ISSN 0378-4347

VOL. 310 NO. 2 OCTOBER 12, 1984

(Biomedical Applications, Vol. 35, No. 2)

THIS ISSUE COMPLETES VOL. 310

JOURNAL OF CHROMATOGRAPHY BIOMEDICAL APPLICATIONS

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 Submission of an article is understood to imply that the article is original and unpublished and is not being considered for publication elsewhere.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:

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)

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

VOL. 310, NO. 2 JOURNAL OF CHROMATOGRAPHY OCTOBER 12, 1984

(Biomedical Applications, Vol. 35, No. 2)

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)

DRUGS OF ABUSE

by LAWRENCE FISHBEIN, *National Center for Toxicological Research, Jefferson, AR, and University of Arkansas for Medical Sciences, Little Rock, AR, U.S.A.*

CHROMATOGRAPHY OF ENVIRONMENTAL HAZARDS VOLUME 4

The main objective of this volume is to provide the analytical and clinical chemist, as well as the forensic toxicologist, with a practical text which is also a literature reference source, of selected descriptive chromatographic procedures that stress the separation, detection and quantification of a spectrum of drugs of abuse, e.g. depressants, narcotics and analgesics, stimulants and hallucinogens, from biological, licit and illicit samples. The principal methods are thin-layer chromatography, gas-liquid chromatography, gas-liquid chromatography-mass spectrometry and high performance liquid chromatography. The procedures described are those which are primarily useful in therapeutic monitoring and pharmacokinetic studies, emergency, clinical, forensic and toxicological analyses and in monitoring in drug abuse screening programs.

Relevant information has been included, where available, regarding the synthesis or isolation from natural sources, the salient chemical, biological and physical properties, as well as the pharmacology, metabolism, pharmacokinetics, disposition and toxicity of the abused drugs. This information is important to investigators in diverse disciplines including biochemistry, clinical chemistry and medicine, pharmacology, toxicology, public health, and others such as clinicians and drug counselors who are all equally concerned with detecting, ameliorating, managing or eliminating the effects of this social and environmental hazard.

CONTENTS: Chapters 1. Introduction. **Part 1. Depressants (including Sedatives, Tranquillizers and Hypnotics).** 2. Alcohol. 3. Barbiturates. 4. Benzodiazepine Antianxiety Agents. 5. Phenothiazines. 6. Ethchlorvynol. 7. Carbamates and Ureides. 8. Antihistaminics. 9. Methaqualone. **Part. 2. Narcotics and Analgesics.** 10. Opium Alkaloids Morphine and Codeine. 11. Heroin. 12. Methadone and alpha-Acetylmethadol. 13. Salicylates. 14. Phenacetin and Acetaminophen. 15. Propoxyphene. 16. Meperidine. **Part 3. Stimulants.** 17. Amphetamine and Methamphetamine. 18. Cocaine. 19. Tricyclic Antidepressants. 20. Methylphenidate and Phenmetrazine. **Part. 4. Hallucinogenic Agents (including Psychotogenic, Psychotomimetic and Psychedelic Drugs).** 21. Marihuana, Cannabis Constituents and Hashish. 22. Psilocin, Psilocybin, Peyote and Mescaline. 23. LSD. 24. Mono- and Dimethoxyamphetamines and 3,4-Methylenedioxyamphetamine. 25. Phencyclidine. 26. Ketamine. **Subject Index.**

All chapters are followed by copious references.

1982 x + 490 pages
US $ 106.50/Dfl. 250.00
ISBN 0-444-42024-x

Chromatography of Environmental Hazards

Vol. 3
Pesticides

1975 xvi + 820 pages
US$ 134.00/Dfl. 315.00
ISBN 0-444-41158-5

"...very useful and the sheer amount of information between these covers is a monument to the energy and enthusiasm of the author".

Pans

Vol. 2
Metals, Gaseous and Industrial Pollutants

1973 xii + 637 pages
US $ 117.00/Dfl. 275.00
ISBN 0-444-41059-7

"The author has accomplished a monumental task in compiling so much data within a 600-page volume ... the book is a worthy addition to the library of the researcher actively involved in this very complex area of chemical analysis."

Journal of Chromatography

Vol. 1
Carcinogens, Mutagens and Teratogens
1972 1st Repr. 1983
vii + 500 pages
US $ 95.75/Dfl. 225.00
ISBN 0-444-40948-3

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 valid in USA and Canada only.

Journal of Chromatography, 310 (1984) 223—231
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2210

CRITICAL REVIEW

GAS CHROMATOGRAPHIC ANALYSIS OF AMINO ACIDS IN PHYSIOLOGICAL FLUIDS: A CRITIQUE

D. LABADARIOS*, I.M. MOODIE and G.S. SHEPHARD

Metabolic Unit, Tygerberg Hospital, Department of Internal Medicine, University of Stellenbosch, and the M.R.C. National Research Institute for Nutritional Diseases, P.O. Box 63, Tygerberg 7505 (Republic of South Africa)

(First received February 22nd, 1984; revised manuscript received April 27th, 1984)

CONTENTS

1. INTRODUCTION

Amino acids are fundamental units in the living organism and the extent to which each is present affects the healthy existence of that living species. Because amino acid levels have such far-reaching effects, the ability to determine these levels accurately and precisely assumes paramount importance in many biochemical and clinical investigations.

Until recently it has been a widely held and accepted view, as judged by the number of publications, that such analyses are best accomplished by the classical ion-exchange technique using an automatic amino acid analyser. Despite the high degree of sophistication attained, however, this method has the inherent disadvantages of requiring high initial capital outlay, high running costs, and of being capable of a very limited application, mainly that of amino

0378-4347/84/$03.00

acid analysis. Furthermore, the actual time for analysis of one sample is approximately 2 to 2½ h, which when added to the time required for sample preparation, is a major limiting factor in sample throughput.

These considerations have prompted various research groups to adopt the technique of amino acid analysis based on gas chromatography (GC), despite the added time required in the preparation of volatile amino acid derivatives. In general, capital and running costs of commercially available equipment are considerably lower, elution time is shorter, and the precision and sensitivity achieved using GC are an improvement on those of the amino acid analyser. Thus relative standard deviations for peak areas in amino acid standard mixtures are frequently quoted to be ± 2% at the 5—10 ng level using the analyser, whereas equivalent figures of below 1% are routinely possible using GC. The sensitivity threshold using spectrophotometric detection of amino acid—ninhydrin products is claimed to be 100 pmol (about 10 ng) and this can be extended to 10 pmol (about 1 ng) using fluorescence detection of *o*-phthaldehyde (OPA) amino acid derivatives. These and lower levels (10—100 pg) can be obtained using GC with electron-capture detection and the sensitivity can be further increased by coupling up to a mass spectrometer. This combination of high-resolution and identification capability cannot be equalled by any other technique at present. In addition the gas chromatograph need not necessarily be dedicated to amino acid analysis but has the capability of being used in a wide range of analyses.

GC analysis of amino acids requires the synthesis of their volatile derivatives. Whilst an alternative [1] to the more widely used esterification—acylation procedure for preparing these derivatives offers the potential advantage of permitting analysis of glutamine and asparagine, the preparative procedure contains sample manipulations which do not lend themselves to a laboratory analytical routine. In addition, there is a major disadvantage in that it requires a second short column for the complete elution of tryptophan, cystine and histidine. Thus, it would appear that the most practicable derivatisation procedure is that described by MacKenzie and Tenaschuk [2], modified by Moodie and co-workers [3, 4] and applied to a micro-scale analysis by Labadarios et al. [5]. However, before embarking upon derivatisation steps, biological samples require some form of purification procedure, the one most widely used being that of ion-exchange, in which biological material, in acid medium, is applied to a small column of cation-exchange resin (see Tables 1—3).

The increasing use of GC in amino acid analysis of physiological fluids has resulted in many publications appearing in the literature and it is this area of interest which will now be reviewed.

2. METHODS

A retrospective literature search was conducted using a direct link to the Medline Search system, the National Interactive Retrieval Service, based at the National Library of Medicine, Bethesda, MD, U.S.A.

By using the interacting search terms (1) amino acid, (2) gas chromatography and (3) analysis in blood, plasma, cerebrospinal fluid, urine or any body tissue, applied only to publication titles, the scope of the search was purposely kept

broad so as to have the best chance of including all relevant publications. The literature was scanned from the beginning of 1971 up to June 1983 (English, French and German language).

A total of 941 references derived from 135 journals were captured of which 36 were found to be relevant to the present exercise; that is they contained details of the attempted purification procedure of all protein amino acids from biological fluids, in order to reduce or eliminate interfering substances prior to quantitative GC determination.

3. RESULTS AND DISCUSSION

For convenience in this discussion, the procedure for determining amino acids in physiological fluids and tissue extracts (Tables 1—3) has been divided into four sections (A—D), each containing stages essential to optimal quantitative analysis. The percentage of the authors, appropriately referenced, who perform these steps is also shown in Tables 1—3.

TABLE 1

PERCENTAGE OF REFERENCED AUTHORS PERFORMING THE APPROPRIATE STEPS IN THE PURIFICATION PROCEDURE OF PLASMA/SERUM PRIOR TO GC ANALYSIS OF AMINO ACIDS

Procedure	Percentage of authors	References
A. Deproteinisation	81	13, 16, 18, 19, 20, 22, 25, 26, 29, 33, 34, 37, 40, 41, 44, 45, 46
B. Ion-exchange "clean-up" step	90	7, 13, 16, 18, 19, 20, 26, 29, 33, 34, 35, 37, 38, 39, 40, 41, 44, 45, 46
Details of:		
1. Resin type, column and resin bed size	52	7, 18, 29, 34, 35, 37, 38, 39, 40, 41, 46
2. Resin equilibration/ regeneration	43	7, 13, 16, 18, 38, 39, 40, 44, 45
3. Sample amount applied to resin	57	7, 13, 18, 20, 29, 34, 38, 39, 40, 41, 44, 45
4. Washing step	57	7, 13, 18, 26, 29, 34, 37, 38, 39, 40, 41, 44
5. Elution rate	33	7, 18, 29, 34, 38, 39, 40
6. Eluent volume	43	7, 18, 29, 34, 37, 38, 39, 40, 41
7. Eluate collected	24	13, 18, 26, 34, 35
C. Recoveries of amino acids:		
1. From column	29	13, 26, 38, 39, 41 46
2. From plasma	33	13, 18, 20, 25, 38, 39, 45
3. Amount used in recovery	43	13, 18, 20, 25, 26, 38, 39, 41, 46
4. Reproducibility of recovery	33	13, 18, 20, 25, 38, 39, 46
D. Reproducibility of derivatisation and chromatography	43	20, 25, 29, 38, 39, 41, 44, 45, 46

TABLE 2

PERCENTAGE OF REFERENCED AUTHORS PERFORMING THE APPROPRIATE STEPS IN THE PURIFICATION PROCEDURE OF URINE PRIOR TO GC ANALYSIS OF AMINO ACIDS

Procedure	Percentage of authors	References
A. Deproteinisation/reflux	67	12, 15, 16, 18, 19, 22, 26, 33, 34, 46
B. Ion-exchange "clean-up" step	93	7, 15, 16, 18, 19, 22, 23, 26, 33, 34, 36, 38, 39, 46
Details of:		
1. Resin type, column and resin bed size	60	7, 18, 22, 23, 26, 34, 38, 39, 46
2. Resin equilibration/regeneration	33	7, 16, 18, 38, 39
3. Sample amount applied to resin	60	7, 15, 18, 22, 23, 34, 36, 38, 39
4. Washing step	53	7, 18, 22, 23, 34, 36, 38, 39
5. Elution rate	40	7, 18, 23, 34, 38, 39
6. Eluent volume	53	7, 18, 22, 23, 34, 36, 38, 39
7. Eluate collected	27	15, 18, 26, 34
C. Recoveries of amino acids:		
1. From column	40	15, 22, 26, 38, 39, 46
2. From urine	27	15, 18, 38, 39
3. Amount used in recoveries	47	15, 18, 22, 26, 38, 39, 46
4. Reproducibility of recoveries	27	18, 38, 39, 46
D. Reproducibility of derivatisation and chromatography	40	12, 15, 23, 38, 39, 46

A careful study of these procedural steps, described in these cited publications, reveals a wide diversity both in reagents used and also in the degree of descriptive experimental detail.

A. Deproteinisation

Deproteinisation has been carried out using a variety of reagents, the most common by far being picric acid, followed by sulphosalicylic acid and trichloroacetic acid, both of which have been used to a similar extent. This is in line with the findings described by Ohara and Ariyoshi [6], who compared the performance of a number of plasma protein precipitating reagents. They conclude that (1) no single precipitant leads to good results for all amino acids, (2) picric acid gave the most reliable results except for the basic amino acids and (3) sulphosalicylic acid should be used when dealing with the latter. The problem of variable recoveries associated with the precipitation step has led various workers [7, 13, 38, 39] to adopt a procedure involving sample dilution with acetic acid rather than deproteinisation. Low yields of some plasma amino acid with this procedure made it unattractive, although the use of a reversed-

TABLE 3

PERCENTAGE OF REFERENCED AUTHORS PERFORMING THE APPROPRIATE STEPS IN THE PURIFICATION PROCEDURE OF CEREBROSPINAL FLUID, AMNIOTIC FLUID, TISSUES AND TISSUE EXTRACTS PRIOR TO GC ANALYSIS OF AMINO ACIDS

Procedure	Percentage of authors	References
A. Deproteinisation/hydrolysis	80	11, 14, 16, 17, 21, 24, 27, 28, 31, 32, 41, 42
B. Ion-exchange "clean-up" step	80	9, 11, 16, 17, 24, 27, 28, 30, 31, 32, 41, 42
Details of:		
1. Resin type, column and resin bed type	73	9, 11, 17, 24, 27, 28, 30, 31, 32, 41, 42,
2. Resin equilibration/regeneration	40	9, 11, 16, 24, 28, 32
3. Sample amount applied to resin	47	9, 27, 28, 30, 31, 41, 42
4. Washing step	53	9, 27, 28, 30, 31, 32, 41, 42
5. Elution rate	27	17, 24, 31, 32
6. Eluent volume	60	9, 11, 27, 28, 30, 31, 32, 41, 42
7. Eluate collected	47	9, 17, 24, 28, 30, 32, 42
C. Recoveries of amino acids:		
1. From column	13	9, 41
2. From tissue extract/fluid	7	31
3. Amount used in recovery	7	41
4. Reproducibility of recovery	7	9
D. Reproducibility of derivatisation and chromatography	33	14, 21, 31, 41, 43

flow technique has recently been claimed to greatly improve quantitative recoveries [7].

B. Ion-exchange clean-up

The use of a single cation-exchange step involving Dowex 50W resin was the most commonly encountered clean-up procedure (23 papers). The degree of cross-linking varied from X-2 to X-12 and the resin mesh sizes from 50—100 to 200—400. Both these parameters may be expected to impart different characteristics upon a resin and may further adversely influence quantitative analysis. The importance of standardisation in the type of resin used is substantiated by experimental work [7] showing the extent to which frequency of divinylbenzene cross-links in the ion-exchange polystyrene matrix influences the recovery of some basic amino acids from the resin bed. Some clearly have not recognised this important aspect and even omit to indicate details related to these parameters. Yet others, in addition to a single-stage clean-up, describe the use of both cation- and anion-exchange resins in a dual-step process.

Details in respect of the amount of resin and column dimensions used are widely divergent and do not appear to be related to the quantity of material subjected to clean-up. Furthermore, it is often not clear whether dimensions, when quoted at all, refer to a column containing the resin or to the resin bed itself. The most frequently used resin bed size varied from 5 to 10 cm in length and about 0.5 cm diameter to which deproteinised, centrifuge sample aliquots of 0.5—5 ml or even as large as 15 ml in the case of tissue extracts are applied. Moreover, columns as large as 15 × 1.5 cm diameter, and as small as 50 mg resin "in a pasteur pipette" are also described though the dimensions of the resultant resin bed are frequently not defined. It is noteworthy that the largest and smallest volumes of material for "clean-up" are not applied to the largest and smallest columns respectively.

Procedures for the preparatory washing and equilibration of resins also vary widely. For example, 7 *M* ammonia solution for a 3-h period followed by washing to neutral with water and regeneration with 3 *M* hydrochloric acid has been used on the one hand whereas other workers do not describe any special washing or equilibration steps.

The present data show that only just over one half of the publications indicate the sample volume or the deproteinised aliquot thereof which is applied to the ion-exchange clean-up column. Moreover, scant, if any, reference is made to the exchange capacity of a particular resin bed, surely another important parameter if quantitative recoveries are considered important.

Washing of unwanted material from the column whilst retaining amino acids on the resin is a step described again in about half the publications though the nature and quantity of washing reagent vary considerably and do not appear to relate to either resin bed or sample volume. It is not stated for instance, or even implied, that experiments have been conducted to establish the optimum column operating parameters.

Details of amino acid elution appear in approximately one third of all the papers. This is somewhat surprising as the final stage of recovering amino acids from the resin is reasonably expected to be the most important. Elution rates vary from 1 drop per 5—10 sec to 16 ml/min.

Most frequently ammonia solutions are used as an eluent, the concentration of which varies considerably (1—9 *M*). Only two publications, by the same group, refer to eluting with 1 *M* ammonia solution. The findings of Boila and Milligan [8], which are in agreement with our experience, that the use of ammonia at concentrations in excess of 2 *M* leads to increasingly variable recoveries of amino acids, must therefore throw considerable doubt upon results obtained from procedures using ammonia in excess of 2 *M*. Moreover, Cancalon and Klingman [9] have shown that major losses occur with methionine, phenylalanine, tyrosine and tryptophan and James [10] has reported major losses of arginine not only during the ion-exchange "clean-up" but also during a simple evaporation of a mixture of arginine in ammonia solution even at a concentration of 1 *M*.

C. Recovery of amino acids

It was expected that laboratories would test the recovery of amino acids

from an ion-exchange column by adding known quantities of standards to the column. Comparison of the eluent composition with that of the original standard would give a measure of column recovery efficiency. Moreover, "spiking" of the original sample with a known quantity of standards and passing it through the column would, by comparison with a similarly treated "unspiked" sample, permit an assessment of the recovery of amino acids from the sample. It was surprising to find that, overall, less than half of the publications carried any account of recovery or of analytical precision.

D. Reproducibility of derivatisation

It was also expected that laboratories would report on the reproducibility of the recoveries together with an assessment of the precision of their derivatisation and chromatographic steps. This is considered to be a fundamentally important omission, in view of the importance of sample preparation and its role in accurate quantitative analysis.

4. CONCLUSION

It is concluded that, despite the important advantages offered by GC in amino acid analysis, the existing sample purification procedure by ion-exchange of biological fluids and tissue extracts is far less than optimal. There appears to be an urgent need for standardisation of the steps involved in such purification, especially in view of the increasing interest in amino acid determination in a variety of scientific disciplines and clinical investigations in particular. The impression should not, however, be gained that amino acid analysis is more precise when performed by amino acid analysers, as recent evidence [47] indicates that even in this area "there is clearly room for improvement". Presumably the authors are referring to their collaborative study of amino acid analysis in which a statistical evaluation, involving the interaction of laboratories, sample preparations and determinations was performed. Coefficient of variation for a given laboratory performing a single analysis on a single preparation varied from 5.6% to 151.2%. In this respect the cited conclusion of Gerritsen and Niederwieser [48], that the accuracy of determination of better than 3% claimed for commercial amino acid analysers is "utopian", may be considered more generally appropriate.

5. SUMMARY

The available methodology for sample purification prior to gas chromatographic analysis of amino acids in physiological fluids and tissue extracts is analysed. It would appear that over the past ten years the method of choice is that of an ion-exchange purification step, and little, if any, progress has been achieved in sample purification procedures. The inherent disadvantages of such a methodology are not only perpetuated but also cast some considerable doubt on the accuracy of the quantitative analysis of amino acids.

REFERENCES

1 P. Hušek, J. Chromatogr., 234 (1982) 381.
2 S.L. MacKenzie and D. Tenaschuk, J. Chromatogr., 97 (1974) 19.
3 I.M. Moodie and J.A. Burger, in R.E. Kaiser (Editor), 4th Int. Symp. Capillary Chromatography, Hindelang, May 3—7, 1981, Hüthig, Bad Dürkheim, 1981, p. 523.
4 I.M. Moodie, G.S. Collier, J.A. Burger and B.C. Werb, J. Sci. Food Agric., 33 (1982) 345.
5 D. Labadarios, G.S. Shephard, I.M. Moodie and E. Botha, S. Afr. J. Sci., 80 (1984) 240.
6 I. Ohara and S. Ariyoshi, Agric. Biol. Chem., 43 (1979) 1473.
7 P. Hušek, G. Herzogová and V. Felt, J. Chromatogr., 236 (1982) 493.
8 R.J. Boila and L.P. Milligan, J. Chromatogr., 202 (1980) 283.
9 P. Cancalon and J.D. Klingman, J. Chromatogr. Sci., 12 (1974) 349.
10 L.B. James, J. Chromatogr., 166 (1978) 333.
11 J.D. Schulman, J.T. Queenan and L. Doores, Amer. J. Obstet. Gynecol., 114 (1972) 243.
12 T.W. Larsen and R.F. Thornton, Anal. Biochem., 109 (1980) 137.
13 E.D. Pellizzari, C. Rising, J.H. Brown, R.W. Farmer and L.F. Fabre, Anal. Biochem., 44 (1971) 312.
14 N. Lachovitzki and B. Bjǒrklund, Anal. Biochem., 38 (1970) 446.
15 R.F. McGregor, G.M. Brittin and M.S. Sharon, Clin. Chim. Acta, 48 (1973) 65.
16 H.N. Tucker and S.V. Molinary, Clin. Chem., 19 (1973) 1040.
17 M. Butler, A. Darbre and H.R.V. Arnstein, Biochem. J., 150 (1975) 419.
18 R.W. Zumwalt, D. Roach and C.W. Gehrke, J. Chromatogr., 53 (1970) 171.
19 R.W. Zumwalt, K.C. Kuo and C.W. Gehrke, J. Chromatogr., 55 (1971) 267.
20 E.D. Pellizzari, J.H. Brown, P. Talbot, R.W. Farmer and L.F. Fabre, Jr., J. Chromatogr., 55 (1971) 281.
21 M.S. Greaves and J.M.H. Moll, Clin. Chem. 22 (1976) 1608.
22 K.M. Williams and B. Halpern, Aust. J. Biol. Sci., 26 (1973) 831.
23 J. Chauhan, A. Darbre and R.F. Carlyle, J. Chromatogr., 227 (1982) 305.
24 J. Gabrýs and J. Koneckie, J. Chromatogr., 182 (1980) 147.
25 S. Yamamoto, S. Kiyama, Y. Watanabe and M. Makita, J. Chromatogr., 233 (1982) 39.
26 A. Doussin and R. Bourdon, Ann. Biol. Clin., 30 (1972) 417.
27 T.P. Stein, G.P. Buzby, M.J. Leskin and J.L. Mullen, Cancer, 49 (1982) 1476.
28 Y. Okano, M. Kataoka, T. Miyata, H. Marimoto, K. Takahama, T. Hitoshi, Y. Kase, I. Matsumoto and T. Shinka, Anal. Biochem., 117 (1981) 196.
29 H. Frank, A. Rettenmeier, H. Welcker, G.J. Nicholson and E. Bayer, Clin. Chim. Acta, 105 (1980) 201.
30 K.F. Faull, J.R. DoAmaral, P.A. Berger and J.D. Barchas, J. Neurochem., 31 (1978) 1119.
31 W.P. Leighton, S. Rosenblatt and J.D. Chanley, J. Chromatogr., 164 (1979) 427.
32 J. Gabrýs and J. Koneckie, J. Chromatogr., 222 (1981) 345.
33 F.S. Collins and G.K. Summer, J. Chromatogr., 145 (1978) 456.
34 J. Desgres, D. Boisson and P. Padieu, J. Chromatogr., 162 (1979) 133.
35 J. Sanchez and L. Enjuanes, Oncology, 25 (1971) 44.
36 P.J. Finlayson, R.K. Christopher and A.M. Duffield, Biomed. Mass Spectrom., 7 (1980) 450.
37 E.E. Kingston and A.M. Duffield, Biomed. Mass Spectrom., 5 (1978) 621.
38 R.F. Adams, J. Chromatogr., 95 (1974) 189.
39 R.F. Adams, F.L. Vandermark and G.J. Schmidt, J. Chromatogr. Sci., 15 (1977) 63.
40 F.E. Kaiser, C.W. Gehrke, R.W. Zumwalt and K.C. Kuo, J. Chromatogr., 94 (1974) 113.
41 A.M. Lewis, C. Waterhouse and L.S. Jacobs, Clin. Chem., 26 (1980) 271.
42 T. Tepper, G.K. van der Hem, G.J. Tuma, L. Arisz and A.J.M. Donker, Clin. Nephrol., 10 (1978) 16.
43 M. Wolfensberger and U. Amsler, J. Neurochem., 38 (1982) 451.
44 H. Frank, A. Rettenmeier, H. Welcker, G.J. Nicholson and E. Bayer, Anal. Chem., 54 (1982) 715.

45 D.E. Matthews, E. Ben-Galim and D.M. Bier, Anal. Chem., 51 (1979) 80.
46 C.W. Gehrke, R.W. Zumwalt and K.C. Kuo, J. Agric. Food Chem., 19 (1971) 605.
47 A.P. Williams, D. Hewitt, J.E. Cockburn, D.A. Harris, R.A. Moore and M.G. Davis, J. Sci. Food Agric., 31 (1980) 474.
48 T. Gerritsen and A. Niederwieser, in H.E. Curtius and M. Roth (Editors), Clinical Biochemistry, Principles and Methods, Vol. 2, De Gruyter, Berlin, New York, 1974, p. 1062.

Journal of Chromatography, 310 (1984) 233—242
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2212

GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC PROFILING WITH NEGATIVE-ION CHEMICAL IONIZATION DETECTION OF PROSTAGLANDINS AND THEIR 15-KETO AND 15-KETO-13,14-DIHYDRO CATABOLITES IN RAT BLOOD

C.R. PACE-ASCIAK* and S. MICALLEF

Research Institute, The Hospital for Sick Children, 555 University Avenue, Toronto M5G 1X8 (Canada) and Department of Pharmacology, University of Toronto (Canada)

(First received February 22nd, 1984; revised manuscript received May 7th, 1984)

SUMMARY

A method was set up in which the primary prostaglandins ($PGF_{2\alpha}$, PGD_2, PGE_2, thromboxane B_2, 6-keto-$PGF_{1\alpha}$ and 6-keto-PGE_1) and their catabolites (15-keto and 15-keto-13,14-dihydro) could be analyzed in the same sample at the same time. The method makes use of long capillary columns (60 m) to resolve the complex mixture during gas chromatography and mass fragmentography to provide the specificity of detection of these products. Selectivity and sensitivity is provided through use of appropriate derivatives (pentafluorobenzyl esters) which allow detection by negative-ion chemical ionization in which high-abundance fragments in the high end of the mass spectrum (M-pentafluorobenzyl) are observed. A purification procedure of whole blood is described involving diethyl ether extraction, C_{18} Sep-Pak chromatography, derivatization into the pentafluorobenzyl—O-methyloxime, C_{18} Sep-Pak and silicic acid chromatography followed by final derivatization into trimethylsilyl ethers for gas chromatographic—mass spectrometric analysis. Recovery of added [^{3}H]$PGF_{2\alpha}$ was 73.8 ± 2.2% (n=10). Sample workup and analysis takes ten days for six samples. The method is sufficiently sensitive for the profiling of a 10-ml sample of whole blood (limit approximately 1 pg/ml; 1-pg injection on column).

INTRODUCTION

Methods for the profiling of all prostaglandins (PGs) in the presence of their catabolites are mostly lacking. It is evident that neither radioimmunoassay nor bioassay are appropriate since the former would entail analysis of each product separately through use of specific antibodies rendering such a method expensive and cumbersome [1—3]; bioassay is also inappropriate as most prostaglandin catabolites lack biological activity [4—7]. Clearly, the problem

0378-4347/84/$03.00

of measuring each product in such a mixture of closely related substances is ideally suited to mass spectrometry (MS) after appropriate resolution of the mixture is performed first by gas chromatography (GC) on long capillary columns. The GC—MS system described in this paper used in the negative-ion chemical ionization (NICI) mode for enhanced sensitivity provides the much needed analytical means to achieve this goal. We describe a method capable of simultaneous measurement of a mixture of the six primary prostaglandins and their 15-keto and 15-keto-13,14-dihydro catabolites (total of eighteen products plus internal standard). Application of this method to the profiling of these products in normal rat blood is also described.

MATERIALS AND METHODS

[9-^{3}H]$PGF_{2\alpha}$ of high specific activity (i.e. 13.7 Ci/nmol) was purchased from New England Nuclear. All prostaglandin standards used in this study were kindly supplied by Dr. J.E. Pike, Upjohn (Kalamazoo, MI, U.S.A.). The 15-keto and 15-keto-13,14-dihydro catabolites of 6-keto-PGE_1 were prepared in our laboratory [8]. Male adult Wistar rats (300 g) were purchased from Camm Research Laboratory Animals (Wayne, NJ, U.S.A.). All solvents were HPLC grade glass-distilled as supplied by Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Sample workup

Arterial blood (10 ml) was withdrawn from lightly anaesthetized (diethyl ether) rats by cardiac puncture into plastic syringes containing 1 ml sodium citrate (3.8%). After gentle mixing, the blood was transferred into glass centrifuge tubes containing 50 ml ethanol. The samples were mixed thoroughly, centrifuged at 1000 *g* for 15 min and 20 ng C22-$PGF_{2\alpha}$ as internal standard and 120,000 cpm [^{3}H]$PGF_{2\alpha}$ (3.6 ng) were added. The ethanol supernatant was evaporated to complete dryness in vacuo. The residue was washed with dry ethanol and the ethanol-soluble fraction was transferred and evaporated again in vacuo. This procedure was repeated several times. The final residue after evaporation of the ethanol was extracted with diethyl ether and acidified (pH 3) water in centrifuge tubes. After neutral washing, the diethyl ether layer was evaporated to dryness and the residue was taken up in 10 ml water—methanol—acetic acid (80:20:0.4, v/v/v) and passed through a C_{18} Sep-Pak cartridge (Waters) prewashed with methanol and water. The Sep-Pak was eluted with water—methanol—acetic acid mixtures of increasing amounts of methanol, i.e. 70:30:0.4 (10 ml), 35:65:0.4 (10 ml) and finally pure methanol (10 ml); the mixture of prostaglandins and their 15-keto and 15-keto-13,14-dihydro catabolites were eluted with water—methanol—acetic acid (35:65:0.4, v/v/v). This fraction was taken to complete dryness in vacuo and derivatized by a modification of the method of Wickrema Sinha et al. [9] into the pentafluorobenzyl (PFB) esters as follows: the sample was taken up in 10 μl methanol and 50 μl acetonitrile, then 2 μl pentafluorobenzyl bromide (Pierce) and 1.5 μl diisopropylethylamine (Aldrich) were added, and the sample was heated at 60°C for 5 min. The solvents were evaporated with a fine stream of nitrogen and the residue was dissolved in 25 μl methoxylamine hydrochloride in pyridine (MOX reagent; Pierce). After standing overnight at room tempera-

ture, the solvent was evaporated with nitrogen and the residue was extracted with 200 μl diethyl ether and 50 μl water. The diethyl ether extract was dried and the residue was dissolved in 10 ml water—methanol (1:1, v/v). The sample was placed on a C_{18} Sep-Pak cartridge prewashed with methanol, water and the same solvent mixture. The prostaglandin mixture was subsequently eluted with water—methanol (15:85, v/v). This fraction was taken to complete dryness in vacuo and the residue was dissolved in benzene and placed on a small Pasteur pipette column filled with a slurry of silicic acid (HA minus 325 mesh, Bio-Rad Labs.) in benzene. After washing the column with benzene, the prostaglandin mixture was eluted with chloroform—methanol (9:1, v/v). This fraction was taken to dryness, transferred to a micro vial (400-μl volume) and 25 μl TRI SIL Z (Pierce) was added. After 5 min at 60°C, the solvent was evaporated with a fine stream of nitrogen and 50 μl ice cold hexane and 20 μl ice cold water were added. The sample was rapidly mixed and the water was frozen by immersing the sample in a dry ice—methanol bath. The sample was stored at −20°C until analysis. Recovery of [^{3}H]$PGF_{2\alpha}$ throughout the procedure was 73.8 ± 2.2% (n=10) (see Fig. 1).

Citrated blood sample or tissue extract	
↓	
Addition of labelled $PGF_{2\alpha}$ (120,000 cpm) and C22-$PGF_{2\alpha}$ standard (20 ng)	Recovery (%) of ^{3}H-$PGF_{2\alpha}$ (n=10)
↓	
1. 5 vols. ethanol	
2. Diethyl ether–H^+ extraction	99.3 ± 3.4
3. C_{18} Sep-Pak, water–methanol–acetic acid (35:65:0.4, v/v/v) fraction	97.8 ± 2.8
4. Derivatization a. Pentafluorobenzyl b. O-Methyloxime	
5. Diethyl ether–water extraction	90.4 ± 1.9
6. C_{18} Sep-Pak, water–methanol (15:85, v/v) fraction	75.3 ± 3.2
7. Silicic acid, chloroform–methanol (9:1, v/v) fraction	73.8 ± 2.2
8. Derivatization a. Trimethylsilyl	
9. Assay by GC–ECD and GC–MS	

Fig. 1. Brief procedure for the purification of a mixture of the primary PGs and their catabolites indicating recoveries of added labelled $PGF_{2\alpha}$.

Instrumentation

Sample analysis was performed by two methods, GC with electron-capture detection (GC—ECD) and GC—MS in the NICI mode making use of single-ion monitoring (SIM) to increase the sensitivity and selectivity of the products under measurement. Both methods employed 60-m capillary columns of fused silica (DB-1, J. & W. Scientific) and hydrogen as carrier gas. The GC—ECD system had a make-up flow of argon—methane (98.35:1.65) while in GC—MS the NICI was operated with 100% methane as reactant gas. The carrier gas was passed through moisture and oxygen traps while detector gases were passed through moisture traps only. The GC conditions were: column

injection temperature 100°C maintained for 1 min, then programmed to 270°C at 30°C/min. This temperature was held for the rest of the run. Samples were injected through a Hewlett-Packard splitless injector into the GC—ECD system (HP 5700 Series) or the GC—MS system (HP 5987A Series). Inlet temperature was maintained at 300°C; detector temperature in GC—ECD was 350°C, in GC—MS 300°C. Source temperature in GC—MS was kept at 150°C. SIM was performed at 3000 electron multiplier voltage.

RESULTS

A sample purification scheme was devised which would permit purification of a mixture of known prostaglandins and their 15-keto and 15-keto-13,14-dihydro catabolites as a group from contaminating substances in blood or other biological fluids. The efficient use of C_{18} Sep-Pak cartridges was explored for this purpose. A procedural scheme was initially devised through use of products with polarity at the extreme end of the mixture of products to be analyzed, i.e. 15-keto-13,14-dihydro-PGE_2 as the least polar product and -$PGF_{2\alpha}$ as the most polar product. The procedure described in Fig. 1 satisfied our requirements for sample cleanliness and good recoveries. To test this scheme, a mixture of authentic prostaglandins and their catabolites (20 ng each) was passed through this purification process and assayed by GC—ECD. All products were recovered in a similar proportion to that in which they were made up (not shown) indicating that the "windows" chosen for each chromatographic step gave good recoveries of the least polar and most polar products of the group of PGs and PG catabolites.

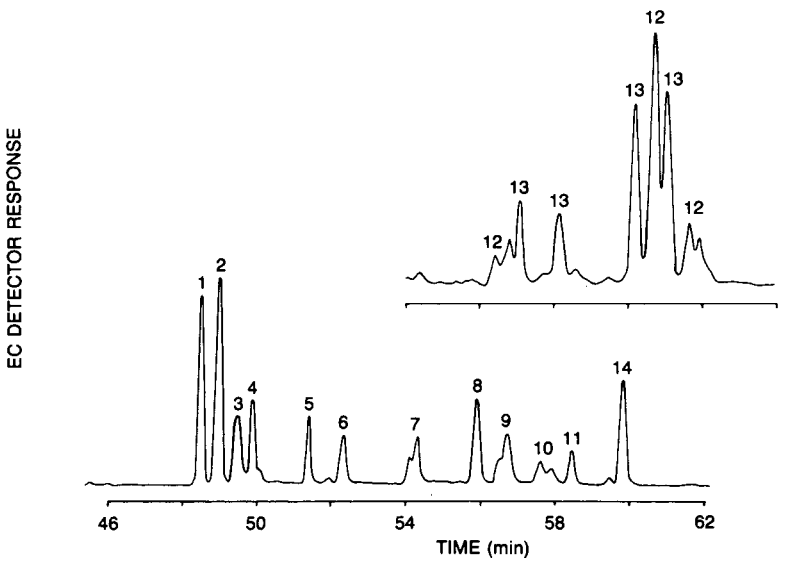


Fig. 2. GC—ECD profiles showing resolution of a standard mixture of primary PGs and their 15-keto and 15-keto-13,14-dihydro catabolites as pentafluorobenzyl—O-methyloxime—trimethylsilyl (PFB—MO—TMS) derivatives on a 60-m fused silica column of DB-1. Hydrogen was used as carrier gas (see Materials and methods for details). The retention times on the axis for the top chromatogram are the same as those for the bottom. For peak identification see Table I.

Fig. 2 shows the type of chromatographic resolution observed on a 60-m capillary column of DB-1 routinely used in our assay. Composite data are shown for GC—ECD analysis to show that the catabolites are easily resolved from the primary PGs making such PG profiling possible. Further specificity of this assay is derived through use of GC—MS for the detection and quantita-

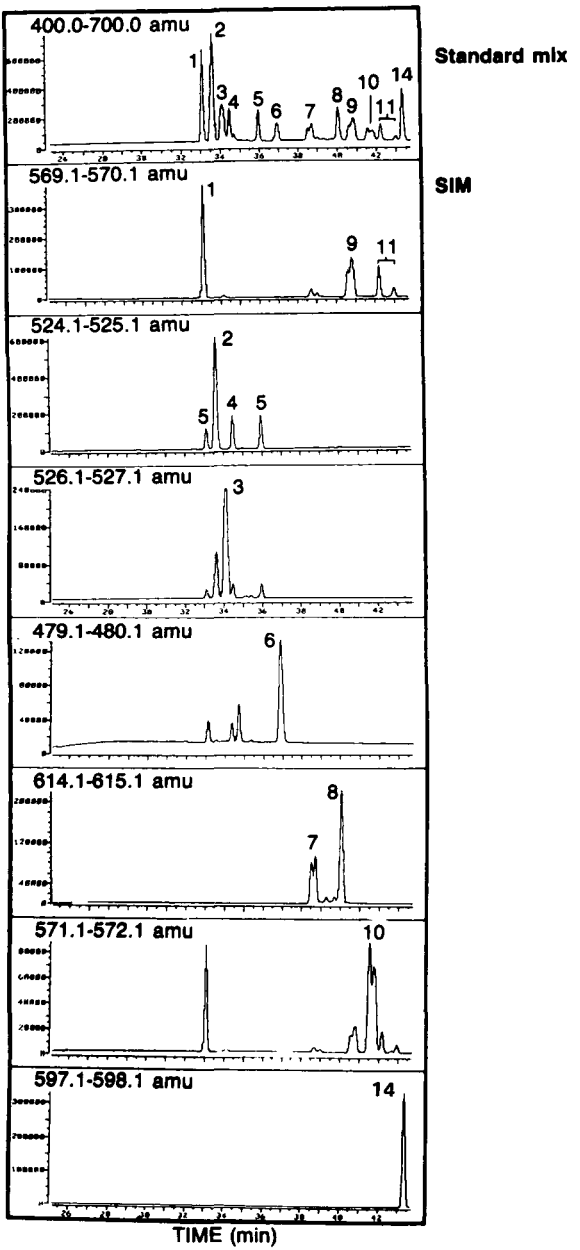


Fig. 3. GC—MS—SIM profiles showing analysis by NICI of a mixture of primary PGs and their catabolites as PFB—MO—TMS derivatives under GC conditions similar to those employed in Fig. 2. For peak identification see Table I.

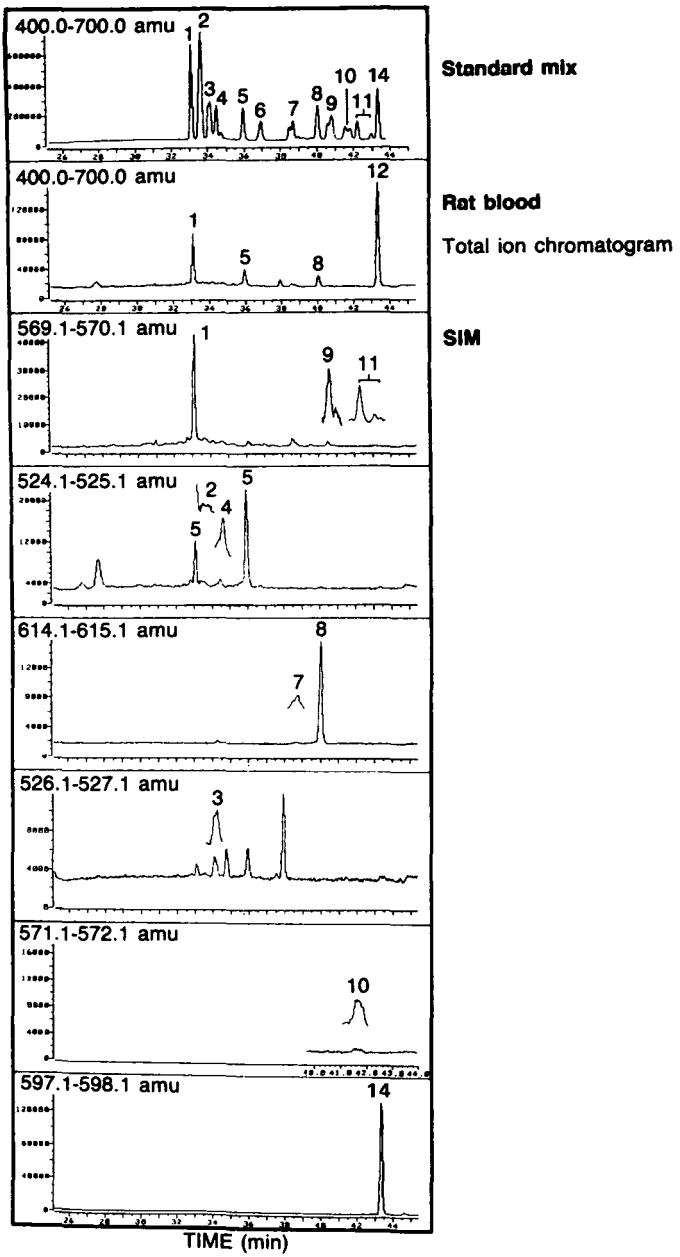


Fig. 4. Application of GC—MS—SIM—NICI to the profiling of PGs and catabolites in rat whole blood. Conditions employed were similar to those in Fig. 3. For peak identification see Table I.

tion of each product. Using NICI detection wherein the mass spectrum of each product is dominated by a single mass peak of high abundance (>50%) due to the loss of the PFB group (M-181) [10], a high degree of specificity and sensitivity is obtained (Fig. 3). Undulations in baseline through minor contamination peaks in tissue extracts are often observed with non-specific assays

such as GC—ECD. The use of specific assays such as the GC—MS assay with SIM, described in this paper, eliminates to a large extent these interferences. The selectivity of GC—MS in the SIM mode with NICI detection is also of benefit in the separation of overlapping peaks. This can be seen by comparison of the $PGF_{2\alpha}$ ion chromatogram (which shows absence of PGE_2) with the PGE_2 ion chromatogram which shows an isomer of PGE_2 overlapping with $PGF_{2\alpha}$ (Fig. 3). Because *m/z* 569 is specific for $PGF_{2\alpha}$ (it is not present in the mass spectrum of PGE_2) and *m/z* 524 is specific for PGE_2 in that it is absent from that of $PGF_{2\alpha}$, a complete resolution of the two products (i.e. one isomer of PGE_2 with $PGF_{2\alpha}$) is obtained despite identical retention times on GC. In a similar fashion, several of the isomers of the 15-keto and 15-keto-13,14-dihydro catabolites of 6-keto-PGE_1 which overlap (see Fig. 2) with 6-keto-$PGF_{1\alpha}$ and its 15-keto and 15-keto-13,14-dihydro catabolites can be completely resolved by SIM with NICI detection since the catabolites of 6-keto-PGE_1 contain mass spectral fragments at *m/z* 524 and *m/z* 526 in contrast to 6-keto-$PGF_{1\alpha}$ (*m/z* 614), 15-keto-6-keto-$PGF_{1\alpha}$ (*m/z* 569) and 15-keto-13,14-dihydro-6-keto-$PGF_{1\alpha}$ (*m/z* 571). SIM profiles to demonstrate this have not been included here to avoid further cluttering of the data presented in Fig. 3.

Application of this method to PG profiling analysis in normal rat whole blood is shown in Fig. 4. Selected ion chromatograms are shown identifying most products searched for as listed in Table I. In most cases, clearly distinct peaks are observed for each product at the identical retention time as authentic standard.

Fig. 5 shows data from the analysis of four samples of rat whole blood. The low levels of thromboxane B_2 indicate the lack of clotting during with-

TABLE I

MAJOR MASS FRAGMENTS OBSERVED IN THE MASS SPECTRA OF PGs AND PG CATABOLITES AS PENTAFLUOROBENZYL—O-METHYLOXIME—TRIMETHYLSILYL DERIVATIVES USING NICI (METHANE REACTANT GAS) DETECTION

Compound	M-181 fragment (*m/z*)	Retention time* (min)
1. $PGF_{2\alpha}$	569	33.1
2. 15-Keto-$PGF_{2\alpha}$	524	33.6
3. 15-Keto-13,14-dihydro-$PGF_{2\alpha}$	526	34.1
4. PGD_2	524	34.4
5. PGE_2	524	35.9
6. 15-Keto-13,14-dihydro-PGE_2	479	36.9
7. Thromboxane B_2	614	38.5
8. 6-Keto-$PGF_{1\alpha}$	614	40.0
9. 15-Keto-6-keto-$PGF_{1\alpha}$	569	40.6
10. 15-Keto-13,14-dihydro-6-keto-$PGF_{1\alpha}$	571	41.7
11. 6-Keto-PGE_1	569	42.2
12. 15-Keto-6-keto-PGE_1	524	43.8**
13. 15-Keto-13,14-dihydro-6-keto-PGE_1	526	43.3, 44.1**
14. C22-$PGF_{2\alpha}$ (internal standard)	597	43.4

*60-m DB-1 capillary column (see Materials and methods for details).

**Retention times of major peaks.

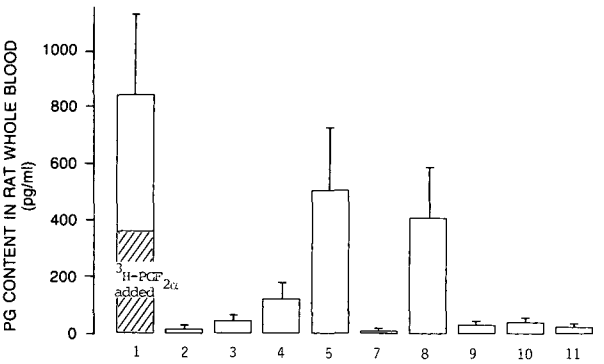


Fig. 5. Quantitative data derived by GC—MS—SIM—NICI of PGs and catabolites in whole blood samples from four rats. Values represent the mean ± S.E.M. For compound identification see Table I.

drawal of blood in citrate. We have found considerable thromboxane B_2 in venous blood from man or rat arterial blood collected in 77 m*M* EDTA (not shown). It is interesting to note that the method is so sensitive that the [^{3}H]$PGF_{2\alpha}$ added for recovery purposes (120,000 cpm = 3.6 ng) is easily detectable. Using authentic $PGF_{2\alpha}$, we have observed that detection of 10 pg injected into the GC—MS instrument is easily possible.

DISCUSSION

We report a method whereby a mixture of prostaglandins and their primary catabolites, i.e. 15-keto and 15-keto-13,14-dihydro, can be purified as a group from a biological sample with subsequent analysis as a group by GC—MS. Analysis is possible through use of a 60-m capillary column where complete resolution of the products can be attained. We have chosen whole blood because we felt that this is most relevant to in vivo studies (physiologic, pharmacologic and pathologic stresses) and because this would provide a stringent test on our methods of purification since samples would have to be quite clean before injection on capillary columns can be made.

We have chosen as derivatives the PFB esters instead of methyl esters because (1) this permits an initial check of samples on a gas chromatograph in our own laboratory equipped with an electron-capture detector prior to analysis by GC—MS and (2) because PFB esters provide exceedingly simple fragmentation patterns (M-PFB represents over 50% of the total abundance) in NICI—MS [10] making these derivatives highly suitable for attaining a high degree of sensitivity when the GC—MS is used in the SIM mode. The additional benefit of the use of PFB esters is that all products containing a carboxyl group are detectable permitting analysis not only of known PGs and their known catabolites but also of hitherto unknown or uncharacterized products. For example, the method is highly suited to prostaglandin catabolism studies since the precursor and all of its products contain a carboxyl group making them visible by ECD with subsequent analysis by NICI—GC—MS. We have recently applied this method of analysis to the study of the catabolism of 6-keto-PGE_1 by rat kidney homogenates and to the analysis of rat blood for this PG and its 15-keto and 15-keto-13,14-dihydro catabolites [8].

The use of PFB esters or oximes in PG analysis by GC—ECD has been reported previously [11] although this method has not found much use because of limitations in sensitivity and selectivity in that mode of analysis. A previous report [12] has shown the suitability of GC—MS in the electron impact (EI) mode for the analysis on 'short' (21 m) columns of the primary prostaglandins of the "2" series despite the shortcomings of the EI mode of analysis in terms of sensitivity. Recently, Blair et al. [10] have shown that PFB esters have excellent properties in GC—MS especially in the NICI mode due to the facile loss of the PFB group. We have applied this method through use of long (60 m) capillary columns to the analysis of all PGs of the "2" series in the presence of their primary catabolites (15-keto and 15-keto-13,14-dihydro). We know of no other method presently employed in the prostaglandin area which allows the analysis of all primary PGs and their catabolites in a single sample. The benefits of profiling methods described in this paper are obvious since information on all products is derived from a single analysis. It is also obvious that analysis of all these products at the same time is required if the effects of specific PG synthesis or catabolism inhibitors are to be assessed in vivo, or in the investigation of drugs which divert the PG biosynthetic pathway into one or other PGs or in pathological conditions where one PG may be formed at the expense of others.

Although use of an internal standard, C22-$PGF_{2\alpha}$, as described in this study for the quantification of PGs has its inherent limitations, this is unavoidable at present because appropriate deuterium-labelled products as internal standards are not commercially available. The purification method employs a "window" wide enough to retain all products including C22-$PGF_{2\alpha}$ for simultaneous analysis by GC—MS.

The sensitivity of the method described here makes it suitable for the quantitation of PGs in whole blood. Although in this study we have used 10 ml of citrated blood, workup of lesser amounts of blood is possible for $PGF_{2\alpha}$, PGD_2, PGE_2 and 6-keto-$PGF_{1\alpha}$ and some of their catabolites. Further studies are in progress to determine the effect of physiologic stresses on levels of this group of PGs in vivo.

ACKNOWLEDGEMENTS

This study was supported by a grant to C.R. Pace-Asciak from the Medical Research Council of Canada (MT-4181). We thank Mr. I. Johnson for assitance in using the mass spectrometer at the University of Toronto and Dr. L. Diosady for permission to use the facility.

REFERENCES

1 I. Alam, K. Ohuchi and L. Levine, Anal. Biochem., 93 (1979) 339.
2 F. Dray, Methods Enzymol., 86 (1982) 297.
3 E. Granström and H. Kindahl, in B. Samuelsson and R. Paoletti (Editors), Advances in Prostaglandin and Thromboxane Research, Vol. 1, Raven Press, New York, 1976, pp. 81—92.
4 S. Ferreira and J.R. Vane, Nature (London), 216 (1967) 868.
5 P.J. Piper, J.R. Vane and J.H. Wyllie, Nature (London), 225 (1970) 600.

6 C.R. Pace-Asciak, M.C. Carrara and K.C. Nicolaou, Prostaglandins, 15 (1978) 999.
7 G.J. Dusting, S. Moncada and J.R. Vane, Brit. J. Pharmacol., 64 (1978) 315.
8 C.R. Pace-Asciak and S. Micallef, Can. J. Biochem. Cell Biol., in press.
9 A.J. Wickrema Sinha, W. Morozowich, W. Hamlin and S.R. Shaw, J. Pharm. Sci., 62 (1973) 1428.
10 I.A. Blair, S.E. Barrow, K.A. Waddell, P.J. Lewis and C.T. Dollery, Prostaglandins, 23 (1982) 579.
11 F.A. Fitzpatrick, D.A. Stringfellow, J. Maclouf and M. Rigaud, J. Chromatogr., 177 (1979) 51.
12 J. Rosello, E. Gelpi, M. Rigaud, J. Durand and J.C. Breton, Biomed. Mass Spectrom., 8 (1981) 149.

Journal of Chromatography, 310 (1984) 243—250
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2207

DERIVATIZATION AND MASS SPECTROMETRIC BEHAVIOUR OF CATECHOLAMINES AND THEIR 3-O-METHYLATED METABOLITES

A.C. TAS*, J. ODINK, M.C. TEN NOEVER DE BRAUW, J. SCHRIJVER and R.J.G. JONK

CIVO-Institutes TNO, Utrechtseweg 48, P.O. Box 360, 3700 AJ Zeist (The Netherlands)

(Received February 9th, 1984)

SUMMARY

A method has been developed for the derivatization of both catecholamines (dopamine, noradrenaline and adrenaline) and their 3-O-methylated metabolites (3-methoxytyramine, normetanephrine and metanephrine) in a single run. The compounds were first incubated with methanolic hydrochloric acid to methylate those compounds that contain a benzylic hydroxyl group and were subsequently converted into their pentafluoropropionyl derivatives. The derivatives thus prepared, showed good gas chromatographic and electron-impact mass spectrometric properties and can be analysed in a single gas chromatographic run.

The effect of the derivatization on exchange reactions in the aromatic ring was investigated because standard compounds with deuterium label in that part of the molecule are often used in isotope dilution measurements. The exchange of deuterium for hydrogen in the aromatic ring under derivatization conditions was found to be limited.

INTRODUCTION

In recent years a variety of analytical methods has been developed for the determination of catecholamines and their 3-O-methylated metabolites [1—7]. Among these methods capillary gas chromatography—multi ion detection mass spectrometry (GC—MID-MS) affords a combination of great selectivity and sensitivity which is not easily obtained by other methods. Moreover, a high accuracy can be obtained with the isotope dilution method if stable isotopically labelled compounds are available.

GC—MS can be used for routine quantitative measurements and for confirmation of the results obtained with other methods. The fact that several compounds can be measured in a single run makes the method appropriate for profiling purposes. Such applications actually require only one derivatization method for a group of related compounds to afford suitable derivatives for a particular ionization method.

0378-4347/84/$03.00

Very recently such a derivatization method has been reported by De Jong and Cramers [8] which enables the extraction of catecholamines and their 3-O-methylated metabolites via the formation of their formyl esters, followed by an additional conversion with *tert.*-butyldimethylsilyl chloride to O-*tert.*-butyldimethylsilyl, N-formate derivatives. In this way profiles are obtained from these compounds, except from the more volatile 3-methoxytyramine.

In this paper a derivatization method is described for converting in one run of dopamine, noradrenaline, adrenaline, 3-methoxytyramine, normetanephrine and metanephrine into derivatives with good electron-impact properties.

It appeared that after this derivatization six compounds can easily be recorded in a single profile.

In many quantitative GC—MID-MS applications deuterium-labelled compounds are used for isotope dilution. With the catecholamines and their 3-O-methylated metabolites deuterium labelling is often positioned on the aromatic ring. In this connection, the stability of the compounds under derivatization conditions with respect to exchange reactions in the aromatic ring was investigated too.

EXPERIMENTAL

Reagents and materials

Analytical grade methanol, deuteromethanol, acetyl chloride and ethyl acetate were obtained from E. Merck (Darmstadt, F.R.G.) and pentafluoropropionic anhydride from Pierce (Rockford, IL, U.S.A.). DL-3,4-dihydroxyphenylalanine, 3-methoxytyrosine, dopamine, adrenaline, noradrenaline, 3-methoxytyramine, metanephrine and normetanephrine were purchased from Sigma (St. Louis, MO, U.S.A.). The compounds were dissolved in methanol (0.5 g/l) and stored at −15°C for a period not exceeding one month.

Solutions of hydrochloric acid in methanol were prepared by addition of acetyl chloride to methanol. A 1 *M* ^{2}HCl solution in deuteromethanol was prepared by addition of acetyl chloride to deuteromethanol.

The derivatization procedure was carried out in screw-capped tubes (7 ml, Sovirel 461151 P).

Derivatization

In order to find the optimal conditions for the synthesis of the benzylic-O-methyl (β-MeO) compounds of noradrenaline (NA), adrenaline (A), normetanephrine (NM) and metanephrine (M), the reaction with methanol–hydrochloric acid was carried out at three different reaction times (0.5, 1 and 4 h) and three hydrochloric acid concentrations (0.5, 1.1 and 2.1 *M*) at room temperature. An additional experiment was carried out, using 1.1 *M* hydrochloric acid at 60°C for 1 h. Although dopamine (DA) and 3-methoxytyramine (3-MT) do not possess a benzylic hydroxyl group, these compounds were also included in the first derivatization step. Yields were estimated from the peak heights in the reconstructed total ion current (RTIC) chromatogram.

A 0.1 ml volume of a methanol solution of catecholamines and 3-O-methylated catecholamines (0.5 g/l) was evaporated to dryness in a stream of nitrogen. A volume of 0.1 ml methanol—hydrochloric acid was added and

the sample was allowed to stand at room temperature. Subsequently, the methanol–hydrochloric acid was evaporated in a stream of dry nitrogen. A 0.1 ml volume of pentafluoropropionic anhydride (PFPA) was added and the sample was heated at 80°C for 15 min. After cooling to 20–30°C the excess reagent was evaporated in a gentle stream of dry nitrogen. A 0.1 ml portion of ethyl acetate containing 4% (v/v) PFPA was added to dissolve the derivatives.

Exchange reactions were carried out by incubating the compounds with 0.1 ml of 1 *M* deuteromethanol–^{2}HCl at room temperature for 1 h.

Gas chromatography–mass spectrometry

A Varian gas chromatograph 3700 coupled by an open split interface [9] to a Finnigan-MAT 212 mass spectrometer was used. The gas chromatograph was equipped with a capillary column (CP Sil 5, 45 m × 0.5 mm I.D.; Chrompack, Middelburg, The Netherlands). Helium (5 ml/min) was used as carrier gas. The column temperature was set at 170°C. The GC injection port and the interface region were maintained at 225°C and 220°C, respectively; the line-of-site temperature was kept at 200°C. The mass spectrometer was operated at an electron energy of 70 eV and an emission current of 1 mA. The ion source temperature was maintained at 220°C. For the identification of the derivatized compounds, spectra were taken in the mass range 23–800 with a scan speed of 1 decade/sec. Data were collected and stored in a Finnigan MAT SS200 computer system.

To investigate the exchange reaction of deuterium for hydrogen, the fragment ion + 1/fragment ion ratios of the derivatives were measured with MID under computer control. These ratios were compared with those from the same derivatives, prepared at the same time with methanol–hydrochloric acid under similar conditions. DA was measured at *m/z* 429/428; NA and A at *m/z* 446/445; 3-MT at *m/z* 297/296; NM and M at *m/z* 314/313.

RESULTS AND DISCUSSION

Optimization of the benzylic-O-methylation

During quality control it was observed that the hydrochlorides of NA, A, NM and M standard compounds dissolved in methanol and stored at 4°C were slowly converted. When the standard solution was stored under this condition for two to four months, all these compounds showed a satelite peak in the gas chromatogram after derivatization with PFPA. Such a conversion was not observed with DA and 3-MT, which indicates that the benzylic hydroxyl group was involved. The satelite peaks were identified by mass spectrometry as the β-MeO-PFP compounds (compounds 4, 6, 9 and 10, Fig. 1).

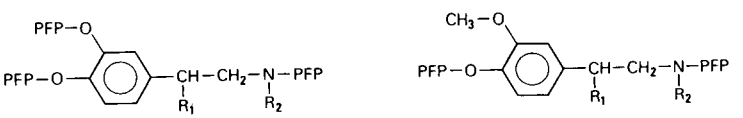


Fig. 1. Derivatives of catecholamines and their 3-O-methylated metabolites: 1 = NA (R_1 = OPFP; R_2 = H); 2 = A (R_1 = OPFP; R_2 = CH_3); 3 = DA (R_1 = H; R_2 = H); 4 = NA (R_1 = OCH_3; R_2 = H); 5 = NM (R_1 = OPFP; R_2 = H); 6 = A (R_1 = OCH_3; R_2 = CH_3); 7 = 3-MT (R_1 = H; R_2 = H); 8 = M (R_1 = OPFP; R_2 = CH_3); 9 = NM (R_1 = OCH_3; R_2 = H); 10 = M (R_1 = OCH_3; R_2 = CH_3).

Compounds 4 and 6 were recently prepared by Martin et al. [10], who studied GC and mass spectral characteristics of these compounds. Arnold and Ford [11] reported the analysis of compounds 9 and 10 in brain tissue, using GC with electron-capture detection. Also the corresponding benzylic-O-ethyl derivatives were applied in a GC—MS assay of human plasma catecholamines [4].

From the literature and our own data it was concluded that the preparation of the β-MeO-PFP compounds would be a useful method for combined derivatization of catecholamines and their 3-O-methylated metabolites.

By varying the incubation time and the hydrochloric acid concentration, the benzylic-O-methylation was found to have a near optimum when the reaction was carried out for 1 h in 1 *M* methanol—hydrochloric acid at 20°C (Table I). Extended reaction times and increased hydrochloric acid concentrations gave slightly higher yields, but on the other hand resulted in some by-product formation. These by-products were identified as the β-chloroethyl analogues of NA and A (R_1 = Cl, Fig. 1). Application of a reaction temperature of 60°C resulted in very low yields, probably caused by an increased β-chloroethyl formation followed by a further decomposition. With none of the compounds could a complete conversion be achieved. Therefore, after reaction with PFPA the "all"-PFP derivatives 1, 2, 5 and 8 were also observed in the RTIC chromatogram (Figs. 1 and 2).

