids, however, are linked to larger molecules, such as phospholipids, glycolipids, lipoproteins, lipopolysaccharides, and lipotechoic acids [1]. Unlike free lipids, which can be extracted with appropriate solvents, bound lipids require acid or alkaline hydrolysis to be released. Bound lipids have been found to differ markedly from free lipids in bacteria such as *Pseudomonas*, *Alcaligenes*, and possibly also *Moraxella* and *Neisseria* (review: ref. 1), and it has been suggested that many other bacteria will appear with similar differences as more data are accumulated, particularly among Gram-negative organisms. We have previously reported on the free fatty acid content as a tool in the chemotaxonomic differentiation between the closely related facultative Gram-negative rods *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* [2, 3]. The present study, which deals with the composition of bound cellular acids in *A. actinomycetemcomitans* and *H. aphrophilus*, establishes the usefulness of differentiating between free and bound acids in these bacteria, and suggests such differentiation as a routine in chemotaxonomic studies.

0378-4347/84/$03.00

MATERIAL AND METHODS

Bacteria

The strains of *A. actinomycetemcomitans* and *H. aphrophilus* investigated, and the sources from which they were obtained, are listed in Table I. Strains ATCC 33389, 33384, 29522, and 19415 were obtained directly from the American Type Culture Collection, the remaining strains through Forsyth Dental Center. The organisms were maintained and mass cultivated as described previously [3].

TABLE I

LIST OF BACTERIA INVESTIGATED

Bacteria	Strain	Source	Site of origin
Actinobacillus actinomycetemcomitans	33384 (9710)	ATCC* (NCTC**)	Lung abscess
	29524	ATCC	Chest aspirate
	29523	ATCC	Blood
	29522	ATCC	Mandibular abscess
	2112	FDC***	Periodontitis
	2097	FDC	Periodontitis
	2043	FDC	Periodontitis
	511	FDC	Periodontitis
	N 27	FDC	Periodontosis
	Y 4	FDC	Periodontosis
Haemophilus aphrophilus	33389 (5906)	ATCC (NCTC)	Endocarditis
	19415 (5886)	ATCC (NCTC)	Endocarditis
	655	FDC	Periodontitis
	654	FDC	Periodontitis

*American Type Culture Collection, Rockville, MD, U.S.A.
**National Collection of Type Cultures, London, U.K.
***Forsyth Dental Center, Boston, MA, U.S.A.

Removal of free fatty acids from whole cells

Lyophilized material from each of two series of bacteria, cultured on different days, was Soxhlet-extracted twice with fresh *n*-hexane [2, 3].

Removal of bound fatty acids from hexane-extracted cells

Lyophilized, hexane-extracted bacterial cells (200 mg) were suspended in 2—3 ml of deionized distilled water and sonicated for 10 min under ice cooling. The suspension was diluted to 10 ml with deionized distilled water and centrifuged (27,000 *g*, 15 min, 4°C). The recovered pellet was freeze dried until constant weight, suspended in 2 ml of 2 *M* hydrochloric acid in anhydrous methanol and incubated in a PTFE-sealed tube with screw cap for 24 h at 85°C. The methanolysis tube was cooled to 20°C, shaken, and its content transferred to a centrifuge tube. After two washings of the methanolysis tube, each time with 2 ml of absolute ethanol, centrifugation was carried out at 48,200 *g* for 15 min at 4°C. The supernatant was pipetted off and filtered through a 0.22-μm Millex®-GS filter (Millipore, Molsheim, France) and recentrifuged. The supernatant was diluted with 4 ml of deionized distilled

water and extracted twice with chloroform, 4 ml each time. The organic phase was washed twice, each time with 2 ml of deionized distilled water, and dried with 400 mg of magnesium sulphate for 30 min, filtered through a filtration paper, and evaporated by a stream of nitrogen while kept on ice. The residue was solubilized in hexane, filtered and analysed by gas chromatography (GC) and gas chromatography—mass spectrometry (GC—MS).

Reference compounds

The ammonium salt of 3-deoxy-D-manno-2-octulosonic acid (KDO) was obtained from Sigma (St. Louis, MO, U.S.A.). Its methyl derivative was synthesized by incubation of KDO in a PTFE-sealed tube in the presence of 2 *M* hydrochloric acid in anhydrous methanol at 85° C for 24 h. The methyl ester of iso-$C_{15:0}$ acid (Larodan Fine Chemicals, Malmö, Sweden) was synthesized by methylation of the acid with BF_3 in methanol. Methyl esters of lauric, myristic, palmitic, and palmitoleic acid were obtained from Supelco (Bellefonto, PA, U.S.A.). The methyl ester of racemic 3-hydroxymyristic acid was synthesized by Reformatsky reaction [4]. After fractional distillation, the ester was saponified for 1 h with 2 *M* potassium hydroxide in aqueous methanol at refluxing temperature. The solution was chilled in water and the crystalline potassium salt of the hydroxy acid recovered by filtration, washed with ethanol at 0° C and dissolved in hot deionized distilled water at 25° C. The aqueous solution was acidified with hydrochloric acid until pH 2 and cooled to 2° C. The insoluble 3-hydroxymyristic acid was recovered by filtration and its methyl ester synthesized by heating the 3-hydroxymyristic acid in anhydrous methanol with 0.2 *M* hydrochloric acid for 3 h at 75° C. The methanol solution was concentrated to 0.2 ml, diluted with 2 ml of deionized distilled water and extracted three times with chloroform. The chloroform-containing solutions were pooled, dried for 30 min with 40 mg of anhydrous magnesium sulphate and filtered through a paper filter which afterwards was washed with chloroform. The chloroform solutions were pooled and evaporated by a stream of nitrogen while kept on ice. GC—MS was used to control the synthesized product.

Gas chromatography

A Sigma 3 gas chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.) furnished with an electronic integrator (Sigma 10) was used. The Chrompack (Middelburg, The Netherlands) CP-Sil 5 CB (polydimethylsiloxane) capillary column used was 25 m × 0.22 mm I.D. with film thickness 0.14 μm and height equivalent of a theoretical plate (HETP) 0.25 mm. Helium served as carrier gas at a flow-rate of 2 ml/min. The pressure at the inlet of the column was 151.5 kPa, and the temperature of the injector and flame ionization detector 220° C. The gas chromatograph was programmed from 120° to 260° C at 5° C/min. The attenuator of the gas chromatograph was set at 8, that of the Sigma data system at −1. Paper-speed was 10 mm/min. The identity of the bacterial fatty acids (methyl esters) was established by direct cochromatography and by GC—MS. Tentative identification was made by comparing their retention times with those of authentic standards.

Gas chromatography—mass spectrometry

The instrument used for combined GC—MS consisted of a Carlo Erba 4200 gas chromatograph, a Micromass 7072F (Vg Micromass, Cheshire, U.K.) mass spectrometer, and a Vg data system 2200. The gas chromatograph was equipped with a glass capillary OV-1 methyl silicone column (20 m × 0.3 mm I.D.). Helium served as carrier gas. The column temperature was programmed from 120°C to 250°C at 5°C/min. Electron-impact ionization spectra were recorded under the following conditions: ionizing energy 70 eV, ionizing current 200 μA, ion-source temperature 240°C, and accelerating voltage 4 kV.

RESULTS

Gas chromatography

The composition of bound acids (methyl esters) of previously hexane-extracted whole cells from *A. actinomycetemcomitans* and *H. aphrophilus* is given in Table II. The major acids included $C_{14:0}$, 3-OH-$C_{14:0}$, $C_{16:1}$, $C_{16:0}$, and KDO. All strains, except *A. actinomycetemcomitans* strain ATCC 29522, yielded more $C_{16:0}$ than $C_{16:1}$ acid. A typical gas chromatogram of the bound acids is shown in Fig. 1. The fatty acid profiles were not so characteristic as to allow differentiation between *A. actinomycetemcomitans* and *H. aphrophilus.*

Gas chromatography—mass spectrometry

The fragmentation pattern of the methyl esters of $C_{14:0}$, $C_{16:1}$ and $C_{16:0}$ was identical with that of the synthetic analogues and in accordance with the McLafferty rearrangement [5] with formation of a 74 *m/e* fragment. Fragmen-

TABLE II

MAJOR BOUND CELLULAR FATTY ACIDS IN *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* AND *HAEMOPHILUS APHROPHILUS*

Values, which are expressed as relative percentage (w/w) of total substances present, are means of four runs on the gas chromatograph. S.D. = 5%.

Bacteria		$C_{12:0}$	$C_{14:0}$	3-OH-$C_{14:0}$	$C_{16:1}$	$C_{16:0}$
Actinobacillus actinomycetemcomitans						
ATCC	33384	0.1	21.7	9.2	21.4	26.6
ATCC	29524	0.1	19.6	21.2	17.0	27.8
ATCC	29523	0.3	21.9	18.4	19.6	26.0
ATCC	29522	0.8	30.8	24.7	16.1	13.2
FDC	2112	0.4	18.3	32.6	13.9	16.7
FDC	2097	0.8	20.1	14.2	21.9	28.5
FDC	2043	0.5	32.8	17.8	16.4	24.9
FDC	511	0.1	20.7	15.5	22.5	30.0
FDC	N 27	0.1	18.0	27.1	14.9	27.0
FDC	Y 4	0.4	24.1	21.8	23.3	29.6
Haemophilus aphrophilus						
ATCC	33389	0.5	19.5	10.0	25.4	31.6
ATCC	19415	0.5	19.7	16.1	16.5	36.0
FDC	655	0.6	26.5	18.0	13.8	31.5
FDC	654	0.7	19.8	20.2	23.4	24.8

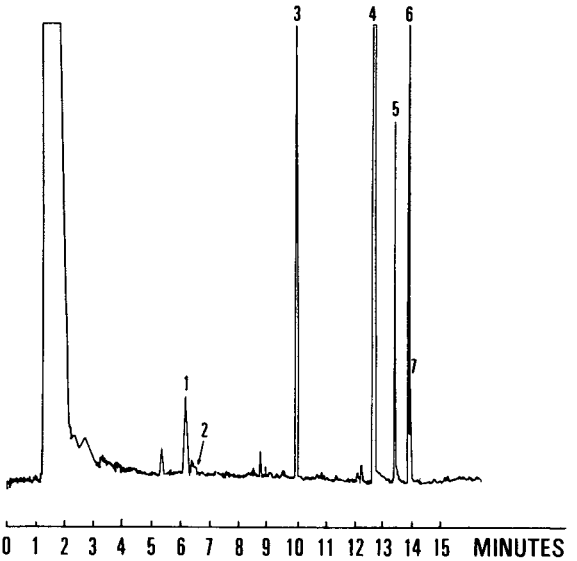


Fig. 1. Typical gas chromatogram of bound acids (methyl esters) from *A. actinomycetemcomitans* and *H. aphrophilus*, as respresented by *A. actinomycetemcomitans* strain FDC 2112. Programme: 120°C to 260°C and 5°C/min. Temperature of the injector and flame ionization detector, 220°C. Attenuator of gas chromatograph, 8, that of Sigma 10 data system, —1. Paper-speed, 10 mm/min. 1 = KDO, 2 = $C_{12:0}$, 3 = $C_{14:0}$, 4 = 3-OH-$C_{14:0}$, 5 = $C_{16:1}$, 6 = $C_{16:0}$, 7 = substance with M^+ 278.

tation of the methyl ester of β-hydroxymyristic acid occurred through formation of the 103 *m/e* fragment [6]. A minor unknown substance with M^+ 278, possibly of chain length C_{17}, was detected in all the bacterial strains investigated.

DISCUSSION

In previous studies on cellular fatty acids in bacteria, fatty acids removed by organic solvents were designated as free lipids, those that remained after this procedure, as bound lipid [7, 8]. The same concepts have been adopted in the present study. After removal of free fatty acids with hexane, four major bound fatty acids, i.e. myristic, palmitic, palmitoleic, and 3-hyroxymyristic acid, were detected in whole cells of *A. actinomycetemcomitans* and *H. aphrophilus* by means of GC, GC—MS, and by comparing our results with existing literature data on fatty acids [5, 6, 9, 10]. The present results agreed with those of Calhoon et al. [11] and Jantzen et al. [12], but varied with those of Braunthal et al. [13] who detected little or no 3-hydroxymyristic acid. The distribution of bound fatty acids was not so specific as to allow differentiation between *A. actinomycetemcomitans* and *H. aphrophilus*. In our GC study on free fatty acids from the very same species, differentiation between *A. actinomycetemcomitans* and *H. aphrophilus* was possible to some extent [3]. It should be emphasized that free fatty acids had been removed completely beforehand, as checked by reextraction of the hexane-extracted cells with chloroform—methanol. Interestingly, iso-$C_{15:0}$ acid, which appeared as a free major fatty

acid in *A. actinomycetemcomitans* strain ATCC 29522 and FDC Y 4, could not be detected among the bound fatty acids. It was also noteworthy that the amount of bound $C_{16:0}$ acid was higher than that of $C_{16:1}$, except in one strain. A reverse relationship prevailed among free $C_{16:0}$ and $C_{16:1}$ acids [3]. Among the bound acids recovered were also KDO, 3-hydroxymyristic acid and an unidentified substance, probably acid of chain length C_{17}. None of these substances were detected as free acids. Exact figures for KDO were not given in the present study due to possible interference during quantification by methanolysis and derivatization products. These aspects have been discussed during quantification of KDO in lipopolysaccharide (LPS) from *A. actinomycetemcomitans* and *H. aphrophilus* [14]. In LPS, KDO links the polysaccharide to lipid A in a ketosidic linkage [15]. Hydroxylated fatty acids in Gram-negative bacteria are largely found in LPS, ornithine lipid, and the polymer poly-β-hydroxybutyrate [1] where they are covalently bound. Hydroxy fatty acids could not be detected in lipids extractable from bacterial cells with lipophilic solvents such as ethanol and methanol—chloroform [7, 8]. Certain species of *Alcaligenes* containing both free and bound hydroxy acids [1] may be exceptions.

Our studies on cellular fatty acids in *A. actinomycetemcomitans* and *H. aphrophilus* demonstrated that free and bound acids differ markedly in these bacteria. Such differences may be significant in the identification and characterization of bacterial cultures. In some extraction methods for bacterial lipids of whole cells, e.g. methanolysis, free and bound acids are removed simultaneously without any differentiation being made. In the currently investigated bacteria, free fatty acids accounted for approximately 90—70% of the total extractable fatty acids, bound fatty acids for about 10—30%. When the content of free fatty acids is that high, these acids may tend to mask bound acids when free and bound acids are examined simultaneously. To provide accurate determination of all the cellular acids present in bacteria, it would appear reasonable as a routine first to screen for the presence of free fatty acids, then for bound acids.

It was recently demonstrated that in patients with localized juvenile periodontitis, *A. actinomycetemcomitans* strains ATCC 29522 and FDC Y 4, which constituted serotype b, were approximately twice as frequent as serotype a or c strains, suggesting a particularly high periodontopathic potential of the serotype b strains [16]. This observation coincided with our demonstration of an extra free major fatty acid, iso-$C_{15:0}$ acid, in the serogroup b strains [3]. It seems possible that screening for free fatty acids in whole cells may also provide information as to potential pathogenic mechanisms in bacterial disease.

Other significant features of the present study were that it allowed methanolysis and methylation to be performed simultaneously, and that additional derivatization of the formed products, including the methyl ester of KDO, was not necessary before GC analysis. Also 3-hydroxymyristic acid (methyl ester) was sufficiently volatile and stable to allow direct analysis. This was in contrast to free β- or α-hydroxy acids which are thermally unstable [17]. We did not detect any myristoleic (*cis*-9-tetradecenoic) acid by means of the present method. β-Hydroxy acids may give αβ-unsaturated acids when boiled with base or acid in the presence of water [17]. When Hase et al. [18]

examined fatty acids of *Fusobacterium nucleatum* LPS, they discovered that alkaline hydrolysis with 4 *M* potassium hydroxide resulted in the production of Δ^2-tetradecenoic acid as an artifact resulting from 3-hydroxytetradecanoic acid. It should also be kept in mind that the fatty acid content and composition determined after acidic hydrolysis of lipids containing 3-hydroxyalkanoic acid may be seriously in error due to acid-catalysed polymerization of 3-hydroxy acid and esterification between hydroxy and non-hydroxy acids [19].

CONCLUSIONS

Bound cellular acids extracted from the bacteria *A. actinomycetemcomitans* and *H. aphrophilus* were determined by GC and GC—MS after free fatty acids had been removed. Bound acids included myristic, palmitic, palmitoleic, 3-hydroxymyristic, and 3-deoxy-D-manno-2-octulosonic acid and possibly its methanolysis products. In chemotaxonomic studies on bacteria it would be reasonable as a routine to differentiate between free and bound fatty acids since they may differ markedly.

ACKNOWLEDGEMENTS

This work was supported in part by the Anders Jahres fond til vitenskapens fremme. The authors gratefully acknowledge the technical assistance of G. Isaksen, J. Vedde, and M.-B. Jørgensen.

REFERENCES

1 M.P. Lechevalier, CRC Crit. Rev. Microbiol., 5 (1977) 109.
2 I. Brondz, I. Olsen and T. Greibrokk, J. Chromatogr., 274 (1983) 299.
3 I. Brondz and I. Olsen, J. Chromatogr., 278 (1983) 13.
4 R.J.W. Cremlyn, in A College Organic Chemistry, Heinemann Educational Books, London, 1970, p. 340.
5 W. Kemp (Editor), Organic Spectroscopy, Macmillan, London, 2nd edn., 1978, pp. 185—218.
6 C.W. Moss and S.B. Dees, J. Chromatogr., 112 (1975) 595.
7 T. Kaneshiro and A.G. Marr, J. Lipid Res., 3 (1962) 184.
8 T. Kaneshiro and A.G. Marr, Biochim. Biophys. Acta, 70 (1963) 271.
9 S. Stenhagen, S. Abrahamsen and F.W. McLafferty (Editors), Atlas of Mass Spectral Data, Wiley, New York, 1969, p. 1564.
10 S. Stenhagen, S. Abrahamsen and F.W. McLafferty (Editors), Register of Mass Spectral Data, Wiley, New York, 1974, pp. 1781—1782.
11 D. Calhoon, W.R. Mayberry and J. Slots, J. Clin. Microbiol., 14 (1981) 376.
12 E. Jantzen, B.P. Berdal and T. Omland, Acta Pathol. Microbiol. Scand. Sect. B, 88 (1980) 89.
13 S.D. Braunthal, S.C. Holt, A.C.R. Tanner and S.S. Socransky, J. Clin. Microbiol., 11 (1980) 625.
14 I. Brondz and I. Olsen, J. Chromatogr., 308 (1984) 19.
15 O. Lüderitz, M.A. Freudenberg, C. Galanos, V. Lehmann, E.T. Rietschel and D.H. Shaw, in F. Bronner and A. Kleinzeller (Editors), Current Topics in Membranes and Transport, Vol. 17, Academic Press, New York, London, 1982, p. 91.
16 J.J. Zambon, J. Slots and R.J. Genco, Infect. Immun., 41 (1983) 19.
17 R.J.W. Cremlyn, in A College Organic Chemistry, Heinemann Educational Books, London, 1970, pp. 216—217.
18 S. Hase, T. Hofstad and E.T. Rietschel, J. Bacteriol., 129 (1977) 9.
19 S.G. Wilkinson, J. Lipid Res., 15 (1974) 181.

Journal of Chromatography, 308 (1984) 289--294
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2092

Note

Rapid analysis of C_{19}-steroid metabolism by high-performance liquid chromatography and in-line monitoring of radioactivity

PER I. LUNDMO* and ARNE SUNDE

The Institute of Cancer Research in Trondheim, Department of Surgery, The Medical Faculty, University of Trondheim, Regionsykehuset, N-7000 Trondheim (Norway)

(Received December 8th, 1983)

Analysis of androgen metabolism has traditionally been based on paper chromatography [1] or thin-layer chromatography (TLC) [2, 3] for the separation and isolation of metabolites. Such assays are generally time-consuming and laborious. Studies of androgen metabolism have therefore often been restricted to a few enzymatic reactions and a limited number of biological specimens at a time. In order to understand the biological role of the numerous steroid-metabolizing enzymes, a large number of assays have to be done simultaneously on the same biological sample [4, 5].

We have devised a fast and reproducible method for the automatic analysis of radioactive metabolites of C_{19}-steroids from incubation studies, based on high-performance liquid chromatography (HPLC) and in-line radiomonitoring (RM). In the present report, this method is compared with a traditional method based on TLC.

MATERIALS AND METHODS

Radioactively labelled steroids were obtained from The Radiochemical Centre (Amersham, U.K.). Unlabelled steroids were supplied by Steraloids (U.S.A.). Labelled steroids were purified as described by Rosness et al. [6]. Scintillation fluid (M299) was obtained from Packard (U.S.A.). From Sigma were obtained: β-NAD, Grade III; NADP, Sigma grade; glucose-6-phosphate; glucose-6-phosphate dehydrogenase, Type XV; and 2,7-dichlorofluorescein. Solvents for HPLC were from Rathburn Chemicals (Walkerburn, U.K.). Ultrapure water was obtained by filtering distilled water through a Gelman Water-I filtration unit (Gelman, Ann Arbor, MI, U.S.A.). All other chemicals and

0378-4347/84/$03.00

solvents were obtained from E. Merck (Darmstadt, F.R.G.) and were of analytical grade.

Incubations

The preparation of homogenates, conditions of incubation and methods for isolation and characterization of steroids have been described in detail previously [3, 6], and only a brief review will be presented here. The tissues were homogenized in Tris—HCl buffer. The enzymatic studies were done on the 800 *g* supernatant fraction. The ^{3}H-labelled steroids used as substrates were dissolved in Tris—HCl buffer together with the cofactors used, and incubations were started by adding tissue homogenate. Incubations were carried out for 5—30 min at 32° C (testis) or at 37° C (prostate and coagulating gland) and were terminated by the addition of 2 ml of ice-cold ethyl acetate. The ethyl acetate contained several reference steroids in a concentration of 15 μg/ml [6]. The incubates were further extracted with ethyl acetate containing no added steroids.

Chromatography

TLC was performed on 20 × 20 cm preformed silica gel plates, obtained from Merck or from Schleicher & Schüll, Dassel, F.R.G. (Table I). The TLC systems have been described previously [3, 6].

The TLC chromatograms were divided into zones corresponding to the reference steroids, and the radioactivity in each zone was measured by liquid scintillation counting (Rackbeta, 1215, LKB, Sweden).

HPLC was performed on a chromatograph consisting of a Constametric III reciprocal pump [Laboratory Data Control (LDC), U.S.A.], a Spectromonitor-III variable-wavelength ultraviolet detector (LDC), and a Refractomonitor-III refractive index (RI) monitor (LDC). The column system consisted of a 50 × 4.6 mm I.D. guard column, dry packed with 40-μm pellicular packing (Pelliguard LC-18; Supelco, U.S.A.) and a 250 × 4.6 mm I.D. reversed-phase analytical column (Supelcosil LC-18; 5-μm spherical packing; Supelco). Development of a separation system for androgens based on reversed-phase HPLC has been published previously [7].

For in-line detection of radioactive metabolites, the eluent from the HPLC column was directly coupled to an SM200 splitter mixer [Nuclear Enterprises

TABLE I

SUMMARY OF CHROMATOGRAPHIC SYSTEMS USED

System No.	Type	Stationary phase	Eluent	Purpose
1*	TLC	Silica Gel 60 (Merck)	Toluene—methanol (9:1)	Separation of 4-ene- and 5α-reduced androgens
2*	TLC	Silica Gel F-1500 (Schleicher & Schüll)	Dichloromethane—ethyl acetate (9:1)	Separation of 5α-reduced androgens
3	HPLC	Supelcosil® LC-18 (Supelco)	Methanol—acetonitrile—water (14:43:43)	Separation of 4-ene- and 5α-reduced androgens
4**	HPLC	Supelcosil® LC-18 (Supelco)	Methanol—acetonitrile—water (33:26:41)	Separation of 5α-reduced androgens

*Two developments [3, 6].
**Ref. 7.

(NE), U.K.], and there continuously mixed with three times its volume of liquid scintillant (scintillator 299, Packard). This mixture was led to an Isoflo (NE) radiomonitor equipped with a flow-cell of 1 ml volume. The content of radioactivity in the flow-cell was normally counted in intervals of 10 sec. The data collected by the radiomonitor were fed to an ABC-80 microcomputer (Scandia-Metric, Norway). This computer was programmed to collect the chromatographic data. The computer performed all peak integrations and the data were presented on a printer (8300, Scandia-Metric) and on a strip-chart recorder.

The eluents used in HPLC were prepared in batches of 10 l. Each batch of eluent was tested and elution volumes of various authentic radiolabelled C_{19}-steroids were recorded.

RESULTS AND DISCUSSION

Separation and quantification of radioactively labelled C_{19}-steroids by a TLC assay and our new HPLC—RM assay gave similar results (Tables II and III). Total time for analysing one sample, including chromatography and quantitation of recovered radioactivity in each isolated fraction, is about 80 min using the TLC assay. In our new HPLC—RM assay, the total analysis time is 10—35 min, depending on the flow-rate in the analytical column. An increase in flow-rate will result in decreased chromatographic resolution and reduced dynamic counting efficiency due to shortened sample residence time in the RM unit.

Each sample analysed by the TLC assay requires about 40 min of manual labour, compared to our new HPLC—RM assay which requires only 2—3 min attendance and manual labour per sample. The HPLC analysis may also be run with an automatic sample injection and computer-based automatic peak integration. This further reduces the manual work required per sample and enables 24 h per day operation giving a high sample through-put.

The static counting efficiency of the radiomonitor is comparable to that of an ordinary liquid scintillation counter. The baseline activity of the detector is normally about 10—15 cpm. Peaks containing about 350 dpm or more of a tritiated compound may be detected and quantitated with good accuracy in our standard assay.

TABLE II

A COMPARISON OF SEPARATION AND QUANTIFICATION OF RADIOLABELLED REFERENCE STEROIDS BY TLC AND HPLC ASSAYS

A mixture of 4-[^{14}C]androstene-3,17-dione ($2.2 \cdot 10^6$ dpm), 17β-hydroxy-4-[^{14}C]androsten-3-one ($5 \cdot 10^5$ dpm) and 17β-hydroxy-5α-[^{14}C]androstan-3-one ($5 \cdot 10^5$ dpm) together with unlabelled reference steroids was divided into eight portions; four of these were analysed by TLC (system 1) and the other four by HPLC (system 3). Values are presented as percentage recovered radioactivity in each fraction (mean ± S.D., $n = 4$).

Isolated steroids	HPLC	TLC
4-Androstene-3,17-dione	68.4 ± 0.5	68.6 ± 0.1
17β-Hydroxy-4-androsten-3-one	15.5 ± 0.3	15.3 ± 0.1
17β-Hydroxy-5α-androstan-3-one	15.4 ± 0.2	15.3 ± 0.2

TABLE III

QUANTIFICATION OF METABOLITES FROM VARIOUS TISSUES BY HPLC AND TLC ASSAYS

(a) 5α-[^{3}H]Androstane-3α,17β-diol (1 μg) was incubated for 15 min at 37°C with a homogenate from the rat ventral prostate (17.0 mg protein). NAD was added as cofactor. (b) 5α-[^{3}H]Androstane-3β,17β-diol (1 μg) was incubated for 5 min at 32°C with a rat testicular homogenate (21.4 mg protein) in the presence of an NADPH-generating system. (c) 17β-[^{3}H]Hydroxy-4-androsten-3-one (1 μg) was incubated for 60 min at 37°C with a homogenate from the rat coagulating gland (10.5 mg protein) in the presence of an NADPH-generating system. The metabolites were extracted as described in Material and methods. The extracts were each divided into eight (c) or ten (a, b) aliquots. Half of the corresponding aliquots were analysed by TLC (a, b: system 2; c: system 1) or by HPLC (system 4). Values are presented as percentage recovered radioactivity in each fraction [mean ± S.D., n = 5 (a, b) n = 4 (c)].

Metabolite	Ventral prostate (a)		Testis (b)		Coagulating gland (c)	
	HPLC	TLC	HPLC	TLC	HPLC	TLC
5α-Androstane-3α,17β-diol	19.5 ± 0.5	18.7 ± 0.7	3.3 ± 0.2	3.8 ± 0.7	1.6 ± 0.1	1.9 ± 0.1
5α-Androstane-3β,17β-diol	—	—	78.0 ± 0.9	81.9 ± 0.3	—	—
3α-Hydroxy-5α-androstan-17-one	—	—	0.46 ± 0.37	0.16 ± 0.09	—	—
3β-Hydroxy-5α-androstan-17-one	—	—	0.60 ± 0.10	1.25 ± 0.16	—	—
17β-Hydroxy-5α-androstan-3-one	80.5 ± 0.5	81.3 ± 1.0	11.7 ± 0.8	11.8 ± 0.1	11.6 ± 0.2	11.0 ± 0.2
5α-Androstane-3,17-dione	—	—	0.72 ± 0.10	0.58 ± 0.20	—	—
17β-Hydroxy-4-androsten-3-one					86.8 ± 0.2	87.0 ± 0.2

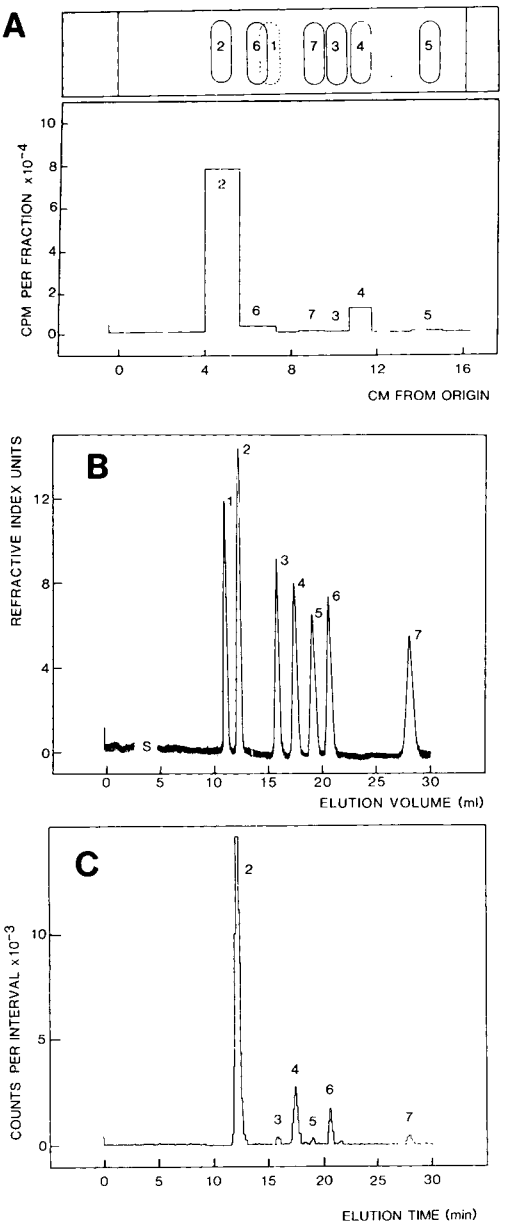


Fig. 1. Comparison of TLC and HPLC—RM separation and quantitation of radiolabelled C_{19}-steroids. Tritiated 5α-androstane-3β,17β-diol was incubated with a testicular homogenate as described in Materials and methods. Radiolabelled metabolites were extracted and separated by TLC or HPLC (systems 2 and 4, respectively, Table I). (A) TLC chromatogram and distribution of radioactivity in isolated fractions determined by liquid scintillation counting (see Materials and methods). (B) HPLC chromatogram of a mixture of authentic C_{19}-steroids. (C) Radiochromatogram of radiolabelled C_{19}-steroids separated by HPLC and quantitated by an Isoflo radiomonitor (see Materials and methods). Peaks: 1 = testosterone; 2 = 5α-androstane-3β,17β-diol; 3 = 3β-hydroxy-5α-androstan-17-one; 4 = 17β-hydroxy-5α-androstan-3-one; 5 = 5α-androstane-3,17-dione; 6 = 5α-androstane-3α,17β-diol; 7 = 3α-hydroxy-5α-androstan-17-one.

An HPLC—RM unit will generate a substantially increased amount of information compared to traditional TLC assays (Fig. 1). In extracts from incubations of radioactive C_{19}-steroids with rat and human prostatic tissues we have observed several minor metabolites yet to be structurally identified.

C_{19}-Steroids display functional group specific solvent selectivity which can be used to increase chromatographic selectivity [8]. We have used such selective solvent effects to gain structural information and thus tentative identification of unknown metabolites by running the chromatographic analysis with different mobile-phase compositions [8].

In conclusion, addition of a radiomonitor to a conventional HPLC system gives a fast and reproducible method for the separation and quantitation of radiolabelled gonadal steroids as well as a variety of other labelled compounds.

ACKNOWLEDGEMENTS

The authors are research fellows of The Norwegian Society for Fighting Cancer and this work was supported by a grant from this society. We thank Professor K.J. Tveter for valuable discussions. We are indebted to N. Nesjan for taking care of the animals, and D. Moholdt for typing this manuscript.

REFERENCES

1 R. Neher, Steroid Chromatography, Elsevier, Amsterdam, 1974.
2 E. Heftmann, Chromatography of Steroids, Elsevier, Amsterdam, 1976.
3 A. Sunde, P. Stenstad and K.B. Eik-Nes, J. Chromatogr., 175 (1979) 219.
4 J.T. Isaacs, W.W. Scott and D.S. Coffey, in G.P. Murphy and A.A. Sandberg (Editors), Prostate Cancer and Hormone Receptors, Alan R. Liss, New York, 1979, pp. 133—144.
5 J.T. Isaacs, C.B. Brendler and P.C. Walsh, J. Clin. Endocrinol. Metab., 56 (1983) 139.
6 P.A. Rosness, A. Sunde and K.B. Eik-Nes, Biochim. Biophys. Acta, 488 (1977) 55.
7 A. Sunde and P.I. Lundmo, J. Chromatogr., 242 (1982) 381.
8 A. Sunde and P.I. Lundmo, in preparation.

Journal of Chromatography, 308 (1984) 295—300
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2064

Note

Application of reversed-phase high-performance liquid chromatography for radioimmunoassay of plasma 18-hydroxycorticosterone

N. IMAIZUMI, S. MORIMOTO*, T. KIGOSHI, K. UCHIDA, H. HOSOJIMA and I. YAMAMOTO

Division of Endocrinology, Department of Internal Medicine, Kanazawa Medical University, Uchinada, Ishikawa 920-02 (Japan)

(First received October 20th, 1983; revised manuscript received January 4th, 1984)

Marked elevation of 18-hydroxycorticosterone (18-OHB), a major by-product of adrenal aldosterone biosynthesis [1, 2], has been found in primary aldosteronism due to an adrenal adenoma [3—7] and with 17α-hydroxylase deficiency [8], but not in patients with idiopathic aldosteronism due to adrenal hyperplasia [5, 6] and with normal and low renin essential hypertension [6]. Thus, measurement of plasma or urinary 18-OHB together with aldosterone has proved very useful in the differential diagnosis of these hypertensive disorders [1—8]. High-performance liquid chromatography (HPLC) has recently been proposed for the isolation of multiple adrenal steroids including 18-OHB in serum [9], urine [10] and adrenal tissue extract [11].

We herein describe a method for the separation of plasma 18-OHB from cross-reacting steroids by reversed-phase HPLC prior to its radioimmunoassay (RIA).

EXPERIMENTAL

Materials and reagents

Methanol was of HPLC grade and all other organic solvents were of analytical grade. Water was distilled and then purified by a Millipore filter assembly including a charcoal and ion-exchange tube. Unlabelled 18-OHB was purchased from Fluka (Buchs, Switzerland) and its labelled form ([1,2-^{3}H]18-OHB, specific activity 42 Ci/mmol) from the Radiochemical Centre, Amersham, U.K. The antiserum of 18-OHB raised against 3-(O-carboxymethyl)-

0378-4347/84/$03.00

oxime—bovine serum albumin conjugate (18-OHB-3-CMO-BSA) was supplied by the Teikoku Hormone Mfg. Co. (Kawasaki, Japan). The characteristic of this 18-OHB-3-CMO-BSA antibody was reported by Ojima and Kambegawa [12], who found it to be highly specific. Other unlabelled steroids for examination of cross-reactivity were obtained from Merck (Darmstadt, F.R.G.). The purity of all unlabelled and labelled steroids was checked by HPLC under the conditions described below.

Instruments

The HPLC system used here was constructed with a Kyowa Model KHP-010 liquid chromatograph (Kyowa Co., Tokyo, Japan) and a Oyo-Bunko Model UVILOG 5 III-A variable-wavelength ultraviolet (UV) spectrophotometer (Oyo-Bunko Co., Tokyo, Japan).

Methods

The total assay procedure consisted of three steps: extraction of steroids, HPLC and RIA.

Extraction. Half a millilitre of plasma was extracted with 20 ml of dichloromethane after adding 1.0 ml of 1 *M* sodium hydroxide. After removal of the upper layer, the dichloromethane layer was washed with 1.0 ml of water and evaporated at 37°C under a stream of nitrogen. The resulting residue was dissolved in methanol—water (2:1, v/v) and then injected for HPLC.

HPLC. Conditions of HPLC are as follows: separation column, Finepack SIL $C_{18\text{-}5}$ (250 mm × 4 mm I.D., particle size 5 μm); precolumn, Fine SIL $C_{18\text{-}10}$ (5 mm × 4 mm I.D., particle size 10 μm); eluent, 0.005 *M* hydrochloric acid—methanol (35:65, v/v); flow-rate, 1.0 ml/min; column temperature, 37°C; monitoring wavelength, 245 nm. The separation of 18-OHB from other UV-absorbing steroids was checked photometrically by the injection of a steroid mixture containing 0.6 nmol of each steroid standard, and the retention time of 18-OHB was measured by the injection of 0.6 nmol of standard 18-OHB alone prior to the analysis of plasma samples. The 18-OHB-containing fraction of plasma extract eluted by HPLC was collected in a tube, neutralized with ammonium hydroxide and evaporated at 37°C under a stream of nitrogen. The resulting residue was subjected to RIA.

RIA. RIA of 18-OHB was performed by the method of Ojima and Kambegawa [12]. Total recovery of 18-OHB throughout the procedure was determined by the addition of about 5000 dpm of the labelled steroid to plasma.