TABLE I

OPTIMIZATION OF THE BENZYLIC-O-METHYLATION OF NA, A, NM AND M

Reaction at room temperature of NA, A, NM and M (50 μg in 100 μl of methanol) at different hydrochloric acid concentrations and reaction times. Yields were estimated by means of the peak heights in the reconstructed total ion current chromatogram after derivatization with PFPA.

HCl concentration (*M*)	Reaction time (h)	Yield (%)			
		NA	A	NM	M
0.5	1	56	47	34	32
1.1	0.5	71	66	40	35
1.1	1	90	75	60	46
1.1	4	93	89	84	67
2.1	1	91	88	63	57

Despite the fact that DA and 3-MT do not react during the β-O-methylation step, the compounds can be included in the first derivatization step, yielding PFP derivatives in the subsequent reaction with PFPA, thus enabling derivatization of six compounds in a single run.

Under the GC conditions applied, all derivatives were well separated. The β-MeO-PFP derivatives showed longer retention times than the corresponding "all"-PFP derivatives. When dissolved in ethyl acetate—4% (v/v) PFPA the derivatives were stable for at least 24 h at ca. 4°C.

Some experiments were carried out to ascertain whether the same derivatization method could be applied to convert 3,4-dihydroxyphenylalanine and 3-methoxytyrosine into their methyl ester PFP derivatives, as was reported by Arnold and Ford [11]. However, under the conditions applied these attempts

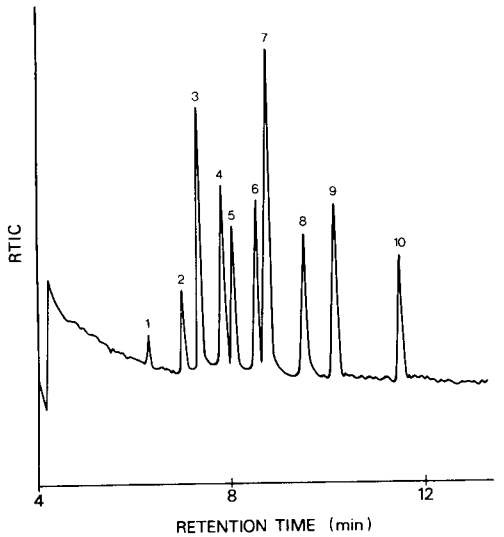


Fig. 2. Reconstructed total ion current (RTIC) chromatogram of a derivatized mixture of catecholamines and 3-O-methylated catecholamines. GC column and conditions are given in the experimental section. Peak numbers refer to the compounds mentioned in the legend to Fig. 1.

remained unsuccessful, probably because the esterification step only resulted in low yields.

Mass spectral characteristics

The sensitivity and selectivity of GC—MID-MS assays depend on the presence of high-intensity fragments or molecular ions in the higher mass region. The β-MeO-PFP derivatives of NA, A, NM and M (compounds 4, 6, 9 and 10, Fig. 1) show very dominant ring-containing fragments at m/z 445 (NA and A) and at m/z 313 (NM and M), which make them suitable for MID under electron-impact conditions. These fragments result from cleavage of the side-chain between the α- and the β-position, with charge retention on the aromatic part of the molecule. As shown in Table II, these fragments account for a considerable part of the total ion current. The excellent mass spectrometric properties of this type of derivative of NM and M have already been described by Martin et al. [10].

The PFP derivatives of DA and 3-MT (compounds 3 and 7, Fig. 1) have their base peaks at m/z 428 and m/z 296, respectively. Both fragments are formed via a McLafferty rearrangement and contain the aromatic ring and the α,β part of the side-chain [12]. In this case ring-deuterated as well as side-chain-deuterated standard compounds can be used for isotope dilution measurements.

The "all"-PFP derivatives of NA, A, NM and M (compounds 1, 2, 5 and 8, Fig. 1) show less favourable fragmentation characteristics. NA and A possess ring-containing fragments of low intensity at m/z 590 and m/z 604, respectively. The less specific side-chain fragments at m/z 176 and m/z 190 dominate. With NM and M the ring-containing fragment at m/z 458 is of medium intensity.

Comparison of the β-MeO-PFP derivatives with the "all"-PFP derivatives

TABLE II

MASSES $m/z > 170$ AS PERCENTAGE OF THE BASEPEAK INTENSITY (B) AND OF THE TOTAL ION INTENSITY (I), BOTH FROM THE MASS RANGE m/z 41—800

	Mass	B	I		Mass	B	I
1. NA	176	21	9	2. A	190	100	40
	590	<1	<1		191	4	2
					604	<1	<1
3. DA	176	54	11	4. NA	176	2	1
	177	3	1		179	5	2
	225	5	1		267	2	1
	253	3	1		299	3	1
	265	8	2		417	2	1
	269	3	1		445	100	37
	281	39	8		446	12	4
	282	3	1		447	3	1
	387	6	1		458	3	1
	428	100	21				
	429	22	5				
5. NM	176	7	1	6. A	179	9	2
	270	6	1		190	24	7
	298	5	1		191	2	1
	311	8	2		267	3	1
	417	7	1		417	2	1
	445	11	2		445	100	28
	458	27	5		446	26	7
	459	5	1		447	3	1
					472	3	1
7. 3-MT	176	4	1	8. M	190	100	35
	255	4	1		191	3	1
	283	19	5		311	2	1
	284	3	1		445	3	1
	296	100	26		458	18	6
	297	16	4		459	3	1
	298	2	1		471	2	1
	459	6	2		472	2	1
9. NM	298	2	1	10. M	190	4	2
	313	100	41		313	100	42
	314	14	6		314	10	4

shows that the former group of derivatives has better mass spectrometric properties, because the ring-containing fragments in the higher mass region are more intense.

Exchange reaction of deuterium for hydrogen in the aromatic ring

Because the robust ^{13}C-labelled compounds are often not commercially available at the required degree of labelling, or not available at all, deuterium-labelled compounds are more frequently used.

With catecholamines and 3-O-methylated catecholamines, deuterium labelling is often carried out in the aromatic ring. Only recently a method has been developed for the synthesis of 3-O-deuteromethylated catecholamines

[13]. These compounds have a good stability under acidic conditions. Although side-chain-deuterated NM and M are commercially available now, their use is limited to those ionization methods that do not split off the side-chain as a fragment. Therefore, we were interested in the stability of compounds with respect to exchange reactions in the aromatic ring.

In this connection, measurements were carried out of the ratios of the fragment ion + 1/fragment ion of derivatives prepared in deuteromethanol—^{2}HCl, and those of derivatives prepared in methanol—hydrochloric acid (Table III). These ratios indicate that NA, A, NM, M and 3-MT show no or hardly any exchange. The loss of ring deuterium label for NM and M, as was observed by Martin et al. [10], was not confirmed in our experiments. With DA a slightly higher exchange rate was observed; the 429/428 ratio was ca. 2.3% higher. When exact measurements are needed, the use of side-chain-labelled DA is to be preferred.

TABLE III

INFLUENCE OF THE DERIVATIZATION ON THE FRAGMENT ION + 1/FRAGMENT ION RATIOS

Reaction at room temperature of catecholamines and 3-O-methylated catecholamines, 50 μg in 100 μl of 1 *M* deuteromethanol—^{2}HCl and 100 μl of 1 *M* methanol—hydrochloric acid, respectively, for 1 h. The fragment ion + 1/fragment ion ratios are given in %. NA and A were measured at *m/z* 446/445; DA at *m/z* 429/428; NM and M at *m/z* 314/313; 3-MT at *m/z* 297/296. The figures given are the mean values ($\bar{x}$) and the standard deviations (S.D.) of eight replicate reactions.

	Deuteromethanol—^{2}HCl		Methanol—HCl		$\Delta\bar{x}$
	$\bar{x}$	S.D.	$\bar{x}$	S.D.	
NA	15.4	0.4	15.1	0.4	+0.3
A	15.3	0.6	15.1	0.1	+0.2
DA	23.0	3.2	20.7	1.1	+2.3
NM	12.6	0.3	12.7	0.3	−0.1
M	12.4	0.5	12.8	0.3	−0.4
3-MT	17.3	0.9	16.7	0.3	+0.6

Our experiments, carried out with unlabelled standard compounds, give an overestimation of the exchange rate of the labelled compounds, since deuterium-labelled compounds are less susceptible to exchange reaction. This isotope effect is caused by the higher activation energy needed for the cleavage of a carbon–deuterium bond [14].

CONCLUSION

A simple derivatization method has been developed for the combined conversion of catecholamines and their 3-O-methylated metabolites. The exchange of deuterium for hydrogen in the aromatic ring was found to occur only to a limited extent.

ACKNOWLEDGEMENTS

We thank Drs. K.D. Bos and J. van der Greef for their stimulating comments.

REFERENCES

1 B.H.C. Westerink and J. Korf, J. Neurochem., 29 (1977) 697.
2 S. Demassieux, L. Corneille, S. Lachance and S. Carrière, Clin. Chim. Acta, 115 (1981) 377.
3 F.A.J. Muskiet, C.G. Thomasson, A.M. Gerding, D.C. Fremouw-Ottevangers, G.T. Nagel and B.G. Wolthers, Clin. Chem., 25 (1979) 453.
4 J.-D. Ehrhardt and J. Schwartz, Clin. Chim. Acta, 88 (1978) 71.
5 Y. Mizuno and T. Ariga, Clin. Chim. Acta, 98 (1979) 217.
6 L.M. Nelson, F.A. Bubb, P.M. Lax, M.W. Weg and M. Sandler, Clin. Chim. Acta, 92 (1979) 235.
7 A.J. Speek, J. Odink, J. Schrijver and W.H.P. Schreurs, Clin. Chim. Acta, 128 (1983) 103.
8 A.P.J.M. de Jong and C.A. Cramers, J. Chromatogr., 276 (1983) 267.
9 M.C. ten Noever de Brauw, J. Chromatogr., 165 (1979) 207.
10 J.T. Martin, J.D. Barchas and K.F. Faull, Anal. Chem., 54 (1982) 1806.
11 E.L. Arnold and R. Ford, Anal. Chem., 45 (1973) 85.
12 M.R. Holdiness, M.T. Rosen, J.B. Justice and D.B. Neill, J. Chromatogr., 198 (1980) 329.
13 F.A.J. Muskiet, S. Pavel, G.J. Stob and H. Elzinga, J. Labelled Compd. Radiopharm., 20 (1983) 627.
14 R. Neeter, N.M.M. Nibbering and Th.J. de Boer, Org. Mass Spectrom., 3 (1970) 597.

Journal of Chromatography, 310 (1984) 251—259
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2204

QUANTIFICATION BY SELECTED ION MONITORING OF PIPECOLIC ACID, PROLINE, γ-AMINOBUTYRIC ACID AND GLYCINE IN RAT BRAIN

Y. OKANO, T. KADOTA, J. NAGATA, A. MATSUDA, S. IJIMA, K. TAKAHAMA and T. MIYATA*

Department of Chemico-Pharmacology, Faculty of Pharmaceutical Sciences, Kumamoto University, 1-5 Oehon-machi, Kumamoto 862 (Japan)

(First received February 9th, 1984; revised manuscript received May 8th, 1984)

SUMMARY

A procedure for the simultaneous analysis of brain pipecolic acid, proline, γ-aminobutyric acid and glycine — amino acids with potent inhibitory actions on the central nervous system — was developed. The identification and quantification of the amino acids were performed with a gas chromatographic—mass spectrometric—computer system using deuterium-labelled amino acids as the internal standards. After separation of the amino acids by high-performance liquid chromatography, the methyl ester heptafluorobutyryl derivatives were prepared. The lower limit of quantification for this method is at the picomole level.

The usefulness of this chromatographic procedure has been demonstrated by measurement of trace amounts of pipecolic acid in rat brain.

INTRODUCTION

The role of γ-aminobutyric acid (GABA) and glycine as inhibitory transmitters in the central nervous system is now fairly well established [1, 2]. Pipecolic acid (piperidine-2-carboxylic acid), a major metabolite of lysine metabolism in rat brain [3, 4], and proline, the lower membered homologue of pipecolic acid, are alicyclic amino acids with potent neuropharmacological activity [5, 6]. In the central nervous system, pipecolic acid and proline have also been suggested to have inhibitory actions resembling those of GABA and glycine [7, 8]. Furthermore, evidence for a neuromodulatory role of pipecolic acid obtained in our laboratory and others included the occurrence [9, 10], degradation [11], distribution [12], accumulation [13], uptake and release system [14—16] in the brain.

In addition, pipecolic acid is likely to have a connection with some neuro-

0378-4347/84/$03.00

pathological diseases in infants with hyperpipecolataemia [17, 18], hyperlysinaemia [19], and the cerebrohepatorenal syndrome of Zellweger [20], which seems to be due to an inborn error in pipecolic acid or lysine metabolism. Abnormally elevated levels of pipecolic acid have been detected in the serum of young patients, and progressive mental retardation is a common symptom of these patients.

A variety of methods are available for the determination of proline, GABA and/or glycine in biological samples. However, the analytical methods for pipecolic acid that have so far been reported are too insensitive to allow convincing micro-quantification of pipecolic acid levels in biological samples [17—23], especially the brain [9, 17, 23]. In a previous study, a combination of ion-exchange column chromatography and gas chromatography—mass spectrometry (GC—MS) was used for the separation and determination of alicyclic amino acids [10].

In the present study, the selected ion monitoring technique using a gas chromatographic—mass spectrometric—computer (GC—MS—COM) system following high-performance liquid chromatographic (HPLC) pre-purification was applied to develop a new method with high specificity and sensitivity for the simultaneous quantification of pipecolic acid, proline, GABA and glycine in rat brain.

EXPERIMENTAL

Materials

L-Pipecolic acid was obtained from Kyowa Hakko (Tokyo, Japan). The following compounds were commercially available: L-proline, GABA and glycine from Kyowa Hakko; DL-[1,2,2′,3,3,4,4,5,5,6,6-$^2H_{11}$]pipecolic acid (deuterium-labelled pipecolic acid, pipecolic acid-d_{11}), L-[2,3,3,4,4,5,5-$^2H_7$]-proline (proline-d_7), γ-[2,2,3,3,4,4-$^2H_6$]aminobutyric acid (GABA-d_6) and [1,1-$^2H_2$]glycine (glycine-d_2) from Merck Sharp & Dohme (Montreal, Canada); heptafluorobutyric anhydride (HFBA), boron trifluoride in methanol complex (BF_3—CH_3OH, 14% boron trifluoride in anhydrous methanol) and formic acid from Wako (Osaka, Japan). Organic solvents were of analytical grade and were further purified by redistillation.

Apparatus

A Shin-Nihon Musen Model NJE-2601 Metabostat System microwave device was used for rapid inactivation of brain enzymes. Microwave fixation was achieved by irradiation of whole animals in an oven delivering 4.5 kW at 2450 MHz. Animals received 1.5 sec of irradiation.

A Waters Assoc. ALC/GPC 244 HPLC system equipped with a semipreparative μBondapak C_{18} column (8—10 μm, 30 cm × 7.8 mm) and a guard column packed with Bondapak C_{18}/Corasil (37—50 μm, 3.8 cm × 3.9 mm) was used for the separation of the amino acid fraction of the brain extracts. The detection system employed was a variable-wavelength ultraviolet (UV) detector (S-310A, Soma Kogaku, Japan).

The quantification of amino acids was performed on a GC—MS—COM system (JMS modified D-100 equipped with a JMA-2000S mass data analysis

system). Separations were made on a 200 × 2 mm I.D. glass column packed with 3% OV-1 on 80—100 mesh Gas-Chrom Q (Gasukuro Kogyo, Tokyo, Japan). The chromatographic conditions were as follows: column temperature 130°C; injection port temperature 250°C; helium flow-rate 40 ml/min. Selected ion monitoring was performed under the following conditions: ion source and separator temperatures 250°C; ionizing voltage 75 eV; trap current 300 μA. The instrument was used in the selected ion monitoring mode. The fragment ions used for monitoring were *m/z* 280.1 for pipecolic acid, *m/z* 289.2 for pipecolic acid-d_{11}, *m/z* 266.1 for proline, *m/z* 273.1 for proline-d_7, *m/z* 254.1 for GABA, *m/z* 260.2 for GABA-d_6, *m/z* 226.1 for glycine, and 228.1 for glycine-d_2. All these compounds were run as the HFB-Me-amino acid derivatives [10, 12].

Computer analysis of data

At the completion of analysis, two data files had been recorded on disc representing some eight separate ion current profiles (*m/z* 226.1, 228.1, 254.1, 260.2, 266.1, 273.1, 280.1, 289.2). A computer program received, as fixed data, the retention time of each peak and its area, together with a position where background intensity could be measured. The ratio of peak area for amino acid in the brain to deuterium-labelled amino acid as an internal standard pair was then calculated. Quantitation limits were estimated from the computer responses generated by the known concentrations of amino acids used in the preparation of the standard calibration curves [24].

Methods

Male Wistar rats (190 ± 10 g, 7 weeks' old) from Kyudo Farm (Kumamoto, Japan) were used. The animals were allowed free access to food (CREA, CE-2) and tap water. The animals were usually sacrificed at 10.00 a.m. The brain from one rat was used for each assay. The brain was removed after microwave irradiation, weighed and homogenized in 5 vols. of ice-cold 5% trichloroacetic acid (TCA) with a homogenizer (Ultra-Turrax, Jankel & Kunkel KG Ika-Werk, F.R.G.). The homogenate was centrifuged at 17,000 *g* for 30 min at 0°C. A 1.0-ml volume of the supernatant (about 10 ml in total volume) was taken up and transferred to a glass-stoppered test tube. Proline-d_7 (5 nmol), GABA-d_6 (100 nmol) and glycine-d_2 (100 nmol) were added to the supernatant taken up as internal standards. Pipecolic acid-d_{11} (5 nmol) was added to the remaining supernatant (about 9 ml).

The TCA solution was washed with an equivalent volume of ethyl acetate for 60 sec using a Vortex-Gemie mixer. The organic layer was removed by aspiration. The procedure was repeated four times. The remaining aqueous layer containing amino acids was evaporated under reduced pressure at 40°C. The residue was dissolved in 1.0 ml of water. For removal of numerous organic compounds, a Sep-Pak C_{18} cartridge (Waters Assoc.) was employed. The cartridge was activated with 5 ml of water, then flushed with 5 ml of methanol. The 1.0-ml aqueous solution containing the brain extract was passed through the cartridge and the eluate containing the amino acids was collected and evaporated. The residue was dissolved in about 250 μl of 0.1% formic acid solution (the mobile phase for HPLC).

A 250-μl volume of the solution was introduced on to the column via a U6K universal injector (Waters Assoc.). The flow-rate was changed after 2.6 min from 3.0 to 1.0 ml/min and was maintained at 1.0 ml/min for an additional 20 min. The detection of authentic amino acids (non-labelled and labelled amino acids) or of those in biological samples was at 205 nm. Retention times of the amino acids were 2.8 min for glycine, 4.1 min for GABA, 5.9 min for proline and 11.7 min for pipecolic acid. The elution fractions at the retention times were collected manually. For example the pipecolic acid fraction was collected between 10.2 and 14.0 min. The volume of eluate was about 3.8 ml.

The eluate was evaporated in a test tube. The residue was dissolved in 1.5 ml of distilled methanol and transferred into a Reacti-vial (Microproduct V vial with PTFE-lined cap, Wheaton Scientific). The solution in the vial was evaporated under a gentle stream of dried nitrogen. Finally, the residue was dried in a vacuum desicator (60 mmHg) over silica gel for 12 h at room temperature.

BF_3—CH_3OH reagent (100 μl) was added to the dried sample and heated for 13 min at 100°C in a Dri-Block heater (Model DB-2H, M&S Instruments, Tokyo, Japan). The vial was cooled to room temperature before being opened. After evaporation to dryness under a stream of dried nitrogen at 80°C, the trace of water was azeotropically removed twice using 100 μl of methylene chloride at 100°C [25]. The residue was dissolved in 250 μl of distilled ethyl acetate, and 100 μl of HFBA were added. The mixture in the vial was heated for 20 min at 150°C in the Dri-Block heater and then gradually cooled to room temperature. The reaction product was evaporated to dryness using a gentle stream of dried nitrogen [26]. The residue was dissolved in 50 μl of ethyl acetate and 0.1—3.0 μl were injected into the GC—MS system.

RESULTS AND DISCUSSION

There are very few studies concerning the determination of pipecolic acids, while several methods, including enzyme assay, GC and GC—MS for GABA [27, 28] and HPLC and automated amino acid analysis for proline and glycine, have been reported [29].

In the present study, selected ion monitoring using a GC—MS—COM system following HPLC was chosen as a possible method for high sensitivity and specificity. This method has some advantages over the procedure previously utilized in our laboratory [10]. The advantage includes higher purification of samples within a short time by the replacement of the ion-exchange column chromatography with a combination of crude and pre-purification using Sep-Pak C_{18} cartridges and sequential HPLC separation. In addition, the sequential HPLC operation is semiautomatic and can visually develop the peaks derived from amino acids. It has been demonstrated that selected ion monitoring is a sensitive quantitative method for pipecolic acid and other amino acids [10, 12].

Mass spectra of HFB-ME derivatives of pipecolic acid, proline, GABA, glycine and internal standards are shown in Figs. 1 and 2. The molecular and base ions of the respective derivatives were *m/z* 339.2 $[(M)^+]$ and *m/z* 280.1 $[(M - COOCH_3)^+]$ for HFB-Me-pipecolic acid and *m/z* 348.2 $[(M)^+]$ and *m/z*

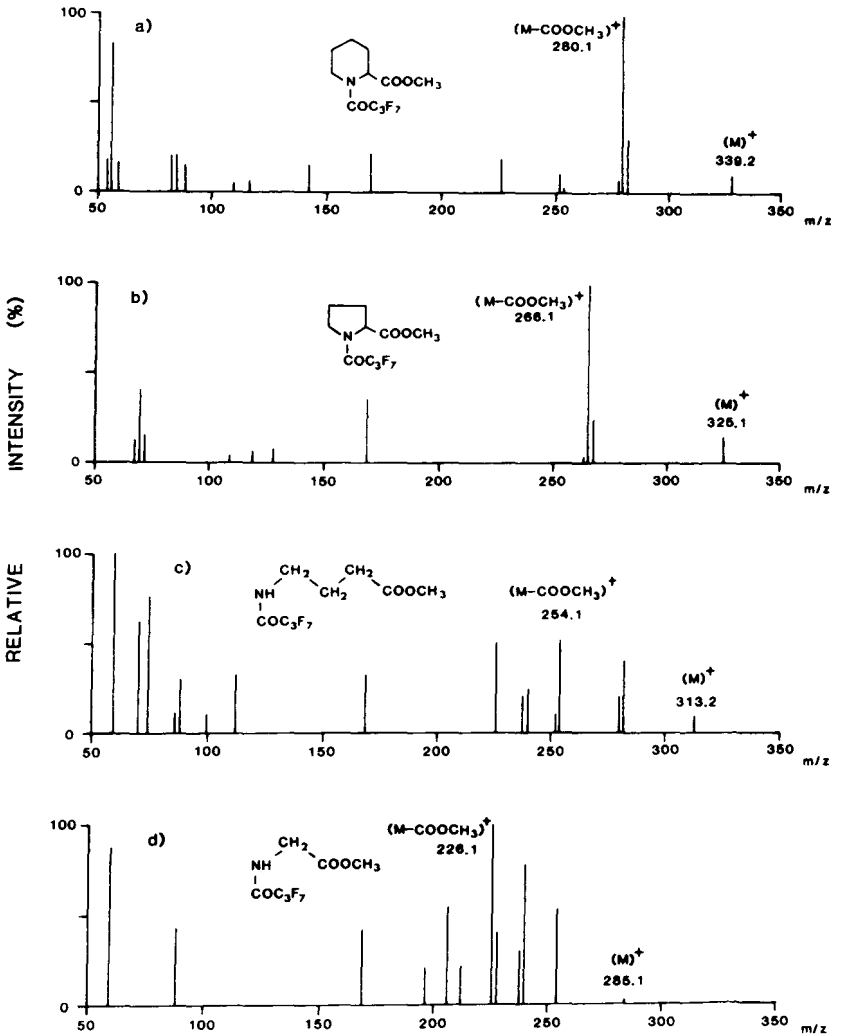


Fig. 1. Mass spectra of authentic HFB-Me derivatives of (a) pipecolic acid, (b) proline, (c) GABA and (d) glycine.

289.2 $[(M - COOCH_3)^+]$ for HFB-Me-pipecolic acid-d_9; *m/z* 325.1 $[(M)^+]$ and *m/z* 266.1 $[(M - COOCH_3)^+]$ for HFB-Me-proline and *m/z* 332.1 $[(M)^+]$ and *m/z* 273.1 $[(M - COOCH_3)^+]$ for HFB-Me-proline-d_7; *m/z* 285.1 $[(M)^+]$ and *m/z* 226.1 $[(M - COOCH_3)^+]$ for HFB-Me-glycine and *m/z* 287.1 $[(M)^+]$ and *m/z* 228.1 $[(M - COOCH_3)^+]$ for HFB-Me-glycine-d_2.

Because the base peak ions described above had the characteristics of the parent compound moieties, they were used for selected ion monitoring. The derivatives of GABA and GABA-d_6 have the molecular peak ion $[(M)^+]$ at *m/z* 313.2 and 319.2, the common base peak ions $[(COOCH_3)^+]$ at *m/z* 59.0, and the other intensive peak ions $[(M - COOCH_3)^+]$ at *m/z* 254.1 and 260.2, respectively. Thus the common base peak ion derived from GABA and GABA-d_6 had a very low molecular weight and did not have the specificity of the

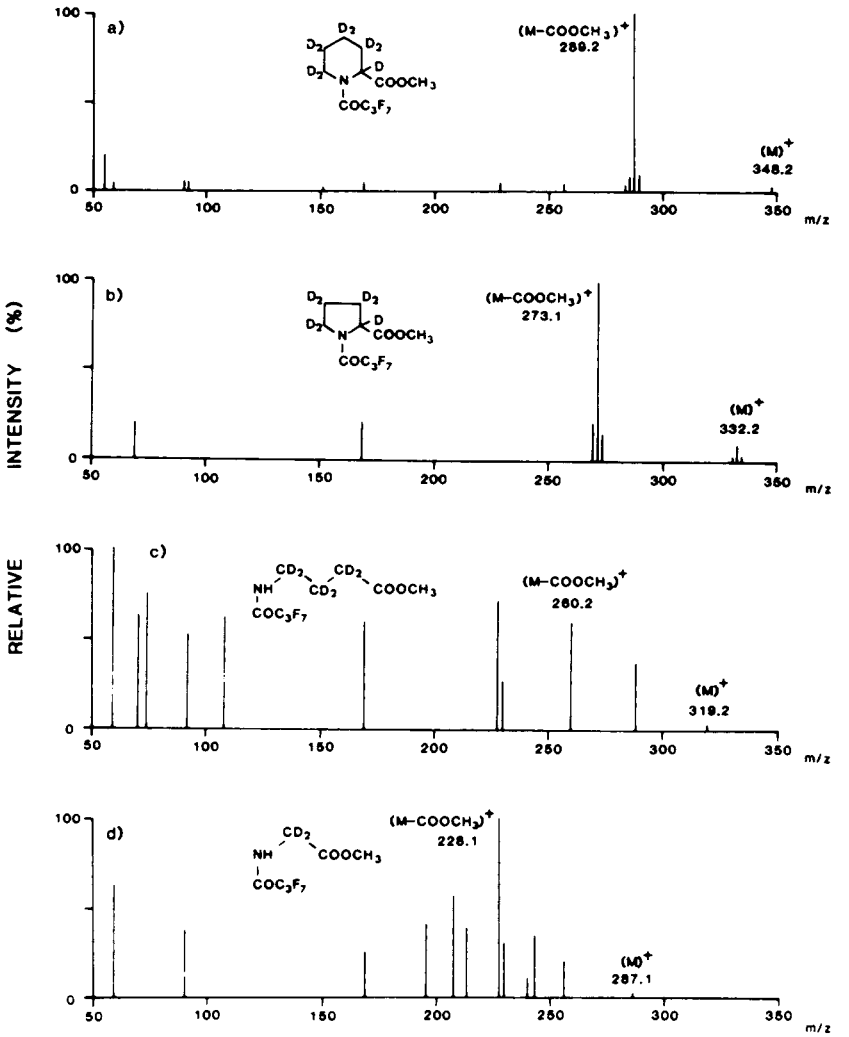


Fig. 2. Mass spectra of authentic HFB-Me derivatives of (a) pipecolic acid-d_{11}, (b) proline-d_7, (c) GABA-d_6 and (d) glycine-d_2.

parent compound moieties. Accordingly, intensive ions with *m/z* 254.1 and 260.2 which had GABA moieties were applied to GABA determination for selected ion monitoring.

Amino acids in brain extract were identified by the selected ion monitoring technique; that is, the ratios of peak area of the base or intensive ions to the molecular ions of HFB-Me-amino acids were measured using the standards and the brain extract samples, respectively. The ratios of *m/z* 280.1 to 339.2 for pipecolic acid were 10.7 ± 0.85 (standard) and 10.8 ± 0.64 (brain); *m/z* 266.1 to 325.1 for proline were 7.87 ± 0.44 (standard) and 7.75 ± 0.76 (brain); *m/z* 226.1 to 285.1 for glycine were 40.0 ± 1.06 (standard) and 38.7 ± 1.88 (brain); *m/z* 254.1 to 313.2 for GABA were 2.52 ± 0.22 (standard) and 2.57 ± 0.39 (brain) (six determinations each). Thus, the ratios were almost the same for the

standard and the brain extract, demonstrating the specificity of this method.

Fig. 3 shows selected ion monitoring recordings obtained from an analysis of pipecolic acid and other amino acids in rat brain extract containing the deuterium-labelled amino acids as the internal standards. The retention times for the HFB-Me derivatives of each amino acid and internal standard were 1.0 min for glycine, 2.4 min for GABA, 3.0 min for proline and 4.0 min for pipecolic acid.

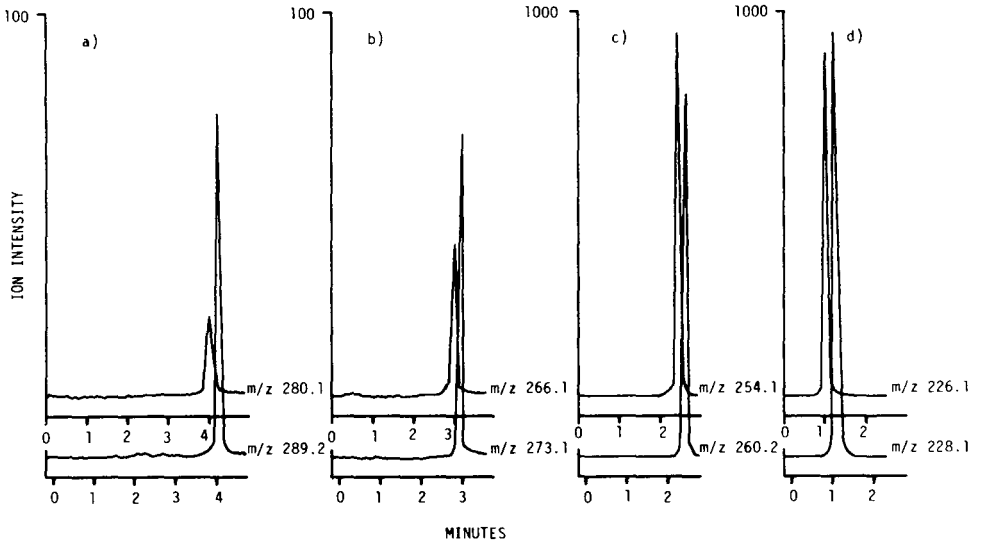


Fig. 3. Selected ion monitoring records of the extract from rat brain with added internal standards: (a) HFB-Me-pipecolic acid (*m/z* 280.1) and HFB-Me-pipecolic acid-d_9 (*m/z* 289.2); (b) HFB-Me-proline (*m/z* 266.1) and HFB-Me-proline-d_7 (*m/z* 273.1); (c) HFB-Me-GABA (*m/z* 254.1) and HFB-Me-GABA-d_6 (*m/z* 260.2); (d) HFB-Me-glycine (*m/z* 226.1) and HFB-Me-glycine-d_2 (*m/z* 228.1).

The amino acid concentrations in rat brain were calculated from the ratio of peak areas of *m/z* 280.1 to 289.2 for pipecolic acid, *m/z* 266.1 to 273.1 for proline, *m/z* 254.1 to 260.2 for GABA and *m/z* 226.1 to 228.1 for glycine using the computer system. Linear calibration curves (r = 0.97—0.99) were obtained with amounts in the nanomole range. The quantitative limits of the amino acids were as low as 5—10 pmol (signal-to-noise ratio = 2—4). The detection limit of the amino acids was about 0.5 pmol.

Pipecolic acid, proline, GABA and glycine levels in rat brain were as follows (mean ± standard error of ten determinations): 0.69 ± 0.04 nmol/g for pipecolic acid, 77.8 ± 13.7 nmol/g for proline, 1.29 ± 0.04 μmol/g for GABA and 1.44 ± 0.07 μmol/g for glycine. The proline, GABA and glycine levels in whole rat brain were similar to those reported by some other investigators [29].

Previously, pipecolic acid had been reported to be present only in the blood [17—22] and urine [17, 19]. However, recent preliminary studies in our laboratory [10, 12] and also by Gatfield et al. [17] and Schmidt-Glenewinkel et al. [23] have indicated that pipecolic acid is present in human and rat brain. The concentration of pipecolic acid in rat brain determined by this study was

one-twentieth that given by Schmidt-Glenewinkel et al. Accordingly, insufficient specificity may be one explanation for the tendency to high pipecolic acid concentrations found in the published papers. Since the chemical behaviour of other alicyclic amino acids (nipecotic acid, isonipecotic acid and hydroxypipecolic acids, etc.) such as the elution pattern in HPLC, column and gas chromatography resembles that of pipecolic acid, other alicyclic amino acids and other contaminants would increase the apparent value of pipecolic acid in tissues.

Thus the present paper describes a highly sensitive and specific method for the detection and assay of pipecolic acid. Furthermore, the method seems to be easily adapted to the simultaneous analysis of physiological, pathological and pharmacologically induced variations of these amino acids especially pipecolic acid in the brain regions, and such studies appear to be useful for investigating their possible physiological and pathological roles.

ACKNOWLEDGEMENTS

A part of this work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan. Thanks are due to Miss M. Kawagoe for her skilful technical assistance.

REFERENCES

1 D.R. Curtis, in P. Krogsgaard-Larsen, J. Scheel-Krüger and H. Kofod (Editors), GABA-Neurotransmitters, Munksgaard, Copenhagen, 1978, p. 17.
2 R. Werman, R.A. Davidoff and M.H. Aprison, Nature, 214 (1967) 681.
3 Y.F. Chang, Biochem. Biophys. Res. Commun., 69 (1976) 174.
4 E. Giacobini, Y. Nomura and T. Schmidt-Glenewinkel, Cell. Mol. Biol., 25 (1980) 1.
5 T. Miyata, K. Kamata, M. Noguchi, Y. Okano and Y. Kasé, Jap. J. Pharmacol., 23 (1973) 81p.
6 Y. Kasé, K. Takahama, T. Hashimoto, J. Kaisaku, Y. Okano and T. Miyata, Brain Res., 193 (1980) 608.
7 K. Takahama, T. Miyata, T. Hashimoto, Y. Okano, T. Hitoshi and Y. Kasé, Brain Res., 239 (1982) 294.
8 K. Takahama, N. Tokutomi, Y. Okano and T. Miyata, in S. Kito, T. Segawa and H.I. Yamamura (Editors). Neurotransmitter Receptor Regulation, Interactions and Coupling to the Effectors, Hiroshima University, Hiroshima, 1983, p. 67.
9 Y. Kasé, M. Kataoka, T. Miyata and Y. Okano, Life Sci., 13 (1973) 867.
10 Y. Okano, M. Kataoka, T. Miyata, H. Morimoto, K. Takahama, T. Hitoshi, Y. Kasé, I. Matsumoto and T. Shinka, Anal. Biochem., 117 (1981) 196.
11 E. Giacobini, in A. Lajtha (Editor), Handbook of Neurochemistry, Vol. 3, Plenum, New York, 1983, p. 583.
12 Y. Okano, T. Kadota, K. Takahama, Y. Kasé and T. Miyata, Jap. J. Pharmacol., 33 (1983) 228p.
13 H. Nishio, J. Ortiz and E. Giacobini, Neurochem. Res., 6 (1981) 1241.
14 Y. Kasé, T. Myata, A. Hirata, H. Morimoto, Y. Okano and T. Takahama, Folia Pharmacol. Jap., 67 (1980) 68p.
15 Y. Nomura, Y. Okuma, T. Segawa, T. Schmidt-Glenewinkel and E. Giacobini, J. Neurochem., 33 (1979) 803.
16 H. Nishio and E. Giacobini, Neurochem. Res., 6 (1981) 835.
17 P.D. Gatfield, E. Taller, G.G. Hinton, A.C. Wallace, G.M. Abdelnour and M.D. Haust, Can. Med. Assoc. J., 99 (1968) 1215.

18 G.H. Thomas, R.H.A. Haslam, M.L. Batshaw, A.J. Capute, L. Niedengard and J.L. Ransam, Clin. Genet., 8 (1975) 376.
19 N.C. Woody and M.B. Pupene, Pediatr. Res., 4 (1970) 89.
20 D.M. Danks, P. Tippett, C. Adams and P. Cambell, J. Pediatr., 86 (1975) 382.
21 R.F. Grimble, Clin. Chim. Acta, 38 (1972) 113.
22 J. Hutzler and J. Dancis, Clin. Chim. Acta, 128 (1983) 75.
23 T. Schmidt-Glenewinkel, E. Giacobini, Y. Nomura, Y. Okuma and T. Segawa, in V. Neuhoff (Editor), 2nd Meeting Eur. Soc. Neurochem., Gottingen, Verlag Chemie, Weinheim, New York, 1978, p. 618.
24 J.J. Rafter, M. Ingelman-Sundberg and J.-A. Gustafsson, Biomed. Mass Spectrom., 6 (1979) 317.
25 J.D. Doherty, O.C. Snead and R.H. Roth, Anal. Biochem., 69 (1975) 268.
26 S.L. MacKenzie and D. Tenaschuk, J. Chromatogr., 97 (1974) 19.
27 Y. Okada, C. Nitsch-Hassler, J.S. Kim, I.J. Bak and R. Hassler, Brain Res., 13 (1971) 514.
28 L. Bertilsson and E. Costa, J. Chromatogr., 118 (1976) 395.
29 L. Battistin, A. Grynbaum and A. Lajtha, Brain Res., 29 (1971) 85.

Journal of Chromatography, 310 (1984) 261—272
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2220

DIFFERENTIATION BETWEEN *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* AND *HAEMOPHILUS APHROPHILUS* BASED ON CARBOHYDRATES IN LIPOPOLYSACCHARIDE

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 February 16th, 1984; revised manuscript received May 14th, 1984)

SUMMARY

In the present study, the closely related facultative, Gram-negative rods, *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*, were distinguished taxonomically by means of their carbohydrate composition in phenol-extracted lipopolysaccharide. Both *A. actinomycetemcomitans* and *H. aphrophilus* lipopolysaccharide contained rhamnose, fucose, galactose, glucose, L-glycero-D-mannoheptose, galactosamine, and glucosamine. The content of galactose was approximately twice as high in lipopolysaccharide from *H. aphrophilus* as in lipopolysaccharide from *A. actinomycetemcomitans*. D-Glycero-D-mannoheptose was detected exclusively in lipopolysaccharide from *A. actinomycetemcomitans* where it constituted 11.8—16.7% of the sugar content. This aldoheptose may therefore serve as a marker for chemotaxonomic differentiation between *A. actinomycetemcomitans* and *H. aphrophilus*. The present study also describes fragmentation of methylheptoside derivatives of trifluoroacetic acid (D-glycero- and L-glycero-D-mannoheptose) from *A. actinomycetemcomitans* as suggested by mass spectrometry.

INTRODUCTION

Lipopolysaccharide (LPS), located in the outer membrane of Gram-negative bacterial cells, represents a unique class of macromolecules. It consists of a lipid portion, lipid A, and a long covalently linked heteropolysaccharide, the core and the O-specific chain [1]. O-Specific polysaccharides, comprising a series of different sugar residues in many combinations and glycosidic linkages, may display great variability between bacterial strains. In the common core,

0378-4347/84/$03.00

where a number of closely related oligosaccharides are found, the degree of structural freedom is lower. The O chain carries the immune determinant structure. Through the core, serologic R specificity is expressed [2]. LPS has been classified into chemotypes based on the content of sugar constituents [3]. In *Salmonella* this chemical classification correlated well with the serological classification of the Kauffman—White scheme [4]. In the present study we have used the sugar composition of LPS for taxonomic differentiation between the closely related dental plaque bacteria *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus*. Identification of these organisms in the routine laboratory is generally done by biochemical tests, but only a few of these have been found useful for differentiation [5]. The present study suggested that LPS may be a useful parameter for accurate distinction between *A. actinomycetemcomitans* and *H. aphrophilus* when conventional methods are insufficient. Accurate identification is urgent when it comes to clarification of a possible association between distinct bacterial species and clinical forms of disease. So far, an association has been suggested between *A. actinomycetemcomitans* and a special form of periodontal disease affecting juveniles (juvenile periodontitis) [6—8]. The relationship between *H. aphrophilus* and periodontal destruction, however, has not yet been determined. It should also be realized that both *A. actinomycetemcomitans* and *H. aphrophilus* may be implicated in a series of extraoral infections, e.g. bacterial endocarditis and miscellaneous abscesses (for review, see ref. 9), where similar problems of differentiation may occur.

The present study also describes fragmentation of trifluoroacetylated (TFA-derivatized) methylglycosides of D-glycero- and L-glycero-D-manno-heptose from *A. actinomycetemcomitans*, as suggested by mass spectrometry. The purpose of this aspect of the study was to make identification of these sugars easier when reference bacteria containing them are not at hand.

MATERIAL AND METHODS

Bacteria

The thirteen strains of *A. actinomycetemcomitans* and *H. aphrophilus* investigated, the sources from which they were obtained, and the procedures for maintenance and cultivation have already been described [10, 11].

Preparation of lipopolysaccharide

LPS from *A. actinomycetemcomitans/H. aphrophilus* strain pairs was isolated by the phenol—water procedure [11]. The preparations from *A. actinomycetemcomitans* strain ATCC 33384 and from *H. aphrophilus* strain ATCC 33389 were made in duplicate from batches cultured at different time periods. All LPS preparations were divided into two parts, each of which was methanolysed and derivatized separately.

Methanolysis

Samples (0.5—1.5 mg) of LPS were methanolysed (2 *M* hydrochloric acid in anhydrous methanol, 2 ml, 24 h, 85°C) [11]. 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 organic phase was washed twice, each time with 4 ml of distilled water, and the water phase twice, with 4-ml batches of chloroform. The water phases were pooled and lyophilized before derivatization for sugar analysis. Fatty acids have already been determined from the pooled organic phases [11].

Derivatization

The lyophilized water phase (0.1—0.2 mg) from each part of the LPS preparations was suspended in 100 or 200 μl of acetonitrile (Rathburn, U.K.). Acetonitrile and trifluoroacetic acid anhydride (Fluka), 1 or 2 ml of each, were then added, and the mixture was transferred to a PTFE-sealed tube with screw cap, which was kept in a water bath for 3 min at 90°C. After cooling the tube to room temperature, its content was diluted 1:1 with acetonitrile. Also synthetic sugars and reference LPS were methanolysed and derivatized as described above.

Reference compounds

Sigma (St. Louis, MO, U.S.A.) provided α-D(+)-fucose, D(+)-galactose, α-D(+)-glucose, D(+)-galactosamine, D(+)-glucosamine, D(+)-mannose, and α-L-rhamnose. Natural galactose, glucosamine, L-glycero-D-mannoheptose, mannose, and rhamnose were identified from LPS (Sigma) of *Escherichia coli* [12] and *Salmonella typhimurium* [13]. D-Glycero-D-mannoheptose was determined from LPS of *Chromobacterium violaceum* [14]. *Ch. violaceum* and N-glucosaminemyristate were generously provided by Drs. O. Lüderitz and U. Meier, Max-Planck-Institut für Immunbiologie, Freiburg, F.R.G.

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 (polydimethylsiloxane) glass capillary column used was 25 m × 0.22 mm I.D. with film thickness 0.14 μm and height equivalent of a theoretical plate 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 200°C. Programme: hold 2 min at 90°C, then 90°C to 260°C at 9°C/min with the attenuator of the Sigma 10 data system at −1. The paper speed was 10 mm/min. The sample (0.2 μl) was delivered as a splitless injection. The identity of the sugars in LPS, prepared by trifluoroacetic acid anhydride derivatization of LPS methanolysates, was established by direct cochromatography and by gas chromatography—mass spectrometry (GC—MS). They were identified tentatively by comparing their retention times with those of authentic standards. From each portion of methanolysed and derivatized LPS, three runs were made on the gas chromatograph. Accordingly, the quantitative values given in Table I are based on means from twelve runs with LPS from ATCC

TABLE I

PERCENTAGE SUGAR COMPOSITION OF PHENOL-EXTRACTED LIPOPOLYSACCHARIDE (S.D. = 5%)

Strain*	Rhamnose	Fucose	Galactose	Glucose	D-Glycero-D-mannoheptose	L-Glycero-D-mannoheptose	Galactosamine + glucosamine
Actinobacillus actinomycetemcomitans							
ATCC 33384 (NCTC 9710)	9.8	5.7	10.1	36.5	14.4	17.8	5.6
ATCC 29524	13.0	10.7	9.3	31.8	11.8	17.1	6.2
ATCC 29522	11.8	6.9	7.1	30.9	12.3	23.0	8.0
FDC 511	6.8	7.0	8.6	29.9	16.7	26.3	4.7
Kilian HK 435	8.3	10.0	9.6	30.1	16.2	21.6	4.1
FDC N 27	8.2	9.2	9.7	32.2	12.3	20.5	7.8
FDC Y 4	13.0	8.8	10.2	31.4	12.5	18.8	5.3
Haemophilus aphrophilus							
ATCC 33389 (NCTC 5906)	7.2	4.6	20.2	40.9		23.0	4.1
ATCC 19415 (NCTC 5886)	8.1	4.8	20.3	40.1		22.9	3.8
FDC 655	8.5	6.0	16.5	34.2		29.5	5.4
FDC 654	6.2	4.5	20.8	33.6		30.0	4.7
FDC 626	10.7	6.7	18.9	33.4		24.4	5.8
FDC 621	6.9	5.0	18.4	38.6		26.4	4.7

*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.

33384 and ATCC 33389, and from six runs with LPS from each of the remaining strains.

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 an OV-1 (methyl silicone) glass capillary column (20 m × 0.3 mm I.D.). Helium was used as carrier gas. The column temperature was programmed with a 2-min hold at 90°C, then from 90°C to 250°C at 9°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. Chemical ionization mass spectra were recorded with isobutane as a reactant gas and with an ionizing energy of 50 eV. 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

The content of carbohydrates in all the LPS preparations from *A. actinomycetemcomitans* and *H. aphrophilus* is given in Table I. The sugar composition of LPS from these two species was so different that *A. actinomycetemcomitans* could easily be distinguished from *H. aphrophilus*. For taxonomic differentiation, D-glycero-D-mannoheptose, which was detected exclusively in LPS from *A. actinomycetemcomitans*, served as a marker. The usefulness of this substance in chemotaxonomy was substantiated by the fact that it constituted a considerable portion of the carbohydrates in LPS from *A. actinomycetemcomitans*. There was also a marked difference in the amount of galactose in LPS from *A. actinomycetemcomitans* and *H. aphrophilus*, galactose being approximately twice as abundant in LPS from *H. aphrophilus* as in LPS from *A. actinomycetemcomitans*. The relationship between galactosamine and glucosamine was approximately 4:1, as measured in LPS from *A. actinomycetemcomitans* strain ATCC 33384.

TABLE II

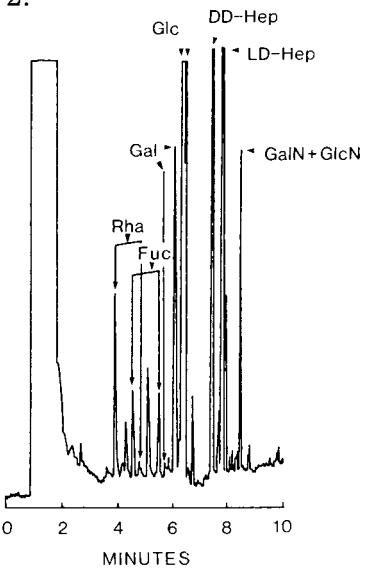
MOLAR RESPONSE RELATIVE TO TRIFLUOROACETYL DERIVATIVES OF GLUCOSE AND GALACTOSAMINE

Trifluoroacetyl derivatives	Relative molar response
Glucose	1.00
Galactosamine	1.00
Galactose	0.90
Fucose	0.86
Rhamnose	0.86
D-Glycero-D-mannoheptose*	1.25
L-Glycero-D-mannoheptose*	1.25

*Prepared by preparative gas chromatography.

Molar response factors of trifluoroacetyl derivatives of the detected sugars have been compared in Table II, using derivatives of glucose and galactosamine as basis.

Typical gas chromatograms of the methanolysed and derivatized sugars from LPS of *A. actinomycetemcomitans* and *H. aphrophilus* are given in Figs. 1 and 2.



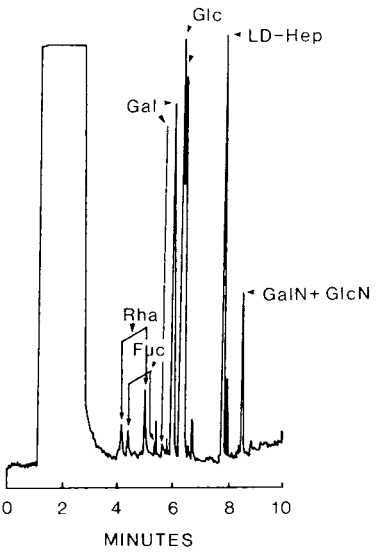


Fig. 1. Typical gas chromatogram of the sugar composition in LPS from *A. actinomycetemcomitans*, as represented by strain ATCC 33384. Abbreviations: Rha, rhamnose; Fuc, fucose; Gal, galactose; Glc, glucose; DD-Hep, D-glycero-D-mannoheptose; LD-Hep, L-glycero-D-mannoheptose; GalN; galactosamine; GlcN, glucosamine.

Fig. 2. Typical gas chromatogram of the sugar composition in LPS from *H. aphrophilus*, as represented by strain ATCC 33389. Abbreviations: Rha, rhamnose; Fuc, fucose; Gal, galactose; Glc, glucose; LD-Hep, L-glycero-D-mannoheptose; GalN, galactosamine; GlcN, glucosamine.

In Fig. 3 the mass spectrum of D-glycero-D-mannoheptose is shown. Fig. 4 gives three possible routes of fragmentation (Schemes 1—3) for methylheptoside derivatives of trifluoroacetic acid (D-glycero- and L-glycero-D-mannoheptose), as suggested by electron-impact mass spectrometry. The following ions and fragments were determined through high-resolution mass spectrometry: *m/e* 69 (CF_3), 97 ($COCF_3$), and 113 ($OCOCF_3$), and the remaining ions (*m/e* 139, 157, 170, 193, 196, 223, 252, 253, 265, 278, 295, 305, 309, 337, 451, 531, and 591) were in accordance with Fig. 4. M^+ was not recorded. The first way of fragmentation (Scheme 1) involves splitting off a heterocyclic ion with *m/e* 451, which eliminates TFA with formation of an *m/e* 337 ion. This ion splits off methanol, forming an ion with *m/e* 305, or TFA, with formation of the 223 ion. Also OCH_3 and $OCOCF_3$ may split off, producing an ion with *m/e* 193. Another possibility (Scheme 2) is the ring opening and migration of $OCOCF_3$ from C-3 to C-1, and cleavage of the C-1—O binding with the formation of an intense ion with *m/e* 157, approximately 25% of the base ion, or cleavage of the C-4—C-5 binding with production of an ion with *m/e* 265.

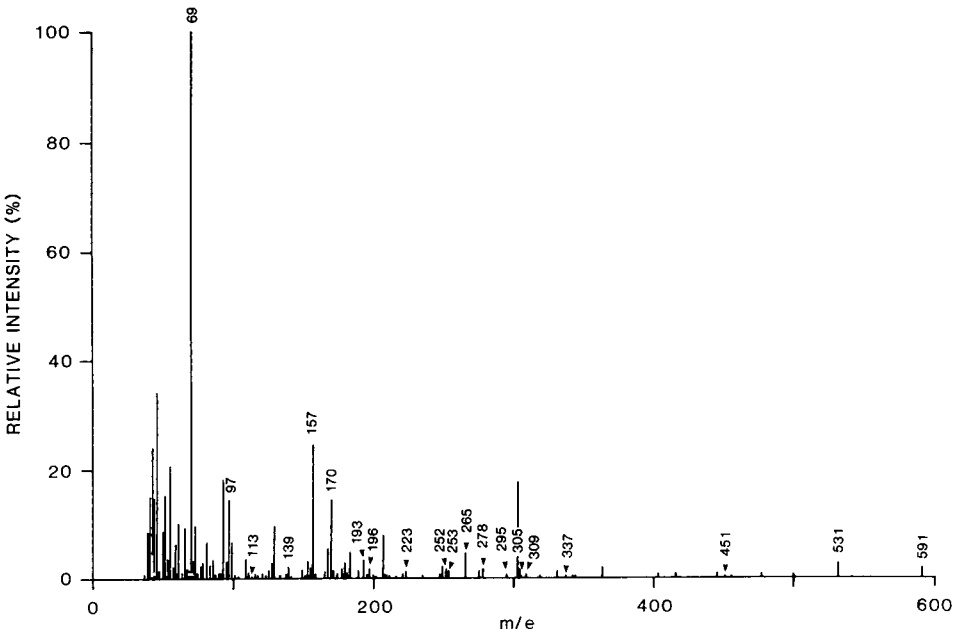


Fig. 3. Mass spectrum of D-glycero-D-mannoheptose.

The cleavage of the C-5—O binding leads to formation of an ion with *m/e* 531, which is fragmented into an ion with *m/e* 253 and 278. The third way of fragmentation (Scheme 3) involves cleavage of the C-1—O and C-2—C-3 bindings with formation of an ion with *m/e* 170 and cleavage of the C-1—C-2 and C-3—C-4 bindings with formation of an ion with *m/e* 252. Formation of the ions with *m/e* 309, 295, and 196 supported the proposals made on fragmentation of D-glycero- and L-glycero-D-mannoheptose.

DISCUSSION

Our results were in accordance with those of Kiley and Holt [15] who detected rhamnose, fucose, glucose, galactose, galactosamine, glucosamine, and heptose in LPS from *A. actinomycetemcomitans* strain FDC N 27 and Y 4. Bryn and Jantzen [16] were able to differentiate between D-glycero- and L-glycero-D-mannoheptose in one strain of *A. actinomycetemcomitans.* As far as we know, the sugars in LPS from *H. aphrophilus* have not previously been examined. Whereas the fatty acids of LPS from *A. actinomycetemcomitans* and *H. aphrophilus* did not provide any source of differentiation between these species [11], the sugar content of their LPS did. The outstanding feature of the present study was the demonstration of D-glycero-D-mannoheptose in phenol—water-extracted LPS from all reference and laboratory strains classified as *A. actinomycetemcomitans.* LPS from none of the examined strains of *H. aphrophilus* contained this aldoheptose. D-Glycero-D-mannoheptose, constituting a considerable portion of the sugar content in LPS from *A. actinomycetemcomitans*, may therefore serve as a marker in the chemotaxonomic differentiation between *A. actinomycetemcomitans* and *H. aphro-*

philus. D-Glycero-D-mannoheptose is a rare [1] but not unique constituent of the LPS core where the predominant heptose is L-glycero-D-mannoheptose [17]. Further, D-glycero-D-mannoheptose is an intermediate in the formation of L-glycero-D-mannoheptose [18], and both sugars are interconverted in the form of their nucleotide diphosphate derivatives by the action of a specific epimerase [19]. D-Glycero-D-mannoheptose has also been detected in LPS from *Haemophilus influenzae* type b [20]. This organism has $C_{15:0}$ as a bound cellular fatty acid [20]. Previously, we have found iso-$C_{15:0}$ acid as a free cellular acid in *A. actinomycetemcomitans* strain ATCC 29522 and FDC Y 4 [10]. The distribution of biological markers such as D-glycero-D-mannoheptose and C_{15} acids in specific strains of *A. actinomycetemcomitans* and type b of *H. influenzae* suggested some relationship between them. It is noteworthy that these strains seem to be more pathogenic in human disease than other strains of *A. actinomycetemcomitans* and *H. influenzae* [21, 22].

SCHEME 1

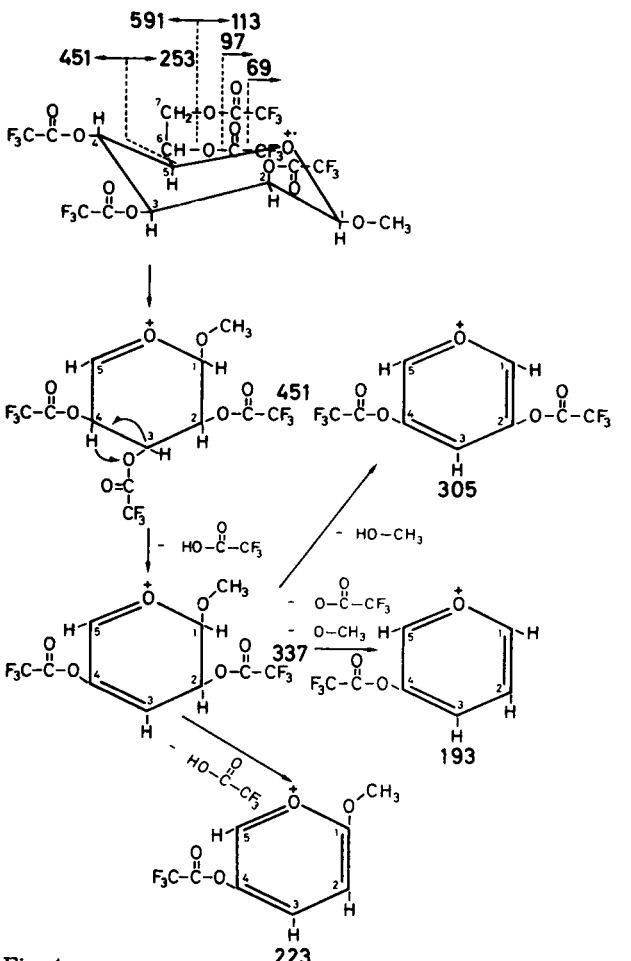
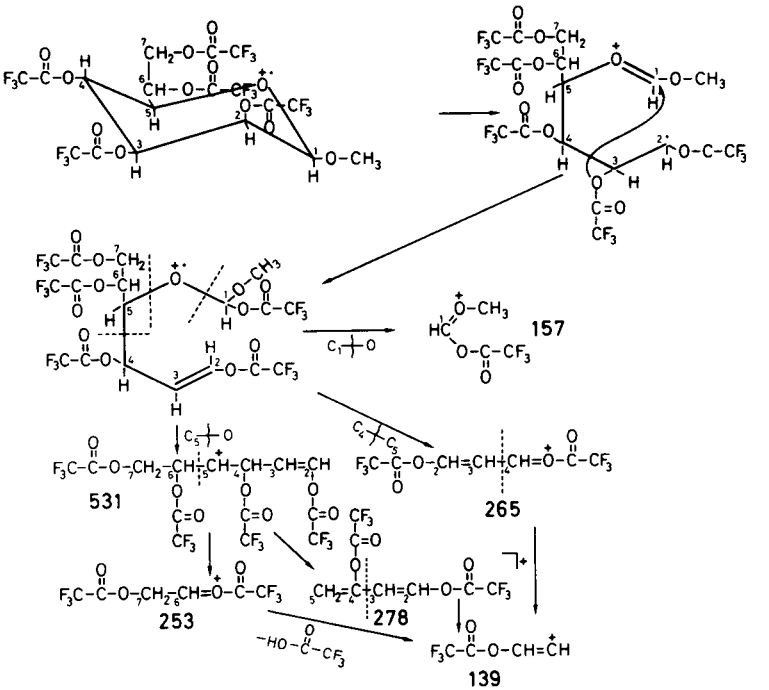


Fig. 4.

SCHEME 2



SCHEME 3

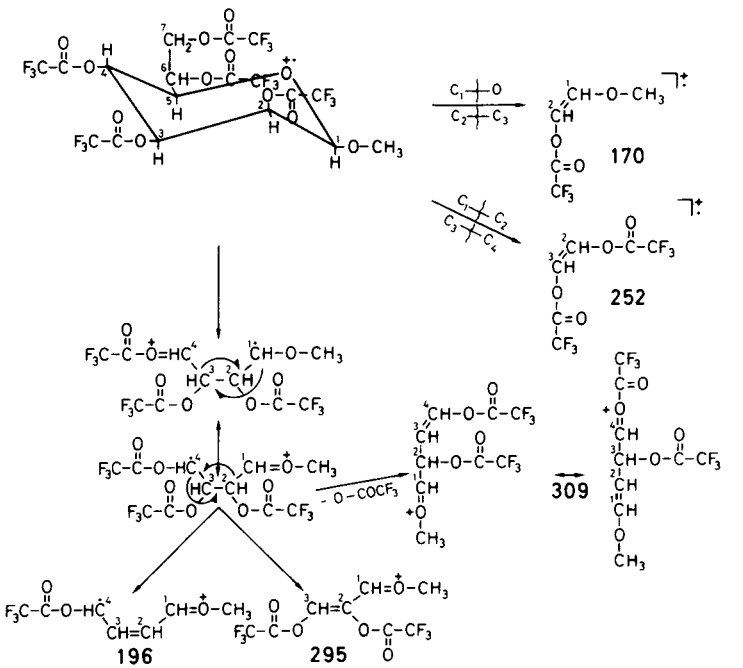


Fig. 4. Three main routes of fragmentation (Schemes 1—3) for methylheptoside derivatives of trifluoroacetic acid (D-glycero- and L-glycero-D-mannoheptose), as suggested by electron-impact mass spectrometry.

LPS from *H. aphrophilus* contained twice as much galactose as LPS from *A. actinomycetemcomitans*. This may reflect interspecies differences in the chemical composition of the O chain, and/or of the core, where galactose is a common component [1].

Previous taxonomic work has suggested that *A. actinomycetemcomitans*, being closely related to *H. aphrophilus*, should be included in the genus *Haemophilus* [23]. Until recently, *A. actinomycetemcomitans* was listed in Bergey's Manual of Determinative Bacteriology [24] as a species incertae sedis, uncertainty especially existing in the relationship to *H. aphrophilus*. The present study, based on the carbohydrate composition of LPS, provided clear evidence that *A. actinomycetemcomitans* and *H. aphrophilus* are distinct species and therefore should be maintained as such in current taxonomy. Even in our first study with free fatty acids in whole cells it was possible to distinguish between *A. actinomycetemcomitans* and *H. aphrophilus* [10]. Other reports based on biochemical characters [5], deoxyribonucleic acid relatedness [9, 25] and cellular proteins [26, 27] support this differentiation, and in a recent edition of Bergey's Manual of Systematic Bacteriology [28] *A. actinomycetemcomitans* was listed as an established species. Even if *Actinobacillus* and *Haemophilus* can be differentiated on the species level, this does not exclude relatedness at genus or family level. Actually, creation of a new family based on *Actinobacillus*, *Haemophilus* and *Pasteurella* has been suggested [9, 29].

The present study demonstrated that LPS is a preparation well fitted for taxonomic differentiation between closely related bacteria. As well as in *Enterobacteriaceae* [30], LPS has been found useful as a taxonomic marker in *Bacteroidaceae* [31].

The sugar content of LPS from *A. actinomycetemcomitans* and *H. aphrophilus* did not vary so much as to allow establishment of different chemotypes within these species. Usually, there is a correlation between the sugar composition and serological specificities of O antigens in LPS. King and Tatum [32] and Zambon et al. [21] were able to divide *A. actinomycetemcomitans* into serogroups which shared a common antigen with *H. aphrophilus*. It cannot be excluded that capsular polysaccharides of *A. actinomycetemcomitans* [33] represent the serotype specific antigens of this species, preventing anti-O or anti-R antibodies from reacting with the LPS antigens. LPS in the aqueous phase would be freed of possible contaminating capsular polysaccharides by our repeated ultracentrifugation [3, 11].

The difference in the sugar composition of LPS from *A. actinomycetemcomitans* and *H. aphrophilus* may have implications as to the virulence of these organisms. For *Fusobacterium* it has been demonstrated that the polysaccharide fraction of LPS stimulates bone resorption in vitro even more than lipid A [34]. Differences in the sugar composition of LPS may therefore confer different potential for induction of bone resorption to *A. actinomycetemcomitans* and *H. aphrophilus*.

Our experiments confirmed that conversion of carbohydrates into their polytrifluoroacetates, which are extremely volatile and polar, is a rapid and dependable analytical method for carbohydrates in a complex mixture [35, 36]. This method has been found superior to previously reported gas—liquid

chromatographic methods based on derivatization to methyl esters, polyacetates, or polytrimethylsilyl ethers (for review, see ref. 37). Methanolysis can in principle yield four different glycosides for each sugar, i.e. α- and β-anomers of both the methyl pyranosides and the methyl furanosides [38]. The relative portion of the different isomers for a given sugar is a function of the methanolysis conditions and usually is not altered by subsequent derivatization of the methyl glycosides. Quantitation of the sugars in the present chromatograms were usually based on multiple peaks, which were reproducible. It can also be made from selected peaks because the ratio between the peaks was stable under the experimental conditions used.

ACKNOWLEDGEMENTS

This work was supported in part by the Anders Jahres Fond til Vitenskapens Fremme and by the Proprietær Christopher Øverland og Hustru Ingeborg Øverlands Legat. The authors are also indebted to Drs. O. Lüderitz and U. Meier, Max-Planck-Institut für Immunbiologie, Freiburg, F.R.G., for generous supply of *Ch. violaceum* and N-glucosaminemyristate, to Dr. M. Kilian, Royal Dental College, Aarhus, Denmark, for *A. actinomycetemcomitans* strain HK 435, and to Cand. Real. A. Reine, Telemark Sentralsjukehus, Skien, Norway, for reading through the manuscript.

REFERENCES

1 O. Lüderitz, M.A. Freudenberg, C. Galanos, V. Lehmann, E.T. Rietschel and D.H. Shaw, Curr. Top. Membr. Transp., 17 (1982) 79.
2 K. Jann and O. Westphal, in M. Sela (Editor), The Antigens, Vol. III, Academic Press, New York, 1975, pp. 1—125.
3 O. Lüderitz, K. Jann and R. Wheat, in M. Florkin and E.H. Stotz (Editors), Comprehensive Biochemistry, Vol. 26A, Elsevier, Amsterdam, 1968, pp. 105—228.
4 F. Kauffmann, The Bacteriology of Enterobacteriaceae, Williams and Wilkins, Baltimore, MD, 1966, pp. 149—225.
5 J. Slots, Arch. Microbiol., 131 (1982) 60.
6 M.G. Newman, S.S. Socransky, E.D. Savitt, D.A. Propas and A. Crawford, J. Periodontol., 47 (1976) 373.
7 J. Slots, H.S. Reynolds and R.J. Genco, Infect. Immun., 29 (1980) 1013.
8 R.L. Mandell and S.S. Socransky, J. Periodontol., 52 (1981) 593.
9 A.C.R. Tanner, R.A. Visconti, S.S. Socransky and S.C. Holt, J. Periodontol. Res., 17 (1982) 585.
10 I. Brondz and I. Olsen, J. Chromatogr., 278 (1983) 13.
11 I. Brondz and I. Olsen, J. Chromatogr., 308 (1984) 19.
12 W. Weidel, Z. Physiol. Chem., 299 (1955) 253.
13 M.L. Osborn, S.M. Rosen, L. Tothfield and B.L. Horecher, Proc. Nat. Acad. Sci. U.S., 48 (1962) 1831.
14 D.A.L. Davies, Nature, 180 (1957) 1129.
15 P. Kiley and S.C. Holt, Infect. Immun., 30 (1980) 862.
16 K. Bryn and E. Jantzen, J. Chromatogr., 240 (1982) 405.
17 G.A. Adams, C. Quadling and M.B. Perry, Can. J. Microbiol., 13 (1967) 1605.
18 V. Lehmann, G. Hämmerling, M. Nurminen, I. Minner, E. Ruschmann, O. Lüderitz, T.-T. Kuo and B.A.D. Stocker, Eur. J. Biochem., 32 (1973) 268.
19 C. Galanos, O. Lüderitz, E.T. Rietschel and O. Westphal, in T.W. Goodwin (Editor), Biochemistry of Lipids II [International Review of Biochemistry, Vol. 14], University Park Press, Baltimore, MD, 1977, pp. 239—335.

20 J.L. Brice, T.G. Tornabene and F.M. LaForce, J. Infect. Dis., 140 (1979) 443.
21 J.J. Zambon, J. Slots and R.J. Genco, Infect. Immun., 41 (1983) 19.
22 W. Frederiksen and M. Kilian, in M. Kilian, W. Frederiksen and E.L. Biberstein (Editors), *Haemophilus, Pasteurella* and *Actinobacillus*, Academic Press, London, New York 1981, pp. 39—55.
23 M. Kilian, J. Gen. Microbiol., 93 (1976) 9.
24 J.E. Phillips, in R.E. Buchanan and N.E. Gibbons (Editors), Bergey's Manual of Determinative Bacteriology, Williams and Wilkins, Baltimore, MD, 8th ed., 1974, pp. 373—377.
25 A.L. Coykendall, J. Setterfield and J. Slots, Int. J. System. Bacteriol., 33 (1983) 422.
26 D.A. Calhoon, W.R. Mayberry and J. Slots, J. Clin. Microbiol., 14 (1981) 376.
27 E. Jellum, V. Tingelstad and I. Olsen, Int. J. System. Bacteriol., (1984) in press.
28 J.E. Phillips, in N.R. Krieg (Editor), Bergey's Manual of Systematic Bacteriology, Williams and Wilkins, Baltimore, MD, 9th ed., 1984, pp. 570—576.
29 W. Mannheim, S. Pohl and R. Holländer, Zentralbl. Bakteriol. Hyg. I. Abt. Orig. A, 246 (1980) 512.
30 H. Nikaido, Int. J. System. Bacteriol., 20 (1970) 383.
31 T. Hofstad, J. Gen. Microbiol., 85 (1974) 314.
32 E.O. King and H.W. Tatum, J. Infect. Dis., 111 (1962) 85.
33 S.C. Holt, A.C.R. Tanner and S.S. Socransky, Infect. Immun., 30 (1980) 588.
34 K. Sveen and N. Skaug, Scand. J. Dent. Res., 88 (1980) 535.
35 M. Vilkas, Hiu-I-Jan, G. Boussac and M.-C. Bonnard, Tetrahedron Lett., 14 (1966) 1441.
36 E. Jantzen, K. Bryn and K. Bøvre, Acta Pathol. Microbiol. Scand. Sect. B, 82 (1974) 753.
37 J. Shapira, T. Putkey, A. Furst and G.A. McCasland, Carbohydr. Res., 25 (1972) 535.
38 D.G. Pritchard and W. Niedermeier, J. Chromatogr., 152 (1978) 487.

Journal of Chromatography, 310 (1984) 273—281
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2202

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR SCREENING DISORDERS OF AROMATIC ACID METABOLISM USING A MULTI-DETECTION SYSTEM

HIDEMI TODORIKI*, TOKISHI HAYASHI and HIROSHI NARUSE

National Center for Nervous, Mental and Muscular Disorders, 2620, Ogawa-Higashi-Machi, Kodaira, Tokyo (Japan)

(First received February 3rd, 1984; revised manuscript received May 18th, 1984)

SUMMARY

This paper describes the use of a high-performance liquid chromatograph equipped with an ultraviolet multi-detection system for the analysis of aromatic acids to help establish a high-risk screening system for disorders of organic acid metabolism. The peak height ratios of about seventy metabolically important aromtic acids have been compiled using the multi-detection system. It may be possible to identify aromatic acids by comparing retention time and peak height ratios. The method was very effective for the diagonisis of disorders of aromatic acid metabolism.

INTRODUCTION

Since the identification of isovaleric acidaemia in 1966 by Tanaka et al. [1] using gas chromatography (GC) and gas chromatography—mass spectrometry (GC—MS), knowledge concerning organic acids in physiological fluids has been greatly extended and the number of types of organic acid disorders is still increasing.

In general, many organic acid disorders show closely similar clinical presentations, such as acidosis, ketosis, vomiting and coma, and their differential diagnosis cannot be made on clinical grounds alone. Most of the diseases reported are characterized by acute life-threatening illness in newborn babies and infants. Moreover, most surviving cases are often physically or mentally handicapped. With early diagnosis and proper therapy, patients have a better chance to survive and achieve normal physical and mental development. It is extremely important, therefore, that studies be carried out

0378-4347/84/$03.00

with a view to establishing methods for the early diagnosis and therapy of organic acid disorders.

Recently, Watts [2] reported that a study of the screening system for organic acid disorders was needed. He reported also that the disease incidence of patients with such organic acid disorders could be about 1 in 10,000, which is roughly the same as the incidence of phenylketonuria in Caucasian populations. Therefore, it will be necessary to employ a high-risk screening system for organic acid disorders, which means screening newborn babies or infants who show some clinical signs of the diseases.

Several analytical methods for organic acids in biological fluids have been reported, including paper chromatography [3], thin-layer chromatography [4], high-performance liquid chromatography (HPLC) [5—9], GC [10] and GC—MS [11—18]. Of these GC—MS has been used in the clinical diagnosis of organic acid disorders. However, the technique is expensive and not convenient for the routine analysis of a large number of clinical samples. Screening for organic acid disorders using GC—MS is only possible in a very limited number of laboratories. If a detection system able to provide good-quality information could be developed, it would be possible to screen large numbers of samples by means of the GC and HPLC methods.

This paper describes an HPLC method using a multi-detection system as a tool in screening for aromatic acid disorders.

EXPERIMENTAL

Apparatus

A Tri Rotar III high-performance liquid chromatograph equipped with three Uvidec 100-II UV spectrometers (Japan Spectroscopic, Tokyo, Japan) was used. Sample injections were performed automatically with a KSST-60 auto sample injector (Kyowa Seimitsu, Tokyo, Japan) connected to the chromatograph. HPLC separation was carried out with a stainless-steel column (250 × 4 mm I.D.) packed with LiChrosorb RP-8 (particle size 5 μm; E. Merck, Darmstadt, F.R.G.) by a balanced density slurry packing method. The operating conditions for HPLC are given in the legend to Fig. 1.

Reagents

All aromatic acids used were purchased commercially from Aldrich (Milwaukee, WI, U.S.A.), Sigma (St. Louis, MO, U.S.A.), Nakarai (Kyoto, Japan) or Wako (Osaka, Japan) and were used without purification. The other reagents and solvents were reagent grade.

Procedure

To a urine sample corresponding to 0.2 mg of creatinine, 200 μl of a 0.1 *M* solution of disodium hydrogen phosphate, containing 100 nmol of 2-hydroxy-3-naphthoic acid as internal standard, were added. The solution was diluted to 2 ml with redistilled water and then washed with 5 ml of ethyl acetate by shaking for 5 min. Urinary aromatic acids were then extracted with 5 ml of ethyl acetate by shaking for 5 min after the addition of 0.6 g of sodium

chloride and 0.5 ml of 2 *M* hydrochloric acid. The ethyl acetate layer was dried over sodium sulphate and evaporated to dryness. The residue was redissolved with two drops of N,N-dimethylformamide and diluted with six drops of 0.05 *M* phosphate buffer (pH 2.50). A 30-μl aliquot of the resulting solution was injected into the HPLC system.

Determination of creatinine

The amount of creatinine in the urine was determined by the Jaffe reaction [19]. Absorbance was measured at 520 nm with a Model UV-150-02 spectrophotometer (Shimadzu Seisakusho, Kyoto, Japan).

RESULTS AND DISCUSSION

In recent years, HPLC has been one of the fastest growing analytical techniques in the world and is used in such areas as analytical, biological and clinical chemistry, etc. This growth is due to the reliability and versatility of the separation. In general, the most popular detectors for HPLC are the UV spectrophotometer, fluorescence spectrophotometer or electrochemical detector. These detectors are highly sensitive for many compounds, but they provide little information about peak components except the retention time. Therefore, it is generally difficult to identify peak components using an HPLC system equipped with one of these detectors. This is a significant disadvantage of an HPLC system, but in order to obtain a more definitive identification of compounds, it will be necessary to develop a new type of HPLC detector, which can provide some qualitative information concerning peak components. In this investigation, a multi-detection system made up of plural detectors (in this study, three single UV detectors) was used to obtain some qualitative information. This system could facilitate the identification of aromatic acids. The three UV detectors were set at 260, 280 and 320 nm, respectively. These wavelengths lie in the UV absorption region of aromatic acids. We examined the retention times and peak height ratios of about seventy authentic samples of aromatic acids using this multi-detection system.

Most of the aromatic acids listed in Table I are normal metabolic intermediates and/or various unusual metabolites, which are known to accumulate in the urine of patients with organic acid disorders. Some groups of aromatic acids gave close retention times. These data suggest that it is not easy to identify peak components from the retention time alone. Table I indicates that even the organic acids that yielded very similar retention times can be distinguished from each other by comparing the two sets of peak height ratios. These peak height ratios must show constant values for each aromatic acid independent of its concentration, since the values represent the ratios of molecular absorption coefficients at 260, 280 and 320 nm. Thus, the data suggest that identification might be achieved by comparing the retention time and the peak height ratios, if the peak component could be considered to be single or almost single.

Tanaka and Hine [10] reported that organic acid disorders are commonly characterized by the urinary excretion of extremely large amounts of certain metabolic intermediate organic acids which are not excreted, or which are

TABLE I

RELATIVE RETENTION TIMES AND THE PEAK HEIGHT RATIOS OF AROMATIC ACIDS USING THE UV MULTI-DETECTION SYSTEM

The relative retention time, t_R (rel), was calculated for each aromatic acid relative to 2-hydroxy-3-naphthoic acid (internal standard). The values PH_{260}, PH_{280} and PH_{320} are peak heights at 260, 280 and 320 nm at 0.16, 0.16 and 0.04 a.u.f.s., respectively.

Acids	t_R (rel)	PH_{260}/PH_{280}	PH_{320}/PH_{280}
Imidazolepyruvic	0.09	0.64	0.23
5-Methylorotic	0.10	0.60	0.13
Orotic	0.10	0.54	0.07
Picolinic	0.11	2.20	0.07
Quinolinic	0.11	0.80	0.66
Isonicotinic	0.11	1.99	0.11
Nicotinic	0.12	3.07	—
Urocanic	0.14	1.07	0.04
Citrazinic	0.14	0.40	0.01
4-Hydroxymandelic	0.17	0.68	0.01
3-Hydroxymandelic	0.23	0.43	0.02
Vanillylmandelic	0.24	0.39	0.03
Homogentisic	0.25	0.15	0.13
Benzoylformic	0.33	4.15	0.15
Protocatechuic	0.37	1.90	0.30
2-Furoic	0.40	8.05	0.05
3,4-Dihydroxyphenylacetic	0.41	0.27	0.02
Mandelic	0.45	13.5	3.20
4-Hydroxyphenyllactic	0.46	0.43	0.03
Xanthurenic	0.48	2.72	3.61
4-Hydroxybenzoic	0.50	2.15	0.03
2,3-Dihydroxybenzoic	0.52	2.55	17.0
Kynurenic	0.53	3.33	13.9
Phenylpyruvic	0.53	1.20	—
4-Hydroxyphenylacetic	0.53	0.46	0.03
5-Hydroxyindoleacetic	0.53	0.61	0.44
2-Methoxymandelic	0.54	0.47	0.03
Vanillyllactic	0.54	0.30	0.03
Phthalic	0.54	0.88	0.05
Quinalidinic	0.54	1.31	18.2
3,4-Dihydroxyhydrocinnamic	0.54	0.23	0.03
Hippuric	0.54	2.89	0.18
3-Hydroxybenzoic	0.57	0.54	1.32
3-Hydroxyphenylacetic	0.57	0.46	0.02
3-Methoxymandelic	0.58	0.45	0.04
Anthranilic	0.59	3.30	19.0
Homovanillic	0.60	0.29	0.03
Caffeic	0.60	0.51	6.53
2-Hydroxyphenylacetic	0.60	0.56	0.02
Vanillic	0.61	1.71	0.14
Terephthalic	0.62	3.20	0.09
Tropic	0.63	22.7	1.00
4-Hydroxyphenylpyruvic	0.64	0.28	4.60
β-Phenyllactic	0.64	10.5	—
3-Hydroxy-4-methoxybenzoic	0.64	1.94	0.31
2-Hydroxyhippuric	0.65	0.86	2.66

TABLE I (continued)

Acids	t_R (rel)	PH_{260}/PH_{280}	PH_{220}/PH_{280}
β-3-Indolelactic	0.69	0.57	0.03
4-Hydroxycinnamic	0.70	0.27	5.30
Benzylmalonic	0.72	0.60	0.04
4-Hydroxy-3-methoxyphenylpyruvic	0.72	0.43	7.00
Ferulic	0.75	0.49	7.26
2-Methoxybenzoic	0.75	0.38	1.16
Salicylic	0.76	0.26	3.83
Benzoic	0.76	0.97	0.02
Veratric	0.77	1.76	0.17
3,4-Dimethoxyphenylacetic	0.77	0.34	0.03
5-Indolecarboxylic	0.78	0.83	0.46
3-Indoleacetic	0.79	0.60	0.02
2-Hydroxycinnamic	0.79	0.56	2.16
5-Methoxyindole-3-acetic	0.79	0.61	0.29
4-Methoxyphenylacetic	0.82	0.49	0.03
3-Methoxyphenylacetic	0.83	0.55	0.05
2-Methoxyphenylacetic	0.84	0.84	0.04
4-Methoxybenzoic	0.85	3.25	0.03
3,4-Dimethoxycinnamic	0.90	0.42	6.70
3-Indolepyruvic	0.91	0.66	11.2
3,4,5-Trimethoxycinnamic	0.93	0.37	6.10
Cinnamic	0.96	0.62	0.09
3-Indolebutyric	1.01	0.55	0.03
3-Phenyl-*n*-butyric	1.03	12.5	0.20
4-Phenyl-*n*-butyric	1.04	10.7	0.06
5-Methylindole-2-carboxylic	1.04	1.92	0.16

excreted only in small amounts into normal urine. Table II shows the lowest aromatic acid levels that are required for identification by HPLC with a multi-detection system. These results were obtained adding three different amounts of authentic aromatic acids to normal urine samples. In this investigation, a normal urine sample which gave relatively intense peaks on the chromatograms was chosen from the fifty normal urine samples investigated. The results suggest that if certain organic acids above 100 μg excreted into a urine corresponding to 0.2 mg of creatinine, almost all of the aromatic acids in Table II could be identified simply by comparing the retention times and the peak height ratios with those of authentic samples in Table I.

The level necessary for peak identification using the multi-detection system is generally much lower than that found in the urine of many aromatic acid disorders. For example, LaDu [20] reported that 1—4 mg of homogentisic acid were excreted into urine corresponding to 0.2 mg of creatinine in cases of alcaptonuria. In another type of organic acid disorder, tyrosinuria, *p*-hydroxyphenylpyruvic acid, *p*-hydroxyphenyllactic acid and *p*-hydroxyphenylacetic acid are increased from 88 to 170 times normal levels [21]. Fig. 1 (left) shows a chromatogram obtained from a urine sample from a 1.5-year-old male with high blood tyrosine (38 mg/dl). The peaks marked A, A′, A″, B and C were extremely large compared with the chromatogram of a normal subject (Fig. 1,

TABLE II

ORGANIC ACID LEVELS AT WHICH IT IS POSSIBLE TO IDENTIFY ORGANIC ACIDS IN URINE

+ = identified, — = not identified.

Acids	Amount added (mg/sample)		
	0.01	0.1	1.0
4-Hydroxymandelic	—	+	+
3-Hydroxymandelic	—	+	+
Vanillylmandelic	+	+	+
Homogentisic	+	+	+
Protocatechuic	+	+	+
3,4-Dihydroxyphenylacetic	—	+	+
4-Hydroxyphenyllactic	—	+	+
Xanthurenic	+	+	+
Kynurenic	+	+	+
4-Hydroxyphenylacetic	—	+	+
5-Hydroxyindoleacetic	+	+	+
Hippuric	—	+	+
3-Hydroxybenzoic	—	+	+
Homovanillic	+	+	+
Caffeic	—	+	+
2-Hydroxyphenylacetic	—	—	+
3-Hydroxy-4-methoxybenzoic	+	+	+
2-Hydroxyhippuric	+	+	+
4-Cumaric	+	+	+
β-3-Indoleacetic	—	+	+
Ferulic	—	+	+
2-Methoxybenzoic	+	+	+
Salicylic	+	+	+
Veratric	—	+	+
5-Indolecarboxylic	+	+	+
2-Cumaric	+	+	+
4-Methoxyphenylacetic	—	+	+
3-Methoxyphenylacetic	—	+	+
Cinnamic	+	+	+

right). The retention time and the peak height ratios of each large peak seem to be close to those of *p*-hydroxyphenylpyruvic acid (A), its degradation products (A′, A″), *p*-hydroxyphenyllactic acid (B) and *p*-hydroxyphenylacetic acid (C) (Table III). It was therefore concluded that the peak components in question were the above-mentioned aromatic acids. Thus, this case was suspected as tyrosinaemia due to a deficiency of *p*-hydroxyphenylpyruvate oxidase on the basis of these results.

A few methods have been reported for the screening of organic acids by means of GC or HPLC. Tanaka and Hine [10] described a GC method of urinary organic acid analysis, which was designed to be used in screening programmes for organic aciduria; they reported that the retention indices, in terms of methylene units (MU), on two kinds of column for 163 metabolites were useful to make a diagnosis of the well defined organic aciduria. We have

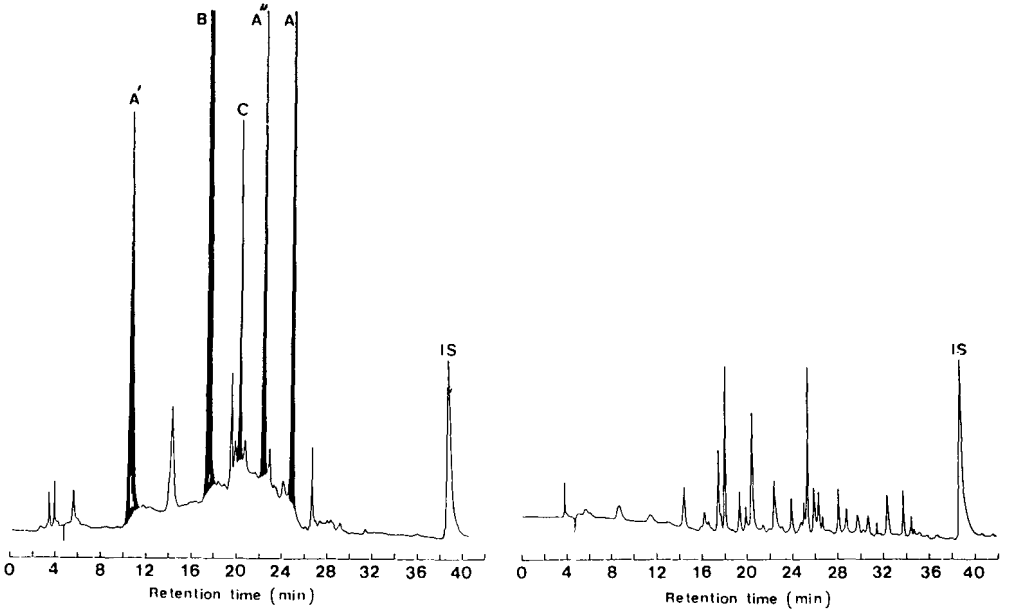


Fig. 1. High-performance liquid chromatograms of aromatic acids in urine from a patient with tyrosinaemia (left) and from a normal adult (right). The relative retention times and the peak height ratios of aromatic acids in urine on the chromatogram are shown in Table III. Operating conditions: column, LiChrosorb RP-8 (5 μm, 250 × 4 mm I.D.); column temperature, 50°C; flow-rate, 1.0 ml/min; solvent A, 0.05 *M* phosphate buffer (pH 2.5); solvent B, 80% acetonitrile; gradient, 0% B to 65% B in 40 min; detection, UV at 280 nm. Each separation was started after reconditioning the column with 150 ml of solvent A. IS = internal standard. For other peaks idenfications see Table III.

TABLE III

RELATIVE RETENTION TIME AND RESPONSE OF URINARY AROMATIC ACIDS FROM A PATIENT WITH TYROSINAEMIA USING THE UV MULTI-DETECTION SYSTEM

The values in parentheses are peak height ratios and retention times of authentic aromatic acids in Table I.

Peak	t_R (rel)	PH_{260}/PH_{280}	PH_{320}/PH_{280}	Acids
A	0.66	0.21	4.76	4-Hydroxyphenylpyruvic acid
	(0.64)	(0.28)	(4.60)	
A'	0.28	0.75	0.37	
	(0.27)	(0.70)	(0.38)	Degradation products of
A''	0.57	0.51	0.35	4-hydroxyphenylpyruvic acid
	(0.58)	(0.51)	(0.33)	
B	0.46	0.45	0.03	4-Hydroxyphenyllactic acid
	(0.46)	(0.43)	(0.03)	
C	0.53	0.46	0.03	4-Hydroxyphenylacetic acid
	(0.53)	(0.46)	(0.03)	

tried the GC method which Tanaka and Hine [10] reported, but their method did not always give satisfactory results for the identification of some kinds of urinary organic acids. The reason why satisfactory results could not be

obtained is that the differences between MU indices obtained with the two types of GC columns were too small to identify many organic acids.

Buchanan and Thoene [7] reported the development of urinary organic acid profiling analysis of urinary organic acids utilizing two columns in series. The R_F values, together with UV absorbance ratios, were used for compound identification. Buchanan and Thoene [7] also described that the dual-column system affords better resolution of urinary organic acids than does either column separately. However, the separation of aromatic acid was not so satisfactory in comparison with results obtained by our method. In order to establish a screening system for organic acid disorders, the identification of more than one hundred organic acids would be required. However, it would be difficult to screen organic acid disorders using the dual-column method because the numbers of the different kinds of organic acids reported were not enough for identification purposes. Also, Buchanan and Thoene [7] presented 200-nm and 230-nm chromatograms obtained from a urine sample from an infant with methylmalonic aciduria. The peak on the 200-nm chromatogram, which is assigned to methylmalonic acid, is not particularly strong and the peak on the 230-nm chromatogram is absent. Therefore, it would seem difficult to obtain the peak height ratios of methylmalonic acid and to diagnose the aliphatic acid disorders using peak height ratios. Moreover, as the aliphatic acids have small molecular absorption coefficients in the UV range compared with those of aromatic acids, the peaks of aliphatic acids must be hidden by those of aromatic acids.

For the purpose of the analysis of the aromatic acid group alone, the present UV multi-detection system might be in most cases capable of spectrophotometrically resolving aromatic acids from aliphatic acids. Moreover, by means of gradient elution techniques, a good separation of the peaks on the chromatogram was achieved. The method in this investigation offers several advantages over the currently available procedure for the identification of urinary aromatic acids and was very effective for the diagnosis of aromatic acid disorders.

CONCLUSION

Organic acid disorders are characterized by acute life-threatening illness in newborn babies and infants. If these disorders are not detected early, they frequently lead to early death, or physical and mental handicaps. However, the limited availability of the GC—MS system has hindered early diagnosis of organic disorders in many areas. For the purpose of developing a new tool for the screening for aromatic acid disorders, a new HPLC detection system, the multi-detection system, was developed, and the HPLC system described above could easily identify the peak components of aromatic acids which were excreted in large amounts into the urine.

ACKNOWLEDGEMENT

This work was supported by Grant No. 83-11 from the National Center for Nervous, Mental and Muscular Disorders of Ministry of Health and Welfare, Japan.

REFERENCES

1 K. Tanaka, M.A. Budd, L. Erfron and K.J. Isselbacher, Proc. Nat. Acad. Sci. U.S., 56 (1966) 236.
2 R.W.E. Watts, in H. Naruse and M. Irie (Editors). Proceedings of the Second International Conference on Neonatal Thyroid Screening and the International Symposium on Neonatal Screening for Inborn Errors of Metabolism, Tokyo, August 16—21, 1982, Excerpta Medica, Amsterdam, 1983, p. 392.
3 J. Nordman and R. Nordman, Advan. Clin. Chem., 4 (1961) 52.
4 J.W.T. Seakins, in J. Stern and C. Toothill (Editors), Organic Acidurias, Churchill Livingstone, Edinburgh and London, 1972, p. 175.
5 I. Molnár and C. Horváth, J. Chromatogr., 143 (1977) 391.
6 D.N. Buchanan and J.G. Thoene, J. Liquid Chromatogr., 4 (1981) 1587.
7 D.N. Buchanan and J.G. Thoene, Anal. Biochem., 124 (1982) 108.
8 A. Rehman, S.C. Gates and J.W. Webb, J. Chromatogr., 228 (1982) 103.
9 E.L. Mattiuz, J.W. Webb and S.C. Gates, J. Liquid Chromatogr., 5 (1982) 2343.
10 K. Tanaka and D.G. Hine, J. Chromatogr., 239 (1982) 301.
11 E. Jellum, Trends Anal. Chem., 1 (1981) 12.
12 S.C. Gates and C.C. Sweeley, Clin. Chem., 24 (1978) 1663.
13 E.C. Horning and H.G. Horning, Clin. Chem., 17 (1971) 802.
14 S.C. Gates, N. Dendraims and C.C. Sweeley, Clin. Chem., 24 (1978) 1674.
15 J.A. Thompson and S.P. Markey, Anal. Chem., 47 (1975) 1313.
16 K. Tanaka, D.E. Hine, T.B. Lynn and T. Lowe, Clin. Chem., 26 (1980) 1874.
17 S.C. Gates, C.C. Sweeley, W. Krivit, D. DeWitt and B.E. Blaisdell, Clin. Chem., 24 (1978) 1680.
18 A.M. Lawson, R.A. Chalmers and R.W.E. Watts, Clin. Chem., 22 (1976) 1283.
19 M.Z. Jaffe, Physiol. Chem., 10 (1886) 391.
20 D.N. LaDu, in J.B. Stunbury, J.B. Wyngarden and D.S. Fredrickson (Editors), The Metabolic Basis of Inherited Diseases, McGraw Hill, New York, 3rd ed., 1982, p. 268.
21 L.A. Goldsmith, in J.B. Stunbury, J.B. Wyngarden and D.S. Fredrickson (Editors), The Metabolic Basis of Inherited Diseases, McGraw Hill, New York, 3rd ed., 1982, p. 287.

Journal of Chromatography, 310 (1984) 283—295
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2200

NON-RADIOCHEMICAL PROCEDURE FOR THE MEASUREMENT OF O-METHYLATION OF THE STEREOISOMERS OF ISOPRENALINE

SUZANNE BARONE*, ROBERT E. STITZEL and RICHARD J. HEAD

Department of Pharmacology and Toxicology, West Virginia University, Morgantown, WV 26506 (U.S.A.)

(First received January 31st, 1984; revised manuscript received May 15th, 1984)

SUMMARY

A non-radiochemical procedure has been developed which permits the separation and measurement of isoprenaline (ISO) and the O-methylated metabolite, 3-methoxyisoprenaline (MeOISO). This methodology employs alumina chromatography and toluene solvent extraction to separate the catecholamine, ISO, from the O-methylated derivative, MeOISO. High-performance liquid chromatography with electrochemical detection has been used to quantify these compounds. The biological application of this procedure includes the evaluation of O-methylation of the stereoisomers of ISO by intact tissues.

INTRODUCTION

Extraneuronal uptake (ENU) and O-methylation by the enzyme catechol-O-methyltransferase (COMT) are mechanisms for the synaptic removal and intracellular inactivation of the neuronally released transmitter, noradrenaline, by effector tissues. Isoprenaline (ISO) has been used commonly as a substrate for examining these two processes. Since this catecholamine is not metabolized by the enzyme monoamine oxidase, but is metabolized by COMT it has only one major metabolite, 3-methoxyisoprenaline (MeOISO). Additionally, ISO is also not a good substrate for neuronal uptake. Thus, this amine appears to be an ideal substrate for studies on ENU since its uptake and metabolite profile is less complicated than that for other catecholamines including the naturally occurring noradrenaline and adrenaline.

Much of the experimentation in this area has involved the use of tritiated *dl*-isoprenaline ([^{3}H]ISO). The methods used in the past for the separation of [^{3}H]ISO and [^{3}H]MeOISO have included thin-layer chromatography [1], solvent extraction [2], and column chromatography [3]. The limitations of

0378-4347/84/$03.00

these experimental approaches lie not within the methodologies themselves, but in the use of radiolabeled substrates. In addition to the high cost of purchase and disposal of radiochemicals, several laboratories have described the presence of impurities as a problem with several substrates including ^{3}H-ISO [2, 4]. Impurities create the additional requirement of employing a purification procedure prior to the use of the radiolabeled substrate.

Although radiochemical procedures are quite sensitive, their use in studies of ENU are severely limited. For example, an examination of the stereospecificity of the ENU system using ISO as substrate cannot be accomplished since ^{3}H-ISO is commercially available only as the racemic mixture. Another drawback associated with the use of radiochemical procedures is the difficulty encountered in attempting to carry out multi-substrate studies. This is due both to the limitations of scintillation spectrometry and to the cross-contamination that can occur when different isotopically labeled compounds are used.

The problems outlined about have prompted us to seek a non-radiochemical procedure for the detection of ISO and MeOISO which approaches the sensitivity of the existing radiochemical methods. Most of the existing non-radiochemical procedures which employ high-performance liquid chromatography (HPLC) to examine O-methylation and COMT activity involve the use of purified rat liver COMT with other catecholamines such as noradrenaline or adrenaline as substrates [5—7]. Since the primary aim of our study was to examine the uptake and O-methylation of ISO by intact vascular tissue, these procedures could not be employed. We present here a newly developed assay procedure employing HPLC with electrochemical detection (ED) that permits the measurement of the O-methylation of ISO which occurs in intact vascular tissue.

EXPERIMENTAL

Apparatus

An HPLC apparatus consisting of a Waters Assoc. M-45 pump solvent delivery system, a Waters U6K universal injector with 1.5-ml injection sample loop, C_{18} filled guard and 10 μBondapak C_{18} stainless-steel reversed-phase columns (30 cm × 3.9 mm I.D.), and radial compression cartridges prepacked with C_{18} (10 μm, 12 cm × 8 mm I.D.) were used for the separation procedure. A Bioanalytical LC-4B amperometric detector with a glassy carbon electrode was used for the electrochemical detection measurements.

Chemicals

d-ISO (bitartrate salt), *l*-ISO (hydrochloride), and 3-methoxytyramine were obtained from Sigma (St. Louis, MO, U.S.A.). 3,4-Dihydroxybenzylamine (hydrobromide) was obtained from Aldrich (Milwaukee, WI, U.S.A.). 3-Methoxyisoprenaline and 3,4-dihydroxy-2-methyl propriophenone were gifts of Boehringer Ingleheim (Ridgefield, CT, U.S.A.) and Upjohn (Kalamazoo, MI, U.S.A.), respectively. Acid alumina AG4 was purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.) HPLC grade methanol, EDTA and acids and salts for the various buffers were reagent grade from Fisher Scientific (Fair Lawn, NJ, U.S.A.).

Incubation procedure

The tissue incubation procedure employed was a modification of that described by Levin [8]. Male New Zealand white rabbits (1.5—3.5 kg) were killed by stunning with a blow to the head followed by exsanguination. The entire length of the thoracic aorta (from heart to diaphragm) was removed and cleaned of adhering blood and fat, slit lengthwise, cut into eight segments, blotted and weighed (25—55 mg segments). The segments were then placed in 1 ml of physiological Krebs solution (117 m*M* NaCl, 4.8 m*M* KCl, 1.2 m*M* KH_2PO_4, 25 m*M* $NaHCO_3$, 2.5 m*M* $CaCl_2$, 5.5 m*M* glucose, 0.03 m*M* EDTA), and oxygenated with oxygen—carbon dioxide (95:5) at 37° C. The tissues were preincubated for 30 min prior to transfer to 1 ml of fresh oxygenated Krebs solution. ISO was then added and the tissues, along with appropriate drug blanks, tissue blanks, and recovery tubes, were incubated for an additional 60 min.

Post-incubation tissue preparation

After the 1-h incubation period, the tissue segments were washed for 1 min in fresh oxygenated Krebs solution, blotted, and placed in 1 ml of 0.4 *M* perchloric acid containing the appropriate internal standards. Dihydroxybenzylamine (DHBA) and 3-methoxytyramine (3-MT) were the internal standards for ISO and MeOISO, respectively. The concentration of internal standard used varied with the incubation substrate concentration. The tissues were then homogenized using a glass—glass homogenizer (Kontes Duall size 20) equipped with a motor-driven pestle. The homogenate was placed in a 1.5-ml capacity polypropylene microcentrifuge tube and centrifuged for 5 min at 3250 *g* (Beckman Microfuge II). The resulting supernatant fluid was then frozen (—5° C) until a precolumn extraction could be performed.

Post-incubation incubate preparation

After the 1-h incubation, 800 μl of the incubation solution were added to tubes containing the appropriate internal standards in 100 μl of 4.0 *M* perchloric acid. The internal standards were made in 0.01 *M* hydrochloric acid, and then diluted to the proper concentration in 4.0 *M* perchloric acid shortly before adding the incubation solution. A 100-μl aliquot of fresh Krebs solution was added to give a final volume of 1 ml and a final perchloric acid concentration of 0.4 *M*. The samples were then vortexed and frozen to await precolumn extraction. It should be noted that a correction factor of 1.25 was applied when calculating the total incubate metabolite concentrations in order to account for the entire 1 ml of original incubation solution.

Precolumn purification

The precolumn purification procedure was designed to separate the substrate, ISO, and the metabolite, MeOISO, into separate fractions.

Catechol fraction (ISO)

The frozen acidified samples of incubation media and/or tissue homogenate extracts were thawed and 200 μl of a sodium metabisulfate—EDTA mixture (0.5 g sodium metabisulfite in 20 ml of 5% EDTA solution) were added; acid alumina (140 mg) also was added. The tubes were vortexed and the pH

adjusted to 8.4 with 3 *M* Tris buffer (pH 10.9). The tubes were shaken (approximately 220 oscillations per min) at a 45° angle for 15 min, and then centrifuged for 2 min at 140 *g*. The resulting supernatant fluid (alumina effluent) was collected and stored on ice for later processing (see *O-Methylated catechol fraction*). The alumina was washed with 5 ml of 0.03 *M* phosphate buffer (pH 7.3) by vortexing and centrifuging; wash solutions were discarded. The wash procedure was then repeated with 3.0 ml of distilled water.

Catechols were eluted from the alumina by washing twice with 300 μl of 1 *M* acetic acid containing 0.01 *M* boric acid. Each elution was carried out by shaking the samples for 10 min and then centrifuging. The eluates were pooled, placed in glass culture tubes, and dried on a Buchler vortex evaporator (approximately 1 h). Once dried, the samples were reconstituted in 200 μl of 0.05 *M* acetic acid and frozen until assay by HPLC.

O-Methylated catechol fraction (MeOISO)

To the alumina effluent (pH 8.4) that was saved from a previous step (see above), 250 μl of sodium borate (2 *M* sodium hydroxide with 1 *M* boric acid) were added. Powdered potassium phosphate (dibasic, anhydrous, 2.5 g) was then added and the samples were vortexed. Toluene (5 ml) was added and the samples were shaken for 15 min. Following centrifugation (140 *g* for 2 min), 4 ml of the toluene phase were transferred to tubes containing 500 μl of 0.5 *M* acetic acid. Fresh toluene (5 ml) was added to the original tubes and the extraction steps (i.e., shaking, centrifugation, and 4.0 ml transfer) were repeated. The resulting 8.5 ml of toluene and the acetic acid were then shaken for 20 min. Following centrifugation, the aqueous layer was frozen (dry ice—methanol) and the toluene layer discarded. The aqueous layer was then thawed and transferred to glass culture tubes and dried in a Buchler vortex evaporator for approximately 1 h. The dried samples were reconstituted in 200 μl of 0.05 *M* acetic acid and were frozen awaiting HPLC assay. It should be noted that a correction factor of 1.25 had to be used when calculating the total O-methylated catecholamine content to account for the entire 10 ml of toluene used in the extraction process.

HPLC—ED assay

Conditions for ISO and DHBA. A Waters reversed-phase C_{18} radial compression module cartridge (12 cm × 8 mm I.D.) was used for the separation of ISO and DHBA. The mobile phase consisted of a citrate—phosphate buffer (0.1 *M* $Na_2HPO_4 \cdot 7H_2O$ with the pH adjusted to 4.0 with 0.1 *M* citrate solution) containing 25—35% methanol. The amount of methanol used was dependent upon the age and amine retention times of individual cartridges. The mobile phase was delivered by the pump at a flow-rate of 2 ml/min. The potential setting which gave optimal detection was 0.70 V.

Conditions for MeOISO and 3-MT. A Waters reversed-phase stainless-steel C_{18} μBondapak (300 mm × 3.9 mm I.D.) column was used for the separation of MeOISO and 3-MT. The citrate—phosphate buffer (pH 4.0) containing 15% methanol served as the mobile phase. The pumping flow-rate of the mobile phase delivery system was 1 ml/min. The optimal detection was achieved at a potential setting of 0.80 V.

Synthesis of d-MeOISO and l-MeOISO. The synthesis of *d*- and *l*-MeOISO was a modification of that of Head et al. [2]. *d*- or *l*-ISO was incubated in the presence of the cofactors S-adenosylmethionine, magnesium chloride and buffered purified rat liver COMT; the enzyme was prepared according to the method of Sole and Hussian [9]. The enzymatic O-methylation reaction was stopped after a 1-h incubation by adding tetraphenyl borate and sodium borate. The *d*- or *l*-MeOISO was then extracted with toluene—isoamyl alcohol and back-extracted over acetic acid. The acid was dried and the samples were reconstituted in an appropriate volume of 0.05 *M* acetic acid prior to analysis by HPLC—ED.

RESULTS AND DISCUSSION

Chromatographic properties

Preliminary studies, using an isocratic solvent system, have shown that ISO and MeOISO can be identified by electrochemical detection and that these substances have reversed-phase column retention times greater than those of the endogenous catecholamines. The prolonged retention times eliminate possible problems of interference by the endogenous catecholamines. However, our results also demonstrated that a simple isocratic solvent system is not altogether suitable for the determination of both ISO and MeOISO in a single sample when employing our method since there was a considerable difference in the retention times between the two compounds. A gradient elution technique could not be considered since drastic shifts in the ED baseline occurred when this form of elution was attempted.

Experiments were caried out with a variety of solvent systems, using both stainless-steel columns and radial compression module cartridges. Two separate conditions (see Experimental), one for ISO and the other for MeOISO, were developed. When these conditions were used, the retention times for the respective isomers of each amine (i.e., *d*- and *l*-ISO approximately 6 min; *d*- and *l*-MeOISO 7 min) were identical. *d*-MeOISO and *l*-MeOISO were synthesized biologically in our laboratory since they were not commercially available as separate compounds (see Experimental). This was done because it was considered important to verify that the retention times and electrochemical properties of the two isomers were identical. This finding assured us that the racemic mixture could be used as the standard for the HPLC—ED assay.

To achieve maximum sensitivity with minimum background current, it was necessary to establish a voltage—response curve for each compound of interest, i.e. *d*- and *l*-ISO, *dl*-, *l*- and *d*-MeOISO. The voltage—response curves for the *d*- and *l*-isomers of ISO are identical (Fig. 1) as were those for the electrochemical measurement of *d*- and *l*-MeOISO (Fig. 2). From the curves in Figs. 1 and 2, it was determined that 0.7 V was the optimal working potential for ISO while 0.8 V was optimal for MeOISO.

Linearity of response of the HPLC—ED assay

After the optimal chromatographic and electrochemical conditions were developed for ISO and MeOISO, it was necessary to determine the linearity of the HPLC—ED response. A range of 1 ng to 1 μg was chosen since studies

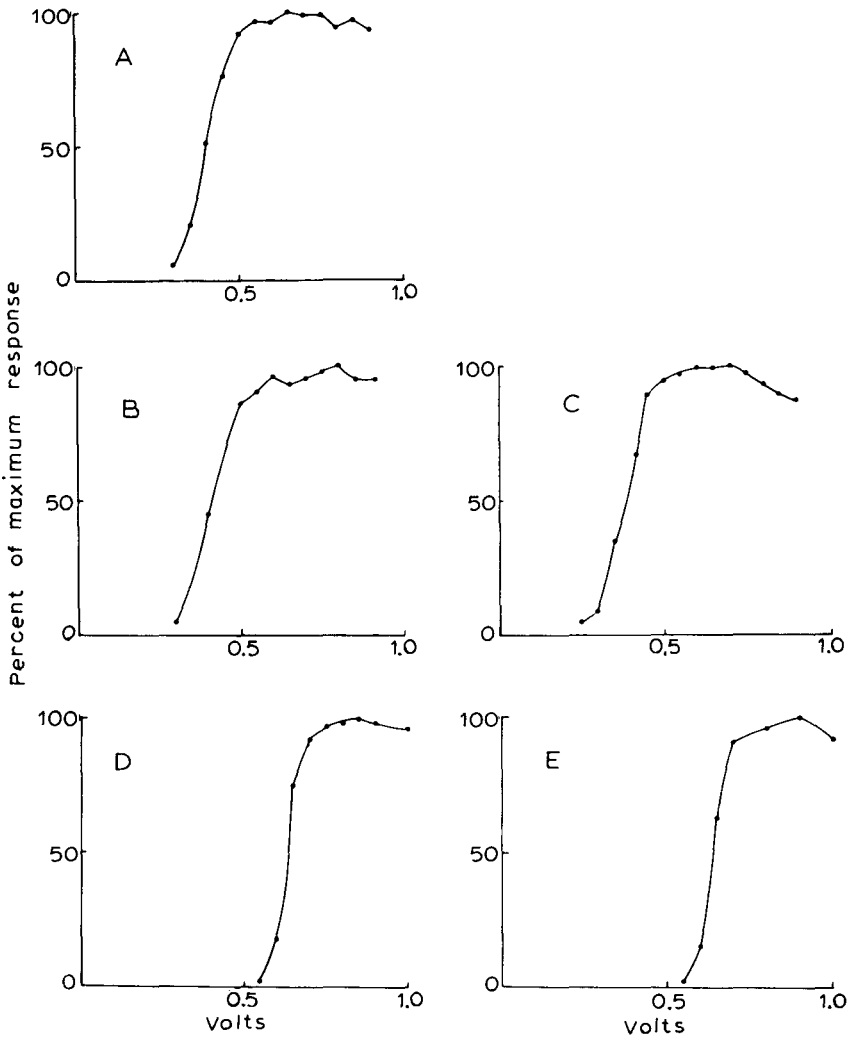


Fig. 1. Voltage—response curves constructed by using a standard concentration of *l*-ISO (A), *d*-ISO (B), DHBA (C), *dl*-MeOISO (D) and 3-MT (E) at various voltage settings.

reported in the literature indicated that this was an appropriate concentration range to use for studies of ENU. The data demonstrate that the responses to increasing amounts of ISO and MeOISO were linear from 1 ng to 1 μg with a correlation coefficient of 0.99. Furthermore, the lower limit of detectability was calculated to be approximately 200 pg.

Precolumn purification

The use of a precolumn purification procedure for the separation of ISO and MeOISO from biological samples was indicated for several reasons. First, our original isocratic HPLC—ED assay could not be used to analyze ISO and MeOISO from a single sample due to the rather large differences in their retention times. A single sample analysis of ISO and MeOISO was also

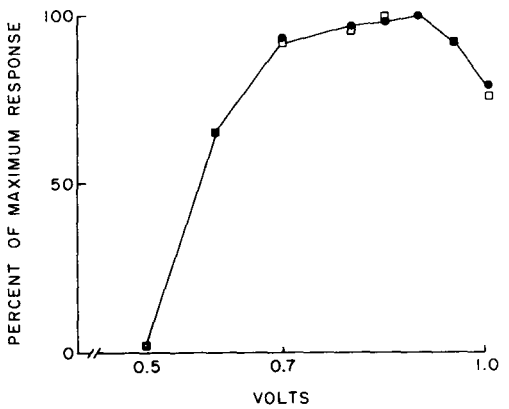


Fig. 2. Voltage—response curves constructed using *d*-MeOISO (•) and *l*-MeOISO (□) synthesized in the laboratory. The response of a standard volume of drug was measured at various voltage settings.

unwarranted due to the difference in concentration of ISO and MeOISO that occurred after a 1-h incubation period. Therefore, a method had to be developed to isolate and separate the ISO from MeOISO prior to HPLC—ED assay. Secondly, since the incubation and extraction solutions obtained after the tissue incubations have a relatively large volume, they are not suitable for direct analysis. The Krebs incubation solutions have a volume of 1 ml and have a considerable inorganic ion content. In addition, the perchloric acid extracts of the tissues also are 1 ml in volume and are extremely acidic in nature. Thus, a precolumn preparation offers a method by which purification and volume—concentration adjustment can be obtained in addition to the separation of ISO from its O-methylated metabolite.

Precolumn isolation of ISO

It has been known for some time that catechol compounds bind to acidic alumina in an alkaline medium. It is also known that the catecholamine compounds are readily oxidized under such high pH conditions, and that it is necessary to add an antioxidant to the tissue acid extracts and the acidified incubation solutions before increasing the pH for the alumina extraction. The antioxidant sodium metabisulfite (NaMBS) in an EDTA solution was chosen. NaMBS is a potent, non-catechol, non-acidic antioxidant that will not interfere or bind to the alumina at basic pH values. Since metal ions can promote the oxidation process, a metal ion chelator, EDTA, also was added prior to alumina extraction. A 3 *M* Tris buffer (pH 10.9) was used to increase the pH of the solutions after the addition of the antioxidant solution and alumina. It was found that a pH range of 8.2—8.4 was critical to ensure maximal catechol—alumina binding. The pH of each sample was individually adjusted during the addition of the Tris buffer. It was also necessary to vortex the sample while adding Tris to ensure consistency of pH throughout final solution.

ISO was removed from the alumina through use of two separate elutions (300 μl each) with 1 *M* acetic acid. The final volume (600 μl), although less than that of the original post-incubation solution, was still considered too large and too strongly acidic for optimal HPLC—ED assay. An additional step in

which the samples were taken to dryness on a Buchler vortex evaporator was added. This permitted the readjustment of final volume in a suitable vehicle solution. It was of concern that during the drying process the catechols may be oxidizing as the acid volume decreased. To avoid the possible oxidation of these compounds boric acid was added to the 1 *M* acetic acid used in the back-extraction. Boric acid binds to catechol groups in alkaline conditions, thus preventing their oxidation. Several concentrations of borate were tested (0.2 an 0.01 *M* borate in 1 *M* acetic acid). Unfortunately, there was a considerable amount of salt residue found after drying in those tubes which contained the highest borate concentration. Some of this residue was lost as the vacuum of the evaporator was turned off (the change in pressure pushed the salt up and out of the tubes, especially when vortexing was vigorous). By using the lower borate concentration, the problem was avoided and the recoveries were improved.

Several acid solutions were examined for use as a sample reconstituting medium (0.04 *M* perchloric acid, 0.05 *M* acetic acid). When assayed on HPLC—ED, blank samples reconstituted in perchloric acid contained a number of peaks, several of which interfered with the peaks of interest. 0.05 *M* Acetic acid was chosen as the reconstitution acid since it was not corrosive and produced no interfering peaks.

Precolumn isolation of MeOISO

Preliminary studies in this laboratory have shown that O-methylated compounds, such as normetanephrine and metanephrine, can be extracted readily from an alkaline medium with chloroform once the aqueous phase was saturated with K_2HPO_4 (2.5 g). Unfortunately, chloroform was found to be unreliable for the extraction of MeOISO from the alkaline effluent. Several solvents and solvent combinations possessing a range of polarities were tested for their ability to extract MeOISO from the salt-saturated alkaline medium. A summary of these results are given in Table I. Toluene, a relatively non-polar solvent, gave the best recovery for MeOISO and provided the best correlation between the recoveries of MeOISO and its internal standard, 3-methoxy-tyramine (3-MT) (internal standards will be discussed in the next section).

TABLE I

SOLVENTS TESTED FOR MeOISO EXTRACTION

Values expressed are the mean ± standard error, $n = 3$.

Solvent	Percentage recovery*	
	MeOISO	3-MT
Chloroform	49.3 ± 8.8	63.3 ± 3.3
Chloroform + 25% ethyl acetate	37.7 ± 1.7	55.3 ± 0.3
Chloroform + 25% heptane	51.0 ± 7.7	63.0 ± 3.0
Methylene chloride	28.7 ± 5.7	23.0 ± 3.0
Toluene	68.7 ± 6.4	69.3 ± 4.6

*Percentage recovery after extraction of standard concentration (1 μg) MeOISO, 0.5 μg 3-MT.

Since toluene rather than chloroform was to be used in the present studies, it was necessary to reexamine the salt conditions used previously when extracting with chloroform (i.e. 2.5 g K_2HPO_4). Differing amounts of potassium phosphate (1.0, 2.0, 2.5, 3.0 g) and sodium chloride (1.0, 2.0, 2.5 g), were tested for their effect on the toluene extraction of MeOISO from the aqueous alkaline medium. The best recovery of MeOISO was obtained when K_2HPO_4 was used, with additions of 2.5 g and 3.0 g giving similar consistent results; 2.5 g (the same amount as that used with chloroform) was chosen.

Two successive 5-ml portions of toluene were used to extract the O-methylated MeOISO from the aqueous salt-saturated phase. The two aliquots were then pooled over 500 μl acid (see below) for back-extraction. During the removal of the 10 ml of solvent (toluene) in the extraction process (i.e., two lots of 5 ml), some the aqueous salt layer was occasionally transferred to the back-extraction acid tube. This seemed to interfere with the back-extraction of MeOISO into the acid. To avoid this problem, 4-ml aliquots of each 5-ml portion of toluene were collected and pooled. A correction factor of 1.25 was then applied to the data.

When 0.1 *M* hydrochloric acid was used in the back-extraction of MeOISO in the preliminary studies, a lower recovery rate and additional peaks on the chromatogram began to appear. A similar decomposition of amines in hydrochloric acid has been documented recently by Head et al. [10]. In view of this breakdown, several concentrations (1 *M*, 0.5 *M*, 0.1 *M*) of acetic acid, an organic rather than a mineral acid, were tested for their effectiveness in back-extraction. 0.5 *M* Acetic acid gave consistent results for the extraction of MeOISO. Since the volume (500 μl) of acid used for the back-extraction was relatively large, it became necessary to dry the samples. The same conditions for drying and reconstitution used for ISO (see above) were employed for MeOISO.

Internal standards

Extraction and separation procedures are subject to loss of compound. To correct for this potential problem, the use of internal standards has been examined. Since we have found that there is no loss of compounds during the incubation, we proceeded to test the next phase of extraction procedure, i.e., experiments were conducted in which standard concentrations of ISO and MeOISO were dried in the vortex evaporator. The results indicated that there was no loss during the drying process. However, further analysis of the overall experimental method suggested that incomplete extraction was still a problem. There was a decrease in the recovery of ISO during the alumina extraction and a decrease in MeOISO recovery during the toluene extraction because of incomplete solvent and/or acid extraction. Since each of these losses occur in different fractions, two internal standards had to be employed.

Isoprenaline internal standard

Various compounds were tested as possible internal standards for ISO. Since the precolumn isolation and purification of ISO involves alumina extraction, catechol compounds capable of being adsorbed to alumina were examined. The catechol compounds tested were: epinine, dopamine, α-methylnoradrenaline,

TABLE II

PERCENTAGE RECOVERY FOLLOWING ALUMINA ABSORPTION AND ELUTION

Values represent mean ± standard error; $n = 8$.

	Recovery (%)
Isoprenaline	42.4 ± 1.8
DHBA	46.6 ± 2.0

2-methyl-3-(3,4-dihydroxyphenylalanine), α-methyldopamine, 3,4-dihydroxyphenylethyleneglycol, and dihydroxybenzylamine (DHBA). DHBA appeared to be the best candidate to serve as an internal standard for ISO since it did not interfere with the electrochemical detection of ISO, and since it is not an endogenous substance. The recovery values for DHBA and ISO from alumina are shown in Table II and are clearly seen to be almost identical.

Methoxyisoprenaline internal standard

The internal standard for the O-methylated fraction, i.e., the one containing MeOISO, had to be an O-methylated catecholamine capable of being extracted

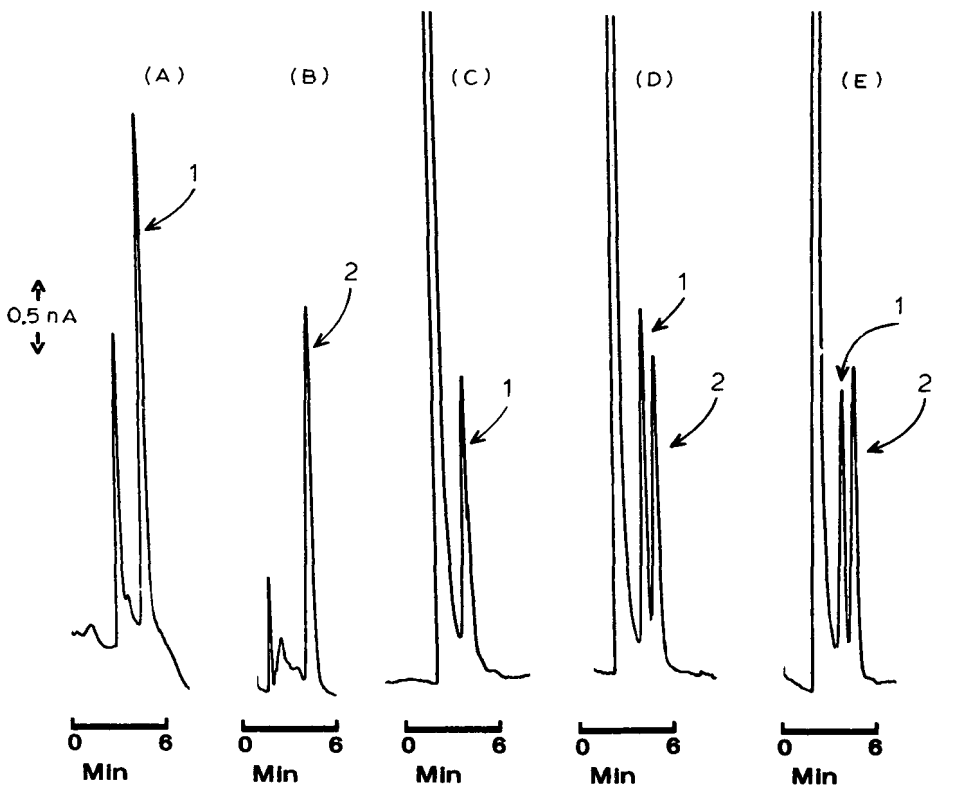


Fig. 3. Chromatograms of the content of MeOISO in the incubation solutions after a 1-h incubation period in the presence or absence of *d*- or *l*-ISO (5 μ*M*) (see Experimental for conditions employed). A, 5 ng 3-MT; B, 10 ng MeOISO; C, aorta no ISO; D, aorta *l*-ISO; E, aorta *d*-ISO. Peaks: 1 = 3-MT; 2 = MeOISO.

with toluene and also having chromatographic properties similar to MeOISO. Normetanephrine and metanephrine chromatographed too near the solvent front and since these compounds are inhibitors of extraneuronal uptake and could possibly be needed in future experiments, they were not considered. 3-Methoxytyramine (O-methylated dopamine) seemed to be a prime candidate since it chromatographed near MeOISO when the HPLC—ED conditions established previously for MeOISO were used. For a voltage—response curve for 3-MT refer to Fig. 1. The toluene extraction of 3-MT also closely followed that of MeOISO. Thus, 3-MT fit all of the criteria necessary to be an ideal internal standard for MeOISO.