RESULTS AND DISCUSSION

We used a reversed-phase chromatographic system to elute 18-OHB alone by a non-gradient method at a relatively early retention time compared with that using a normal-phase chromatographic system. The mean recoveries (± S.D.) of 18-OHB in five plasma samples before and after HPLC in this method were 89.7 ± 5.3% (range, 83.6—94.7%) and 69.9 ± 5.1% (range, 63.4—75.7%), respectively. The mean recovery after HPLC in our method was slightly higher than that (55.3 ± 4.4%) reported by Schöneshöfer et al. [9], who used a

normal-phase chromatographic system. A chromatogram with UV detection of steroid standards is shown in Fig. 1a. The separation of 18-OHB from other steroids such as aldosterone, cortisol, corticosterone, deoxycorticosterone, 18-hydroxydeoxycorticosterone (18-OHDOC) and progesterone was satisfactory. A chromatogram with UV detection of the extract of a normal plasma sample is shown in Fig. 1b. 18-OHB was eluted at the retention time of 6.53

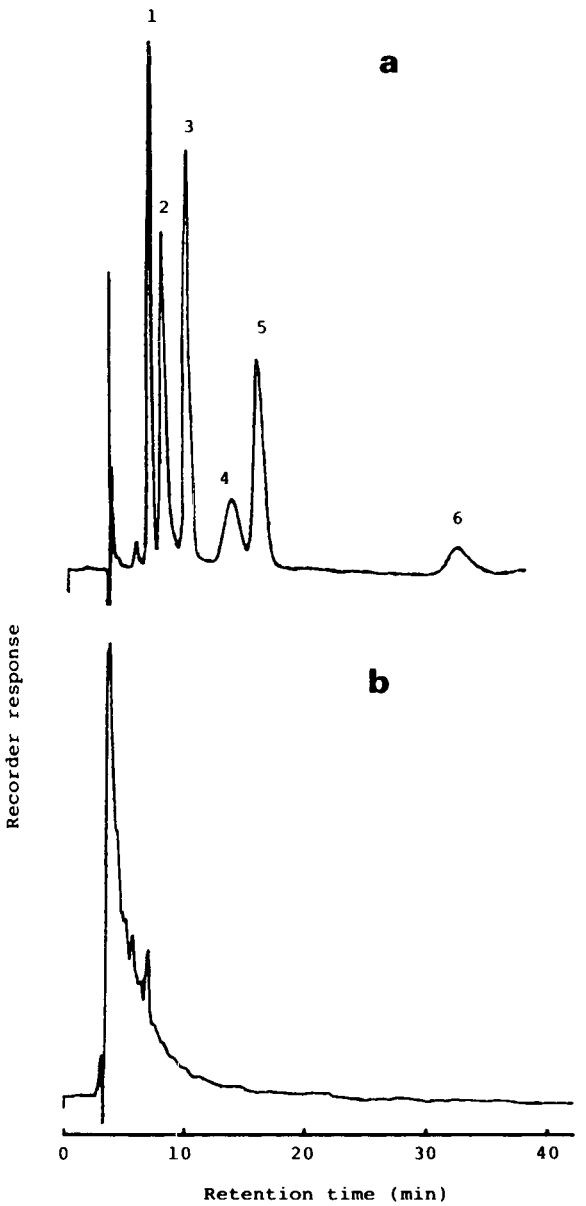


Fig. 1. UV-detected chromatograms of a mixture of steroid standards (a) and of the dichloromethane extract of a normal plasma sample (b). Peaks: 1 = aldosterone and cortisol, 2 = 18-OHB, 3 = corticosterone, 4 = 18-OHDOC, 5 = deoxycorticosterone, 6 = progesterone.

TABLE I

PRECISION OF THE RIA OF 18-OHB

	Pooled plasma	Plasma 18-OHB (nmol/l)					C.V. (%)
		1	2	3	4	Mean ± S.D.	
Intra-assay	A	0.35	0.29	0.26	0.25	0.29 ± 0.04	13.6
	B	1.10	1.23	1.31	1.00	1.16 ± 0.12	10.3
	C	1.99	1.80	2.25	1.89	1.98 ± 0.17	8.6
Inter-assay	A	0.35	0.38	0.25		0.33 ± 0.05	16.5
	B	1.09	1.30	0.97		1.12 ± 0.14	12.2
	C	1.99	1.85	2.36		2.07 ± 0.22	10.4

min after its injection. The coefficient of variation for the retention time of 18-OHB ranged from 0.2 to 0.9%.

The detection limit of the RIA of 18-OHB, defined as the mean blank measurement plus 2.5 S.D. (2.5 × standard deviation of zero bound) [13], was 0.016 pmol. Intra- and inter-assay coefficients of variation are summarized in Table I. The intra-assay coefficient of variation ranged from 8.6 to 13.6% and the inter-assay coefficient of variation ranged from 10.4 to 16.5%. The residue of neutralized eluate did not affect the RIA of 18-OHB (Fig. 2). The pattern of immunoreactivities of standard 18-OHB and plasma extract arising against the 18-OHB antiserum is shown in Fig. 3. The portion showing the maximum immunoreactivity of the plasma extract corresponded with that of the standard 18-OHB. Although the 18-OHB antiserum used here has been reported to be highly specific [12], the multiple non-specific immunoreactive

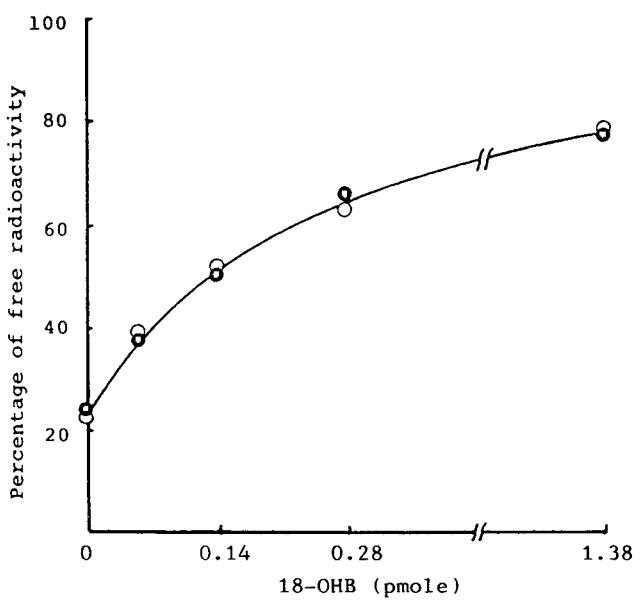


Fig. 2. Calibration curves for the standard 18-OHB alone (•) and for the standard 18-OHB added to the dried residue of 1.5 ml of neutralized eluent (○).

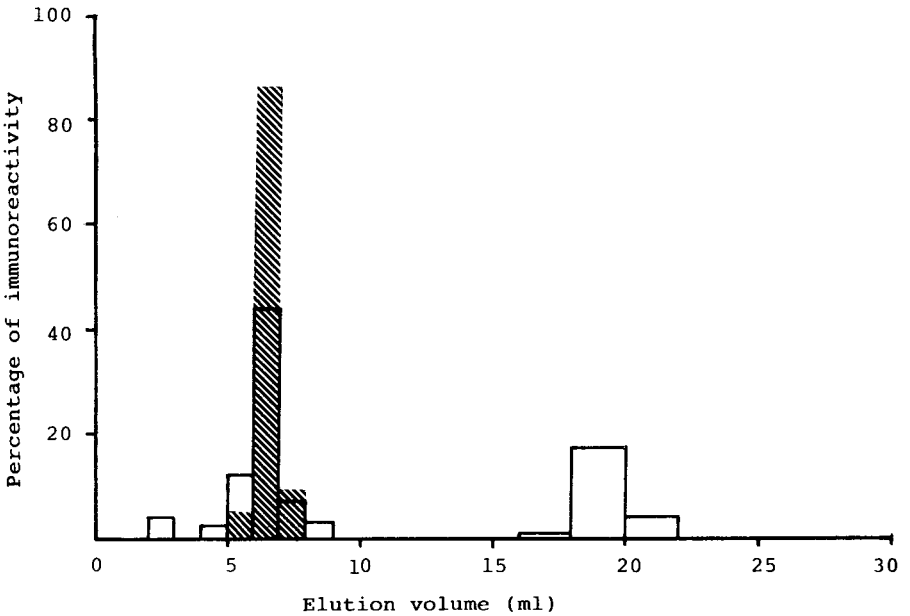


Fig. 3. Patterns of immunoreactivity arising in the HPLC eluates of the standard 18-OHB (hatched area) and of the dichloromethane extract of a normal plasma sample (open area). Values are plotted as the percentage of total immunoreactivity. Fractions of the eluate were collected every 1.0 ml for the first 10 ml and every 2.0 ml thereafter.

portions including the presumed 18-OHDOC one also were found in normal plasma as reported by Schöneshöfer et al. [9]. When interference of the steroids eluted around 18-OHB was tested in the assay system by adding 0.28 μmol of aldosterone, cortisol or corticosterone to 0.28 nmol of 18-OHB, no interference of these steroids was observed.

Plasma 18-OHB levels were measured at 08:00 h after overnight recumbency in fourteen normal subjects and at 12:00 h after 2-h recumbency in the six normal subjects while they were on an ad libitum sodium intake. The plasma 18-OHB level decreased from 0.59 ± 0.24 (S.D.) nmol/l (range, 0.26—0.88 nmol/l) to 0.42 ± 0.14 (S.D.) nmol/l (range, 0.22—0.63 nmol/l), suggesting some circadian rhythm. These normal levels of 18-OHB measured by the present method closely resembled those reported previously in the literature [9, 12, 14, 15]. Plasma 18-OHB levels at 08:000 h after overnight recumbency in five patients with primary aldosteronism due to an adrenal adenoma ranged from 2.62 to 5.83 nmol/l and were similar to those reported by Biglieri and Schambelan [5].

In conclusion, the method described here presents a simple, rapid and reliable isolation of plasma 18-OHB prior to its RIA. The preceding chromatographic isolation of plasma 18-OHB makes its RIA highly appropriate for clinical use.

REFERENCES

1 S. Ulick, J. Clin. Endocrinol. Metab., 43 (1976) 92—96.

2 R. Fraser and C.P. Lantos, J. Steroid Biochem., 9 (1978) 273—286.
3 S. Ulick, Amer. J. Cardiol., 38 (1976) 814—824.
4 T.M. Connolly, P. Vecsei, D. Haack, K.-H. Kohl, S. Abdelhamid and A. Ammenti, Klin. Wochenschr., (Suppl. I) 56 (1978) 173—181.
5 E.G. Biglieri and M. Schambelan, J. Clin. Endocrinol. Metab., 49 (1979) 87—91.
6 R. Fraser, C. Beretta-Piccoli, J.J. Brown, A.M.M. Cumming, A.F. Lever, J.J. Morton and J.I.S. Robertson, Hypertension, (Suppl. I) 3 (1981) I87—I92.
7 S. Abdelhamid, P. Vecsei, D. Haack, K.-H. Gless, D. Walb, P. Fiegel and K. Lightwald, J. Steroid Biochem., 14 (1981) 913—920.
8 C.E. Kater, E.G. Biglieri, N. Brust and B. Chang, J. Clin. Endocrinol. Metab., 55 (1982) 295—302.
9 M. Schöneshöfer, A. Fenner and H.J. Dulce, J. Steroid Biochem., 14 (1981) 377—386.
10 M. Schöneshöfer, B. Weber and H.J. Dulce, J. Chromatogr., 227 (1982) 492—496.
11 M.J. O'Hare, E.C. Nice and M. Capp, J. Chromatogr., 198 (1980) 23—39.
12 M. Ojima and A. Kambegawa, Tohoku J. Exp. Med., 132 (1980) 75—85.
13 R. Ekins and B. Newman, Acta Endocrinol. (Copenhagen) Suppl., 147 (1970) 11—36.
14 V.I. Martin, C.R.W. Edwards, E.G. Biglieri, G.P. Vinson and F.C. Bartter, Steroids, 26 (1975) 591—604.
15 A. Wilson, P.A. Mason and R. Fraser, J. Steroid Biochem., 7 (1976) 611—613.

Journal of Chromatography, 308 (1984) 301—305
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2075

Note

High-performance liquid chromatographic determination of plasma catecholamines during α-methyldopa therapy

G.P. JACKMAN*, C.J. ODDIE, H. SKEWS and A. BOBIK

Clinical Research Unit, Alfred Hospital and Baker Medical Research Institute, Commercial Road, Prahran, Victoria 3181 (Australia)

(Received December 8th, 1983)

α-Methyldopa [L-3-(3′,4′-dihydroxyphenyl)-2-methylalanine, Aldomet, αMD] is commonly used in the treatment of essential hypertension. In animals, the drug lowers blood pressure by reducing sympathetic activity [1]. Direct evidence for this mechanism in man is lacking since it is not possible to measure plasma noradrenaline (NE) levels, one of the most useful indices of sympathetic activity in man [2], when subjects are on αMD therapy.

Following oral administration of therapeutic doses of αMD (250—500 mg), plasma αMD concentrations may exceed those of NE by 10,000-fold [3, 4]. This high concentration of αMD competitively inhibits the enzyme catechol-O-methyltransferase, thereby greatly lowering the sensitivity of radioenzymatic assays for NE. With high-performance liquid chromatographic (HPLC) assays, analysis is limited to times at which plasma αMD concentrations are low because of the problem of resolving the high αMD concentrations from NE [3].

The aim of this study was to provide an HPLC assay for plasma NE which could also be used for patients on αMD therapy.

EXPERIMENTAL

Reagents

Noradrenaline bitartrate (NE), adrenaline bitartrate (E) and 3,4-dihydroxybenzylamine hydrobromide (DHBA) were obtained from Sigma (St. Louis, MO, U.S.A.). α-Methylnoradrenaline (αMNE) was a gift from Sterling-Winthrop Research Institute (Rensselaer, NY, U.S.A.). α-Methyldopa was obtained from Merck, Sharpe & Dohme (Australia) (South Granville, Australia). Bio-Rex 70, 50—100 mesh (Na^+), cation-exchange resin (Bio-Rad Labs., Richmond, CA,

0378-4347/84/$03.00

U.S.A.) was packed into polypropylene columns (4 × 1 cm, Bio-Rad Labs.) and washed three times with 1 *M* hydrochloric acid and 1 *M* sodium hydroxide, once with 1 *M* sodium acetate, pH 6.5, and once with 0.01% disodium EDTA before use. Alumina (Merck, Darmstadt, F.R.G.) was activated by the method of Anton and Sayre [5]. Water for HPLC was redistilled from alkaline potassium permanganate. All other reagents were of analytical grade.

Samples

Blood containing αMD and its metabolites was obtained from two sources: (1) supine, hypertensive subjects treated with αMD (500 mg per os); and (2) rabbits 3 h after a dose of αMD (50 mg/kg intravenously).

For precision studies, plasma was obtained from humans and rabbits under resting conditions and when sympathetic activity was reduced or increased. In humans, resting sympathetic activity was achieved by having the subjects lie in a supine position for 30 min; reduced activity was achieved pharmacologically by administering prazosin, clonidine and atropine [6], and increased activity by exercise on a bicycle ergometer [7]. In rabbits, resting, reduced and increased activity conditions were achieved respectively by the rabbit sitting quietly in its box, by intracisternal administration of αMD (600 μg/kg) [8], and by subjecting the animal to moderate noise stress.

Blood was collected into polypropylene tubes containing a solution of glutathione (30 mg/ml) and EGTA (100 mg/ml), pH 6.8—7.0, in the proportion of 20 μl per ml blood. Blood samples were immediately centrifuged at 4° C and the plasma separated and stored at —20° C.

Extraction of catecholamines

Plasma (2 ml) or distilled water (2 ml) containing catecholamine standards is added to 5 ml of 0.1% disodium EDTA, 0.5 ml of 1 *M* sodium acetate, pH 6.5, and 4 ng DHBA (internal standard). The solutions are passed through the Bio-Rex 70 columns, the resin is washed with 10 ml distilled water and both effluents are discarded. Catecholamines are eluted from the column with 1 ml of 0.7 *M* sulphuric acid and 3.5 ml of 2 *M* ammonium sulphate containing 0.1% disodium EDTA. The eluate is adjusted to pH 8.6 with 3 ml of 1 *M* Tris—HCl buffer, pH 8.6, containing 2% disodium EDTA; 100 mg activated alumina are added and the samples shaken for 3 min. After centrifugation, the supernatant is aspirated and the alumina washed three times with 10 ml distilled water. The alumina is transferred to a small filtration apparatus, excess water is removed by centrifugation and the catecholamines are eluted with 0.2 ml of 0.2 *M* perchloric acid. The eluate (50—100 μl) is assayed by HPLC.

Chromatography

Catecholamines in the eluate are separated by HPLC using a 150 × 4.6 mm I.D., 5-μm particle size, Spherisorb ODS2 reversed-phase column (Phase Separations, Queensferry, U.K.). The mobile phase is 0.1 *M* sodium dihydrogen phosphate, 2 m*M* sodium heptanesulphonate, 0.001% disodium EDTA, pH 5.0. Flow-rate is 1.5 ml/min. Catecholamines are detected at a glassy carbon electrode (Model LC-5) using a Model LC-4A detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.). The potential is set at 0.50 V vs. Ag/AgCl.

Detector response is quantitated using a Model 308 computing integrator (Laboratory Data Control, Riviera Beach, FL, U.S.A.), and is expressed as the ratio of the peak height for each catecholamine relative to the internal standard, DHBA.

Analysis of α-methyldopa

Plasma αMD concentrations are measured by a method based on that of Ong et al. [9]. Plasma is deproteinated with perchloric acid and an aliquot of the diluted supernatant injected directly for HPLC. The chromatographic system is the same as above, using a potential of 0.65 V.

RESULTS AND DISCUSSION

To minimise interference of αMD in the plasma assay for NE, catecholamines were first isolated from plasma onto a weak cation-exchange resin (Bio-Rex 70). We have previously shown that αMD is not retained by this resin under conditions which successfully isolate catecholamines from urine [10]. Incorporation of this chromatographic step prior to alumina adsorption of the catecholamines and subsequent HPLC removes more than 99% of the αMD originally present in plasma. This now allows αMD (retention time 4.6 min) and NE (retention time 6.4 min) to be well resolved from each other even when the concentration differential between the two is high (Fig. 1). In the two representative chromatograms shown (Fig. 1B and C), plasma originally contained 1.04 and 4.2 μg/ml αMD whilst NE levels were 388 pg/ml and 137 pg/ml, respectively. Peak plasma αMD concentrations in humans following a therapeutic dose of αMD (500 mg per os) range from 1.4 to 5.5 μg/ml at 3 to 4 h post dose [4, 11] indicating that the assay can be used over the entire therapeutic range for αMD.

Despite the incorporation of an additional step to separate αMD from the catecholamines, overall recoveries for the catecholamines in the assay are still relatively good. Recoveries of NE, αMNE and E from plasma averaged 48, 46 and 46%, respectively. For DHBA the recovery was slightly higher averaging 55%. Recoveries of catecholamines added to distilled water or plasma were similar. Regression lines for the calibration curves for NE were $y = 0.00063x + 0.0061$ ($r^2 = 0.997$), $y = 0.00066x + 0.158$ ($r^2 = 0.995$) and $y = 0.00065x + 0.105$ ($r^2 = 0.997$) for distilled water, human and rabbit plasma, respectively, where y represents the peak height ratio of NE/DHBA and x represents the catecholamine concentration (pg/ml). The slopes of the lines are not significantly different ($P > 0.05$). The slopes of regression lines similarly obtained for αMNE and E were also not significantly different.

Because NE occurs endogenously in plasma, assay specificity for NE was validated by comparing the effect of oxidation voltage on detector response over the range 0.2—0.6 V vs. Ag/AgCl. No difference was found between NE extracted from plasma and NE standard, indicating that the chromatographic peak identified as NE in plasma extracts is due to endogenous NE. It was not possible to carry out this procedure for E because the levels of this catecholamine were always below the limit of sensitivity in our samples. No interfering peak for αMNE occurred in chromatograms, indicating that the assay is valid for measuring this metabolite of αMD.

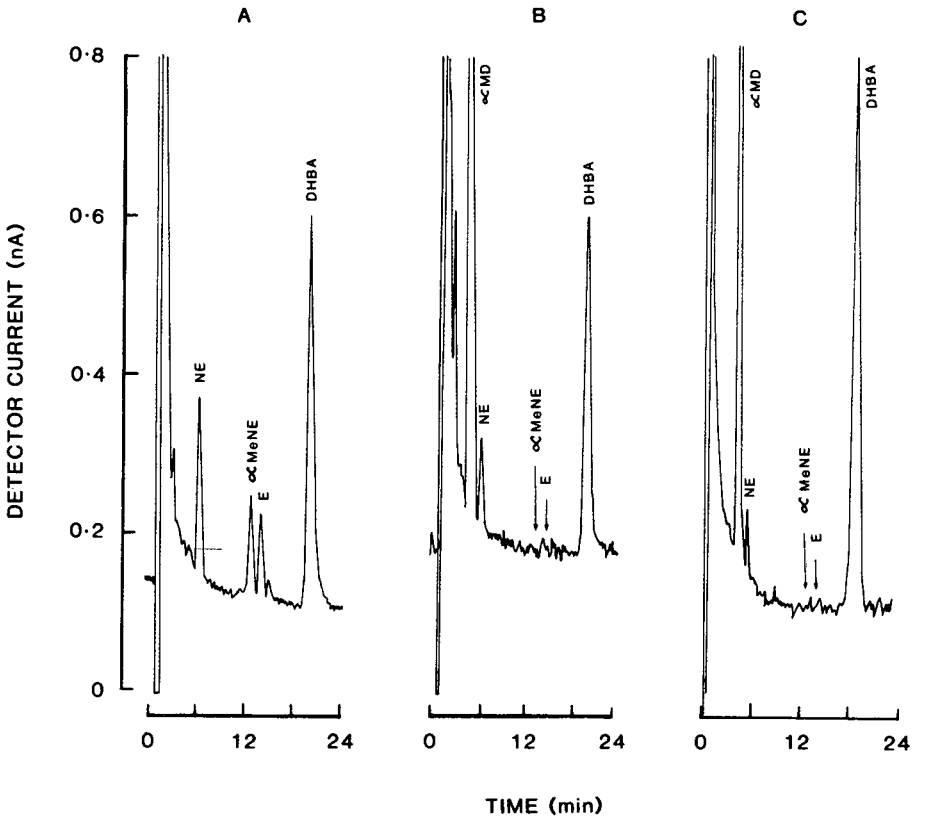


Fig. 1. Chromatographic traces of: (A) standard catecholamines, NE, αMNE and E (500 pg/ml), and internal standard, DHBA (4 ng), extracted from distilled water; (B) extract of human plasma 4 h following a dose of αMD (500 mg, per os), containing 388 pg/ml NE; and (C) extract of rabbit plasma 3 h following αMD administration (50 mg/kg, intravenously), containing 137 pg/ml NE.

TABLE I

PRECISION OF THE ASSAY

Sample	Relative sympathetic activity	Plasma NE		
		Mean ± S.D. (pg/ml)	*n*	C.V. (%)
Human	Low	171 ± 13.5	5	7.9
	Normal	224 ± 8.9	6	4.0
	High	1252 ± 38.7	6	3.1
Rabbit	Low	92.8 ± 6.3	6	6.8
	Normal	231 ± 6.9	6	3.0
	High	570 ± 20.7	6	3.6

The limits of sensitivity for NE, αMNE and E are 30, 50 and 70 pg/ml respectively when 2 ml plasma are assayed. Assay sensitivity may be further increased by using 4 ml plasma without modifying the method.

Assay precision for NE was established by replicate analysis of human and rabbit plasma samples over the concentration range 90—1200 pg/ml. This represents the entire range of catecholamine levels normally encountered. Coefficients of variation were 7—8% for levels below 200 pg/ml and 3—4% for higher NE levels (Table I). These values compare favourably with other HPLC or radioenzymatic methods [12—15].

Following αMD administration, no αMNE could be detected in plasma from either humans or rabbits. This finding is in agreement with the work of Jenner et al. [3] in which αMNE was only detected during severe exercise. It would appear that αMNE is not released in significantly large amounts from sympathetic nerves under resting conditions. This finding is consistent with αMNE reducing sympathetic activity by activating central noradrenergic neurones [1] as well as inhibiting NE release from sympathetic nerves by activating peripheral presynaptic α_2 adrenoceptors [16].

In summary, we have developed an assay for plasma catecholamines which is applicable to samples taken over the entire therapeutic range of αMD. The method can also be used in human and animal studies elucidating the mechanism of action of αMD.

ACKNOWLEDGEMENTS

We would like to thank Miss P. Scott and the clinical staff of the Clinical Research Unit for assistance in obtaining the rabbit and human samples.

REFERENCES

1 W. Kobinger, Rev. Physiol. Biochem. Pharmacol., 81 (1978) 40—100.
2 D.S. Goldstein, R. McCarty, R.J. Polinsky and J.J. Kopin, Hypertension, 5 (1983) 552—559.
3 D.A. Jenner, M.J. Brown and F.J.M. Lhoste, J. Chromatogr., 224 (1981) 507—512.
4 A.J. Barnett, A. Bobik, V. Carson, J.S. Korman and A. McLean, Clin. Exp. Pharmacol. Physiol., 4 (1977) 331—339.
5 A.H. Anton and D.F. Sayre, J. Pharmacol. Exp. Ther., 138 (1962) 360—375.
6 G.L. Jennings, M.D. Esler and P.I. Korner, Lancet, ii (1980) 166—169.
7 G.L. Jennings, A. Bobik, E.T. Fagan and P.I. Korner, Brit. J. Clin. Pharmacol., 7 (1979) 245—256.
8 E. Badoer, G.A. Head and P.I. Korner, J. Cardiovasc. Pharmacol., 5 (1983) 760—767.
9 H. Ong, S. Sved and N. Beaudoin, J. Chromatogr., 229 (1982) 433—438.
10 G.P. Jackman, Clin. Chem., 27 (1981) 1202—1204.
11 G.P. Jackman, C.J. Oddie, H. Skews and A. Bobik, unpublished results.
12 H. Hallman, L.O. Farnebo, B. Hamberger and G. Jonsson, Life Sci., 23 (1978) 1049—1052.
13 P. Hjemdahl, M. Daleskog and T. Khan, Life Sci., 25 (1979) 131—138.
14 D.S. Goldstein, G. Feverstein, J.L. Izzo, I.J. Kopin and H.R. Keiser, Life Sci., 28 (1981) 467—475.
15 J.D. Peuler and G.A. Johnson, Life Sci., 20 (1977) 625—636.
16 K. Starke, Rev. Physiol. Biochem. Pharmacol., 77 (1977) 2—124.

Journal of Chromatography, 308 (1984) 306—309
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2107

Note

Quantitative analysis of ascorbic acid-2-sulfate by high-performance liquid chromatography with electrochemical detection

C.S. TSAO*, M. YOUNG and S.M. ROSE

Linus Pauling Institute of Science and Medicine, 440 Page Mill Road, Palo Alto, CA 94306 (U.S.A.)

(First received December 2nd, 1983; revised manuscript received February 6th, 1984)

Ascorbic acid-2-sulfate (AAS) was found in brine shrimp cysts, in the urine of humans, monkeys, rats and guinea pigs [1—4] and in rat bile [5] and tissues [6, 7]. AAS has been reported to be one of the metabolites of ascorbic acid (AA, vitamin C). It has been suggested that AAS was involved in some physiological functions [4].

AA and AAS have been quantitatively differentiated by their different rates of osazone formation of the oxidized AA and AAS with 2,4-dinitrophenylhydrazine [8] and by their different rates of oxidation with 2,6-dinitrophenolindophenol and $KBrO_3$ [9]. Paper chromatography [1, 3, 5, 10—13] and thin-layer chromatography [1, 3, 5, 11—17] have been commonly used for the separation of these two substances in microgram quantities. High-voltage paper electrophoresis [5] has also been used. AAS has been quantitated by measuring the UV absorption of the eluent from a liquid chromatography column after isolation from animal tissues [1, 10, 18—20]. High-performance liquid chromatography (HPLC) with an ultraviolet (UV) detector has also been used for the separation of AA and AAS [21].

HPLC has been used for the measurement of AA with anion-exchange columns [21—27], LiChrosorb-NH_2 [28—31] reversed-phase C_{18} [24] and ion-pair reversed-phase C_{18} [32—34] columns with UV detection [27—31, 34, 35] and electrochemical detection [22—26, 32, 33].

In the present study, AAS is analyzed using reversed-phase HPLC with electrochemical detection. AAS has been shown to oxidize at an applied potential of 0.88 V vs. Ag/AgCl [33]. An ion-pairing agent, octylamine, is added to the acetate mobile phase. The procedure is simple and rapid and involves only homogenization of the sample, centrifugation and direct quanti-

0378-4347/84/$03.00

tation by HPLC. The method is reliable, highly sensitive, and can be used to detect accurately as low as 2 ng of AAS in a sample.

EXPERIMENTAL

HPLC

The equipment and instrumentation were thoroughly described by Pachla and Kissinger [22, 33]. All chromatograms were obtained using commercially available components and an amperometric detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.). The analytical column was a 7.5 cm × 4.6 mm I.D. Ultrasphere ODS 3-μm, Altex prepacked column (Cat. No. 244 254). The eluent which consisted of 30 m*M* acetic acid—acetate (22 m*M* glacial acid and 8 m*M* sodium acetate) plus 1.5 m*M* octylamine in ethanol—water (9.5:90.5) was partly degassed by a simple water aspirator before use. A Milton Roy pump (Model 396) equipped with a pulse dampener was used to pump the eluent through the system at a constant flow-rate of 0.7 ml/min. The injector valve was a Rheodyne injection valve (Model 7125). The detector electrode was packed with a wax-graphite paste, CP-W (BioAnalytical Systems). The potential of the chromatographic detector was set at 0.91 V vs. Ag/AgCl reference electrode. The concentration of AAS in the samples was determined by comparing the peak height or the area to that of a calibration standard.

Preparation of solutions

The stock AA or AAS solution was prepared freshly with 50 m*M* perchloric acid and diluted immediately before use. Urine samples were diluted 1:10 with cold 50 m*M* perchloric acid and analyzed immediately. A volume of 5 μl was injected into the HPLC column.

RESULTS

We have obtained chromatograms of AAS at detector potentials ranging from 0.85 to 1 V vs. Ag/AgCl. When the HPLC was run with an applied potential of 1 V vs. Ag/AgCl, the chromatograms of a urine sample showed the occurrence of many interfering substances in the sample, and a detector potential of 0.85 V vs. Ag/AgCl was not sufficient for the detection of very small quantities of AAS in the urine. Thus, an applied potential of 0.91 V vs. Ag/AgCl was used for the operation of the chromatographic detector, which would allow the electro-oxidation of AAS but would prevent the oxidation of many interfering substances in the urine unrelated to AAS analysis.

The ion-pair complex derived from AAS is relatively strongly retained on the column. Although AAS elutes in a relatively broader band, we found that either peak heights or peak areas may be used for the construction of the calibration curve. A calibration in terms of peak height or peak area versus nanogram amounts of AAS is linear for the concentration range of 2—50 ng AAS. The precision of this method was checked by multiple analysis on a single urine sample containing AAS. Typically, the relative standard deviation for six measurements is calculated to be better than 3%. We found that AAS in solution is much more stable than AA.

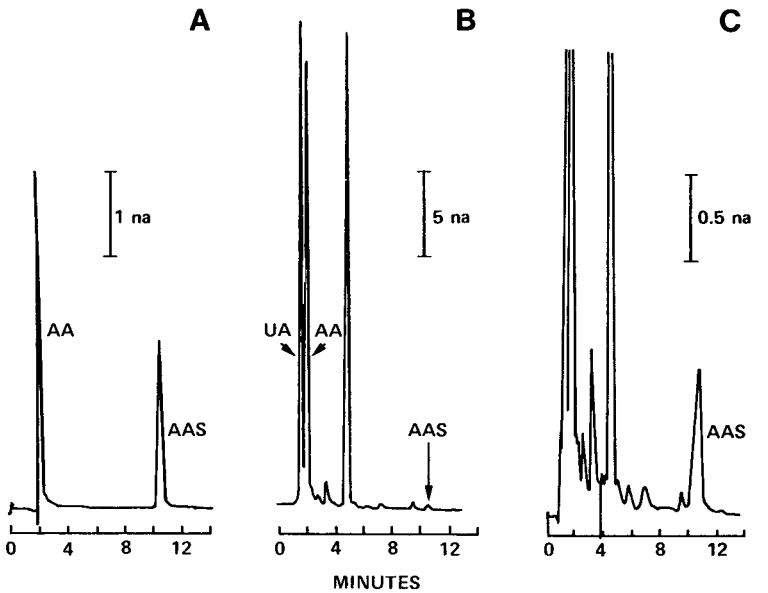


Fig. 1. Chromatograms for L-ascorbic acid (AA) and ascorbic acid-2-sulfate (AAS) in standard and in human urine samples, diluted 1:10, 5 μl injected. (A) 10 ng AA and 20 ng AAS standard injected; (B) urine sample containing uric acid (UA), AA and AAS; (C) urine sample, increased detector sensitivity and with 8 ng AAS standard addition. Conditions: Altex C_{18} reversed-phase 7.5 cm × 4.6 mm stainless-steel column; mobile phase: 30 m*M* acetate buffer plus 1.5 m*M* octylamine in ethanol—water (9.5:90.5) at a flow-rate of 0.7 ml/min; applied potential: 0.91 V vs. Ag/AgCl.

The advantages of the electrochemical detector are obvious in the analysis of biological samples. The UV detector for the detection of AAS [21] is far less sensitive and the chromatograms show interfering UV-absorbing components in biological samples [28—30].

The daily excretion of AAS in human urine has been reported to be 30—60 mg of the dipotassium salt [3, 35]. Fig. 1 illustrates the use of our method on the detection of AAS on urine samples. A parallel experiment of AA and AAS standards is shown in Fig. 1A for a chromatographic comparison. The chromatogram of the urine sample (Fig. 1B) shows the presence of uric acid (UA), AA and AAS as well as some unidentified electrochemically active compounds and the chromatography column is able to separate them. Two unidentified substances with elution times of 15 and 25 min, respectively are not shown in the figure. When the chromatograms were obtained under the same conditions but at a lower flow-rate of the mobile phase (0.5 ml/min), UA and AA were completely resolved. Fig. 1C shows the chromatogram of the urine sample with increased detector sensitivity and with standard AAS addition. The urine donor was a 28-year-old male subject. We found that 5 μl of the diluted urine sample contained 66 ng of AA and 1.5 ng of AAS. Thus, the concentrations of AA and AAS in the original urine of this subject were calculated to be 132 mg/l and 3 mg/l of the free acid, respectively.

REFERENCES

1 C.G. Mead and F.S. Finamore, Biochem., 8 (1969) 2652.
2 J.E. Halver, R.R. Smith, B.M. Tolbert and E.M. Baker, Ann. N. Y. Acad. Sci., 258 (1975) 81.
3 E.M. Baker, D.C. Hammer, S.C. March, B.M. Tolbert and J.E. Canham, Science, 173 (1971) 826.
4 B.M. Tolbert, M. Downing, R.W. Carlson, M.K. Knight and E.M. Baker, Ann. N. Y. Acad. Sci., 258 (1975) 48.
5 D. Hornig, H. Gallo-Torres and H. Weiser, Biochim. Biophys. Acta, 320 (1973) 549.
6 R.O. Mumma and A.J. Verlangieri, Biochim. Biophys. Acta, 273 (1972) 249.
7 D. Hornig, Ann. N. Y. Acad. Sci., 258 (1975) 103.
8 E.M. Baker, D.C. Hammer, J.E. Kennedy and B.M. Tolbert, Anal. Biochem., 55 (1973) 641.
9 M. Terada, Y. Watanabe, M. Kunitomo and E. Hayashi, Anal. Biochem., 84 (1978) 604.
10 D.W. Lillard, Jr. and P.A. Seib, ACS Symp. Ser., 77 (1978) 1.
11 A.J. Verlangieri and R.O. Mumma, Atherosclerosis, 17 (1973) 37.
12 A.D. Bond, B.W. McClelland, J.R. Einstein and F.J. Finamore, Arch. Biochem. Biophys., 153 (1972) 207.
13 P.A. Seib, Y.-T. Liang, C.-H. Lee, R.C. Hoseney and C.W. Deyoe, J. Chem. Soc. Perkin Trans 1, (1974) 1220.
14 D. Hornig, F. Weber and O. Wiss, Z. Klin. Chem. Klin. Biochem., 12 (1974) 62.
15 R.R. Muccino, R. Markezich, G.G. Vernice, C.W. Perry, J. Cupano and A.A. Liebman, Methods Enzymol., 62 (1979) 39.
16 H. Hatanaka, Y. Ogawa and F. Egami, J. Biochem., 75 (1974) 861.
17 H. Hatanaka and F. Egami, J. Biochem., 80 (1976) 1215.
18 S.C. March, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 31 (1972) 705.
19 Y. Akada, S. Kawano, T. Kondo, T. Sato and Y. Tanase, Yakugaku Zasshi, 97 (1977) 106.
20 M. Tsujimura, H. Yoshikawa, T. Hasegawa and T. Suzuki, Vitamins, 15 (1977) 207.
21 W.N. Bigler and D.M. Kelly, Ann. N. Y. Acad. Sci., 258 (1975) 70.
22 L.A. Pachla and P.T. Kissinger, Anal. Chem., 48 (1976) 364.
23 K.V. Thrivikrama, C. Refshang and R.N. Adams, Life Sci., 15 (1974) 1335.
24 R. Stillman and T.S. Ma, Mikrochim. Acta, (1974) 641.
25 K. Brunt and C.H. P. Bruins, J. Chromatogr., 172 (1979) 37—47.
26 C.S. Tsao and S.L. Salimi, J. Chromatogr., 224 (1981) 477—480.
27 L.W. Doner and K.B. Hicks, Anal. Biochem., 115 (1981) 225.
28 N. Arakawa, M. Otsuka, T. Kurata and C. Inagaki, J. Nutr. Sci. Vitaminol., 27 (1981) 1.
29 M. Otsuka, T. Kurata, E. Suzuki, N. Arakawa and C. Inagaki, J. Nutr. Sci. Vitaminol., 27 (1981) 9.
30 M.H. Bui-Nguyên, J. Chromatogr., 196 (1980) 163.
31 R.C. Rose and D.L. Nahrword, Anal. Biochem., 114 (1981) 140.
32 C.S. Tsao and S.L. Salimi, J. Chromatogr., 245 (1982) 355.
33 L.A. Pachla and P.T. Kissinger, Methods Enzymol., 62 (1979) 15.
34 J.W. Finley and E. Duang, J. Chromatogr., 207 (1981) 449.
35 E.M. Baker, J.E. Kennedy, B.M. Tolbert and J.E. Canham, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 31 (1972) 705.

Journal of Chromatography, 308 (1984) 310—315
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2078

Note

High-performance liquid chromatographic determination of imidazole in biological fluids

P. CREMONESI*, G. GALIMBERTI, O. COLETTI and G. SPORTOLETTI

Research Group Italfarmaco, V. le F. Testi 330, 20126 Milan (Italy)

(First received October 12th, 1983; revised manuscript received January 18th, 1984)

Since the discovery of the role of thromboxane A_2 [1] and prostacyclin PGI_2 [2] in the regulation of platelet function, there has been considerable interest in the property of imidazole derivatives that inhibit thromboxane synthetase activity [3]. Consequently a series of 1-alkyl or alkylaryl imidazoles have been studied [4—6]. Most of these derivatives are active at low concentrations, and determination of the plasma levels requires the use of sensitive methodology such as a labelled compound [7] or high-performance liquid chromatography (HPLC) [8].

Imidazole (I) itself is a competitive inhibitor of thromboxane synthetase with a K_i of 10^{-4} *M* [9]. Imidazole 2-hydroxybenzoate (II, see Fig. 1) is a derivative which may exert this activity when administered, because of its content of I, and it has shown anti-inflammatory activity in animal experiments [10]. It therefore became interesting to have a method of determination of I in biological fluids as a tool for the study of its pharmacokinetics and metabolism.

The high solubility of I in water does not allow the use of a two-phase extraction (water and organic solvent) for the isolation of I from biological fluids and, to our knowledge, no methodology for analysis in biological materials has been reported in the literature. In addition, the same methodology should be

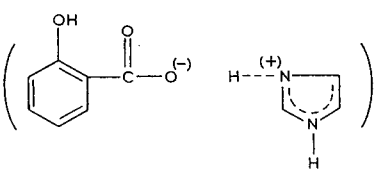


Fig. 1. Chemical structure of imidazole 2-hydroxybenzoate (II).

0378-4347/84/$03.00

useful for the determination of other drugs that can produce I as a metabolite.

Because of the low content of I expected in biological fluids after the administration of II, many of the analytical procedures in the literature [11—13] are unsuitable due to their low sensitivity. We report a sensitive and specific method based on HPLC for monitoring I in biological fluids.

MATERIALS AND METHODS

Analytical grade reagents and spectrophotometric grade eluents were used. Imidazole and salicylic acid were purchased from Merck (Darmstad, F.R.G.) and II was prepared following the procedure previously described [14].

HPLC determination

The HPLC apparatus was a Jasco Model Twincle equipped with a 25-μl loop injector and an ultraviolet (UV) detector (Uvidec 100, equipped with an 8-μl cell). Several columns packed with C_{18} or C_8 reversed phases were assayed with different t_R responses for I.

Using plasma extracts, the Finepack Sil C_{18} column (250 mm × 4.6 mm I.D., 10 μm, reversed-phase C_{18}, Jasco) gave the highest efficiency and was therefore used in determining I in the biological fluid extracts.