Validity of total method

After the entire assay procedure (incubation, precolumn separation, and HPLC—ED assay) was developed, it was necessary to verify the methodology using segments of rabbit thoracic aorta incubated with 4.7 μM of *d*- or *l*-ISO. Sample chromatographs obtained after the extraction of incubate MeOISO, tissue MeOISO and tissue ISO are shown in Figs. 3—5. Chromatograph C on all three figures demonstrates that the ISO and MeOISO peaks were absent when the tissues were not incubated with ISO. Furthermore,there was no interference produced by any endogenous compound.

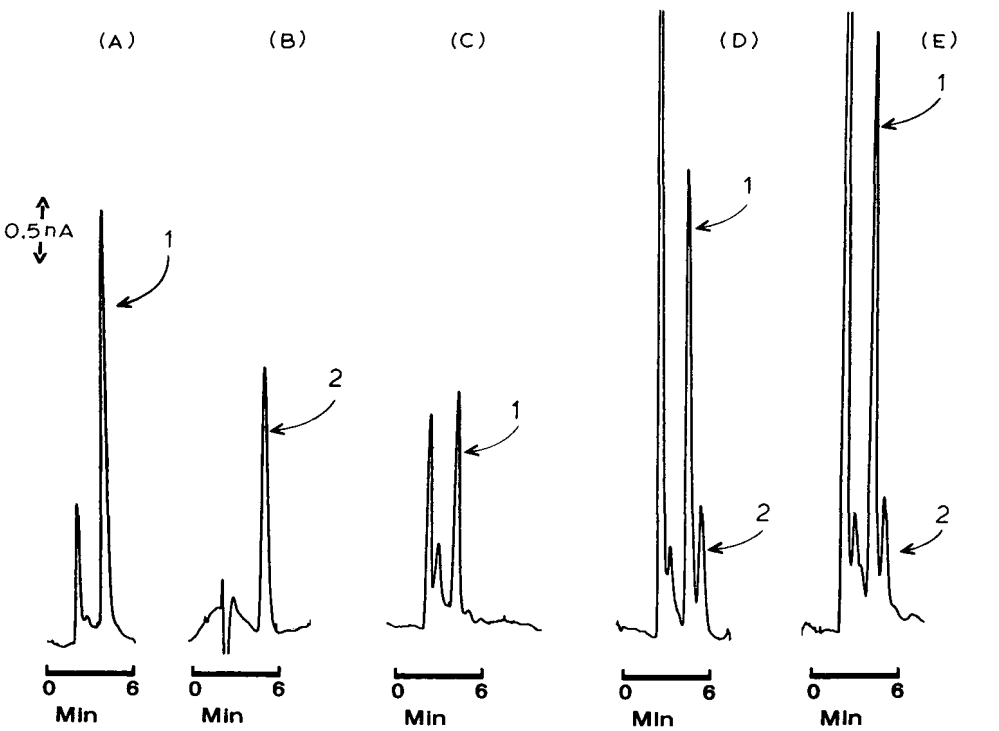


Fig. 4. Chromatograms of the content of MeOISO in the tissue following a 1-h incubation period in the presence or absence of *d*- or *l*-ISO (5 μM) (see Experimental for conditions employed). A, 5 ng 3-MT; B, 10 ng MeOISO; C, aorta no ISO; D, aorta *l*-ISO; E, aorta *d*-ISO. Peaks: 1 = 3-MT; 2 = MeOISO.

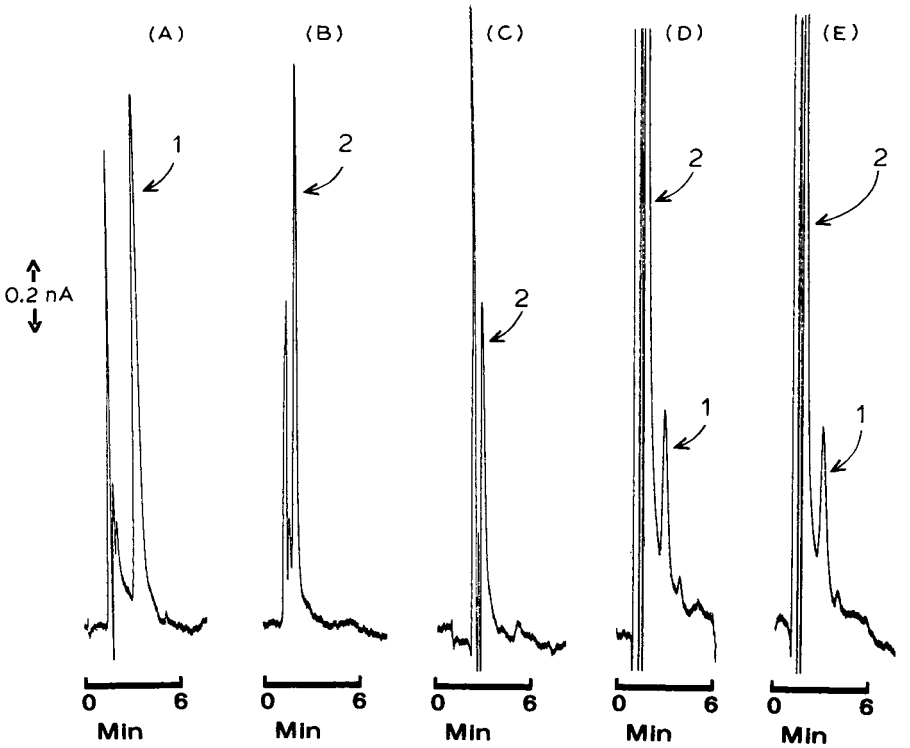


Fig. 5. Chromatograms of the content of ISO in the tissue after a 1-h incubation period in the presence or absence of *d*- or *l*-ISO (5 μM) (see Experimental for conditions employed). A, 5 ng ISO; B, 2 ng DHBA; C, aorta no ISO; D, aorta *l*-ISO; E, aorta *d*-ISO. Peaks: 1 = ISO; 2 = DHBA.

TABLE III

INCUBATE MeOISO (μg/g) AFTER INCUBATION WITH U0521 (10 μg/ml)

	n	Control	U0521
d-ISO	10	2.99 ± 0.30	< 0.30 ± 0.02*
l-ISO	9	4.11 ± 0.24	< 0.36 ± 0.03*

*$p < 0.01$.

In order to verify whether the substance found in the incubation medium and tissue after ISO incubation was actually MeOISO, the known COMT inhibitor U0521 was added prior to addition of ISO to the bath. The incubate MeOISO content after U0521 treatment decreased dramatically and fell below the level of detectability (Table III).

The results above appear to verify that the methodology developed will provide a valid approach to the study and detection of the metabolism of ISO in the intact rabbit thoracic aorta.

ACKNOWLEDGEMENTS

This work was supported in part by NIH Grants 1K04-HLO 1128, 5 T32 GM07039 and the West Virginia University Biomedical Research Fund.

REFERENCES

1 R.J. Head, R.J. Irvine and J.A. Kennedy, J. Chromatogr. Sci., 14 (1976) 578.
2 R.J. Head, I.S. de la Lande, R.J. Irvine and S.M. Johnson, Blood Vessels, 17 (1980) 229.
3 K.H. Graefe, F.J.E. Stefano and S.Z. Langer, Biochem. Pharmacol., 22 (1973) 1147.
4 P. Hjemdahl, M. Dakeskog and T. Kahan, Life Sci., 25 (1979) 131.
5 R.T. Borchardt, M.F. Hegazi and R.L. Schowen, J. Chromatogr., 152 (1978) 255.
6 M.T.I.W. Schüsler-van Hees and G.M.J. Beijersbergen van Henegouwen, J. Chromatogr., 196 (1980) 101.
7 S. Koh, M. Arai, S. Kawai and M. Okamoto, J. Chromatogr., 226 (1981) 461.
8 J. Levin, J. Pharmacol. Exp. Ther., 190 (1974) 210.
9 M. Sole and M. Hussian, Biochem. Med., 18 (1977) 301.
10 R.J. Head, J. Hemstead and B. Berkowitz, Blood Vessels, 19 (1982) 135.

Journal of Chromatography, 310 (1984) 297—305
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2208

FAECAL LIPID CHROMATOGRAPHY

I. QUANTITATIVE DETERMINATION WITH CHROMARODS

E. PEUCHANT, G. COVI and R. JENSEN*

Department of Biochemistry, Saint-André Hospital, 1, Rue Jean-Burguet, 33075 Bordeaux Cedex (France)

(First received February 9th, 1984; revised manuscript received April 17th, 1984)

SUMMARY

A new and original method is proposed for the qualitative and quantitative analysis of faecal lipids by thin-layer chromatography and detection through the flame ionization detector of an analyser (the Iatroscan TH 10). This method enables the rapid quantification of the different faecal lipid classes, including cholesterol, with great accuracy and reproducibility. In-series operations are possible with easy manipulation.

INTRODUCTION

Faecal lipid measurement is of the greatest interest in clinical biology for the appreciation of assimilation diseases [1,2]. Several techniques have been used for this analysis: gravimetric [3,4], volumetric [5,6], spectrophotometric [7—9], chromatographic [10—12] and colorimetric [13—15]. These methods are time-consuming, impractical, unsuitable for serial analysis and involve manipulations with offensive material. The method proposed here eliminates certain difficulties by using Folch reagent [16] for extraction, thin-layer chromatography (TLC) on Chromarods for the separation of different lipid classes and flame ionization detection (FID) for quantification.

MATERIALS

Reagents

All reference compounds were purchased from Sigma (St. Louis, MO, U.S.A.). The other reagents, all analytical grade, were obtained from E. Merck (Darmstadt, F.R.G.).

0378-4347/84/$03.00

Apparatus and operating conditions

An Iatroscan TH 10 Mark II analyser (Iatron Labs., Tokyo, Japan), described by Sipos and Ackman [17], was used equipped with a recorder electronic stepping integrator IRC 1 B (Intersmat, France). FID was performed with a hydrogen flow-rate of 160 ml/min. Clean outside air was supplied by an aquarium pump at a flow-rate of 2100 ml/min. The intensity of the current proportional to the organic compounds during pyrolysis was recorded. The recorder chart speed was proportional to the Chromarod scanning speed in the flame which was 0.32 cm/sec. The recorder speed was 0.20 cm/sec and the attenuation rate was 14 mV.

METHODS

Extraction procedure

The faecal lipid extract was recovered by extraction from 1 g of homogenized faeces to which 50 ml of Folch reagent (chloroform—methanol, 2:1, v/v) had been added. Two sonifications made optimal extraction possible. A 0.9% sodium chloride solution (10 ml) was added to the mixture obtained to eliminate the impurities in the upper phase. The lower phase containing the total lipids was separated from the upper phase by decantation, and then filtered. An aliquot (20 ml) was evaporated under reduced pressure and the dry residue was dissolved in 2 ml of chloroform.

Chromatography

The Chromarods used for TLC were cylindrical quartz rods 152 × 0.9 mm, covered with a sintered silica gel, type S II (5 μm particle size). Prior to use, the Chromarods were activated in the oven at 110°C for 2 h and then were passed through the FID. A 1 μl volume of faecal extract was spotted on the Chromarod 1 cm from one of the ends. In order to ensure identical conditions, another rod was spotted with a standard mixture containing a known amount of each lipid class. The two rods were then placed in a special rod holder frame. This frame was then placed in a glass tank lined with filter paper which had been saturated for 20 min with a solvent mixture benzene—chloroform—formic acid (35:15:1, v/v/v). The migration time was 30 min at 22—25°C. On removal from the tank, the rods were dried at 60°C for 5 min and transferred to the scanning frame of the analyser. Every peak was identified on the basis of retention time and in comparison with the standard mixture analysed under the same conditions. The detector response for the standard mixture was used to calculate the concentration of lipids in the biological sample.

RESULTS

Linearity of detector response

The study was performed using a standard mixture containing increasing amounts from 1 to 10 mg/ml of each lipid: L-α-phosphatidylcholine, monostearyl-*rac*-glycerol, D-1,2-dipalmitin, cholesterol, stearic acid, tristearin and cholesteryl stearate. Each standard mixture was analysed with the Iatroscan ten times during one week. The mean of the peak squares and the

extreme values calculated by the integrator that were obtained from the different concentrations of each lipid fraction, led us to examine the function $Y = f(X)$ where Y represents the concentration (mg/ml) and X the square (μV/sec), in order to find the best correlation coefficient (R). The quality of the linear relationship between the theoretical values and those calculated by the integrator was thus obtained using this function. As shown in Table I, the function $Y = A + BX + CX^2$ gave the best correlation coefficient.

Fig. 1 shows the quadratic regression lines obtained for each lipid fraction. Since the value of C is very small (for example, $20 \cdot 10^{-11}$), the detector response must be considered as linear, and thus suitable for analysing small amounts of lipids similar to those found in faecal extracts.

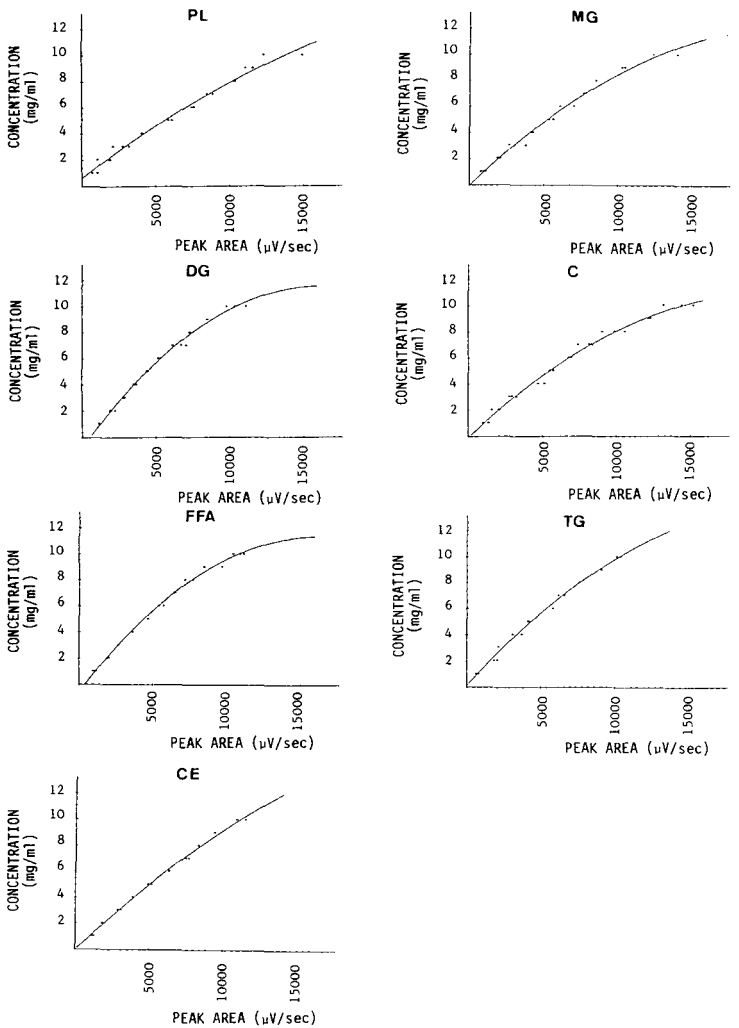


Fig. 1. Quadratic regression lines obtained for each lipid fraction by plotting concentrations versus peak areas.

TABLE I

DETECTOR RESPONSE: STUDY OF DIFFERENT FUNCTIONS $Y = f(X)$ GIVING THE BEST CORRELATION COEFFICIENT FOR EACH LIPID FRACTION

Lipid fraction*	Correlation coefficient (R)							
	$Y = A+BX$	$Y = A + \frac{B}{X}$	$\frac{1}{Y} = A + \frac{B}{X}$	$Y = A+B\sqrt{X}$	$Y = Ae^{BX}$	$Y = AX^B$	$Y = A+(B \log X)$	$Y = A+BX+CX^2$
PL	0.98	0.58	0.89	0.97	0.85	0.97	0.89	0.99
MG	0.98	0.59	0.96	0.98	0.84	0.99	0.90	0.99
DG	0.97	0.73	0.99	0.99	0.83	0.98	0.95	0.98
C	0.97	0.70	0.93	0.99	0.81	0.97	0.94	0.99
FFA	0.98	0.73	0.98	0.99	0.85	0.99	0.95	0.99
TG	0.99	0.56	0.96	0.98	0.85	0.98	0.89	0.99
CE	0.99	0.73	0.98	0.99	0.87	0.99	0.94	0.99

*For abbreviations see Fig. 6.

TABLE II

REPRODUCIBILITY OF MEASUREMENTS OF THE LIPID CLASSES IN A STANDARD MIXTURE CONTAINING 5 mg/ml OF EACH LIPID FRACTION AND IN A BIOLOGICAL SAMPLE

Results are given by peak area values expressed as mean ± S.D. and C.V. of thirty determinations over three days.

Lipid fraction*	Standard mixture		Biological sample	
	Mean ± S.D. (mV/sec)	C.V. (%)	Mean ± S.D. (mV/sec)	C.V. (%)
PL	56.25 ± 0.86	1.53	72.42 ± 1.03	1.42
MG	54.11 ± 0.95	1.76	2.77 ± 0.53	2.10
DG	45.19 ± 0.87	1.93	5.03 ± 0.09	1.79
C	57.06 ± 0.88	1.54	136.49 ± 1.14	0.84
FFA	46.24 ± 0.92	1.99	70.89 ± 1.25	1.76
TG	41.05 ± 0.86	2.10	6.39 ± 0.13	2.03
CE	47.07 ± 0.96	2.04	2.59 ± 0.06	2.32

*For abbreviations see Fig. 6.

Reproducibility

The reproducibility of the measurement of all lipid classes was expressed by the standard deviation (S.D.) and the coefficient of variation (C.V.) calculated from the analysis of a pure standard mixture and a sample of faecal lipid extract. Three series of ten rods were analysed over a three-day period according to the method described. From the results presented in Table II it can be seen that the accuracy and the reproducibility of the method are satisfactory.

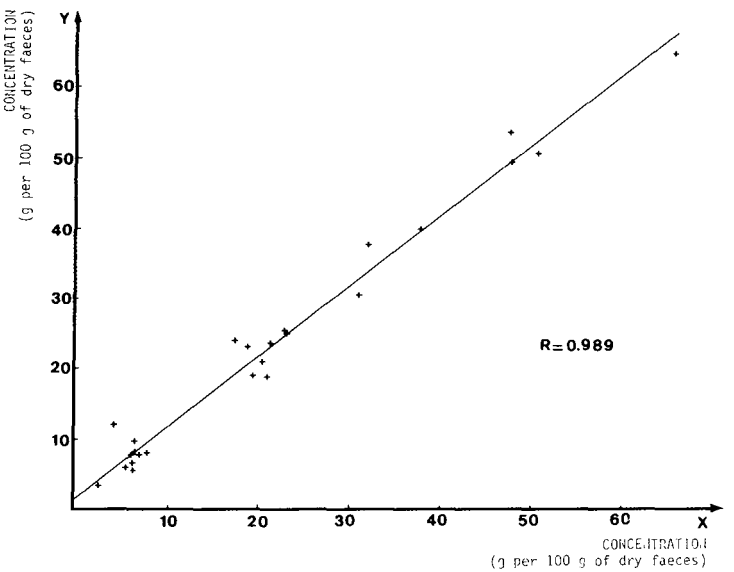


Fig. 2. Analysis of total lipids (PL, MG, DG, FFA, TG, CE) using the Van den Kamer method (abscissa) and chromatography with Chromarods: correlation between the methods.

Comparison between Iatroscan and Van den Kamer's techniques

The results obtained by the proposed method and those obtained with a classical acidimetric method, such as Van den Kamer's, on faecal extracts from hospitalized subjects were compared. The weights of total lipids, expressed in g per 100 g of dried stool, analysed by the two techniques are compared in Figs. 2 and 3. The measurement of dried stool was obtained using the

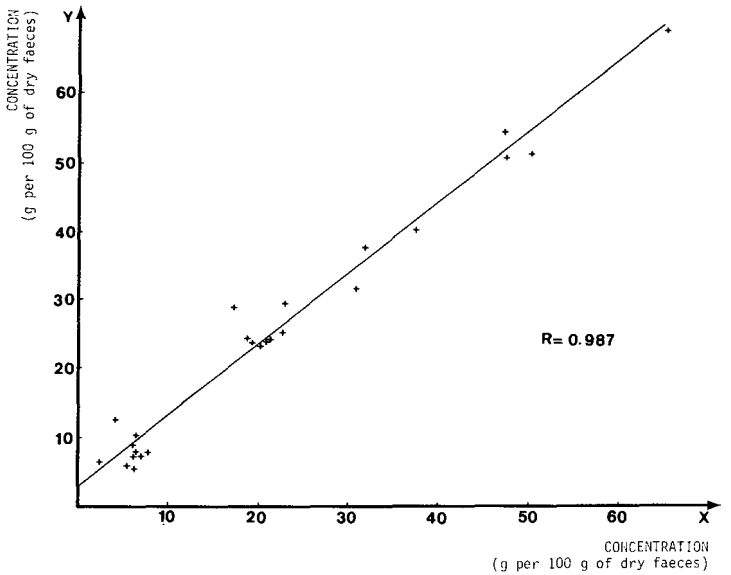


Fig. 3. Analysis of total lipids by both methods: the chromatography method including cholesterol in total lipids as opposed to the acidimetric (Van den Kamer) method (abscissa).

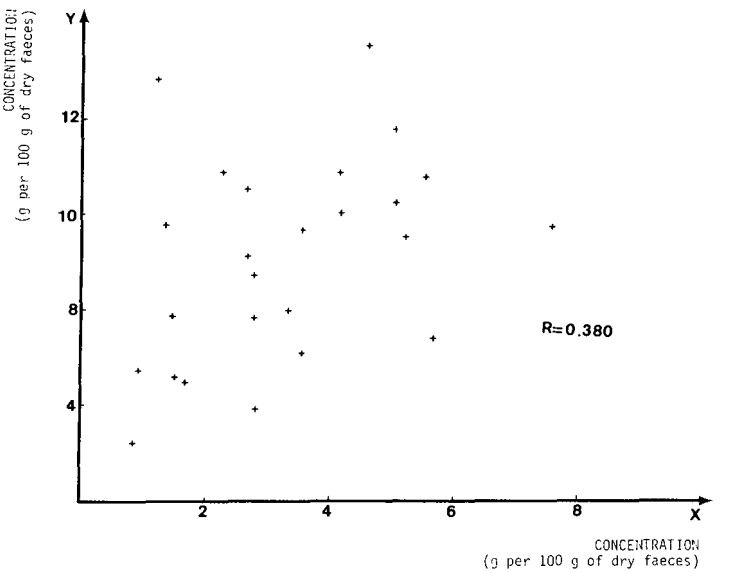


Fig. 4. Study of the correlation between both methods of analysis of polar and neutral fats. Van den Kamer's method: abscissa.

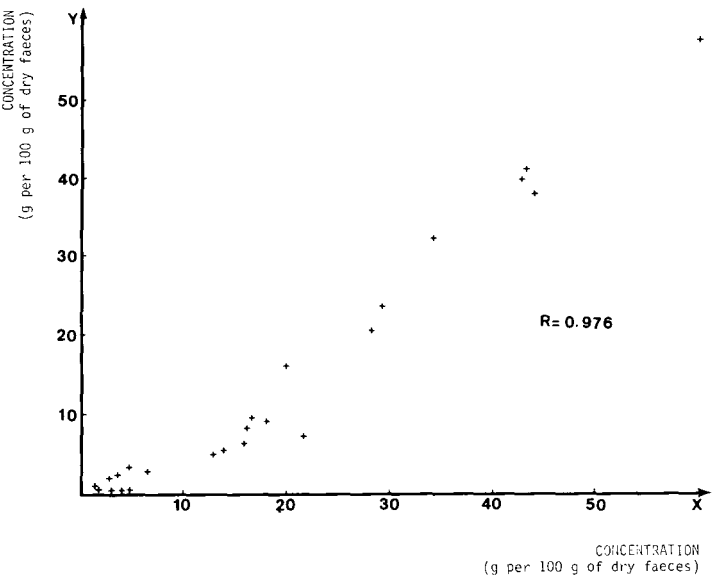


Fig. 5. Study of the correlation between both methods of analysis of free fatty acids. Van den Kamer's method: abscissa.

gravimetric method after infrared treatment or with the Karl Fisher method [18], water being extracted with Folch reagent. The correlation of the analysis of the different lipid classes between these two techniques is presented in Figs. 4 and 5.

DISCUSSION

For development, all other authors have used a solvent composed of diethyl ether, hexane and an organic acid such as formic acid [19,20] in different proportions. The effects of the proportion of this solvent were very noticeable on the resolution of some lipid classes, in particular free fatty acids and triglycerides [21], separation of which was very difficult to perform. As Fig. 6 shows, the migration solvent proposed in the proportion described above enables total separation of faecal lipids.

Fig. 2 shows that, for total lipid analysis, the correlation between the classical technique and the proposed method is quite satisfactory. Moreover, cholesterol contained in the unsaponifiable fraction was not analysed with the acidimetric method and so was not included in total faecal lipids. The correlation of the total faecal lipids between the Van den Kamer method and the proposed method, where cholesterol is included, can be seen in Fig. 3. The correlation coefficient can be seen to decrease slowly; therefore, cholesterol represents a substantial fraction because a solid food diet can produce as much as 3 g of cholesterol excreted in the faeces. To compare the different lipid fractions, the proposed method was used to calculate hydrolysed fats corresponding to free fatty acids, and both polar and neutral fats corresponding to phospholipids, glycerides, and cholesteryl esters; on the other hand, the acidimetric technique allows the evaluation of only the two latter

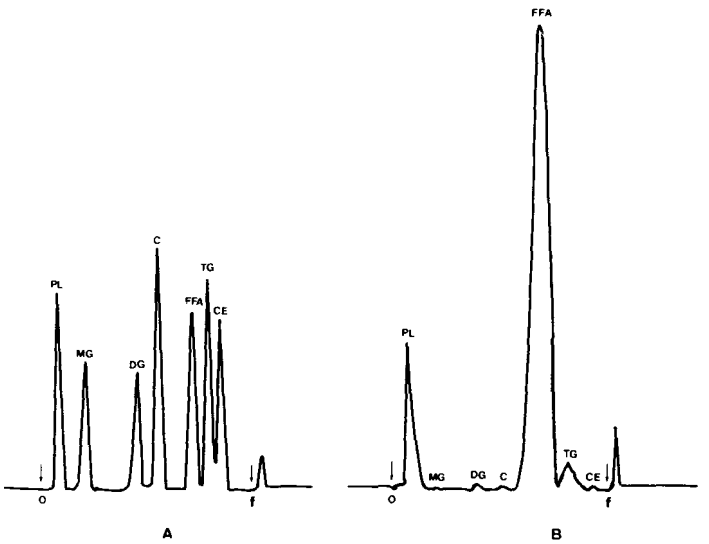


Fig. 6. Chromatograms showing the separation of neutral lipid classes on Chromarods with solvent benzene—chloroform—formic acid (35:15:1). (A) Separation of standard mixture. Peak designation: PL = L-α-phosphatidylcholine; MG = monostearyl-*rac*-glycerol; DG = D-1,2-dipalmitin; C = cholesterol; FFA = stearic acid; TG = tristearin; CE = cholesteryl stearate. (B) Separation of a human faecal extract. Peak designation: PL = phospholipids; MG = monoglycerides; DG = diglycerides; C = cholesterol; FFA = free fatty acids; TG = triglycerides; CE = cholesteryl esters. o = start; f = front.

lipid fractions. Unlike total lipids, the correlation of the results obtained for the analysis of polar and neutral fats on the two methods is very poor (Fig. 4). This is for three reasons. First, extractions performed with the classical method are not homogeneous for the different lipid fractions because of their various amphipathic properties. The water in faeces modifies the separation coefficient, so our procedure extraction mobilizes all the water while making all the lipids soluble. Secondly, when acid hydrolysis is being performed, some esterified lipids are hydrolysed into free fatty acids, and this overestimates their values. Thirdly, the quantification process used in the classical method is debatable. The polar and neutral fats have very different molecular weights and are expressed in tristearin equivalents; this tends largely to undervalue them.

The technique proposed here eliminates these disadvantages by individualizing every lipid fraction representing polar and neutral faecal fats. However, the correlation of the free fatty acid analysis between the two methods is quite sound (Fig. 5). These two methods may therefore be used to analyse molecules with identical molecular weights expressed by the same unit (stearic acid equivalent). The only difference is that the classical method does have a tendency to overestimate the free fatty acid values.

Lipid chromatographic analysis is of interest for analytical and diagnostic reasons. As regards the analytical aspect, this method gives more exact and more realistic results than the classical acidimetric methods: every lipid fraction can be analysed separately, in particular cholesterol, which is something that many classical methods cannot do although it represents a fraction that is not negligible.

Moreover, ten different stools can be analysed at the same time. This makes in-series operation possible.

The resulting method is very quick, effective, and does not need any manipulation other than extraction with Folch reagent and the spotting of the lipid extract on the Chromarods. The study of the reproducibility in the biological sample indicates that it is as good for the low amounts of separated lipids as for the high.

This method has such a degree of precision that it could be used as a reference technique to calibrate apparatus like the Infraalyzer (Technicon), which performs total faecal lipid analysis by near infrared reflectance analysis (NIRA) [22,23]. The quality of the results obtained by the NIRA technique depends only on the precision of the calibration set [24] that the TLC—FID technique permits. As for biological diagnosis, the quality of the results obtained and the ease of their interpretation could be of valuable assistance to clinicians.

CONCLUSION

A simple, rapid and accurate technique used for in-series operation is proposed for the analysis of the different faecal lipid classes. The great precision of this method means that it could be used as a reference technique to create a calibration set.

REFERENCES

1 F.S. Fouweather, Brit. J. Exp. Pathol., 7 (1926) 7.
2 E.E. Wollaeger, M.W. Comfort, J.F. Wier and A.E. Osterberg, Gastroenterology, 6 (1946) 83.
3 M. Kumagawa and K. Suto, Biochem. Z., 8 (1908) 212.
4 M.A. Bowers, P.K. Lund and J.C. Mathies, Clin. Chim. Acta, 2 (1964) 314.
5 J.H. van den Kamer, H. ten Bokkel Huinink and H.A. Weyers, J. Biol. Chem., 177 (1959) 347.
6 J.Rousselet, in P. Loiseleur (Editor), Techniques de Laboratoires, Vol. II, Masson, Paris, 3rd ed., 1963, p. 273.
7 J.S. Amenta, Clin. Chem., 16 (1970) 339.
8 I. Bang, Biochem. Z., 91 (1918) 235.
9 F.W. Fales, Clin. Chem., 17 (1971) 513.
10 W. Erb and E. Böhle, Z. Klin. Chem. Klin. Biochem., 6 (1968) 379.
11 R. Gilly, R. Geyssel and M. David, Pediatrie, 20 (1965) 805.
12 F. Matthys, A. Christophe and G. Verdank, Clin. Chim. Acta, 36 (1972) 341.
13 N. Zolner and K. Kirsch, Z. Gesamte Exp. Med., 135 (1962) 1713.
14 M. le Towska and L. Tomaszewski, Pol. Tyg. Lek., 28 (1973) 784.
15 L. Tomaszewski, Clin. Chim. Acta, 61 (1975) 113.
16 J. Folch, M. Lees and C.H. Sloane-Stanley, J. Biol. Chem., 226 (1957) 497.
17 J.C. Sipos and R.G. Ackman, J. Chromatogr. Sci., 16 (1978) 443.
18 D. Buffangeix, R. Jensen and G. Covi, Clin. Chem., 22 (1976) 1351.
19 M. Tanaka, T. Itoh and H. Kaneko, Yukagaku, 25 (1976) 263.
20 M. Tanaka, T. Itoh and H. Kaneko, Yukagaku, 26 (1977) 454.
21 J.K.G. Kramer, R.C. Fouchard and E.R. Farnworth, J. Chromatogr., 198 (1980) 279.
22 M.O. Gravina, in Journée d'Information sur l'Infraalyzer Physiologie, Paris, 1983, Technicon, Paris, in press.
23 C. Mashio, E. Tomei, B. Leonardi and E. Cardinale, in Journée d'Information sur l'Infraalyzer Physiologie, Paris, 1983, Technicon, Paris, in press.
24 M.S. Day and F.R.D. Fearn, Infraalyzer for Food Analysis, Technicon, London, 1982.

Journal of Chromatography, 310 (1984) 307—317
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2198

GAS CHROMATOGRAPHIC QUANTITATION OF METHOXYPHENAMINE AND THREE OF ITS METABOLITES IN PLASMA

S.D. ROY, G. McKAY, E.M. HAWES and K.K. MIDHA*

College of Pharmacy, University of Saskatchewan, Saskatoon, Saskatchewan S7N 0W0 (Canada)

(First received January 24th, 1984; revised manuscript received May 24th, 1984)

SUMMARY

Sensitive gas chromatographic procedures for the determination of methoxyphenamine and three of its metabolites in plasma have been developed. The metabolites were measured using an electron-capture detector. This simple procedure is based on the precipitation of protein from a 1-ml plasma sample with 10% trichloroacetic acid, followed by aqueous derivatization with pentafluorobenzoyl chloride at pH 9.2 and a single-step cyclohexane extraction. The lower limit of detection for the N-desmethyl, O-desmethyl and aromatic 5-hydroxy metabolites of methoxyphenamine were 1.6, 3.1 and 2.2 ng ml^{-1}, respectively, with coefficients of variation less than 10%. The poor electron-capture response of fluorinated derivatives of methoxyphenamine necessitated the use of nitrogen—phosphorus detection. Extractive derivatization with pentafluorobenzoyl chloride, without the need for protein precipitation, enabled quantitation of methoxyphenamine down to 3.8 ng ml^{-1} from a 2-ml aliquot of plasma. In a pilot study involving healthy volunteers who received a single oral dose of methoxyphenamine hydrochloride plasma concentration could be followed in all three subjects for at least 24, 32, 12 and 4 h for methoxyphenamine and the O-desmethyl, 5-hydroxy and N-desmethyl metabolites, respectively.

INTRODUCTION

Methoxyphenamine [MP, 1-(2-methoxyphenyl)-2-methylaminopropane] is a β_2 stimulant used clinically in the treatment of asthma and other allergic conditions. It is metabolized in man and animals by at least three distinctly different metabolic pathways [1—4], which include N-desmethylation, O-desmethylation and hydroxylation at the 5-position of the benzene ring [4]. Inter-individual and species differences in the excretion of metabolites resulting from O-desmethylation and/or N-desmethylation have been observed in a preliminary report [3]. These differences were based on urine analysis by gas

0378-4347/84/$03.00

chromatography (GC) with a flame-ionization detector [5]. In this method, MP and N-desmethylmethoxyphenamine (NDMP), O-desmethylmethoxyphenamine (ODMP) and N,O-didesmethylmethoxyphenamine were analyzed as their trifluoroacetyl derivatives following extraction of the urine samples. However, 5-hydroxymethoxyphenamine (5HMP) has not been quantitated in any of the studies reported so far.

In order to investigate the pharmacokinetics of MP, GC assays for the quantitative determination of MP and its three primary metabolites in plasma were developed. These methods, which are described here, require a 2-ml plasma sample for MP analysis and a 1-ml plasma sample for the simultaneous determination of the three metabolites. The methods are rapid and have adequate sensitivity to determine MP, ODMP, NDMP and 5HMP at concentrations of 3.8, 3.1, 1.6 and 2.2 ng ml^{-1} in plasma, respectively, with coefficients of variation less than 10%.

EXPERIMENTAL

Materials

Pentafluorobenzoyl chloride (PFBCl), distilled cyclohexane, triethanolamine and ethyl acetate were analytical grade. MP hydrochloride was generously donated by Upjohn Canada (Don Mills, Canada).

ODMP, NDMP and 5HMP were synthesized in these laboratories [4]. Phenethylamine (PA) and 4-methoxyphenethylamine (4MPA) (Aldrich, Montreal, Canada) were used as the internal standards for GC using nitrogen—phosphorus detection (NPD) and GC using electron-capture detection (ECD), respectively. All other solvents and chemicals were analytical grade and used without further purification. All glassware used for sample preparation were silanized. The cyclohexane—triethanolamine (CH—TEA) extraction solvent was prepared by refluxing cyclohexane with small amounts of triethanolamine for 2 h, cooling and separating the two phases [6].

The ammonium buffer was a saturated ammonium chloride solution adjusted to pH 9.4 with undiluted ammonia.

Instrumentation and chromatographic conditions

A Hewlett-Packard Model 5840A gas chromatograph equipped with a nitrogen—phosphorus detector was used for the analysis of MP. The silanized glass column, 122 × 0.2 cm I.D., was packed with 3% OV-225 on Gas-Chrom W (100—120 mesh, DMCS-washed). The column was conditioned for 24 h at 285°C at a low helium flow-rate. Temperature was programmed from 185°C, 3.5 min hold-time, to 235°C at a rate of 15°C min^{-1}. The injection port and the detector temperature were maintained at 280°C and 300°C, respectively. The helium gas flow-rate was 30 ml min^{-1}.

For the analysis of metabolites, a Hewlett-Packard Model 5790A gas chromatograph equipped with a ^{63}Ni detector was used. The chromatographic conditions using the above 3% OV-225 column were: injection port temperature 300°C; detector temperature 300°C; column oven programmed from 210°C, 2.1 min hold-time, to a final temperature of 285°C for 2.65 min at 20°C min^{-1}. The carrier gas (argon—methane, 95:5, v/v) flow-rate was 35 ml min^{-1}.

The gas chromatographic—mass spectrometric (GC—MS) analysis was carried out on a V.G. Micromass 7070E mass spectrometer interfaced via a single-stage glass-jet separator to a Hewlett-Packard 5790A gas chromatograph operated under the same conditions as described above. The instrument was operated in the electron-impact (EI) mode at 70 eV and emission current of 200 μA. The GC—MS interface and ion source were held constant at 280°C and 220°C, respectively. Data was collected using a V.G. 2025 data system.

Internal standards

Stock solutions of PA hydrochloride (50 μg ml^{-1} as the free base) and 4MPA hydrochloride (50 μg ml^{-1} as the free base) were prepared every six months in distilled deionized water and stored at 4°C.

Preparation of standard curves

Stock solutions [MP (16 μg ml^{-1}), ODMP (8 μg ml^{-1}), NDMP (8 μg ml^{-1}) and 5HMP (5.8 μg ml^{-1})] were made every six months in distilled deionized water and stored at 4°C. For the preparation of standard curves, appropriate dilutions of these solutions were mixed in distilled deionized water and the appropriate volumes added to 1 or 2 ml of fresh blank plasma for metabolites and MP, respectively. These standard plasma samples for MP and metabolite determinations were then treated in an identical fashion as unknown samples.

Plasma level study

Three healthy male volunteers weighing 65, 70 and 78 kg were fasted overnight and then orally administered 60.3 mg of MP hydrochloride (extemporaneously prepared capsule) with 250 ml of water. Blood samples were drawn at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 8, 12, 24, 28 and 32 h in heparinized evacuated tubes (Vacutainers, Becton and Dickinson, Mississauga, Canada), centrifuged (TJ6 centrifuge, Beckman Instruments, Toronto, Canada) and the separated plasma was stored at −20°C until analysis. During collection of the venous samples, care was taken to avoid contact of the blood with the rubber stoppers of the evacuated tubes.

Extraction and derivative formation

Methoxyphenamine determination in plasma: extractive pentafluorobenzoylation. To a 10-ml PTFE-lined screw-capped test tube were added in turn a 2-ml plasma sample, either spiked or from a MP-dosed volunteer, 20 μl of internal standard solution (6.25 μg ml^{-1}) containing 125 ng of PA, 200 μl of NH_4^+/NH_4OH buffer, 5 ml of CH—TEA and 10 μl of PFBCl (0.5% in cyclohexane). The tube was tightly capped and mixed (Evapomix Fisher Scientific, Edmonton, Canada) for 10 min and centrifuged (Fisher Safety Centrifuge, Fisher Scientific) at 400 *g* for 3 min. The organic phase was transferred to another tube and evaporated under nitrogen at 65°C in a dry bath. The residue was reconstituted in 50 μl of ethyl acetate. Aliquots of 1 μl were analyzed by GC—NPD.

Metabolite determination in plasma: pentafluorobenzoylation in aqueous media. To a 1-ml plasma sample either spiked or from a dosed volunteer were added 20 μl of internal standard solution (2.5 μg ml^{-1}) containing 50 ng of

4MPA, 1 ml water and 1 ml of 10% trichloroacetic acid to precipitate the plasma proteins. The 10-ml tube was tightly capped and centrifuged (TJ6 centrifuge) at 1720 *g* for 10 min. The supernatant was transferred to another 10-ml tube and added sequentially were 300 μl of saturated sodium carbonate solution to neutralize the trichloroacetic acid, 1 ml of 10% sodium bicarbonate solution and 1 μl of PFBCl. The tube was shaken vigorously for 5 min and allowed to stand for 30 min at room temperature. The aqueous layer was extracted with 5 ml of cyclohexane by mixing on an Evapomix for 10 min followed by centrifugation at 400 *g* for 3 min. The organic phase was transferred to a clean tube and evaporated in a dry bath under nitrogen. The dried residue was reconstituted with 50 μl of anhydrous ethyl acetate by mixing on a Vortex mixer for 5 sec. Aliquots (1 μl) of the resulting solution were analyzed by GC—ECD.

Recovery study

For the determination of recovery, four replicate samples at levels of 120, 100, 50, 5, 50 and 125 ng ml^{-1} for MP, ODMP, 5HMP, NDMP, 4MPA and PA, respectively, were spiked in fresh blank plasma and run through the appropriate procedure as described above. The external standard method was employed and the peak height ratios obtained for the extracted samples were compared with those of fresh standards prepared in organic solvents.

Temperature dependence study

MP (400 ng) and each of its metabolites (80 ng) were derivatized with PFBCl as described above. These benzoyl derivatives were analyzed with the column oven temperature programmed from 200°C to 270°C at a rate of 12°C min^{-1}. The detector temperature was varied at 25°C installments from 150°C to 300°C. The effect of detector temperature on the ECD response of each compound at a fixed concentration was examined by plotting the peak area against detector temperature.

RESULTS AND DISCUSSION

N-Pentafluorobenzoyl derivatives of primary and secondary alkylamine drugs and doping agents have been prepared for their sensitive analysis by GC—ECD [6—11]. Pentafluorobenzoylation not only facilitates gas chromatography, it also provides electron-capturing ability to these amines, which is generally much greater to that obtained with derivatizing agents such as trifluoroacetic anhydride, pentafluoropropionic anhydride and heptafluorobutyric anhydride [7—9]. Therefore, for the sensitive analysis of MP and three of its metabolites, N- and N,O-pentafluorobenzoyl derivatives were prepared. Generally, amine compounds are extracted from biological fluids with organic solvents before acylation. More recently extractive acylation, especially benzoylation with PFBCl, has been found to be a very efficient method for the analysis of doping agents [6], because it combines extraction and derivatization in one step. This extractive pentafluorobenzoylation, when applied to the GC—ECD or GC—NPD analysis of MP, gave recoveries of 99.9% from plasma (Table I) with no interferences from endogenous plasma constituents. The metabolites

TABLE I

RECOVERY OF METHOXYPHENAMINE, METHOXYPHENAMINE METABOLITES, AND THE INTERNAL STANDARDS FROM PLASMA

Compound added	Amount added to 1 ml plasma (ng)	Amount recovered (ng)	*n*	Percentage recovery (mean ± S.D.)
Methoxyphenamine	120	119.9	4	99.9 ± 2.6
Phenethylamine	125	89.87	4	71.9 ± 1.2
O-Desmethylmethoxyphenamine	100	65.80	4	65.8 ± 3.6
5-Hydroxymethoxyphenamine	50	29.65	4	59.3 ± 1.9
N-Desmethylmethoxyphenamine	5	2.53	4	50.7 ± 3.4
4-Methoxyphenethylamine	50	45.50	5	91.0 ± 3.0

of MP were analyzed by adapting an aqueous derivatization procedure [12] in which the pentafluorobenzoyl derivatives of phenols are formed in aqueous medium by the addition of sodium bicarbonate and PFBCl. These derivatives can then be conveniently extracted in high yields with organic solvents. This overcomes the problems associated with the poor recoveries of phenolic amines from biological medium. However, in the analysis of MP metabolites in plasma, the aqueous O- and N-derivatization was performed after the precipitation of plasma proteins with 10% trichloroacetic acid. In the presence of plasma proteins, the phenolic amines did not react efficiently with PFBCl. This protein precipitation improved the recoveries about four-fold as compared to when protein precipitation was not carried out. Aqueous derivatization was found to be highly pH-dependent. Therefore, the control of pH was important in order to have reproducible recoveries of the derivatized phenolic amines. Pentafluorobenzoylation of phenols is known to be facilitated above pH 9, therefore, in the present study, after protein precipitation, derivatization in an aqueous medium was carried out at around pH 9.4 in the presence of sodium bicarbonate.

Formation of N- and O-pentafluorobenzoyl derivatives was checked by GC—MS (Table II) under the conditions described in Experimental; MP and its N-desmethyl metabolite were both converted into their N-monoacyl deivatives, while ODMP and 5HMP yielded N,O-dipentafluorobenzoyl derivatives. Each of these derivatives gave a mass spectrum with the appropriate molecular ions and diagnostic ion. Rationalization of these diagnostic ions in terms of the structures of the N- and N,O-pentafluorobenzoyl derivatives of MP, NDMP, ODMP and 5HMP can be readily elucidated from literature reports [4, 6, 10, 11].

Detector temperature was observed to have significant effect on the electron-capturing abilities of the PFB derivatives of MP and the three metabolites. The PFB derivative of MP showed maximum electron-capturing response at a temperature of 150°C (Fig. 1) and this response decreased considerably as the detector temperature was raised, especially at temperatures of 200°C and above. However, the PFB derivatives of NDMP, ODMP and 5HMP each showed one maximum in the range 175—200°C and, after a minimum in the range 225—250°C, a subsequent rise in their ECD response up to the maximum

TABLE II

TABULATION OF PRINCIPAL FRAGMENTATION PATHWAYS OF PFB DERIVATIVES OF METHOXYPHENAMINE AND ITS THREE METABOLITES

Methoxyphenamine	$M^{+\cdot}$ 373 (0.2)	M−121$^+$ 252 (53)	M−178$^+$ 195 (100)	M−206$^+$ 167 (9)	M−225$^+$ 148 (47)	M−282$^+$ 91 (19)	
N-Desmethylmethoxyphenamine	$M^{+\cdot}$ 359 (0.7)	M−121$^+$ 238 (21)	M−164$^+$ 195 (100)	M−192$^+$ 167 (12)	M−211$^+$ 148 (97)	M−268$^+$ 91 (42)	
O-Desmethylmethoxyphenamine	$M^{+\cdot}$ 553 (0.1)	M−225$^+$ 328 (0.8)	M−301$^+$ 252 (100)	M−358$^+$ 195 (100)	M−386$^+$ 167 (15)	M−436$^+$ 117 (4)	M−462$^+$ 91 (1)
5-Hydroxymethoxyphenamine	$M^{+\cdot}$ 583 (1)	M−225$^+$ 358 (10)	M−331$^+$ 252 (68)	M−388$^+$ 195 (100)	M−416$^+$ 167 (15)	M−466$^+$ 117 (3)	M−492$^+$ 91 (2)

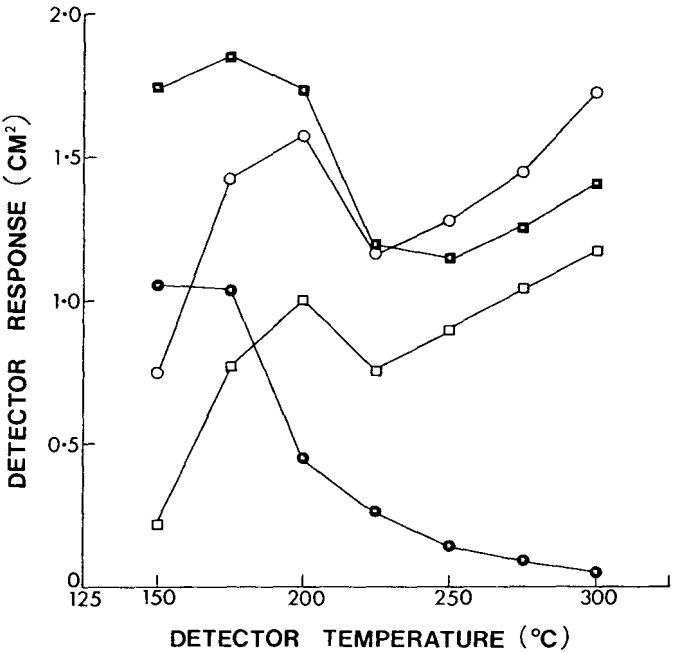


Fig. 1. Relationship between ECD response and detector temperature for methoxyphenamine PFB (•), N-desmethylmethoxyphenamine PFB (▪), O-desmethylmethoxyphenamine di-PFB (○) and 5-hydroxymethoxyphenamine di-PFB (□).

examined temperature of 300°C. In each case both the maximum and the subsequent rise occurred at temperatures where the MP derivative had negligible ECD response. Due to the significant reduction in ECD response of the MP derivative at detector temperatures above 150°C, it became necessary to analyze MP separately from its metabolites, and hence, a GC—NPD assay was developed for the analysis of MP from plasma. The phenomenon of a lower ECD response at higher detector temperatures for secondary amine compounds lacking other derivatizable functional groups as compared to their analogous primary amine derivatives, has also been observed for other secondary amines such as ethylamphetamine [11] and fenfluramine [9].

N- and N,O-pentafluorobenzoyl derivatives of MP, NDMP, ODMP, 5HMP and the internal standards phenethylamine and 4-methoxyphenethylamine gave sharp symmetrical peaks. Fig. 2A shows a typical chromatogram obtained by processing control blank plasma, without the internal standard, through the GC—ECD assay. The extraneous peak observed at a retention time of 7.8 min did not interfere with the assay as it elutes after the peaks due to the derivatives of the metabolites and the internal standard. A chromatogram obtained when the GC—ECD method was applied to spiked human plasma (1 ml) containing 25 ng ml^{-1} each of NDMP, ODMP, 5HMP and 50 ng ml^{-1} internal

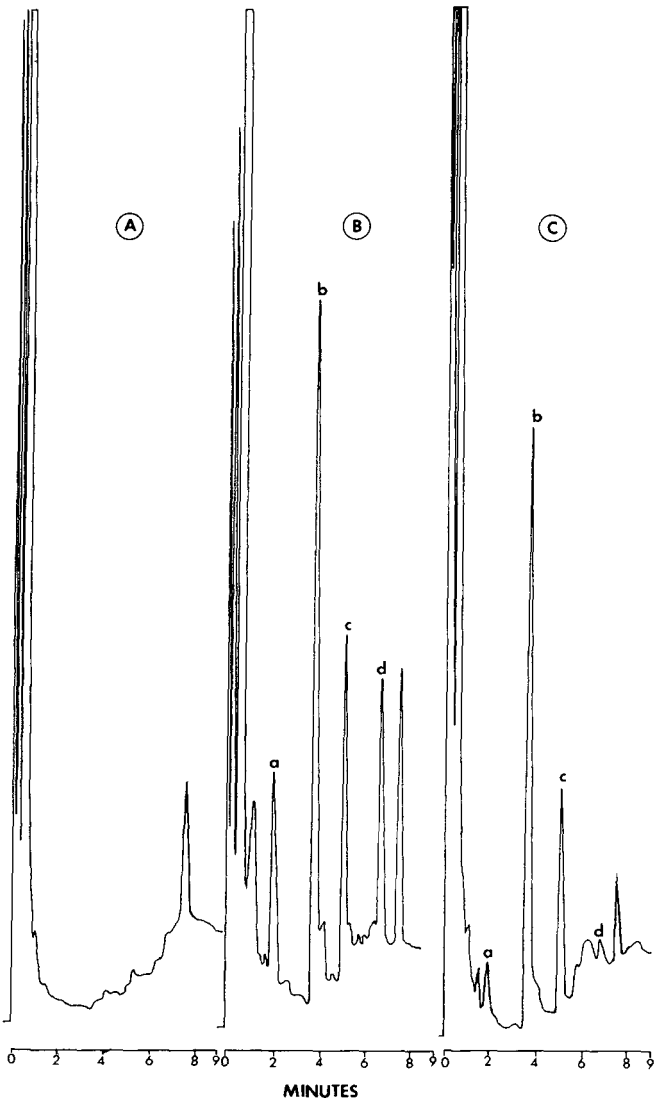


Fig. 2. Electron-capture chromatograms of extracts from 1 ml of plasma. A, Blank plasma; B, plasma spiked with N-desmethylmethoxyphenamine (a), O-desmethylmethoxyphenamine (c), 5-hydroxymethoxyphenamine (d) and internal standard (b); C, plasma sample from a volunteer 4 h after oral administration of methoxyphenamine hydrochloride.

standard (4 MPA) is shown in Fig. 2B. The chromatogram of the extract from a plasma sample (1 ml) from blood withdrawn from a male volunteer (78 kg) at 4 h after oral administration of 60.3 mg of MP · HCl is shown in Fig. 2C. GC analysis time was 9.0 min and 2.4, 22.3 and 2.6 ng ml^{-1} NDMP, ODMP and 5HMP, respectively, were estimated in this sample.

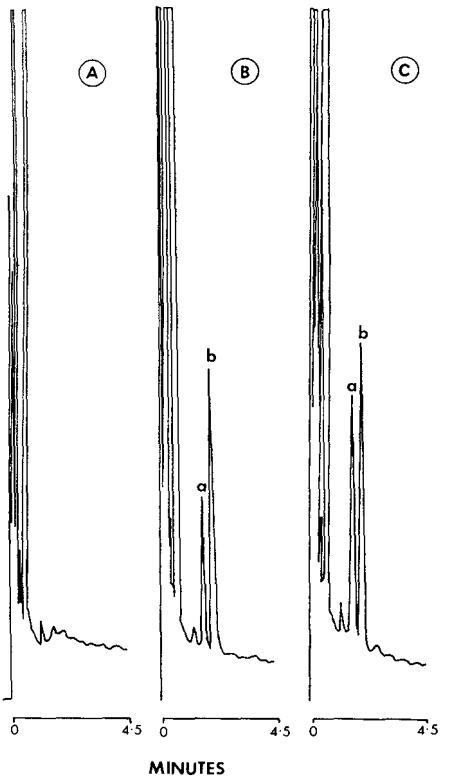


Fig. 3. Chromatograms with nitrogen-selective detection of extracts from 2 ml of plasma. A, Blank plasma; B, plasma spiked with methoxyphenamine (a) and internal standard (b); C, plasma sample from a volunteer 4 h after oral administration of methoxyphenamine hydrochloride.

Fig. 3A shows a typical chromatogram obtained by processing control blank plasma, without the internal standard, through the GC—NPD assay of unmetabolized MP. There were no endogenous peaks which interfered with the peaks due to the N-pentafluorobenzoyl derivatives of MP (t_R 2.4 min) and the internal standard PA (t_R 2.8 min). Also shown (Fig. 3B) is a chromatogram obtained when the GC—NPD method was applied to spiked human plasma (2 ml) containing 60 ng ml^{-1} MP and 125 ng ml^{-1} PA. Fig. 3C shows a GC—NPD chromatogram of a plasma sample (2 ml) from blood withdrawn from a healthy male volunteer (70 kg) 1 h after oral administration of 60.3 mg of MP · HCl. GC analysis time was less than 4.0 min and 89 ng ml^{-1} MP was found in this sample.

Column selection was found to be critical for separation of the N-pentafluorobenzamides of MP and NDMP. Initial attempts to resolve these

derivatives on non-polar to polar columns such as OV-1, OV-17, OV-25 and OV-210 were unsuccessful. These two derivatives could only be separated on the polar stationary phase OV-225, which was thermally stable with minimal drift [13] in the GC—ECD analysis of the metabolites at column oven temperatures of 210—285°C. However, this drift of baseline became more significant in the GC—NPD analysis of MP, although the column oven temperatures were lower than those used in the metabolite assay by GC—ECD. This problem of baseline drift, which may be due to bleed from a cyano column contaminating the nitrogen—phosphorus detector, was overcome by preconditioning the column just before analysis at 285° C for 6 h with a low flow of helium.

TABLE III

CALIBRATION CURVE DATA FOR METHOXYPHENAMINE GC—NPD

Concentration (ng ml $^{-1}$)	n	Mean peak height ratio	S.D.	C.V. (%)
3.75	3	0.036	0.002	5.7
7.5	7	0.073	0.005	6.4
15	7	0.145	0.007	5.3
30	7	0.292	0.014	5.3
60	7	0.584	0.027	4.9
120	7	1.122	0.053	4.7

$Y = 0.0093X - 0.006$ ($r^2 = 0.99$)

TABLE IV

CALIBRATION CURVE FOR O-DESMETHYLMETHOXYPHENAMINE GC—ECD

Concentration (ng ml $^{-1}$)	n	Mean peak height ratio	S.D.	C.V. (%)
3.125	5	0.056	0.005	8.9
6.25	5	0.107	0.003	2.8
12.5	5	0.198	0.019	9.7
25	5	0.364	0.024	6.6
50	5	0.683	0.036	5.3
100	5	1.350	0.076	6.1

$Y = 0.0132X + 0.024$ ($r^2 = 0.99$)

TABLE V

CALIBRATION CURVE DATA FOR N-DESMETHYLMETHOXYPHENAMINE GC—ECD

Concentration (ng ml $^{-1}$)	n	Mean peak height ratio	S.D.	C.V. (%)
1.563	4	0.0289	0.008	2.7
3.125	5	0.0433	0.003	6.7
6.25	5	0.0830	0.002	3.3
12.5	5	0.1644	0.010	6.1
25	5	0.3364	0.020	6.0

$Y = 0.0132X + 0.003$ ($r^2 = 0.99$)

TABLE VI

CALIBRATION CURVE DATA FOR 5-HYDROXYMETHOXYPHENAMINE GC—ECD

Concentration (ng ml^{-1})	n	Mean peak height ratio	S.D.	C.V. (%)
2.22	4	0.0550	0.002	3.3
4.44	4	0.1096	0.008	7.0
8.88	4	0.2000	0.004	2.2
17.76	4	0.3775	0.010	2.6

$Y = 0.0205X + 0.019$ ($r^2 = 0.99$)

The accuracy and preccision of the GC—NPD assay of MP are demonstrated in Table III. Tables IV—VI show the accuracy and precision for the GC—ECD assays of ODMP, NDMP and 5HMP, respectively. The calibration curves were linear in the concentration ranges 3.8—120 ng ml^{-1} for MP, 3.1—100 ng ml^{-1} for ODMP, 1.6—25 ng ml^{-1} for NDMP and 2.2—17.8 ng ml^{-1} for 5HMP, while their respective overall coefficients of variation in these ranges were 5.4%; 6.6%; 5.0% and 3.8%.

The overall recoveries of MP, ODMP, 5HMP, NDMP and the internal standards are given in Table I. The mean recoveries of MP, NDMP, ODMP and 5HMP were found to be 99.9 ± 2.6%, 50.7 ± 3.4%, 65.8 ± 3.6% and 59.3 ± 1.9%, respectively. The internal standards 4MPA and PA gave overall recoveries of 91.0 ± 3.0% and 71.9 ± 1.2%, respectively.

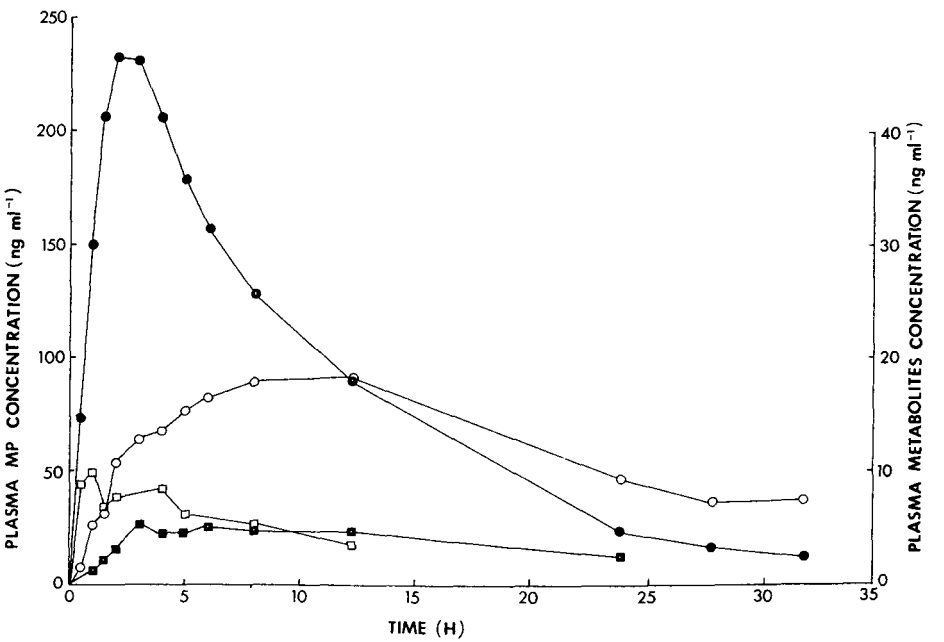


Fig. 4. Plasma concentration—time profiles for methoxyphenamine (•), O-desmethylmethoxyphenamine (○), N-desmethylmethoxyphenamine (▪) and 5-hydroxymethoxyphenamine (□) obtained from a volunteer (65 kg) who received a single oral dose of methoxyphenamine hydrochloride.

The application of the present methods to the determination of MP and its metabolites in plasma is shown in Fig. 4. The sensitivity of the GC—NPD assay of MP was such that plasma concentrations could be followed up to 24 h in all three subjects who were each orally administered 60.3 mg of MP · HCl. Also, the GC—ECD assay allowed the anlaysis of ODMP in plasma samples collected as late as 32 h after MP · HCl administration, whereas due to their lower plasma levels, NDMP and 5HMP could only be quantitated for 4 and 12 h, respectively, in all three subjects following single doses of MP · HCl.

In summary, GC methods are reported for the quantitative analysis of MP and three of its metabolites in plasma. The GC—NPD method for MP and the GC—ECD method for NDMP, ODMP and 5HMP are sensitive and reproducible and will be used to determine the pharmacokinetics of the drug in healthy volunteers following single oral doses.

ACKNOWLEDGEMENT

The support of the Medical Research Council of Canada (Grant No. MA-6950) is gratefully acknowledged.

REFERENCES

1 K.K. Midha, J.K. Cooper, I.J. McGilveray, R.T. Coutts and R. Dawe, Drug Metab. Dispos., 4 (1976) 568.
2 K.K. Midha and R.T. Coutts, J. Chromatogr., 144 (1977) 280.
3 T. Inoue, T. Niwaguchi and M. Emoto, Kagaku Keisatsu Kenkyusho Hokoku, Hokagaku Hen, 33 (1980) 89.
4 G. McKay, J.K. Cooper, E.M. Hawes, S.D. Roy and K.K. Midha, Xenobiotica, 13 (1983) 257.
5 T. Niwaguchi and T. Inoue, J. Chromatogr., 161 (1978) 223.
6 F.T. Delbeke, M. Debackere, J.A.A. Jonckheere and A.P. De Leenheer, J. Chromatogr., 273 (1983) 141.
7 S.B. Matin and M. Rowland, J. Pharm. Sci., 61 (1972) 1235.
8 A.C. Moffat, E.C. Horning, S.B. Matin and M. Rowland, J. Chromatogr., 66 (1972) 255.
9 K.K. Midha, J.K. Cooper and I.J. McGilveray, J. Pharm. Sci., 68 (1979) 557.
10 K.K. Midha, I.J. McGilveray and J.K. Cooper, Can. J. Pharm. Sci., 14 (1979) 18.
11 K.K. Midha, J.K. Cooper, D. Gagné and K. Bailey, J. Anal. Toxicol., 3 (1979) 53.
12 N. Nose, S. Kobayashi, A. Tanaka, A. Hirose and A. Watanabe, J. Chromatogr., 125 (1976) 439.
13 G.L. Dadish, W. Vycudilik and G. Machata, Forensic Sci., 10 (1977) 205.

Journal of Chromatography, 310 (1984) 319—326
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2216

IMPROVED GAS—LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF BACLOFEN IN PLASMA AND URINE

GREGORY KOCHAK* and FRANK HONC

Research Department, Pharmaceuticals Division, Ciba-Geigy Corporation, Ardsley, NY 10502 (U.S.A.)

(First received February 15th, 1984; revised manuscript received April 28th, 1984)

SUMMARY

A simple, rapid and sensitive assay for baclofen analysis has been developed. Baclofen and the internal standard are analyzed by gas—liquid chromatography with electron-capture detection after esterification of the carboxyl group to the butyl ester and acylation of the amino group to the pentafluoropropionylamide. Recovery from biological matrixes is accomplished by ion-pair extraction. The limit of quantitation of the entire assay as stated is about 10 ng/ml baclofen in plasma.

INTRODUCTION

Baclofen (Lioresal®) is a centrally acting muscle relaxant which is indicated for the alleviation of signs and symptoms of spasticity resulting from multiple sclerosis, particularly for the relief of flexor spasms and concomitant pain, clonus and muscular rigidity.

A sensitive and specific analytical procedure is necessary for use in bioavailability and pharmacokinetic studies. Following administration of single oral 10-mg commercial tablets resulting plasma concentrations are 30—50 ng/ml at 10 h.

Reported methods [1, 2] for the analysis of baclofen, γ-amino-β-(*p*-chlorophenyl)butyric acid, utilized adsorption of baclofen onto charcoal, XAD-2, or Dowex 50W-X4 resin for recovery from biological matrixes. Recovered baclofen was analyzed by gas—liquid chromatography (GLC) with electron-capture detection following derivatization to the butyl ester and heptafluorobutyrylamide or mass fragmentography.

An improved method for the determination of baclofen in plasma and urine has been developed. Recovery of baclofen and the internal standard, γ-amino-β-

0378-4347/84/$03.00

(2,4-dichlorophenyl)butyric acid from biological matrixes is accomplished by ion-pair extraction with heptanesulfonic acid directly into an organic phase. Analysis is by GLC with electron-capture detection following derivatization to the butyl ester and pentafluoropropionylamide. The use of an ion-pair extraction and formation of the pentafluoropropionylamide derivative results in significant improvement in selectivity, throughput time, reproducibility, and sensitivity compared to the previous GLC method [1] and comparable sensitivity to the other method [2].

EXPERIMENTAL

Preparation of reagents and sources

Heptanesulfonic acid, 90 m*M*, in 1 *M* phosphate buffer pH 3 was prepared by dissolving 68.995 g of sodium phosphate monobasic (J.T. Baker, Phillipsburg, NJ, U.S.A.), 2.5 ml of 85% orthophosphoric acid (Fisher Scientific, Pittsburgh, PA, U.S.A.) and 9.9 g heptanesulfonic acid, sodium salt (Eastman, Rochester, NY, U.S.A.) in 500 ml of distilled water.

Butanolic hydrochloric acid was prepared by mixing 5 ml of 1-butanol with 0.25 ml acetylchloride (both from MCB, Gibbstown, NJ, U.S.A.).

Borate buffer pH 10 was prepared by dissolving 24.6 g boric acid, 29.8 g potassium chloride and 14.1 g sodium hydroxide (all obtained from J.T. Baker) in 1 l of distilled water.

Pentafluoropropionic anhydride (Pierce, Rockford, IL, U.S.A.) was mixed with ethyl acetate (MCB) to give a concentration of 28.6% (v/v).

The following solvents were mixed (v/v) to the indicated concentrations: 40% 1-butanol in dichloromethane (MCB); 20% tertiary amyl alcohol (MCB) in ethyl acetate; 20% dichloromethane in diethyl ether; 2% acetone (both from J.T. Baker) in hexane (MCB); and methanol absolute (J.T. Baker) was used as supplied.

Preparation of standard solutions and calibration standards

The internal standard was prepared by dissolving γ-amino-β-(2,4-dichlorophenyl)butyric acid (Ciba-Geigy, Basle, Switzerland) in 0.1 *M* hydrochloric acid (J.T. Baker) to a concentration of 1 μg/ml.

The baclofen standard solution was prepared by dissolving γ-amino-β-(*p*-chlorophenyl)butyric acid (Ciba-Geigy, Summit, NJ, U.S.A.) in 0.1 *M* hydrochloric acid to a concentration of 1 μg/ml. Standard solutions were refrigerated and remained stable for at least six months.

Calibration standards were prepared by spiking pooled human plasma or urine with the prepared standard solutions.

Sample preparation and recovery

Ion-pair extraction. A 100-μl aliquot of internal standard solution is added to 1 ml plasma or 20 μl urine diluted in 1 ml distilled water, followed by 1 ml heptanesulfonic acid solution, 4 drops of 1 *M* phosphoric acid, 2.5 ml distilled water, and 0.5 ml of 40% butanol in dichloromethane. The resultant pH of this mixture should be 2.5—3.0. The mixture is shaken for 10 min on a horizontal mechanical shaker and centrifuged 10 min. The protein precipitate will

compress at the bottom in the tube and the clear supernatant is transferred into a clean 13-ml centrifuge tube.

The supernatant is extracted twice with 4 ml of 20% tertiary amyl alcohol in ethyl acetate by shaking on a horizontal shaker for 15 min. Both organic phases are pooled and evaporated to dryness under a stream of nitrogen at 45°C.

Derivatization. The dry residue is dissolved in 0.5 ml of butanolic hydrochloric acid, heated at 100°C for 15 min and evaporated to dryness under nitrogen at 45°C. The dry residue is dissolved in 5 ml of 20% dichloromethane in diethyl ether and the solution is extracted with 2 ml of 0.05 *M* sulfuric acid by shaking for 10 min. After a short centrifugation the organic phase is discarded. To the aqueous phase 2 ml of borate buffer pH 10 are added and extracted into 5 ml of 2% acetone in hexane by shaking 10 min. After a short centrifugation 4 ml of the organic phase are evaporated to dryness under nitrogen.

A 0.2-ml aliquot of 28.6% pentafluoropropionic anhydride in ethyl acetate is added to the residue and reacted for 1 h at room temperature. Unreacted reagent is evaporated to dryness and the tube washed with 0.2 ml methanol in order to destroy left-over reagent. The methanol is evaporated and the dry residue dissolved in 1 ml toluene (or heptane) of which 3 μl are injected on column.

Chromatographic conditions and instrumentation

The chromatographic system consisted of a Varian Model 3700 gas chromatograph with a ^{63}Ni (8 μCi) electron-capture detector and Perkin-Elmer Model 023 strip-chart recorder. The column used was a 15 m × 2 mm I.D. glass column packed with 3% OV 225 on Chromosorb W HP 80—100 mesh (Supelco, Bellefonte, PA, U.S.A.). The following temperatures were used: column 218°C, injector 240°C, detector 350°C. Nitrogen was used as the carrier gas at a flow-rate of 32 ml/min. The sensitivity of the electrometer was set at $1 \cdot 10^{-12}$ A.f.s. and attenuation 64.

RESULTS AND DISCUSSION

Chromatography

The retention times of the derivatives of baclofen and the internal standard were 3.1 and 4.2 min, respectively. Typical chromatograms obtained from control and spiked plasma are shown in Fig. 1. Chromatograms obtained from control and spiked urine are shown in Fig. 2.

Derivative formation

Baclofen (I, Fig. 3) and the internal standard are converted easily into their butyl esters (II) by heating with butanolic hydrochloric acid. Subsequent acylation of the primary amine was achieved by reaction of the ester with pentafluoropropionic anhydride. The structure of the resulting baclofen derivative (III) has been verified by mass spectrometry.

Derivatization of baclofen to the methyl ester and then transesterification to the butyl ester as previously reported [1] was found not to be necessary.

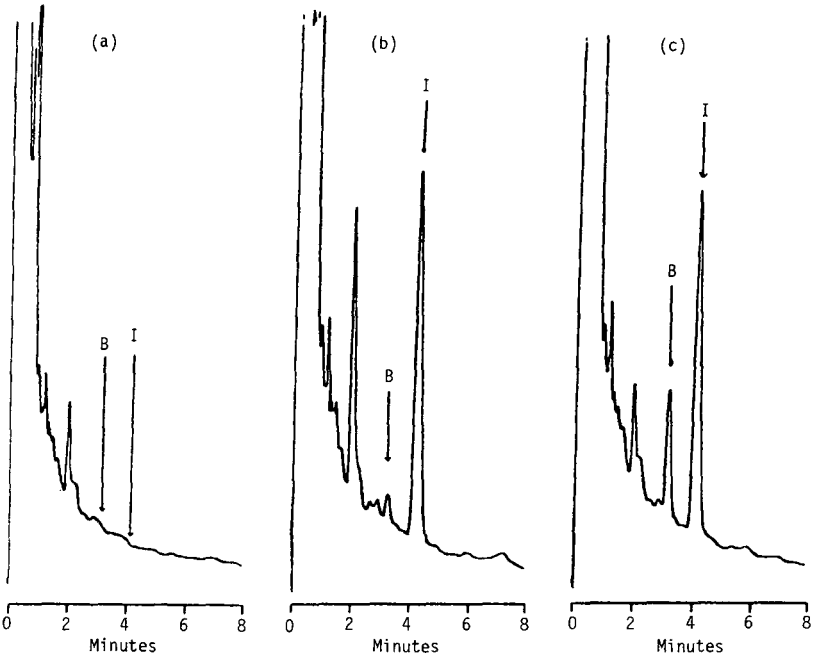


Fig. 1. Typical chromatograms from plasma extract. (a) Blank pooled plasma extract from 1 ml plasma; (b) plasma extract and reaction products from 1 ml plasma spiked with 20 ng/ml baclofen and 100 ng/ml internal standard; (c) plasma extract and reaction products from 1 ml plasma spiked with 100 ng/ml baclofen and 100 ng/ml internal standard. Peaks: B = baclofen reaction product; I = internal standard reaction product.

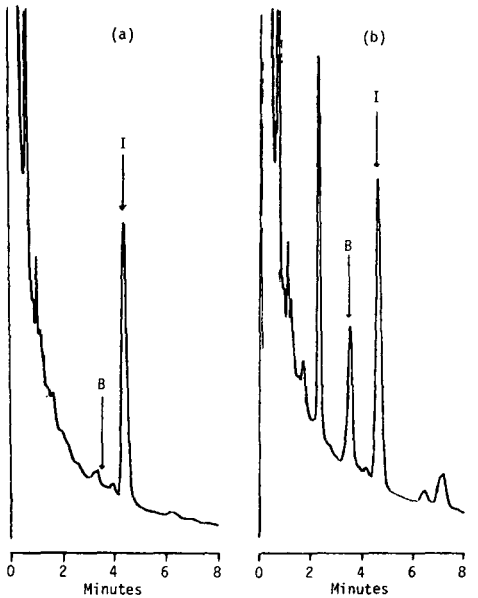
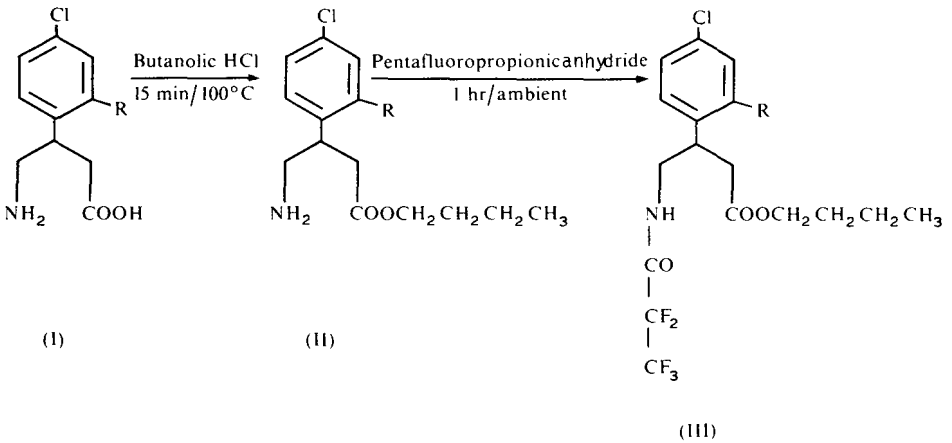


Fig. 2. Typical chromatograms from urine extract. (a) Pooled urine extract from 20 μl urine containing 5 μg/ml internal standard; (b) urine extract and reaction products from 20 μl urine spiked with 5 μg/ml baclofen and 5 μg/ml internal standard. Peaks: B = baclofen reaction product; I = internal standard reaction product.



(I) (II) (III)

Fig. 3. Scheme showing formation of *n*-butyl esters and the N-pentafluoropropionyl derivatives of baclofen (R = H) and the internal standard (R = Cl).

Direct formation of the butyl ester was facile and rapid. The throughput time was decreased by 50% for the acylation step used in this method compared to that previously reported [1]. The N-pentafluoropropionyl derivatives exhibited superior chromatographic performance and throughput time compared to the previously reported N-heptafluorobutyryl derivatives [1]. The N-pentafluoropropionyl derivatives resulted in increased sensitivity both in terms of peak area and cleaner baseline chromatography.

Ion-pair extraction

The ion-pair extraction produced a cleaner extract from plasma due to the increase in selectivity for baclofen and the internal standard. This resulted in an overall increase in sensitivity because of the improved signal-to-noise ratio. Throughput time was decreased by at least 30% compared to charcoal and resin adsorption recovery methods [1, 2]. The extraction efficiency was 35.3 ± 6.3% (n = 14) over the range 20—800 ng/ml. The extraction efficiency decreased if the plasma or urine was not adjusted to a pH below 3. This was due to ionization of the carboxylic groups on baclofen and the internal standard. Ion-pair extraction of urine samples was found to be necessary. Urine sample volumes of 20 μl resulted in an interference under the baclofen peak of approximately 8% peak height relative to a 100-ng baclofen sample. No interference was detected relative to the internal standard.

Linearity of calibration curves

The standard curve for plasma was linear in the range 10—800 ng/ml employing a sample volume of 1 ml and a single standard curve. The standard curve regression was determined as follows: peak height ratio = 0.00313 (concentration baclofen) + 0.00221, r = 0.9999. However, for samples in the range 5—20 ng/ml a separate calibration curve is suggested in order to prevent bias in the regression slope and intercept. Similarly the urine calibration curve was linear in the range 1—40 μg/ml using 20-μl sample volumes. The standard curve regression was determined as follows: peak height ratio = 0.00445 (concentration baclofen) — 0.05876, r = 0.9989.

Selectivity

Control plasma (drug-free) showed no detectable interferences (Fig. 1). Control urine samples (20 μl) showed only a single very low level interference under the baclofen peak (Fig. 2). Due to the high concentration of baclofen in urine (microgram range) after the administration of a single 20-mg oral dose this level of interference is considered negligible. Larger urine sample volumes, however, should only be used cautiously due to the increasing percentage of interference relative to baclofen in the sample.

Since only 3—6% of a dose of baclofen is excreted renally as metabolites in man [3] no detectable metabolic interferences are anticipated. Plasma (1 ml) and urine (20 μl) samples from subjects receiving baclofen indeed showed no detectable interferences with baclofen or the internal standard.

Accuracy and precision

The results of within-day accuracy and precision for the determination of baclofen in independent plasma samples are presented in Table I. The coefficients of variation (C.V.) ranged from 25.0% (n = 3) at 5 ng/ml to 1.4% (n = 4) at 50 ng/ml. Mean absolute error ranged from 20% at 5 ng/ml to 1% at 100 ng/ml. Similarly within-day accuracy and precision for urine determination utilizing 20-μl urine samples are presented in Table II. The C.V. values ranged from 5.0% (n = 3) at 800 ng per 20 μl to 2.8% (n = 3) at 100 μg per 20

TABLE I

WITHIN-DAY ACCURACY AND PRECISION FOR DETERMINATION OF BACLOFEN IN PLASMA BY GLC

Baclofen added (ng/ml)	Baclofen found (ng/ml)				No. of samples	Mean ± S.D.	C.V. (%)	Mean absolute error ± S.D. (%)
5	3	5	4		3	4 ± 1.0	25.0	20 ± 20.0
10	11	11	9	12	4	11 ± 1.3	11.8	13 ± 5.0
20	27	25	22	22	4	24 ± 2.4	10.0	20 ± 12.2
50	44	43	44	43	4	44 ± 0.6	1.4	11 ± 3.5
100	99	101	103	100	4	101 ± 1.7	1.7	1 ± 1.3
200	209	216	204	214	4	211 ± 5.4	2.6	5 ± 2.7
400	441	437	400	441	4	430 ± 19.9	4.6	7 ± 5.0
800	795	748	817	767	4	782 ± 30.4	3.9	3 ± 2.5

TABLE II

WITHIN-DAY ACCURACY AND PRECISION FOR DETERMINATION OF BACLOFEN IN URINE BY GLC

Baclofen added (ng per 20 ml)	Baclofen found (ng per 20 ml)			No. of samples	Mean ± S.D.	C.V. (%)	Mean absolute error ± S.D. (%)
20	22	23	21	3	22 ± 1.0	4.5	10 ± 5.0
100	94	95	90	3	93 ± 2.6	2.8	7 ± 2.6
800	821	768	848	3	812 ± 40.7	5.0	4 ± 1.7

TABLE III

DAY-TO-DAY PRECISION FOR DETERMINATION OF BACLOFEN IN PLASMA AND URINE BY GLC

Day	Regression slope	Regression intercept	No. of standards per curve	Correlation coefficient (r)
*Plasma**				
1	0.00313	0.00221	8	0.9999
2	0.00310	0.00588	7	0.9998
3	0.00336	0.00409	8	0.9995
4	0.00319	0.03487	27	0.9992
5	0.00343	0.03777	7	0.9980
Average	0.00324	0.01696		0.9993
S.D.	0.00015	0.01775		0.0008
C.V. (%)	4.6	104.7		0.1
Urine				
1	0.00445	—0.05876	6	0.9989
2	0.00421	—0.01768	12	1.0000

*Over a period greater than two weeks.

μl. Mean absolute error ranged from 10% at 20 ng per 20 μl to 4% at 800 μg per 20 μl.

Day-to-day variability is demonstrated for a period in excess of two weeks by the change in slope of standard curves (Table III). For plasma determinations of baclofen the C.V. for the regression slope is 4.6% (n = 5) indicating a high degree of reproducibility. The regression slopes for the urine standard curves (Table III) were reproducible for two curves obtained on separate days, two days apart.

Sensitivity

Based on the use of a single calibration curve and the reported instrumental conditions the overall limit of quantitation is 10 ng/ml for the plasma assay and 1 μg/ml for the urine assay. For plasma analysis improved accuracy and precision would be expected utilizing an additional calibration curve in the 5—20 ng/ml range.

Stability of plasma samples

Spiked 50 and 400 ng/ml plasma samples were frozen and stored at —15°C for up to 148 days. The average (± S.D.) recovery at 47 days was 102.6% (n = 2) and at 148 days was 93.5 ± 7.8% (n = 4). These values indicate the stability of baclofen plasma samples for at least 148 days.

CONCLUSION

An improved method for the determination of baclofen in biological matrixes has been developed which provides a sensitive and selective analytical procedure for use in bioavailability and pharmacokinetic studies. This method

provides at least a two-fold increase in sensitivity as well as significant increases in selectivity and throughput time of analysis over previously described methods.

REFERENCES

1 P.H. Degen and W. Riess, J. Chromatogr., 117 (1976) 399.
2 C. Swahn, H. Beving and G. Sedvall, J. Chromatogr., 162 (1979) 433.
3 J.W. Faigle, personal communication.

Journal of Chromatography, 310 (1984) 327—333
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2191

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF TIAPROFENIC ACID AND ITS METABOLITES IN PLASMA AND URINE BY DIRECT INJECTION*

F. JAMALI*, A.S. RUSSELL, B.W. BERRY and C. LEHMANN

Faculty of Pharmacy and Pharmaceutical Sciences, and Division of Rheumatology, The University of Alberta, Edmonton, Alberta T6G 2N8 (Canada)

(First received October 28th, 1983; revised manuscript received May 4th, 1984)

SUMMARY

A rapid, convenient, sensitive and selective reversed-phase high-performance liquid chromatographic method was developed to measure tiaprofenic acid, its reduced and oxidized metabolites and their conjugates in biological fluids. The method involved direct injections of plasma and urine samples into the chromatograph before and after alkaline hydrolysis of the conjugates. Concentrations as low as 0.5 μg/ml of the drug in plasma and urine were quantifiable. The method was suitable for analysis of tiaprofenic acid and its metabolites in biological fluids after administration of therapeutic doses. Several other non-steroidal anti-inflammatory drugs which were applied to the system did not interfere with the assay.

INTRODUCTION

Tiaprofenic acid (Surgam®, Roussel) is a new non-steroidal anti-inflammatory drug (NSAID) which is under clinical trial in North America. The drug is a potent inhibitor of prostaglandin synthesis [1] and, despite its short half-life ($t_{1/2}$), seems to be as effective as other NSAIDs with longer $t_{1/2}$ if given 200 mg three times a day [2]. Tiaprofenic acid (TA) and its reduced (R) and oxidized (O) metabolites (Fig. 1) have been measured in biological fluids using a thin-layer chromatographic—spectrometric method [3]. More recently, a high-performance liquid chromatographic (HPLC) method describing extraction and determination of TA in plasma of a man was reported [4]. In

*Presented in part at the Research Symposium and Research Conference of the Association of Faculties of Pharmacy of Canada, Montreal, P.Q., May 1983.

0378-4347/84/$03.00

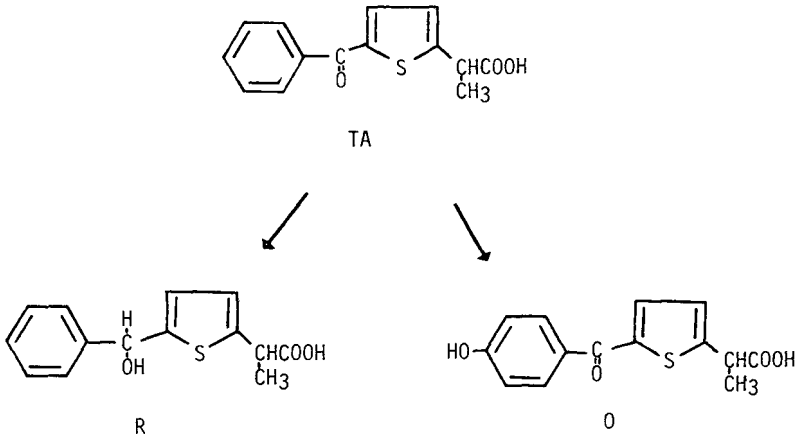


Fig. 1. Chemical structure of tiaprofenic acid (TA) and of its reduced, α-(5-benzyl alcohol-2-thienyl)propionic acid (R), and oxidized, α-[5-(4-hydroxybenzoyl)-2-thienyl]propionic acid (O).

this paper we report a convenient, selective and sensitive method for measuring TA, R and O and their conjugates in biological fluids.

EXPERIMENTAL

Apparatus

The HPLC system (Waters Sci., Mississauga, Canada) consisted of a Model 6000A pump, a Wisp autosampler, a Model 481 variable-wavelength ultraviolet (UV) detector, a Model 730 data module and a 10-cm reversed-phase column (Novapac C_{18}; 5-μm Radial-Pak cartridge, Waters Sci.). A 5-cm guard column (Whatman, Clifton, NJ, U.S.A.) packed with 10-μm C_{18} material was utilized throughout the experiment. The mobile phase, methanol—water—acetic acid (57:40:3) was pumped at a flow-rate of 1.5 ml/min and pressure of 3.5 MPa after filtering through 45-μm filters (Millipore, Mississauga, Canada).

UV spectra of TA, O and R in mobile phase showed maximum absorbances at 305, 254 and 315 nm, respectively. They all showed considerable absorbance at 254 nm while negligible absorbance was detected for R at 305 and 315 nm. Specimens were tested at 254 and 315 nm to examine the presence of the drug and its metabolites. For quantitation purposes, however, plasma samples were monitored at 315 nm and urine samples at 254 nm.

Chemicals

The drug and its metabolites were gifts from Roussel (Montreal, Canada). Naproxen (Syntex, Palo Alto, CA, U.S.A.) was used as internal standard (IS). Salicylic acid (BDH Chemicals, Toronto, Canada), piroxicam (Pfizer, Kirkland, Canada), ketoprofen (Poulenc, Montreal, Canada), fenprofen (Eli Lilly, Toronto, Canada), flurbiprofen (Boots, U.K.) and etodolac (Ayerst, Montreal, Canada) were examined for their elution time using the developed HPLC conditions. All organic solvents were of analytical grade. Water was distilled and deionized.

Sample preparation

To 100—200 μl of plasma or urine were added either 50 μl of water or 50 μl of 1 *M* sodium hydroxide, 50 μl of IS solution (0.5 mg/ml in methanol) and 300 μl of acetonitrile. Samples were vortexed, centrifuged at high speed (Fisher micro-centrifuge, Model 235A) for 2 min, filtered through 2-μm disposable filter tips (Supelco, Bellefonte, PA, U.S.A.) and transferred into vials containing micro-inserts (250 μl). Depending on the concentration of samples 10—200 μl of the preparations were injected into the chromatograph. To quantify the plasma and urine contents, four sets of solutions containing different concentrations of TA (1, 2, 3, 5, 10, 20, 40, 50 and 100 μg/ml) and half of the above concentrations of R and O were prepared. To 50 μl of the above solutions were added 50 μl of blank plasma and after mixing, the preparations were treated as described for plasma and urine samples. The peak area ratios (TA/IS, O/IS and R/IS) were calculated and plotted versus corresponding concentrations. The slope, intercept and correlation coefficient of the resulting standard line were calculated using a regression equation. Samples were always tested against a set of three freshly prepared standard solutions.

Subjects, dosages and sample collections

Two patients (one male and one female) with rheumatoid arthritis (RA) and two healthy male subjects participated in the study voluntarily. Patients were amongst the participants in a clinical trial and under therapy with TA (200 mg t.i.d.). Blood (4—5 ml) and urine (total output) samples were collected from patients during the first daily dosing interval at 0, 0.25, 0.50, 0.75, 1, 2, 3, 4, 5, 6 and 8 h and 0, 1, 3, 5, 7 and 8 h, respectively. Healthy subjects took 400 mg TA 1.5 h before breakfast and their urine samples were taken hourly from 0 to 6 h and then 8, 10, 12 and 24 h post-dosing.

Blood samples were drawn into dry heparinized tubes, centrifuged and the plasma fraction was separated. The volume and pH of urine samples were recorded after collection. The specimens were either tested immediately or kept frozen until the day of analysis. Volunteers were under no other drug therapy at least one week prior to and during the experiment.

Concentrations of TA in plasma and urinary excretion rates of each component were plotted versus time. The slopes of the terminal phases of curves were calculated using a curve-fitting program (Model 41C Hewlett-Packard, Corvallis, OR, U.S.A.).

Stability tests

Solutions of TA were prepared in plasma (16 mg/l), urine (24 mg/l), methanol (22 mg/l) and 1 *M* sodium hydroxide (26 mg/l) and kept at room temperature, 4°C and −20°C. Samples were taken for twelve days to four months, freshly prepared solutions of naproxen were added and were injected into the chromatograph.

RESULTS AND DISCUSSION

Fig. 2 depicts chromatograms of blank plasma and that of a patient 8 h after administration of 200 mg TA. The drug and IS appeared 7.8 and 10.9 min after

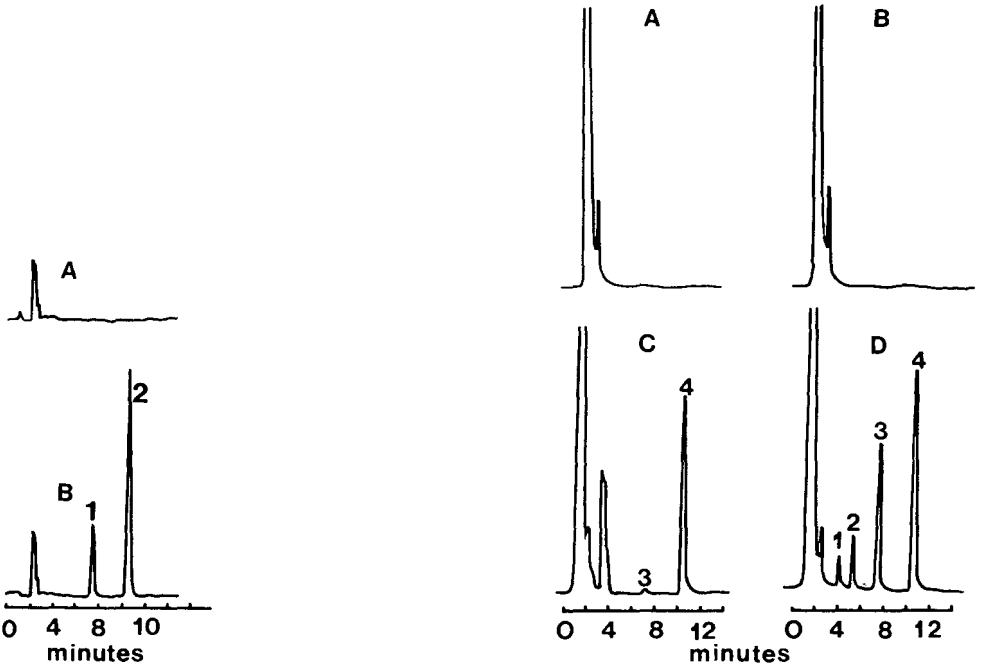


Fig. 2. Chromatograms of blank plasma (A) and plasma of a patient (B) 8 h after a 200-mg dose of tiaprofenic acid (1). Peak 2 is internal standard.

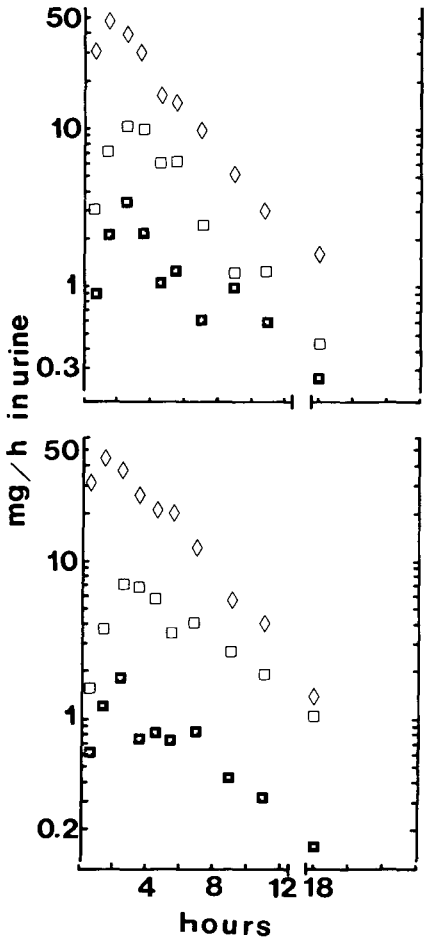
Fig. 3. Chromatograms of blank urine (A and B) and urine of a subject (C and D) 8 h after a 400-mg dose of tiaprofenic acid (3) before (A and C) and after (B and D) alkaline hydrolysis. Peaks 1, 2 and 4 represent reduced and oxidized metabolites and internal standard, respectively.

injection into the instrument, respectively, and no interfering peaks were observed. Although no interfering peak appeared at 305 nm (TA maximum absorbance) a more steady baseline was observed when samples were monitored at 315 nm. None of the metabolites were found in plasma. In urine samples, however, different pictures were observed (Fig. 3). No significant amount of intact TA, R and O were found in urine samples analyzed immediately after collection. Instead, a cluster of few peaks appeared 3 to 4 min after injection (Fig. 3). These peaks seem to be conjugates of TA, R and O. Pottier et al. [3] have reported conjugates of TA in urine. However, due, perhaps, to problems inherent in their assay procedure, they did not separate the intact drug and metabolites from their conjugates. Our direct injection assay method provided a clear picture of TA metabolism in man. Similar to probenecid, naproxen and ketoprofen [5], TA seems to be excreted mainly as ester conjugates. These conjugates are so unstable that their hydrolysis to intact drug takes place even in the bladder and results in conflicting interpretation of data [5]. Conversion of conjugates to TA, R and O was observed even during storage of urine samples at 4°C and after freezing and thawing.

After addition of alkali to the urine of patients, TA, R and O were instantly and completely hydrolyzed to intact compounds (Fig. 3). Moreover, the addition of alkali was accompanied by appearance of a transient red color

which was clearly noticeable when TA concentrations greater than 5 mg/l were present in urine. The color intensity appeared to be concentration-dependent. The nature of the color producing reaction was not studied. Retention times for R and O were 3.8 and 5.6 min, respectively. No interfering peak was eluted from blank urine (Fig. 3).

An excellent correlation was observed between the peak area ratios and concentrations of the components. For urine the best-fit lines through the points were described by $Y = 0.070X - 0.005$, $Y = 0.054X - 0.002$ and $Y = 0.036X - 0.001$ for TA, R and O, respectively. For TA in plasma $Y = 0.084X + 0.040$ was observed. Correlation coefficients of greater than 0.998 were observed for all lines. Concentrations as low as 0.5 μg/ml of TA, R and O in plasma and urine were quantifiable. The coefficients of variation (C.V.) varied from 2% to 5% within the examined range.



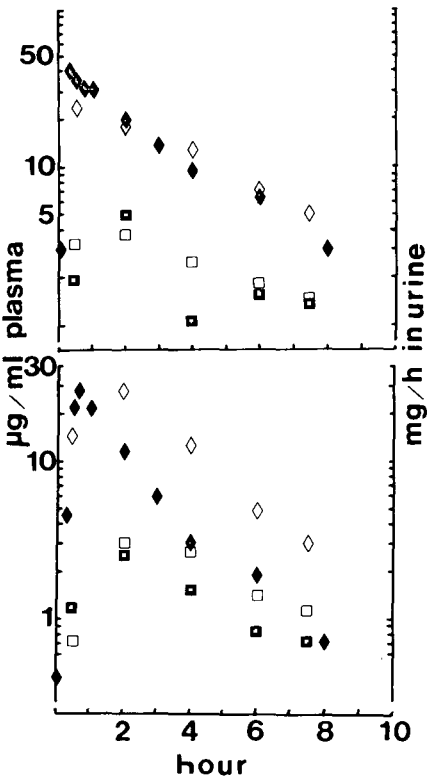


Fig. 4. Urinary excretion rates of tiaprofenic acid (◇) and of its reduced (□) and oxidized (■) metabolites after alkaline hydrolysis in healthy subjects who took 400-mg doses of the drug.

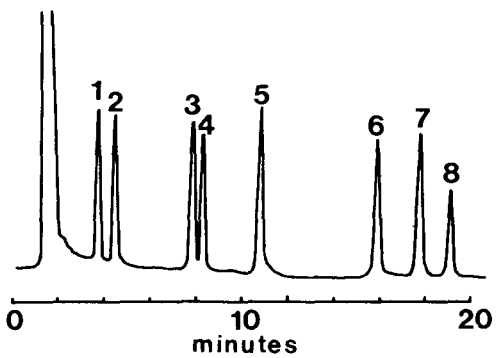
Fig. 5. Plasma tiaprofenic acid (♦) and urinary excretion rates of the conjugates of the acid (◇) and of its reduced (□) and oxidized (■) metabolites in patients under therapy with 200 mg t.i.d.

In Fig. 4, plasma TA concentration—time curves are shown. Our observations agree with those of Ward et al. [4] who reported an HPLC method for determination of TA in plasma of a healthy subject. Their sample preparation method involved extraction with chloroform in acidic medium, evaporation and reconstitution in mobile phase. The method presented here does not require extraction, and, therefore, decreases the preparation time and increases precision. Although our sample contained TA concentrations > 0.5 μg/ml, levels as low as 0.25 μg/ml were also quantifiable with acceptable reproducibility (C.V. < 9%).

After hydrolysis of conjugates of TA, R and O, their urinary excretion rates were plotted versus time as shown in Fig. 4 and 5. The slopes of excretion rate plots were parallel with those of TA concentration—time curves. A total of 76.16—79.50% of the dose, consisting of 59.32—60.60% TA, 14.30—14.35% R and 2.54—4.56% O conjugates, was recovered from urine of healthy subjects. Since patients were at the steady-state drug concentration, the cumulative amount of the drug excreted in one dosing time-interval was considered as total recovery from a given dose. They excreted 67.30—72.24% of the given dose as conjugates of TA (53.46—54.34%), R (8.04—10.65%) and O (5.81—6.57%).

Plasma TA concentration—time curves appeared as multiexponential curves. The half-lives of the terminal phase varied from 2.00 to 2.65 h and values from plasma data were similar to those calculated from urinary data.

Under our HPLC conditions, salicylic acid, piroxicam, ketoprofen, naproxen, fenprofen, flurbiprofen and etodolac had elution times of 3.7, 4.5, 8.3, 10.9, 16.0, 17.8 and 19.2 min, respectively (Fig. 6). Although peaks representing salicylic acid and piroxicam were clearly detectable in the presence of TA metabolites, their peak distances were not long enough to permit quantitation of the components in urine. In plasma, however, since none of the TA metabolites are present, determination of TA in the presence of all the above NSAIDs is possible.



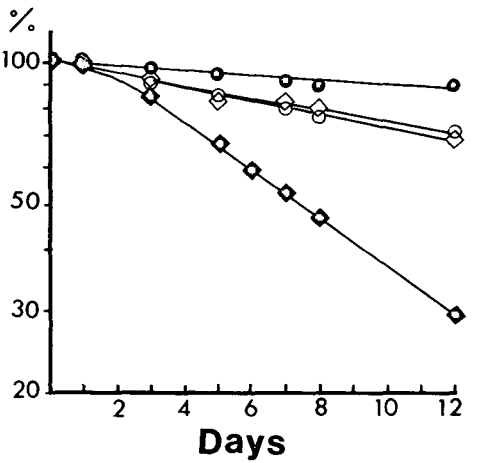


Fig. 6. Blank plasma spiked with salicylic acid (1), piroxicam (2), tiaprofenic acid (3), ketoprofen (4), naproxen (5), fenprofen (6), flurbiprofen (7) and etodolac (8).

Fig. 7. Percent tiaprofenic acid remaining in methanol (•), plasma (◇), urine (○) and 1 *M* sodium hydroxide (◆) at room temperature as a function of time.

No changes in retention times of components and separation properties of the analytical column were observed during the experiment indicating a relatively long useful column life.

TA seems to be stable for at least four months if stored frozen (−20°C) regardless of the nature of the solvent. At 4°C, the drug was stable in methanol, plasma and urine for at least two weeks but not in 1 *M* sodium hydroxide. In the latter solvent, 50% of the added TA was decomposed in twelve days.

Fig. 7 depicts stability of TA in methanol, plasma, urine and 1 *M* sodium hydroxide at room temperature. The drug seems to have limited stability at room temperature in all solvents. The decomposition products of TA appeared as four sharp peaks 6.9, 8.3, 11.3 and 28.2 min after injection.

The method presented here is sensitive and specific, and requires less preparation time than that reported by Ward et al. [4]. It also provides a clear picture of the urinary excretion of TA and its metabolites. The method can be used to measure plasma TA concentrations in presence of other NSAIDs.

ACKNOWLEDGEMENTS

C.L. and B.W.B. were recipients of the Alberta Heritage Foundation for Medical Research Summer Studentship. We thank the staff of station 35, University of Alberta Hospital for their assistance in providing patients specimens.

REFERENCES

1 R. Deraeat, S. Jouquey, F. Delevllee and M. Flahaut, Eur. J. Pharmacol., 61 (1980) 17.
2 E.C. Huskisson and G. Katona, Rheumatology, An Annual Review, Vol. 7, S. Karger, Basel, 1982, pp. 143, 159, 164, 173.
3 J. Pottier, D. Berlin and J.P. Raynaud, J. Pharm. Sci., 66 (1977) 1030.
4 G.T. Ward, J.A. Stead and M. Freeman, J. Liquid Chromatogr., 5 (1982) 165.
5 R.A. Upton, J.N. Buskin, R.L. Williams, N.H.G. Holford and S. Riegleman, J. Pharm. Sci., 69 (1980) 1254.

Journal of Chromatography, 310 (1984) 335—342
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2193

DETERMINATION OF ETHYLENEDIAMINEPLATINUM(II) MALONATE IN INFUSION FLUIDS, HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

W.J.F. VAN DER VIJGH*, F. ELFERINK and G.J. POSTMA

Research Laboratory of Internal Medicine, Free University Hospital, De Boelelaan 1117, 1081 HV Amsterdam (The Netherlands)

and

J.B. VERMORKEN and H.M. PINEDO

Department of Oncology, Free University Hospital, De Boelelaan 1117, 1081 HV Amsterdam (The Netherlands)

(First received December 8th, 1983; revised manuscript received May 8th, 1984)

SUMMARY

A selective and convenient high-performance liquid chromatographic assay was developed for ethylenediamineplatinum(II) malonate (JM-40) in plasma ultrafiltrate and urine. A μPorasil silica column (30 cm) was used with acetonitrile—water (90:10, v/v) as the mobile phase and the elution of compounds was monitored by ultraviolet absorbance at 214 nm. A linear dynamic range of at least three decades (1—1000 μg/ml) was achieved. The detection limit in plasma ultrafiltrate was 0.35 μg/ml. The stability of JM-40 was determined in 0.9% sodium chloride, 5% glucose, plasma ultrafiltrate and urine. More stable drug solutions were obtained with 5% glucose than with 0.9% sodium chloride. JM-40 was also determined in plasma ultrafiltrate and urine samples of one patient receiving short-term infusions of the drug. In plasma ultrafiltrate unmetabolized JM-40 was detected during the first 5 h after administration and had a half-life of 21.3 ± 1.6 min. The parent drug was excreted in the urine in rapidly decreasing amounts. Eighteen per cent of the dose was recovered as unmetabolized drug during the first 6 h.

INTRODUCTION

Since the discovery of the antineoplastic activity of cisplatin [1, 2], many analogues of this platinum complex have been synthesized in the search for a

0378-4347/84/$03.00

compound with a better therapeutic index [3]. The second-generation platinum compound ethylenediamineplatinum(II) malonate (JM-40) (see Fig. 1) was selected by the EORTC New Drug Development Committee for phase I studies on account of its antitumour activity and lack of emetic effect in the ferret [4, 5]. The drug is presently under clinical investigation.

In general, pharmacokinetic studies of cisplatin and its analogues have been performed using total platinum levels in clinical samples determined by flameless atomic absorption spectrophotometry (AAS) [6—8]. This method is very sensitive, but cannot differentiate between the original platinum complex and its degradation products. Moreover, it cannot be used on-line with a high-performance liquid chromatographic (HPLC) system.

The purpose of our study was to develop a selective HPLC procedure using ultraviolet (UV) absorbance detection for the quantification of JM-40. The applicability of this method is demonstrated by the analysis of JM-40 in infusion solutions and body fluids.

EXPERIMENTAL

Materials

The chemicals used were of analytical grade. The mobile phase consisted of acetonitrile (LiChrosorb®, Merck, Darmstadt, F.R.G.) and double-distilled water (90:10, v/v). Glucose, used for the stability experiments, was of Ph. Eur. quality (Brocacef, Amsterdam, The Netherlands). Chemically pure JM-40 (99.8% by elemental analysis) which was used for in vitro experiments was kindly provided by Johnson Mathey (Reading, Berkshire, U.K.). JM-40 ampoules (50 mg of JM-40 per 10 ml of water), used for intravenous administration to patients, were supplied by Bristol Myers (Brussels, Belgium).

An ultrafiltrate of pooled plasma samples from healthy individuals was prepared with Amicon Centriflo® ultrafiltration membrane cones, CF 50A. The model MPS-1 micropartition system provided with YMT filters (both from Amicon, Oosterhout, The Netherlands) was used for the filtration of plasma samples from patients.

Instrumentation

The chromatographic system consisted of a Model 6000A solvent delivery system, a 30 × 0.39 cm column packed with μPorasil, 10 μm (both from Waters, Etten-Leur, The Netherlands) and an LDC UV III monitor Model 1203 with a fixed wavelength of 214 nm (Charles Goffin, De Bilt, The Netherlands). For the stability experiments, 10-μl samples were injected with a WISP 710B autoinjector equipped with a datamodule and a system controller (all from Waters). Plasma ultrafiltrates and urine samples of patients were injected with a Valco Universal Inlet equipped with a 10-μl loop (Chrompack, Middelburg, The Netherlands). The chromatograms were recorded with a Model PM 8251 strip-chart recorder (Philips, Eindhoven, The Netherlands). UV spectra were determined with a Model 25 spectrophotometer (Beckman Instruments, Mijdrecht, The Netherlands).

Total platinum concentrations in plasma, plasma ultrafiltrate and urine samples were determined with a Model 5000A atomic absorption spectro-

photometer at 265.9 nm. The atomic absorption instrumentation included a HGA 500 graphite furnace, an AS 40 autosampler and a PRS printer-sequencer (all from Perkin-Elmer, Gouda, The Netherlands).

Methods

The stability of JM-40 was determined at ambient temperature. A freshly prepared solution of JM-40 in water was used as the external standard. Plasma ultrafiltrate and urine samples from healthy volunteers and solutions of 0.9% sodium chloride and 5% glucose were spiked with a concentrated stock solution of JM-40 to obtain a final drug concentration of 100 μg/ml. After preparing the solutions, 10-μl aliquots were periodically introduced into the chromatographic system without prior purification.

JM-40 was administered to the patients immediately after dilution of the pharmaceutic preparation in 300 ml of 5% glucose. Doses of 300 and 400 mg/m^2 were given as intravenous infusions of 12 and 25 min duration, respectively. The interval between subsequent drug administrations was one month. Blood samples were drawn at the end of infusion and 10, 20, 30, 60, 90, 120, 150, 210, 240 and 360 min thereafter. Urine was obtained from one patient every 2 h. All samples were immediately processed and analysed within 30 min after collection.

RESULTS AND DISCUSSION

Chromatography

Several stationary phases, including silica and silica-bonded phase packings (—CN, $-NH_2$, $-NO_2$) were examined. Elution was carried out using various combinations of acetonitrile and water. Silica columns offered a wide range of retention times for JM-40 allowing a choice of various eluents. An optimal separation of JM-40 from endogeneous constituents present in plasma ultrafiltrate and urine was obtained with a 30-cm μPorasil column and a mobile phase consisting of acetonitrile—water (90:10, v/v). Fig. 1 shows representative chromatograms of plasma ultrafiltrate and urine from a patient before and after administration of JM-40. The chromatographic conditions for the separation of JM-40 from endogeneous compounds are comparable to those used for CBDCA [*cis*-diamine-1,1-cyclobutanedicarboxylatoplatinum(II)] [9]. Although JM-40 lacks a cyclobutane group, its polarity may be similar to that of CBDCA due to the ethylene group at the amine site. Higher proportions of water in the eluent resulted in decreased resolution of JM-40 from plasma ultrafiltrate and urine constituents. The retention volume of 25 ml required a flow-rate of 2 ml/min. The use of a 10-μl injection volume was a compromise between detection limit and resolution; a larger injection volume caused a reduction in the number of theoretical plates from 4000 with 10 μl to 3000 with 20 μl.

The UV absorption spectrum (500—190 nm) of JM-40 in eluent showed a maximum at 200 nm with a molar absorptivity of $5.35 \cdot 10^3$ l mol^{-1} cm^{-1}. A comparable value ($5.05 \cdot 10^3$ l mol^{-1} cm^{-1}) was obtained at 214 nm corresponding to the fixed wavelength of the UV detector.

The relationship between peak height and concentration of aqueous JM-40 solutions was linear over a range of 1—1000 μg/ml on a log—log scale (r =

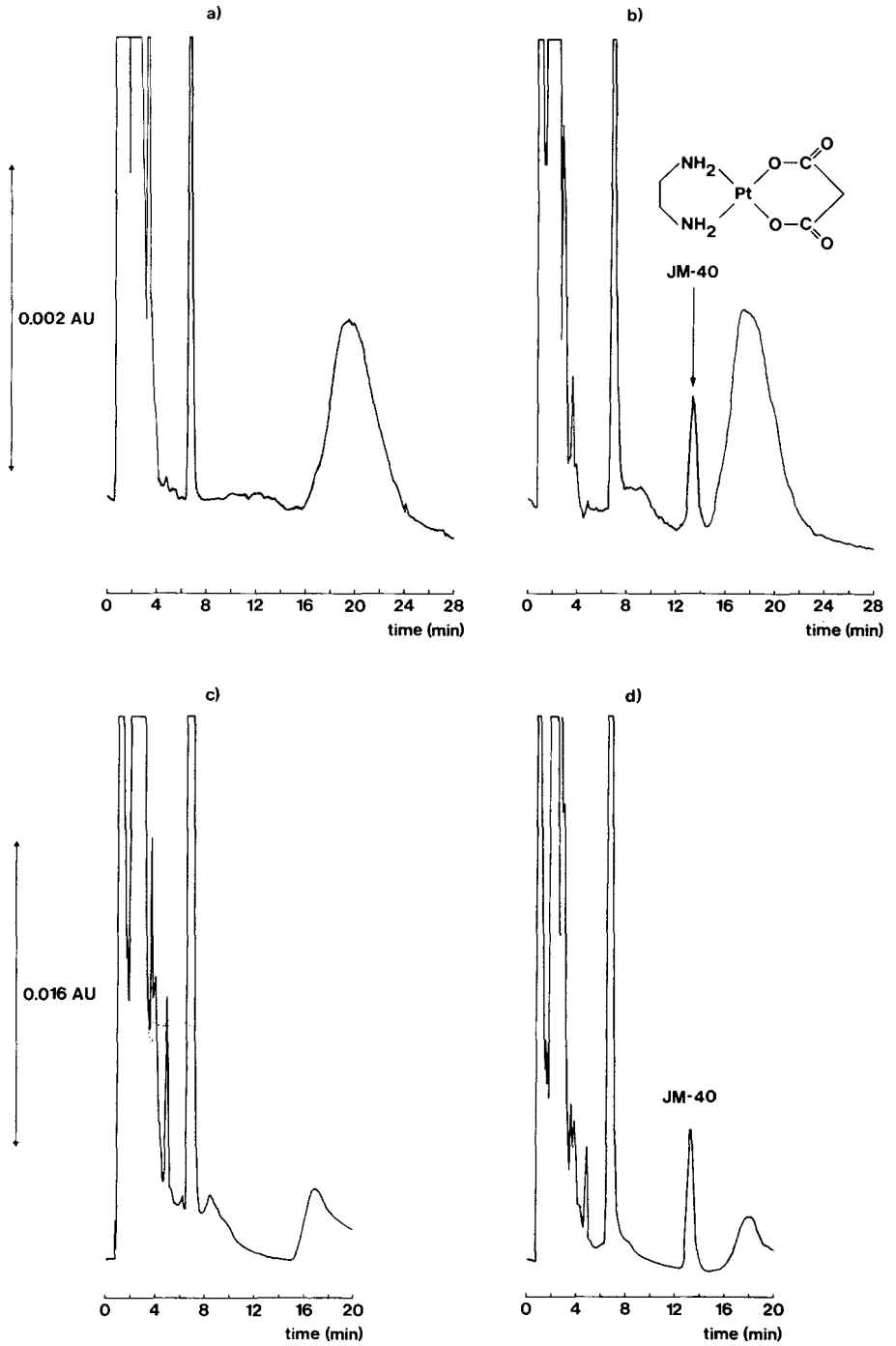


Fig. 1. Chromatograms of plasma ultrafiltrate and urine from a patient. (a) Ultrafiltrate before and (b) 90 min after administration of 300 mg/m² JM-40 (concentration 8.5 μg/ml); (c) urine before and (d) 2—4 h after administration of 400 mg/m² JM-40 (concentration 54.4 μg/ml). Column: μPorasil, 10 μm, 30 × 0.39 cm. Mobile phase: acetonitrile—water (90:10, v/v). Flow-rate: 2 ml/min. UV detection at 214 nm.

0.999, n = 6). Concentrations of JM-40 in biological fluids were calculated by linear interpolation of daily prepared standard curves for aqueous drug solutions. The sensitivity was $13.6 \cdot 10^{-3}$ absorbance units per μg. A practical detection limit of 0.35 μg/ml in plasma ultrafiltrate was achieved at a signal-to-noise ratio of 3:1. The recovery of JM-40 from the HPLC system was determined with AAS by the ratio of the platinum content of the JM-40 peak and the amount of platinum originally present in the sample injected. Using 10-μl samples containing 90.5 μg/ml JM-40, drug recovery from plasma ultrafiltrate, urine and aqueous solutions was 91.3 ± 3.5% (n = 6), 91.3 ± 2.7% (n = 3) and 90.9 ± 3.9% (n = 4), respectively. The overall precision of the assay, determined with an aqueous solution of 103 μg/ml JM-40 over three subsequent days, was 1.6% (n = 7). Repeated analysis of 50 μg/ml JM-40 in plasma ultrafiltrate, freshly prepared before each injection, revealed a mean within-day precision of 1.8% (three days, n = 6 per day) and an overall precision of 3.5% (n = 18).

Stability

The stability of JM-40 was determined in acetonitrile—water (9:1, v/v), 0.9% sodium chloride, 5% glucose, plasma ultrafiltrate and urine at ambient temperature. Parent JM-40 was determined periodically after preparation of the solutions. The results of these experiments are shown in Fig. 2.

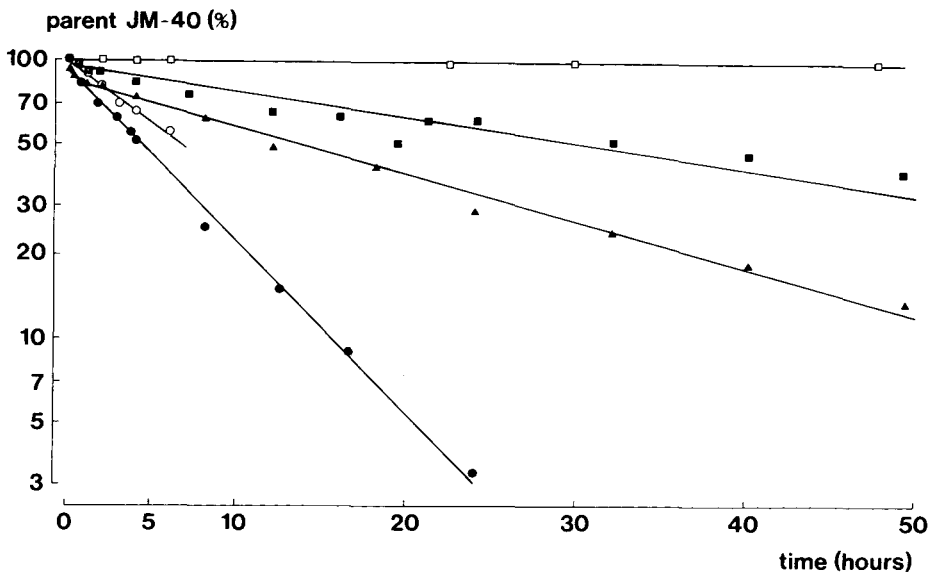


Fig. 2. Degradation curves for JM-40 in 5% glucose (□), 0.9% sodium chloride (■), plasma ultrafiltrate (▲), urine (●) and mobile phase (○).

In the mobile phase JM-40 had a half-life of 6.9 h (r = 0.994). This indicates that 2.1% of the injected drug may be lost during its 12.4-min residence in the column. JM-40 appeared to be stable in 5% glucose, confirming that 5% glucose is an appropriate vehicle for the administration of JM-40 to patients. Conversely, the drug appeared the be much less stable in saline, possibly due to

an exchange between the malonate group and the chloride ion [10]. The overall half-life in saline was 36.3 h (r = 0.95), although a half-life of 16.2 h (r = 0.97) was obtained during the first 4 h.

The reliability of the quantitation of JM-40 in body fluids is determined by the stability of JM-40 in these fluids. Fig. 2 shows that JM-40 disappears rapidly in plasma ultrafiltrate and urine, indicating the need for immediate analysis of patient samples. Half-lives of JM-40 in plasma ultrafiltrate and urine were 17.7 h (r = 0.994) and 4.9 h (r = 0.998), respectively. However, during the first 2 h, shorter half-lives of 10 h (r = 0.92) in plasma ultrafiltrate and 3.2 h (r = 0.97) in urine were obtained.

JM-40 concentrations in plasma ultrafiltrate could be determined within 30 min, at which point decomposition was limited to about 3%. Instability of JM-40 in urine was more severe. Therefore, decomposition during the stay of the drug in the bladder and during sample handling should be considered.

Pharmacokinetics

The assay was applied in the analysis of parent JM-40 in plasma ultrafiltrate and urine samples of a patient who received JM-40 in doses of 300 mg/m^2 and 400 mg/m^2. Fig. 3 shows the concentration—time curves for parent JM-40 in plasma ultrafiltrate compared to total platinum in plasma and plasma ultrafiltrate during the first 8 h after both infusions. The concentrations in plasma ultrafiltrate represent the amounts of non-protein bound forms of the

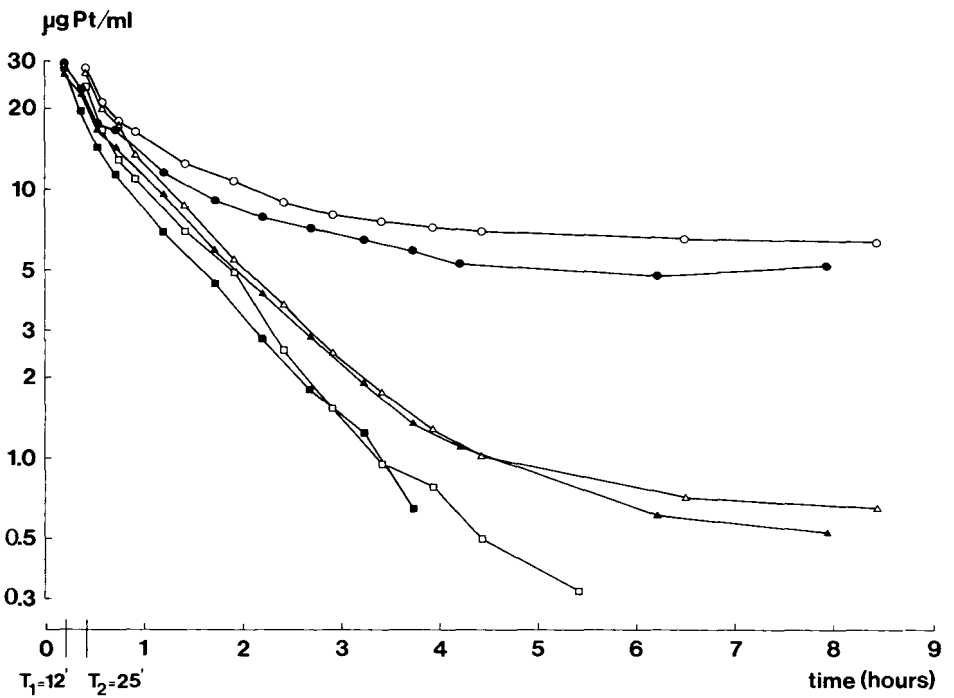


Fig. 3. Concentration—time curves of total platinum in plasma (●, ○), total platinum in plasma ultrafiltrate (▲, △) and parent JM-40 in plasma ultrafiltrate (■, □) in one patient after infusion of 300 mg/m^2 in T_1 = 12 min (closed symbols) and 400 mg/m^2 in T_2 = 25 min (open symbols).

metabolized and the parent drug. These species are thought to be responsible for the therapeutic effect, since protein-binding of platinum compounds is irreversible [11] and generates inactive adducts [12]. The concentration—time curves show three exponential terms for total platinum in plasma and plasma ultrafiltrate and two exponential terms for parent JM-40, due to distribution and elimination. The occurrence of more than two terms may indicate that metabolites of JM-40 are formed, which is supported by the presence of a third phase in the curve of total platinum in plasma ultrafiltrate in contrast to that of parent JM-40. Initial half-lives of distribution of parent JM-40 in plasma ultrafiltrate and of total platinum in plasma and plasma ultrafiltrate were 21.3, 28.3 and 28.6 min, respectively, when measured over a time interval of 0—20 min and without correction for subsequent phases.

Table I shows parent JM-40 and total platinum in plasma ultrafiltrate as a percentage of total platinum in plasma. These data indicate that in contrast to cisplatin [7, 13] non-protein-bound platinum can still be detected up to 24 h after administration of JM-40. Even 4 h after administration, about 50% of total platinum in plasma ultrafiltrate represents parent drug.

The results of the determination of JM-40 in patients' urine during the second cycle are given in Table II. The analysis was limited to urine samples collected during short time intervals because of possible drug degradation in the bladder. Most of the platinum excreted in the urine during the first 2 h was

TABLE I

PARENT JM-40 AND TOTAL PLATINUM IN PLASMA ULTRAFILTRATE AS PERCENTAGE OF TOTAL PLATINUM IN PLASMA

Results represent the mean of two cycles in one patient.