Elution times of I were found to be strongly dependent on the pH and ionic strength of the eluent. Satisfactory results were obtained with 0.003 *M* potassium phosphate buffer—methanol (60:40, v/v) titrated at pH 7.2 with phosphoric acid.

The UV absorbance maximum reported for I is at 207 nm (with log ϵ = 3.70), thus the detector was set at 210 nm for monitoring.

Extraction procedure

To 1 ml of plasma, 2.5 ml of acetonitrile were added. The sample was vortexed to favour the precipitation of the protein moiety and after centrifuging at 750 *g* for 10 min, the clear supernatant solution (A) was quantitatively transferred to a conical flask and evaporated to dryness at 35°C under a nitrogen stream. To the residue 200 μl of eluent were added and the opalescent solution was centrifuged at 750 *g* for 5 min; 25 μl of the solution were injected into the HPLC apparatus. When the concentration was higher than 10^{-4} *M*, 25 μl of solution A were used for analysis. Urine samples were diluted twenty times with eluent and a 25-μl volume was injected.

Calibration curves

The calibration curve was made with 1 ml of plasma sample containing up to 10 μg of I. The samples were treated following the above procedure.

The calibration curve for urine was made with 1 ml of centrifuged urine diluted twenty times with the eluent and containing up to 3 μg of I.

Plasma levels and renal excretion

The applicability of the HPLC method for determining I in biological fluids was checked after administration of II to rats. Animals weighing about 250 g were used, and 50 mg/kg of II were administered per os in aqueous solution. Blood was collected at 15, 30, 60, and 120 min.

The same experiment was made on another group of animals and the urine was collected over the period of 0—1, 1—4, 4—8, and 8—24 h after administration. The plasma and urine samples could be stored at −20° C until analysed.

Following a similar blood collection schedule, plasma samples were obtained from human adult volunteers who had taken one 750-mg tablet of II.

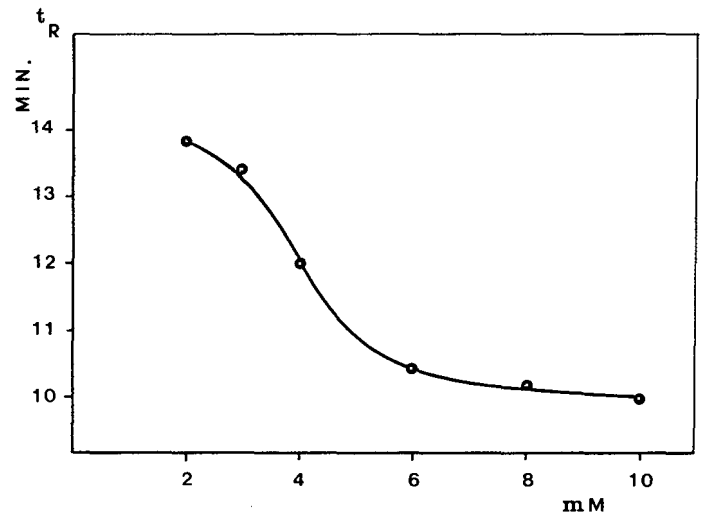


Fig. 2. Dependence of t_R of imidazole on ionic strength. Eluent, phosphate buffer (different molarities)—methanol titrated at pH 7.2 with phosphoric acid; flow-rate, 0.9 ml/min; detector, UV 210 nm.

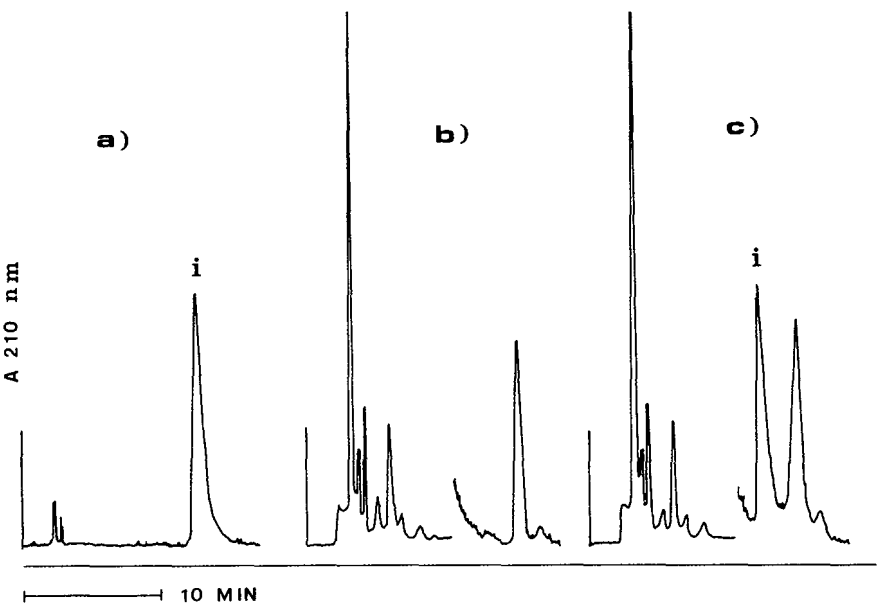


Fig. 3. Chromatograms of imidazole (a), control serum (b) and control serum with 1 μg/ml imidazole added (c). Eluent, phosphate buffer (0.003 *M*)—methanol (60:40, v/v) titrated at pH 7.2 with phosphoric acid; flow-rate, 0.9 ml/min; detector, UV 210 nm.

RESULTS AND DISCUSSION

I can be detected by HPLC using UV absorption in the far region of the UV spectrum where a strong absorption of other plasma components can interfere. Therefore the selectivity of the determination is based mainly on the efficiency of the chromatographic phase and the eluent. The t_R of I was dependent on the pH and ionic strength of the eluent. As Fig. 2 shows, I can be eluted between 10 and 14 min by varying the molarity of the buffer. This allowed the choice of the appropriate molarity of buffer to "drive" the elution of imidazole in the region where the plasma components have low interference. Useful conditions for the plasma samples were found to be 3 m*M* phosphate buffer, as shown in Fig. 3.

The lowest detectable amount was 10 ng for pure imidazole and 25 ng for imidazole in plasma.

Calibration curve

The determination of I added to plasma showed a linear response up to 100 μg/ml with a recovery of 85 ± 5% in the range 0.3—3 μg/ml and 97 ± 7% in the range 10—100 μg/ml.

Plasma levels and renal excretion

The plasma levels of I, after oral administration of II, in rats (50 mg/kg) and in man are exemplified in Fig. 4a and b. The two curves show that I is rapidly absorbed and is largely excreted in unmodified form as Table I illustrates.

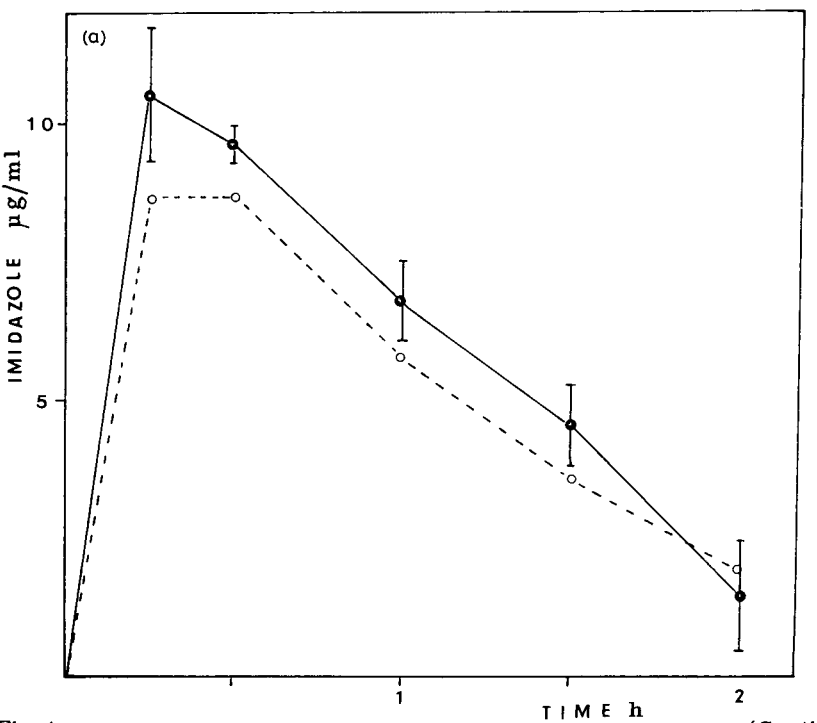


Fig. 4.

(Continued on p. 314)

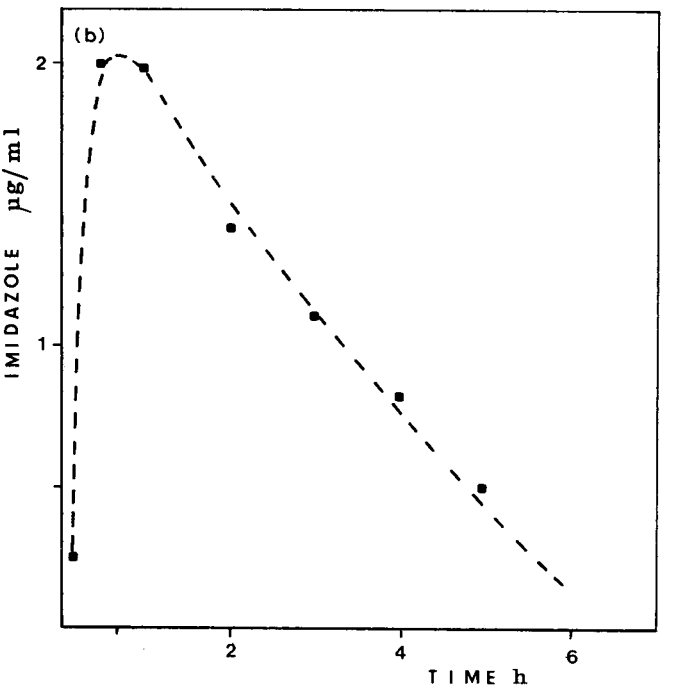


Fig. 4. (a) Plasma samples of imidazole as a function of time. (•) 50 mg/kg of II, and (○) 17 mg/kg of imidazole, administered to rats; plasma was obtained from citrated (10%) blood. Same conditions as Fig. 3. (b) Plasma levels of imidazole as a function of time. Conditions: 750 mg of II were administered to human volunteers; plasma was obtained from citrated (10%) blood. Same chromatographic conditions as Fig. 3.

TABLE I

HPLC DETERMINATION OF IMIDAZOLE IN URINE

Dosage*	Time intervals (h)	R_1** (%)	± S.D.	± S.E.	R_2*** (%)
A	0— 1	9.08	2.89	1.67	—
A	1— 4	2.65	1.41	0.82	—
A	4— 8	9.16	6.83	3.94	—
A	8—24	1.75	2.27	1.60	22.64
B	0— 1	8.18	5.06	2.92	—
B	1— 4	8.70	0.85	0.49	—
B	4— 8	2.43	2.59	1.49	—
B	8—24	9.36	8.21	4.74	28.67

*Administration of II to rats: 50 mg/kg per os (A); 50 mg/kg intravenously (B).
**R_1 = percentage recovery for each time interval.
***R_2 = percentage recovery for 24 h.

CONCLUSIONS

Several methods are reported in the literature for the determination of imidazole derivatives, utilized as drugs, that can be extracted from biological fluids by organic solvents in a two-phase system. For imidazole this procedure did not give good results. With the HPLC methodology reported here, I can be determined in plasma samples with a minimum detectable concentration of $4.5 \cdot 10^{-6}$ *M*. The determination can be applied also to biological fluids other than plasma and is useful for drugs that can give I as a product.

REFERENCES

1 M. Hamberg, J. Svensson and B. Samuelsson, Proc. Nat. Acad. Sci. U.S., 72 (1975) 2994.
2 S. Moncada, R. Gryglewski, S. Bunting and J.R. Vane, Nature, 236 (1976) 663.
3 P. Needleman, A. Raz, J.A. Ferrendelli and M. Minkes, Proc. Nat. Acad. Sci. U.S., 74 (1977) 1716.
4 H.H. Tai and B. Yuan, Biochem. Biophys. Res. Commun., 80 (1978) 236.
5 T. Yoshimoto, S. Yamamoto and O. Hayashi, Prostaglandins, 16 (1978) 529.
6 K. Iizuka, K. Akahane, D. Momase, M. Nakazawa, T. Tanouchi, M. Kawamura, I. Ohyama, I. Kajiwara, Y. Iguchi, T. Okada, K. Taniguchi, T. Miyamoto and M. Hayshi, J. Med. Chem., 24 (1981) 1139.
7 Anonymus, Drugs of the Future, 7 (1982) 460.
8 A.M. Tyler, C.A. Saxton and M.J. Parry, Lancet, i (1981) 629.
9 S. Moncada, S. Bunting, K. Mullane, P. Thorogood, J.R. Vane, A. Raz and P. Needleman, Prostaglandins, 13 (1977) 611.
10 P. Pagella, O. Bellavite, S. Agozzino, G. Donà, P. Cremonesi and F. De Santis, Arzneim.-Forsch., 33 (1983) 716.
11 H. Nakamura, S. Tsuzuki, Z. Tamura, R. Yoda and Y. Yamamoto, J. Chromatogr., 200 (1980) 324.
12 J.D. Ramsey, T.D. Lee, M.D. Ossleton and A.C. Moffat, J. Chromatogr., 184 (1980) 185.
13 S.K. Wadman, P.K. DeBree, C. Van der Heiden and F.J. Sprang, Clin. Chim. Acta, 31 (1971) 215.
14 G. Sportoletti, U. S. Pat., 4,329,340 (1982).

Journal of Chromatography, 308 (1984) 316—321
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2059

Note

Simple single-step high-performance liquid chromatographic method for the separation of cyclooxygenase and lipoxygenase enzyme metabolites of arachidonic acid

CAROLYN S. COCKRELL and EARL F. ELLIS*

Department of Pharmacology and Toxicology, Box 613, MCV Station, Medical College of Virginia, Richmond, VA 23298-0001 (U.S.A.)

(First received September 14th, 1983; revised manuscript received January 3rd, 1984)

Arachidonic acid (AA) is metabolized in numerous biological systems by the cyclooxygenase and lipoxygenase enzymes. The products of these pathways comprise several groups of metabolites including the primary prostaglandins (PGs) E_2, F_{2a} and D_2, thromboxane (Tx) A_2 and its stable hydrolysis product TxB_2, PGI_2 and its stable hydrolysis product 6-keto-PGF_{1a}, hydroxylated derivatives of AA (hydroxyeicosatetraenoic acids, HETEs), and leukotrienes (LTs), which have in addition to the AA-derived portion of the molecule all or part of the tripeptide glutathione [1].

Since both enzymatic pathways are functional in many cell types, individual samples are often analyzed for both cyclooxygenase and lipoxygenase products. The current approach to leukotriene separation is high-performance liquid chromatography (HPLC) with quantitation often dependent on biological assay or UV spectrometry [1, 2]. Thin-layer chromatography (TLC) and HPLC are the methods of choice for separating PGs and HETEs with additional quantification by radioimmunoassay or gas chromatography—mass spectrometry. HPLC of arachidonic acid metabolites has been recently reviewed by Hamilton and Karol [1].

To our knowledge there is no published simple single-step method for the simultaneous separation of the metabolites of both enzymatic pathways. Rouzer et al. [3] use HPLC to separate cyclooxygenase and lipoxygenase products of two types of pulmonary macrophages but must rechromatograph the fractions containing PGs to achieve separation of the various PGs. Several TLC systems allowing rapid separation and identification of metabolites of both pathways have been reported. One fails to resolve TxB_2 from PGE_2 satisfactorily and 15-HETE from 12- and 5-HETE [4] while others do not

0378-4347/84/$03.00

address the separation of LTs from other lipoxygenase [5] or cyclooxygenase products [6].

We report here a combination of solvent systems that separate 6-keto-PGF_{1a}, TxB_2, PGE_2, PGF_{2a}, PGD_2, HHT, LTB_4/LTC_4, LTD_4, 5-HETE, 12-HETE, 15-HETE and AA in a single 100-min HPLC run. The value of this method is two-fold. First, it reduces time in sample analysis by reducing the likelihood of needing additional chromatography, which in turn minimizes sample loss. Secondly, it can be performed without a separate solvent programming device, and, since a standard reversed-phase column is used, a radial compression device [7] is not required.

EXPERIMENTAL

Materials

Acetonitrile and methanol (distilled in glass) were purchased from Burdick & Jackson Labs. (Media, PA, U.S.A.). Water was deionized and purified with a Milli-R/Q system (Millipore, Bedford, MA, U.S.A.). Tritium-labeled standards were purchased from New England Nuclear (Boston, MA, U.S.A.) with the exception of [^{3}H]12-hydroxy-5,8,10-heptadecatrienoic acid (HHT) which was synthesized by washed platelets from radiolabeled AA.

Chromatography

The HPLC apparatus consists of a single Waters 6000A pump (Milford, MA, U.S.A.) a Rheodyne Model 7125 injector (Cotati, CA, U.S.A.) and a 30 cm × 3.9 mm μBondapak C_{18} 10-μm particle size reversed-phase column and a phenyl Corasil packed guard column (Waters).

Elution was accomplished in less than 100 min with three different solvents. Flow-rate was 2 ml/min throughout and 1-min fractions were collected and counted in a scintillation counter. Samples were dissolved in 2 ml of solvent A, water—acetonitrile—benzene—acetic acid (767:230:2:1, v/v/v/v), prior to injection. Solvent A was pumped for 48 min before switching to solvent B, methanol—water—acetic acid (650:350:0.1, v/v/v, pH 4.2). Solvent B continued for 42 min before switching to solvent C, methanol—water—acetic acid (900:100:0.1, v/v/v), which was pumped for 10 min.

Blood platelets

Nine volumes of blood were drawn from the femoral vein of pentobarbital (30 mg/kg) anesthetized cats into one volume of 77 m*M* sodium EDTA and washed platelets prepared according to the method of Hamberg et al. [8]. Broken platelets were prepared by freezing and thawing the washed platelet suspension three times. Aliquots (475 μl) of the washed platelets were incubated in an aggregometer (Payton Assoc., Buffalo, NY, U.S.A.) at 37°C with constant stirring for 1 min before any additions were made. Platelets received 5 μl of a solution which contained 1 μCi [^{3}H]AA (62 Ci/mmol, New England Nuclear) and 0.5 μg of unlabeled AA (Nu Chek Prep., Elysian, MN, U.S.A.) as the sodium salt. The platelets and AA were incubated for 5 min. Aggregation is not induced by this concentration of AA. For some experiments indomethacin (Sigma, St. Louis, MO, U.S.A.) and sodium car-

bonate were weighed out (1:3, w/w), dissolved in distilled water and added to the platelets 1 min before AA to produce a final indomethacin concentration of 3 μg/ml of platelets. The pH of the indomethacin stock solution was measured to ensure it did not exceed 8, since alkaline solutions destroy indomethacin [9].

Following the 5-min incubation of the platelet suspensions the radiolabeled products were extracted and separated by HPLC. First the protein in each platelet incubate was precipitated with 300 μl of acetone and the pH adjusted to 3 with 0.4% formic acid. The incubate was then extracted twice with 3 ml of ethyl acetate. The ethyl acetate extract was dried under a stream of nitrogen, dissolved in 2 ml of chloroform and filtered with a 0.45-μm Millipore filter (Gelman, Ann Arbor, MI, U.S.A.). The chloroform was then removed with nitrogen and the residue dissolved in 2 ml of solvent A.

RESULTS AND DISCUSSION

The retention times of authentic standards separated by this HPLC method are seen in Fig. 1. Solvent A separates cyclooxygenase products, including PGD_2 and PGE_2. The same solvent run in like manner on a Waters Fatty Acid Analysis reversed-phase column does not resolve these two metabolites. Solvent B separates lipoxygenase products. However, note that LTB_4 and LTC_4 co-elute. Changes in solvent pH did not change this co-elution, nor did the use of a gradient from solvents A to B. It has been reported by others that LTB_4 and LTC_4 can be separated using a Nucleosil C_{18} column and a solvent system

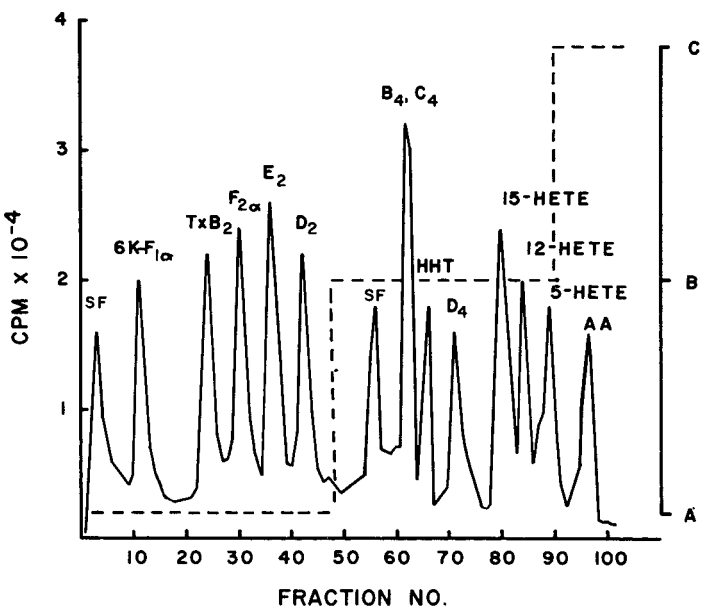


Fig. 1. Single-step HPLC separation of PGs, LTs, HETEs and AA. The labeled peaks are retention times of authentic standards. A μBondapak C_{18} column is used with a flow-rate of 2 ml/min (1-min fractions). The solvent composition is given in the text. The dashed line indicates the time and manner in which the solvents are changed.

similar to solvent B but at a higher pH [6]. Elution of AA is readily accomplished with solvent C. Some investigators have reported poor or variable recoveries of leukotrienes using a C_{18} column [2]. Table I shows that the mean recovery of ^3H standards from a C_{18} column using our method was 82%. We feel that in light of the highly effective separation that this method accomplishes, these recoveries are acceptable. We might also note that this HPLC system can be more fully automated with the use of two pumps and a solvent switching device or a third pump used in conjunction with a Waters System Controller. Gradients can be run from one solvent to the next if desired, but retention times increase accordingly.

TABLE I

RECOVERY OF ^3H-LABELED STANDARDS

Compound	Recovery (%) (n = 3)
6-keto-PGF_{1a}	83
TxB_2	82
PGF_{2a}	57
PGE_2	79
PGD_2	90
LTB_4	80
LTC_4	85
LTD_4	72
15-HETE	79
12-HETE	74
5-HETE	78
AA	90
Mean	82

This HPLC solvent system has been applied successfully in our laboratory to study blood platelet metabolism of [^3H]AA. When washed, whole blood platelets are incubated for 5 min with [^3H]AA and the products extracted and run on this HPLC system a product profile is obtained, as shown in Fig. 2. We have used this HPLC system to compare the metabolism of AA in whole versus broken platelets. Table II shows that in whole cells 30% of the products were cyclooxygenase products, with approximately equal distribution between TxB_2 and HHT. The lipoxygenase product 12-HETE was twice as abundant as the cyclooxygenase products while only 9% of the [^3H]AA was not metabolized.

The percent distribution of TxB_2, HHT, 12-HETE and AA produced by broken cells was significantly different from the percent distribution of the same products in whole cells ($P < 0.01$, Student's t-test). With broken platelets, 12-HETE was half as abundant as in whole platelets while the percent cyclooxygenase products and unutilized AA both increased. The reason for this difference may be related to the fact that lipoxygenase is a soluble enzyme and in broken cells would be dispersed into the incubation buffer, thus decreasing its local concentration. This would allow more arachidonate to be

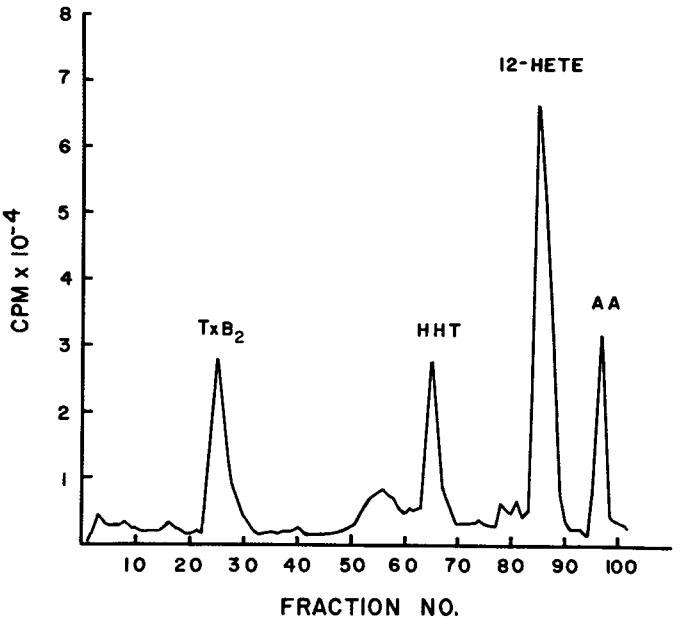


Fig. 2. Single-step HPLC separation of the cyclooxygenase and lipoxygenase products of whole washed cat platelets incubated with [^{3}H]AA.

TABLE II

METABOLISM OF [^{3}H]ARACHIDONIC ACID BY WHOLE OR BROKEN PLATELETS AND THE EFFECT OF INDOMETHACIN

	n	Product distribution of ^{3}H (mean ± S.E.M., %)			
		TxB_2	HHT	12-HETE	AA
Whole cells	4	13.5 ± 0.6	16.3 ± 1.4	61.3 ± 1.1	9.0 ± 1.0
Broken cells	3	14.0 ± 3.0	19.0 ± 4.8	32.6 ± 1.6	34.4 ± 7.9
Broken cells plus indomethacin (3 μg/ml)	6	0.4 ± 0.1	1.1 ± 0.1	48.2 ± 2.6	50.2 ± 2.6

utilized by the microsomal enzyme, cyclooxygenase, and also result in a decreased overall utilization of AA during the 5-min incubation period. In order to demonstrate that our experimental system is capable of detecting the effect of inhibitors, we pretreated broken platelets with the cyclooxygenase inhibitor, indomethacin. Indomethacin virtually abolished the cyclooxygenase products TxB_2 and HHT and increased the percent distribution in both 12-HETE and AA.

In summary, we have reported a relatively efficient HPLC method that uses only a single pump and column to achieve separation of twelve major AA metabolites. This capability will become increasingly important in light of the extensive work being done on the interrelationship of the cyclooxygenase and lipoxygenase pathway.

ACKNOWLEDGEMENTS

This work is supported by NS 12587 and a Grant-in-Aid from the American Heart Association. We thank Mrs. R. Harris for typing this manuscript.

REFERENCES

1 J.G. Hamilton and R.J. Karol, Progr. Lipid Res., 21 (1982) 155.
2 S.A. Metz, M.E. Hall, T.W. Harper and R.C. Murphy, J. Chromatogr., 233 (1982) 193.
3 C.A. Rouzer, W.A. Scott, A.L. Hamill and Z.A. Cohn, J. Exp. Med., 155 (1982) 720.
4 S.J. Harris and N.L. Baenziger, Prostaglandins, 25 (1983) 733.
5 K.C. Srivastava and K.K. Awasthi, J. Chromatogr., 275 (1983) 61.
6 W.R. Mathews, J. Rokach and R.C. Murphy, Anal. Biochem., 118 (1981) 96.
7 T. Eling, R. Warnock, D. Dick and B. Trainer, Prostaglandins Med., 5 (1980) 345.
8 M. Hamberg, J. Svensson, T. Wakabayashi and B. Samuelsson, Proc. Nat. Acad. Sci. U.S., 71 (1974) 345.
9 S.H. Curry, E.A. Brown, H. Kuck and S. Cassin, Can. J. Physiol. Pharmacol., 60 (1982) 988.

Journal of Chromatography, 308 (1984) 322—328
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2076

Note

Isolation and purification of HLA-DR antigens from tumour cells by affinity chromatography and chromatofocusing

D. BERNARD

Laboratoire d'Immunologie, Centre Jean Perrin, 30, Place Henri Dunant, B.P. 392, 63011 Clermont-Ferrand Cedex (France)

J.C. MAURIZIS*

INSERM U 71, Rue Montalembert, 63005 Clermont-Ferrand Cedex (France)

and

Ph. CHOLLET, J. CHASSAGNE and R. PLAGNE

Laboratoire d'Immunologie, Centre Jean Perrin, 30, Place Henri Dunant, B.P. 392, 63011 Clermont-Ferrand Cedex (France)

(Received December 8th, 1983)

The major histocompatibility complex (MHC) controls the expression of several membrane proteins in human and other mammals.

Owing to the high degree of polymorphism of these antigens, each organism can recognize its own proteins very specifically. This plays an essential role in the interactions of the immune system. The HLA-DR region of the MHC, localized in the short arm of the 6th chromosome controls antigen expression (Ia-like), whose importance in immunological reactions has been demonstrated [1—4]. Ia-like antigens are glycoproteins composed of two subunits of a molecular weight of 34,000 (α-chain) and 28,000 (β-chain). Their presence has been demonstrated in the cellular lineages of the immune system: B lymphocytes, T lymphocytes and macrophages [5]. This antigen can be expressed in the lactating mammary gland [6] and in certain malignant cells such as melanoma [7].

Apart from lactation, the normal mammary gland does not express this

0378-4347/84/$03.00

antigen. However, certain breast epithelioma may express Ia-like antigens. In fact, by analogy with the lactating gland, it would be interesting to relate the percentage of prolactin receptors on mammary tumours to the expression of Ia antigens on these same tumours. The setting up of such a relationship would involve a quantitative method of determining Ia protein on tumour cells. Until now, different published works have attempted to identify the protein, and have only led to the determination of its presence or absence on a given cell [8, 9].

The procedure of isolating the Ia-like antigen, which different authors have described [8, 9], consists of purifying membrane glycoproteins by affinity chromatography on lectin, immunoprecipitation with monoclonal antibody immune complex fixation on *Staphylococcus aureus* or protein A—Sepharose and protein release by immune complex dissociation. With this procedure, the antigen can be identified, but the proportion of glycoproteins it represents cannot be evaluated quantitatively.

To this purpose, a technique has been developed which combines the double labelling (^{131}I, ^{125}I) of membrane proteins and antibodies, and isolation of the immune complex by affinity chromatography and chromatofocusing. Thus a purified and doubly labelled immune complex has been obtained which allows a quantitative evaluation of the antigen linked to the antibody.

EXPERIMENTAL

Tumour cells

Cells were obtained by enzymatic dissociation [10]. Solid tumours are cut into thin strips then incubated at 37°C for 2 h in an RPMI 1640 medium containing 10% foetal calf serum, 0.4% collagenase I, and 0.002% deoxyribonuclease I. The number of viable cells is determined by trypan blue exclusion.

Products

Sephadex G-25, lentil-lectin—Sepharose 4B, gel PBE 9-4, and polybuffer 9—6 were from Pharmacia (Uppsala, Sweden), protein A—Ultrogel was from IBF (Villeneuve la Garenne, France), Nonidet P 40 from Serva (Heidelberg, F.R.G.), and glucose, glucose oxidase, and lactoperoxidase were from Boehringer (Mannheim, F.R.G.). The monoclonal human anti-Ia antibody produced in the mouse was from the Ortho-Pharmaceutical Corporation (Raritan, NJ, U.S.A.). ^{125}I and ^{131}I were from the Atomic Energy Commissariat (France). The Bolton and Hunter ^{125}I reagent was from Amersham (U.K.)

Cell labelling with ^{131}I

The cell suspension, at a concentration of 10^7 cells per ml in 0.05 *M* phosphate buffer pH 7.2 containing 0.15 *M* sodium chloride (PBS) was added to 10 units of lactoperoxidase, 10 units of glucose oxidase, 100 μg of glucose, and 1 mCi of $Na^{131}I$. After 10 min incubation at room temperature, the reaction was blocked by the addition of 50 μl of a tyrosine solution (0.5 *M*) in PBS.

Solubilization of membrane proteins

The suspension of labelled cells was centrifuged for 10 min at 600 *g*. The cells were then washed twice in PBS and suspended in 1 ml of PBS containing 2% Nonidet P 40, for 15 min at 4° C with gentle stirring.

The suspension was then centrifuged for 30 min at 30,000 *g*. The protein concentration was measured using the method of Lowry et al. [11]. Labelled proteins were purified on a small Sephadex G-25 column (5 cm × 1 cm), eluted with PBS.

Purification of glycoproteins on Lens culinaris lectin—Sepharose 4B column

The labelled proteins were placed at the top of a *Lens culinaris* lectin—Sepharose 4B column (10 × 0.9 cm) that had previously been equilibrated with 2—3 vols. of PBS.

The column was eluted at 15 ml/h with 30 ml of PBS, then with 15 ml of PBS containing 2% α-methylmannoside, which selectively desorbs glycoproteins. Fractions of 1 ml were collected by an automatic collector and counted in a dual-channel autogamma spectrometer. The fractions containing glycoproteins were concentrated up to a volume of 1 ml by ultrafiltration (Immersible C × 10, Millipore).

Antibody labelling with ^{125}I

The procedure of Bolton and Hunter was used [12]. First, 100 μCi of Bolton and Hunter reagent ^{125}I (2000 Ci/mmol) in 10 μl of benzene were evaporated under nitrogen in a small glass tube. Then 40 μl of a solution containing 80 μg of monoclonal anti-Ia antibody in PBS pH 7.2 were added. The reaction was left to take place for 10 min at 4° C, and was then blocked by adding 20 μl of 0.05 *M* borate buffer pH 8.2 containing 0.5 *M* glycine. The labelled antibody was purified by chromatography on a small column (3 × 0.5 cm) of Sephadex G-25. Its specific activity was 0.2—0.4 μCi/μg.

Immmunoprecipitation

The solution of ^{131}I-labelled proteins obtained as previously described was incubated at room temperature by stirring with 80 μg of ^{125}I-labelled antibodies.

Isolation of immune complex on protein A—Ultrogel

The reaction medium was fixed on to a protein A—Ultrogel (6 × 0.9 cm) column previously equilibrated with 2—3 vols. of PBS. The column was eluted at 15 ml/h with 20 ml of PBS, then with 10 ml of 0.025 *M* glycine buffer pH 2.6 which desorbed the doubly labelled immune complex.

Purification of immune complex by chromatofocusing

The fractions containing the immune complex previously dialysed against 0.025 *M* ethanolamine buffer pH 9.4 were fixed on the top of a PBE 9-4 gel column (30 × 0.9 cm) equilibrated with 0.025 *M* ethanolamine buffer pH 9.4. The elution was conducted at 10 ml/h with 150 ml of polybuffer 9—6 pH 6. Fractions of 2 ml were collected with an automatic fraction collector and counted in a dual-channel autogramma spectrometer.

RESULTS AND DISCUSSION

The solubilization of cellular membranes yielded 2—4 mg of labelled proteins per 10^7 cells. The specific activity of these proteins was 20—40 μCi/mg. The extraction yield of glycoproteins on *Lens culinaris* lectin—Sepharose 4B was 2—4% in agreement with results obtained on T lymphocytes [9]. The immunoprecipitation of the Ia glycoprotein by monoclonal antibody led to the formation of an immune complex that could not be isolated quantitatively by ultracentrifugation.

In these conditions, the method of choice for isolating the immune complex is affinity chromatography on protein A, a ligand specific to immunoglobulins. Fig. 1 shows the elution pattern obtained by chromatography on protein A—Ultrogel of the reaction medium after immunoprecipitation of ^{131}I-labelled glycoproteins with monoclonal antibody labelled with ^{125}I. The PBS buffer eluted only the ^{131}I proteins, while the glycine buffer pH 2.6 eluted doubly labelled proteins. This result shows that all of the immune complex was bound to protein A. The elution yield of ^{125}I was 85%; 8—12% of glycoproteins were eluted simultaneously with the antibody. In order to test the possible non-specific link to protein A—Ultrogel, labelled membrane glycoproteins underwent chromatography in the same conditions; 85—90% of the proteins were eluted by the PBS buffer, and 2—6% by the glycine buffer pH 2.6. This shows that some glycoproteins can be linked to protein A—Ultrogel at pH 7.2, and released at an acidic pH. It is obvious that the immune complex eluted by the glycine buffer is greatly contaminated by proteins that are not specifically linked to the gel.

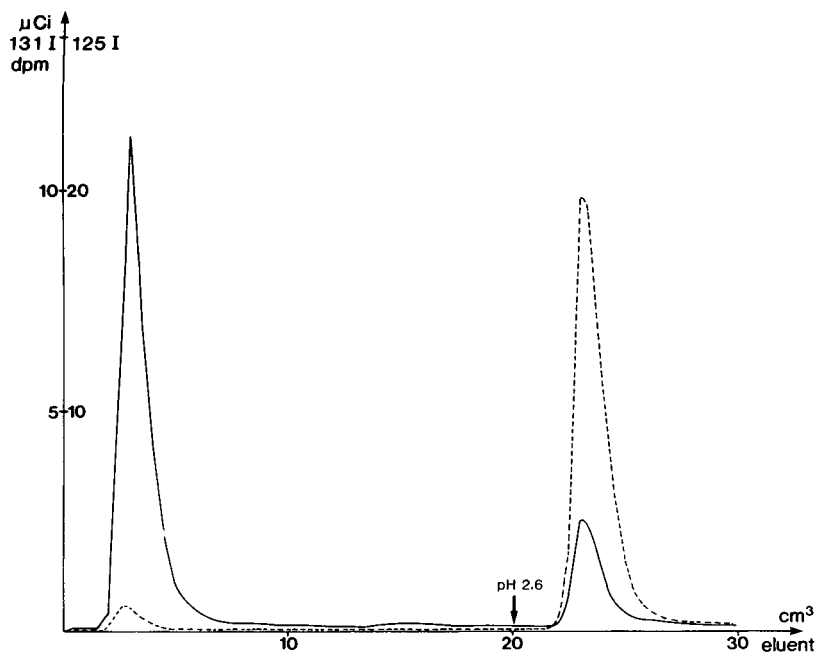


Fig. 1. Separation of the Ia—anti-Ia immune complex from solubilized membrane glycoproteins on protein A—Ultrogel column. Eluent 1: PBS pH 7.2. Eluent 2: 0.025 *M* glycine buffer pH 2.6. (——), ^{131}I; (- - - -), ^{125}I.

The final purification method of the immune complex was chromatofocusing. Indeed, antigen—antibody reactions lead to the polymerization of proteins reaching very high molecular weights, which inhibit gel filtration and polyacrylamide gel electrophoresis. A method that used the separation of proteins as a function of their isoelectric point, irrespective of their molecular weight, appeared to lead to a satisfactory purification of the immune complex. The isoelectric chromatofocusing consists of eluting an anionic ion-exchange column equilibrated at an alkali pH with a buffer containing an ampholyte mixture (polybuffer) with an acidic pH. During elution, a pH gradient will form between the pH of the gel and the buffer pH. Since the isoelectric point of γ-globulins falls between 7.3 and 6.3 [13], a pH gradient of 9 to 6 (polybuffer 9—6) was chosen. The protein solution was poured on to the column with a pH of 9.4. The negatively charged proteins were fixed on to the gel. They will be selectively released each time the pH gradient reaches the pH of each protein. Thus the proteins are focalised in a very small volume of eluent, which makes very fine separation possible.

Fig. 2 shows an example of the purification of the immune complex isolated on protein A—Ultrogel. The fraction containing the antibody was eluted at a very narrow peak, at pH 7.15. The other labelled fractions only contained ^{131}I. The purity of the doubly labelled immune complex was tested as follows: the glycoproteins were incubated with the ^{125}I-labelled antibody in the presence of 10^6 T lymphocytes. The T lymphocytes contain the Ia antigen in their membrane proteins. If this protein is specifically linked to the antibody, the

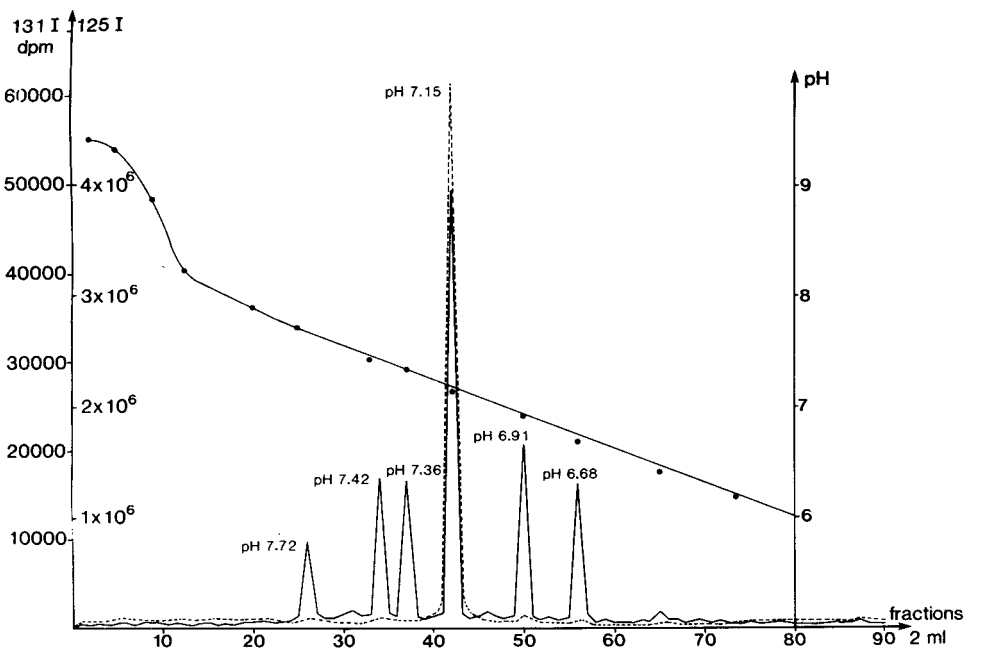


Fig. 2. Elution pattern of doubly labelled immune complex obtained from breast tumour cells on PBE 9.4 gel column, eluted at a flow-rate of 10 ml/h with polybuffer 9—6 pH 6. (——), ^{131}I; (- - - -), ^{125}I; (•——•), eluent pH.

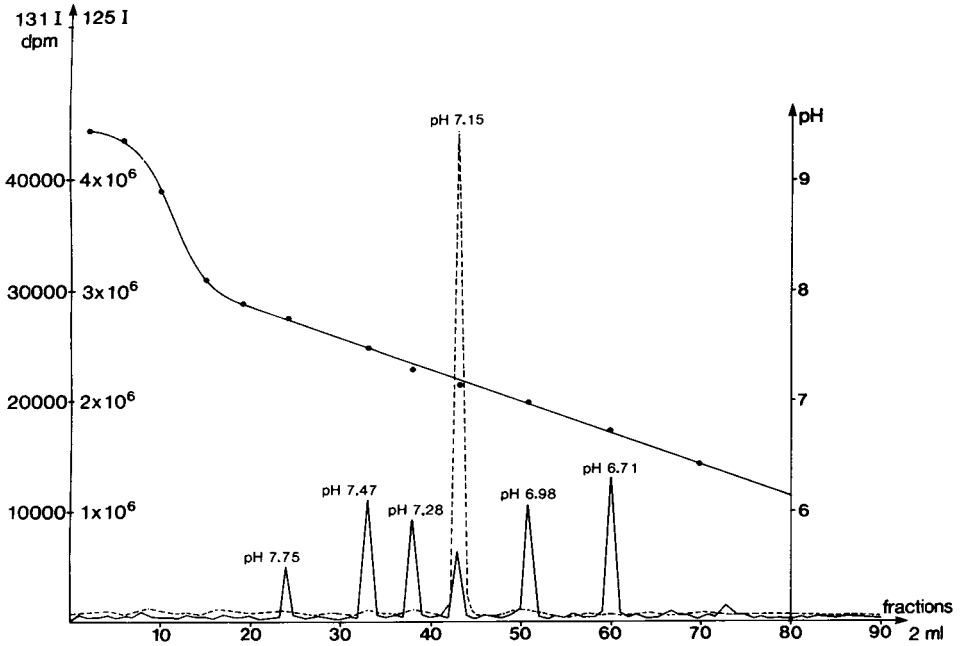


Fig. 3. Elution pattern of doubly labelled immune complex obtained from breast tumour cells after displacement of the equilibrium by leukemic lymphoid cells. Other conditions as in Fig. 2.

TABLE I

PURIFICATION OF THE Ia ANTIGEN—ANTI-Ia IMMUNE COMPLEX FROM 10^7 TUMOUR CELLS

Results are expressed as μg of protein.

	Labelling and solubilization	*Lens culinaris* lectin—Sepharose 4B	Protein A—Ultrogel	Chromatofocusing
^{131}I-labelled proteins	2000—4000	60—200	5—24	1.8—6
^{125}I-labelled antibody	80		68	54

equilibrium of the reaction of ^{131}I-labelled antigen with ^{125}I-labelled antibody must be displaced in the presence of the non-labelled antigen, and, consequently, the amount of ^{131}I linked to the antibody must decrease. Fig. 3 shows that in the presence of lymphocytes the doubly labelled eluted fraction at pH 7.2 had its ^{131}I activity markedly decreased, while the activity of the other ^{131}I-labelled fractions was not modified. These results show that the doubly labelled fraction contained the Ia—antibody immune complex. Since the specific activity of the ^{131}I-labelled proteins is known, the mass of Ia antigen linked to the antibody can be deduced. Table I shows the results of the purification of the Ia

antigen from solubilized membrane proteins; 1.8—6 μg of Ia were obtained in this way from 2000—4000 μg of protein. The antibody yield was 85% after chromatography on protein A—Ultrogel, and 67% after chromatofocusing.

Thus, a method to measure the amount of Ia antigen present in membrane proteins is available for mammary tumour cells. This may provide a way to evaluate the extent to which the expression of this antigen is a function of hormonal induction.

REFERENCES

1 D.L. Mann, S.H. Abelson and D.B. Amos, Nature, 259 (1976) 145.
2 T.A. Springer, J.F. Kaufman, C. Terhost and J.L. Stromnger, Nature, 268 (1977) 213.
3 D.G. Snary, W.F. Barnstable, P.N. Bodmer and M.J. Grumpton, Scand. J. Immunol., 6 (1977) 439.
4 J.W. Uhr, J.D. Capra, E.J. Vitetta and R.G. Cook, Science, 206 (1979) 292.
5 M.F. Greaves, W. Verbi, H. Festenstein, C. Papasteriades, D. Jaraquemada and A. Maymard, Eur. J. Immunol., 9 (1979) 356.
6 L. Klareskog, U. Forsum and P.A. Peterson, Eur. J. Immunol., 10 (1980) 958.
7 P.G. Natali, C. De Martino, V. Quaranta, A. Bigotti, M.A. Pellegrino and S. Ferrone, Immunogenetics, 12 (1981) 409.
8 D.J. Charron and H.O. McDevitt, J. Exp. Med., 152 (1980) 185.
9 M. Letarte and J. Falk, J. Immunol., 128 (1982) 217.
10 H.K. Slocum, Z.P. Pavelic and Y.M. Rustum, in S.E. Salmon (Editor), Cloning of Human Tumor Stem Cells, Alan R. Liss, New York, 1980, p. 339.
11 R.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
12 A.E. Bolton and W.M. Hunter, Biochem. J., 133 (1973) 529.
13 E.J. Cohn, Blood, 3 (1948) 471.

Journal of Chromatography, 308 (1984) 329—333
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2093

Note

Sensitive method for the determination of diclofenac in human plasma by gas chromatography—mass spectrometry

H. KADOWAKI*, M. SHIINO and I. UEMURA

NRI Life Science, Kajiwara, Kamakura, Kanagawa 247 (Japan)

and

K. KOBAYASHI

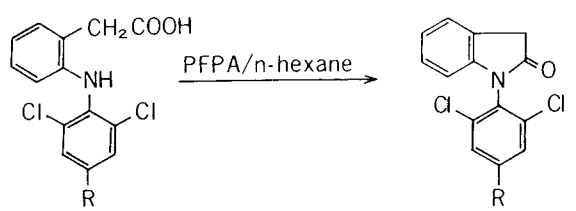
R & D Ciba-Geigy (Japan), Miyuki-cho, Takarazuka 105 (Japan)

(First received August 25th, 1983; revised manuscript received February 6th, 1984)

Diclofenac sodium (Voltaren®) is a widely used anti-inflammatory and analgesic agent.

Procedures have been described for the determination of unchanged diclofenac (Fig. 1, I) in biological materials by gas chromatography with electron-capture detection [1—6] and by liquid chromatography [7], but the lowest limit of detection attainable with these methods is 2 ng/ml of plasma [1].

These existing methods were consequently unsuitable for the measurement of the presumably very low concentrations of diclofenac reached in human



(I) R=H diclofenac (II) R=H
(III) R=OCH_3 internal standard (IV) R=OCH_3

Fig. 1. Formation of the indolinone derivatives.

0378-4347/84/$03.00

plasma after cutaneous application of 1% diclofenac sodium cream, and a more sensitive procedure therefore had to be developed for pharmacokinetic studies of this formulation.

The method proposed is capable of detecting diclofenac levels down to 0.2 ng/ml of plasma by gas chromatography—mass spectrometry (GC—MS) after conversion of diclofenac to the indolinone derivative (Fig. 1, II) through dehydration and cyclization with pentafluoropropionic anhydride (PFPA), as shown in Fig. 1.

EXPERIMENTAL

Materials

Diclofenac sodium (Voltaren active substance), 4′-methoxydiclofenac (internal standard; Fig. 1, III), and the indolinone derivative of diclofenac were supplied by Ciba-Geigy (Basle, Switzerland). Benzene and *n*-hexane were used as manufactured for pesticide-residue analysis by Wako (Tokyo, Japan), and ultrapure grade chloroform was obtained from Kanto Chemical Co. (Tokyo, Japan). PFPA was purchased from Gaskuro Kogyo (Tokyo, Japan). Other reagents used were all of analytical grade, manufactured by Wako.

Gas chromatography—mass spectrometry

A Shimadzu-LKB 9000 gas chromatograph—mass spectrometer and a Shimadzu 9060S multiple-ion-detector peak matcher were used. For GC, a glass column (0.5 m × 3 mm I.D.) was packed with silicone OV-1, 3% Chromosorb W AW DMCS, 80—100 mesh. The temperatures of the injection port, column oven, separator and ion source were set at 270° C, 200° C, 270° C and 290° C, respectively. Helium was used as carrier gas at a flow-rate of 30 ml/min.

For mass fragmentography, the parent ions *m/z* 277 of diclofenac and *m/z* 307 of the 4′-methoxydiclofenac derivative were monitored, and the gain ratio was adjusted at *m/z* 277/307 (1000/15). Accelerating voltage was set at 3.5 kV, electron-ionization energy at 20 eV, trap current at 60 μA, entrance slit width at 0.3 mm and collector slit width at 0.6 mm.

Extraction procedure

A sample of 1.0 ml plasma was taken, and 50 μl of 4′-methoxydiclofenac dissolved in methanol (22.0 ng/μl) were added as internal standard. After stirring for 2—3 sec, 1.0 ml of 1 *M* phosphoric acid and 7 ml of benzene were added as extraction solvent; the mixture was then shaken for 15 min and centrifuged (1870 *g*, 5 min). The benzene layer was taken, and 1.0 ml of 0.08 *M* sodium carbonate buffer (pH 9.6) added; the tubes were shaken for 10 min, then centrifuged. After the benzene layer had been aspirated and discarded, 1.0 ml of 1 *M* phosphoric acid and 7 ml of benzene were added; the mixture was shaken for 15 min and then centrifuged. The benzene layer was removed and evaporated to dryness in a water-bath at 50° C under a nitrogen stream. To the residue, 1.0 ml of *n*-hexane and 100 μl of PFPA were added, and the reaction mixture was left to stand at room temperature for 30 min. Upon the completion of the reaction, the resultant indolinone derivatives were dried in a water-bath at 40° C under a nitrogen stream. For GC—MS, 25 μl of

chloroform were added to the residue, 1—2.5 μl of which were injected for measurement.

Calibration curve

Diclofenac (0.5, 1.0, 2.4, 4.9 and 9.7 ng in a volume of 1.0 ml) was added to blank human plasma samples, and extraction was performed as described above. The peak-height ratio of the diclofenac derivative (*m/z* 277) to the internal standard derivative (*m/z* 307) in the mass fragmentogram was plotted against the concentration of added diclofenac, and a calibration curve prepared using the least-squares method.

RESULTS AND DISCUSSION

Mass fragmentography

In the mass spectra (Fig. 2) of the indolinone derivatives of diclofenac and internal standard, the parent ion peaks (*m/z* 277, *m/z* 307) are both base peaks.

Fig. 3 shows a mass fragmentogram obtained from a spiked human plasma sample. No interference peak derived from plasma was noted. The calibration curve of diclofenac in human plasma showed good linearity in the range of measurement. Table I summarizes the precision and accuracy of the determination procedure. The recovery of diclofenac in the extraction step from plasma was 83.6 ± 5.1% (n = 6).

Sensitivity

The limit of determination of diclofenac in this analysis using a 1-ml plasma sample was 0.2 ng/ml which is ten times lower than that of the most sensitive methods hitherto available. For the cyclization of diclofenac, Geiger et al. [1]

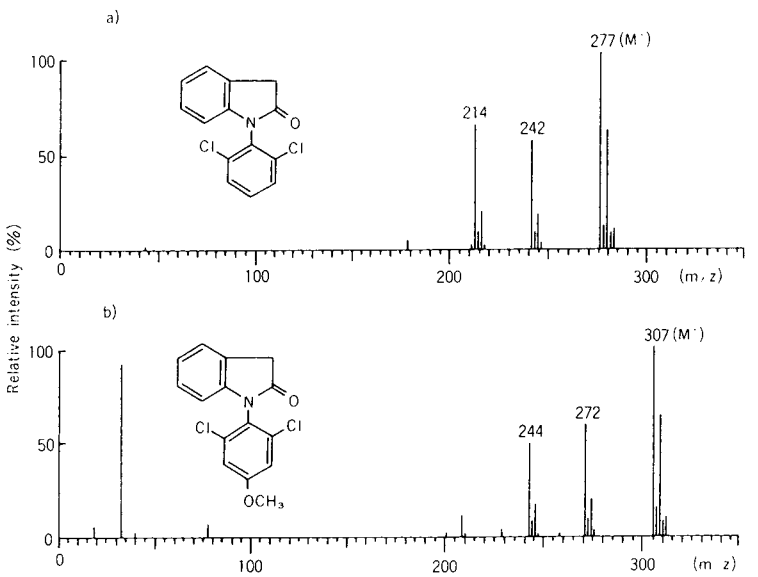


Fig. 2. Electron-impact mass spectra (20 eV) of indolinone derivatives: (a) diclofenac; (b) 4'-methoxydiclofenac (internal standard).

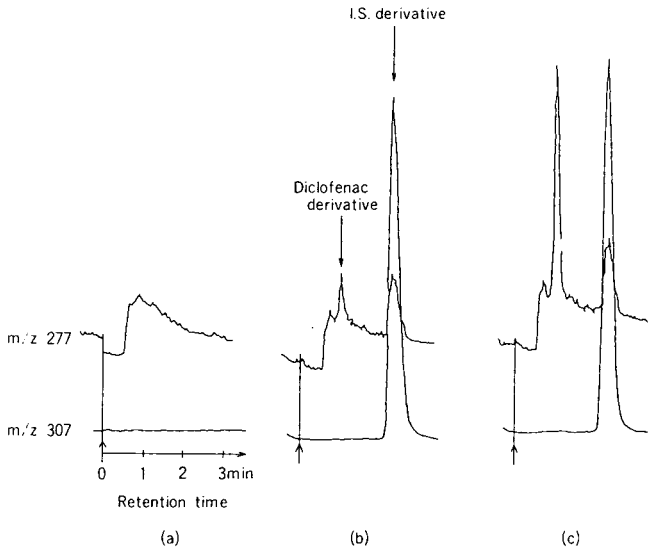


Fig. 3. Mass fragmentograms obtained from human plasma: (a) blank plasma; (b) 0.5 ng/ml diclofenac and internal standard(I.S.) in blank plasma; (c) 2.4 ng/ml diclofenac and internal standard in blank plasma.

TABLE I

PRECISION AND ACCURACY OF THE DETERMINATION OF DICLOFENAC IN HUMAN PLASMA

Actual concentration (ng/ml)	Found concentration (mean ± S.D., n = 6) (ng/ml)	C.V. (%)
0.2	0.23 ± 0.02	8.7
1.0	0.98 ± 0.05	5.1
1.9	1.92 ± 0.11	5.7
4.9	4.74 ± 0.28	5.9
7.4	7.41 ± 0.38	5.1

described a method using sulphuric acid in trifluoroethanol, but this method was unsuitable because of the appearance of an interference peak derived from plasma in the mass fragmentogram and the long time required for the cyclization reaction (more than 75 min). Further, in the method of extractive methylation published by Schweizer et al. [8] and Schneider and Degen [6], 4'-hydroxydiclofenac, a main metabolite, forms the same reaction product as 4'-methoxydiclofenac and so the method could not be applied. In the present study, PFPA [9] proved the most appropriate reagent, giving a rapid cyclization with no side-reaction and being easily removed afterwards. In addition, trifluoroacetic anhydride was tried but found unsatisfactory because of the appearance of an interference peak derived from plasma in the mass fragmentogram. PFPA was very reactive, forming by-products (considered to be enol esters) when no solvent was used, but none when n-hexane was used as solvent. The reaction was complete in 30 min at room temperature, and the indolinone

derivatives produced were stable at room temperature for at least four days. In the extraction procedure, a buffer solution at pH 9.6 was used in preference to 1 *M* sodium hydroxide for the back-extraction of diclofenac into the aqueous phase, this having proved a more effective means of removing interference peaks in the mass fragmentogram.

In case diclofenac cannot be determined down to 0.2 ng/ml in this analysis owing to contamination with other drugs taken simultaneously, it has been confirmed that a mass fragment is obtained without any interference peak if the cyclization products are redissolved in *n*-hexane and washed with sodium hydroxide solution.

Application

Diclofenac sodium (1%) cream was applied 28 times to the back of a healthy volunteer (2.5 g per 250—300 cm^2, three times daily), and blood samples were taken repeatedly. The results are shown in Fig. 4. Diclofenac was detectable in

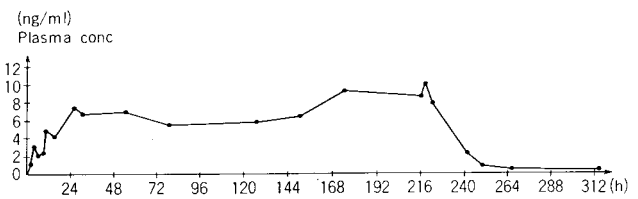


Fig. 4. Plasma concentration of diclofenac obtained after topical application of diclofenac sodium to the back of a human volunteer, three times daily (07:00, 15:00 and 23:00 h).

the plasma (1.3 ng/ml) 2 h after the initial application (in the morning of the first day); the plasma concentration subsequently remained steady between 6 and 10 ng/ml, decreasing to 0.4 ng/ml 98 h after the last application (in the morning of the tenth day). The described method is consequently well suited for studies of the percutaneous absorption of diclofenac sodium.

REFERENCES

1 U.P. Geiger, P.H. Degen and A. Sioufi, J. Chromatogr., 111 (1975) 293.
2 M. Ikeda, M. Kawase, M. Hiramatsu, K. Hirota and S. Ohmori, J. Chromatogr., 183 (1980) 41.
3 J. Kozaya, K. Tada and F. Shimojo, Clin. Rep., 6 (1972) 34.
4 D.B. Jack and J.V. Willis, J. Chromatogr., 223 (1981) 484.
5 M. Ikeda, M. Kawase, T. Kishie and S. Ohmori, J. Chromatogr., 223 (1981) 486.
6 W. Schneider and P.H. Degen, J. Chromatogr., 217 (1981) 263.
7 S.A. Said and A.A. Sharaf, Arzneim.-Forsch., 31 (1981) 2089.
8 A. Schweizer, J.V. Willis, D.B. Jack and M.J. Kendall, J. Chromatogr., 195 (1980) 421.
9 S. Ahuja, J. Pharm. Sci., 65 (1976) 163.

Journal of Chromatography, 308 (1984) 334—340
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2071

Note

Determination of diethylcarbamazine in blood using gas chromatography with alkali flame ionization detection*

SHEELA NENE**, B. ANJANEYULU and T.G. RAJAGOPALAN**,*

Hindustan Ciba-Geigy Research Centre, Goregaon, Bombay 400 063 (India)

(First received October 20th, 1983; revised manuscript received January 11th, 1984)

Diethylcarbamazine, 1-diethylcarbamyl-4-methylpiperazine, (DEC), is an antifilarial drug showing good microfilaricidal activity. Pharmacokinetic studies on this drug also are few probably due to lack of sufficient sensitivity of the methods reported in the literature for the determination of DEC concentration in biological fluids, viz. spectrophotometric methods [1—3] and the more recent improved gas chromatographic (GC) methods [4, 5].

The present report describes a GC procedure using an alkali flame ionization detector, for the quantitative determination of DEC in blood samples. The method can be used for measuring 50 ng of DEC (free base) and above in blood with a good degree of precision and accuracy. 1-Diethylcarbamyl-4-ethylpiperazine was used as the internal standard in the analytical procedure.

EXPERIMENTAL

Solvents, standards and reagents

Reagent grade toluene, methylene chloride, and methyl ethyl ketone were obtained from E. Merck (Bombay, India); hexane and ethyl acetate were from Sarabhai M. Chemicals (Baroda, India). Hexane and toluene were purified as described earlier [6].

All other chemicals used were of analytical reagent grade. Diethylcarbamyl chloride, N-methylpiperazine and ethyl *p*-toluene sulphonate were purchased from Fluka (Buchs, Switzerland).

*Contribution No. 686 from Hindustan Ciba-Geigy Limited, Research Centre, Goregaon, Bombay, India.

**Present address: Richardson Hindustan Ltd., Tiecicon House, Dr. E. Moses Road, Bombay 400 011, India.

0378-4347/84/$03.00

Apparatus

The gas chromatograph was a Varian Model 2700 equipped with an alkali flame ionization detector. Nitrogen was used as the carrier gas at a flow-rate of 40 ml/min. Hydrogen and air flow-rates were 40 and 268 ml/min, respectively. The range and attenuator settings corresponded to $4 \cdot 10^{-12}$ A for full scale deflection. The column was operated at 180°C, the injector at 200°C, and the detector at 270°C. Peak height measurements were used to quantitate the chromatograms. The samples (3 μl) were injected on-column using a 10-μl syringe with a long needle.

The glass column (2.7 m × 4 mm I.D.) was packed with 5% SP-2401 DB (base deactivated) on 80—100 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). The packed column was conditioned, silylated using Silyl 8 (Pierce, Rockford, IL, U.S.A.) and primed using authentic standards.

Standards

DEC (Fig. 1, IIIa) and the internal standard (Fig. 1, IIIb) were conveniently prepared by the general carbamylating procedures [7] using diethylcarbamyl chloride and N-monoalkyl piperazines (Fig. 1, IIa and IIb). IIb was synthesized by a procedure different from that reported [8]. The free bases (Fig. 1, IIIa and IIIb) were converted to the corresponding crystalline citrate salts by reacting with equimolar quantities of citric acid in acetone solution.

The general synthetic procedures are outlined in Fig. 1.

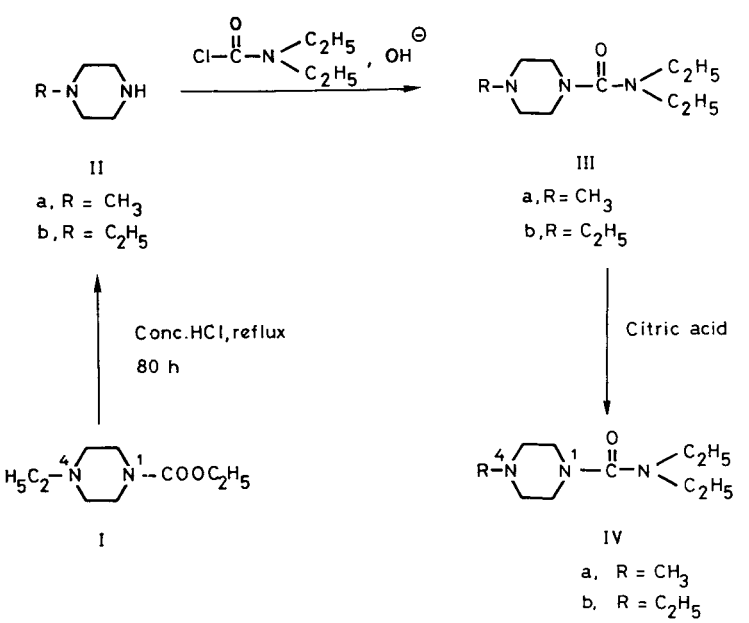


Fig. 1. Summary of reactions used in the synthesis of DEC and internal standard.

Method

Step 1. DEC citrate (1 mg) and 1 mg of internal standard citrate were dissolved separately in 50 ml of methanol; 1 ml of this stock solution was

diluted with methanol to 10 ml. The stock solutions were kept refrigerated and dilutions from stock solutions were made fresh.

Step 2. A 100-μl volume of the diluted internal standard corresponding to 200 ng of internal standard citrate and 50, 100, 150 and 200 μl of the diluted DEC solution corresponding to 100, 200, 300 and 400 ng of DEC citrate were pipetted into a set of silanized glass-stoppered tubes. Then 1 ml of blood was added and the tubes were swirled by hand. This was followed by the addition of 3 ml of 0.1 *M* sodium hydroxide and after gentle mixing the contents of the tube were allowed to equilibrate for 5 min at room temperature. Then 3 ml of an ethyl acetate—methyl ethyl ketone (4:1, v/v) mixture was added. The stopper was sealed with a drop of water and the tube was mounted horizontally on a reciprocal shaker and shaken at full speed for 5 min.

Step 3. The tube was removed from the shaker and centrifuged at 4000 *g* for 10 min. The organic layer was transferred to a 10 ml glass-stoppered tube and the extraction was repeated as described above.

Step 4. The free bases in the pooled organic layer were back-extracted into citric acid by the addition of 1 ml of 0.1 *M* citric acid using a 10 min shaking period on a reciprocal shaker. The tube was centrifuged and the organic layer was aspirated and discarded.

Step 5. The aqueous layer was washed first with 2 ml of ethyl acetate, followed by 2 × 3 ml of hexane, and the organic phases were discarded.

Step 6. The aqueous layer was basified by the addition of 1 ml of 2 *M* sodium hydroxide and extracted three times using a mixture of toluene—methylene chloride (5:2, v/v). The pooled organic layer was evaporated to dryness under a gentle stream of nitrogen and resuspended in 100 μl of acetone. A 2—4 μl aliquot was injected on-column in duplicate. Peak height ratios were calculated by dividing the peak height of DEC by the height of the internal standard peak. Calibration curves were constructed by plotting the peak height ratio as a function of DEC concentration. Ten to twelve samples were analysed for each calibration point. This calibration curve was used subsequently to calculate unknown concentrations of DEC in blood.

RESULTS AND DISCUSSION

Use of 1-diethylcarbamyl-4-ethylpiperazine as the internal standard in the quantitation of DEC was largely responsible for the success of the analytical method. The internal standard was ideal in its extraction and GC resolution behaviour when compared to DEC. The availability of pure internal standard and DEC paved the way for optimizing the response of the alkali flame ionization detector.

Among the solvents and solvent mixtures tried, the solvent pair ethyl acetate—methyl ethyl ketone (4:1, v/v) was found to be superior in extracting DEC from blood. This extract was unsuitable for direct GC and therefore several clean-up procedures were tried. Back-extraction of the bases into citric acid and successive washings with ethyl acetate and hexane followed by re-extraction using the solvent mixture toluene—methylene chloride emerged as the ideal purification step. The overall recovery of DEC and the internal standard added to blood ranged from 45% to 50%.

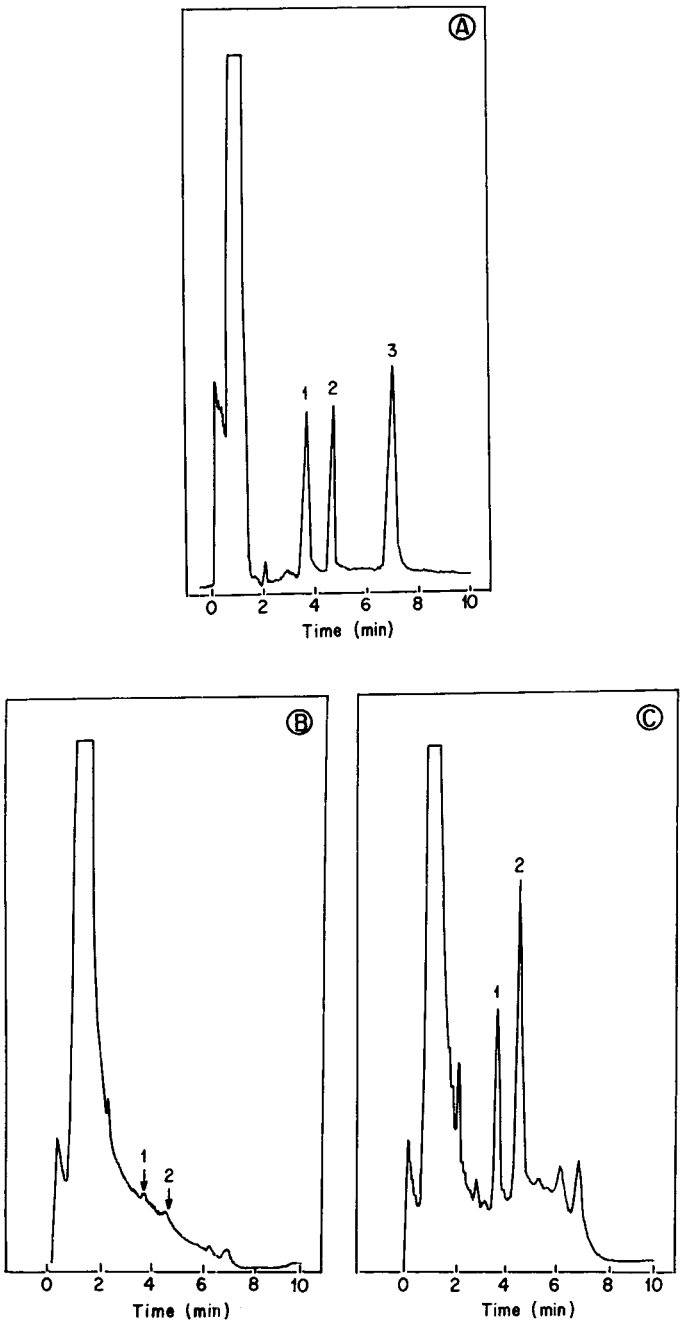


Fig. 2. (A) Separation of DEC, internal standard and desethyl metabolites of DEC. (B) Chromatogram of human blood blank. (C) Human blood sample spiked with 100 ng of DEC free base. Peaks: 1 = DEC; 2 = internal standard; 3 = desethyl DEC.

TABLE I

PRECISION OF THE METHOD APPLIED TO SPIKED HUMAN BLOOD SAMPLES

Amount of DEC (free base) added (ng/ml)	Amount found (ng/ml) (mean ± S.D.; n = 4)	Precision/reproducibility (C.V., %)
58.2	60.75 ± 3.7	6.09
77.6	79.75 ± 4.0	5.02
126.1	127.5 ± 6.5	5.1
174.6	171.5 ± 6.6	3.85

Fig. 2 illustrates the separation of DEC and some of its major metabolites [9] along with the internal standard. The mono- and di-desethyl metabolites of DEC overlap and therefore cannot be separated under the conditions used in the present study, but they are completely separable from both DEC and the internal standard.

Typical gas chromatograms obtained with the method using blood samples are also shown in Fig. 2. The calibration curve constructed by adding known amounts of DEC and a constant amount of internal standard to blood was

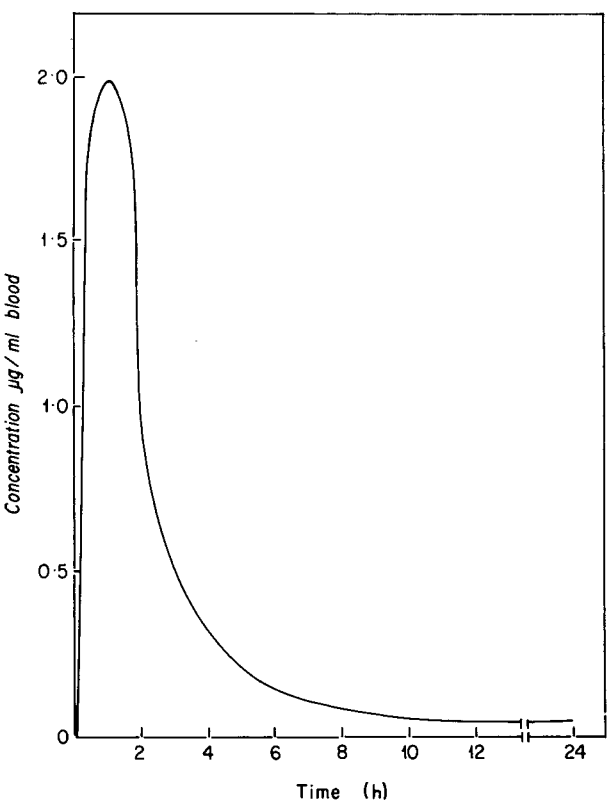


Fig. 3. Concentration of DEC in dog blood after the administration of an oral dose of 200 mg DEC (banocide).

linear in the range 50—200 ng DEC (free base). The equation for the calibration curve from blood was calculated and found to be $Y = 0.0061X + 0.025$ with an excellent correlation ($r = 0.999$). Table I shows the results obtained on spiked blood samples. The results demonstrate good reproducibility of the method.

The specificity and sensitivity of the method for pharmacokinetic studies was checked by an in vivo experiment in a 12-kg mongrel dog following oral administration of 200 mg of DEC citrate (Banocide®). The results of this study are shown in Fig. 3. A C_{max} of 2.03 μg/ml was reached in 1 h followed by a very rapid decline to 0.094 μg/ml at 8 h. The levels beyond this time were around 50 ng/ml, the minimum detection limit of the present method. The

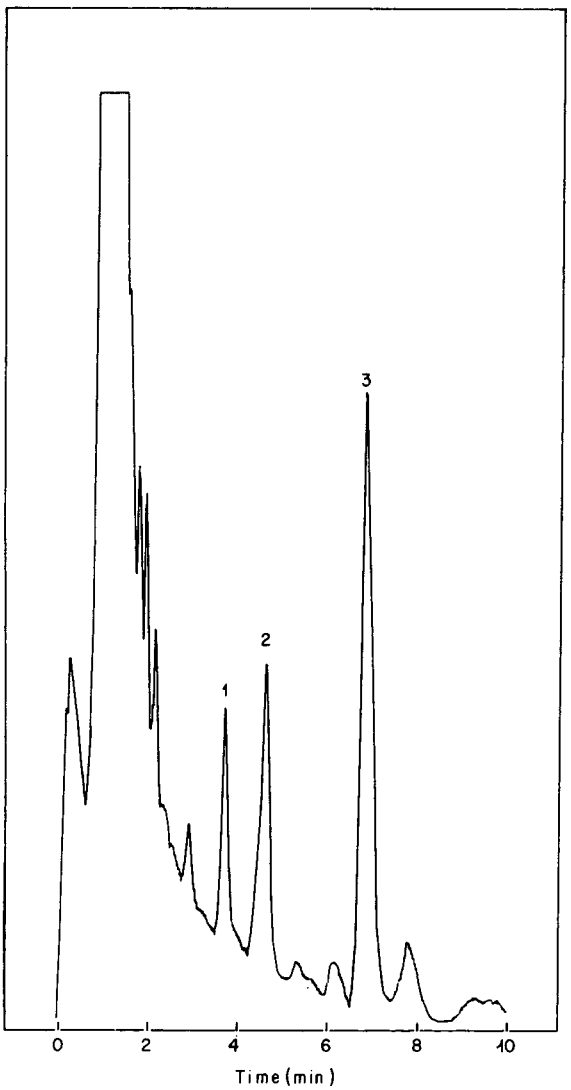


Fig. 4. Chromatogram of a steady-state human blood sample. 1 = DEC; 2 = internal standard; 3 = desethyl metabolites of DEC.

suitability of the method for measuring steady-state levels of DEC in man was ascertained by determining DEC levels in one subject after seven days on a 100-mg t.d.s. dosage. A level of 0.605 μg/ml was found on the eighth day. Studies are in progress to establish potential interference if any from other concomitantly prescribed drugs.

Fig. 4. presents the gas chromatogram of the steady-state blood sample. Besides DEC considerable amounts of the desethyl metabolites are present in the blood and their non-interference with the reported method of assay of DEC in blood is also quite apparent.

Experiments on the application of this analytical method to study single- and multiple-dose pharmacokinetics of this antifilarial drug are in progress.

REFERENCES

1 M. Lubran, Brit. J. Pharmacol., 5 (1950) 210.
2 K.N. Rao and D. Subrahmanyam, Indian J. Med. Res., 58 (1970) 746.
3 M. Ramachandran, Indian J. Med. Res., 61 (1973) 864.
4 J.A. Bogan, Analyst (London), 102 (1977) 56.
5 G.D. Allen T.M. Goodchild and B.C. Weatherley, J. Chromatogr., 164 (1979) 521.
6 T.G. Rajagopalan, B. Anjaneyulu, V.D. Shanbag and R.S. Grewal, J. Chromatogr., 224 (1981) 265.
7 S. Kushner, L.M. Brancone, R.I. Hewitt, W.L. McEwen, Y. Subba Row, H.W. Stewart, R.J. Turner and J.J. Denton, J. Org. Chem., 13 (1948) 144.
8 T. Fujii and H. Watanabe, Chem. Abstr., 50 (1956) P 7883i.
9 J.K. Faulkner and K.J.A. Smith, Xenobiotica, 1 (1971) 321.

Journal of Chromatography, 308 (1984) 341—344
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2095

Note

Determination of nanogram amounts of dicyclomine with gas chromatography and nitrogen-selective detection

EUGENIO BERETTA* and GIANNI VANAZZI

Research Laboratories of Gruppo Lepetit S.p.A., Via Durando, 38, 20158 Milan (Italy)

(First received December 2nd, 1983; revised manuscript received January 31st, 1984)

Dicyclomine · HCl [β-(diethylamino)ethyl 1-cyclohexylcyclohexanecarboxylate hydrochloride] is a parasympatholytic agent, employed for many years in the symptomatic treatment of gastrointestinal disorders [1, 2]. In a comparative study of the bioavailability of three marketed dicyclomine preparations the pioneering gas chromatographic (GC) method of Meffin et al. [3], albeit very sensitive, appeared unsuitable for routine use because of the too laborious preparation of the sample extract to be injected. Therefore an assay procedure was developed which involved extraction and purification steps followed by GC measurement with a nitrogen-selective detector.

EXPERIMENTAL

Materials

Dicyclomine · HCl (Fig. 1, I) was supplied by Merrell (Cincinnati, OH, U.S.A.). Chlorcyclizine · HCl (Fig. 1, II), the internal standard, was purchased from Pfalz & Bauer (Flushing, NY, U.S.A.). All the other chemicals were obtained from Merck (Darmstadt, F.R.G.). The organic solvents were distilled

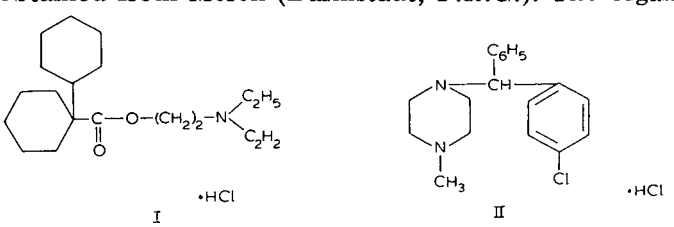


Fig. 1. Chemical structures of dicyclomine · HCl (I) and chlorcyclizine · HCl (KI), internal standard.