Time after administration (h)	Total platinum in plasma ultrafiltrate (μg/ml)	Parent JM-40 in plasma ultrafiltrate (μg/ml)
0	93.4	90.4
0.5	82.8	67.0
1	75.8	57.9
2	46.4	31.6
3	26.5	15.8
4	17.6	7.1*
8	10.2	
24	9.3	

*Second cycle only.

TABLE II

PARENT JM-40 EXCRETED IN URINE AFTER A DOSE OF 400 mg/m²

Time interval (h)	JM-40 (μg/ml)	Volume (ml)	Percentage of dose	Percentage of total platinum
0—2	88.8	1000	11.7	73.9
2—4	54.4	620	4.5	53.0
4—6	48.8	270	1.7	39.4

parent JM-40 (73.9%). After 6 h 18% of the dose was excreted as parent JM-40, while total platinum accounted for 28.6% of the dose. At 24 h, 39.0% of the dose was excreted. The cumulative platinum excretion in urine after administration of cisplatin was 24.4% and 28.4% of the dose at 6 and 24 h, respectively [13]. The excretion of a higher percentage of the dose over 24 h after JM-40 may be explained by the prolonged presence of non-protein-bound platinum species in plasma after administration of this drug.

ACKNOWLEDGEMENTS

This study was supported by a grant from The Netherlands Cancer Foundation (K.W.F.) No. VU 83-7, which allowed an integrated phase I study of JM-40 within the Pharmacokinetic and Metabolism Group of the EORTC. We thank Mrs. I. Klein for the analysis of total platinum.

REFERENCES

1 B. Rosenberg, L. Van Camp, J.E. Trosko and V.H. Mansour, Nature (London), 222 (1969) 385.
2 M. Rozencweig, D.D. Von Hoff, R. Abele and F.M. Muggia, in H.M. Pinedo (Editor), Cancer Chemotheraphy, Vol. 1, Excerpta Medica, Amsterdam, 1976, p. 107.
3 K.R. Harrap, in F.M. Muggia (Editor), Cancer Chemotheraphy, Vol. 1, Martinus Nijhoff, Boston, MA, 1983, p. 171.
4 R.J. Speer, H. Ridgway, L.M. Hall, D.P. Stewart, K.E. Howe, D.Z. Lieberman, A.D. Newman and J.M. Hill, Cancer Chemother. Rep. Part I, 59 (1975) 629.
5 W.T. Bradner, W.C. Rose and J.B. Huftalen, in A.W. Prestayko, S.T. Crooke and S.K. Carter (Editors), Cisplatin, Current Status and New Developments, Academic Press, New York, 1980, p. 171.
6 A.F. Leroy, M.J. Wehling, H.L. Sponseller, W.S. Friauf, R.E. Solomon and R.L. Dedrick, Biochem. Med., 18 (1977) 184.
7 J.B. Vermorken, W.J.F. van der Vijgh, I. Klein, H.E. Gall and H.M. Pinedo, Eur. J. Cancer Clin. Oncol., 19 (1982) 1069.
8 G.A. Curt, J.J. Grygiel, B.J. Corden, R.E. Ozols, R.B. Weiss, D.T. Tell, Ch.E. Myers and J.M. Collins, Cancer Res., 43 (1983) 4470.
9 S.J. Harland, D.R. Newell, Z.H. Siddik, R. Chadwick, A.H. Calvert and K.R. Harrap, Cancer Res., 44 (1984) 1693.
10 S.D. Cutbush, R. Kuroda, S. Neiddle and A.B. Robins, Inorg. Biochem., 18 (1983) 213.
11 A.J. Repta, D.F. Long, in A.W. Prestayko, S.T. Crooke and S.K. Carter (Editors), Cisplatin, Current Status and New Developments, Academic Press, New York, 1980, p. 285.
12 A.B. Robins, Chem. Biol. Interact., 38 (1982) 349.
13 J.B. Vermorken, W.J.F. van der Vijgh, I. Klein, A.A.M. Hart, H.E. Gall and H.M. Pinedo, Cancer Treat. Rep., 68 (1984) 505.

Journal of Chromatography, 310 (1984) 343—352
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2195

RAPID AND SENSITIVE DETERMINATION OF ACETYLSALICYLIC ACID AND ITS METABOLITES USING REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

R. JAMES O'KRUK, MICHAEL A. ADAMS and RICHARD B. PHILP*

Department of Pharmacology and Toxicology, The University of Western Ontario, London, Ontario N6A 5C1 (Canada)

(First received January 4th, 1984; revised manuscript received May 14th, 1984)

SUMMARY

A rapid and sensitive high-performance liquid chromatographic technique was developed for the simultaneous determination of gentisic acid, salicyluric acid, acetylsalicylic acid and salicylic acid in plasma and serum. The method involved a single deproteinization step and separation using a reversed-phase column eluted with a buffered methanol (35%) mobile phase. Detection was achieved with a variable-wavelength ultraviolet detector set at 235 nm and a given chromatographic analysis could be completed in less than 10 min. The method was tested in both human and animal (rat) models given a single dose of acetylsalicylic acid.

INTRODUCTION

The analgesic, anti-inflammatory and antipyretic actions of acetylsalicylic acid (ASA) have resulted in the widespread and frequent use of this drug. Most recently, it is being employed as an antithrombotic agent based on its ability to inhibit platelet function [1]. In man, ASA is rapidly hydrolysed to salicylic acid (SA) by non-specific esterases found in many tissues. SA is eliminated from the body by renal excretion and by hepatic biotransformation to salicyluric acid (SU), salicylic phenolic glucuronide, salicylic acyl glucuronide and gentisic acid (GA) [2].

Various methods have been reported for quantifying ASA and its metabolites in different biological media. These include the standard colorimetric [3] and fluorometric [4] methods and the more modern analysis by gas—liquid chromatography [5] and high-performance liquid chromatography (HPLC) [6—13]. Of these analytical methods only HPLC presently offers the required

0378-4347/84/$03.00

level of specificity, sensitivity and simplicity needed to analyse large numbers of samples in a short period of time.

Of many HPLC methods which analyse various combinations of ASA and its metabolites in different biological fluids [6—13], four employ a time-consuming solvent extraction and nitrogen evaporation process [6—10]. Others, which analyse plasma following deproteinization detect GA, SU and SA but not ASA [11, 12]. Only one method has measured ASA and its major metabolites GA, SU, ASA and SA in plasma without solvent extraction [13]. Here we describe a modification of this method for the simultaneous determination of GA, SU, ASA and SA in serum and plasma. The method improves separation and reduces sample analysis time to less than 10 min.

EXPERIMENTAL

Chemicals and solvents

The salicylate standards ASA, SA, GA and SU were obtained from Sigma (St. Louis, MO, U.S.A.). The internal standard 3,4,5-trimethoxybenzaldehyde (TMB) was purchased from Aldrich (Milwaukee, WI, U.S.A.). Chromatographic mobile phases were prepared with double-distilled water in HPLC grade methanol (Fisher Scientific, Fair Lawn, NJ, U.S.A.). The buffering agent was HPLC grade potassium dihydrogen phosphate from Fisher Scientific. All solvents were filtered and degassed prior to use. All other chemicals were analytical grade.

Chromatographic conditions

The chromatographic system consisted of a Model 110A solvent pump (Beckman Instruments, Berkeley, CA, U.S.A.), a Model 7120 injector (Rheodyne, Cotati, CA, U.S.A.) with a 20-μl loop, a guard column, 40 mm × 2 mm I.D. packed with Co:Pell ODS, particle size 30—38 μm (Whatman, Clifton, NJ, U.S.A.) and a reversed-phase C_8 column (octyl siloxane bonded phase on silica; 250 mm × 4.6 mm I.D.), particle size 10 μm (Alltech Assoc., Deerfield, IL, U.S.A.) at ambient temperature. The effluent was monitored using a variable-wavelength detector UV-50 (Varian Instruments, Palo Alto, CA, U.S.A.). Quantitation was accomplished with a Model 3380A integrator (Hewlett-Packard, Palo Alto, CA, U.S.A.).

Conditions for optimal separation of the compounds of interest were studied using a mobile phase consisting of methanol and 0.1% potassium dihydrogen phosphate buffer in which the methanol and final pH of the mobile phase were systematically modified. These studies indicated an optimal mobile phase concentration of 35% methanol, pH 3.9 ± 0.1. The flow-rate was 2 ml/min and the effluent was monitored at 235 nm. Standard curves were constructed based on peak area ratios obtained by internal standardization. The line of best fit was measured using a least-squares linear regression method.

Preparation of plasma and serum samples

Blood samples (500 μl) were collected into chilled test tubes containing 5 mg/ml potassium fluoride (25%), to prevent ASA hydrolysis. For the analysis of plasma, blood samples were mixed with 1.0% disodium ethylenediamine-

tetraacetate (EDTA, 1:10 parts blood), then centrifuged at 750 *g* for 15 min and the plasma was separated. For serum preparation, blood samples were allowed to coagulate, then centrifuged at 9000 *g* for 4 min and the serum was separated.

Aliquots of plasma or serum (200 μl) were mixed with 20 μl of a 30% perchloric acid solution containing the internal standard TMB (0.02%) in 1.5-ml polypropylene Eppendorf micro test tubes (Brinkmann Instruments, Westbury, NY, U.S.A.) followed by 200 μl of methanol (spiked with standards or drug-free). The mixture was then vortexed for 2 min and centrifuged at 9000 *g* for 4 min. A 20-μl sample of the clear supernatant was injected into the chromatograph.

For standardization, freshly prepared methanolic solutions of GA, SU, ASA and SA ranging in concentration from 1.0 to 480 μg/ml were added to drug-free rat or human sera.

Preparation of urine samples

Equal volumes of urine and hydrochloric acid (10 *M*) were mixed in a 1.5-ml polypropylene test tube and deep frozen (−80°C) until analysis. After thawing, 400-μl samples were transferred to 2-ml glass ampoules, flushed with nitrogen and immediately sealed. The ampoules were heated at 120°C for 3 h to hydrolyse the conjugates. On cooling, the contents of the ampoules were vortexed and 20 μl of the hydrolysate mixed with 20 μl of internal standard (0.02%, TMB), 180 μl of double-distilled water and 200 μl of methanol (spiked with standards or drug-free). The mixture was clarified by centrifugation at 9000 *g* for 2 min. A 20-μl sample of the supernatant was injected into the chromatograph. For analysis of urine the wavelength was changed from 235 to 313 nm as preliminary studies revealed that an endogenous peak co-chromatographed with SA at the lower wavelength. Freshly prepared standard solutions were made from SA (1—100 μg/ml) dissolved in methanol and added to drug-free hydrolysed urine.

RESULTS AND DISCUSSION

The method reported here improves resolution of the four compounds (GA, SU, ASA, SA) over the original technique of Rumble et al. [13] and the analysis time was reduced to less than 10 min following preparation of the supernatant from the deproteinized sample.

Optimal conditions for the separation of GA, SU, ASA and SA were studied in preliminary experiments. Based on these results (Figs. 1 and 2) we chose as mobile phase methanol—0.1% potassium dihydrogen phosphate buffer pH 3.9 (35:65).

TMB was chosen as the internal standard in the present assay as it eluted closely after, and was completely resolved from, the compounds of interest. Blank serum, plasma and urine showed no peaks that interfered with TMB.

All four salicylate compounds and the internal standard were monitored at various wavelengths ranging from 205 to 240 nm. A wavelength of 235 nm was found to be most suitable for monitoring all the compounds simultaneously.

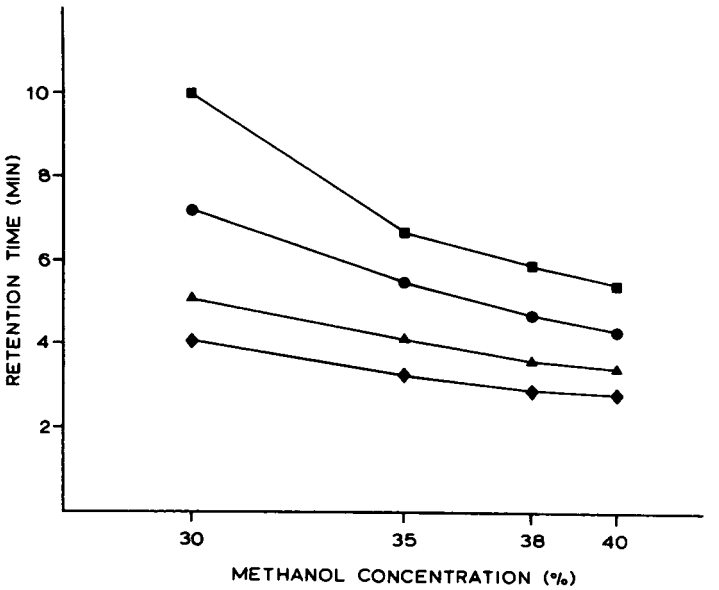


Fig. 1. Effect of methanol concentration on the retention times of GA (♦), SU (▲), ASA (●) and SA (■) at a constant pH (3.9 ± 0.2).

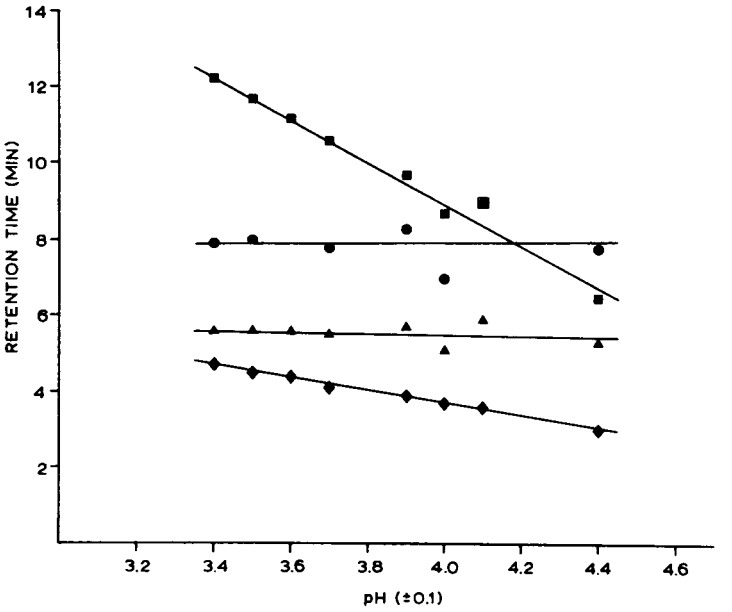


Fig. 2. Effect of pH on the retention times of GA (♦), SU (▲), ASA (●) and SA (■) at a constant methanol concentration (35%).

Sample preparation followed the simple perchlorate deproteinization procedure of Rumble et al. [13] but other agents were tested for their ability to precipitate protein. These included acetonitrile, trichloroacetic acid and formic acid. We elected to use perchloric acid in the present assay although the

other agents were also effective. However, the use of perchloric acid requires a high-speed centrifuge. If unavailable, acetonitrile may be a better choice as the deproteinizating agent [14]. The extraction of salicylate compounds from the precipitated protein gave consistent recoveries (80—86%).

Fig. 3 depicts a chromatogram of GA, SU, ASA, SA and TMB (10 μg/ml) and compares chromatograms of blank serum and spiked serum. Retention times of GA, SU, ASA, SA and TMB were 3.3, 4.1, 5.5, 6.7 and 8.7 min, respectively. The blank serum and plasma (Figs. 3 and 7) gave a baseline that was virtually free of extraneous interfering peaks after the initial solvent front. Only one endogenous peak that could interfere with low levels of SU (< 2 μg/ml) was sometimes observed at 3.9 min.

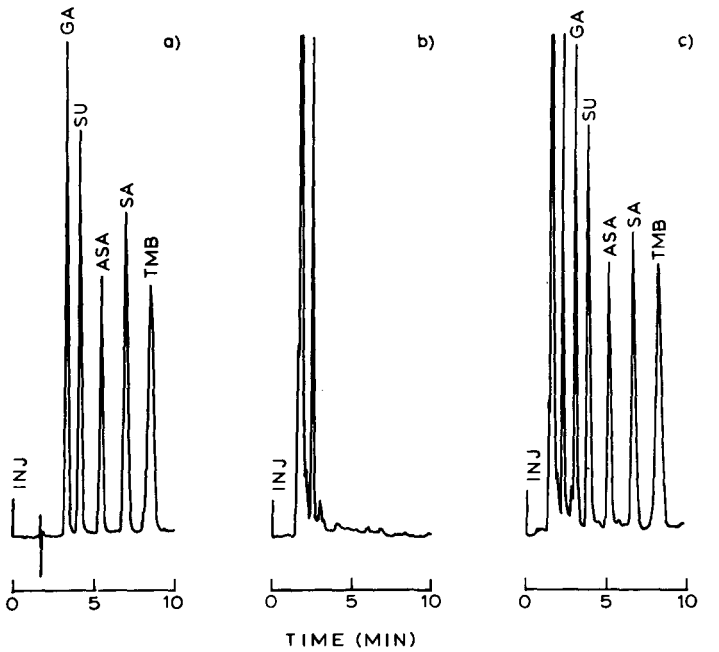


Fig. 3. Chromatograms of (a) methanol spiked with 10 μg/ml each of GA, SU, ASA, SA and TMB, (b) blank serum and (c) serum spiked with 10 μg/ml each of GA, SU, ASA, SA and TMB.

The calibration curves for serum ASA and SA were linear in the range 1—500 μg/ml while those of GA and SU were linear from 1 to 60 μg/ml. The regression equations when peak area ratios were taken were: GA, $Y = 0.054X + 0.007$, $r = 0.9932$; SU, $Y = 0.065X - 0.028$, $r = 0.9933$; ASA, $Y = 0.054X + 0.007$, $r = 0.9984$; SA, $Y = 0.063X + 0.012$, $r = 0.9979$. The limit of detection, defined as a peak at least three times the height of the baseline noise measured over a 2-min interval, for GA, SU, ASA and SA were 0.3, 0.8, 0.3 and 0.2 μg/ml, respectively.

The reproducibility of the method was investigated by analysing six serum replicates of each compound at a concentration of 45 μg/ml. The coefficient of variation for the normalized peak area ratios for GA, SU, ASA and SA were 5.2, 3.2, 3.9, and 4.2, respectively.

TABLE I

RELATIVE RETENTION TIMES OF BENZOIC ACID DERIVATIVES

Relative retention time*	Compound
0.08	Sulphosalicylic acid
0.25	3,4-Dihydroxybenzoic acid
0.27	4-Aminobenzoic acid (PABA)
0.32	Benzoylaminoacetic acid (hippuric acid)
0.34	Gentisic acid
0.34	4-Aminosalicylic acid
0.40	2,3-Dihydroxybenzoic acid
0.40	2,6-Dihydroxybenzoic acid
0.41	4-Hydroxybenzoic acid
0.46	3-Hydroxybenzoic acid
0.47	2,4-Dihydroxybenzoic acid
0.47	2-Acetylbenzoic acid
0.48	Salicyluric acid
0.68	Acetylsalicylic acid
0.83	Salicylic acid
1.00	3,4,5-Trimethoxybenzaldehyde (ISTD)
> 1.4	4-Methylbezoic acid (*p*-toluic acid)
≫ 1.4	4-Methylsalicylic acid

*Based on retention time of ISTD.

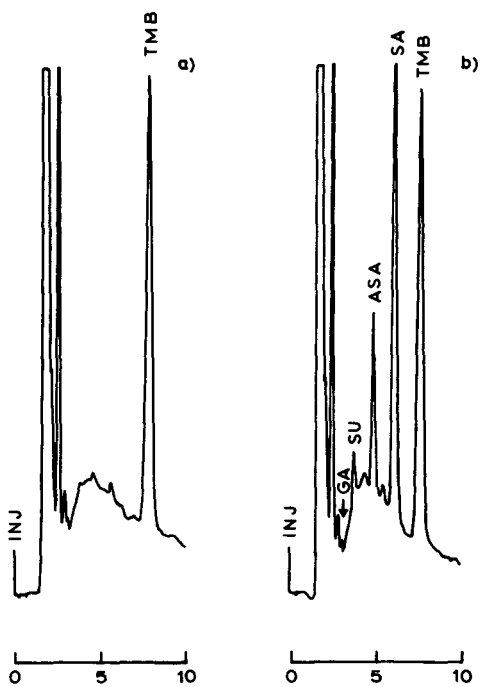


Fig. 4. Chromatograms of (a) blank human plasma containing TMB and (b) human plasma 30 min after administration of 1.3 g aspirin orally.

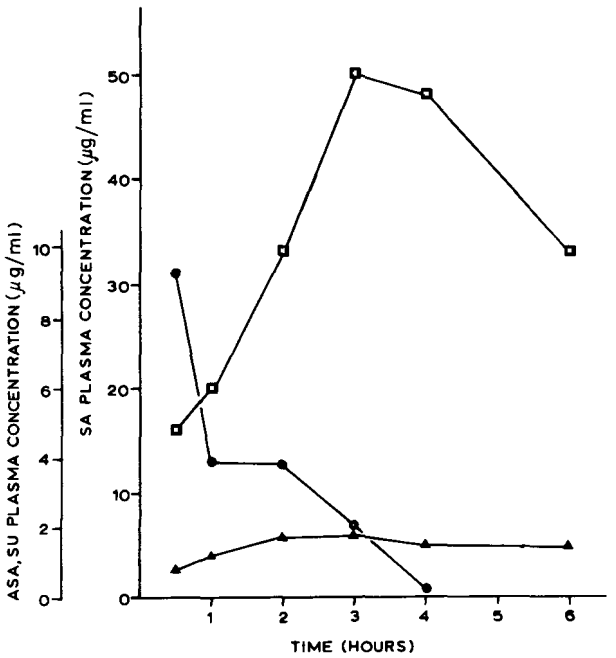


Fig. 5. Concentrations of SA (■), ASA (●) and SU (▲) in human plasma following an oral dose of four 325-mg tablets of ASA. GA was quantifiable at 0.5 and 4 h (0.4 and 0.5 μg/ml, respectively) but values fell below the limit of detection (0.3 μg/ml) at the other sampling times.

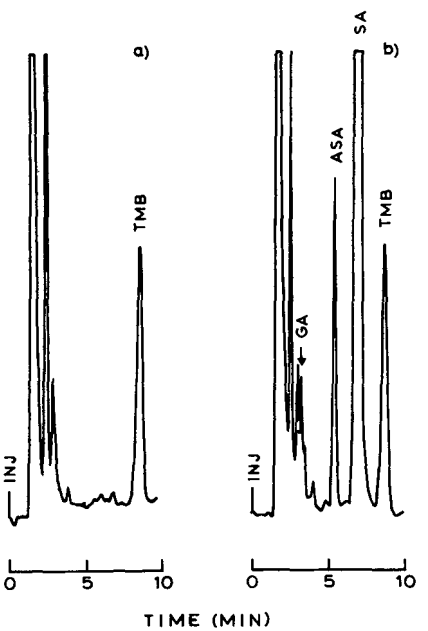


Fig. 6. Chromatograms of rat serum before (a) and 30 min after (b) the administration of 200 mg/kg ASA intravenously. Samples were filtered before being injected onto the chromatograph.

Several benzoic acid derivatives were tested in this system for interference with ASA and its metabolites and the results are shown in Table I.

To accurately determine the concentration of ASA, it was necessary to collect blood into chilled tubes containing an esterase inhibitor such as potassium fluoride to prevent rapid hydrolysis [15]. In addition the samples were analysed as quickly as possible or deep-frozen until analysis to minimize the extent of ASA hydrolysis.

When blood was collected with 30 I.U./ml heparin (Organon, Toronto, Canada) as the anticoagulant, the preservative, benzyl alcohol, was found to co-chromatograph with SU. This could be averted with heparinized blood collection tubes or by using other anticoagulants such as EDTA.

The versatility of the method was tested on human and animal (rat) models. Rats were given ASA by intravenous infusion (200 mg/kg) whereas one human subject was given four 325-mg tablets of ASA by oral administration. Blood samples were obtained from each model at various time periods and analysed for ASA and its metabolites. Urine from rats was also analysed.

Fig. 4 compares chromatograms of human plasma from the same subject before and after the ingestion of a single oral dose of 1.3 g of ASA. The plasma concentration—time profiles of GA, SU, ASA and SA are shown in Fig. 5 from

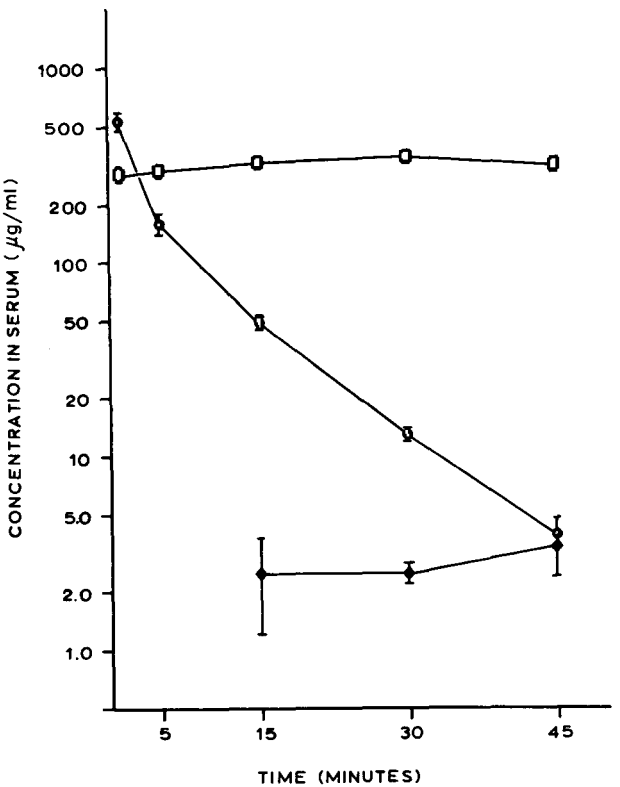


Fig. 7. Time course (mean ± S.D.) of GA (♦), ASA (●) and SA (■) concentrations in rat serum (n = 3) following a 200 mg/kg intravenous dose of ASA. Values of GA were below the limit of detection (0.3 μg/ml) at 1 and 5 min.

samples collected at 0.5, 1, 2, 3, 4 and 6 h. These results are comparable to profiles found by others at similar doses [11, 13, 16, 17]. The maximum concentration of SA (50 μg/ml) was lower than expected but this may be due to the pronounced inter-subject differences in metabolism experienced by normal subjects receiving the same dose of ASA [18].

Fig. 6 shows typical chromatograms of serum before and after a rat was treated with 200 mg/kg ASA intravenously. The time course of serum concentrations (mean ± S.D.) of GA, ASA and SA is shown in Fig. 7. The serum half-life of ASA (7—8 min) determined from these experiments was shorter than the plasma half-life for man (15 min) [19]. Salicyluric acid was not detected in serum in the present study, although SU has been reported in rat urine by Nelson et al. [20]. However, based on an excretion rate of 1 μg/min for SU in the rat [20], serum levels would be close to or below the limits of detection of SU (0.8 μg/ml) in this assay.

Chromatograms of rat urine collected before and after ASA, 200 mg/kg intravenously, are shown in Fig. 8. The retention times of SA and TMB were 6.0 and 7.5 min, respectively. No interfering peaks were seen in blank urine samples. The calibration curve for urine SA was linear in the range 1—100 μg/ml. The regression equation obtained from data based on rat urine samples was $Y = 0.034X - 0.016$, $r = 0.9960$. The coefficient of variation determined from peak area ratios was 2.1% and the detection limit was 1.6 μg/ml.

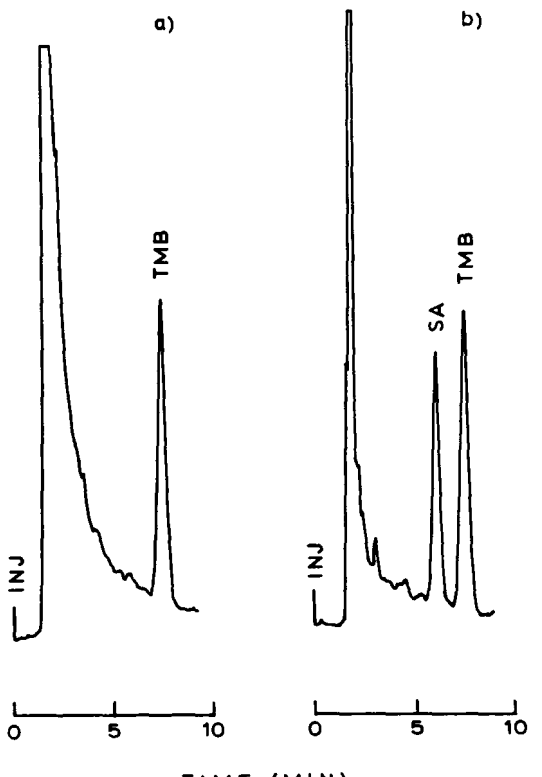


Fig. 8. Chromatograms of (a) blank rat urine containing TMB and (b) rat urine 50 min after administration of 200 mg/kg ASA intravenously. The total salicylate level (mean ± S.D.) was 1.25 ± 0.72 mg/ml ($n = 3$).

ASA is almost completely excreted by the kidneys (99%), as SA or its four major metabolites, GA, SU and the two conjugates salicylic phenolic glucuronide and salicylic acyl glucuronide [21]. Therefore, it is important to completely hydrolyse the glucuronide conjugates in the urine in order to determine total salicylate.

In a previous paper from this laboratory [22], a slightly modified version of this HPLC technique was employed to determine salicylate levels in human serum following ingestion of 325 mg of ASA. This HPLC method is currently being used in a pharmacokinetic study of ASA in doses as low as 1.7 mg/kg, intravenously in an arterial thrombosis rat model.

CONCLUSION

This HPLC technique offers a simple and rapid assay for the determination and quantitation of GA, SU, ASA and SA in biological fluids. The method is highly sensitive and can quantify samples to concentrations as low as 0.2 μg/ml. The assay incorporates a single deproteinization step followed by a rapid chromatographic analysis time of less than 10 min. This method was found suitable for pharmacokinetic studies of ASA and its major metabolites in animals and man.

ACKNOWLEDGEMENTS

This research was supported by the Ontario Heart Foundation. The authors wish to thank Dr. D.J. Freeman for his advice in preparing the manuscript.

REFERENCES

1 J.W. Burch and P.W. Majerus, Semin. Hematol., 16 (1979) 196.
2 L.S. Cohen, Semin. Thromb. Hemostasis, 2 (1976) 146.
3 I.A. Muni, J.L. Leeling, R.J. Helms, N. Johnson, J.J. Bare and B.M. Phillips, J. Pharm. Sci., 67 (1978) 289.
4 G. Graham and M. Rowland, J. Pharm. Sci., 61 (1972) 1219.
5 J.P. Tischio, J. Pharm. Sci., 65 (1976) 1530.
6 G.W. Peng, W.A.F. Gadalla, V. Smith, A. Peng and W.L. Chiou, J. Pharm. Sci., 67 (1978) 710.
7 L.Y. Lo and A. Bye, J. Chromatogr., 181 (1980) 473.
8 S.M. Douidar and A.E. Ahmed, J. Clin. Chem. Clin. Biochem., 20 (1982) 791.
9 J.N. Micelli, M.K. Aravind, S.N. Cohen and A.K. Done, Clin. Chem., 25 (1979) 1002.
10 J.N. Buskin, R.A. Upton and R.L. Williams, Clin. Chem., 28 (1982) 1200.
11 B.E. Cham, D. Johns, F. Bochner, D.M. Imhoff and M. Rowland, Clin. Chem., 25 (1979) 1420.
12 D.L. Maulding and J.F. Young, J. Pharm. Sci., 69 (1980) 1224.
13 R.H. Rumble, M.S. Roberts and S. Wanwimolruk, J. Chromatogr., 225 (1981) 252.
14 W.L. Chiou, M.A.F. Gadalla and G.W. Peng, J. Pharm. Sci., 67 (1978) 182.
15 M. Rowland and S. Riegelman, J. Pharm. Sci., 56 (1967) 717.
16 R.A. Seymour and M.D. Rawlins, Brit. J. Clin. Pharmacol., 13 (1982) 807.
17 G.G. Graham, G.D. Champion, R.O. Day and P.D. Paull, Clin. Pharmacol. Ther., 22 (1977) 410.
18 G. Levy, Drug Metab. Rev., 9 (1979) 3.
19 M. Rowland and S. Reigelman, J. Pharm. Sci., 57 (1968) 1313.
20 E. Nelson, M. Hanano and G. Levy, J. Pharmacol. Exp. Ther., 153 (1966) 159.
21 E.L. Alpen, H.G. Mandel, V.W. Rodwell and P.K. Smith, J. Pharmacol. Exp. Ther., 102 (1951) 150.
22 R.B. Philp and M.L. Paul, Prostaglandins Leukotrienes Med., 11 (1983) 131.

Journal of Chromatography, 310 (1984) 353—360
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2214

DETERMINATION OF BROMOPRIDE IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

R.R. BRODIE*, L.F. CHASSEAUD and L. ROONEY

Department of Metabolism and Pharmacokinetics, Huntingdon Research Centre, Huntingdon (U.K.)

(First received April 6th, 1984; revised manuscript received May 17th, 1984)

SUMMARY

Bromopride was measured in plasma and urine using reversed-phase high-performance liquid chromatography employing ultraviolet absorption detection. The limit of detection in plasma was 2 ng/ml, sufficient for pharmacokinetic studies of the drug. Plasma concentrations of bromopride reached mean peak levels (55 ng/ml) at 1 h after single oral doses of 20 mg and declined with a half-life of 4.9 h. Less than a mean of 10% of an oral dose was excreted unchanged in the urine. The assay could also be used to measure metoclopramide in these bio-fluids.

INTRODUCTION

Bromopride [4-amino-5-bromo-N-(2-diethylaminoethyl)-2-methoxybenzamide], Viaben® (Fig. 1) is an anti-emetic drug which has a selective effect on the basal motility of the gastro-intestinal tract and is used in the treatment of certain gastro-intestinal tract diseases [1, 2]. It is the bromo-analogue of metoclopramide.

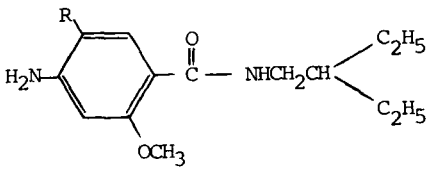


Fig. 1. Chemical structure of bromopride (R = Br) and metoclopramide (R = Cl).

Until recently, few analytical methods were available of sufficient specificity and sensitivity to measure these compounds in biological fluids. Metoclopramide has been measured by gas—liquid chromatography (GLC)

0378-4347/84/$03.00

[3, 4] as a heptafluorobutyryl derivative with a limit of detection of 5 ng/ml, and by high-performance liquid chromatography (HPLC) in an adsorption mode [5] and in a reversed-phase mode [6—8] with limits of detection of 8—10 ng/ml.

Bromopride has also been measured by GLC [9] as a heptafluorobutyryl derivative and by HPLC [9, 10] using gradient elution with limits of detection of 5—10 ng/ml. Under isocratic reversed-phase HPLC conditions, bromopride and metoclopramide elute as broad tailing peaks (Fig. 2).

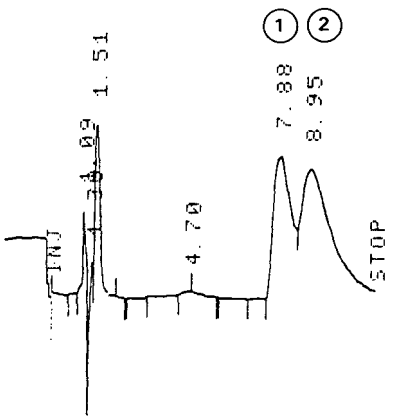


Fig. 2. Chromatogram of metoclopramide (1) and bromopride (2) under reversed-phase conditions in the absence of alkylammonium ions. Chromatographic conditions: column: μBondapak C_{18} (30 cm × 0.39 cm I.D.); flow-rate: 2 ml/min; solvent system: 40% (v/v) acetonitrile in phosphate buffer (0.1%, pH 7.5); detector: ultraviolet absorption (275 nm).

This paper describes a newly developed HPLC procedure for the measurement of bromopride under isocratic reversed-phase conditions using alkylammonium ions in the mobile phase to modify retention. Sokolowski and Wahlund [11] have previously shown that alkylammonium ions added to the mobile phase compete with sample ammonium ions for adsorption sites on the stationary phase, thus eliminating peak tailing and decreasing the retention of hydrophobic ammonium compounds under reversed-phase conditions. Metoclopramide is used as an internal standard and bromopride concentrations as low as 2 ng/ml can be detected. The method could also be used for the measurement of metoclopramide in plasma and urine.

EXPERIMENTAL

Materials and reagents

All reagents were of analytical grade and all inorganic reagents were prepared in freshly glass-distilled water. Acetonitrile was HPLC—far UV grade (Fisons Scientific Apparatus, Loughborough, U.K.). N,N-Dimethyl-3-chloropropylamine hydrochloride was obtained from Koch-Light Labs. (Colnbook, U.K.). Bromopride base and metoclopramide hydrochloride, used as internal standard, were supplied by Delagrange International (Paris, France), as were potential metabolites of bromopride, bromopride N-oxide, monodesethylbromopride and 4-amino-5-bromo-2-methoxybenzoic acid.

Standard solutions of bromopride base and metoclopramide hydrochloride were prepared at a concentration of 1 mg/ml in acetonitrile. Stock solutions were further diluted with acetonitrile to give concentrations of bromopride of 10 μg/ml and 1 μg/ml and metoclopramide of 10 μg/ml. All standard solutions were stored at 4°C in the dark under which conditions they were stable for several weeks.

Sample preparation procedures

Plasma samples (2 ml) or urine samples (200 μl) were transferred into conical centrifuge tubes and mixed with internal standard solution containing 60 ng metoclopramide hydrochloride for plasma samples or 120 ng for urine samples. The samples were extracted twice with diethyl ether (5 ml, freshly redistilled) in the presence of 0.5 *M* sodium borate buffer (1 ml, pH 10.5) using a rotary mixer for 10 min. The ether layers were separated after centrifugation, combined and extracted with 0.1 *M* hydrochloric acid. The samples were again mixed for 10 min, centrifuged and the diethyl ether layer was removed and discarded. The acid layers were then made alkaline by the addition of 0.5 *M* borate buffer (1 ml) and re-extracted with diethyl ether (5 ml). After mixing and centrifugation, the diethyl ether layers were carefully transferred to conical tubes and evaporated to dryness at 37°C under a stream of nitrogen. The residues were washed to the bottom of the tubes with a small volume of diethyl ether which was also evaporated to dryness.

The residues were dissolved in mobile phase (100 μl) and injected into the liquid chromatograph.

Calibration procedures

Samples of control (drug-free) plasma (2 ml) were mixed with bromopride at concentrations equivalent to 2, 5, 10, 20, 40, 50, 80 and 110 ng/ml and with internal standard at a fixed concentration of 30 ng/ml.

Samples of control (pre-dose) urine (200 μl) were mixed with bromopride at concentrations equivalent to 20, 50, 100, 200, 400 and 500 ng/ml and with internal standard at a fixed concentration of 600 ng/ml.

When concentrations of bromopride in urine exceeded 500 ng/ml, samples were diluted with control urine before measurement.

All calibration standards were submitted to the sample preparation procedures previously described.

Instrumentation

The liquid chromatograph consisted of a M6000A pump and WISP™ autosampler (Waters Assoc., Cheshire, U.K.) coupled to an LC-3 variable-wavelength detector (Pye Unicam, Cambridge, U.K.) operated at 275 nm and 0.01 a.u.f.s. Chromatograms were recorded on a 3380A computing integrator (Hewlett-Packard, Hitchin, U.K.) using an attenuation of 8 for plasma samples and 16 for urine samples. Peak height measurements were performed manually.

Chromatography

Chromatography was performed in a reversed-phase mode with alkylammonium ions in the mobile phase to act as a modifier of the retention of bromopride and internal standard (metoclopramide).

The mobile phase consisted of 40% (v/v) acetonitrile in phosphate buffer (0.1%, w/v) containing N,N-dimethyl-3-chloropropylamine hydrochloride (0.12%, w/v); the final pH was adjusted to 7.9 using 4 *M* sodium hydroxide solution.

The column used for the analysis was constructed of stainless steel (25 cm × 0.46 cm I.D.) and packed with Zorbax® C_8 (mean particle diameter 6 μm) (Dupont, Stevenage, U.K.). A pre-column (7 cm × 0.2 cm I.D.) constructed of stainless steel and dry-packed with pellicular Co:Pell® ODS (particle diameter 24—37 μm) (Whatman, Maidstone, U.K.) was installed in series in front of the analytical column to protect it from contamination. A mobile phase flow-rate

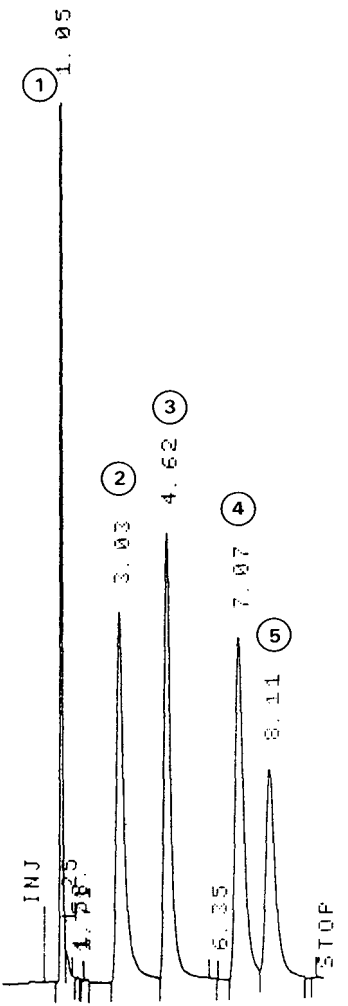


Fig. 3. Chromatogram of bromopride and its metabolites. Peaks: 1 = 4-amino-5-bromo-2-methoxybenzoic acid; 2 = bromopride N-oxide; 3 = N-desethylbromopride; 4 = metoclopramide (internal standard); 5 = bromopride. Chromatographic conditions: column: Zorbax C_8 (25 cm × 0.46 cm I.D.); flow-rate: 2.2 ml/min; solvent system: 40% (v/v) acetonitrile in phosphate buffer (0.1%, w/v) containing N,N-dimethyl-3-chloropropylamine hydrochloride (0.12%, w/v) pH 7.9; detector: ultraviolet absorption (275 nm), 0.01 a.u.f.s.

was adjusted between 2.2 and 2.7 ml/min to give retention times for bromopride and metoclopramide of approximately 8 and 7 min, respectively. Potential metabolites of bromopride, 4-amino-5-bromo-2-methoxybenzoic acid, N-desethylbromopride and bromopride N-oxide, were well resolved and did not interfere in the chromatography (Fig. 3).

Studies in human subjects

Plasma and urine samples were obtained from six human volunteer subjects dosed orally with 20 mg of bromopride as a solution formulation and analysed by the foregoing procedures. These volunteer studies were conducted under conditions similar to those described by Brodie et al. [12].

TABLE I

BETWEEN-ASSAY PRECISION MEASUREMENTS OF BROMOPRIDE IN PLASMA

Concentration of bromopride (ng/ml)	Peak height ratio ($\frac{\text{bromopride}}{\text{internal standard}}$)							Mean (± S.D.)	C.V. (%)
.2	0.06	0.10	0.09	0.05	0.06	0.07	0.06	0.07 (0.02)	29
5	0.13	0.19	0.15	0.14	0.13	0.15	0.17	0.15 (0.02)	13
10	0.29	0.28	0.27	0.22	0.26	0.28	0.26	0.27 (0.02)	7
20	0.59	0.47	0.56	0.51	0.58	0.57	0.49	0.54 (0.05)	9
40	1.12	1.18	1.13	1.11	1.11	1.14	1.20	1.14 (0.04)	4
50	1.28	1.33	1.39	1.42	1.30	1.40	1.49	1.37 (0.07)	5
80	2.26	2.30	2.35	2.38	2.38	2.41	2.23	2.33 (0.07)	3
110	3.26	3.23	3.19	3.22	3.33	3.18	3.29	3.24 (0.05)	2

TABLE II

BETWEEN-ASSAY PRECISION MEASUREMENTS OF BROMOPRIDE IN URINE

Concentration of bromopride (ng/ml)	Peak height ratio ($\frac{\text{bromopride}}{\text{internal standard}}$)						Mean (± S.D.)	C.V. (%)
20	0.03	0.03	0.04	0.04	0.05	0.04	0.04 (0.008)	20
50	0.08	0.09	0.07	0.09	0.09	0.07	0.08 (0.010)	13
100	0.15	0.14	0.14	0.15	0.17	0.16	0.15 (0.012)	8
200	0.28	0.30	0.28	0.32	0.27	0.27	0.29 (0.020)	7
400	0.57	0.56	0.53	0.55	0.61	—	0.56 (0.030)	5
500	0.71	0.70	0.68	0.68	0.77	0.77	0.72 (0.042)	6

RESULTS AND DISCUSSION

Precision and accuracy of measurement

Extraction and measurement were repeated on seven occasions at each concentration over the selected calibration range in plasma and on six occasions at each concentration in urine. The between-assay precision of the method, as indicated by the coefficient of variation (C.V.) of peak height ratio measurements of drug to internal standard ranged from ± 29% at 2 ng/ml to ± 2% at 110 ng/ml for plasma samples (Table I) and from ± 20% at 20 ng/ml to ± 6% at 500 ng/ml for urine samples (Table II).

The calibration line for bromopride in plasma constructed from seven replicate measurements at eight concentrations was linear ($Y = 0.0293 - 0.020X$, $r = 0.999$) as was that for bromopride in urine, constructed from six replicate measurements at six concentrations ($Y = 0.0014X - 0.009$, $r = 0.996$). The accuracy of the method as indicated by the standard errors of the fitted least-squares regression lines was ± 2.5 ng/ml for the plasma line and ± 23 ng/ml for the urine line.

Recovery

The recoveries (extraction efficiencies) of bromopride and internal standard from plasma were 82 ± 4% S.D. and 77 ± 6% S.D., respectively, and from urine were 77 ± 2% S.D. and 82 ± 5% S.D., respectively. The bromopride concentration ranges studied were 10—50 ng/ml in plasma and 50—500 ng/ml in urine.

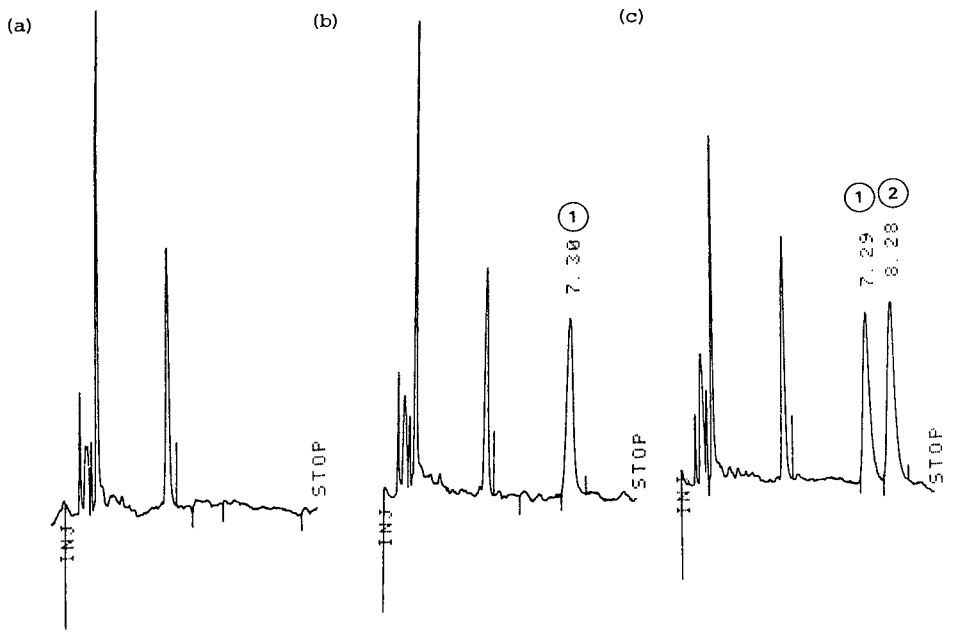


Fig. 4. Chromatograms of (a) pre-dose plasma; (b) pre-dose plasma containing added internal standard; and (c) 2-h post-dose plasma containing bromopride at a concentration of 36 ng/ml. Peaks: 1 = internal standard; 2 = bromopride. Conditions as described for Fig. 3.

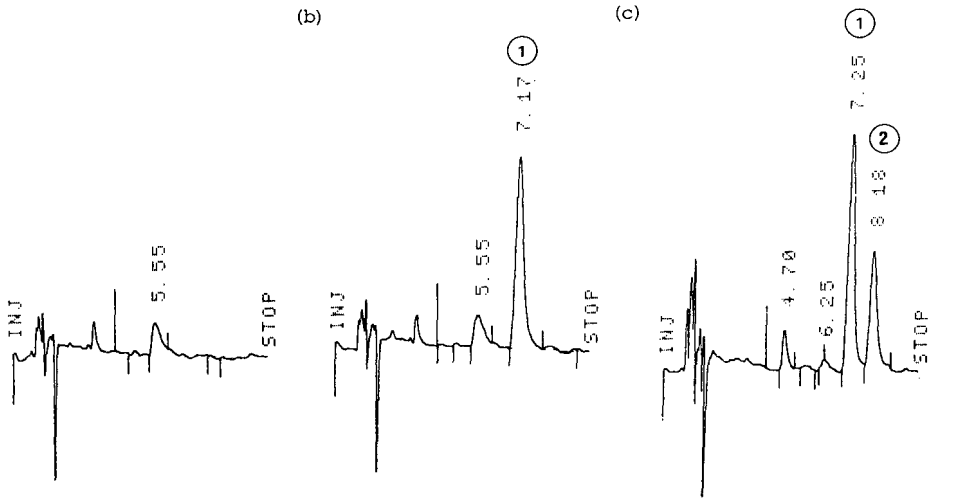


Fig. 5. Chromatograms of (a) pre-dose urine; (b) pre-dose urine with internal standard; and (c) urine collected during 0.5—1 h after dosing containing bromopride at a concentration of 336 ng/ml. Peaks: 1 = internal standard; 2 = bromopride. Conditions as described for Fig. 3.

Selectivity and limit of detection

No interfering peaks with the same retention times as bromopride or metoclopramide were present in pre-dose plasma (Fig. 4) or urine (Fig. 5) samples. Potential metabolites of bromopride were well resolved; however, 4-amino-5-bromo-2-methoxybenzoic acid and bromopride N-oxide were not extracted under the conditions used. N-Desethylbromopride was not detected in plasma samples contrary to data published elsewhere [9].

TABLE III

MEAN PLASMA BROMOPRIDE CONCENTRATIONS IN SIX HUMAN SUBJECTS AFTER SINGLE ORAL DOSES OF 20 mg IN SOLUTION

Time after dosing (h)	Concentration of bromopride (ng/ml ± S.D.)
0.5	32 ± 21
0.75	51 ± 28
1	55 ± 27
1.5	53 ± 30
2	54 ± 23
2.5	49 ± 22
3	47 ± 20
3.5	41 ± 20
4	38 ± 19
5	33 ± 17
6	29 ± 14
8	23 ± 14
10	16 ± 11
12	13 ± 9
14	9 ± 6
24	4 ± 4

The lowest calibration standard of 2 ng/ml was taken as the limit of detection in plasma and this represented a signal-to-noise ratio of 2:1.

This present bromopride assay procedure is more sensitive and simpler than previously published procedures, in that no derivatisation stage is required in the assay, and chromatography can be performed under isocratic conditions, which allows a greater throughput of samples to be analysed in a given time.

Concentrations of bromopride in human plasma and urine

After single oral doses of 20 mg of bromopride administered as a solution to six healthy human subjects, a peak of mean plasma concentrations of 55 ng/ml was reached at 1 h after dosing (Table III). After reaching peak levels, plasma concentrations declined apparently monoexponentially with a mean half-life of 4.9 ± 0.7 h S.D. A total of 1.87 ± 0.86 mg S.D. (9.4% dose) of bromopride was excreted unchanged in urine during 48 h after dosing.

ACKNOWLEDGEMENT

We are grateful to Delagrange International, Paris, France, for the opportunity to conduct these studies.

REFERENCES

1 P. Monille and G. Cheymol, Ann. Pharm. Fr., 35 (1977) 53.
2 L. de Angelis, Drugs Today, 14 (1978) 193.
3 L.M. Ross-Lee, M.J. Eadie, F. Bochner, W.D. Hooper and J.H. Tyrer, J. Chromatogr., 183 (1980) 175.
4 K.W. Riggs, J.E. Axelson, D.W. Rurak, D.A. Hasman, B. McErlane, M. Bykma-Howell, G.H. McMorland, R. Ongley and J.D.E. Price, J. Chromatogr., 276 (1983) 319.
5 L. Teng, R.G. Bruce and L. Kay Dunning, J. Pharm. Sci., 66 (1977) 1615.
6 W. Block, A. Pingoud, M. Khan and P. Kjellerup, Arzneim.-Forsch., 31 (1981) 1041.
7 D.N. Bateman, R. Gokal, T.R.P. Dodd and P.G. Blain, Eur. J. Clin. Pharmacol., 19 (1981) 437.
8 G.B. Bishop-Freudling and H. Vergin, J. Chromatogr., 273 (1983) 453.
9 E. Grassi, G.L. Passetti and A. Trebbi, in A. Frigerio (Editor), Recent Developments in Mass Spectrometry in Biochemistry and Medicine, Vol. 1, Plenum, New York, 1978, p. 99.
10 P.W. Lücker, W. Tinhof, N. Wetzelsberger, W. Weyers and R. Brodbeck, Arzneim.-Forsch., 33 (1983) 453.
11 A. Sokolowski and K.-G. Wahlund, J. Chromatogr., 189 (1980) 299.
12 R.R. Brodie, L.F. Chasseaud, A. Darragh, R.F. Lambe, L. Rooney and T. Taylor, Biopharm. Drug Dispos., submitted for publication.

Journal of Chromatography, 310 (1984) 361—371
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2215

REVERSED-PHASE PAIRED-ION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE SEPARATION AND QUANTIFICATION OF MULTIPLE BLEOMYCIN CONGENERS

ROBERT P. KLETT, JAMES P. CHOVAN* and ILENE H.R. DANSE*

Department of Pharmacological Sciences, State University of New York at Stony Brook, Health Sciences Center, BHS T-8, Stony Brook, NY 11794 (U.S.A.)

(First received December 14th, 1983; revised manuscript received June 4th, 1984)

SUMMARY

A rapid, linear gradient chromatographic technique for separating and quantifying copper(II)-chelated bleomycin congeners is described. This method is also capable of (1) separating divalent from trivalent metal chelates; (2) determining the purity of many chemically modified bleomycins; and (3) assaying bleomycin hydrolase activity on complex mixtures. Quantification at 280 nm is sensitive to 50 pmol and is linear at least up to 10 nmol per injection.

INTRODUCTION

The phleomycin (PHLM) and bleomycin (BLM) groups of antitumor antibiotics were originally isolated by Maeda et al. [1] and Umezawa et al. [2]. These atypical glycopeptides (Fig. 1) are known to chelate both divalent and trivalent transition metal ions [3]. Although isolated from their respective *Streptomyces* cultures as the Cu(II) complexes, the most active form in vitro is Fe(II)—BLM, which rapidly degrades DNA producing both single and double strand breaks and releasing free bases and the recently described base propenals [4]. Several other radioactive metal chelates have been formulated for use as tumor imaging agents, the ^{57}Co complex having been studied most [5].

Both BLM and PHLM are isolated as mixtures of congeners, differing only by the structure of a C-terminal group (see Fig. 1 and Table I). In fact, most of the experimentation on these antibiotics has utilized mixtures, since pure

*Present address: Environmental Health Associates, Inc., 520 Third Street, Suite 208, Oakland, CA 94607, U.S.A.

0378-4347/84/$03.00

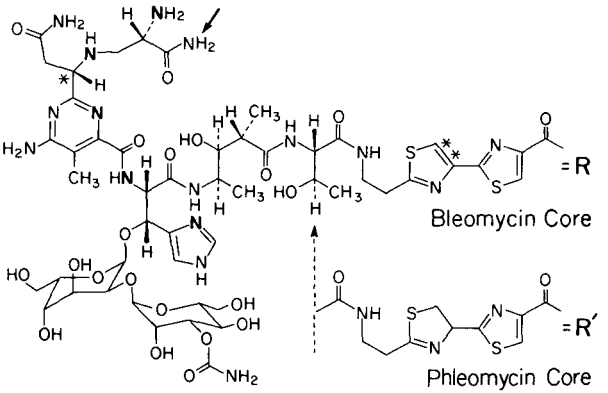


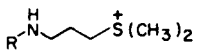
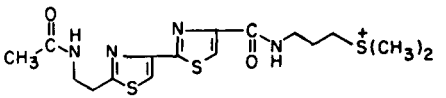
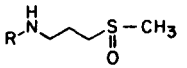
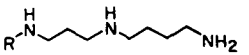
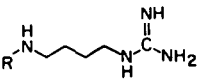
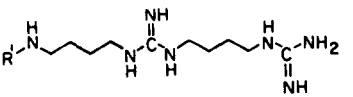
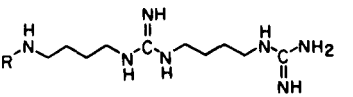
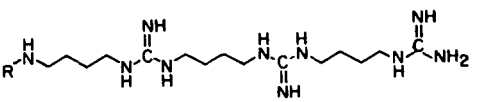
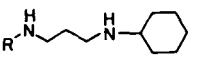
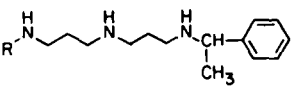
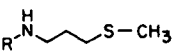
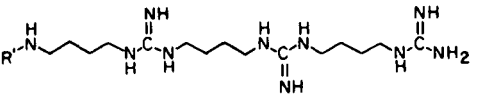
Fig. 1. The structures of the bleomycin (R) and phleomycin (R′) cores. BLMs differ by the specific group attached to the thiazole carboxylate (see Table I), usually with a peptide bond, although esters have also been synthesized. The PHLM core differs from BLM only by reduction of the double bond at the paired asterisks [6]. The single asterisk indicates an *R*-configuration; epi-BLMs have an *S*-configuration at this site [7]. The darkened nitrogens are the proposed [3] sites of metal coordination. The arrow points to the amide group removed by BLM hydrolase [8], resulting in a desamido-BLM congener. Iso-BLMs have 2-O-carbamoyl-mannose sugars, rather than the 3-O-derivative shown [9].

congeners are not readily available. The clinically used BLM is regulated [10] to be 60—70% BLM A_2, 25—32% BLM B_2, and less than 10% of other bleomycins. This mixture is said to have a higher therapeutic index than the individually pure substances, many of which appear to have unacceptable levels of pulmonary toxicity and/or antitumor activity [11]. Many new analogues have been prepared semisynthetically in an attempt to improve anticancer activity while reducing pulmonary side-effects [12]. These studies have shown that the BLMs vary widely in all of their biological activities, including DNA cleaving ability, antitumor activity, and systemic side-effects. We have found that the ability to induce pulmonary fibrosis is directly related to the structure of the terminal moiety [13—16]. Although it is presumed that, with regard to this effect, the distinction between congeners is expressed at the level of DNA [17], only one study on uptake and intracellular distribution has been reported [18]; this utilized only BLM A_2, and recoverable radioactivity was minimal. Thus, the search for new bleomycins has focused on the C-terminal moiety, without truly knowing the intracellular site of action.

In our studies of the cellular mechanism of cytotoxicity, we proposed to synthesize radiolabeled congeners of high specific activity. Thus we required a rapid and highly sensitive method of analysis. Unfortunately, published high-performance liquid chromatographic (HPLC) methods proved too cumbersome, not specific enough, or irreproducible [19—25]. Therefore, we have now developed a method for the separation and quantification of BLM congeners, which remedies these difficulties and which also is capable of separating the PHLMs. This is the subject of this communication.

TABLE I

CAPACITY FACTORS (k') OF SOME BLEOMYCIN AND PHLEOMYCIN CONGENERS

Bleomycin or phleomycin congener	Terminal structure	k'
Bleomycin or phleomycinic acid	R–OH, R'–OH	1.0
BLM B_1'	R-NH_2	2.9
BLM A_2		3.5
BLM A_2-BT		3.7
BLM A_1		4.2
BLM A_5		4.3
BLM B_2		6.1
PHLM E		6.9
BLM B_4		8.0
BLM B_6		9.2
BLM CHP		10.4
BLM PEPP		11.8
BLM DM-A_2		12.7
PHLM G		13.4

EXPERIMENTAL

Materials

Bleomycin (as Blenoxane®, lot FOX04, expiration date September, 1981)

was obtained through Drs. William Bradner and Stanley Crooke as a gift from Bristol Labs. (Syracuse, NY, U.S.A.). A single lot was used throughout these studies. Additional gifts from Bristol included BLMs A_2, B_2, A_1, and A_5. The BLM A_2-bithiazole C-terminus [BLM A_2-BT; 2′-(2-acetamidoethyl)-2,4′-bithiazole-4-(3-dimethylsulfonio)-propylcarboxamide] was chemically synthesized by Mr. Mark Levin and Dr. Sidney Hecht of the Department of Chemistry, University of Virginia (Charlottesville, VA, U.S.A.). Dr. Hecht also kindly supplied bleomycinic acid. PHLMs E and G, as well as BLMs B_4, B_6, CHP, and PEPP, were gifts from Drs. Noel K. Hart and Alan Lane (CSIRO, Australia). BLM B_1' was provided by Dr. Tomohisa Takita of the Institute of Microbial Chemistry (Tokyo, Japan). (See Fig. 1 and Table I for the structures of these compounds.) None of the above standards, except BLMs B_1' and A_2-BT (and BLMs A_2 and B_2 prepared by ion-exchange chromatography [23] in our laboratory), were greater than 90% pure as judged by the HPLC procedures described below. The remaining congeners presented herein were 70—85% pure, and the major peak was taken to be the authentic BLM or PHLM.

HPLC-grade water and methanol were obtained through Fisher Scientific (Springfield, NJ, U.S.A.) or Milli-Q-purified (Millipore) water was used. Pentanesulfonic acid, sodium salt, was purchased from Aldrich (Milwaukee, WI, U.S.A.). Ultrapure indium chloride was obtained from Alfa Products (Danvers, MA, U.S.A.), and all other reagents were purchased from Fisher.