0378-4347/84/$03.00

before use; in particular, diethyl ether shortly before. All the glassware (tubes, pipettes) was previously washed twice with ethyl acetate containing 5% methanol and heated at 500°C for 4 h. Dichlordimethylsilane (Merck), 5% in toluene (Merck), was employed as silanizing agent for the conical evaporation tubes. The standard solutions contained 5 mg of I and/or II in 10 ml of methanol. From these suitably diluted working solutions were prepared.

Extraction procedure

A 1-ml volume of plasma and 6 μl of internal standard solution (30 ng) were transferred to a 100 × 16 mm screw-capped centrifuge glass tube. After the addition of 0.5 ml of 1 *M* sodium hydroxide the sample was extracted with 5 ml of diethyl ether for 5 min in a horizontal shaker and then centrifuged. The extract was transferred to another glass tube and shaken with 1 ml of 1 *M* hydrochloric acid for 3 min and centrifuged. The upper phase was discarded by suction, whereas the aqueous phase was washed by shaking with 3 ml of diethyl ether for 3 min. The upper layer was aspirated and discarded, then the aqueous phase was alkalinized with 1.2 ml of 1 *M* sodium hydroxide, extracted with 5 ml of diethyl ether for 5 min and centrifuged. The upper phase was transferred to a 120 × 24 mm silanized glass conical tube and evaporated to dryness under a nitrogen stream. The residue was taken up with 10 μl of ethyl acetate containing 5% (v/v) methanol for injection into the chromatograph.

Chromatographic system

A coiled glass column (1.8 m × 2 mm I.D.) packed with 5% OV-225 on 80—100 mesh Chromosorb HP Hewlett-Packard (Cernusco sul Naviglio, Milan, Italy) was mounted in a Hewlett-Packard 5830 A computer-controlled gas chromatograph equipped with a nitrogen-selective detector and connected with a 18850 A terminal. Working conditions: temperatures, 250°C (injector), 245°C (oven) and 300°C (detector); gas flow-rates, 3 ml/min (hydrogen), 49 ml/min (air) and 30 ml/min (nitrogen, carrier gas). The operating conditions were programmed into the system through the terminal which also controlled them and produced a report containing the chromatogram and the determination results (peak areas and retention times of I and II).

Calculations

The detector response factors to be used in the calculations came from spiked control human plasma samples (30 ng of both I and II in 1 ml), processed as above along with the samples to be assayed (one for every five unknown samples). Aliquots of an ethyl acetate—methanol (95:5) solution containing 30 ng of both I and II were injected before and after a series of unknown samples (at least twenty) to obtain the reference values for the response factor and the retention times.

RESULTS AND DISCUSSION

The clean-up steps on the plasma sample provided an extract with minute interfering peaks at the retention times of I and II (5.2 and 8.9 min, respectively). The time of the analysis was prolonged until 30 min to permit the exit of

TABLE I

RECOVERY OF DICYCLOMINE ADDED TO CONTROL PLASMA IN REPLICATE (n = 3) ANALYSIS

Dicyclomine added (ng/ml)	Dicyclomine found (ng/ml, ± S.D.)	Relative S.D. (%)	Recovery (%)
5	6.4 ± 0.6	9.4	128
10	11.2 ± 0.6	5.4	112
20	20.7 ± 1.8	8.7	104
40	39.0 ± 1.4	3.6	98
80	76.3 ± 6.4	8.4	95

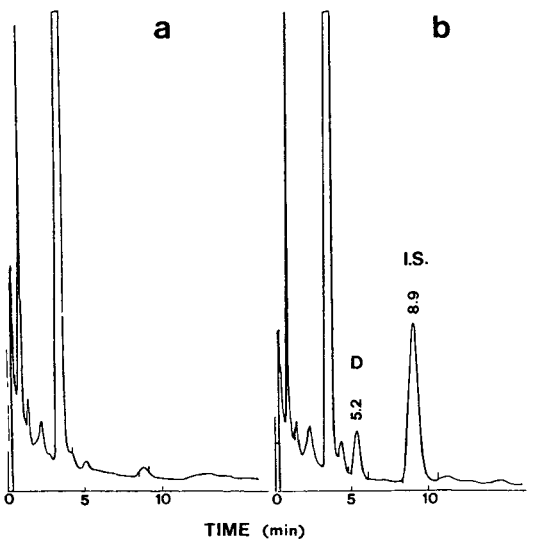


Fig. 2. Chromatograms of human plasma extracts. (a) Control plasma; (b) 3-h plasma from a subject receiving 20 mg of I, containing 18 ng/ml dicyclomine (D) and 30 ng/ml chlorcyclizine (I.S., internal standard).

a peak with a retention time of 24 min which proved to give trouble in the subsequent tracing when not previously removed. For the construction of a calibration curve control human plasma was spiked with I over a concentration range from 5 to 80 ng/ml of dicyclomine base and II (30 ng/ml of chlorcyclizine base). Triplicates for each concentration were submitted to the assay (Table I).

The results showed that the response was linear, the determination was satisfactorily reproducible (relative standard deviation from 3.5 to 9.2%) and that the recovery ranged from 95 to 128% for the concentrations tested. The recovery exceeding 100% for the low concentrations might be attributed to a small amount of interfering material that was found at the retention time of I (Fig. 2a).

The regression equation for the straight line calculated from the values in Table I was $Y = 1.8472X + 0.9307$ with $r = 0.9953$. The limit of quantitation of the method was 5 ng/ml; nevertheless, it was possible to detect dicyclomine

in samples at concentrations less than 5 ng/ml. The reproducibility of the method in the chromatographic system was tested on ten diverse samples containing 30 ng of both I and II, separately processed on two consecutive days (5 + 5) and showed a relative standard deviation of 3.4%.

In order to minimize the loss of I and/or II for adsorption on the column, two or three control human plasma extracts were injected just before the unknown sample series was assayed.

Although the supplier of the nitrogen-selective detector recommended avoiding the use of OV-225 (nitrogen-containing phase), the overall system proved to give reliable results with noticeable savings in time when the work was restarted by maintaining the collector bead overnight at a temperature high enough to prevent contamination from the column bleed and under the same flow-rates of air and hydrogen as during working operations.

Under the conditions described the method was successfully employed for dicyclomine determinations on 200 plasma samples from six healthy volunteers who received 20 mg of drug orally in a cross-over study of its bioavailability from drops, tablets and gel preparations.

REFERENCES

1 B.B. Brown, C.R. Thompson, G.R. Klahm and H.W. Werner, J. Amer. Pharm. Assoc. Sci. Ed., 39 (1950) 305.
2 D.T. Chamberlin, Gastroenterology, 17 (1951) 224.
3 P.J. Meffin, G. Moore and J. Thomas, Anal. Chem., 45 (1973) 1964.

Journal of Chromatography, 308 (1984) 345—349
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2096

Note

Gas chromatographic determination of bevantolol in plasma

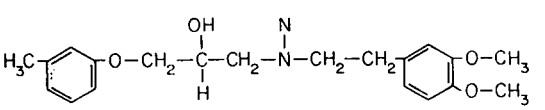
EDWARD J. RANDINITIS*, C. NELSON and A.W. KINKEL

Pharmacokinetics/Drug Metabolism Department, Warner-Lambert/Parke-Davis Pharmaceutical Research, Ann Arbor, MI 48106 (U.S.A.)

(First received December 8th, 1983; revised manuscript received February 2nd, 1984)

Bevantolol hydrochloride (Fig. 1) is a cardioselective β-blocking agent currently undergoing clinical trials for its antihypertensive activity [1, 2]. In order to study its pharmacokinetics and to accurately assess the biopharmaceutic properties of several formulations, it was necessary to develop an analytical method suitable for the determination of bevantolol in the submicrogram range in plasma. In addition, plasma concentrations of total (free plus glucuronide) bevantolol were also required. A gas chromatographic method (GC) utilizing electron-capture detection was developed and is described here. Typical results are also supplied to demonstrate the applicability of the method.

BEVANTOLOL



INTERNAL STANDARD

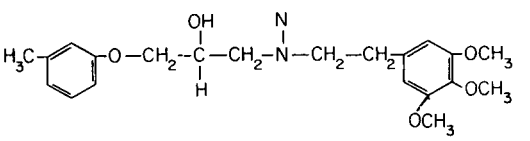


Fig. 1. Chemical structures of bevantolol and internal standard.

EXPERIMENTAL

Materials

Ethyl acetate and diethyl ether were anhydrous ACS reagent grade. Hexane

0378-4347/84/$03.00

was UV spectroquality grade. Chloroform was freshly redistilled in glass prior to use. Sulfuric acid, 1 *M*, was prepared from concentrated sulfuric acid. Acetate buffer at pH 5.2 (2 *M*) and carbonate buffer at pH 8.9 (2 *M*) were also used. Heptafluorobutyric anhydride (HFBA) was purchased from Pierce (Rockford, IL, U.S.A.). Glucuronidase—sulfatase was purchased from Calbiochem (LaJolla, CA, U.S.A.). Bevantolol hydrochloride and internal standard, 1-(3-methylphenoxy)-3-{[(3,4,5-trimethoxyphenyl)ethyl]amino}2-propanol (see compounds 1 and 10 in ref. 3), were synthesized in the Warner-Lambert/Parke-Davis Pharmaceutical Research Laboratories (Ann Arbor, MI, U.S.A.).

Stock solutions of bevantolol and internal standard (100 μg/ml) were prepared in 0.1 *M* hydrochloric acid. These were diluted with water to 10 μg/ml and 1 μg/ml. Standard curves were prepared by adding 0.05, 0.1, 0.25, 0.4, and 0.5 ml of the 1 μg/ml standard solution to 0.5 ml of plasma for free bevantolol in plasma, or, 0.05, 0.1, 0.2, and 0.3 ml of the 10 μg/ml standard solution to 1 ml of plasma for the total bevantolol in plasma determinations.

Apparatus

An Orion Model 601 pH meter and Varian Models 2100 and 3700 gas chromatographs equipped with electron-capture detectors were used. A 2 m × 2 mm I.D. glass column packed with 3% OV-1 coated on 100—120 mesh Gas-Chrom Q (Supelco, Bellafonte, PA, U.S.A.) was used for the assay of both free and total bevantolol in plasma. The injection port and electron-capture detector temperatures were 240°C and 325°C, respectively. The column was maintained at 265°C isothermal until the peaks of interest eluted, then temperature-programmed to 265°C at 15°C/min and maintained at 265°C for 2 min. This was necessary to elute the endogenous peaks in plasma extracts which would interfere with subsequent injections. Nitrogen was used as the carrier gas at a flow-rate of about 50 ml/min. A Shimadzu C-RIA recording integrator set to measure peak heights and peak height ratios was used to quantitate the results.

Extraction

For the assay of plasma or standards for free bevantolol, 0.4 ml of the internal standard solution (1 μg/ml), 0.5 ml of 1 *M* sulfuric acid, and 10 ml of diethyl ether are added to 0.5 ml of plasma. The mixture is shaken for 15 min on a reciprocating shaker, centrifuged, and the ether layer discarded. Carbonate buffer, pH 8.9 (1 ml) is added to the aqueous phase and the pH adjusted to pH 8.9 if needed and 12 ml of chloroform added. This mixture is shaken on a reciprocating shaker for 20 min, centrifuged, and the aqueous layer discarded. The organic layer is transferred to a clean glass-stoppered tube and evaporated to dryness at 65°C with the aid of a current of air. Ethyl acetate (1 ml) and 50 μl of HFBA are added, the tubes stoppered, and heated at 60°C for 20 min to convert the compounds to their heptafluorobutyrates. The stopper is removed and the ethyl acetate and excess HFBA evaporated at 60°C with the aid of a current of air. After complete removal of the HFBA, 1 ml of hexane is added and 1—2 μl injected onto the GC column.

For the assay of total (free plus glucuronide) bevantolol in plasma, 0.3 ml of the 10 μg/ml solution of internal standard is added to 1 ml of plasma to which

has been added 1 ml of acetate buffer, pH 5.2, and 25 μl glucuronidase—sulfatase. This mixture is incubated at 37°C overnight to free the bevantolol from its glucuronide prior to extraction. A 12-h incubation time was shown by previous experiments to allow complete hydrolysis. After hydrolysis, 0.5 ml of 1 *M* sulfuric acid is added and the procedure is continued as in the free bevantolol procedure.

RESULTS AND DISCUSSION

Typical chromatograms for plasma extracts for free and total bevantolol are shown in Figs. 2 and 3. The retention times for bevantolol and internal standard are 7.5 and 9.5 min, respectively. The peak at the retention time of

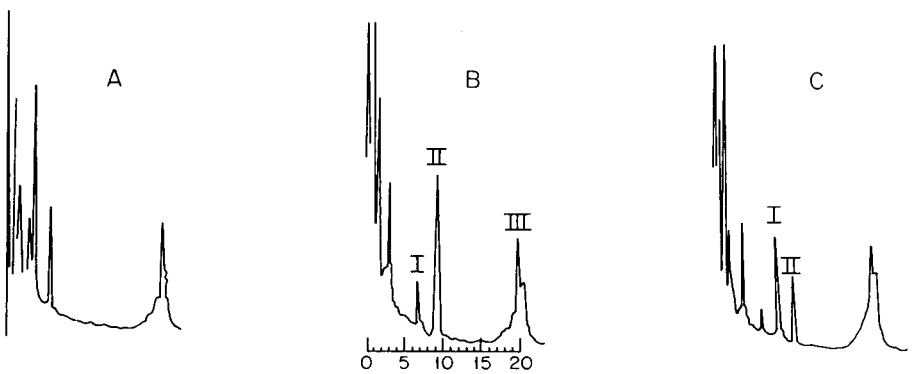


Fig. 2. Typical chromatograms of plasma extracts for free bevantolol. A = Blank plasma; B = spiked plasma, 100 ng/ml; C = plasma sample, 30 min following a 100-mg oral dose of bevantolol, concentration = 620 ng/ml. Peaks: I = bevantolol; II = internal standard; III = due to temperature programming.

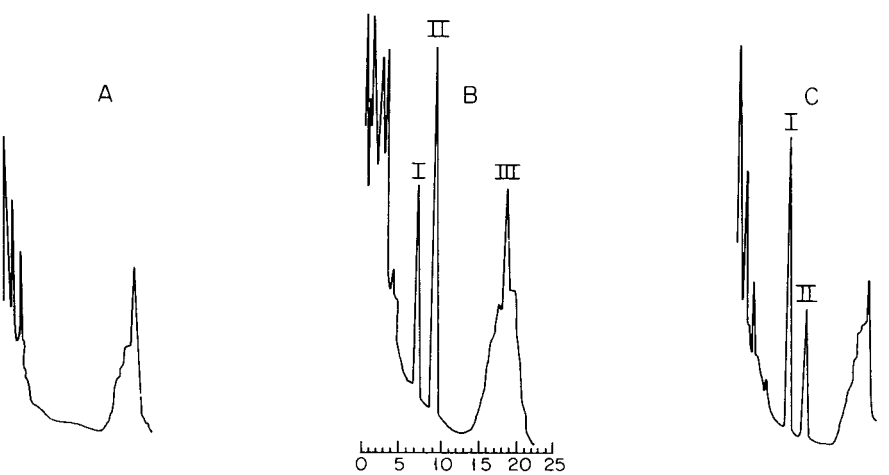


Fig. 3. Typical chromatograms of plasma extracts for total bevantolol. A = Blank plasma; B = spiked plasma, 250 ng/ml; C = plasma sample, 3 h following a 100-mg oral dose of bevantolol, concentration = 970 ng/ml. Peaks: I = bevantolol; II = internal standard; III = due to temperature programming.

15—20 min is due to the temperature programming necessary to prevent interferences from occurring in subsequent chromatograms.

Extraction recovery as measured against a non-extracted solution of bevantolol was about 70% and consistent over the range of concentrations studied. The minimum detectable concentrations were determined to be about 10 ng/ml of plasma for free bevantolol and about 25 ng/ml for total bevantolol, by three times the standard deviation of the lowest concentration studied in the precision studies. Minimum quantifiable concentrations were determined to be about 20 ng/ml and 50 ng/ml, respectively. This sensitivity is adequate to monitor the plasma concentrations of bevantolol for 24 h following the oral administration of a single 100-mg dose.

Calibration curves were linear ($r^2 > 0.99$) from 20 ng/ml to concentrations of at least 3 μg/ml of plasma. The results of the precision studies of both free and total bevantolol in plasma are presented in Table I. Validation of the method for free bevantolol was accomplished on each of three separate runs in triplicate while that on the total bevantolol in plasma was performed in a single run with six replicates of each concentration. Precision was assessed by the determination of percent relative standard deviation (R.S.D.) on a within-run as well as overall basis. The R.S.D. was less than 10% in all cases and therefore the method was considered valid.

The method has been utilized in several pharmacokinetic and biopharmaceutic studies assessing various formulations of bevantolol. Complete details of these studies will be reported elsewhere. As a demonstration of the applicability of the method, the mean plasma concentrations of six subjects administered a single 100-mg tablet of bevantolol are presented in Fig. 4. Maximum plasma concentrations of free bevantolol were reached in about 1 h, after which the plasma concentrations decreased biexponentially with an effective half-life of about 1.6 h.

TABLE I

PRECISION STUDIES OF FREE AND TOTAL BEVANTOLOL IN PLASMA (ng/ml)

Values between parentheses represent percent R.S.D.

Added	Back-calculated			
	1 (n = 3)	2 (n = 3)	3 (n = 3)	Overall (n = 9)
Free bevantolol				
100	111 (7.0)	110 (5.9)	104 (5.3)	108 (6.2)
200	206 (4.1)	211 (5.2)	203 (2.2)	207 (3.9)
500	488 (5.8)	495 (3.1)	506 (5.0)	496 (4.4)
800	770 (0.4)	776 (2.2)	784 (1.7)	776 (1.6)
1000	1014 (1.2)	993 (0.3)	998 (1.4)	1002 (1.3)
Total bevantolol (n = 6)				
500	508 (5.2)			
1000	1031 (3.2)			
2000	2013 (3.3)			
3000	2982 (1.2)			

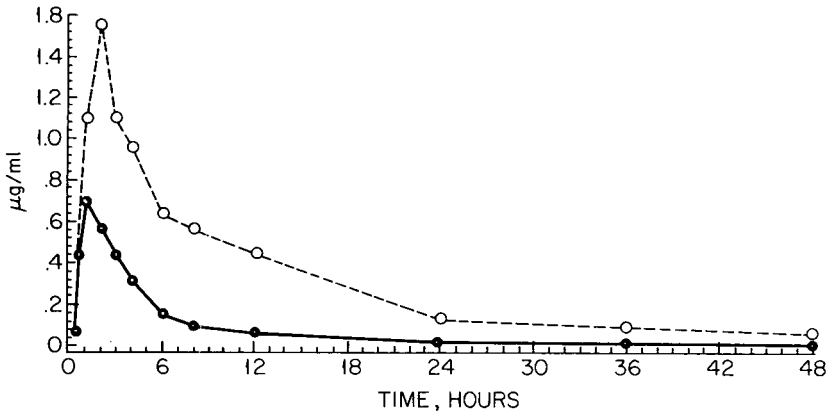


Fig. 4. Mean plasma concentration—time profile of free (•—•) and total (○- -○) bevantolol following the oral administration of a single 100-mg tablet of bevantolol to six subjects.

A method to assess the urinary excretion of bevantolol and its metabolites has also been developed and validated in these laboratories. The method and the metabolic scheme for bevantolol will be reported separately.

In addition, a liquid chromatographic method which lends itself better to automation is currently used in these laboratories. This method will be reported in a separate communication.

CONCLUSIONS

A GC method for the determination of free and total (free plus glucuronide) bevantolol in plasma has been developed and validated. The method is reproducible (R.S.D. < 10%) with minimum quantifiable concentrations of 20 and 50 ng/ml of plasma for free and total bevantolol, respectively. The method has been utilized in several pharmacokinetic and biopharmaceutic studies; the results of one such study is presented to demonstrate the applicability of the method.

REFERENCES

1 S.G. Hastings, R.D. Smith, R.M. Corey, A.D. Essenberg, C.E. Pettway and D.K. Tessman, Arch. Int. Pharmacodyn. Ther., 226 (1977) 81.
2 A.D. MacKay, H.R. Gribbon, C.J. Baldwin and A.E. Tattersfield, Clin. Pharmacol. Ther., 29 (1981) 1.
3 M.L. Hoefle, S.G. Hastings, R.F. Meyer, F.M. Corey, A. Holmes and C.D. Stratton, J. Med. Chem., 18 (1975) 148.

Journal of Chromatography, 308 (1984) 350—353
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2098

Note

Determination of ibuprofen by high-performance liquid chromatography

M.K. ARAVIND*, J.N. MICELI and R.E. KAUFFMAN

*Department of Pediatrics and Pharmacology, Wayne State University School of Medicine and *Children's Hospital of Michigan, Division of Clinical Pharmacology and Toxicology, 3901 Beaubien, Detroit, MI 48201 (U.S.A.)*

(First received October 12th, 1983; revised manuscript received January 18th, 1984)

Ibuprofen is a non-steroidal, anti-inflammatory, antipyretic and analgesic drug [1]. It is used predominantly in the treatment of adult and juvenile rheumatoid arthritis [2, 3], relief of pain from dysmenorrhea [4] and for the treatment of fever [5]. Due to the growing popularity and increased use of ibuprofen, the need for routine therapeutic monitoring of this drug has also increased.

Previously published methods for analysis of ibuprofen have included gas chromatography [6—9] and high-performance liquid chromatography (HPLC) [10—13]. The gas chromatographic assays require a minimum of 1 ml of serum, and are probably not well suited for routine ibuprofen monitoring in the laboratory. HPLC methods appear to offer the most useful methodologies for routine clinical determination of ibuprofen. The purpose of this report is to describe a new HPLC procedure using 50 μl of sample for the routine quantitation of ibuprofen, which makes it well suited for pediatric patients.

MATERIALS AND METHODS

Chromatography

Analysis was performed on a Perkin-Elmer Series II HPLC instrument equipped with an LC75 UV-VIS variable-wavelength detector interfaced with a Sigma 10 data system (Perkin-Elmer, Norwalk, CT, U.S.A.). The data system provided a read-out of the digitally integrated area under the peaks, determined the retention times, and calculated response factors for ibuprofen and the internal standard. All assays were performed using a 3-μm Rainin Microsorb 10 cm × 4.6 mm C_{18} reversed-phase column (Rainin Instrument, Woburn, MA,

0378-4347/84/$03.00

U.S.A.) maintained at 50°C. The flow-rate was 1.5 ml/min and the effluent was monitored at 220 nm.

Reagents

Ibuprofen analytical standard was supplied by Boots Pharmaceuticals (Shreveport, LA, U.S.A.). 5-Ethyl-5-*p*-tolylbarbituric acid (ETBA), used as the internal standard, was obtained from Applied Science (State College, PA, U.S.A.). All other chemicals and organic solvents were HPLC or reagent grade.

The mobile phase consisted of 35% acetonitrile in 0.1 *M* sodium acetate. The pH was adjusted to 6.4 with a few drops of glacial acetic acid. This solution was freshly prepared and degassed under vacuum just prior to use. A 1.0 *M* sodium acetate (pH 4.6) solution was also prepared in a similar manner.

The stock standard of ibuprofen (1.0 g/l) was prepared by dissolving an appropriate amount of ibuprofen in methanol. Working standards were prepared fresh in drug-free sera from the stock standard to yield concentrations from 1.0—100 mg/l. The internal standard was added to the extraction solvent to yield a final concentration of 1.0 mg/l.

Procedure

A 50-μl aliquot of standard, control, or patient serum was placed in a 1.5-ml Eppendorf centrifuge tube. To each tube, 100 μl of 1.0 *M* sodium acetate (pH 4.6) was added and mixed. This was followed by addition of 1.0 ml of the extraction solvent (ethyl acetate) containing ETBA. The tubes were vortexed vigorously for at least 1 min and then centrifuged for 3 min at 21,000 *g* in a Brinkman table-top microcentrifuge. The upper organic phase was transferred to a clean glass tube (75 × 10 mm) and evaporated to dryness at 40°C under nitrogen. The dried sample residue was reconstituted with 50 μl methanol, 10 μl of which was injected onto the column.

Within-run precision was evaluated by assaying a prepared ibuprofen serum pool and day-to-day precision was evaluated by analyzing samples on consecutive days. Stability studies were also conducted using a pool of serum to which known quantities of ibuprofen were added. Aliquots of these samples were frozen at −10°C and analyzed over a period of 18 weeks.

RESULTS AND DISCUSSION

Table I shows the results of within-run and day-to-day precision. Table II shows that there was no appreciable change in the concentration of the drug under these conditions. The accuracy of the method was further validated by analysis of five blind check samples supplied by Boots Pharmaceuticals. The concentrations ranged from 0.0 to 60 mg/l ibuprofen as tabulated in Table III. The correlation coefficient between known and measured concentrations was 0.999.

Fig. 1 shows typical chromatograms of: (A) drug-free serum containing the internal standard; (B) drug-free serum reconstituted with 20 mg/l of ibuprofen and the internal standard; and (C) a patient sample which was taken 3 h after oral ingestion of 400 mg ibuprofen. The determined concentration in this

TABLE I

PRECISION OF SERUM IBUPROFEN ANALYSIS

	Within-run	Day-to-day
Amount added (mg/l)	20.0	50.0
Amount obtained		
Mean (mg/l)	20.1	49.7
S.D.	0.6	1.5
C.V. (%)	3.0	3.0
Number of analyses	10.0	13.0

TABLE II

STABILITY OF IBUPROFEN

Samples were stored frozen at −10° C.

Day	Value (mg/l)	Day	Value (mg/l)
1	9.2	60	9.7
4	9.5	95	10.5
5	10.0	98	10.1
8	10.6	122	9.9
11	11.1	126	10.0
Mean (mg/l)	10.1		
S.D.	0.6		

TABLE III

DETERMINATION OF IBUPROFEN BLIND-CHECK SAMPLES

Sample	Values obtained (mg/l)	Actual concentration (mg/l)
A	9.7	10.0
B	40.5	40.0
C	Not detected	0.0
D	20.2	20.0
E	59.9	60.0

$r = 0.999$

sample was 19.1 mg/l. The retention times for internal standard and ibuprofen were 3.0 and 4.0 min, respectively. The concentration of ibuprofen, calculated from the integrated area under the peaks, was linearly related to the internal standard area over the concentration range from 1.0 to 100 mg/l. The mean recovery of ibuprofen from serum samples was 95%.

The optimal wavelength (220 nm) for detection of ibuprofen was employed which increased sensitivity, selectivity, and decreased sample size requirements. Previously reported HPLC procedures require at least 0.5 ml of serum volume for analysis, ten times the amount required for this assay. In addition, the internal standard is incorporated in the extraction solvent allowing one pre-chromatography step (extraction and drying) for sample preparation thereby minimizing dilution and manipulation errors.

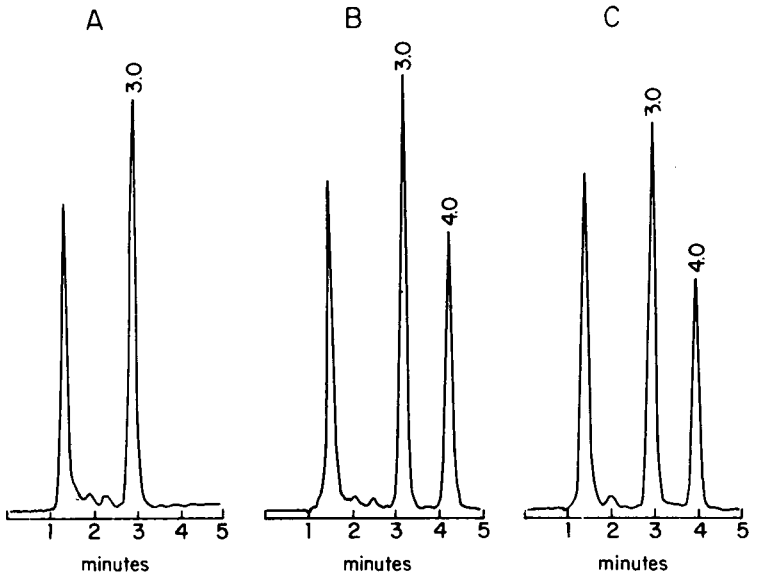


Fig. 1. (A) Drug-free serum containing the internal standard; (B) drug-free serum reconstituted with 20 μg/ml ibuprofen and the internal standard; and (C) a patient sample which was taken 3 h after an oral dose of 400 mg ibuprofen. Retention times: internal standard = 3.0 min; ibuprofen = 4.0 min.

The assay is sensitive to 1 mg/l and linear to 100 mg/l. This encompasses the range of therapeutic concentrations, reported to be from 1.0 to 42 mg/l [7]. The sample size of 50 μl makes it an assay ideally suited for pediatric patients. Gentamycin, tobramicin, chloramphenicol, salicylates, and acetaminophen did not interfere with the assay. The method is readily adaptable for routine therapeutic monitoring in those laboratories equipped with HPLC systems.

ACKNOWLEDGEMENT

This study was supported in part by Boots Pharmaceuticals, Shreveport, LA, U.S.A.

REFERENCES

1 R.J. Flower, S. Moncada and J.R. Vane, in A.G. Gilman, L.S. Goodman and A. Gilman (Editors), The Pharmacological Basis of Therapeutics, Macmillan, New York, 1980, p. 710.
2 T.G. Kantor, Ann. Int. Med., 91 (1979) 877.
3 F.E. Sheldrake and B.M. Ansell, Curr. Med. Res. Opin., 3 (1975) 604.
4 W.Y. Chan, M.Y. Dawood and F. Fuchs, Amer. J. Obstet. Gynecol., 135 (1979) 102.
5 S. Simila, K. Kouvalainen and S. Keinsmen, Scand. J. Rheumatol., 5 (1976) 81.
6 D.G. Kaiser and G.J. Vangiessen, J. Pharm. Sci., 63 (1974) 219.
7 D.G. Kaiser and R.S. Martin, J. Pharm. Sci., 67 (1978) 627.
8 L.P. Hackett and L.J. Dusci, Clin. Chim. Acta, 87 (1978) 301.
9 D.J. Hoffman, J. Pharm. Sci., 66 (1977) 749.
10 J.L. Shimek, N.G.S. Rao and S.K. Wahba Khalil, J. Pharm. Sci., 70 (1981) 514.
11 A. Ali, S. Kazmi and F.M. Plakogiannis, J. Pharm. Sci., 70 (1981) 944.
12 G.L. Kearns and J.T. Wilson, J. Chromatogr., 226 (1981) 183.
13 G.F. Lockwood and J.G. Wagner, J. Chromatogr., 232 (1982) 335.

Journal of Chromatography, 308 (1984) 354—358
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2060

Note

Therapeutic drug monitoring using high-speed liquid chromatography and rapid sample preparation: an assay for serum theophylline

JAY L. BOCK*, STANLEY LAM and ARTHUR KARMEN

Department of Laboratory Medicine, Albert Einstein College of Medicine, Bronx, NY 10461 (U.S.A.)

(First received October 12th, 1983; revised manuscript received December 13th, 1983)

For routine serum drug measurements, chromatography often has the drawbacks of lengthy analysis and tedious sample preparation. Recently the speed of liquid chromatography (LC) has been increased, through use of smaller-sized silica particles for column packing and low-volume components to minimize band spreading [1]. However, sample requirements for such systems may be more stringent. Microscale protein precipitation with acetonitrile is a convenient preparation technique for conventional high-performance liquid chromatography (HPLC) [2], but Kabra and co-workers [3, 4] found that deproteinized serum causes clogging of a high-speed system.

Lam and co-workers [5, 6] have previously devised a method for serum deproteinization that uses zinc sulfate in combination with methanol or acetonitrile. It yields a visibly clearer supernatant than does either solvent alone. In the present communication we describe an application of high-speed LC for serum theophylline, using this precipitation method followed by chromatography on a short, 3-μm reversed-phase column.

MATERIALS AND METHODS

LC apparatus

We used a Series Two pump, an LC-85 UV detector having a 2.4-μl flow-cell and a 135-msec response time, and a 32 × 4.6 mm reversed-phase column packed with 3-μm C_{18}-bonded silica, all from Perkin-Elmer. Sample application was either manual, with a Rheodyne injector, or automatic with a Perkin-Elmer ISS-100 sampling system. In either case we used a 10-μl sample loop and a

0378-4347/84/$03.00

Perkin-Elmer bypass coil, which maintains column flow during valve switching to minimize pressure fluctuations during injection.

Sample preparation

To 50 μl of serum we added 10 μl of zinc sulfate solution (10%, w/v), followed by 75 μl of methanol containing 20 μg/ml of the internal standard, 8-chlorotheophylline. Each sample was mixed by vortexing for several seconds, then centrifuged for 2 min at 1000 *g*.

Sample analysis

A 10-μl volume of supernatant was injected onto the column. Elution was at 2.0 ml/min, ambient temperature, with mobile phase containing 50 ml acetonitrile, 30 ml tetrahydrofuran, and 0.5 ml glacial acetic acid per liter, pH adjusted to 4.9 with sodium hydroxide. Following detection at 273 nm, theo-

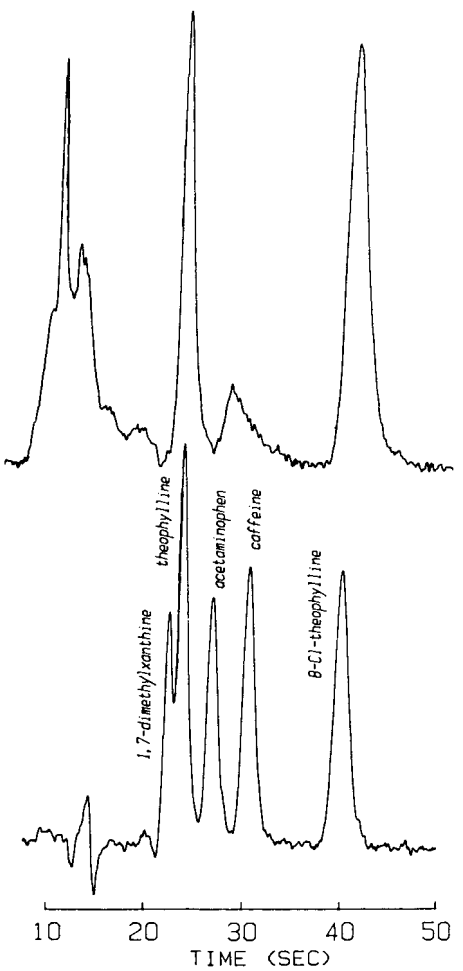


Fig. 1. High-speed LC of (bottom) an aqueous mixture of xanthines and acetaminophen, approximately 10 μg/ml each and (top) de-proteinized serum from a patient on theophylline, concentration measured at 12 μg/ml. Detection at 273 nm, 0.04 a.u.f.s.

phylline was determined from its relative peak height. Standards were prepared in theophylline-free serum.

Correlation with immunoassay

Ninety patient samples assayed by this LC procedure were also tested by an enzyme immunoassay (EMIT, Syva). The EMIT assays were performed using a Rotochem Model R2A centrifugal analyzer (American Instrument Company).

RESULTS

Fig. 1 shows chromatograms of an aqueous mixture of xanthines and acetaminophen, and of de-proteinized serum from a patient on theophylline. Although chromatography is complete in only 40 sec, the quality of separation, in terms of peak widths relative to retention times, is similar to that seen with conventional systems [2, 7]. Baseline resolution of theophylline from caffeine and acetaminophen is achieved, and the resolution from 1,7-dimethylxanthine (paraxanthine) is sufficient to avoid significant interference from this caffeine metabolite [8]. Serum specimens from twenty individuals not on theophylline showed no peaks in the region of theophylline or 8-chlorotheophylline. Substances tested for interference, which were found not to interfere, include theobromine, salicylate, phenobarbital, phenytoin, carbamazepine, procainamide, N-acetyl procainamide, quinidine, acetazolamide, trimethoprim, sulfamethoxazole and ampicillin.

Standard curves exhibited good linearity over a concentration range of 5—40 μg/ml, with correlation coefficients of 0.995—0.998. Precision was determined by repeat assays of a 15 μg/ml serum standard. Within-run coefficient of variation (C.V.) was 2.7% (n = 13), and between-day C.V. was 4.6% (n = 12). Good agreement was achieved between the LC assay and enzyme immunoassay by EMIT, as shown in Fig. 2.

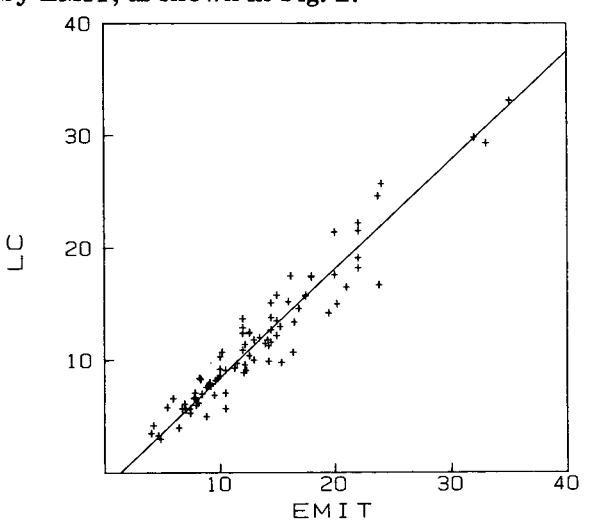


Fig. 2. Correlation between high-speed LC assay of theophylline and immunoassay by EMIT, in 90 patient samples submitted for analysis. Concentrations are in μg/ml. Regression line is LC = 0.970 × EMIT — 1.26, r = 0.959, standard error = 1.24.

During the useful life of each column, samples were injected at about 1-min intervals, and no significant fluctuations in the baseline were observed. However, after about 200 injections deterioration of resolution became noticeable. This deterioration was not related to the use of de-proteinized serum, as it also occurred with repeated injections of an aqueous standard (Fig. 3). It was also found that any sudden changes in flow had a deleterious effect on the performance of the column. Reversing the direction of flow through the column, or changing the packing material at the top, did not give any improvement. The pressure on the column, which was initially about 14 MPa, had generally increased to 20—30 MPa after 200 injections.

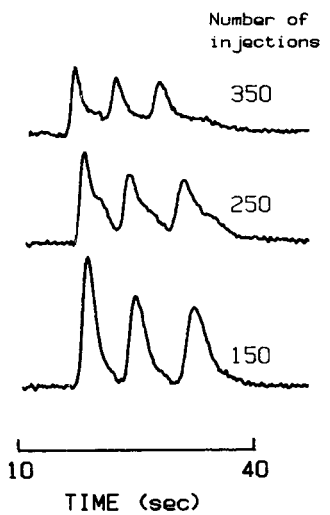


Fig. 3. Deterioration of chromatographic resolution after multiple injections, on a brand new column, of an aqueous standard. The standard contained (in order of elution) theophylline, caffeine, and 8-chlorotheophylline, each 10 μg/ml. The vertical scales for the three chromatograms are identical.

DISCUSSION

Serum theophylline is commonly measured using standard HPLC systems, which give analysis times of several minutes [9]. The procedure described here shortens the chromatographic analysis to 40 sec and uses a rapid method of sample preparation. The time saving not only allows large batches of samples to be processed quickly, but also makes it much easier to re-standardize frequently and perform quality control. For urgent specimens a technician can run the analysis in duplicate along with two standards or controls, and still have results within 10 min.

This procedure appears to be comparable to standard HPLC procedures for theophylline in precision, accuracy, and freedom from interferences. Although the sharpness of peaks enhances sensitivity, this is counterbalanced by a loss in signal-to-noise due to the small flow cell and the dilution that occurs in sample preparation. Reagent expense is minimal, but the economy of this method is currently limited by short lifetime of the column. We could not

pinpoint the cause of column deterioration, but believe it results from mechanical damage to the 3-μm silica. The absence of a more rigorous clean-up step in sample preparation did not appear to have any negative effect on the lifetime of the columns or performance of the system. In contrast, when serum protein precipitation is performed with acetonitrile alone, clogging of a high-speed LC system may result [3, 4].

In summary, the feasibility of high-speed LC for serum drug analysis, using microscale protein precipitation for sample preparation, has been demonstrated. Routine use of this approach would be facilitated by means for increasing the lifetime and decreasing the cost of 3-μm reversed-phase columns.

ACKNOWLEDGEMENTS

We wish to acknowledge the expert technical assistance of Ms. Alice Erling and Ms. June Pieratti.

REFERENCES

1 J.L. DiCesare, M.W. Dong and L.S. Ettre, Introduction to High-Speed Liquid Chromatography, Perkin-Elmer, Norwalk, CT, 1981.
2 J.W. Nelson, A.L. Cordry, C.G. Aron and R.A. Bartell, Clin. Chem., 23 (1977) 124.
3 P.M. Kabra and L.J. Marton, Clin. Chem., 28 (1982) 687.
4 P.M. Kabra, M.A. Nelson and L.J. Marton, Clin. Chem., 29 (1983) 473.
5 S. Lam, S. Rosenbaum and A. Karmen, Clin. Chem., 26 (1980) 963.
6 S. Lam and A. Karmen, Clin. Chem., 28 (1982) 1670.
7 N. Weidner, D.N. Dietzler, J.H. Ladenson, G. Kessler, L. Larson, C.H. Smith, T. James and J.M. McDonald, Amer. J. Clin. Pathol., 73 (1980) 79.
8 J.H.G. Jonkman, R.A. de Zeeuw and R. Schoenmaker, Clin. Chem., 28 (1982) 1987.
9 K.D. Thakker and L.T. Grady, in K. Florey (Editor), Analytical Profiles of Drug Substances, Vol. 11, Academic Press, New York, 1982, pp. 1—44.

Journal of Chromatography, 308 (1984) 359—362
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2099

Note

Determination of oxycodone in human plasma by high-performance liquid chromatography with electrochemical detection

JENNY J. SCHNEIDER, EDWARD J. TRIGGS* and DAVID W.A. BOURNE

Department of Pharmacy, University of Queensland, St. Lucia, Queensland 4067 (Australia)

IAN D. STEPHENS

Mater Misericordiae Public Hospital, South Brisbane, Queensland (Australia)

and

ANN M. HAVILAND

Department of Pharmacy, University of Queensland, St. Lucia, Queensland 4067 (Australia)

(First received November 16th, 1983; revised manuscript received January 27th, 1984)

Oxycodone (14-hydroxydihydrocodeinone) has similar pharmacological properties to morphine and is often used in the treatment of severe pain. Existing methods for the determination of oxycodone in plasma include gas chromatography (GC) with electron-capture [1] or nitrogen-specific detection [2]. While nitrogen-specific detection eliminates the derivatisation step required in electron-capture detection, 5 ml of plasma are needed to detect 2 ng/ml oxycodone. Using the high-performance liquid chromatographic (HPLC) method described in this report, no derivatisation is required and 2 ng/ml oxycodone can be detected using only 2 ml of plasma.

EXPERIMENTAL

Reagents

Stock solutions of oxycodone (1 μg/ml) (Endo Labs., Gordon, Australia) and the internal standard methadone (2 μg/ml) (NBSL, Canberra, Australia) were prepared monthly in methanol and stored at 4°C. Methanol and acetonitrile

0378-4347/84/$03.00

were HPLC grade (Waters Assoc., Brisbane, Australia). Potassium dihydrogen orthophosphate (used in the mobile phase), sodium hydrogen carbonate and sodium carbonate (both used in the carbonate buffer) were AR grade (Ajax Chemicals, Sydney, Australia).

Glassware

Tubes used in the extraction procedure were previously washed with chromium trioxide (Chromerge, Manostate, NY, U.S.A.) in sulphuric acid AR grade (Ajax Chemicals), rinsed thoroughly with distilled water, dried, silanised with 1% hexamethyldisilazine (Ajax Chemicals) in diethyl ether, rewashed in distilled water and dried.

Chromatographic apparatus and conditions

Reversed-phase HPLC was performed using a Model M45 solvent delivery pump (Waters Assoc., Milford, MA, U.S.A.), a Rheodyne Model 7125 injection valve (Rheodyne, Cotati, CA, U.S.A.) equipped with a 40-μl loop, and an RP-8 10-μm column (Brownlee Labs., Santa Clara, CA, U.S.A.). An Omniscribe recorder (Houston Instruments, Austin, TX, U.S.A.) was used in conjunction with an amperometric detector system (Bioanalytical Systems, West Lafayette, IN, U.S.A.). The electrochemical cell contained a glassy carbon working electrode and an Ag/AgCl reference electrode. The working electrode was maintained at an applied voltage of 1.20 V. The chromatography was performed at ambient temperature with a mobile phase of 0.01 *M* potassium dihydrogen orthophosphate—methanol—acetonitrile (20:30:50) at a flow-rate of 1 ml/min. The recorder was set on 10 mV with a chart-speed of 2.5 mm/min.

Extraction method

To 1 ml (or 2 ml) of plasma were added 50 μl of methadone stock solution (internal standard), 500 μl carbonate buffer (pH 9.6) and 6 ml butyl chloride (Burdick & Jackson Labs., Muskegon, MI, U.S.A.). After the mixture was shaken for 15 min on a mechanical shaker (100 rpm), the organic layer was separated by centrifugation (5 min, 2000 *g*) and transferred to a tube containing 3 ml of 0.2 *M* hydrochloric acid. This mixture was shaken for 15 min, centrifuged and the aqueous layer transferred to a tube containing three drops of 60% sodium hydroxide and 6 ml butyl chloride. After shaking for a further 15 min and centrifuging, the organic layer was transferred to a clean tube and the solvent evaporated to dryness at 50° C under a stream of nitrogen. The residue was reconstituted in the HPLC mobile phase (60 μl) and 40 μl were injected into the HPLC system.

Determination of oxycodone in unknown samples

Oxycodone standards (2—200 ng/ml) were prepared by spiking 1-ml or 2-ml aliquots of pooled drug-free plasma with appropriate volumes of oxycodone stock solution. The plasma standards were then extracted and the peak height ratios of oxycodone/methadone (O/M) obtained from the chromatograms were plotted against original oxycodone concentrations. Unknown plasma samples were spiked with the same amount of internal standard and assayed. The oxycodone concentrations of unknown samples were determined from the calibration curve using their peak height ratios (O/M).

RESULTS AND DISCUSSION

Typical chromatograms are shown in Fig. 1. Retention times for oxycodone and methadone are 6.4 minutes and 16 min, respectively. The glassy carbon electrode produced a linear response in the range of 2—200 ng/ml oxycodone in plasma when operated at an applied voltage of 1.2 V. The intra-assay coefficients of variation of the method (C.V.) were 4.1% and 3.6% at oxycodone concentrations of 5 ng/ml (n = 7) and 50 ng/ml, (n = 9), respectively.

The extraction efficiency was 74 ± 3.28% for oxycodone and 80.2 ± 7.15% for methadone (n = 9 for each case). Drugs with similar pharmacological properties were injected and their retention times noted. Three commonly used drugs, fentanyl, phenoperidine and meperidine, had retention times of 6.0, 6.0 and 6.8 min, respectively. To ensure these drugs did not interfere with this assay blank plasma samples were spiked with 100 ng of fentanyl, phenoperidine or meperidine, 100 ng oxycodone and methadone. After extraction and analysis, no interference with the chromatography and quantitation of oxycodone was observed.

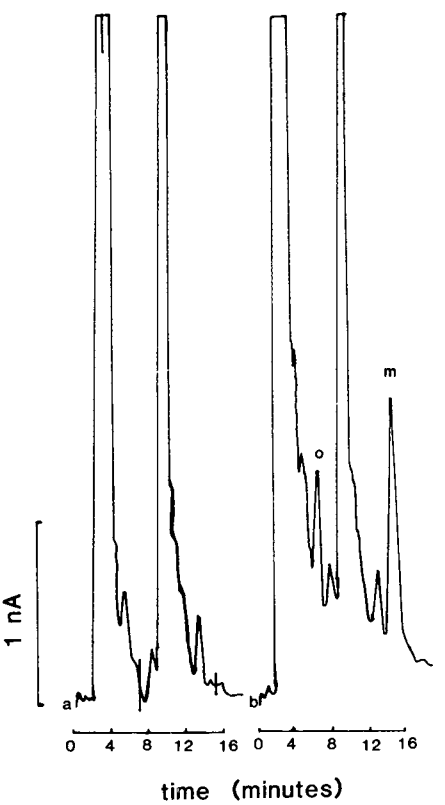


Fig. 1. Chromatograms of 1 ml human plasma taken (a) just prior to administration of oxycodone and (b) 8 h after oral administration of 25 mg oxycodone. Estimated plasma oxycodone concentration is 26 ng/ml. Peaks: o = oxycodone; m = methadone.

Previous methods for the determination of oxycodone in plasma have all used GC. GC with electron-capture detection requires extraction and formation of a volatile oxycodone derivative prior to measurement. In order to determine the pharmacokinetics of oxycodone in human plasma after administration of a single therapeutic dose to normal subjects, repeated blood sampling is required. Using GC with nitrogen-specific detection, 5-ml plasma samples are required at each sampling time. HPLC with electrochemical detection requires only 2-ml plasma samples. Because the total volume of blood withdrawn from a subject is greatly reduced, this method could also be used to study the pharmacokinetics of oxycodone in patients where it is difficult to take very large volumes of blood.

ACKNOWLEDGEMENT

This work was supported by a grant from the Queensland Cancer Fund.

REFERENCES

1 S.H. Weinstein and J.C. Gaylord, J. Pharm. Sci., 68 (1979) 527.
2 N.L. Renzi and J.N. Tam, J. Pharm. Sci., 68 (1979) 43.

Journal of Chromatography, 308 (1984) 363—369
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2101

Note

High-performance liquid chromatographic method for the determination of pyrimethamine and its 3-N-oxide metabolite in biological fluids

M.D. COLEMAN, G. EDWARDS* and G.W. MIHALY

Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool L69 3BX (U.K.)

R.E. HOWELLS

Department of Parasitology, Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA (U.K.)

and

A.M. BRECKENRIDGE

Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool L69 3BX (U.K.)

(First received December 19th, 1983; revised manuscript received February 8th, 1984)

Pyrimethamine—sulphonamide combination therapy is a first choice in areas of chloroquine resistant *Plasmodium falciparum* malaria. The pharmacokinetics of pyrimethamine are poorly understood due to the lack of selectivity of previous analytical methods [1—3].

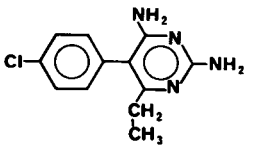
This paper describes a selective and sensitive microanalytical method for the determination of pyrimethamine in plasma and urine. It is also suitable for the simultaneous determination of pyrimethamine 3-N-oxide (Fig. 1), which has recently been identified as a metabolite of pyrimethamine in the rat [4].

MATERIALS AND METHODS

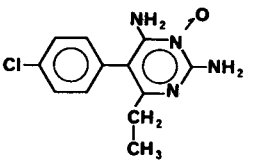
Chemicals

Pyrimethamine base, 2,4-diamino-5-(*p*-chlorophenyl)-6-ethylpyrimidine, and pyrimethamine 3-N-oxide were supplied by Wellcome U.K. (Beckenham, U.K.).

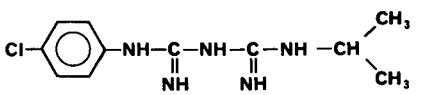
0378-4347/84/$03.00



I



II



III

Fig. 1. Structural formulae of pyrimethamine (I), pyrimethamine 3-N-oxide (II) and internal standard (III).

Proguanil hydrochloride (Fig. 1), the internal standard, was supplied by ICI Pharmaceuticals (Alderley Edge, U.K.). Ammonia solution (0.88 specific gravity) and orthophosphoric acid were obtained from British Drug Houses (Poole, U.K.). Octanesulphonic acid was supplied by Aldrich (Gillingham, U.K.). All other reagents and solvents were HPLC grade (Fisons, Loughborough, U.K.).

Chromatography

The method was developed on a Spectra-Physics liquid chromatograph. The system consisted of an SP 8770 solvent delivery system, an SP 8750 organiser module equipped with a Rheodyne injection system, and an SP 8300 fixed-wavelength UV absorbance detector fitted with a 254-nm source. The separation was carried out on a Partisil ODS reversed-phase column, 10 μm particle size, from HPLC Technology (Wilmslow, U.K.). The mobile phase consisted of water—acetonitrile—methanol (55:35:10, v/v) containing octanesulphonic acid (0.013 *M*) as an ion-pair reagent, buffered to pH 3.5 with orthophosphoric acid, and flowing at 1.8 ml/min.

Sample treatment procedure

To samples of plasma or urine (10—45 μl) contained in 1.5-ml capacity microcap tubes (L.I.P. Equipment and Services, West Yorkshire, U.K.) were added a methanolic solution of the internal standard (20 μg/ml; 0—20 μl added) and ammonia solution (500 μl). The mixture was extracted twice with ethyl acetate (800 μl) by vortex mixing.

After separation, the combined organic phases were evaporated to dryness under nitrogen at 45°C and reconstituted in methanol (20 μl). A 5—18 μl aliquot was injected onto the column.

Calibration curves, analytical precision and recovery

Standard curves in the range 0.5—20 μg/ml were prepared by adding known quantities of pyrimethamine and pyrimethamine 3-N-oxide to drug-free plasma or urine containing the internal standard (20 μg/ml). Samples were analysed as described above, and the peak height ratio of compound to internal standard was plotted against the corresponding weight ratio. Peak height ratios of experimental samples were also determined and the concentrations calculated from the standard curves.

Recovery of pyrimethamine, its 3-N-oxide, and the internal standard were estimated by comparing the peak height obtained from an extracted plasma sample with that from a methanolic solution containing the same amount of each compound. The intra- and inter-assay precision data were determined for both pyrimethamine and its 3-N-oxide by replicate assays of the same sample.

Calculations

Coefficients of variation (C.V.) for calculation of assay precision were calculated from the ratio of the standard deviation to the mean. Plasma elimination half-life was calculated by regression analysis of the post-distributive log linear portion of the plasma concentration versus time curve. Data are presented as mean ± S.D.

Animal studies

Two groups, A and B, of five mice (male TFW, mean weight 25 g) were provided with a Dixons 41B diet and drinking water ad libitum. Mice of both groups were dosed with pyrimethamine base (75 mg/kg intraperitoneally) which was suspended in 'Tween 80' (5%, v/v). Blood samples were obtained from mice in group A, whereas mice in group B were housed in individual metabolism cages throughout the study period to facilitate complete urine collection.

In group A, blood samples (40—60 μl) were removed from the tail vein at 1, 2, 4, 6, 8, 10, 12, 24, 26.5, 31 h into heparinized micro haematocrit tubes of 20-μl capacity (Hawksley, Lancing, U.K.) which were then heat-sealed. Following centrifugation (11,600 *g*, 4 min) volumes of plasma (20—40 μl) were accurately measured using a 50-μl capacity syringe (Hamilton, Reno, NV, U.S.A.) and placed in microcap tubes. Total urine was collected serially for four days from mice in group B, the volume recorded, and an aliquot placed into a microcap tube. All samples were stored at −20° C until time of analysis.

RESULTS AND DISCUSSION

Chromatograms of extracts of drug free plasma, spiked plasma, and plasma from a mouse dosed with pyrimethamine, are shown in Fig. 2. The corresponding chromatograms of urine extracts are shown in Fig. 3. Pyrimethamine, pyrimethamine 3-N-oxide, and the internal standard, proguanil, were resolved, with retention times of 9.25, 6, and 13.5 min, respectively. The minimum detectable concentration (defined as a peak 3 times baseline noise at 0.0025 a.u.f.s.) in a 20-μl plasma sample was 330 ng/ml for

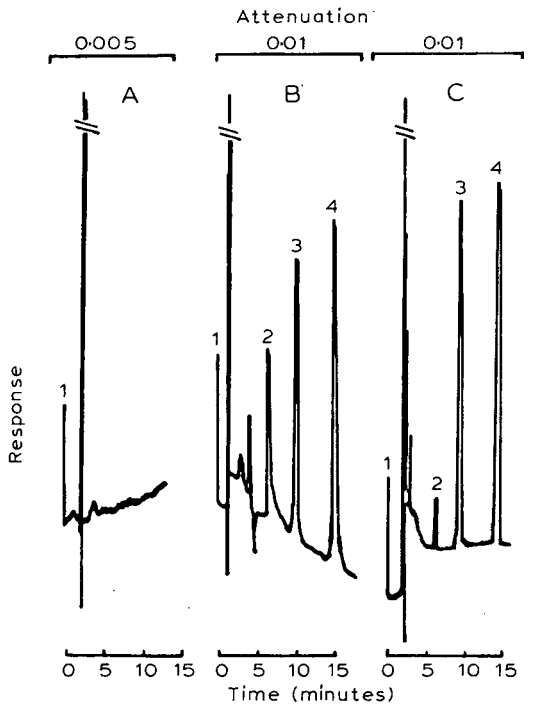


Fig. 2. High-performance liquid chromatograms for (A) a blank plasma extract; (B) a spiked plasma extract (pyrimethamine concentration = 10 μg/ml, pyrimethamine 3-N-oxide concentration = 10 μg/ml); and (C) an extract of a plasma sample taken from a mouse dosed with pyrimethamine at 75 mg/kg (pyrimethamine concentration = 11.7 μg/ml, pyrimethamine 3-N-oxide concentration = 3.0 μg/ml). Peaks: 1 = injection event; 2 = pyrimethamine 3-N-oxide; 3 = pyrimethamine; and 4 = internal standard.

pyrimethamine and 600 ng/ml for the 3-N-oxide metabolite. Analytical recoveries of pyrimethamine, its 3-N-oxide and internal standard were 91.2 ± 5.9%, 82.0 ± 5.1% and 79.0 ± 6.4%, respectively. All calibration curves showed linearity (r = 0.99) for pyrimethamine and pyrimethamine 3-N-oxide in both plasma and urine. The intra- and inter-assay precision data for drug and metabolite in both plasma and urine are summarized in Table I. There was little variation in pyrimethamine assays, with coefficients of variation below 9%. By contrast coefficients of variation of pyrimethamine 3-N-oxide assays were slightly greater at the low concentrations tested.

The assay was shown to be selective, as it was free from chromatographic interference from endogenous material and the antimalarial drugs chloroquine, amodiaquine, cycloguanil, primaquine, sulphadiazine and sulphadoxine; although it was noted, that with some batches of the ODS column used, the resolution between chloroquine and pyrimethamine was incomplete. Therefore, the proposed method would be well suited for pyrimethamine determination, where these other antimalarials were being co-administered, as in combination therapy or studies of drug metabolism interactions.

Sensitive gas—liquid chromatographic (GLC) methods have previously been reported [5, 6]. However they would not be able to quantify N-oxide metab-

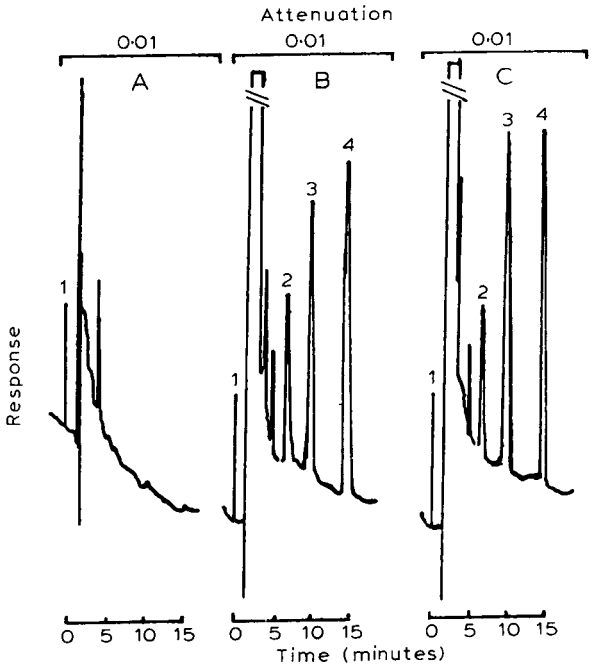


Fig. 3. High-performance liquid chromatograms for (A) a blank urine extract; (B) a spiked urine extract (pyrimethamine concentration = 10 μg/ml, pyrimethamine 3-N-oxide concentration = 10 μg/ml); and (C) an extract of a urine sample from a mouse which received pyrimethamine at 75 mg/kg (pyrimethamine concentration = 12.3 μg/ml, pyrimethamine 3-N-oxide concentration = 8.1 μg/ml). Peaks: 1 = injection event; 2 = pyrimethamine 3-N-oxide; 3 = pyrimethamine; and 4 = internal standard.

TABLE I

INTRA- AND INTER-ASSAY COEFFICIENTS OF VARIATION FOR PYRIMETHAMINE AND ITS 3-N-OXIDE (n = 5)

Compound	Plasma			Urine		
	Concentration (μg/ml)	Intra-assay C.V. (%)	Inter-assay C.V. (%)	Concentration (μg/ml)	Intra-assay C.V. (%)	Inter-assay C.V. (%)
Pyrimethamine	10	4.8	8.5	100	2.7	3.8
	2	3.0	8.0	10	7.0	5.4
Pyrimethamine 3-N-oxide	5	5.0	2.1	10	4.8	11.0
	1	6.0	10.0	2	7.0	11.9

olites of pyrimethamine, as the thermal instability of these molecules [7] makes them unsuitable for GLC analysis. Furthermore, as N-oxides readily revert to the parent drug at elevated temperatures [8], the accuracy of pyrimethamine determination in such methods may be compromised. These disadvantages do not apply to the HPLC method described in this report.

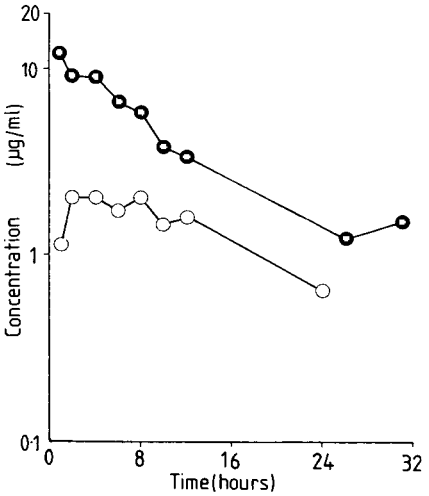


Fig. 4. Representative semi-logarithmic plot of plasma levels of pyrimethamine (•—•) and pyrimethamine 3-N-oxide (○—○) against time, obtained from a single mouse following 75 mg/kg pyrimethamine.

In the present study the assay was used to examine the pharmacokinetics of pyrimethamine in the mouse, which is a convenient and widely used animal model for the study of malaria. A typical log plasma concentration versus time curve is shown in Fig. 4. Plasma levels of pyrimethamine were observed to reach a maximum level of 12.3 μg/ml at 1 h after dosing, after which they decayed with a terminal phase half-life of 7.9 ± 5.9 h, which is in broad agreement with the only previous report [9]. Pyrimethamine 3-N-oxide attained a peak plasma level of 2 μg/ml at 2 h, falling below detectable levels by 26.5 h. Urinary excretion of pyrimethamine over 96 h was 41.8 ± 22.2 μg or 2.3% of the dose. Excretion of pyrimethamine 3-N-oxide was 7.0 ± 5.0 μg or 0.4% of the dose.

The proposed assay has been shown to be suitably selective and sensitive for the quantitation of both pyrimethamine and pyrimethamine 3-N-oxide. As a microanalytical method, it is well suited to analysis of small volumes of plasma and urine, such as those obtained in the chemotherapy of experimental rodent malaria.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council, the Wolfson Foundation, the National Health and Medical Research Council of Australia, and the U.N.D.P./World Bank/World Health Organization Special Programme for Research and Training in Tropical Diseases. We should also like to thank Mrs. C. Clarke for typing the manuscript.

REFERENCES

1 R.A. Ahmad and M.J. Rogers, Brit. J. Clin. Pharmacol., 10 (1980) 519.

2 C.R. Jones and S.M. Ovenell, J. Chromatogr., 163 (1979) 179.
3 U. Timm and E. Weidekamm, J. Chromatogr., 230 (1982) 107.
4 J.P. Hubbell, M.L. Henning, M.E. Grace, C.A. Nichol and C.W. Sigel, in J.W. Gorrod (Editor), Biological Oxidation of Nitrogen, Elsevier/North Holland Biomedical Press, Amsterdam, 1978, pp. 177—182.
5 M. Bonini, F. Mokofio and S. Barazi, J. Chromatogr., 224 (1981) 332.
6 C.R. Jones, P.R. Ryle and B.C. Weatherley, J. Chromatogr., 224 (1981) 492.
7 A.C. Cope and W.A. Le Bel, J. Amer. Chem. Soc., 82 (1960) 4656.
8 J.K. Faulkner and K.J.A. Smith, Xenobiotica, 2 (1972) 59.
9 J.C. Cavallito, C.A. Nichol, W.D. Brenckman, R.L. De Angelis, D.R. Stickney, W.S. Simmons and C.W. Sigel, Drug Metab. Dispos., 6 (1978) 329.

Journal of Chromatography, 308 (1984) 370—375
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2103

Note

Measurement of xamoterol in plasma and urine by high-performance liquid chromatography

C.J. ODDIE*, G.P. JACKMAN and A. BOBIK

Clinical Research Unit, Alfred Hospital, and Baker Medical Research Institute, Commercial Road, Prahran, Victoria 3181 (Australia)

(First received December 8th, 1983; revised manuscript received January 18th, 1984)

Xamoterol, 1-(4-hydroxyphenoxy)-3-[2-(4-morpholinecarboxamino)-ethylamino]-2-propanol, hemifumarate, ICI 118,587, Corwin (Fig. 1), is one of a structurally related series of new, orally active drugs being investigated for use in the management of heart failure in man. Preliminary human and animal studies have shown that xamoterol is a highly selective, partial beta-adrenoceptor agonist [1—3].

We have recently studied the intravenous pharmacokinetics of xamoterol, the time course of its cardiovascular effects and their relationship to dose [4]. For this study, it was necessary to determine plasma and urinary concentrations of xamoterol. The present report describes the analytical procedure developed to measure xamoterol in plasma and urine.

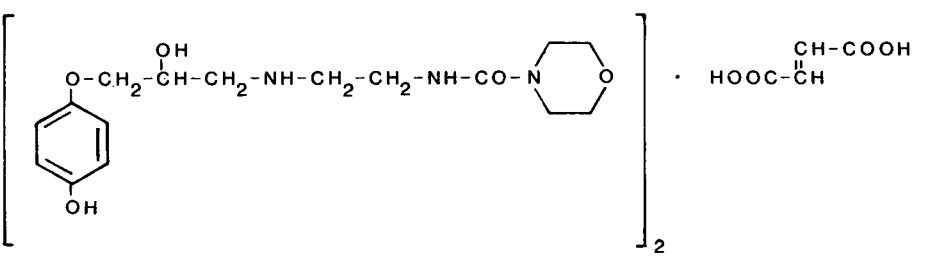


Fig. 1. Structural formula of xamoterol hemifumarate.

METHODS

Reagents

Xamoterol hemifumarate was generously supplied by Imperial Chemical

0378-4347/84/$03.00

Industries, U.K. Prenalterol hydrochloride (*S*-(—)-(4-hydroxyphenoxy)-3-isopropylamino-2-propanol hydrochloride) was generously supplied by Ciba-Geigy (Basle, Switzerland). Stock solutions (1 mg/ml) of both compounds were prepared in 0.1 *M* hydrochloric acid and stored at 4° C. Working solutions were prepared in 0.01 *M* hydrochloric acid in the range 800—12.5 ng/ml. Acetonitrile, 190-nm HPLC grade, was obtained from Waters Assoc. (Milford, MA, U.S.A.). Water for HPLC was redistilled from alkaline potassium permanganate. All other reagents were of analytical grade.

Bio-Rex 70 (50—100 mesh), Na^+, cation-exchange resin (Bio-Rad Labs., Richmond, CA, U.S.A.) was packed into polypropylene columns (4 × 1 cm, Bio-Rad Labs.) and washed sequentially with 3 *M* hydrochloric acid, 3 *M* sodium hydroxide, 3 *M* acetic acid, 1 *M* ammonium acetate, pH 6.5 and distilled water.

Chromatography

High-performance liquid chromatography (HPLC) was carried out using a Model 5000 liquid chromatograph fitted with a 100-μl universal loop injector (Varian Assoc., Palo Alto, CA, U.S.A.). A Spherisorb ODS 5-μm column (25 cm × 4.6 mm I.D.) (Laboratory Data Control, Riviera Beach, FL, U.S.A.) was used. The mobile phase was acetonitrile—0.01 *M* perchloric acid (15:85) at a flow-rate of 2 ml/min. Detection was by fluorescence using an FS-970 fluorimeter (Schoeffel, Westwood, NJ, U.S.A.) fitted with a deuterium arc source. Wavelength of excitation was 190 nm; wavelength of emission was selected by a Corning 7-60 glass filter with bandpass of 320—400 nm.

Biological samples

Blood was collected from normal subjects 0—8 h following a rapid intravenous infusion of xamoterol (100 μg/kg) into heparinised tubes on ice and the plasma separated and frozen at —20° C until assay. Drug-free blood was similarly collected from healthy human subjects on no medication. Plasma from patients on medication with potential for interference in the assay was also tested.

Urine was collected at 0—2, 2—4, 4—8 and 8—24 h from the xamoterol study subjects above. Sodium metabisulphite (0.1%) or 5 *M* hydrochloric acid were used as preservative and aliquots of the urine were stored at —20° C until assay.

Experimental procedure

To a 1-ml aliquot of plasma or 0.1-ml aliquot of urine were added 40 ng prenalterol as internal standard, 5 ml 0.1% disodium EDTA and 0.1 ml of 1 *M* ammonium acetate, pH 6.5. The solution was passed through a washed Bio-Rex 70 column and the resin washed twice with 10 ml distilled water; all effluents being discarded. Xamoterol and prenalterol were eluted from the column with 3 ml of 1 *M* ammonium hydroxide. The eluate was dried under vacuum at 50° C using a vortex evaporator (Buchler Instruments, Fort Lee, NJ, U.S.A.). The dried eluate was reconstituted in 0.25 ml of 0.1 *M* perchloric acid on ice. The sample was transferred to a 1.5-ml tapered tube and centrifuged at 10,000 *g* for 10 min at 4° C (Zentrifuge 5412, Eppendorf, Hamburg, F.G.R.). A 100-μl aliquot of the supernatant was taken for HPLC analysis.

The calibration curve was prepared by processing 1-ml aliquots of subject control plasma or drug-free plasma or 0.1-ml aliquots of subject control urine to which 0—800 ng xamoterol was added through the experimental procedure.

RESULTS AND DISCUSSION

A relatively simple procedure has been developed for the measurement of xamoterol in plasma and urine. Initial purification of xamoterol was obtained by using a weak cation-exchange resin, a technique successfully applied for other ethanolamine derivatives such as urinary metanephrines [5, 6]. Use of the resin was preferential to solvent extraction because xamoterol is not readily extracted by non-polar solvents such as diethyl ether and the use of more polar solvents would be likely to pose chromatographic problems. Xamoterol, like prenalterol [7], used as the internal standard, exhibits good fluorescence on low-wavelength excitation, thereby enabling sensitive and selective detection with HPLC.

The chromatographic system developed provided good resolution of xamoterol and prenalterol from endogenous plasma contaminants. Retention times were 6.9 min for xamoterol and 5.8 min for prenalterol. Sample chromatograms for standards and plasma and urinary extracts from subjects, before and following administration of xamoterol, are shown in Fig. 2.

Recoveries from plasma and urine were similar, averaging 82 ± 2% (mean ± S.E.) for xamoterol and 81 ± 2% for prenalterol. Linear and reproducible standard curves over the range 0—800 ng/ml were obtained with a mean equation, $y = (0.013 \pm 0.001)x - (0.020 \pm 0.010)$, $r^2 = 0.999$, $n = 21$, where y represents the peak height ratio of xamoterol/prenalterol and x represents the concentration of xamoterol hemifumarate in ng/ml.

The limits of sensitivity of the assay are 1 ng/ml for plasma and 10 ng/ml for urine, the difference being due to the sample size (1 ml for plasma versus 0.1 ml for urine). Accuracy and precision of the assay were determined by replicate analysis of plasma to which 1000, 100, 2.5 and 0 ng xamoterol were added. The results are given in Table I. The slope of the regression line for nanogram amounts xamoterol added versus nanogram amounts xamoterol obtained was 1.00 and the intercept was —0.094.

Analysis of patient control plasma or urine gave non-detectable assay blanks and no interference was seen from drugs such as diazepam, nitrazepam and quinidine or from those used in the clinical study, prazosin, clonidine and atropine [4]. The method was also successfully used to measure compounds structurally similar to xamoterol i.e. salbutamol and fenoterol (unpublished observations).

Following a 5-min intravenous infusion of xamoterol (100 μg/kg) into normal subjects, peak plasma concentrations of the drug ranging from 300 to 700 ng/ml were obtained on cessation of the infusion, then declined with an apparent half-time of 2—3 h (Fig. 3, upper panel). Xamoterol was excreted in urine predominantly as the unchanged drug. An average of 60% of the administered dose was excreted in the first 2-h collection period and a total of 82% for the 24-h collection period (Fig. 3, lower panel).

In summary, the method developed permits the sensitive and selective assay

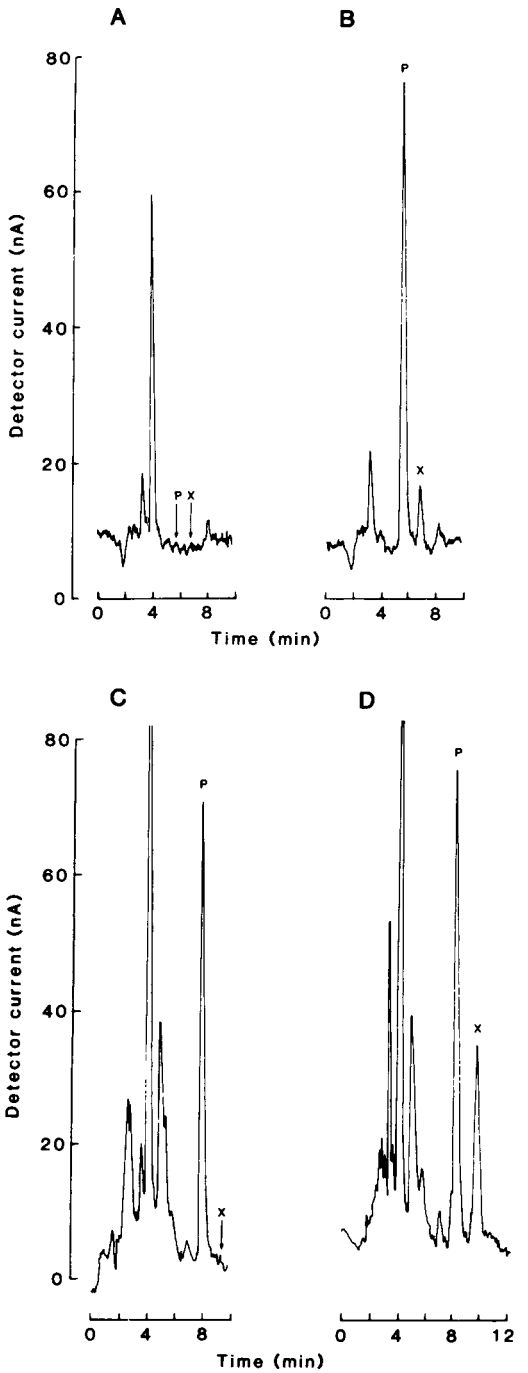


Fig. 2. Chromatographic traces of (A) extract of subject control plasma; (B) extract of subject plasma following a dose of xamoterol (100 μg/kg, intravenously) containing 5.3 ng/ml xamoterol; (C) extract of subject control urine with internal standard added; (D) extract of subject urine following a dose of xamoterol as in (B) containing 353 ng/ml xamoterol. Peaks: X = xamoterol; P = prenalterol, internal standard.

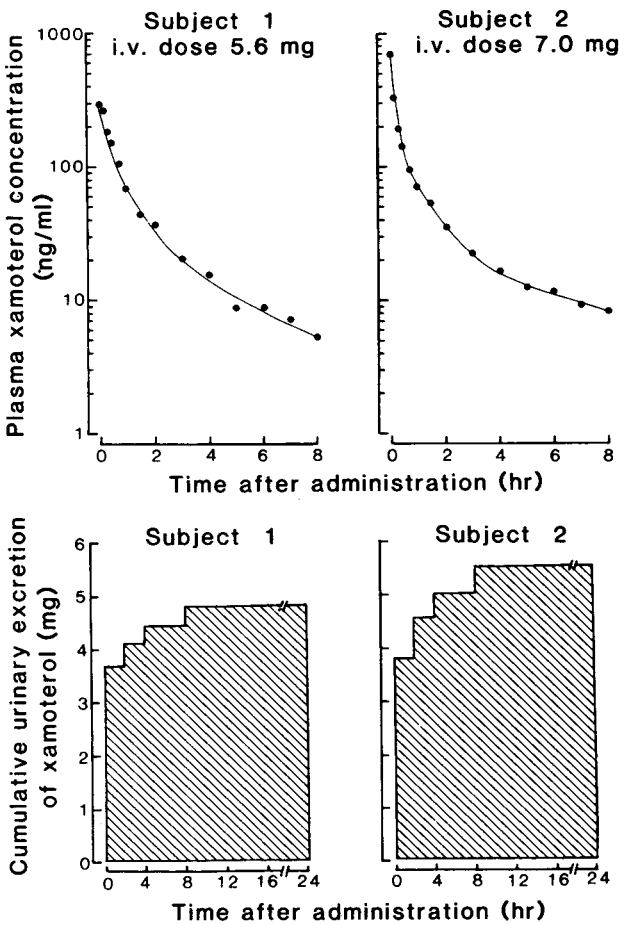


Fig. 3. Upper panel: the decline in plasma xamoterol concentrations immediately following cessation of an infusion of xamoterol (100 μg/kg, intravenously) in two typical subjects. Lower panel: cumulative urinary excretion of xamoterol in the same subjects following the xamoterol infusion.

TABLE I

ACCURACY AND PRECISION OF THE ASSAY (n = 6)

Xamoterol added (ng/ml)	Xamoterol obtained (ng/ml)	
	Mean ± S.D.	C.V. (%)
1000	1000 ± 28.7	2.9
100	98.7 ± 4.25	4.3
10	10.6 ± 0.64	6.1
2.5	2.71 ± 0.14	5.0
0	0 ± 0	—

of xamoterol in biological samples. The ion-exchange procedure shows a wider applicability than solvent extraction, increasing the potential use of the method for the analysis of similar drugs.

ACKNOWLEDGEMENT

The assistance of the clinical staff of the Clinical Research Unit, Alfred Hospital, is gratefully acknowledged.

REFERENCES

1 J.J. Barlow, B.G. Main, J.A. Moors, A. Nuttall and H.M. Snow, Brit. J. Pharmacol., 67 (1979) 412P.
2 H.F. Marlow, A.G. Wardleworth and L.M. Booth, Brit. J. Clin. Pharmacol., 13 (1982) 269P—270P.
3 A. Nuttall and H.M. Snow, Brit. J. Pharmacol., 77 (1983) 381—388.
4 G. Jennings, A. Bobik, C.J. Oddie and R. Restall, Clin. Pharmacol. Ther., (1984) in press.
5 R.E. Shoup and P.T. Kissinger, Clin. Chem., 23 (1977) 1268—1274.
6 G.P. Jackman, Clin. Chim. Acta, 120 (1982) 137—142.
7 C.J. Oddie, G.P. Jackman and A. Bobik, J. Chromatogr., 231 (1982) 473—477.