Chromatographic equipment and technique

A Waters Assoc. (Milford, MA, U.S.A.) high-performance liquid chromatography system was utilized. This consisted of U6K injector, 440 dual-channel UV detector, 660 solvent programmer, 730 data module, M6000A and M-45 pumps, and a 300 × 3.9 mm μBondapak C_{18} column (10-μm particle size) preceded by a 23 × 3.9 mm guard column packed with C_{18} Corasil (particle size, 30—38 μm).

Solvent A (water) and solvent B (methanol) each contained 5.0 m*M* pentanesulfonic acid, sodium salt, and 0.5% (v/v) glacial acetic acid. The pH of solvent A was adjusted to 4.3 with concentrated ammonium hydroxide, while solvent B was used without further additions. Each was passed through a 0.2-μm filter and sonicated for 15 min at the beginning of every day.

The standard procedure was a linear gradient of 28—48% solvent B in solvent A run over 45 min at a flow-rate of 1.5 ml/min with a resulting pressure development of about 1.6 MPa (2200 p.s.i.). Detection was at 280 nm, and all chromatograms shown herein were obtained under these conditions. Except for BLM A_2-BT and as indicated, copper sulfate was added in equimolar amounts or in slight excess.

Bleomycin quantification

Stock solutions of known weights of Cu(II)-chelated BLM A_2 or B_1' (greater than 98% pure as judged by the HPLC procedure outlined above) were prepared in HPLC-grade water, and the absorbance at 292 nm of a diluted sample was determined on a Zeiss MQ3 spectrophotometer. The extinction coefficient of $1.74 \cdot 10^4\ M^{-1}\ cm^{-1}$, defined for Cu(II)—BLM A_2 by Dabrowiak et al. [26], was utilized to determine the concentration of the specific BLM.

Dilutions of these stocks were made, as appropriate, so that a convenient volume (10—20 μl) could be injected. Triplicate samples were injected in the range of 50 pmol to 10 nmol, and the average peak area was determined for each set.

Separation of divalent and trivalent chelates

Several metals were found to form stable chelates under these chromatographic conditions, and the trivalent chelates could be separated from the divalent chelates. As an example, solutions of Blenoxane and the chlorides of Cu(II) and In(III) were prepared separately in unbuffered HPLC-grade water, and combined just prior to injection.

Bleomycin hydrolase analysis

Mouse liver was extracted by the method of Yoshioka et al. [28], except that dialysis of the crude extract was performed over 3 h with two changes of buffer at 4°C. Immediately after dialysis, 200 μl of the extract (about 50 μg protein) were incubated with 50 μl of either metal-free BLM B_2 or Blenoxane, lot FOX04, in 0.1 *M* sodium phosphate (pH 7.2), both at 0.3 mg/ml, for various times at 37°C in a shaking water bath. The reaction was stopped by addition of 0.25 ml of ice-cold HPLC solvent B, and after 15 min on ice the mixture was centrifuged at 15,000 *g*. The supernatant was collected, a slight excess of copper sulfate added, and the solutions were either chromatographed immediately or stored at 4°C until analyzed.

Chemical modifications of bleomycin A_2

BLM A_2 as the Cu(II) chelate was purified to greater than 98% homogeneity by CM-Sephadex C-25 ion-exchange chromatography [23]. BLM demethyl-A_2 (BLM DM-A_2) was prepared by pyrolysis at 100°C for 18 h as originally outlined by Fujii et al. [29] and more recently by Roy et al. [30]. BLM DM-A_2 was remethylated to BLM A_2 using methyl iodide [29,30]. BLM A_1 was synthesized from BLM DM-A_2 by treatment with hydrogen peroxide [29]. We also attempted to form BLM epi-A_2 [7] and BLM iso-A_2 [9], but yields were too poor for definitive analysis. This chemistry is discussed further below.

RESULTS AND DISCUSSION

Chromatography of metal(II)— and metal(III)—bleomycin congeners

The chromatographic procedure described in Experimental allows the separation and quantitation of BLM congeners in the form of their metal ion chelates. Fig. 2 shows the elution profile of the clinically used mixture in the metal-free form (Fig. 2A) and after conversion to the corresponding Cu(II) chelates (Fig. 2B). For analytical purposes, the requirement for metal complexation does not constitute a serious drawback, since their formation is rapid, complete (K_d of about 10^{-11} *M* [3]), and can even proceed while BLM is retained on the column. Excess metal ions do not interfere with the chromatographic separation since they elute in the void volume.

The Cu(II)—BLMs and —PHLMs are especially suitable for this analysis since they are stable under our assay conditions (pH 4.3), whereas the Zn(II)—

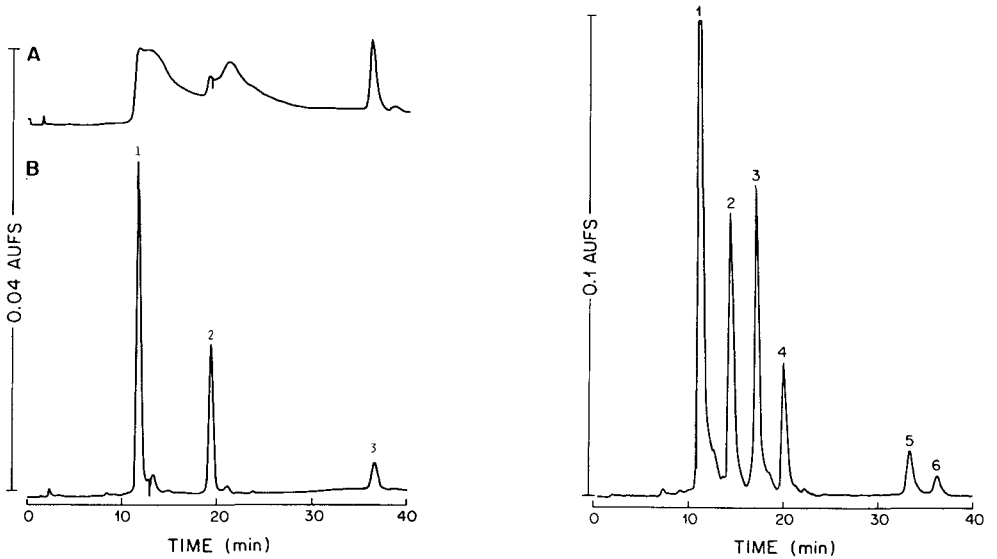


Fig. 2. Chromatographic profiles of the same amount of a Blenoxane mixture (lot FOX04) as (A) the metal-free drug and (B) the Cu(II) chelate. Conditions in this and all other figures are as given in Experimental. The terminal moieties of the indicated BLMs are given in Table I. Peaks: 1 = Cu(II)—BLM A_2; 2 = Cu(II)—BLM B_2; 3 = Cu(II)—BLM DM-A_2.

Fig. 3. Separation of Cu(II) and In(III) mixtures of Blenoxane (lot FOX04). Peaks: 1 = Cu(II)—BLM A_2; 2 = In(III)—BLM A_2; 3 = Cu(II)—BLM B_2; 4 = In(III)—BLM B_2; 5 = Cu(II)—BLM DM-A_2; 6 = In(III)—BLM DM-A_2.

BLMs are not [3]. Co(II) and Fe(II) complexes undergo oxidation to Co(III) and Fe(III) in the presence of oxygen [27], and long incubation periods are required before stable and uniform chelates are obtained. In addition, equimolar Cu(II) displaces Fe(III) [but not Co(III)] in the complex, so that its presence could interfere with results. In(III) chelates are also formed rapidly and are suitable for analysis in this system. The retention times of In(III) complexes are uniformly increased, due to the additional positive charge, and can be separated from the corresponding Cu(II) chelates, as seen in Fig. 3. The relative amount of Cu(II) to In(III) chelate shown in this chromatogram is 2:1, and quantitation of any of the congeners is not altered by the presence of either excess copper chloride or indium chloride.

Chromatography of Cu(II)—BLM and —PHLM congeners

The different BLM and PHLM congeners listed in Table I with their k' values were analyzed as the Cu(II) chelates. The void volume of our system is 3.77 ml, so that the k' is defined as $k'_i = V_i/3.77 - 1$, where V_i is the volume from time of injection to that of elution of the ith peak. Although several of these BLMs and PHLMs were only 70—85% pure by this method, we do not attribute this to instability of the compounds, since samples stored at room temperature in unbuffered HPLC-grade water for longer than one year gave the same chromatographic profiles as when freshly prepared. From the results shown in Table I, several conclusions can be drawn. (1) The negatively charged

bleomycinic acid has relatively little interaction with the octadecyl groups of the chromatographic column. However, fairly minor changes in the C-terminal group (e.g., BLM B_1') drastically influence the retention time. This is confirmed by the k' determined for the terminal fragment BLM A_2-BT, which is eluted very near the corresponding intact congener. (2) As originally seen for ion-exchange chromatography [31], the A group BLMs, with the exception of BLM DM-A_2, generally are eluted before the B group, indicating in both cases that there is less formal charge density at the termini of the A group. However, these results are also consistent with either a greater interaction of the A group terminal moieties with the core portion of BLM (± metal) or lower lipophilicity. (3) Within each group, the greater the charge of the end-piece the greater the ion-pairing and thereby the longer the retention time. The notable exception is BLM DM—A_2, whose methylsulfide group might interact non-ionically with silane and/or silanol groups, as well as the C_{18} portion of the column. The retention of BLMs A_1, CHP, and PEPP is also consistent with the greater effect of lipophilicity over ion-pairing of the terminal group.

A typical separation of an artificial mixture of ten Cu(II)-complexed BLM congeners is shown in Fig. 4, obtained with the standard chromatographic conditions. This specific combination can be separated adequately on a μBondapak C_{18} within 25 min by increasing the gradient slope (data not shown), but some of the smaller, undefined peaks, contaminants in the reference compounds, seen in Fig. 4 are no longer resolved. However, for practical purposes, the Blenoxane (lot FOX04) mixture can be analyzed much more rapidly by increasing the gradient slope and/or by starting the gradient at a higher percentage of solvent B. In addition, preliminary results using a Novapak C_{18} (3.9 × 150 mm, 5-μm particle size) indicate about 50% solvent reduction with no significant loss of resolution.

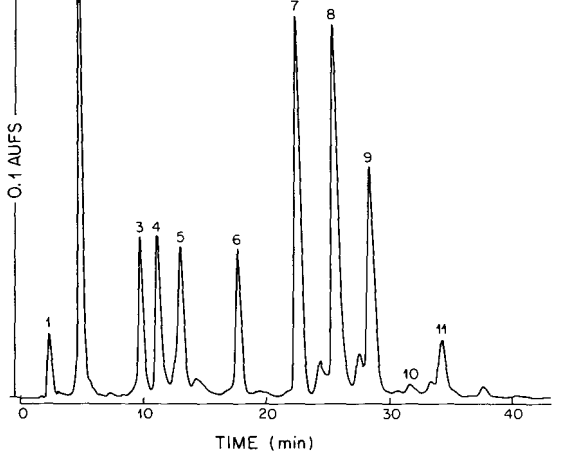


Fig. 4. Chromatogram of ten different Cu(II)—BLMs. The sturctures of the terminal groups are given in Table I. Peaks: 1 = free Cu(II); 2 = bleomycinic acid; 3 = BLM B_1'; 4 = BLM A_2; 5 = BLM A_5; 6 = BLM B_2; 7 = BLM B_4; 8 = BLM B_6; 9 = BLM CHP; 10 = BLM PEPP; 11 = BLM DM-A_2.

Quantitative aspects

The area under the absorbance curve (AUC) for BLM detected at 280 nm is linearly related to the amount injected over a range of 50 pmol to 10 nmol, or, in this system over a concentration range of 25 n*M* to 10 m*M*. For BLM A_2, automated integration gave AUC = $2.38 \cdot 10^{12} X - 129$ (r = 0.9995), while for BLM B'_1, AUC = $2.33 \cdot 10^{12} X - 41$ (r = 0.9991). The standard deviations of every triplicate sample injected were less than 0.5% by this method, and spot checks with a planimeter gave similar results. There is no detectable difference in estimation accuracy when different volumes containing the same amount of a single BLM are injected, but peak broadening with a large volume of a complex mixture might be expected to affect results. However, several combinations of different BLMs in 1—100 μl injected volumes gave the same AUC for each congener as they did when they were injected alone in similar volumes. Of course, the AUC is the same for all congeners with identical chromophores, but not, for instance, for BLM PEPP or the PHLMs (Table I and Fig. 1), whose terminal groups contribute to absorbance at 280 nm.

We have chosen measurement at this wavelength since the baseline absorbance at 254 nm (about 15% more sensitive) is less stable during the gradient and affects accuracy. A variable-wavelength detector set at 292 nm also would be expected to increase sensitivity by about 10%. The most exact detection method (down to about 1.0 pmol) should be by utilizing the fluorescence properties of BLM, as reported for the analysis of BLM hydrolase [32,33], but we have not explored these possibilities. However, since bithiazole fluorescence is being measured [34], this is not applicable for the PHLM. Furthermore, this method is comparable in sensitivity to published radioimmunoassay (RIA) [35,36] and enzymeimmunoassay (EIA) [37,38] techniques with much greater specificity and simplicity. In addition, recovery from tissue extracts (as in the BLM hydrolase assay) or sera from several species (data not shown) is essentially 100%, with no UV interference observed.

Bleomycin hydrolase analysis

Fig. 5 shows the usefulness of this system for the analysis of the action of BLM hydrolase on the Blenoxane mixture. If only one congener is to be used as a substrate (e.g., BLM A_2 or B_2), simply increasing the slope of the solvent gradient can reduce the analysis time to less than 15 min. There is no difference in chromatographic results due to storage in 50% methanol at 4°C or room temperature over twenty days, the longest time tested. Total recovery of original BLM and/or the corresponding desamide analogue(s), regardless of reaction time in the range 0—4 h, is always 100 ± 5%. In addition, the 3-h dialysis effectively removed low-molecular-weight material, so that concentration with a CF-25 Centriflo cone (Amicon, Lexington, MA, U.S.A.) was not found to be necessary to prevent loss of activity during overnight dialysis, as indicated by Yoshioka et al. [28].

This enzyme is known to interact with the various BLMs at different rates [39], BLM B_2 being affected most, as can be seen in Fig. 5. Since BLM is presently used clinically as a mixture, and since there is no guarantee that different tumors inactivate each BLM to the same relative extent, we believe that the mixture should be used to analyze the level of activity of this enzyme.

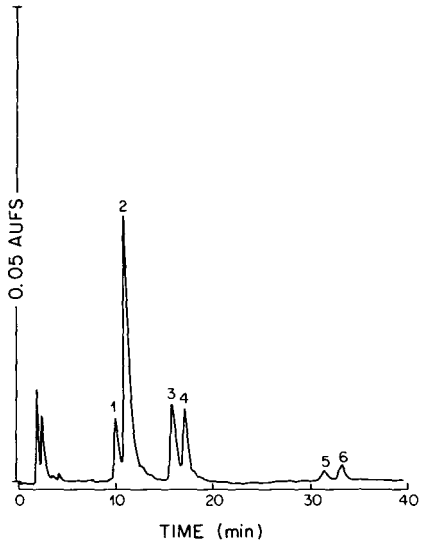


Fig. 5. Assay of BLM hydrolase activity in mouse liver extracts using Blenoxane (FOX04), as indicated in Experimental. Structures of the following identifiable peaks can be defined by referring to Fig. 1 and Table I. Peaks: 1 = BLM desamido-A_2; 2 = BLM A_2; 3 = BLM desamido-B_2; 4 = BLM B_2; 5 = BLM desamido-DM-A_2; 6 = BLM DM-A_2.

This would be especially important when comparing relative rates in cells which respond differently to drug treatment. This technique, therefore, gives an additional dimension to published methods of determining BLM hydrolase activity [28,32,33,40] and provides more useful inactivation rates. Our calculations for mouse liver (1 h incubation) indicate about 25% less activity when the complex is used as compared to BLM B_2 alone [5.6 versus 7.7 μg BLM (mg protein)$^{-1}$ min^{-1}].

Chemical modifications of bleomycin A_2

Fig. 6 shows the analysis of some of the published chemical modifications of BLM A_2. If Cu(II)—BLM A_2 (greater than 98% pure as shown in Fig. 6A) is subjected to pyrolysis at 100°C for 18 h, about 95% is converted to BLM DM-A_2 (Fig. 6B). This peak has a shoulder, just prior to that of authentic BLM DM-A_2, which we attribute to the formation of BLM epi- or iso-DM-A_2. However, preliminary attempts to form these analogues in sufficient yields for analysis were not successful, so that we cannot at this time adequately define these alternate peaks.

Oxidation of BLM DM-A_2 with hydrogen peroxide [29] produces the predicted BLM A_1 (Fig. 6C), and the alternative form(s) is seen to carry over in relatively the same amount. If, however, the BLM DM-A_2 formed in Fig. 6B is remethylated with methyl iodide [29,30], the chemistry does not appear to be as specific. Although the amounts of BLM A_2 and the additional form produced are considerable, these account for no more than 60% of the products. It also appears that BLM A_1 is formed along with other undetermined products. This result is consistent with our analyses of two lots of commercial [^{3}H]S-CH_3-BLM A_2 prepared by this method [29,30], in which 70% and 60%, respectively, of the label was separated from the authentic BLM A_2 peak (data not shown).

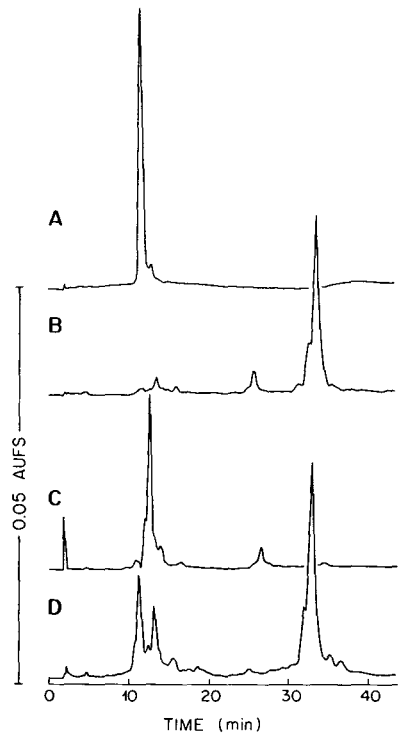


Fig. 6. Chemical modifications of BLM A_2. The chemistry is described in Experimental. A, BLM A_2; B, BLM DM-A_2; C, BLM A_1; D, BLM remethylated-DM-A_2.

CONCLUSIONS

We have developed a procedure which precisely separates many of the common BLMs and/or PHLMs and is also readily adaptable to the analysis of the chemical purity of newly synthesized derivatives of this class of antibiotics. The sensitivity of 50 pmol and the ease of sample preparation make this technique suitable for studies of tissue distribution, metabolism, and pharmacokinetics of specific BLMs, as well as mixtures of different congeners. Moreover, the method may be easily modified to provide increased separation capacity for complex mixtures, or shortened analysis time for samples of simpler composition.

ACKNOWLEDGEMENTS

This work was supported in part by grants from the National Heart, Lung, and Blood Institute Division of Lung Diseases (HL23874-05 TOX) and from the American Cancer Society's Robert A. Sullivan Memorial Grant for Cancer Research (CH-252).

We are grateful to all who have provided specific BLM/PHLM congeners for our use, and to Mrs. Karen Grodsky for her help in the preparation of this manuscript.

REFERENCES

1 K. Maeda, H. Kosaka, K. Yagishita and H. Umezawa, J. Antibiot. Ser. A, 9 (1956) 82.
2 H. Umezawa, K. Maeda, T. Takeuchi and Y. Okami, J. Antibiot. Ser. A, 19 (1966) 200.
3 J.C. Dabrowiak, J. Inorg. Biochem., 13 (1980) 317.
4 L. Giloni, M. Takeshita, F. Johnson, C. Iden and A.P. Grollman, J. Biol. Chem., 256 (1981) 8608.
5 P. Raban, J. Brousil and P. Svihovcova, Eur. J. Nucl. Med., 4 (1979) 191.
6 T. Takita, Y. Muraoka, T. Yoshioka, A. Fujii, K. Maeda and H. Umezawa, J. Antibiot., 25 (1972) 755.
7 Y. Muraoka, H. Kobayashi, A. Fujii, M. Kunishima, T. Fujii, Y. Nakayama, T. Takita and H. Umezawa, J. Antibiot., 29 (1976) 853.
8 H. Umezawa, S. Hori, T. Sawa, T. Yoshioka and T. Takeuchi, J. Antibiot., 27 (1974) 419.
9 Y. Nakayama, M. Kunishima, S. Omoto, T. Takita and H. Umezawa, J. Antibiot., 26 (1973) 400.
10 Code of Federal Regulations, Food and Drugs, Title 21, part 450.10a, U.S. Government Printing Office, Washington, DC, 1980.
11 H. Umezawa, GANN Monogr. Cancer Res., 19 (1976) 3.
12 A. Matsuda, O. Yoshioka, K. Ebihara, H. Ekimoto, T. Yamashita and H. Umezawa, in S.K. Carter, S.T. Crooke and H. Umezawa (Editors), Bleomycin: Current Status and New Developments, Academic Press, New York, 1978, p. 299.
13 I.H. Raisfeld, Toxicol. Appl. Pharmacol., 56 (1980) 326.
14 I.H. Raisfeld, Toxicol. Appl. Pharmacol., 57 (1981) 355.
15 I.H. Raisfeld, J.P. Chovan and S. Frost, Life Sci., 30 (1982) 1391.
16 I.H. Raisfeld, P. Chu, N.K. Hart and A. Lane, Toxicol. Appl. Pharmacol., 63 (1982) 351.
17 M. Takeshita, L.S. Kappen, A.P. Grollman, M. Eisenberg and I.H. Goldberg, Biochemistry, 20 (1981) 7599.
18 M. Miyaki, T. Ono, S. Hori and H. Umezawa, Cancer Res., 35 (1975) 2015.
19 W.C. Eckelman, W.J. Rzeszotarski, B.A. Siegel, H. Kubota, M. Chelliah, J. Stevenson and R.C. Reba, J. Nucl. Med., 16 (1975) 1033.
20 W.J. Rzeszotarski, W.C. Eckelman and R.C. Reba, J. Chromatogr., 124 (1976) 88.
21 T.T. Sakai, J. Chromatogr., 161 (1978) 389.
22 G.K. Shiu, T.J. Goehl and W.H. Pitlick, J. Pharm. Sci., 68 (1979) 232.
23 Y. Muraoka, in S..M. Hecht (Editor), Bleomycin: Chemical, Biochemical, and Biological Aspects, Springer, New York, 1979, p. 92.
24 G.K. Shiu and T.J. Goehl, J. Chromatogr., 181 (1980) 127.
25 A. Aszalos, J. Crawford, P. Vollmer, N. Kantor and T. Alexander, J. Pharm. Sci., 70 (1981) 878.
26 J.C. Dabrowiak, F.T. Greenway, W.E. Longo, M. van Husen and S.T. Crooke, Biochim. Biophys. Acta, 517 (1978) 517.
27 R.M. Burger, S.B. Horwitz, J. Peisach and J.B. Wittenberg, J. Biol. Chem., 254 (1979) 12299.
28 O. Yoshioka, N. Amano, K. Takahashi, A. Matsuda and H. Umezawa, in S.K. Carter, S.T. Crooke and H. Umezawa (Editors), Bleomycin: Current Status and New Developments, Academic Press, New York, 1978, p. 35.
29 A. Fujii, T. Takita, K. Maeda and H. Umezawa, J. Antibiot., 26 (1973) 398.
30 S.N. Roy, G.A. Orr, C.F. Brewer and S.B. Horwitz, Cancer Res., 41 (1981) 4471.
31 H. Umezawa, Y. Suhara, T. Takita and K. Maeda, J. Antibiot. Ser. A, 19 (1966) 210.
32 J.S. Lazo, C.J. Boland and P.E. Schwartz, Cancer Res., 42 (1982) 4026.
33 J.S. Lazo and C.J. Humphreys, Proc. Nat. Acad. Sci. U.S., 80 (1983) 3064.
34 M. Chien, A.P. Grollman and S.B. Horwitz, Biochemistry, 16 (1977) 3641.
35 A. Broughton and J.E. Strong, Cancer Res., 36 (1976) 1418.
36 M.K. Elson, M.M. Oken and R.B. Shafer, J. Nucl. Med., 18 (1977) 296.
37 K. Fujiwara, M. Yasuno and T. Kitagawa, Cancer Res., 41 (1981) 4121.
38 K. Fujiwara, M. Isobe, H. Saikusa, H. Nakamura, T. Kitagawa and S. Takahashi, Cancer Treat. Rep., 67 (1983) 363.
39 H. Umezawa, Prog. Biochem. Pharmacol., 11 (1976) 18.
40 W.E.G. Muller, A. Totsuka, R.K. Zahn and H. Umezawa, Z. Krebsforsch., 83 (1975) 151.

Journal of Chromatography, 310 (1984) 372—378
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2192

Note

Computer-optimized normal-phase high-performance liquid chromatographic separation of *Corynebacterium poinsettiae* carotenoids

A.S. KESTER*

Department of Biological Sciences, North Texas University, Denton, TX 76203 (U.S.A.)

and

R.E. THOMPSON*

Departments of Chemistry and Biochemistry, North Texas State University/Texas College of Osteopathic Medicine, Denton, TX 76203 (U.S.A.)

(First received December 8th, 1983; revised manuscript received May 14th, 1984)

Modern high-performance liquid chromatography (HPLC) owes much of its usefulness to the wide variety of mobile phase components available. Once the mobile phase components have been selected, one is left with the arduous task of simultaneously manipulating several variables, in order to arrive at a mobile phase composition which will produce an acceptable resolution of components. Therefore, a computer algorithm has been developed for optimizing a mobile phase composition to produce a satisfactory resolution of sample components according to a rigorously defined numerical evaluation of that resolution.

The optimization of the separation of a complex mixture, frequently encountered in biological samples, and typified by the carotenoid sample with which this communication is concerned, is correspondingly more difficult than simple two- or three-component samples. The difficulty stems in part from the fact that frequently the investigator does not know the exact number of components in the sample. In this paper we describe an approach to this problem which consists of first selecting a finite number of resolutions of major components of the sample such that they (1) represent apparently difficult

*Present address: Abbott Laboratories, 1921 Hurd, Irving, TX 75061, U.S.A.

0378-4347/84/$03.00

separations and (2) are somewhat evenly spaced throughout the chromatogram. Secondly, the optimization algorithm is allowed to progress toward the ultimate goal of producing resolution factors of 1.5 for each selected resolution.

Carotenoids are produced by a variety of plants, animals and microorganisms where they most often exist as mixtures. Norgard et al. [1] detected nine pigments by thin-layer chromatography in extracts of *Corynebacterium poinsettiae*, the organism used in this study. The six major pigments were identified as a C_{40} hydrocarbon, lycopene; a C_{45} alcohol, C.p. 482 (2-isopentenyl-3,4-dehydrorodopin); a C_{50} diol, C.p. 470 (3,4,3′,4′-tetrahydrobacteriorubrin); a C_{50} diol, C.p. 496 (bisanhydrobacterioruberin); a C_{50} diol, C.p. 473 [1′-hydroxy-1′2′-dihydro-2-isopentenyl-2′-(hydroxyisopentenyl) torulene]; and a C_{50} diol, C.p. 450 [2-(dihydroxyisopentenyl)-2-isopentenyl-β-carotene]. They also partially characterized mono-ol alcohols, C.p. 450 and C.p. 473 and a pigment designated C.p. 435 by their absorption maxima in light petroleum or acetone. The C.p. 450 and C.p. 473 alcohols gave monoacetates after acetylation.

EXPERIMENTAL

Materials

HPLC grade hexanes, acetone, and methylene chloride (Baker, Phillipsburg, NJ, U.S.A.) were used in the mobile phase for HPLC. Solvents and reagents for extraction of pigments and thin-layer chromatography (TLC) were reagent grade.

Chromatography

Separations were performed on a Waters Assoc. (Milford, MA, U.S.A.) μPorasil silica column (30 cm × 3.9 mm, 10 μm particle size). A Waters liquid chromatograph was equipped with an M6000A pump, a U6K syringe injector, and a Model 450 variable-wavelength ultraviolet (UV) detector. A Sargent (Dallas, TX, U.S.A.) Model SR strip-chart recorder was used. Injections (10 μl) of carotenoid sample dissolved in hexane were made throughout the optimization. The detector was set at 450 nm with a sensitivity of 0.04 a.u.f.s.

Optimization algorithm

The simplex optimization algorithm first presented by Spindley et al. [2], and later modified by Nelder and Mead [3] was used with one major modification: quadratic interpolations, as suggested by Routh et al. [4] were implemented in our FORTRAN package as previously described [5].

The objective function which was used to evaluate the quality of the separation is shown in eqn. 1.

$$F_{obj} = \sum_{i=1}^{N-1} [10(1.5 - R_s)]^2 \qquad (1)$$

where the summation is over adjacent peaks, N is the total number of peaks to be resolved, and R_s is the resolution of the i^{th} pair of peaks as conventionally

defined [6]. This particular objective function was designed as an empirical approach to a complex problem. The obvious non-Gaussian peak shapes, differing peak sizes, and similar elution times make the application of a more rigorous separation function of questionable value. While an exhaustive comparison of objective functions such as that carried out by Weyland et al. [7] would have been instructive, the simple, practical function shown in eqn. 1 was chosen in view of the complex sample mixture and the simple goals of this particular separation. The problem of peak-crossing would have been relatively easy to identify considering the differing peak sizes; however, we saw no evidence of it throughout the optimization. Considering the complexity of the carotenoid mixture, their existence at multiple optima is virtually inevitable.

The three-component mobile phase represented two degrees of freedom, so the parameters optimized were:

$$\alpha_1 = f_1 \tag{2}$$

$$\alpha_2 = \frac{f_2}{1 - f_1} \tag{3}$$

where f_1 was the volume fraction hexanes and f_2 was the volume fraction acetone. The volume fraction methylene chloride was calculated by difference (eqn. 4).

$$f_3 = 1 - f_1 - f_2 \tag{4}$$

Both parameters (α_i) were constrained over the region (0, 1).

These three mobile phase components were selected because of their differing chemical properties (hydrocarbons and methylene chloride are recommended for normal-phase selectivity by Snyder et al. [8], carotenoid solubility and successful application to carotenoid separations by TLC [1].

Organism

Corynebacterium poinsettiae ATCC 9682, obtained from the American Type Culture Collection, was maintained on Tryptic Soy Agar (TSA) slants (Difco Laboratories, Detroit, MI, U.S.A.) and was subcultured every three weeks.

Culture conditions

Seed flasks, 50 ml of half-strength Trypticase Soy Broth (TSB) (Difco) per 250-ml flask, were inoculated from slant cultures and incubated for 24 h at 30°C on a rotary shaker (Eberbach) (160 rpm) with continous lighting from cool white fluorescent lamps positioned 1 m above the surface. Flasks (4 l) containing 1 l of half strength TSB were inoculated with 10 ml of the seed flask culture and incubated under the same conditions as the seed flasks. Cells were harvested by centrifugation at 7700 *g* for 15 min and washed once with water before extraction.

Extraction of pigments

Cells were extracted four to five times with methyl alcohol. The pooled extracts were saponified by adding an equal volume of 10% aqueous potassium

hydroxide and allowing the mixture to stand for 1 h at 22—24°C. Saponified pigments were then extracted with light petroleum (b.p. range 35—60°C) which was then washed free of alkali with water and dried over anhydrous sodium sulphate. The extract was then evaporated to dryness in a rotary vacuum evaporator and the residue redissolved in 1—3 ml of either light petroleum or hexane.

Calculation of pigment levels

Pigment levels in crude extracts were estimated assuming an $E_{cm}^{1\%}$ of 2500 at the absorption maximum of the extract, normally 478 nm. For C.p. 496 a molar extinction coefficient of 182,000 was used [6].

TLC

For identification of C.p. 496 resolved by HPLC, the pigments in the crude extract were first purified by TLC. A volume of 200 μl of crude pigment (0.3 μg/μl) in light petroleum was banded on silica gel G plates (0.25 mm thick) and resolved in light petroleum—acetone (80:20, v/v); before banding the plates were heat-activated at 100°C for 15 min. C.p. 496 (R_F 0.50) was scraped off and the pigments eluted with methanol. The methanol was then evaporated to dryness and the residue redissolved in acetone or light petroleum. The pigment was identified by comparison of the absorption maxima in these solvents with those reported by Norgard et al. [6].

HPLC

Normally, 10 μl of crude pigment (0.3 μg/μl) in hexane were injected. The level used for C.p. 496 in determining linearity of response for quantitation was 0.025 μg/μl. The pigments were resolved with hexane—acetone—methylene chloride (11.35:1.73:1.00, v/v/v). Instrument conditions were: chart-speed, 2.54 cm/min; flow-rate, 2 ml/min; sensitivity, 0.04 a.u.f.s.; detection, absorption maximum of sample.

RESULTS AND DISCUSSION

The particular objective function selected for this optimization was constructed so that the best possible cumulative separation would be represented by resolutions of 1.5 for each pair of adjacent peaks. The ten peaks whose separations were to be optimized were selected from the sixteen identifiably separable components of the sample so that the optimization process would concentrate on potentially difficult separations of prominent compounds more-or-less evenly distributed throughout the chromatogram.

Table I shows the mobile phase compositions used during the optimization process. The objective function values were calculated from elution data for ten of the peaks. Experiment C-10 represents the overall best resolution and the chromatogram is shown in Fig. 1. Resolution of adjacent peaks is clearly variable as would be expected from the fact that the information from nine simultaneous separations is accumulated in a single number. The separations are summarized in Table II. Mobile phase C-10 results in poor separations of peaks 2 and 3, peaks 6 and 7, and peaks 7 and 8. However, in each of these cases a major peak was being resolved from a minor shoulder.

TABLE I

OPTIMIZATION PROGRESS

See the Experimental section for sample injection details and definition of F_{obj}

Point	Hexanes*	Acetone*	Methylene chloride*	F_{obj}	Resulting from**
C-1	0.800	0.200	—	904	Initial
C-2	0.750	0.100	0.150	444	Initial
C-3	0.800	0.100	0.100	356	Initial
C-4	0.763	0.043	0.194	—	Short contraction
C-5	0.787	0.154	0.060	352	Long contraction
C-6	0.837	0.134	0.029	232	Reflection
C-7	0.818	0.165	0.017	432	Short contraction
C-8	0.806	0.123	0.071	251	Long contraction
C-9	0.856	0.106	0.038	592	Reflection
C-10	0.804	0.142	0.054	202	Long contraction

*All values are expressed as volume functions uncorrected for molar volumes.
**See ref. 4 for a description of this application of the simplex algorithm.

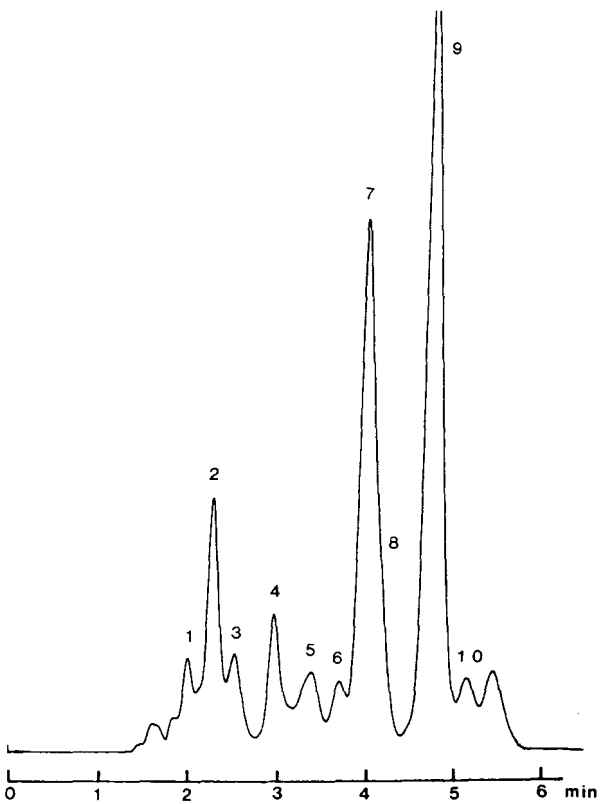


Fig. 1. Resolution of *C. poinsettiae* pigments using mobile phase C-10 (see Table I). The peaks used for the optimization correspond to the peak numbers in Table II.

TABLE II

INDIVIDUAL PEAK ELUTION DATA FOR OPTIMIZED MOBILE PHASES

See the Experimental section for sample injection details and definition of R_s; t_R = retention time; mobile phase compositions (C-10 and C-9) are defined in Table I.

Peak	C-10		C-9	
	t_R (min)	R_s	t_R (min)	R_s
1	2.02		2.47	
		1.34		1.85
2	2.39		3.08	
		0.74		1.03
3	2.62		3.48	
		1.64		2.19
4	3.17		4.43	
		1.37		3.43
5	3.71		6.10	
		1.05		1.29
6	4.14		6.91	
		0.78		1.19
7	4.43		7.66	
		0.77		1.10
8	4.75		8.50	
		1.86		1.88
9	5.52		10.01	
		1.41		2.47
10	6.14		11.91	

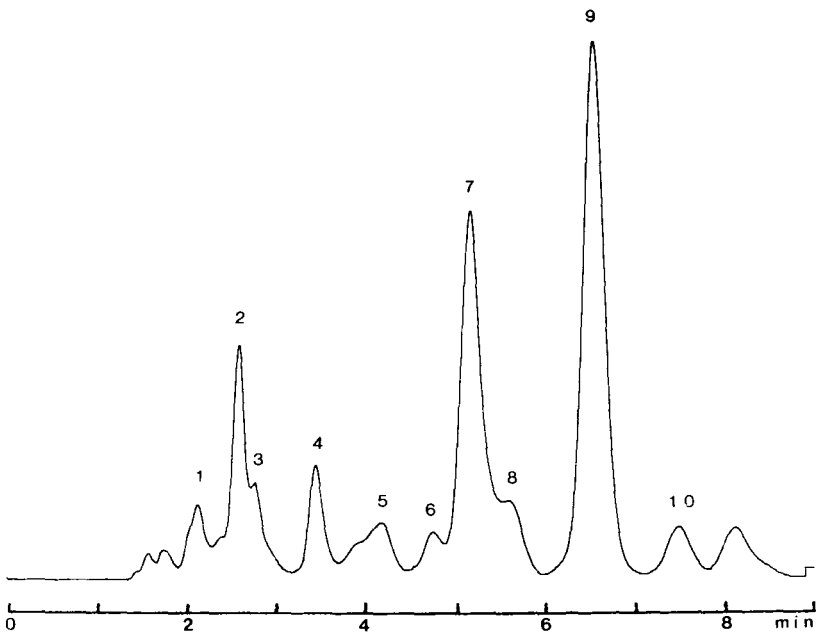


Fig. 2. Resolution of *C. poinsettiae* pigments using mobile phase C-9 (see Table I). The peaks used for the optimization correspond to the peak numbers in Table II.

Mobile phase C-9 shown in Table I succeeded in spreading the peaks to a greater extent than any of the other mobile phases. In particular the first part of the chromatogram was better resolved as shown by the individual R_s values listed in Table II. The chromatogram itself is reproduced as Fig. 2. The reason for the unsatisfactory objective function was that while resolution of the three difficult pairs improved over that obtained with mobile phase C-10, five of the other seven resolutions were significantly greater than the optimal 1.5 and

therefore added to the value of the objective function. This particular mobile phase (predictably) resulted in almost double the elution times produced by mobile phase C-10. It is also noteworthy that peak 1 was resolved into two peaks and a new peak appeared between peaks 7 and 8. Of course, it is quite possible that certain uses to which carotenoid separations are applied might require the resolution provided by mobile phase C-9 and the increased analysis time prove inconsequential. This is, therefore, offered as an alternative mobile phase.

Pigment C.p. 496 was purified by TLC (R_F 0.50) as described in Experimental. With mobile phase C-10, peak 7 eluted at the same time as this pigment. Peak height can be used to quantitate this pigment over the range 0.1—0.7 μg. Reproducibility was found to be better than ± 1.5%.

ACKNOWLEDGEMENTS

The authors would like to express their appreciation to Ms. Karen Oray for preliminary HPLC experiments, to Dr. D.L. Dunn for valuable HPLC procedural discussions as well the suggestion to apply eqn. 1, and to Mr. Tyler Tull (Waters Assoc.) for allowing the use of his liquid chromatograph for this work. Funding for this project was provided in part by NTSU Faculty Research.

REFERENCES

1 S. Norgard, A.J. Aasen and S. Liaaen-Jenson, Acta Chem. Scand., 24 (1970) 21.
2 W. Spindley, S.R. Hext and F.R. Himsworth, Technometrics, 4 (1962) 441.
3 J.A. Nelder and R. Mead, Comput. J., 7 (1965) 308.
4 M.W. Routh, P.A. Swartz and M.B. Denton, Anal. Chem., 49 (1977) 1422.
5 D.L. Dunn and R.E. Thompson, J. Chromatogr., 264 (1983) 264.
6 L.R. Snyder and J.J. Kirkland, Introduction to Modern Liquid Chromatography, Wiley-Interscience, New York, 2nd ed., 1979, p. 34.
7 J.W. Weyland, C.H.P. Bruins, H.J.G. Debets, B.L. Bajema and D.A. Doornbos, Anal. Chim. Acta, 153 (1983) 93.
8 L.R. Snyder, J.L. Glajch and J.J. Kirkland, J. Chromatogr., 218 (1981) 299.

Journal of Chromatography, 310 (1984) 379—385
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2194

Note

Analysis of vanillylmandelic acid, homovanillic acid and 5-hydroxyindoleacetic acid in human urine by high-performance liquid chromatography and fluorometry

J.P.M. WIELDERS* and Chr. J.K. MINK

Department of Clinical Chemistry, St. Annadal Hospital, P.O. Box 1918, 6201 BX Maastricht (The Netherlands)

(First received December 28th, 1983; revised manuscript received May 16th, 1984)

Determination of 3-methoxy-4-hydroxymandelic acid (vanillylmandelic acid, VMA) in urine is a diagnostic aid for the detection of phaeochromocytoma. Probably the most well known and still widely used method was presented by Pisano et al. [1]. Recently, urinary 3-methoxy-4-hydroxyphenylglycol (MHPG) quantification has also been considered valuable either for detection of phaeochromocytoma or in the diagnosis of mental pathology and its treatment [2, 3]. Neuroblastomas and ganglioneuromas are generally accompanied [3] by increased urinary levels of 3-methoxy-4-hydroxyphenylacetic acid (homovanillic acid, HVA). Intestinal tumours, which secrete large amounts of serotonin, are often discovered by the enhanced urinary excretion of 5-hydroxyindoleacetic acid (5-HIAA).

A number of papers have been devoted to the quantification of urinary VMA by high-performance liquid chromatography (HPLC), alone or in combination with HVA or other metabolites (see refs. 4—12, for example). Nearly all authors seem to agree on one aspect of the analysis: the complexity of the urine sample is best dealt with by solvent extraction of acidified urine (eventually followed by re-extraction) and by using the specificity of an electrochemical detector or a post-column or pre-column reaction. Anion-exchange extraction or solid—solvent extraction of VMA has been described and ultraviolet (UV) absorbance or fluorescence detection has been applied by a few authors.

However, measuring one or just a few components of the sample makes it impossible to obtain a "fingerprint" of the whole sample, which may be very valuable for screening purposes and for the elucidation of rare diseases. In a

0378-4347/84/$03.00

recent paper Thomasson et al. [13] compared the analytical performance of five methods for VMA analysis, including Pisano's method, gas—liquid chromatography (GLC) with flame ionization detection, and an HPLC method with electrochemical detection [9]. The HPLC method was the method of choice for VMA analysis, but the overall catecholamine metabolite profile was more informative with the GLC method.

We present an HPLC method that directly uses a purified urine sample, omitting laborious solvent extraction procedures. VMA or other components of interest are not isolated from the crude sample, but only those very lipophilic components that would disturb reversed-phase chromatography (due to their large capacity ratios) are removed. Hence, the "fingerprint" is hardly damaged.

MATERIALS AND METHODS

Urine samples (24 h) were collected in glass or polyethylene bottles containing 10 ml of 6 mol/l hydrochloric acid. Samples were stored at 4°C prior to analysis.

Standard solution was artificial urine (see below) containing weighed amounts of VMA (Sigma, St. Louis, MO, U.S.A.; Cat. No. H-0131), 5-HIAA (Sigma; Cat. No. H-8876) and HVA (Sigma; Cat. No. H-1252) at concentrations of 50 μmol/l, 25 μmol/l and 50 μmol/l, respectively. The pH of the standard solution was adjusted to 4.0 with 6 mol/l hydrochloric acid and 25-ml aliquots in polyethylene bottles were stored at −20°C in the dark.

Artificial urine was prepared by dissolving 20 g of urea, 2 g of creatinine, 10 g of sodium chloride, 1.7 g of potassium sulphate, 1.2 g of $MgSO_4 \cdot 7H_2O$, 2.3 g of $K_2HPO_4 \cdot 3H_2O$ and 1.4 g of KH_2PO_4 per litre of purified water. The pH was about 6.5 and the artificial urine was stored at −20°C.

Sample-purification aids were Sep-Pak C_{18} sample preparation cartridges (Waters Assoc., Milford, MA, U.S.A.) and disposable membrane filters, 25 mm in diameter, 0.45 μm pore size (SM 11306; Sartorius, Göttingen, F.R.G.). Cartridges were handled as suggested by the manufacturer.

Mobile phase A was an aqueous sodium citrate buffer pH 3.55, 0.05 mol/l (0.05 mol/l is the sum of citrate and citric acid concentrations). Mobile phase B was a mixture of acetonitrile—aqueous sodium citrate buffer pH 5.25, 0.05 mol/l (5:95, v/v). Mobile phase C was a mixture of acetonitrile—aqueous sodium citrate buffer pH 5.0, 0.05 mol/l (3:97, v/v).

HPLC-grade acetonitrile was from Rathburn Chemicals (Walkerburn, U.K.). Reagent-grade water was delivered by our Milli-Q deionization unit (Millipore, Bedford, MA, U.S.A.). Tris(hydroxymethyl)aminomethane, citric acid and tri-sodium citrate were obtained from E. Merck (Darmstadt, F.R.G.). Reagents not specified were of analytical grade.

Sample and standard preparation

The urine sample or standard solution was adjusted to pH 6.0 with 3 mol/l Tris. A portion of ca. 5 ml was gently passed through a C_{18} cartridge and a membrane filter, yielding a clear, colourless or nearly colourless solution. The first few millilitres of eluate and filtrate were discarded. The filtrate was brought to pH 4.0 with 2.0 mol/l citric acid. Samples prepared this way were either directly analysed or stored at −20°C.

For quantitative analysis of 5-HIAA and HVA, the urine was adjusted to pH 5.0 and passed through the membrane filter. The filtrate is either directly analysed or stored at −20° C.

Chromatographic analysis

Two mobile phases were employed at a total flow-rate of 2.0 ml/min. The pump system consisted of a Model 6000A pump, a Model 45 pump and a Model 680 Gradient Controller (all from Waters Assoc.). Instead of running the programme depicted in Fig. 1, equivalent results could be obtained when solvent switching was carried out manually using only the 6000A pump.

A 20-μl sample was injected using a Model 7125 valve (Rheodyne, Cotati, CA, U.S.A.). The column was a Radial-Pak C_{18} (10-μm particles, 10 cm × 8 mm; Waters Assoc.) pressurized by a so-called Z-module (Waters Assoc.). A corresponding guard column was inserted. Detection was performed by a Model SFM 23/B spectrofluorometer (Kontron, Zürich, Switzerland) equipped with a 20-μl flow-through cell thermostated at 25° C. Excitation was set at 285 nm, emission was detected at 315 nm. The resulting signal was displayed on a Kipp BD 40 recorder (Kipp & Zonen, Delft, The Netherlands). Peak areas and retention times were calculated by a Minigrator computing integrator (Spectra Physics, Mountain View, CA, U.S.A.).

A complete run took about 16 min for VMA quantification and 5-HIAA and HVA screening, and about 24 min for quantification of 5-HIAA and HVA. For the analysis of hippuric acid and *m*-methylhippuric acid, a variable-wavelength UV detector (Model 450; Waters Assoc.) was coupled after the fluorometric detector.

Concentrations were calculated by the well known external standard method. Minor differences in volume expansion of sample and standard caused by the sample preparation procedure could be neglected. A suitable internal standard method is under investigation.

EXPERIMENTS AND DISCUSSION

Method development

Several authors have described isocratic reversed-phase chromatography at low pH or gradient elution with decreasing eluent polarity for VMA analysis in urinary extracts (see, for example, refs. 2, 5, 8, 9). For the simultaneous determination of VMA and other metabolites more sophisticated chromatographic principles have been used. Breebaart and Grave [12] employed reversed-phase ion-pair chromatography plus a methanol gradient with direct injection of filtered urine samples. We have tested several of these approaches and found either a limited resolving power for the whole urine fingerprint or an excessive time for regeneration needed after each run. The latter holds especially for complex chromatographic systems based upon ion-pair formation with, for example, tetraalkylammoniumbromide and a gradient in the eluent's polarity. The operational system we selected is a biphasic approach based upon the pK_a values of VMA, 5-HIAA and HVA. As a result VMA is eluted at pH 3.6 while 5-HIAA and HVA are eluted at pH 5.2 and in the presence of 5% (v/v) acetonitrile. Adaptation of the column to a shift in pH and/or organic solvent

content occurs obviously much faster than the equilibration with, for example, tetrabutylammonium bromide.

In order to isolate VMA, HVA and 5-HIAA from urine, we tested several approaches of solvent—solvent or solvent—solid extraction and the use of ion-exchange procedures. Instead of this, it was found much more convenient to use Sep-Pak C_{18} cartridges, not for isolation of components of interest, but to get rid of those strongly lipophilic sample constituents that would demand rigid column-cleaning procedures after each direct injection of filtered urine using reversed-phase chromatography. Since we use these cartridges only to capture disturbing lipophilic components, the sample injected still contains the majority of the original anions, cations and other polar components. This approach is obviously advantageous for screening purposes.

Chromatographic experiences

The chromatograms shown in Fig. 1 represent typical urine samples. The conditions selected allow VMA quantification and a semiquantitative determination of 5-HIAA and HVA amongst others. The urinary constituents nominated were identified by means of their retention times and their

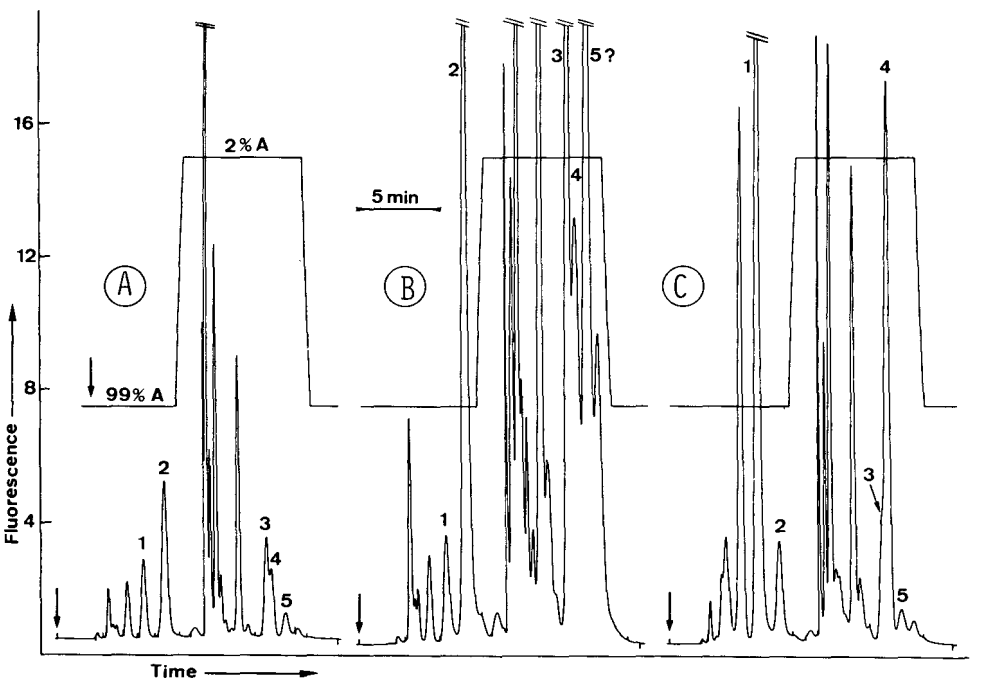


Fig. 1. (A) Chromatogram obtained from a "normal" urine sample (1830 ml per 24 h) containing 14.4 μmol/l VMA (1), tyrosine (2), 5-HIAA (3), HVA (4) and tryptophan (5). The biphasic elution is visualized by the jump from 99% mobile phase A—1% mobile phase B to 2% mobile phase A—98% mobile phase B. The time of injection is indicated by an arrow. Fluorescence intensity is given in arbitrary units. (B and C) Chromatograms from two pathological urine samples. Peaks are identified as in A. (B) Urine sample (1020 ml per 24 h) containing a normal VMA concentration of 22.5 μmol/l, but elevated levels of tyrosine, 5-HIAA and HVA. (C) Urine of a female child (360 ml per 24 h) containing a largely increased VMA level of 154 μmol/l plus an increased HVA excretion.

coelution with added pure components in at least two different chromatographic systems. For a fast (less than 12 min per analysis) VMA quantification it is adequate to increase the acetonitrile content of mobile phase B to 30% (v/v). A 3-min wash with mobile phase B immediately after the elution of VMA effectively cleans the column. However, we prefer the type of analysis represented in Fig. 1A, allowing a screening for 5-HIAA and HVA excretion. The occasional samples containing an increased amount of HVA and/or 5-HIAA and the rare urine samples submitted specially for quantitative analysis of HVA and/or 5-HIAA were subjected to analysis under different chromatographic conditions. Combining VMA, HVA and 5-HIAA into one analysis was possible by selecting an appropriate gradient. However, we found it a waste of time to run such extended programme for each VMA analysis requested.

The analysis of 5-HIAA and HVA in urine is presented in Fig. 2. Mobile phase C was used at a flow-rate of 2 ml/min. An increase to 30% (v/v) of the acetonitrile content for mobile phase C was useful for rapid cleaning of the column between analyses. For some samples the use of a modified mobile phase C, at pH 5.25 and containing 2% (v/v) acetonitrile, was found advantageous.

Preliminary experiments have demonstrated the applicability of this VMA analysis for the simultaneous determination of hippuric acid and *m*-methylhippuric acid. Optimization of such an analysis could start from the use of mobile phase B and UV absorbance measurements near 230 nm.

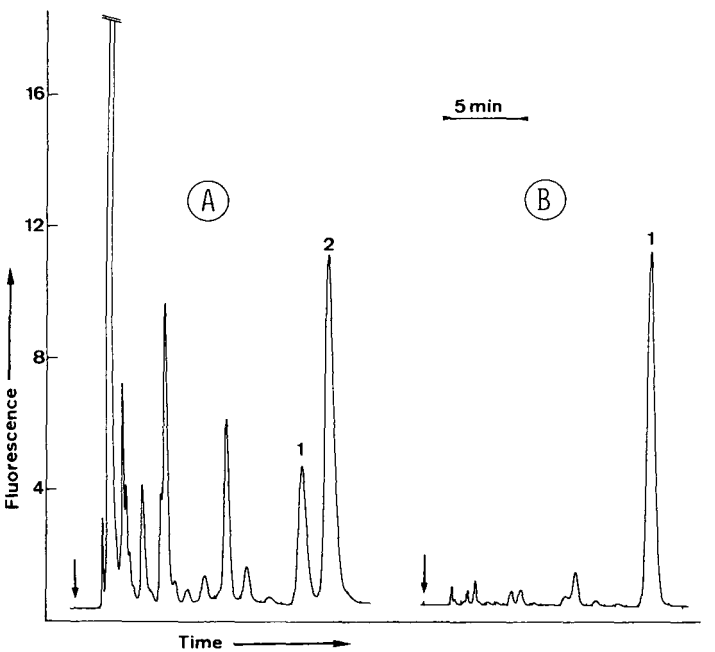


Fig. 2. (A) Quantitative analysis of 5-HIAA and HVA in the urine analysed in Fig. 1C. The urine of this 3-year-old girl (360 ml per 24 h) contained a normal 5-HIAA level (peak 1) of 25.6 μmol/l and a largely increased concentration of HVA (2) of 140.5 μmol/l. A case of ganglioneuroma was finally diagnosed. (B) Chromatogram of a diluted (1:39, v/v) urine of an adult male (940 ml per 24 h) excreting large amounts of 5-HIAA (1). In the diluted sample a concentration of 62.7 μmol/l was measured, hence the daily excretion was about 2668 μmol.

Analytical performance

On comparing the results for four samples prepared for VMA analysis as described in Materials and methods with the results obtained by directly injecting the same fresh, untreated urine samples, we found recoveries of approximately 98%, 87% and 50% for 12 μmol/l VMA, 18 μmol/l 5-HIAA and 56 μmol/l HVA, respectively. The recoveries for 5-HIAA and HVA using the procedure especially developed for their quantification was about 97% for both components. For VMA analysis the inter-assay coefficient of variation was 5.5%, calculated from the analysis of the same urine sample in ten consecutive batches. The intra-assay coefficient of variation for 5-HIAA and HVA analysis was found to be 3.2% and 2.9%, respectively (n = 5). Linearity was excellent for VMA, HVA and 5-HIAA in the concentration range tested (up to 200 μmol/l).

Comparison with Pisano's VMA analysis

We compared our HPLC method with the method of Pisano et al. [1] using urine blanks. For 27 urine samples the HPLC method found 26 ± 17% less than the concentration determined by Pisano's method. However, it is well known that several components interfere with Pisano's spectrophotometric method, apparently increasing the VMA concentration (see ref. 13, for example). The largest differences were in general associated with the most "crowded" chromatograms, suggesting such interferences for Pisano's method. An artificial increase in the HVA concentration of a urine sample by 28 μmol/l caused a 65% increase in the absorbance of the urine blank but no increase in the VMA concentration, as measured by Pisano's method. However, an increase of 15 μmol/l for MHPG (still a physiological amount) raised the VMA concentration (Pisano's method) from 16 to 34 μmol/l. The inter-assay coefficient of variation for Pisano's method was 9.1% (n = 10). Our HPLC method did not suffer from positive or negative interferences due to the presence of, for example, HVA, 5-HIAA, MHPG and vanillin in the urine.

Exploration of the fingerprint

This part of our study is still receiving much attention. Constituents of the urine samples that have been identified (in order of increasing elution time) are VMA, tyrosine, 5-HIAA, HVA and tryptophan. Physiological concentrations of MHPG can be quantified as well but require another sample preparation procedure. In due time we will expand the diagnostic usefulness of our approach.

CONCLUSION

An HPLC method for quantification of urinary VMA, HVA and 5-HIAA is presented, which includes a fast sample preparation method without extraction procedures and the ability to screen a number of physiologically important metabolites in one and the same analysis.

ACKNOWLEDGEMENTS

We thank Mr. H.J.G. Groothuyzen, Mr. L.M.L. Habets and Mr. Th.J.M. Gransier for their technical assistance and valuable practical suggestions.

REFERENCES

1 J.J. Pisano, J.R. Crout and D. Abraham, Clin. Chim. Acta, 7 (1962) 285—291.
2 A.M. Krstulovic, M. Zakaria, K. Lohse and L. Bertani-Dziedzic, J. Chromatogr., 186 (1979) 733—748.
3 C.R.J. Ruthven and M. Sandler, in S.S. Brown, F.L. Mitchell and D.S. Young (Editors), Chemical Diagnosis of Disease, Elsevier, Amsterdam, 1979, Ch. 24.
4 A. Yoshida, M. Yoshioka, T. Sakai and Z. Tamura, J. Chromatogr., 227 (1982) 162—167.
5 J.L. Morrisey and Z.K. Shihabi, Clin. Chem., 25 (1979) 2043—2045.
6 S.J. Soldin and J.G. Hill, Clin. Chem., 26 (1980) 291—294.
7 W. Bauersfeld, U. Diener, E. Knoll, D. Ratge and H. Wisser, J. Clin. Chem. Clin. Biochem., 20 (1982) 217—220.
8 K. Fujita, K. Maruta, S. Ito and T. Nagatsu, Clin. Chem., 29 (1983) 876—878.
9 P. Moleman and J.J.M. Borstrok, Clin. Chem., 29 (1983) 878—881.
10 K. Yamada, E. Kayama, Y. Aizawa, K. Oka and S. Hara, J. Chromatogr., 223 (1981) 176—178.
11 T.G. Rosano and H.H. Brown, Clin. Chem., 25 (1979) 550—554.
12 K. Breebaart and H. Grave, Tijdschr. Ned. Ver. Klin. Chem., 8 (1983) 13—16 (in Dutch).
13 C.G. Thomasson, B.G. Blijenberg, G.A.M. Eilers and B. Leijnse, J. Clin. Chem. Clin. Biochem., 21 (1983) 417—427.

Journal of Chromatography, 310 (1984) 386—389
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2217

Note

Simultaneous determination of fifteen steroid hormones from a single serum sample by high-performance liquid chromatography and radioimmunoassay

G. EIBS

Department of Pediatrics, Division of Pediatric Endocrinology, Klinikum Charlottenburg, Free University Berlin, Heubnerweg 6, D-1000 Berlin 19 (F.R.G.)

and

M. SCHÖNESHÖFER*

Institute for Clinical Chemistry and Clinical Biochemistry, Klinikum Charlottenburg, Free University Berlin, Spandauer Damm 130, D-1000 Berlin 19 (F.R.G.)

(First received February 16th, 1984; revised manuscript received May 4th, 1984)

The precise biochemical diagnosis of inborn errors of steroid biosynthesis requires the estimation of the steroidal substrates and products of all the enzymes involved in steroid biosynthesis [1, 2]. Generally, the diagnosis of those inborn defects is the subject of paediatric endocrinology, and therefore analytical methods have to provide estimates of multiple steroids from small samples [3, 4].

In the present communication, we describe the evaluation of a routine-suited method, which allows the simultaneous estimation of fifteen steroid hormones from a single, small-volume sample. These steroid hormones are progesterone (P), androstenedione (AD), pregnenolone (PL), 5α-dihydrotestosterone (DHT), dehydroepiandrosterone (DHEA), testosterone (T), 11-deoxycorticosterone (DOC), 17-hydroxyprogesterone (17-OHP), 17-hydroxypregnenolone (17-PL), 11-deoxycortisol (S), 18-hydroxy-11-deoxycorticosterone (18-OH-DOC), corticosterone (B), aldosterone (Aldo), cortisol (F), and 18-hydroxycorticosterone (18-OH-B). In principle, the procedure involves high-performance liquid chromatography (HPLC) followed by radioimmunological quantitation [4]. Using the present method, the reference ranges of all steroid hormones from children 4—10 years' old were determined.