Journal of Chromatography, 308 (1984) 376—381
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2074

Note

High-performance liquid chromatography of propanidid in rat plasma

UNTO NOUSIAINEN*

Department of Physiology, University of Kuopio, P.O. Box 6, SF-70211 Kuopio 21 (Finland)

and

OLAVI RAATIKAINEN

Kuopio Regional Institute of Occupational Health, Kuopio (Finland)

(First received December 2nd, 1983; revised manuscript received January 13th, 1984)

Propanidid [3-methoxy-*p*-(N,N-diethylcarbamoylmethoxy)phenylacetic acid *n*-propyl ester] is used for intravenous anaesthesia in man [1—3]. The extremely short duration of action of propanidid is a result of rapid hydrolysis of the ester linkage by tissue esterases [3—5]. The metabolites have been shown to be anaesthetically inactive [6]. Serum concentrations of propanidid have previously been determined by photometric [3, 7] and gas chromatographic [8] methods. The photometric method lacks specificity and sensitivity, and the gas chromatographic technique is relatively time-consuming. Moreover, these methods require several millilitres of blood and cannot be applied for determination of propanidid in microvolumes of plasma.

In the present report we describe a new and rapid high-performance liquid chromatographic (HPLC) method for the determination of propanidid in plasma. The method can quantify propanidid in 0.1 ml of plasma and is applicable for studies such as the experimental pharmacokinetics of propanidid.

MATERIALS AND METHODS

Chemicals

Propanidid in injectable (Propantan®, 50 mg/ml) and in pure (B.P. 1980) form was obtained from Leiras (Turku, Finland), and it was used without

0378-4347/84/$03.00

further dilution. The internal standard α-naphthyl propionate was purchased from Sigma (St. Louis, MO, U.S.A.). Acetonitrile (Orion, Espoo, Finland) and water obtained from Milli-Q system (Millipore, Bedford, MA, U.S.A.) were of HPLC grade, and they were degassed under vacuum immediately before use. Other solvents and chemicals were of reagent grade.

Liquid chromatography and mass spectrometry

A liquid chromatograph, Altex Model 420, equipped with Altex Model 110A pumps, a Rheodyne 7125 injector (20 μl), a Kratos UV-spectroflow monitor SF 770 and a monochromator GM 770 was used. Separations were achieved on an Ultrasphere ODS (Beckman, Irvine, CA, U.S.A.) column, 4.6 × 250 mm (particle size 5 μm) and a mobile phase consisting of water—acetonitrile (30:70, v/v) at a flow-rate of 1.0 ml/min. Propanidid and internal standard were detected at ambient room temperature at 280 nm (see Fig. 3). The propanidid peaks were identified using a Shimadzu PR-1 spectrophotometer and a JEOL JMS-D 300 mass spectrometer equipped with a JMA 2000 mass data analysis system. The conditions for the mass spectrometer were ionization current 300 μA and ionization potential 70 eV, and the instrument was calibrated using perfluorokerocine.

Extraction procedure

Plasma was separated by centrifuging the blood at 800 *g* for 15 min. For both assay calibration and in vivo studies plasma samples (0.1 ml) were transferred to 90 mg of sodium chloride, 0.05 ml of α-naphthyl propionate (10 μg/ml in acetonitrile) and 0.3 ml of acetonitrile, and they were shaken horizontally (Desaga, Heidelberg, F.R.G.) for 30 min. Samples containing 1.0 ml of plasma, 900 mg of sodium chloride, 0.05 ml of α-naphthyl propionate (100 μg/ml in acetonitrile) and 3.0 ml of acetonitrile were prepared in a similar manner. After centrifugation at 2000 *g* for 15 min the acetonitrile layer was collected and used for the determination of propanidid.

Standard curve and assay calibration

A standard curve was prepared by adding known amounts of propanidid and internal standard to acetonitrile and determining the peak ratios of propanidid to internal standard. The recovery of propanidid from rat plasma was determined by comparing the amounts resulting from spiked plasma samples (1.0 ml and 0.1 ml) to those obtained from standard measurements. Inter-assay and intra-assay standardizations were performed with 1.0 ml and 0.1 ml of rat plasma using samples spiked with 5, 10 and 20 μg/ml propanidid. The inter-assay precision was carried out over a two-month period.

Animal study

During light ether anaesthesia the tail vein and the portal vein of male Wistar rats (weight 300 ± 35 g) were cannulated. Propanidid (25 mg/kg) was injected into the tail vein during 10 sec. Blood samples (0.20—0.25 ml) were collected from the portal vein 15, 30, 45, 60, 90, 120 and 180 sec after the injection, in Eppendorf microcentrifuge tubes containing 20% sodium citrate and 0.01 ml of 0.1 *M* diisopropylfluorophosphate (DFP). The tubes were carefully revolved

several times and stored at ambient room temperature for further extraction.

RESULTS AND DISCUSSION

Fig. 1 shows the separation and quantitation of propanidid (peak I) in rat plasma using α-naphthyl propionate (peak II) as an internal standard. In the chromatogram obtained after extraction of 1.0 ml of blank plasma, no additional peaks are seen which could interfere with the determination of propanidid and α-naphthyl propionate (Fig. 1A). Fig. 1B represents the quantitation of propanidid from plasma spiked with propanidid and α-napththyl propionate. Fig. 1C is a chromatogram obtained after extracting 0.1 ml of plasma from a rat 30 sec after the intravenous injection of propanidid (25 mg/kg). Propanidid and the internal standard were well separated with retention times of 4.4 and 6.9 min, respectively.

The peak eluting at 4.4 min in HPLC was identified by mass spectrometry. The structure and the fragmentation of propanidid in the mass spectrometer are presented in Fig. 2. The most prominent ions were the fragments *m/e* 114 (F_1), 100 (F_2) and 72 (F_3), accompanied by the molecular ion *m/e* 337. The mass spectra of pure propanidid and the plasma eluate were similar with respect to the dominant ions. Identification was also made by comparing ultraviolet spectra of the peak (t_R = 4.4 min) and propanidid standard (Fig. 3), which gave identical results. These studies proved that the peak eluting 4.4 min is due to propanidid with no interference from its metabolites.

The calibration curves were obtained using rat plasma spiked with 2—40 μg/ml propanidid and 5 μg/ml α-naphthyl propionate. There was a good correlation between the amount of propanidid added to rat plasma and the

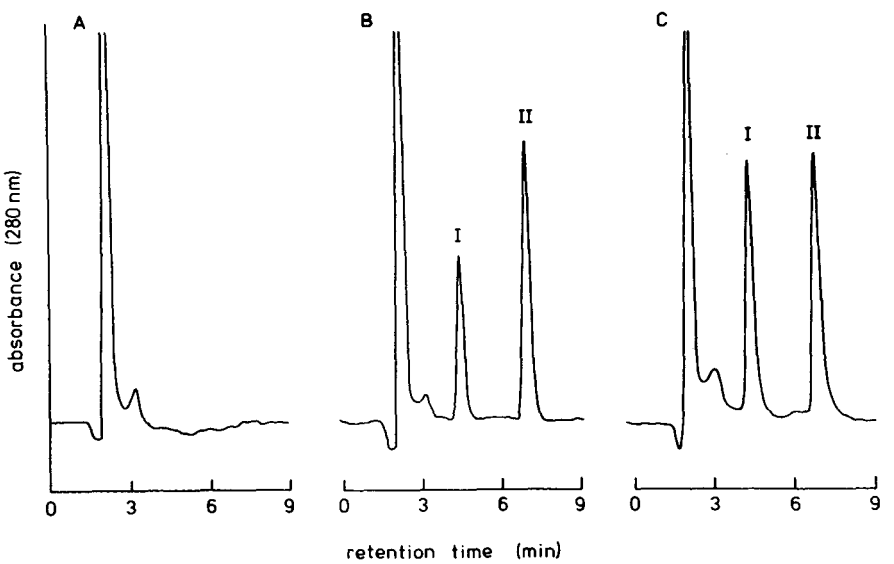


Fig. 1. Chromatograms of rat plasma: (A) blank plasma; (B) plasma spiked with propanidid (10 μg/ml) and α-napththyl propionate (5 μg/ml) as an internal standard; (C) plasma obtained from a rat 30 sec after the injection of propanidid (25 mg/kg, intravenously) and spiked with internal standard. Peaks: I = propanidid; II = α-naphthyl propionate.

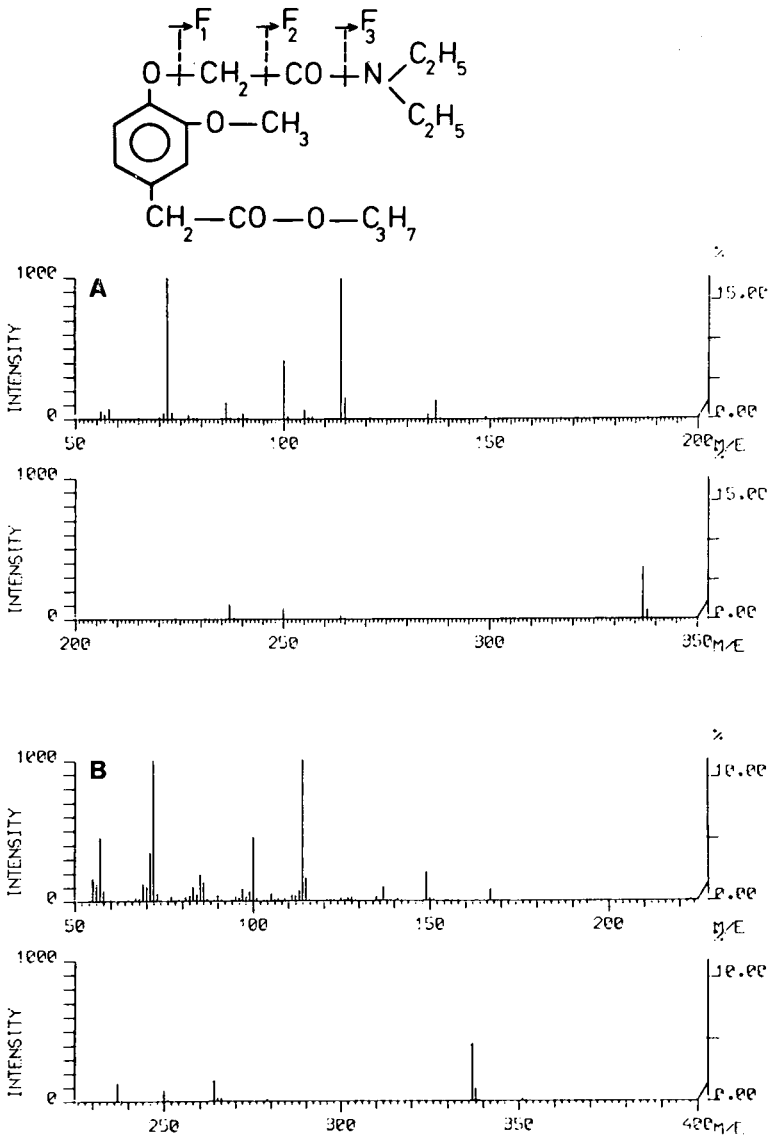


Fig. 2. Mass spectra of the pure propanidid (A) and the eluate (B) at 70 eV.

amount detected in the samples of both 1.0 ml and 0.1 ml plasma. The regression equations of these data are $Y = 17.280X - 0.04$ ($r^2 = 0.9999$) and $Y = 17.909X - 0.05$ ($r^2 = 0.9998$), respectively. The curves passed through the origin and were linear from 1 to 50 μg/ml. The present method could quantify up to 1 ng of propanidid with reasonable accuracy at 100 ng/ml. The intra-assay variations determined using 1.0 ml and 0.1 ml of spiked rat plasma were 1.2 and 2.0% ($n = 10$), and the inter-assay variations 3.0 and 5.9% ($n = 10$), respectively.

The plasma concentration—time curve for propanidid in rats (25 mg/kg,

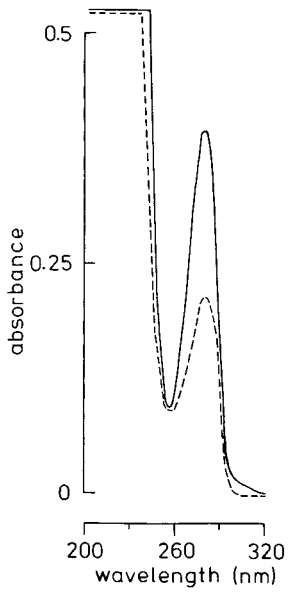


Fig. 3. Ultraviolet spectra of pure propanidid (50 μg/ml) (——) and the eluate (25 μg/ml) (— — —).

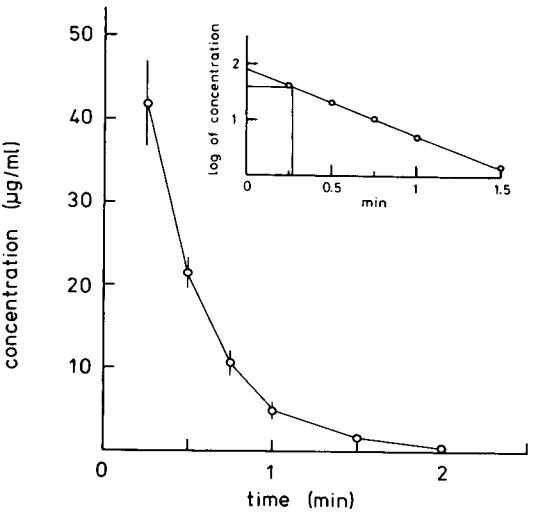


Fig. 4. Plasma concentration—time curve (mean ± S.E.) in rats (n = 15) following a parenteral administration of propanidid (25 mg/kg, intravenously).

intravenously) is shown in Fig. 4. The half-life (mean ± S.D.) of propanidid was 0.27 ± 0.08 min (n = 15). As has been assumed previously [3—5], the esterases in blood play an important role in the degradation of propanidid. The rapid hydrolysis of propanidid must be taken into account when collecting the blood samples. To avoid spontaneous hydrolysis of propanidid, we used DFP as an esterase inhibitor because it inhibits the activities of all B-esterases [9]. We noticed that plasma cholinesterase (butyrylthiocholine iodide as substrate) and carboxylesterase (p-nitrophenylacetate as substrate) were inhibited 80—90% and 40—50%, respectively, after treatment of the blood with DFP. The stability of propanidid and the internal standard in plasma samples were tested under the experimental conditions described above. No significant decrease in concentration of these two substances was observed during a follow-up period of 60 min.

The applicability of heptane [3] as the extraction medium was also tested. The half-life of propanidid in vitro was determined by incubating hepatic microsomes (0.25 mg protein per ml in a final volume of 2.0 ml of Tris—HCl buffer pH 8.2) in the presence of propanidid (5 μg/ml). The extraction of propanidid with 6.0 ml of acetonitrile or heptane showed similar half-lives of 7.0 and 7.8 min, respectively. However, heptane was not suitable for extraction of propanidid in small plasma volumes because of the poor linearity and the low recovery of different concentrations of the drug. Plasma also tended to precipitate during the extraction with this method, which caused difficulties in collecting representative samples for the analysis. These studies suggest that acetonitrile is superior to heptane for the extraction of propanidid in micro-volumes.

The present method is more selective than the photometric method [3, 7] and more sensitive than the gas chromatographic technique [8], and only microvolumes of plasma are required for reliable determinations. No pre-purification or concentration of samples before injection into the HPLC system were necessary. HPLC can be applied for the analysis of propanidid in other biological samples and hydrolysis of propanidid is easily detectable also in vitro conditions. Thus, this HPLC method offers a new way to study the function of tissue esterases both in vivo and in vitro.

ACKNOWLEDGEMENTS

The authors wish to thank Ph. Lic. Terttu Vartiainen for performing the mass spectrometry and Ms. Riitta Venäläinen for technical assistance.

REFERENCES

1 A. Doenicke, Acta Anaesthesiol.Scand. Suppl., XVII (1965) 21.
2 R.S.J. Clarke and J.W. Dundee, Anesth. Analg. (Cleveland), 45 (1966) 250.
3 A. Doenicke, I. Krumey, J. Kugler and J. Klempa, Brit. J. Anaesth., 40 (1968) 415.
4 W. Junge and K. Krisch, Crit. Rev. Toxicol., 3 (1975) 371.
5 J.R. Boyce, F.J. Wright, F.W. Cervenko, S.P. Pietak and S. Faulkner, Anesthesiology, 45 (1976) 629.
6 W. Wirth and F. Hoffmeister, in K. Horatz, R. Frey and M. Zindler (Editors), Die intravenöse Kurznarkose mit dem neuen Phenoxyessigsäurederivat Propanidid (Epontol®), Springer Verlag, New York, 1965, p. 17.
7 H. Kurz, Arch. Exp. Pathol. Pharmakol., 255 (1966) 33.
8 N.N. Dementieva, M.I. Kuleshova, A.D. Plokhoi and N.A. Tsvetkova, Farmatsiya (Moscow), 26 (1977) 50.
9 K. Krisch, in P.D. Boyer (Editor), The Enzymes, Vol. 5a, Academic Press, New York, London, 3rd edn., 1971, p. 43.

Journal of Chromatography, 308 (1984) 382—386
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2082

Note

High-performance liquid chromatographic analysis of flordipine in human plasma

MARC ROSENBERG and R. LESLIE CHOI*

Research and Development Division, Revlon Health Care Group, 1 Scarsdale Road, Tuckahoe, NY 10707 (U.S.A.)

(First received October 12th, 1983; revised manuscript received January 20th, 1984)

Flordipine {diethyl-1,4-dihydro-2,6-dimethyl-1-[2-(4-morpholinyl)ethyl]-4-[2-(trifluoromethyl)phenyl]-3,5-pyridinedicarboxylate} is a new cardiovascular agent that is orally active in animal models [1, 2]. It is currently undergoing clinical trials in humans for use in essential hypertension [3]. This paper describes a procedure, consisting of solvent extraction and reversed-phase high-performance liquid chromatography (HPLC), for the measurement of flordipine in human plasma. This general approach has been used by Pietta et al. [4] for another dihydropyridine compound, nifedipine.

EXPERIMENTAL

Apparatus

A liquid chromatograph (Model 8000B, Spectra-Physics, Santa Clara, CA, U.S.A.), equipped with a variable-wavelength UV detector (Model 770, Schoeffel Instruments, Westwood, NJ, U.S.A.) was used for the assay. The column was a Zorbax ODS, 25 cm × 4.6 mm, 5 μm particle size (DuPont, Wilmington, DE, U.S.A.).

Reagents

Flordipine was synthesized by Revlon Health Care Group, Research and Development Division (Tuckahoe, NY, U.S.A.). Hexane (Burdick and Jackson, Muskegon, MI, U.S.A.), was glass-distilled. Acetonitrile (Fisher Scientific, Fairlawn, NJ, U.S.A.) was HPLC grade. Water was deionized and glass-distilled. All other reagents used were of analytical grade.

0378-4347/84/$03.00

Chromatographic conditions

The mobile phase consisted of acetonitrile—0.005 *M* potassium phosphate buffer (pH 7.0) (60:40, v/v). Its flow-rate was 1.5 ml/min. The column oven temperature was set at 55°C. The wavelength of detection was 238 nm.

Preparation of standards

Flordipine standards, ranging between 25 ng/ml and 200 ng/ml, were prepared by adding appropriate aliquots of a 1.0 μg/ml solution of flordipine in acetonitrile to glass culture tubes (16 × 150 mm) with PTFE-lined screw caps. After evaporation of the acetonitrile to dryness with a stream of nitrogen, 1.0 ml of heparinized human plasma was added to each tube and the tube contents were mixed by vortexing. A tube containing 1.0 ml of plasma served as a blank. Equivalent standards were prepared without addition of plasma to provide a comparison for recovery of drug extracted from plasma.

Extraction

To each plasma standard, or 1 ml of plasma sample, were added 100 μl of distilled triethylamine. The use of triethylamine was found to yield more reproducible extractions of flordipine. The tubes were mixed by vortexing and the samples were extracted with 10 ml of hexane by shaking for 10 min in a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.). The phases were separated by centrifugation at 4°C and the tubes placed in a dry ice—acetone bath to selectively freeze the lower aqueous layer. The hexane extracts were then transferred to conical tubes and evaporated to dryness with a stream of nitrogen. The residues were reconstituted with 75 μl of mobile phase and a 50-μl aliquot was injected onto the column.

Calculation of results

The height of the flordipine peak was measured and calibration curves were constructed by plotting the peak height vs. the flordipine concentration of the standards. The linear regression line was determined and flordipine sample concentrations were calculated from their peak heights using the calibration curve.

RESULTS AND DISCUSSION

Chromatographic properties

Representative chromatograms of extracted blank and standard plasmas are shown in Fig. 1. Chromatograms obtained from human plasma drawn before and after an oral dose of flordipine are presented in Fig. 2. The retention time of flordipine was 9.1 min which was identical to that of a solution of the pure compound injected directly. The peak at 7.6 min in Fig. 2B appears to be a metabolite, yet to be identified, since it was present in post-dose, but not pre-dose, samples. There were no interfering peaks in control or pre-dose human plasma samples with the same retention times as flordipine or the possible metabolite. The possible interference of other drugs, such as β-blockers, diuretics and digitalis glycosides, which could be co-administered with flordipine, has not yet been investigated.

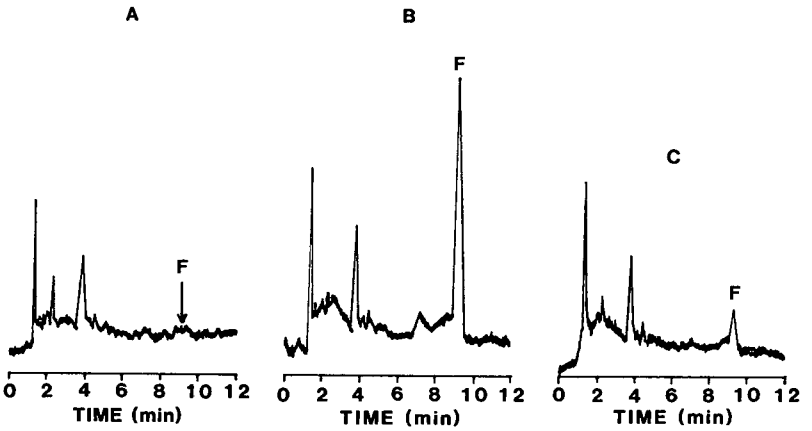


Fig. 1. Chromatograms of extracts of plasma. (A) Blank plasma (F indicates expected retention time of flordipine); (B) and (C) plasma containing 200 ng/ml and 25 ng/ml flordipine (F), respectively.

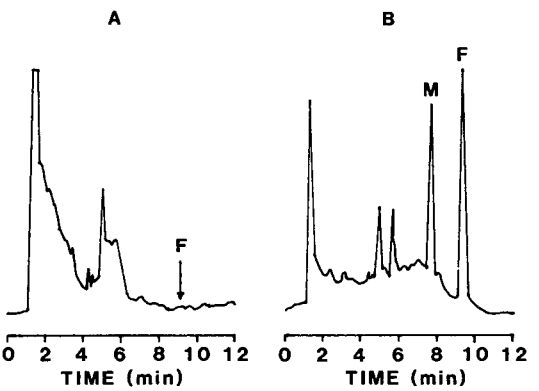


Fig. 2. Chromatograms of extracts of human plasma samples obtained before (A) and 2 h after (B) administration of 200 mg flordipine. F indicates location of the flordipine peak, which was not present in (A) and was determined equivalent to 145 ng/ml in (B). M indicates a possible metabolite peak, since it was present in (B) but not (A).

Precision and reproducibility

Standards of 25, 50, 100 and 200 ng/ml flordipine were assayed in duplicate on three different days to determine the precision and reproducibility of the assay. The composite coefficients of variation (% C.V.) for the peak heights ranged between 6.0% at 25 ng/ml and 6.3% at 200 ng/ml (Table I).

Linearity of calibration curves

Calibration curves obtained by plotting the peak height of flordipine vs. the flordipine concentration were linear over the concentration range of 25 ng/ml to 200 ng/ml. For the composite calibration curve (n = 3) the correlation coefficient was 0.996 and the regression equation was $y = 0.0201x + 0.136$, where y = peak height (cm) and x = concentration (ng/ml).

TABLE I

REPRODUCIBILITY OF THE METHOD

Concentration (ng/ml)	*n*	Mean peak height (cm)	Coefficient of variation (%)
25	6	0.633	6.0
50	5	1.13	6.2
100	5	2.17	6.0
200	5	4.15	6.3

Accuracy and lower limit of quantitation

The accuracy of the method was determined by assaying plasma flordipine standards of 25, 50, 100 and 200 ng/ml. Table II shows that the assay was accurate to 1% or better within the concentration range studied. The coefficient of variation of the calculated concentrations ranged from 15% at 25 ng/ml to 1% at 200 ng/ml. The lower limit of quantitation of the assay was considered to be 25 ng/ml, a level at which the assay was accurate and fairly precise.

TABLE II

ACCURACY OF MEASUREMENT OF FLORDIPINE ADDED TO PLASMA

Theoretical concentration (ng/ml)	*n*	Calculated concentration (Mean ± S.D.) (ng/ml)	C.V. (%)	Difference from theoretical (%)
25	6	24.8 ± 3.6	15	−0.8
50	5	49.8 ± 4.8	10	−0.4
100	5	101 ± 2	2	+1.0
200	5	199 ± 2	1	−0.5

Extraction efficiency

Comparison of the peak height measurements of extracted plasma standards with those of standards dissolved in mobile phase gave an estimate of the extraction efficiency for flordipine. The recovery over three days for the four concentrations averaged 89.4% with a 1.2% relative standard deviation.

Clinical samples

The HPLC procedure described herein has been used for the assay of human plasma samples obtained from subjects who received oral doses of flordipine in an ascending dose tolerance study. Fig. 3 shows the plasma flordipine concentration as a function of time after dose in a subject who received a 200-mg oral dose. The HPLC method presented for the assay of flordipine in plasma is precise and linear and is suitable for the analysis of clinical samples.

ACKNOWLEDGEMENTS

We thank Dr. Samuel Ringel for providing the clinical samples and Ms. Surinder Mangat for performing the analyses of the clinical samples.

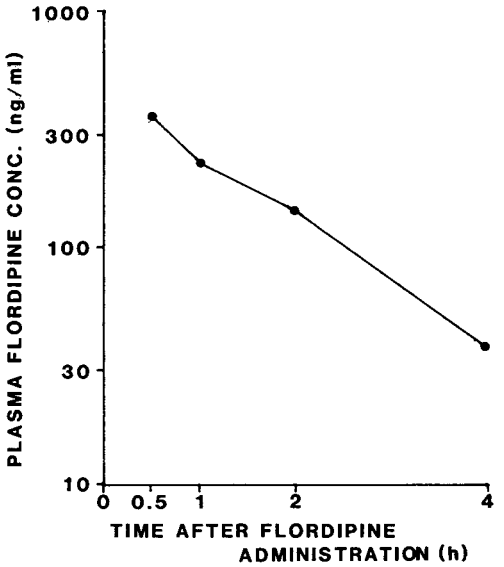


Fig. 3. Plasma concentrations of flordipine in a human subject who received a 200-mg oral dose.

REFERENCES

1 W. Mann, P. Wolf, R. Smith and B. Loev, Pharmacologist, 24 (1982) 137.
2 H.H. Shlevin, J.A. Barrett, R.D. Smith, P.S. Wolf and T.P. Pruss, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 42 (1983) 1291.
3 S.M. Ringel, A. Darragh, J. Rosenthal, F.S. Caruso and R.A. Vukovich, Clin. Pharmacol. Ther., 33 (1983) 231.
4 P. Pietta, A. Rava and P. Biondi, J. Chromatogr., 210 (1981) 516.

Journal of Chromatography, 308 (1984) 387—392
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2088

Note

High-performance liquid chromatography of haloperidol in serum at the concentrations achieved during chronic therapy

A. McBURNEY* and S. GEORGE

Department of Clinical Pharmacology, Groby Road Hospital, Leicester, LE3 9QE (U.K.)

(First received September 27th, 1983; revised manuscript received December 12th, 1983)

Haloperidol, 4-[4-(*p*-chlorophenyl)-4-hydroxypiperidino]-4′-fluorobutyrophenone, is a widely prescribed neuroleptic which is used in both adults and children for the treatment of acute and chronic psychotic syndromes and abnormal movements. Therapeutic monitoring of haloperidol is indicated for the following reasons; (1) there is no direct relationship between daily doses and serum levels at steady state; (2) saturation kinetics may apply at higher doses; (3) commonly associated drugs or disease states may alter the drugs pharmacokinetic parameters; (4) drug disposition is faster in children than adults; (5) optimum serum concentrations vary between pathological syndromes and age groups; and (6) side effects and adverse reactions are clearly related to haloperidol serum concentrations [1].

To date all the accepted methods for monitoring haloperidol have used gas chromatography (GC) and large volumes of serum [2]. A recent electron-capture GC method required only 1 ml of plasma or serum, but the selectivity of the assay was not reported [3]. The present method overcomes the use of such high sample volumes, is simpler to perform and is of equal or higher sensitivity. It has been used to monitor haloperidol serum concentrations in various psychiatric patients and the results of these determinations in 28 patients are presented.

EXPERIMENTAL

Materials and reagents

Haloperidol and the internal standard, fenethazine [10-(2-dimethylamino) ethylphenothiazine·hydrochloride], were obtained from Janssen Pharmaceuticals (Marlow, U.K.) and Rhone-Poulenc (Paris, France), respectively. The inter-

0378-4347/84/$03.00

nal standard was used as a 1 mg/l solution in methanol. The haloperidol metabolites 4-(*p*-chlorophenyl)-4-hydroxypiperidine and 4-fluorobenzoyl propionic acid were obtained from Aldrich (Gillingham, U.K.). Heptane, methanol and acetonitrile were all HPLC grade (Fahrenheit Lab. Supplies, Rotherham, U.K.). Sulphuric acid, isoamyl alcohol, sodium hydroxide and potassium bromide were all analytical reagent grade (BDH, Poole, U.K.).

High-performance liquid chromatography (HPLC)

The solvent delivery system was a constant-flow reciprocating pump (Laboratory Data Control, ConstaMetric III pump) and sample injection was performed using a Rheodyne Model 7125 syringe-loading valve fitted with a 100-μl sample loop. Stainless-steel tubing (0.25 mm I.D.) was used to connect the outlet port of the valve to the analytical column, a stainless-steel tube 30 × 0.5 cm I.D., packed with LiChrosorb Si 60 (5 μm particle size) (Analytical Supplies, Derby, U.K.), which was used at ambient temperature (normally 22°C). The

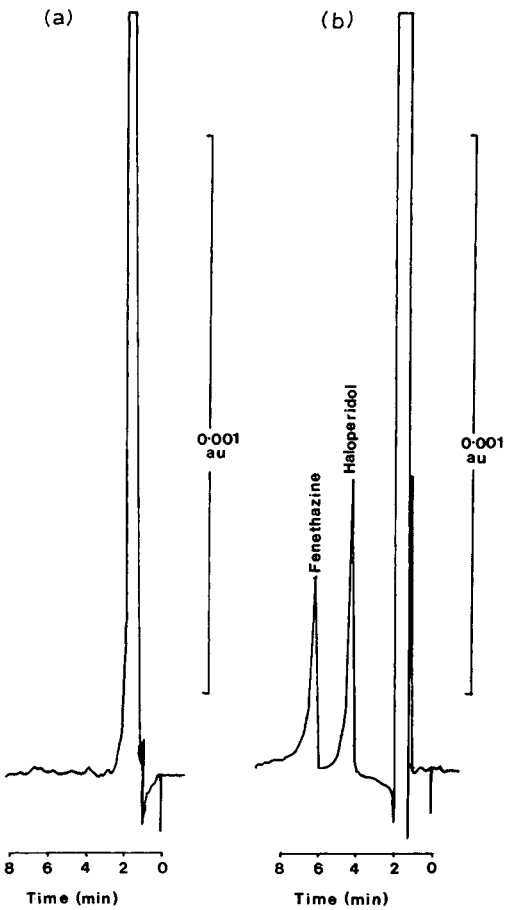


Fig. 1. Chromatograms obtained on analysis of an extract of (a) drug-free human plasma without internal standard; (b) serum from a patient treated with haloperidol, 30 mg/day; the serum haloperidol was found to be 28.7 ng/ml. Conditions were as described in the text.

TABLE I

RETENTION TIMES RELATIVE TO FENETHAZINE OF HALOPERIDOL AND OTHER COMPOUNDS ON A MICROPARTICULATE SILICA COLUMN

Compound	Relative retention time
Amitriptyline	1.41
Nortriptyline	1.89
Imipramine	1.66
Desipramine	1.97
Protriptyline	2.25
Phenytoin	0.20
Phenobarbitone	0.17
Ethosuximide	0.18
Primidone	0.20
Carbamazepine	0.23
Procyclidine	2.03
Chlorpromazine	1.40
Fenethazine	1.00
Haloperidol	0.66
Mianserin	0.74
Trimipramine	1.00
Sulthiame	0.20
Benztropine	>3.00
Metabolite 1*	>3.00
Metabolite 2**	>3.00

*4(*p*-Chlorophenyl)-4-hydroxypiperidine.
**4-Fluorobenzoyl propionic acid.

column effluent was monitored at 244 nm at a sensitivity of 0.002 a.u.f.s. (Laboratory Data Control SpectroMonitor III, Model 1204A). The mobile phase was methanol—acetonitrile (10:90) containing 2 m*M* potassium bromide and this was degassed by sonication for 15 min before use. The flow-rate was 2 ml/min.

Using this system the separation of haloperidol and fenethazine obtained is illustrated in Fig. 1. The relative retention times of these compounds and some additional drugs on this system are given in Table I.

Extraction from serum

Serum (2 ml) was pipetted into a 10-ml conical centrifuge tube and internal standard solution added (30 μl of 1 μg/ml fenethazine in methanol). The tube was vortex-mixed for 30 sec, the contents were made alkaline with 2 *M* sodium hydroxide (1 ml) and 5 ml heptane—isoamyl alcohol (98.5:1.5) added. The contents of the tube were vortex-mixed for 10 sec and the tube was centrifuged at 1000 *g* for 10 min. The organic phase was transferred to a 15-ml centrifuge tube and the extraction repeated. The combined organic extracts were acidified with 0.005 *M* sulphuric acid, (2 ml) and vortex-mixed for 60 sec. On separation of the phases, the organic phase was aspirated off and 1.8 ml of the sulphuric acid were transferred to a 5-ml centrifuge tube. The acid extract was made alkaline with 1 *M* sodium hydroxide (200 μl) and extracted with 500 μl heptane—isoamyl alcohol (98.5:1.5) by vortex-mixing for 30 sec. Following

separation of the organic and aqueous phases by centrifugation at 1000 *g* for 5 min, 150 μl of the organic phase was transferred to a small (Dreyer) test-tube (R.W. Jennings, Nottingham, U.K.), and evaporated to dryness under nitrogen at 60°C. The extract was reconstituted in methanol (50 μl) and 40 μl injected into the chromatograph. Analyses were performed in duplicate and the mean result taken.

Instrument calibration

Standard solutions containing haloperidol at 5, 10, 20, 30 and 50 ng/ml were prepared in expired blood bank plasma by dilution of a 1 mg/ml solution of this compound in methanol.

On analysis of these solutions, the ratio of the peak height of drug to the peak height of the internal standard, when plotted against drug concentration, was linear between 5 and 50 ng/ml and passed through the origin of the graph.

RESULTS AND DISCUSSION

Reproducibility and accuracy

The intra-assay coefficients of variation (C.V.) measured from replicate analyses (n=6) of standard solutions prepared in blood bank plasma were as follows; at 2 ng/ml haloperidol, C.V. = 10.0%; at 20 ng/ml, C.V. = 8.8%.

Overall recovery of haloperidol was determined over the concentration range 0—30 ng/ml using concentrations of 0, 5, 10, 20 and 30 ng/ml in duplicate estimations. Overall recovery of drug relative to the internal standard, fenethazine, was 104.6%.

Selectivity

No endogenous sources of interference have been observed. The chromatogram obtained on analysis of a specimen of drug-free human plasma is illustrated in Fig. 1a and that from a patient treated with haloperidol in Fig. 1b. Analyses of specimens with and without the internal standard fenethazine have not revealed any endogenous compound which could co-elute with this standard.

As haloperidol is frequently given together with other centrally acting drugs, the potential interference from a number of such drugs was investigated. The retention times of these compounds are given in Table I. Of the compounds examined, only trimipramine was found to pose a problem as it co-eluted with the internal standard, fenethazine.

Sensitivity

The limit of sensitivity of the assay was 2 ng/ml. The intra-assay C.V. at this concentration was 10.0% (n=6).

Haloperidol could be detected using a fixed-wavelength UV detector at 254 nm but at this wavelength sensitivity was reduced by almost 35%.

Serum concentrations in psychiatric patients

Haloperidol serum concentrations were determined in 28 patients receiving the drug for a variety of psychiatric conditions. Blood samples were drawn be-

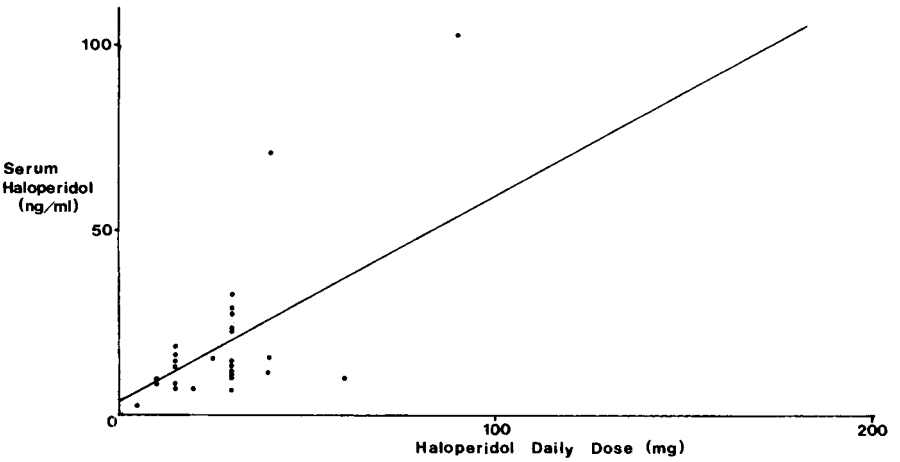


Fig. 2. The relation between steady-state serum concentrations of haloperidol (Y) and daily dose of haloperidol (X) in 28 psychiatric inpatients. The regression line was $Y = 0.56\ X + 3.84$ and the correlation coefficient $r = 0.793$ ($p<0.001$).

TABLE II

SERUM HALOPERIDOL CONCENTRATIONS IN PATIENTS RECEIVING THE DRUG FOR TREATMENT OF MANIA AND SCHIZOPHRENIA

Subject	Mania		Subject	Schizophrenia	
	Dose (mg/day)	Serum concentration (ng/ml)		Dose (mg/day)	Serum concentration (ng/ml)
1	25	15.8	13	30	15.1
2	30	11.6	14	10	9.2
	20	7.0			
			15	15	15.5
3	40	12.1			
	60	10.5	16	30	28.7
4	15	7.6	17	15	13.4
5	15	7.7	18	30	10.6
	30	28.5			
	30	33.0			
6	30	7.2			
7	4.5	2.6			
8	40	16.0			
9	15	18.9			
10	30	23.2			

tween 1 and 4 h post dose. The results as the average of duplicate estimations are shown in Fig. 2 in relation to the total daily dose of haloperidol. Although there was a good correlation between the two variables (r=0.793, $p<0.001$), serum haloperidol concentrations varied up to five-fold in patients given the

same daily dose. For patients given 15 mg/day the serum concentrations ranged from 7.6 to 18.9 ng/ml and for patients receiving 30 mg/day the range was 7.2 to 33.0 ng/ml. It was observed that the greatest variation occurred in samples drawn during the first 2 h post dose, suggesting individual variations in absorption of drug may be responsible for this wide range in serum concentration.

The results of haloperidol determinations in patients given the drug for the control of mania and schizophrenia are shown in Table II. No attempts were made to assess the clinical effect of the drug in relation to the serum concentration.

CONCLUSIONS

The method described here has been found to be suitable for the measurement of serum haloperidol concentrations achieved during therapy and may be useful in single-dose pharmacokinetic studies. Only 2 ml of serum are required and the method is simpler than GC methods to perform in that it is not necessary to silanize glassware, the calibration is linear and passes through zero, the extraction procedure is faster and the level of sensitivity is equal to GC methods which require 5 ml of serum. A recent GC method [3] quoted a limit of sensitivity of 0.5 ng/ml based on the extraction of 1 ml plasma. However, the selectivity of this method was not assessed and the assay protocol involved heating the sample on a boiling-water bath for 1.25 h in order to release protein-bound drug. The current HPLC method is a significant improvement on a previous HPLC method described for the analysis of pure drug solutions only which had a limit of sensitivity of 50 ng/ml [4]. Further HPLC methods using a reversed-phase mode of operation have been described [5,6]. These methods gave equivalent limits of sensitivity to the method described here but one report [5] had a longer analysis time, did not utilise an internal standard during the extraction procedure and gave no indication of the selectivity of the assay whilst the second method [6] employed two internal standards in order to circumvent problems associated with the selectivity of the assay. The HPLC methodology described here using normal-phase liquid chromatography avoids the use of buffered eluents and the concomitant problems associated with the effects of pH variations on the separation parameters. It has been shown to be a rapid, reliable and selective method for the analysis of haloperidol in patients receiving chronic therapy.

REFERENCES

1 P L. Morselli, G. Bianchetti and M. Dugas, Ther. Drug Monitor., 4 (1982) 51.
2 G. Bianchetti, in A. Richens and V. Marks (Editors), Therapeutic Drug Monitoring, Churchill Livingstone, London, 1981, p. 307.
3 F.O. Holley, J.R. Magliozzi, D.R. Stanski, L. Lombrozo and L.E. Hollister, Clin. Pharmacol. Ther., 33 (1983) 477.
4 D.S. Greene, Drug Develop. Indust. Pharm., 5 (1979) 127.
5 K. Miyazaki, T. Arita, I. Oka, T. Koyama and I. Yamashita, J. Chromatogr., 223 (1981) 449.
6 P.I. Jatlow, R. Miller and M. Swigar, J. Chromatogr., 227 (1982) 233.

Journal of Chromatography, 308 (1984) 393—397
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2087

Note

Simultaneous determination of trimethadione and its metabolite in rat and human serum by high-performance liquid chromatography

EINOSUKE TANAKA*, SHIGENOBU HAGINO, TAKEMI YOSHIDA and YUKIO KUROIWA

Department of Biochemical Toxicology, School of Pharmaceutical Science, Showa University, Hatanodai 1-5-8, Shinagawa-ku, Tokyo (Japan)

(First received December 8th, 1983; revised manuscript received January 23rd, 1984)

Recently we reported a sensitive method for the determination of low concentrations of trimethadione (TMO) and its metabolite, 5,5-dimethyl-2,4-oxazolidinedione (DMO), by using gas chromatography (GC) with flame-thermionic detection (FTD) [1]. In addition, a pharmacokinetic study using this sensitive method was carried out in carbon tetrachloride-treated rats [1]. However, GC methods for the determination of TMO and DMO in rat plasma normally are time-consuming.

In this present study, we report the development of a rapid and selective high-performance liquid chromatographic (HPLC) method for the simultaneous analysis of TMO and DMO in rat and human serum.

EXPERIMENTAL

Materials

TMO was purified from commercial powder containing 66.7% TMO (Mino-Aleviatin® , Dainippon, Osaka, Japan). DMO was purchased from Tokyo Kasei (Tokyo, Japan), acetonitrile from Wako (Tokyo, Japan), α-methyl-α-propyl-succinimide from Aldrich (Milwaukee, WI, U.S.A.) and PIC®-B_5 (low UV) from Waters Assoc. (Milford, MA, U.S.A.). All other chemicals were reagent grade.

Extraction procedure

To 50 μl of serum (or standard) in a 2.5-ml tube were added 50 μl of methyl alcohol with α-methyl-α-propylsuccinimide as internal standard. The tube was

0378-4347/84/$03.00

shaken for 2 min and centrifuged at 1500 *g* for 5 min. A 10- or 20-μl aliquot was injected into the chromatograph.

High-performance liquid chromatography

A high-performance liquid chromatograph (Model 633A, Hitachi, Tokyo, Japan) with a variable-wavelength ultraviolet (UV) spectrophotometer (Hitachi) and recorder (Model 056, Hitachi) was employed. The column was a reversed-phase type (Shodex® ODSpak F-411A, 4.6 mm × 150 mm, 5 μm). The detector was set at 200 nm and the mobile phase was water (85 ml), acetonitrile (15 ml) and PIC®-B_5 (3.5 ml). All analyses were performed at room temperature.

Standards

Standard solutions of TMO and DMO (1 mg/ml) were prepared in water and stored at 4° C. These solutions were then diluted as necessary to prepare the appropriate serum standards for each drug and assay run. The internal standard, α-methyl-α-propylsuccinimide, was prepared in methyl alcohol (100 μg/ml) and stored at 4° C. Peak height ratios of TMO and DMO to α-methyl-α-propylsuccinimide were determined.

Recovery and reproducibility

Drug standards were added to drug-free serum in amounts equivalent to 0.01—200 μg/ml. Recovery of drug from serum after protein precipitation was determined at concentrations of 0.1, 0.5, 1.0, 10.0, 50.0, 100.0 and 200.0 μg/ml serum by comparing the peak heights with those obtained for aqueous solutions containing the known concentrations of TMO and DMO. Reproducibility was determined for the same concentration range by quadruplicate analysis of samples at each concentration.

Gas chromatography

Analysis was carried out with a Shimadzu GC-7A equipped with a flame-thermionic detector (Kyoto, Japan). The glass column (50 cm × 2.6 mm I.D.) was packed with 5% PEG 6000 on 80—100 mesh Chromosorb W HP (Chromato Supply, Tokyo, Japan). The column oven temperature was raised from 100° C to 190° C at a rate of 16° C/min and held at 190° C for 5 min. The injection port and detector were at 210° C. The carrier gas was helium at a flow-rate of 50 ml/min.

Animal studies

After overnight fasting, male Wistar rats (Japan Laboratory Animals, Tokyo, Japan), weighing 195—230 g, received 100 mg/kg TMO in 2 ml of water by gavage. Blood samples were obtained from the jugular vein at 0.5, 1.0, 2.0, 4.0, 8.0, 12.0, 24.0, 48.0 and 72 h after administration.

Human studies

The subjects were normal healthy male volunteers, with a mean age of 30.4 years (22—51 years) and mean weight of 65.3 kg (54—76 kg). After overnight fasting, the subjects received an oral dose of 4 mg/kg TMO powder with

100 ml of water. Blood samples were obtained from an arm vein at 0.5, 1.0, 2.0, 6.0, 12.0, 48.0, 72.0, 96.0, 120.0, and 480.0 h after administration.

Pharmacokinetic studies

Concentration—time curves for TMO and DMO were drawn on semi-logarithmic scales. The half-life ($T_{½}$) and elimination rate constant (K_{el}) were calculated by linear regression analysis. The apparent volume of distribution (V_d) was calculated from the ratio of the administered dose to the plasma concentration extrapolated to time zero. The area under the curve (AUC) was calculated by the trapezoidal rule, and area to finite time was added by integration (C_t/K_{el}), where C_t is the last value of the TMO concentration. K_{el} was calculated from the equation $K_{el} = 0.693/T_{½}$.

Metabolic clearance (Cl) was calculated according to the equation $Cl = 0.693V_d/T_{½}$.

For statistical analysis a paired Student's t-test was used.

RESULTS AND DISCUSSION

Fig. 1. shows chromatograms of DMO, TMO and α-methyl-α-propyl-succinimide (internal standard). The results indicate that there was good separation among DMO, TMO and internal standard. The retention times for DMO, TMO and I.S. were 3.4, 5.5 and 12.7 min, respectively. Table I shows an extraction recovery of DMO and TMO between 1 μg/ml and 200 μg/ml from serum. The calibration graphs showed a linear relationship between the peak heights of TMO or DMO to the internal standard in the concentration range 1—200 μg/ml (TMO, $r = 0.995$; DMO, $r = 0.997$). No interfering peaks appeared

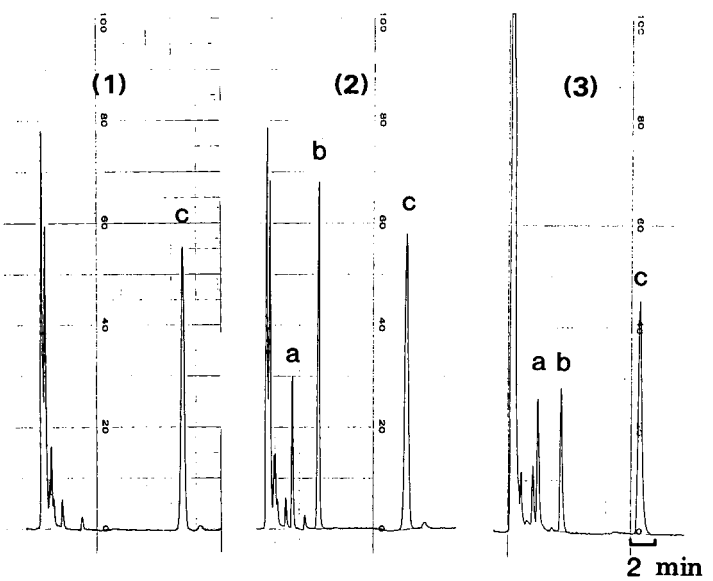


Fig. 1. Chromatograms of TMO and DMO after oral administration of TMO to rat (100 mg/kg) and human (4 mg/kg): (1), serum blank; (2) rat serum; (3) human serum. Peaks: a = DMO, b = TMO, c = internal standard (α-methyl-α-propylsuccinimide).

TABLE I

RECOVERY OF TMO AND DMO FROM SERUM

Amount added (μg/ml)	TMO		DMO	
	Amount found (μg/ml, mean ± S.E.)*	Recovery (%)	Amount found (μg/ml, mean ± S.E.)*	Recovery (%)
1	0.97 ± 0.01	97	0.96 ± 0.01	96
10	10.2 ± 0.33	102	10.1 ± 0.41	101
50	49.1 ± 0.28	98	48.7 ± 0.28	97
100	99.9 ± 2.18	99	98.3 ± 1.41	98
200	198.2 ± 3.16	99	197.1 ± 2.01	98

*$n = 4$.

when phenobarbital, phenytoin, pentobarbital, acetazolamide, carbamazepine or primidone, which are usually administered to patients in combination with TMO, were added to serum. This method was capable of measuring at least 0.1 μg/ml TMO and 0.5 μg/ml DMO. From these results it is reasonable to note that the method presented for the determination of TMO and DMO would be useful even if a lower dose of the drug was administered to animals.

Rat or human serum samples were analysed for DMO and TMO by the method described here and by gas chromatography (GC). The results are shown in Fig. 2 which shows excellent agreement between the two procedures: TMO: $r = 0.998$, $Y = 1.01X - 0.288$; DMO: $r = 0.997$, $Y = 1.01X - 0.301$.

Table II shows the pharmacokinetic parameters following the oral administration of TMO to rats and humans. The serum concentration of TMO in rats reached its peak at 0.5—1 h, and serum DMO reached its peak at around 9 h and then gradually decreased, whereas the serum concentration of TMO in humans reached its peak at 0.5—1 h, and serum DMO reached its peak at around 60—72 h and then gradually decreased. The patterns of serum TMO

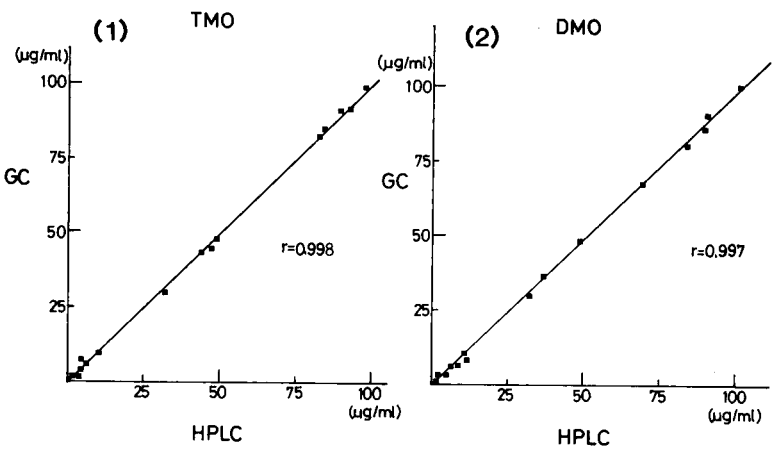


Fig. 2. Correlation between serum TMO (1) and DMO (2) concentrations assayed by HPLC and GC. TMO: $r = 0.998$, $Y = 1.01X - 0.29$; DMO: $r = 0.997$, $Y = 1.01X - 0.30$. $n = 15$.

TABLE II

PHARMACOKINETIC PARAMETERS* FOLLOWING THE ORAL ADMINISTRATION OF TMO TO RATS AND HUMANS

Each value indicates the mean ± S.E. Rats and humans received, respectively, 100 mg/kg and 4 mg/kg TMO orally.

	$T_{1/2}$ (h)	V_d (l/kg)	Cl (l/kg/h)	AUC (μg/ml/h)
Rat	2.66 ± 0.36	0.647 ± 0.013	0.039 ± 0.018	386.5 ± 5.5
Human	10.21 ± 0.69	0.648 ± 0.032	0.043 ± 0.003	100.1 ± 9.5

*$T_{1/2}$ = half-life; V_d = apparent volume of distribution; Cl = metabolic clearance; AUC = area under the curve.

and DMO levels in rats were quite similar to those reported previously by the GC method [2]. These data suggest that the new HPLC method is rapid, sensitive and selective for the simultaneous analysis of TMO and DMO in rat and human serum.

ACKNOWLEDGEMENT

The authors are very grateful to Mr. Hiroyuki Kato for his assistance in the experimental work.

REFERENCES

1 E. Tanaka, H. Kinoshita, T. Yoshida and Y. Kuroiwa, J. Chromatogr., 272 (1983) 380.
2 E. Tanaka, H. Kinoshita, T. Yoshida and Y. Kuroiwa, Pharmacology, 25 (1982) 202.

Journal of Chromatography, 308 (1984) 398—401
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2063

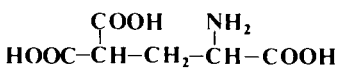
Letter to the Editor

An improved method for the determination of γ-carboxyglutamic acid in human urine by high-performance liquid chromatography

Sir,

γ-Carboxyglutamic acid (Gla, Fig. 1) has been found as a constituent of several vitamin K-dependent, calcium-binding proteins, and free Gla has been demonstrated to be excreted in urine [1]. Since urinary Gla is a final degradation product of Gla-containing proteins [2], its measurement supplies useful clinical information regarding bone metabolism and the blood coagulation systems [3]. Recently, we developed a rapid and sensitive high-performance liquid chromatographic (HPLC) method for the determination of Gla using pre-column derivatization with *o*-phthalaldehyde (OPA) and a silica-based anion-exchange column [4]. During the course of assay of urinary Gla by this method, the need for a suitable internal standard which reacts with OPA to form a fluorescent product but which is not present in biological samples became evident. It was also desirable to minimize the time-dependent decomposition of the OPA derivative of Gla in urine, so that the procedure could be made suitable for routine analysis using an autosampler. The purpose of this communication is to report an improved method which solves these problems.

The present method consists of an alkaline pretreatment of urine samples and an internal standardization method using djenkolic acid (Fig. 1), as described below. A 0.5-ml aliquot of human urine was mixed with an equal volume of 5 *M* potassium hydroxide and the mixture was hydrolyzed at 110°C for 24 h as described previously [5]. The hydrolysate was ice-cooled, spiked



γ-Carboxyglutamic Acid (Gla)

Djenkolic Acid

Fig. 1. Structures of γ-carboxyglutamic acid and djenkolic acid.

with 0.1 ml of djenkolic acid (Tokyo Kasei Industries, Tokyo, Japan) in 0.01 *M* potassium hydroxide (0.1 mg/ml), and then neutralized by dropwise addition of 70% perchloric acid. After standing for 30 min on ice, the solution was centrifuged at 10,000 *g* for 5 min to remove a potassium perchlorate precipitate and the supernatant was mixed with about 30 mg of charcoal to decolourize it. The mixture was filtered through a 0.45-μm filter (Cat. No. E-251, Gelman Science Japan Ltd., Tokyo, Japan) and the filtrate was analyzed by HPLC according to the method described previously [4] using identical conditions, except that the solvent flow-rate was set at 1.5 ml/min instead of 2.0 ml/min.

In our previous work, urine was directly derivatized with OPA-reagent and then analyzed by HPLC [4]. As shown in Fig. 2, the fluorescence of OPA-Gla in urine was decreased by 32% at 3 h after the derivatization. In contrast, when the urine was hydrolyzed in alkali as described above and then derivatized with OPA, the fluorescence of OPA-Gla decreased by only 6% within 3 h. The same result was also obtained with Gla in bone hydrolyzate. These results suggest that the rapid decomposition of OPA-Gla in intact urine is probably caused by peptides or proteins in the urine. The alkaline hydrolysis releases Gla from protein-bound forms, and free Gla is very stable under the conditions used [6]. Thus, pretreatment of urine samples by alkaline hydrolysis was adopted in the standard procedure.

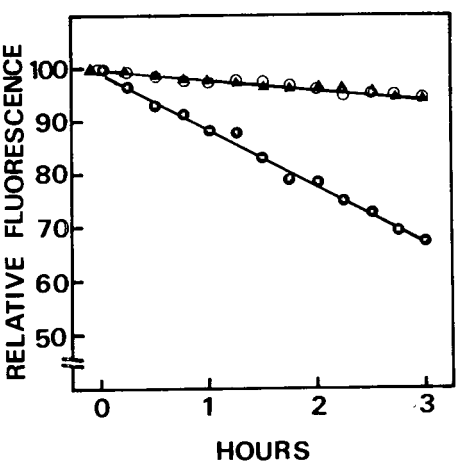


Fig. 2. Kinetics of the fluorescence decay of OPA-Gla in various samples at room temperature. (•), Human urine; (○), an alkaline hydrolyzate of the human urine; (▲), an alkaline hydrolyzate of rat bone. These samples were derivatized with OPA and analyzed by the HPLC method as described in the text.

Fig. 3 shows chromatograms of urine before and after the alkaline hydrolysis. Under the HPLC conditions used, Gla is eluted last of all the protein-constituent amino acids because it has the most acidic nature [4]. The alkaline hydrolysis resulted in the disappearance of peptide peaks and no peak was observed later than that of Gla in the alkaline hydrolyzate of urine. It is noteworthy that the hydrolysis improves the sharpness of the Gla peak. In this method, djenkolic acid was used as an internal standard for the analysis of

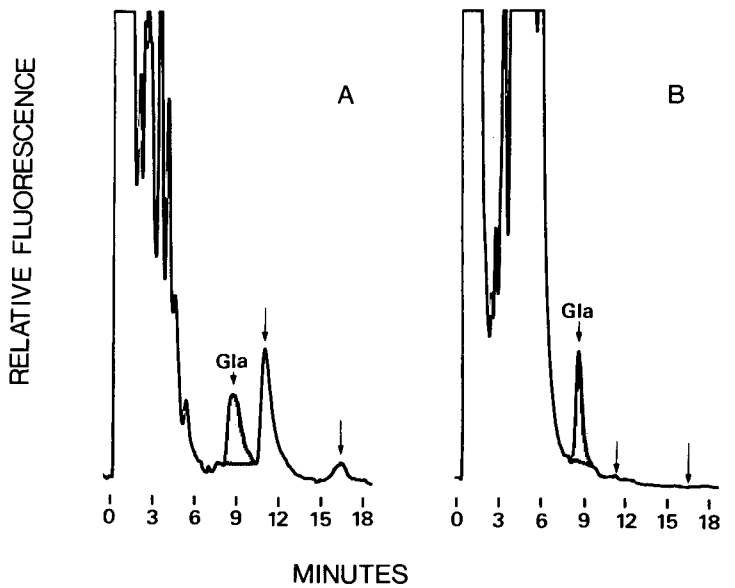


Fig. 3. Chromatograms of (A) human urine and (B) an alkaline hydrolyzate of the urine. The arrows represent peptide peaks which disappeared after the alkaline hydrolysis.

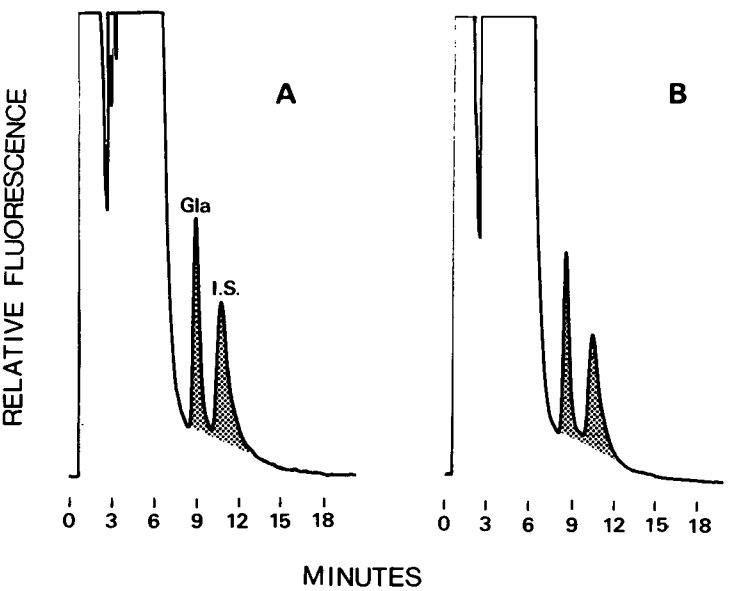


Fig. 4. Chromatograms of an alkaline hydrolyzate of human urine at (A) 5 min and (B) 13 h after fluorescence derivatization.

urinary Gla. This novel amino acid has been found so far only in djenkolic beans (*Pithecolobium lobatum*) and causes so-called djenkol poisoning when the beans are eaten [7]. As shown in Fig. 4, the internal standard was eluted 2 min later than Gla, and there is no interference of endogenous materials in the hydrolyzed urine with the internal standard peak, as described above. As shown in Fig. 5, the fluorescence of the OPA derivative of the internal standard

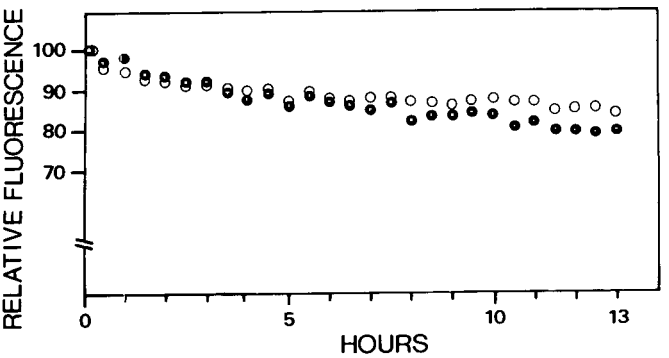


Fig. 5. Kinetics of the fluorescence decay of (•) derivatized Gla and (○) the internal standard in an alkaline hydrolyzate of human urine at room temperature. The urine sample was prepared as described in the text and analyzed by the standard HPLC method.

decayed slowly, at almost the same rate as that of Gla in a hydrolyzate of urine. During 13 h after the derivatization, the coefficient of variation of the peak area ratio of Gla/internal standard in the sample was found to be 4.5% (n = 27). These data indicate that this internal standard is suitable for the precise and reproducible analysis of urinary Gla using our HPLC method.

The present communication has demonstrated that a simple and specific procedure consisting of alkaline pretreatment and internal standardization facilitates the automated determination of urinary Gla by means of the HPLC method described previously [4]. This internal standardization method is also applicable in the analysis of Gla in protein samples and other biological tissues. We believe that the technique provides a useful tool in clinical and biochemical studies requiring the analysis of Gla.

ACKNOWLEDGEMENT

The authors thank Dr. M. Tomoeda of Eisai Company, for valuable assistance and discussions.

Department of Drug Metabolism and Pharmacokinetics, Tsukuba Research Laboratories, Eisai Company Ltd., Tokodai, Toyosato-machi, Tsukuba-gun, Ibaragi (Japan)

MANABU KUWADA*
KOUICHI KATAYAMA

1 J.W. Suttie (Editor), Vitamin K Metabolism and Vitamin K-Dependent Proteins, University Park Press, Baltimore, MD, 1979, p. 592.
2 D.V. Shah, J.K. Tews, A.E. Harper and J.W. Suttie, Biochim. Biophys. Acta, 539 (1978) 209.
3 J.B. Lian, L.M. Pachman, C.M. Gundberg and M.C. Maryjowski, Arthritis Rheum., 25 (1982) 1094.
4 M. Kuwada and K. Katayama, Anal. Biochem., 131 (1983) 173.
5 M. Kuwada and K. Katayama, Anal. Biochem., 117 (1981) 259.
6 P.V. Hauschka, Anal. Biochem., 80 (1977) 212.
7 A.G. van Veen and A.J. Hyman, Rec. Trav. Chim. Pays-Bas, 54 (1935) 493.

(First received September 7th, 1983; revised manuscript received January 6th, 1984)

Related articles published in *Journal of Chromatography*, Vols. 288—291

Volume 288

Volume 289

Author Index

Subject Index

Erratum

J. Chromatogr., 306 (1984) 257—268
Page 259, line 28, "Downers Grove, IL, U.S.A.)." should read "of [^{3}H]-isoproterenol sulphate".

Vol. 308

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

MEETINGS

THIRD INTERNATIONAL SYMPOSIUM ON THE BIOLOGICAL OXIDATION OF NITROGEN IN ORGANIC MOLECULES, LONDON, U.K., AUGUST 12–17, 1984

The above mentioned symposium will be held at Chelsea College, University of London, London, U.K.

Contributions are invited on the following topics:

Occurrence of N-oxygenated products – either naturally occurring or as metabolites of xenobiotics.

Analysis and characterisation of N-oxygenated products derived from biological systems.

Mechanism(s) and enzyme system(s) involved in the N-oxidation process.

Pharmacological and Toxicological consequences of the biological oxidation of nitrogen in organic molecules.

All enquiries to: Dr. J.W. Gorrod, Chelsea College, University of London, Manresa Road, London SW3 6LX, U.K.

CAPILLARY CHROMATOGRAPHY – 3rd INTERNATIONAL SYMPOSIUM, TARRYTOWN, NY, U.S.A., OCTOBER 22–23, 1984

The 3rd International Symposium on Capillary Chromatography will be held October 22 and 23, 1984, at the Westchester Marriott Hotel in Tarrytown, NY, U.S.A. The symposium will consist of invited and submitted papers on all aspects of capillary chromatography given by leading authorities from throughout the world. Informal discussions will permit the free exchange of ideas on various current questions related to these techniques and their applications. There will also be an exhibition of chromatography instrumentation. Abstracts of 200 words should be submitted by May 21, 1984, to: Dr. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A.

6th INTERNATIONAL SYMPOSIUM ON BIOAFFINITY CHROMATOGRAPHY AND RELATED TECHNIQUES, PRAGUE, CZECHOSLOVAKIA, SEPTEMBER 1–6, 1985

The above symposium is being sponsored by the Institute of Organic Chemistry and Biochemistry of the Czechoslovak Academy of Sciences and the Chromatography Section of the Czechoslovak Chemical Society. The symposium will focus upon the following topics:

1. Theory and recognition in bioaffinity chromatography
2. Solid supports and effect of coupling

3. Electromigration processes in bioaffinity techniques
4. Application of bioaffinity techniques in gene engineering
5. Biomedical application of bioaffinity chromatography
6. Reactive dyes in chromatographic separation and analysis
7. Techniques related to affinity chromatography
8. Large-scale applications of bioaffinity chromatography and related techniques

The official language of the symposium will be English. The papers presented at the symposium will be published in a special issue of the Journal of Chromatography, Biomedical Applications. During the symposium there will be held an exhibition of instruments and books on chromatography.

All inquiries concerning the symposium and the correspondence should be addressed to Dr. J. Turková, Institute of Organic Chemistry and Biochemistry ČSAV, Flemingovo n. 2, CS-166 10 Prague 6, Czechoslovakia. Tel.: (422) 324541, int. 080.

CALENDAR OF FORTHCOMING EVENTS

July 29–Aug. 3, 1984 Washington, DC, U.S.A.	**36th National Meeting of the American Association for Clinical Chemistry** Contact: American Association for Clinical Chemistry, 1725 "K" Street, NW, Washington, DC 20006, U.S.A.
Aug. 12–17, 1984 London, U.K.	**3rd International Symposium on the Biological Oxidation of Nitrogen in Organic Molecules** Contact: Dr. J.W. Gorrod, Chelsea College, University of London, Manresa Road, London SW3 6LX, U.K.
Aug. 21–24, 1984 Colombo, Sri Lanka	**Analytical Chemistry in Development** Contact: Centre for Analytical Research and Development, Department of Chemistry, University of Colombo, Colombo, Sri Lanka; or, Trace Analysis Research Centre, Chemistry Department, Dalhousie University, Halifax, N.S. B3H 4J1, Canada.
Aug. 26–31, 1984 Philadelphia, PA, U.S.A.	**188th National Meeting of the American Chemical Society** Contact: A.T. Winstead, American Chemical Society, 1155 16th Street, NW, Washington, DC 20036, U.S.A.
Aug. 26–Sept. 1, 1984 Cracow, Poland	**EUROANALYSIS V – 5th European Conference on Analytical Chemistry** Contact: Professor Zygmunt Kowalski, Secretary-General, Euroanalysis V, Academy of Mining and Metallurgy, Mickiewicza 30, 30-059 Kraków, Poland. (Further details published in Vol. 261, No. 3.)
Aug. 27–31, 1984 Göttingen, F.R.G.	**Electrophoresis 84. 4th International Meeting of the Electrophoresis Society** Contact: Dr. V. Neuhoff, Max-Planck-Institut für experimentelle Medizin, Hermann-Rein-Strasse 3, D-3400 Göttingen, F.R.G. Tel.: 0551-303248.
Sept. 2–6, 1984 Hradec Králové, Czechoslovakia	**4th International Symposium on Isotachophoresis – ITP 84** Contact: ITP 84, Dr. Z. Prusik, C.Sc., Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Flemingovo nám. 2, CS-166 10 Praha 6, Czechoslovakia. (Further details published in Vol. 272, No. 2.)
Sept. 3–6, 1984 Ghent, Belgium	**International Symposium on Quantitative Luminescence Spectrometry in Biomedical Sciences** Contact: Dr. W. Baeyens, Symposium Chairman, Laboratoria voor Farmaceutische Chemie en voor Ontleding van Geneesmiddelen, Rijksuniversiteit Gent, Harelbekestraat 72, B-9000 Ghent, Belgium. (Further details published in Vol. 305, No. 2.)

Sept. 10–14, 1984 Szeged, Hungary	**Advances in Liquid Chromatography (4th Annual American–Eastern European Symposium on Liquid Chromatography; and, International Symposium on Thin-Layer Chromatography with Special Emphasis on Overpressured Layer Chromatography)** Contact: Dr. Huba Kalász, Department of Pharmacology, Semmelweis University of Medicine, P.O. Box 370, Budapest 1445, Hungary; or, Dr. E. Tyihák, Research Institute for Plant Protection, P.O. Box 102, Budapest 1525, Hungary. (Further details published in Vol. 278, No. 2.)
Sept. 23–28, 1984 Philadelphia, PA, U.S.A.	**11th Annual Meeting of the Federation of Analytical Chemistry and Spectroscopy Societies** Contact: R.F. Hirsch, Division of Analytical Chemistry, American Chemical Society, 304 Beach Wood, Orange, NJ 07050, U.S.A.
Oct. 1–5, 1984 Nürnberg, F.R.G.	**15th International Symposium on Chromatography** Contact: Gesellschaft Deutscher Chemiker, Abteilung Fachgruppen, Postfach 90 04 40, Varrentrappstrasse 40–42, D-6000 Frankfurt (Main), F.R.G. (Further details published in Vol. 281.)
Oct. 22–23, 1984 Tarrytown, NY, U.S.A.	**3rd International Symposium on Capillary Chromatography** Contact: Dr. A. Zlatkis, Chemistry Department, University of Houston, TX 77004, U.S.A.
Oct. 24–26, 1984 Montreux, Switzerland	**3rd Workshop on LC–MS and MS–MS** Contact: Prof. Dr. R.W. Frei, Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. (Further details published in Vol. 276, No. 1.)
Nov. 13–16, 1984 New York, NY, U.S.A.	**23rd Annual Eastern Analytical Symposium** Contact: Dr. S. David Klein, EAS Publicity, Merck & Co., Inc., P.O. Box 2000/R80L-106, Rahway, NJ 07065, U.S.A. Tel.: (201) 846-1582.
Nov. 19–24, 1984 Barcelona, Spain	**EXPOQUIMIA 84 – Salón Internacional de la Quimica** Contact: EXPOQUIMIA, Feria de Barcelona, Barcelona 4, Spain.
Nov. 22–24, 1984 Barcelona, Spain	**14th Annual Symposium on Analytical Chemistry of Pollutants** Contact: 3rd International Congress on Analytical Techniques in Environmental Chemistry/EXPOQUIMIA, Av. Reina Ma. Christina, Palacio No. 1, Barcelona 4, Spain. Tel.: 223.31.01; telex: 50458 FOIMB-E.
Nov. 22–24, 1984 Barcelona, Spain	**3rd International Congress on Analytical Techniques in Environmental Chemistry** Contact: 3rd International Congress on Analytical Techniques in Environmental Chemistry/EXPOQUIMIA, Av. Reina Ma. Christina, Palacio No. 1, Barcelona 4, Spain. Tel: 223.31.01: Telex: 50458 FOIMB-E. (Further details published in Vol. 285, No. 1.)
Dec. 10–12, 1984 Baltimore, MD, U.S.A.	**4th International Symposium on HPLC of Proteins, Peptides, and Polynucleotides** Contact: Shirley E. Schlessinger, Symposium Manager, Fourth International Symposium on HPLC of Proteins, Peptides, and Polynucleotides, 400 East Randolph, Chicago, IL 60601, U.S.A. Tel.: (312) 527-2011. (Further details published in Vol. 285, No. 1.)
Feb. 25–March 1, 1985 New Orleans, LA, U.S.A.	**36th Pittsburgh Conference and Exposition on Analytical Chemistry and Applied Spectroscopy** Contact: Linda Briggs, Pittsburgh Conference, 437 Donald Road, Dept. J-005, Pittsburgh, PA 15235, U.S.A.
April 28–May 3, 1985 Miami Beach, FL, U.S.A.	**189th National Meeting of the American Chemical Society** Contact: Meetings Department, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.

July 1–5, 1985 Edinburgh, Scotland, U.K.	**9th International Symposium on Column Liquid Chromatography** Contact: J.H. Knox, Department of Chemistry, University of Edinburgh, Edinburgh EH9 3JJ, Scotland, U.K.
Sept. 1–6, 1985 Prague, Czechoslovakia	**6th International Symposium on Bioaffinity Chromatography and Related Techniques** Contact: Dr. J. Turková, Institute of Organic Chemistry and Biochemistry ČSAV, Flemingovo No. 2, CS-166 10, Prague 6, Czechoslovakia. Tel.: (422) 324541, int. 080.
Sept. 5–8, 1985 Birmingham, U.K.	**Flow Analysis III – An International Conference on Flow Analysis** Contact: Flow Analysis III, Dr. A.M.G. Macdonald, Department of Chemistry The University, P.O. Box 363, Birmingham B15 2TT, U.K. (Further details published in Vol. 288, No. 2.)
Sept. 8–13, 1985 Chicago, IL, U.S.A.	**190th National Meeting of the American Chemical Society** Contact: Meetings Department, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.
Sept. 9–13, 1985 Manchester, U.K.	**30th International Congress of Pure and Applied Chemistry** Contact: The Royal Society of Chemistry, Burlington House, London W1V 0BN, U.K.
Nov. 11–16, 1985 Yalta, U.S.S.R.	**5th Danube Symposium on Chromatography** Contact: Dr. L.N. Kolomiets, The Scientific Council of Chromatography, Academy of Sciences of the U.S.S.R., Institute of Physical Chemistry, Lenin-Prospect 31, Moscow 117312, U.S.S.R. (Further details published in Vol. 281.)

NEW BOOKS

Advances in chromatography, Vol. 23, edited by J.C. Giddings, E. Grushka, J. Cazes and P.R. Brown, Marcel Dekker, New York, Basel, 1984, XVIII + 249 pp., price SFr. 140.00, ISBN 0-8247-7075-7.

New approaches in liquid chromatography *(Proceedings of the 2nd Annual American–Eastern European Symposium on Advances in Liquid Chromatography, Szeged, June 16–18, 1982) (Analytical Chemistry Symposia Series,* Vol. 16), edited by H. Kalász, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, *ca.* 300 pp, price US$ 67.25 (U.S.A. & Canada), Dfl. 175.00 (rest of world), ISBN 0-444-99642-7.

Instrumental liquid chromatography – A practical manual on high-performance liquid chromatographic methods, by N.A. Parris, Elsevier, Amsterdam, Oxford, New York, Tokyo, 2nd, completely revised edition, 1984, XIV + 432 pp., price Dfl. 225.00, US$ 86.50, ISBN 0-444-42061-4.

Steric exclusion chromatography of polymers (Chromatographic Science Series, Vol. 25), edited by J. Janča, Marcel Dekker, New York, Basel, 1984, XVIII + 329 pp., price SFr. 153.00, ISBN 0-8247-7065-X.

HPLC analysis of biological compounds. A laboratory guide (Chromatographic Science Series, Vol. 26), by W.S. Hancock and J.T. Sparrow, Marcel Dekker, New York, Basel, 1984, X + 361 pp., price SFr. 110.00, ISBN 0-8247-7140-0.

Chemistry and biochemistry of the amino acids, edited by G.C. Barrett, Chapman & Hall, London, 1984, ca. 600 pp., price ca. £50.00, ISBN 0-412-23410-6.

Mycotoxins – Production, isolation, separation and purification, edited by V. Betina, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, VIII + 528 pp., price Dfl. 295.00, US$ 113.50, ISBN 0-444-42289-7.

Clinical experiences with Norcuron® (Org. NC 45, Vecuronium Bromide) *(Workshop, Geneva, April 21–22, 1983; Current Clinical Practice,* Vol. 11), edited by S. Agoston, W.C. Bowman, R.D. Miller and J. Viby-Mogensen, Excerpta Medica, Amsterdam, New York, 1983, 221 pp., price Dfl. 120.00, US$ 46.25, ISBN 0-444-90379-8.

PUBLICATION SCHEDULE FOR 1984

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	D 1983	J	F	M	A	M	J	J	A	
Journal of Chromatography	282	283 284/1	284/2 285/1	285/2 285/3 286 287/1	287/2 288/1 288/2 289	290 291 292/1	292/2 293 294	295/1 295/2 296	297	The publication schedule for further issues will be published later
Chromatographic Reviews		300/1					300/2			
Bibliography Section		304/1	304/2			304/3				
Biomedical Applications		305/1	305/2	306	307/1	307/2	308	309/1		

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 264, No. 3, pp. 491–494. A free reprint can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For review articles, see page 2 of cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography*. Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.,* Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Summary. Full-length papers and Review articles should have a summary of 50–100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet.* If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the layout of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts.* Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication".

Proofs. One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

Reprints. Fifty reprints of Full-length papers, Short communications and Notes will be supplied free of charge. Additional reprints can be ordered by the authors. An order form containing price quotations will be sent to the authors together with the proofs of their article.

Advertisements. Advertisement rates are available from the publisher on request. The Editors of the journal accept no responsibility for the contents of the advertisements.