0378-4347/84/$03.00

EXPERIMENTAL

Materials

Reagent-grade chemicals and solvents were used throughout. Extrelut® was purchased from E. Merck (Darmstadt, F.R.G.); plastic syringes (20 ml) used as extraction columns were from Pharmaseal Labs. (Glendale, CA, U.S.A.). Radioactive steroids were from New England Nuclear (Boston, MA, U.S.A.). Other solvents, reagents and accessories used for radioimmunoassay (RIA) were as previously described [4].

High-performance liquid chromatography

The high-performance liquid chromatograph was from Hewlett-Packard (Model 1084 B). The polar bonded phase (DIOL®, 5 μm, 250 × 4.6 mm) was from Knauer, Berlin, F.R.G. Gradient mode was used for elution.

HPLC system I. Solvent A = *n*-hexane; solvent B = *n*-hexane—isopropanol (75:25, v/v). The gradient was run from 20% to 100% of B within 40 min; flow-rate 1.3 ml/min. The temperature of the column oven was 40°C. Ultraviolet (UV) detection was at 254 nm.

HPLC system II. Solvent A = *n*-hexane; solvent B = *n*-hexane—isopropanol (85:15, v/v). The gradient was run from 15% to 100% of B within 30 min; other conditions were as in system I.

Methods

Blood was drawn by venipuncture from nineteen children aged 4—10 years, who had no signs of endocrine diseases. Serum was stored until analysis.

Serum samples (1 ml) were spiked with trace amounts of ^{3}H-labelled isotopes of all steroids. Steroids were extracted from serum samples into 5 ml of diethyl ether using the solid-phase (Extrelut) technique [5]. The evaporated organic extracts were redissolved in *n*-hexane—isopropanol (85:15, v/v) and subjected to automatic HPLC using system I. The times for collecting individual steroids were calibrated by a run of UV—visible amounts or of tritiated steroids prior to each assay [4]. In a first run, fractions were collected containing the steroids P, AD, 17-PL, S, 18-OH-DOC, B, Aldo, F and 18-OH-B. The steroids PL, DHT, DHEA, T and DOC were collected in a common fraction. This latter fraction was evaporated, redissolved in *n*-hexane—isopropanol (95:5, v/v) and again subjected to HPLC using system II.

Quantitation of steroids from the individual organic fractions by RIA and computer evaluation have been described previously [4].

RESULTS AND DISCUSSION

The efficiency of the solid-phase extraction step was better than 95% for all steroids when checked with ^{3}H-recovery measurement.

The HPLC system I (upper chromatogram of Fig. 1) had been successfully applied for the efficient separation of eleven steroid hormones in a single run [4]. From the chromatogram of all the fifteen steroids under study, however, it is evident that the more unpolar steroids PL, DHT, DHEA, T and DOC are not sufficiently resolved from each other by HPLC system I. Since the amount

of serum may become problematic in the paediatric laboratory, it was necessary to cumulatively collect the unresolved non-polar steroids from the first run and to subject them to a second system which provides sufficient resolution of these steroids (lower chromatogram of Fig. 1).

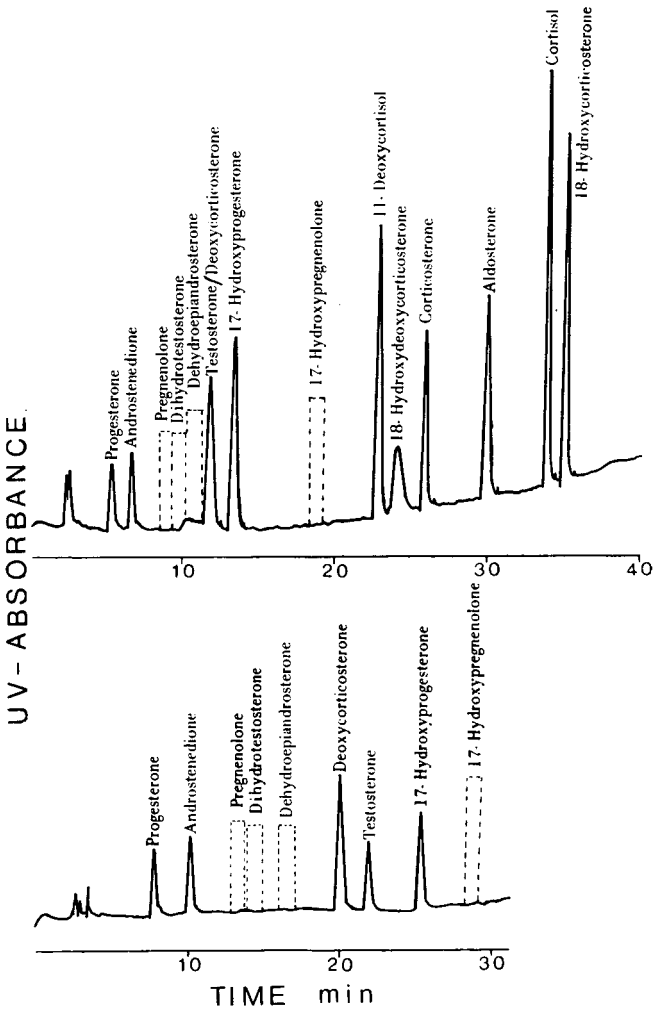


Fig. 1. Chromatograms of steroid standards. Amount of each steroid injected was 750 ng. Steroids not detectable by UV absorbance were localized by ^{3}H-labelled steroid measurement (dotted lines). Upper chromatogram with HPLC system I. Lower chromatogram with HPLC system II.

The analytical variables, such as recovery, sensitivity, specificity, precision, accuracy and practicability, were comparable to those obtained in the eleven-steroid assay [4]. Due to the double chromatography, the overall recoveries of the non-polar steroids PL, DHT, DHEA, T and DOC were slightly lower than those of the other steroids.

The reference ranges of steroid concentrations in serum from 4—10-year-old children are listed in Table I.

TABLE I

CONCENTRATIONS OF FIFTEEN STEROID HORMONES IN SERUM SAMPLES FROM NINETEEN CHILDREN (THIRTEEN GIRLS, SIX BOYS) AGED 4—10 YEARS

Steroid	Median (nmol/l)	Range (nmol/l)
Progesterone	1.29	1.00—1.50
Androstenedione	1.13	0.76—1.34
17-Hydroxyprogesterone	0.57	0.43—0.78
17-Hydroxypregnenolone	3.61	1.76—4.45
11-Deoxycortisol	0.89	0.72—1.46
18-Hydroxydeoxycorticosterone	0.07	N.D.*—0.92
Corticosterone	9.47	6.74—33.77
Aldosterone	0.28	0.14—0.46
Cortisol	167.74	117.25—215.74
18-Hydroxycorticosterone	0.67	0.09—1.03
Pregnenolone	2.30	1.30—3.60
5α-Dihydrotestosterone	0.7	N.D.—1.74
Dehydroepiandrosterone	2.33	0.33—3.57
11-Deoxycorticosterone	0.16	0.03—0.38
Testosterone	0.16	N.D.—2.47

*N.D. = not detectable due to insufficient assay sensitivity.

All subjects were on a random diet and had no signs of endocrine or systematic diseases. The median as well as the lower and upper limits have been calculated according to a logarithmic distribution of values. The serum concentrations of the steroids measured are comparable with the results of previous publications [6—8].

ACKNOWLEDGEMENTS

The skillful technical assistance of Mrs. A. Fenner and B. Weber is gratefully acknowledged.

REFERENCES

1 M. Finkelstein and J.M. Schäfer, Physiol. Rev., 59 (1979) 353.
2 M.I. New and L.S. Levine, Clin. Biochem., 14 (1981) 258.
3 W.G. Sippell, F. Bidlingmaier, H. Becker, T. Brunig, H. Dörr, H. Hahn, W. Golder, G. Hollmann and D. Knorr, J. Steroid Biochem., 9 (1981) 63.
4 M. Schöneshöfer, A. Fenner and H.J. Dulce, J. Steroid Biochem., 14 (1981) 377.
5 M. Schöneshöfer and A. Fenner, J. Clin. Chem. Clin. Biochem., 19 (1981) 71.
6 P.A. Lee and C.J. Migeon, J. Clin. Endocrinol. Metab., 41 (1975) 556.
7 W.G. Sippell, H.G. Dörr, F. Bidlingmaier and D. Knorr, Pediatr. Res., 14 (1980) 39.
8 P.A. Lee, T. Xenakis, J. Winer and S. Matsenbaugh, J. Clin. Endocrinol. Metab., 43 (1976) 775.

Journal of Chromatography, 310 (1984) 390—395
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2213

Note

Histamine assay in tears by fluorescamine derivatization and high-performance liquid chromatography

A. BETTERO

Department of Pharmaceutical Sciences, University of Padova, Padova (Italy)

M.R. ANGI and F. MORO

Department of Ophthalmology, University of Padova, Padova (Italy)

and

C.A. BENASSI*

Department of Pharmaceutical Sciences, University of Padova, Padova (Italy)

(First received January 13th, 1984; revised manuscript received May 10th, 1984)

Histamine has been found in human tears at a level of about 10 ng/ml [1], an amount significantly increased in allergic conjunctivitis [2]. Histamine release is regarded as responsible for the characteristic clinical pattern of lachrymation, itching, oedema and vasodilation present in allergic conjunctival reactions.

The histamine content of tears has been determined by means of radioenzymatic assay [3—5], which requires extraction procedures and the use of a highly purified enzyme in order to enhance sensitivity [6, 7]. Several methods for histamine assay in biological fluids have been reported, involving gas chromatography [8], low-pressure liquid chromatography [9], mass spectrometry [10, 11] and high-performance liquid chromatography (HPLC) after derivatization of histamine with various reagents [12—16].

The aim of this investigation was to make available a fast and reliable method for histamine determination (1) in a small tear volume (5—10 μl), (2) at picomole levels, (3) without sample pretreatment [17], (4) by fluorescamine derivatization [18], (5) by HPLC technique and fluorimetric measurement of the characteristic fluorophore easily separable from analogous imidazole,

0378-4347/84/$03.00

amino acid and amine derivatives, (6) with a procedure requiring only a few minutes.

The method appears particularly useful since the histamine content of tears can be considered — as an alternative to the currently available irritation tests — as a possible index of conjunctival irritation, and it allows the evaluation of the effects of chemicals, drugs and cosmetics to which subjects have been topically exposed [19].

EXPERIMENTAL

Apparatus

We used a Perkin-Elmer S-3b liquid chromatograph equipped with a Rheodyne 7125 injector valve, an ASVI automatic switching valve and LS-4 fluorimetric detector set at excitation and emission wavelengths of 390 nm and 480 nm, respectively.

A Sigma 15 data station was employed. Separation was achieved with a C_8 (10 μm) Nucleosil or RP-8 (10 μm) Merck column and a pre-column fitting with LiChroprep RP-8 (20—40 μm) using the mobile phase acetonitrile—phosphate buffer (potassium dihydrogen phosphate) 0.004 *M* at pH 3.5 (65:35) in isocratic elution at a flow-rate of 0.5 ml/min.

Reagents and materials

Fluorescamine and histamine were obtained from Sigma (St. Louis, MO, U.S.A.). The HPLC-grade solvents were filtered (0.45 μm) and degassed by sonication before use. Fluorescamine stock solution was 20 mg in 100 ml of acetonitrile; histamine stock solution was 1 mg/ml in water. Sodium borate solution was 0.2 *M* at pH 9.1. All reagents were stored at 4°C.

Sample preparation

Standards. The stock solution of histamine was diluted in water to concentrations ranging from 0.05 to 20.0 ng/ml.

Samples. Tears (20-μl samples) were collected from healthy volunteers and allergic conjunctivitis patients by placing a polystyrene capillary tube in the lower fornix of the conjunctiva.

All samples were kept in polystyrene tubes at −20° C prior to assay.

Sample derivatization

Sodium borate buffer (40 μl) and 10 μl of tears were mixed under vigorous agitation with 50 μl of fluorescamine solution. Aliquots of 20 μl of the resulting solution were injected into the HPLC system.

RESULTS AND DISCUSSION

After fluorescamine derivatization, stopped-flow analysis was performed to obtain accurate excitation and emission wavelength values for the histamine fluorophore, which depend on the mobile phase used (Fig. 1).

Fig. 2 shows representative chromatograms of histamine detection in standard solution (20 ng/ml) and in normal (5.2 ng/ml) and allergic conjunctivitis (50.6 ng/ml) human tear samples. Peaks were characterized by the method of

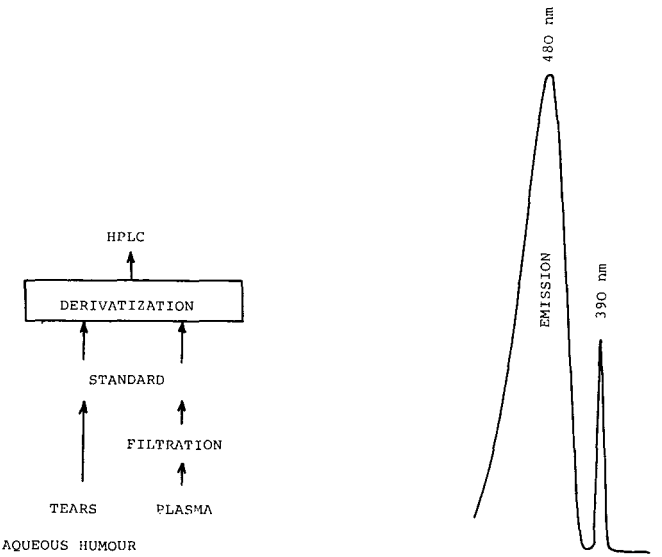


Fig. 1. Sample preparation steps and excitation—emission spectrum of histamine obtained by stopped-flow after fluorescamine derivatization.

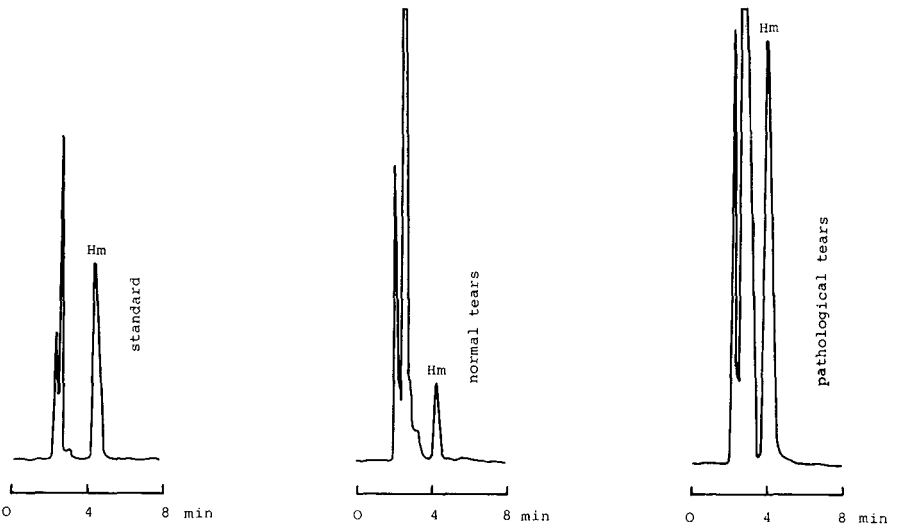


Fig. 2. Chromatograms of histamine (Hm) from standard solution (20 ng/ml) and from normal (5.2 ng/ml) and pathological (50.6 ng/ml) human tear samples. Injection volume 20 μl. Mobile phase: 0.004 *M* phosphate buffer adjusted to pH 3.5 and acetonitrile (35:65) at a flow-rate of 0.5 ml/min.

standard addition and by scanning the excitation and emission spectra of the peak that eluted at the same retention time as the standard using the stopped-flow technique ($k' = 0.91$). Since a reduced injection volume is preferable in routine analysis, where large numbers of samples are handled, the effect of injection volume on resolution was investigated for peaks eluting close to the solvent front. Consequently all detections were carried out by injecting 20 μl of each derivatized sample.

The stability of the histamine fluorophore was tested for at least 3 h by repeated analyses of standard and single tear samples at intervals of 10 min. The reproducibility was 1.5% (relative standard deviation, n = 20).

Fig. 3 shows the calibration curves of histamine in standard solution and added to a human tear sample. The correlation and the similar slopes of the curves confirm the usefulness of the method, which can be applied directly to other biological fluids as reported in Fig. 4.

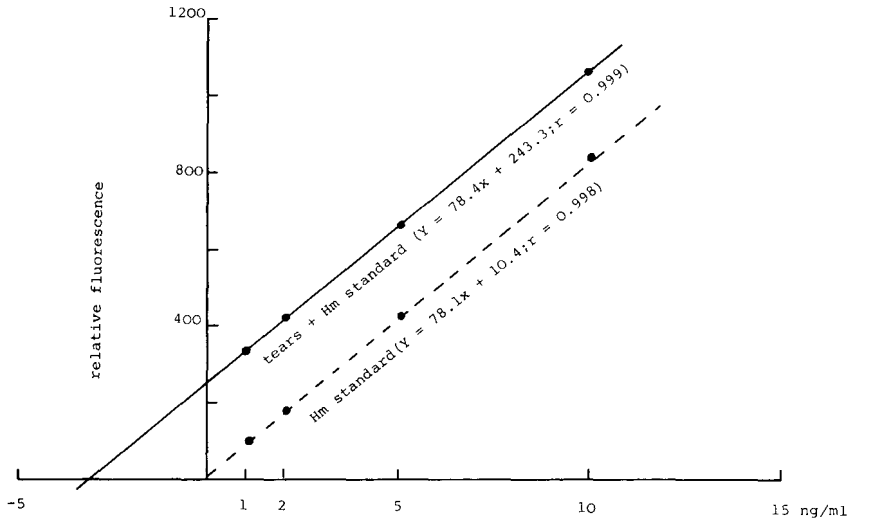


Fig. 3. Calibration curves of histamine (Hm) in standard solution (- - -) and in a human tear sample by standard addition method (——).

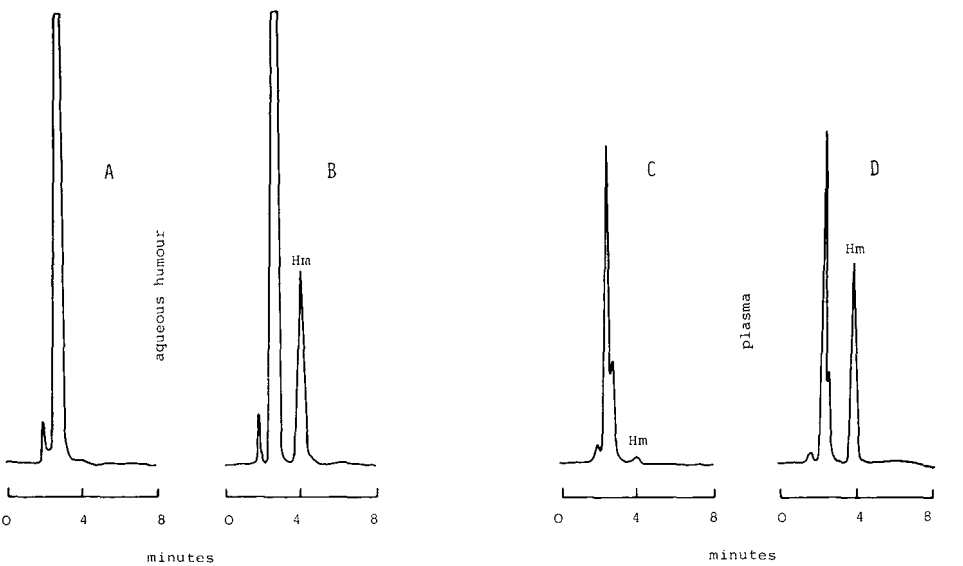


Fig. 4. Chromatograms of 10 μl of human aqueous humour and plasma samples obtained directly (A, C) and by standard addition (B, D) as described in the text. Injection volume 20 μl. Histamine (Hm) detection: not detected in aqueous humour (A), 0.61 ng/ml in plasma (C).

The results show, moreover, that the histamine content of tears can be determined without using deproteinizing agents. The combined action of acetonitrile and the pH of the mobile phase allows retention of the protein fraction in the pre-column system without memory effects [17]. The influence of the pre-column on the capacity factor is negligible; the packing material has to be replaced when the resolution decreases in relation to the number of injected samples. Even though the post-column derivatization reaction has several advantages, the pre-column technique is preferred as it is a simple system suitable for rapid analytical purposes.

The fluorescamine derivatization of histamine occurs rapidly at pH 8.5—9.0 giving a highly fluorescent pyrrolidone derivative. All amino acids and amines also react with fluorescamine; however, in our experimental conditions they were not retained by the column and in particular imidazole derivatives did not interfere with the histamine fluorophore.

The investigation was completed by direct electron-impact mass spectrometry of histamine and its methyl derivatives normally present in biological fluids, before and after fluorescamine derivatization. Preliminary results show (1) the absence of N-methylhistamine in normal human tears, and (2) increased stability of histamine after its fluorescamine derivatization (Bettero et al.) [20].

The present method, as well as other reported HPLC procedures [15, 16], allows selective and sensitive histamine measurements after pre-column derivatization and fluorimetric detection.

The use of fluorescamine—acetonitrile as derivatizing reagent presents several advantages for a rapid and accurate histamine evaluation in biological fluids and particularly in tears where small sample volumes are available: (1) acetonitrile allows sample homogeneity, (2) derivatization occurs instantaneously, (3) fluorophore behaviour can be easily studied by mass spectrometry, (4) only a few microlitres of sample are required, and (5) the total analysis time is less than 5 min.

In conclusion, the method appears particularly suitable for the rapid evaluation of histamine in tears at picomole levels in both allergic or chemically induced conjunctivitis.

ACKNOWLEDGEMENT

We wish to thank Dr. Franca Ceccarini, I.S.F., for her kind collaboration.

REFERENCES

1 M.B. Abelson, N.A. Soter, M.A. Simon, J. Dohlman and M.R. Allansmith, Amer. J. Ophthalmol., 83 (1977) 417.
2 M.B. Abelson, R.S. Baird and M.R. Allansmith, Ophthalmology, 87 (1980) 812.
3 M.A. Beaven, S. Jakobsen and Z. Horakova, Clin. Chim. Acta, 37 (1972) 91.
4 S.H. Snyder, R.J. Baldessarini and J. Axelrod, J. Pharmacol. Exp. Ther., 153 (1966) 544.
5 M.J. Brown, P.W. Ind, P.J. Barnes, D.A. Jenner and C.T. Dollery, Anal. Biochem., 109 (1980) 142.
6 K.M. Werburg, R.R. Bowsher and D.P. Henry, Life Sci., 32 (1983) 2855.

7 M.R. Allansmith, R.S. Baird, E.J. Higgenbotham and M.B. Abelson, Amer. J. Ophthalmol., 190 (1980) 719.
8 H. Navert, J. Chromatogr., 106 (1975) 218.
9 A. Yamatodani, T. Seki, M. Taneda and H. Wada, J. Chromatogr., 144 (1977) 141.
10 H. Mita, H. Yasueda and T. Shida, J. Chromatogr., 181 (1980) 153.
11 J.J. Keyzer, H. Breukelman, H. Elzinga, B.J. Koopman, B.G. Wolthers and A.P. Bruins, Biomed. Mass Spectrom., 10 (1983) 480.
12 M. Roth, Anal. Chem., 43 (1971) 880.
13 Y. Tsuruta, K. Kohashi and Y. Ohkura, J. Chromatogr., 224 (1981) 105.
14 C. Buteau, C.L. Duitschaever and G.C. Ashton, J. Chromatogr., 212 (1981) 23.
15 G. Skofitsch, A. Saria, P. Holzer and F. Lembeck, J. Chromatogr., 226 (1981) 53.
16 J.C. Robert, J. Vatier, B.K. Nguyen Phuoc and S. Bonfils, J. Chromatogr., 273 (1983) 275.
17 A. Bettero, C. Costa and G. Allegri, Progress in Tryptophan and Serotonine Research, Walter de Gruyter, Berlin, New York, 1984, pp. 469–472.
18 S. Udenfriend, S. Stein, P. Böhlen, W. Dairman, W. Leingruber and M. Weigele, Science, 178 (1972) 871.
19 A. Bettero, M.R. Angi, F. Galiano and C.A. Benassi, Food Chem. Toxicol., (1984) in press.
20 M.R. Angi, A. Bettero and C.A. Benassi, Agents Actions, (1984) in press.

Journal of Chromatography, 310 (1984) 396—400
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2219

Note

High-performance liquid chromatographic separation of serum erythrotropin and erythropoietin

L.F. CONGOTE

Endocrine Laboratory, Royal Victoria Hospital and Department of Medicine, McGill University, Montreal H3A 1A1 (Canada)

(Received April 6th, 1984)

The development of bioassays of erythropoietic activity using the incorporation of [^{3}H] thymidine into erythroid cells has been praised as one of the most sensitive and simple methods for the bioassay of erythropoietin [1—3]. Unfortunately, this method may detect the erythrotropins, a new family of erythroid cell-stimulating factors which were originally isolated from fetal calf intestine [4]. The erythrotropins stimulate globin chain synthesis and thymidine incorporation into acid-insoluble materials in fetal bovine liver cells and act synergistically with erythropoietin in cultures of rat liver cells [4, 5]. Fetal bovine serum contains large amounts of an erythrotropin which is similar to the intestinal erythrotropin I [6]. It is then essential to develop a method for the separation of serum erythrotropin and erythropoietin in order to quantitate both peptides using the thymidine incorporation assays. In this paper we describe the reversed-phase high-performance liquid chromatographic (HPLC) separation of serum erythrotropin and sheep plasma erythropoietin and show how the fetal bovine assay is particularly sensitive for the detection of serum erythrotropin in samples of human blood.

MATERIALS AND METHODS

Sheep plasma erythropoietin was obtained from Connaught Labs. (Toronto, Canada). Serum erythrotropin was isolated from fetal bovine serum (Flow Labs., Rockville, MD, U.S.A.) using a method to be described in detail elsewhere [6] but which consists essentially of the reversed-phase extraction of serum and the purification of erythrotropin by reversed-phase and gel permeation HPLC as described for the isolation of the intestinal peptides [4]. In some

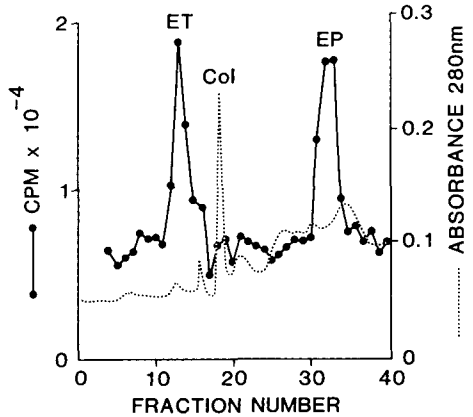
0378-4347/84/$03.00

experiments samples of human cord blood or human fetal plasma were analyzed directly by HPLC without reversed-phase extraction. A small volume of serum or plasma was acidified with 8 vols. of ice-cold 0.1% (v/v) trifluoroacetic acid (TFA), centrifuged at 10,000 *g* for 10 min and the supernatant was applied to the columns. Usually 80—100 μl of serum were used but in some instances larger amounts of serum, up to a total protein content of 10 mg as measured by the method of Bradford [7] were applied onto the columns.

The separation of erythropoietin and erythrotropin was carried out with two μBondapak C_{18} columns (Waters) and a C_{18} silica precolumn (Whatman No. 6561-403) connected in series as described in detail for globin chain separation [8]. The column was washed at room temperature with a mixture of acetonitrile—water—TFA (280:720:1) for 15 min at a flow-rate of 1.5 ml/min. The sample to be analyzed was pumped onto the columns. In some cases 10 μg coproporphyrin I (Sigma) was added as a marker to better localize the fractions containing erythrotropin. The fractions were eluted from the column using a linear gradient for 40 min starting with a mixture of acetonitrile—water—TFA (280:720:1) and finishing with 600:400:1. Fractions of 1.5 ml were collected. Each fraction was mixed with 0.5 ml acetonitrile containing 2-mercaptoethanol and TFA in order to obtain a final concentration of 3 m*M* mercaptoethanol and 0.1% TFA. Aliquots (or the complete fractions) were evaporated using a Speedvac evaporator (Savant) and suspended in 0.75 ml of F-12 medium [4]. Aliquots of 0.25 ml were used for the thymidine incorporation bioassay as described previously [4].

RESULTS AND DISCUSSION

Although the erythrotropins have been isolated using extraction and separation procedures in the presence of strong acids, it is known that erythropoietin has sialic acid residues which may be destroyed under acidic conditions [9]. However, the in vitro activity of asialoerythropoietin should remain unchanged. We found that sheep plasma erythropoietin fractionated on reversed-phase HPLC columns was able to stimulate globin chain synthesis and uridine uptake in cell cultures of fetal bovine liver [10]. We also found that thymidine incorporation into acid-insoluble materials in cell cultures of fetal bovine liver is a very sensitive bioassay for both erythropoietin and erythrotropins [4, 5]. Furthermore, these factors have different retention times on reversed-phase HPLC and therefore it should be possible to separate them using the appropriate gradients of acetonitrile in the presence of TFA. Fig. 1 shows the chromatogram of a mixture of bovine serum erythrotropin and sheep plasma erythropoietin separated with two C_{18} μBondapak columns as indicated in Materials and methods. A 7.5-mg amount of the reversed-phase extract from fetal bovine serum was purified by reversed-phase and gel permeation HPLC (steps I and II of ref. 4). The purified erythrotropin was mixed with 10 μg coproporphyrin I and 5 mg step III sheep plasma erythropoietin (3 U/mg) in 0.3 ml of 0.2% TFA and pumped directly onto the columns. Aliquots of 30 μl (1/67 of each fraction) were evaporated and used for the thymidine bioassay as previously described [4, 5]. Fig. 1 shows that there is an excellent separation of serum erythrotropin (ET) and erythropoietin (EP) in less than 40 min.



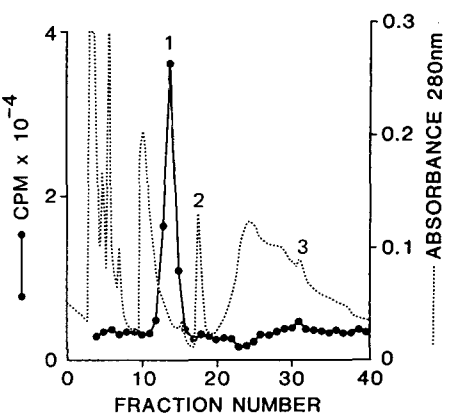


Fig. 1. Separation of erythrotropin (ET) prepared from fetal bovine serum and step III sheep plasma erythropoietin (EP) using two reversed-phase columns as indicated in the text. Coproporphyrin I (CoI) was added as an internal marker for the localization of ET and can be used to separate the fractions containing ET from those containing EP. The bioactivity is expressed as cpm of [^{3}H]thymidine incorporated into acid-insoluble materials in cell cultures of fetal bovine liver.

Fig. 2. Human cord blood serum was acidified and pumped directly onto two reversed-phase columns as indicated in the text. The bioactivity was measured as indicated in the legend of Fig. 1. 1 = Bioactivity corresponding to ET; 2 = coproporphyrin I; 3 = erythropoietin.

Furthermore, the addition of coproporphyrin I (CoI) can be very useful because its pink color can be used as a marker to localize erythrotropin and erythropoietin and as an internal standard to test the correctness of the chromatographic conditions.

The very large amounts of erythrotropin present in fetal bovine serum and human cord blood [6, 11] suggest that it should be possible to carry out a direct HPLC analysis of very small serum samples without a previous reversed-phase extraction. Fig. 2 shows the separation of 10 mg human cord blood serum which was acidified with 0.1% TFA as indicated in Materials and methods and applied to the column. Aliquots of 30 μl were used for the assay. The absorbance peak eluting after the fraction with thymidine incorporation activity corresponds to coproporphyrin I. The fraction which stimulated thymidine incorporation had the same retention time as bovine erythrotropin. Note that there is practically no bioactivity eluting in the positon of sheep erythropoietin. This has been also observed in other samples of cord blood. It is unlikely that human plasma erythropoietin (which is not commercially available) would elute at a complete different position from that of sheep plasma erythropoietin. The apparent absence of detectable amounts of erythropoietin could be explained by high lability of the human hormone but is most likely due to the very low amounts of erythropoietin in newborns [12], which is usually 30—40 mU/ml. Samples of 100 μl serum or less should be at the limits of the sensitivity of the bioassay. The amounts of erythropoietin indicated above were measured using cell culture assays with mice liver erythroid cells [12]. These determinations probably reflect the amounts of erythropoietin rather than erythrotropin for two reasons. First, the assays are carried out

in media supplemented with serum, this means in the presence of erythrotropin. Secondly, it is possible that erythroid cells from mice and probably other rodents are less sensitive to erythrotropin than fetal bovine cells, as has been shown in the case of rat liver cells [5]. Direct analysis of blood samples by HPLC will be particularly valuable in those cases where the amounts of blood available are very small. This is the case of a fetal blood plasma sample obtained from a fetus at the time of prenatal diagnosis of thalassemia, as illutrated in Fig. 3. A 100-μl aliquot of plasma was acidified with TFA and pumped directly onto the columns. Aliquots of 1 ml (one half of the fractions) were used for the bioassay. The thymidine incorporation stimulating activity is again associated with the elution position of erythrotropin and practically no activity is associated with the elution position of erythropoietin. It is interesting to see the presence of an inhibitor of thymidine incorporation eluting after erythrotropin. It remains to be seen if this inhibitor is typical of blood samples at midterm or a single case observed in this plasma sample.

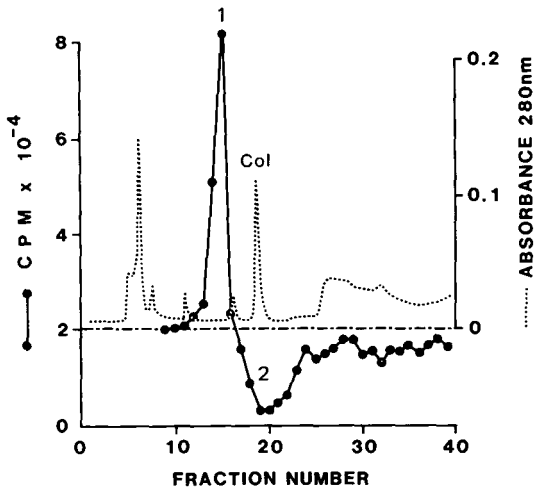


Fig. 3. Human fetal plasma taken from a fetus at midterm for prenatal diagnosis of thalassemia was acidified and pumped directly onto two reversed-phase columns as indicated in the legend of Fig. 2. The dotted line parallel to the abscissa indicate the control value which was observed in cell cultures without serum. CoI = coproporphyrin I; 1 = bioactivity corresponding to ET; 2 = inhibitor.

The chromatographic separation of serum erythrotropin and erythropoietin described above will be useful for the simultaneous determination of both erythroid cell stimulating factors with the very sensitive in vitro bioassays of thymidine incorporation. Furthermore, direct HPLC analysis of serum or plasma will be useful for the detection of erythrotropin. Further experiments indicate that this method may be applied for samples of adult patients. However, it is very important to eliminate proteins precipitated with TFA by centrifugation and to use a precolumn as indicated for globin chains [8] in order to prevent damage of the expensive reversed-phase columns.

NOTE ADDED IN PROOF

Human urinary erythropoietin has a similar retention time to sheep plasma erythropoietin in the HPLC system described above.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. E. Hamilton and T. Perry for supplying the fetoscopy sample and Ms. Susan James for reading the manuscript. The excellent technical assistance of Johanne Theberge is gratefully acknowledged. This work was supported by the Medical Research Council of Canada (MT-6072). The author is a Chercheur Boursier of the Fonds de la recherche en sante du Quebec.

REFERENCES

1 N.C. Brandan, P.M. Cotes and J. Espada, Brit. J. Haematol., 47 (1981) 461.
2 G. Krystall, Exp. Hematol., 11 (1983) 649.
3 T.R.J. Lappin, I. Rich and E. Goldwasser, Exp. Hematol., 11 (1983) 661.
4 L.F. Congote, Biochem. Biophys. Res. Commun., 115 (1983) 477.
5 L.F. Gongote, Prog. Clin. Biol. Res., in press.
6 L.F. Congote, Anal. Biochem., in press.
7 M. Bradford, Anal. Biochem., 72 (1976) 248.
8 L.F. Congote, Blood, 57 (1981) 75.
9 T. Kikaya, C.K.H. Kung and E.J. Goldwasser, J. Biol. Chem., 252 (1977) 5558.
10 L.F. Congote, Can. J. Biochem. Cell Biol., 62 (1984) 143.
11 L.F. Congote, Excerpta Med. Int. Congr. Ser., 652 (1984) 442.
12 T. Saito, A. Urabe, K. Uramoto, Y. Hashimoto and F. Takaku, Brit. J. Haematol., 54 (1983) 53.

Journal of Chromatography, 310 (1984) 401—406
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2197

Note

Measurement of prolidase activity in erythrocytes using isotachophoresis

HIROAKI MIKASA

Department of Chemistry, Kochi Medical School, Oko-cho, Nankoku-shi, Kochi 781-51 (Japan)

JIRO ARATA

Department of Dermatology, Kochi Medical School, Oko-cho, Nankoku-shi, Kochi 781-51 (Japan)

and

HIROYUKI KODAMA*

Department of Chemistry, Kochi Medical School, Oko-cho, Nankoku-shi, Kochi 781-51 (Japan)

(First received December 28th, 1983; revised manuscript received May 10th, 1984)

Prolidase deficiency with iminopeptiduria is a relatively rare inherited disorder characterized by clinical features such as chronic recurrent infections, mental retardation, splenomegaly, and skin lesion. Since 1968, several cases of prolidase deficiency have been described [1—12].

We also reported in previous papers [7—9] that a patient with mental retardation and chronic recurrent ulcers on the legs and soles of the feet excreted massive amounts of iminopeptides in her urine due to hereditary prolidase deficiency.

The measurement of prolidase (EC 3.4.13.9) activity, which hydrolyses dipeptides containing proline or hydroxyproline as the C-terminal amino acid, has been carried out by several methods [8, 11, 13—15].

A new method for the measurement of prolidase activity has been developed using an isotachophoretic analyser [16—19], and carried out by measuring simultaneously the substrate, glycine-proline (Gly-Pro), and product, glycine

0378-4347/84/$03.00

(Gly), in the reaction mixture. This method was also compared with Chinard's method [15] in which he measured the enzyme activity by determining proline in the reaction mixture. The results obtained with isotachophoresis and Chinard's method agree well.

The isotachophoretic method presented here has several advantages over previously described techniques, and has been applied to the measurement of prolidase activity in erythrocytes from a patient with iminopeptiduria and her mother.

MATERIALS AND METHODS

Materials

Gly-Pro, Gly-Hyp, Val-Pro and Leu-Pro (Gly = glycine, Pro = proline, Hyp = hydroxyproline, Val = valine, Leu = leucine) were obtained from Sigma. All other chemicals used were of analytical grade. Venous blood was taken into heparinized tubes from a patient with iminopeptiduria, her mother, and rat. The heparinized blood was mixed with an equal volume of 6% dextran (molecular weight 200,000—300,000; Wako, Osaka, Japan) in saline. After standing for 60 min at room temperature, the upper suspension of leucocytes was separated carefully from the underlayer of erythrocytes. Erythrocytes were washed three times with 2 vols. of physiological saline. Washed erythrocytes were haemolysed by repeated freezing and thawing. The lysate was dialyzed overnight at 4° C against 0.05 *M* Tris—HCl buffer, pH 7.4.

Preincubation

Erythrocyte lysate was diluted 1:10 (human lysate) and 1:2 (rat lysate) with 0.05 *M* Tris—HCl buffer (pH 7.4) containing 1 m*M* manganese chloride and preincubated for 1 h at 37° C

Enzymatic reaction

A 0.5-ml volume of substrate solution containing 1 m*M* manganese chloride and 10 m*M* iminopeptide in 0.05 *M* Tris—HCl (pH 7.4) was added to an aliquot of 0.5 ml of preincubated lysate and incubated for 30 min at 37° C. The reaction was stopped by heating for 5 min in boiling water. After centrifugation the supernatant was used for estimation of Gly-Pro, glycine and proline. A blank was run under the same conditions. Proline was determined by Chinard's method. Gly-Pro and glycine were simultaneously determined using an isotachophoretic analyser.

Instrumentation

The capillary apparatus was a Shimadzu IP-1B isotachophoretic analyser (Shimadzu, Kyoto, Japan). The separations were carried out in a capillary tube, 20 × 0.5 cm I.D., which was maintained at a constant temperature of 20° C. The detector cell had an I.D. of 0.5 mm and length 0.05 mm. The leading electrolyte consisted of 10 m*M* hydrochloric acid and 2-amino-2-methyl-1-propranol (pH 7.5). The terminal electrolyte was 10 m*M* γ-aminobutyric acid and barium hydroxide (pH 10.90). The chart speed was 10 mm/min; migration current was 75 μA.

RESULTS AND DISCUSSION

Isotachophoretic analyses of authentic Gly-Pro, Gly-Hyp, glycine and proline were carried out under the conditions described in Materials and methods. The two zones of Gly-Pro and Gly-Hyp overlapped under the analytical conditions and were detected as the same zone; proline could not be detected. However it has been ascertained that Gly-Pro, glycine and hydroxyproline are easily

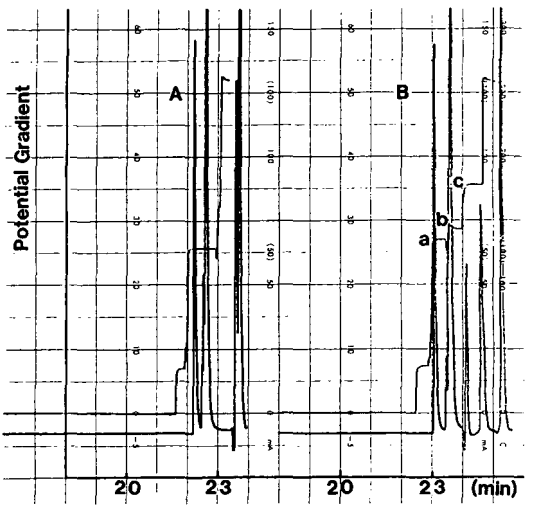


Fig. 1. Isotachophoretic runs of authentic Gly-Pro (A), Gly-Hyp (B, a), glycine (B, b) and Hyp (B, c). Analytical conditions were as follows. The leading electrolyte was 0.01 *M* hydrochloric acid and 2-amino-2-methyl-1-propanol (pH 7.5). The terminal electrolyte was 0.01 *M* γ-aminobutyric acid and barium hydroxide (pH 10.9). The chart speed was 10 mm/min. The migration current was 75 μA.

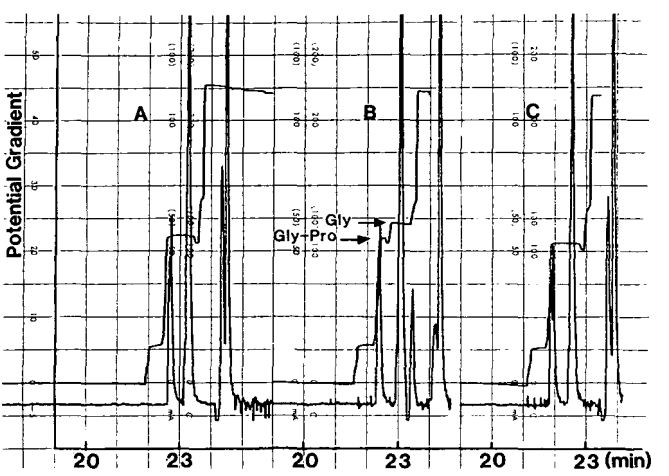


Fig. 2. Isotachophoretic runs of reaction mixture of the mother's erythrocyte lysate at 0 min (A) and 60 min (B) incubation time and of the erythrocyte lysate of a patient with imino peptiduria at 60 min incubation time (C). A 4-μl volume of each reaction mixture was analysed using an isotachophoretic analyser under the same conditions as in Fig. 1.

separated as shown in Fig. 1. Therefore, prolidase activity in erythrocytes using isotachophoresis was measured by determining Gly-Pro as the substrate and glycine as the product in the reaction mixture.

Isotachophoretic runs of prolidase activity in erythrocytes of a patient with iminopeptiduria and her mother are shown in Fig. 2.

The rate of hydrolysis of Gly-Pro in erythrocyte lysate from the mother was about 70% with an incubation time of 60 min under our experimental conditions (Fig. 2B). On the other hand, no prolidase activity in the erythrocyte

TABLE I

COMPARISON OF PROLIDASE ACTIVITY IN RED CELLS OF A PATIENT WITH IMINOPEPTIDURIA AND OF HER MOTHER, DETERMINED BY CHINARD'S METHOD AND AN ISOTACHOPHORETIC ANALYSER

Values of proline, glycine and Gly-Pro represent mean ± S.D. (μmol/ml) obtained in each experiment (n = 3).

Incubation time (min)	Chinard's method		Isotachophoresis			
	Proline		Glycine		Gly-Pro	
	Patient	Mother	Patient	Mother	Patient	Mother
0	0	0	0	0	5.0	5.0
15	0	1.31 ± 0.14	0	1.43 ± 0.14	4.98 ± 0.01	3.31 ± 0.11
30	0	2.06 ± 0.12	0	2.05 ± 0.09	4.97 ± 0.01	2.73 ± 0.08
60	0	3.08 ± 0.08	0	3.31 ± 0.10	4.95 ± 0.01	1.56 ± 0.04

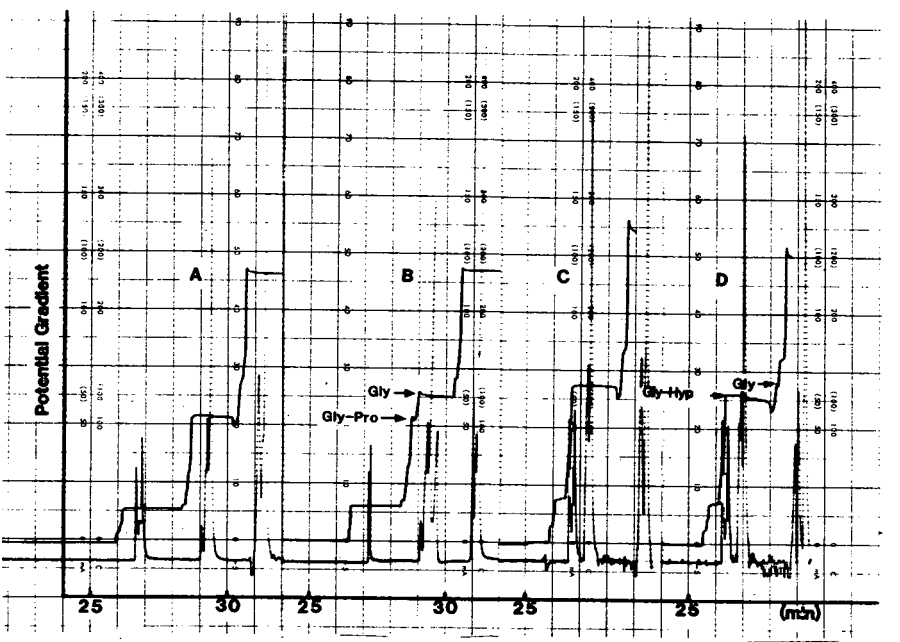


Fig. 3. Isotachophoretic runs of a reaction mixture of rat erythrocyte lysate. Gly-Pro (5 mM; A and B) and Gly-Hyp (5 mM; C and D) were used as substrates. Incubation times were 0 min (A and C) and 60 min (B and D).

lysate from the patient with iminopeptiduria was found (Fig. 2C). These results agree well with results reported previously by Umemura [8].

A comparison of the determination of prolidase activity in erythrocyte lysates from a patient with iminopeptiduria and from her mother using Chinard's method and the isotachophoretic analyser is shown in Table I. The two methods gave almost the same values, and the increase in glycine and the decrease in Gly-Pro determined by the isotachophoretic analyser also gave a good agreement. These results indicate that this method can be suitably utilized for the measurement of enzyme activity such as prolidase in erythrocytes.

In addition to Gly-Pro, several iminopeptides (Gly-Hyp, Leu-Pro, Val-Pro) were tested as substrate for prolidase in rat erythrocytes (Figs. 3 and 4).

Isotachophoretic runs of Gly-Pro and Gly-Hyp to determine prolidase activity in rat erythrocytes are shown in Fig. 3. When Gly-Pro was used as substrate, it was almost completely hydrolysed during the incubation time of 60 min, but hydrolysis of Gly-Hyp was about 6% that of Gly-Pro, as reported previously by Myara et al. [20].

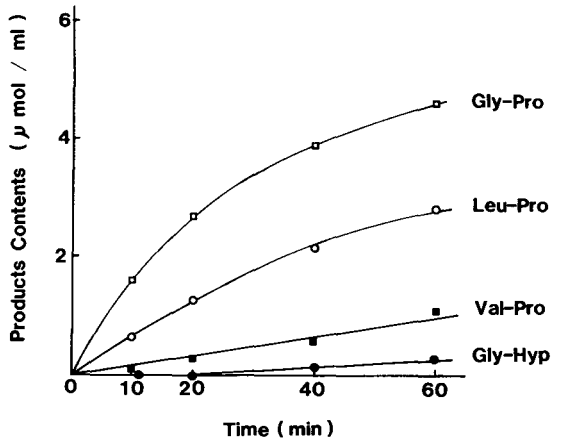


Fig. 4. The relationship between incubation time and several iminopeptides on prolidase of rat erythrocyte lysate. The reaction conditions are described in Materials and methods.

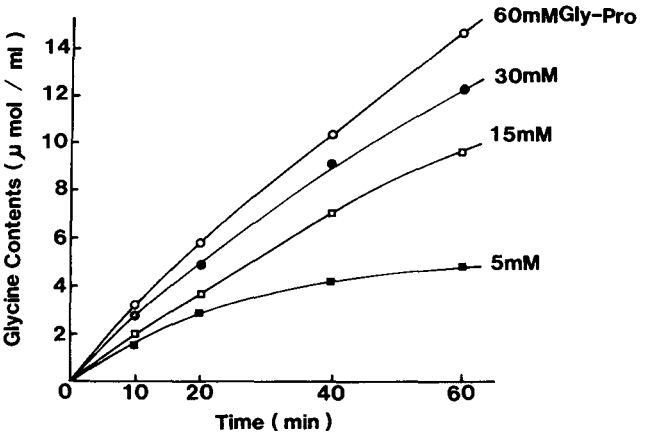


Fig. 5. Studies of incubation time at several Gly-Pro concentrations. The reaction conditions are described under Materials and methods.

The results of studies of incubation time at several concentrations of Gly-Pro are shown in Fig. 5. Under the conditions used, the release of glycine and proline by prolidase of rat erythrocytes, to a final Gly-Pro concentration of 60 m*M*, was linear up to 60 min incubation time.

The isotachophoresis presented here can simultaneously estimate both substrate and product, and was very useful in measuring prolidase activity in erythrocytes of a patient with iminopeptiduria and of a normal human.

REFERENCES

1 I.S. Goodman, C.C. Solomons, F. Muschenheim, A.C. McIntyre, B. Miles and D. O'Brien, Amer. J. Med., 45 (1979) 152—159.
2 M.R.N. Buist, J.J. Strandholm, F.J. Bellinger and G.N. Kennaway, Metabolism, 21 (1972) 1113—1123.
3 F.S. Jackson, W.A. Dennis and M. Greenberg, Can. Med. Assoc. J., 113 (1975) 759—763.
4 F.G. Powell, A.M. Rasco and M.R. Maniscalo, Metabolism, 23 (1974) 505—513.
5 F.K. Faull, M.G. Schier, P. Schlesinger and B. Halpern, Clin. Chim. Acta, 70 (1976) 313—321.
6 J.L. Scheffield, P. Schlesinger, F.K. Faull, J.B. Halpern, M.G. Schier, H.G.R. Cotton, J. Hammond and M.D. Danks, J. Pediatr., 91 (1977) 578—583.
7 H. Kodama, S. Umemura, M. Shimomura, S. Mizuhara, J. Arata, Y. Yamamoto, A. Yasutake and N. Izumiya, Physiol. Chem. Phys., 8 (1976) 463—474.
8 S. Umemura, Physiol. Chem. Phys., 10 (1978) 279—283.
9 J. Arata, S. Umemura, Y. Yamamoto, M. Hagiyama and N. Nohara, Arch. Dermatol., 115 (1979) 62—67.
10 M. Isemura, T. Hanyu, F. Gejyo, R. Nakazawa, R. Igarashi, S. Matsuo, K. Ikeda and Y. Sato, Clin. Chim. Acta, 93 (1979) 401—407.
11 C. Charpentier, K. Dagbovie, M. Larregue, W.A.R. Johnstone and A. Lemmonier, J. Intern. Metab. Dispos., 4 (1981) 77—78.
12 A. Ogata, S. Tanaka, T. Tomoda, E. Murayama, F. Endo and I. Kikuchi, Arch. Dermatol., 117 (1981) 689—694.
13 L. Josefsson and T. Lindberg, Biochim. Biophys. Acta, 105 (1965) 149—161.
14 F. Endo and I. Matsuda, Hum. Genet., 56 (1981) 351—359.
15 P.F. Chinard, J. Biol. Chem., 199 (1952) 91—95.
16 L. Arlinger, Biochim. Biophys. Acta, 393 (1975) 396—403.
17 H. Miyazaki and K. Katoh, J. Chromatogr., 119 (1976) 369—383.
18 H. Kodama and S. Uasa, J. Chromatogr., 163 (1979) 300—303.
19 H. Mikasa, K. Sasaki and H. Kodama, J. Chromatogr., 190 (1980) 501—503.
20 I. Myara, C. Charpentier and A. Lemmonier, Clin. Chim. Acta, 125 (1982) 193—205.

Journal of Chromatography, 310 (1984) 407—411
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2209

Note

Plasma cyclophosphamide assay by selective ion monitoring

C. LARTIGUE-MATTEI, J.L. CHABARD, C. TOUZET, H. BARGNOUX, J. PETIT and J.A. BERGER*

Laboratoire de Chimie Analytique de la Faculté de Pharmacie, Unité No. 71 de l'I.N.S.E.R.M. et Centre Régional de Lutte Contre le Cancer, Boite Postale No. 38, 63001 Clermont-Ferrand Cedex (France)

(First received February 29th, 1984; revised manuscript received May 22nd, 1984)

Cyclophosphamide (CPH) is a cytostatic drug widely used in oncology. Reported work on this agent deals mainly with its pharmacokinetic behaviour [1—3] and metabolism [4,5] when administered alone. Clinically, other drugs may be associated with CPH, particularly other antineoplastics, analgesics, hypnotics, corticoids and antiemetics, but the influence of these associations on the pharmacokinetics of CPH has not been extensively studied [6,7]. A highly specific analytical method for cyclophosphamide assay is required, as the associated drugs are liable to interfere. To date, assay methods have involved radiolabelling [1], which is clinically inconvenient, gas chromatography (GC), either simple [2,8] or coupled with mass spectrometry (MS) [4,9], or high-performance liquid chromatography [5]. For gas—liquid chromatographic analysis, samples must be derivatized to avoid decomposition of CPH. We describe here a method of assay of CPH in plasma by selected monitoring of two characteristic ions, $m/z = 307$ and $m/z = 309$. This method is highly specific and very sensitive.

EXPERIMENTAL

Assays were carried out using a Hewlett-Packard 5710 A gas chromatograph connected to a Hewlett-Packard 5970 A selective mass detector set up for selected ion monitoring. Separation was carried out on a fused-silica capillary column (Hewlett-Packard SP-2100, 25 m × 0.2 mm I.D., film thickness 0.2 μm) at 240°C. Helium was used as carrier gas. The injection port temperature was 250°C. Plasma samples (0.2 ml) were spiked with isophosphamide (IPH) (structure given in Fig. 1) as internal standard (20 μg/ml) and analysed by the

0378-4347/84/$03.00

procedure described by Van den Bosch and De Vos [8], leaving out the purification of the extracts with hexane which proved unnecessary given the high specificity of the selected ion monitoring method. The plasma aliquots were extracted three times with 1 ml of ethyl acetate after addition of 1 ml of 0.6 *M* sodium hydroxide, by shaking on a vortex shaker for 3 min and spinning at 1600 *g* for 5 min. The extracts were evaporated to dryness in a stream of nitrogen at ambient temperature. The residue was taken up in 100 μl of ethyl acetate and 50 μl of trifluoroacetic anhydride and left for 30 min at 60°C [8]. The solution was evaporated to dryness under nitrogen and the residue taken up in 200 μl of ethyl acetate; 1 μl of this solution was injected into the chromatograph using a Ros capillary injector.

Mass spectra

Mass spectra of the trifluoroacetyl derivatives of CPH and IPH were recorded using a Hewlett-Packard 5985 B GC—MS system fitted with an SE-30 fused-silica capillary column (25 m × 0.30 mm I.D.) and a Ros capillary injector. These spectra are shown in Fig. 1. The base peak at m/z = 307 and the ion at m/z = 309 of relative intensity 30% are common to both derivatives. These were chosen for quantitative analysis. Selected ion monitoring recordings of plasma extracts are given in Fig. 2.

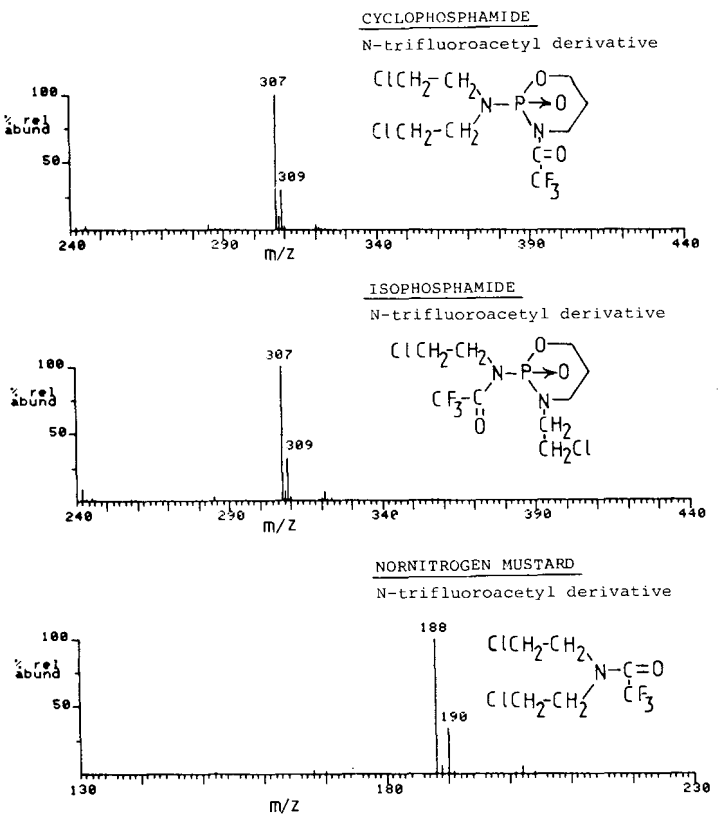


Fig. 1. Structures and electron-impact mass spectra of N-trifluoroacetyl derivatives of cyclophosphamide, isophosphamide and nornitrogen mustard.

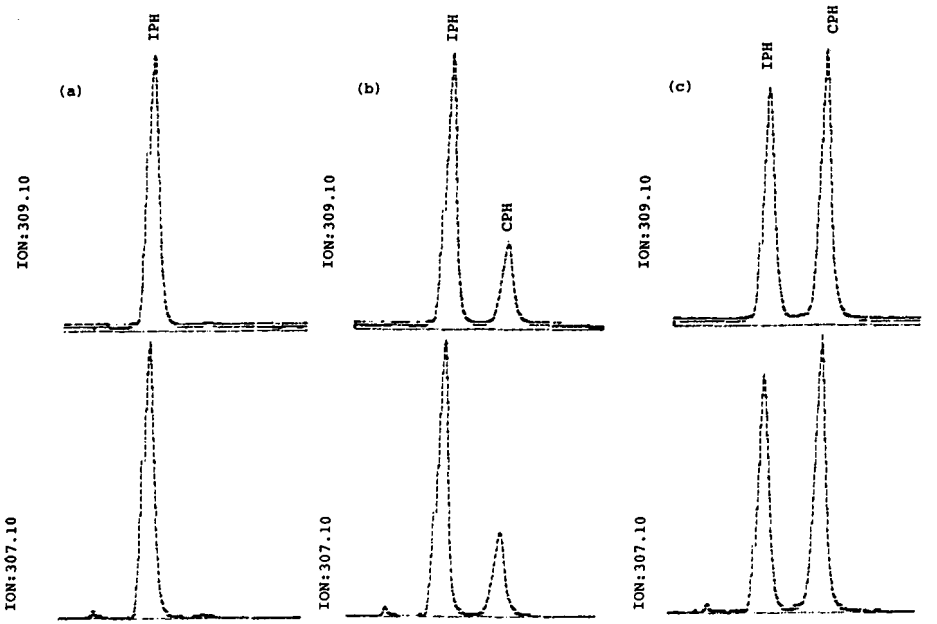


Fig. 2. Selected ion monitoring records for plasma extracts: (a) blank plasma, (b) 2.5 μg/ml cyclophosphamide, (c) 10.0 μg/ml cyclophosphamide.

Calibration

A calibration plot was obtained for each series of assays by adding varying amounts of CPH (0.2—50.0 μg/ml) and a constant amount of IPH (20.0 μg/ml), to control plasma aliquots, and analysing them as above.

RESULTS AND DISCUSSION

Assessment of the method

Extraction yield. CPH extraction efficiency was determined using control plasma samples spiked with IPH (20.0 μg/ml). CPH was added either before or after extraction with ethyl acetate. Comparison of the peak area ratios of unextracted CPH to extracted CPH gave CPH extraction yields of 89.7 ± 3.6% at 2.5 μg/ml ($n=5$), 96.3 ± 1.9% at 10.0 μg/ml ($n=5$) and 98.4 ± 6.0% at 25.0 μg/ml ($n=5$). The IPH extraction yield, similarly determined (unextracted IPH/extracted IPH), was 94.2 ± 5.7% at 20.0 μg/ml.

Sensitivity. Under the operating conditions described above, the sensitivity threshold was about 0.2 μg/ml. This threshold can, however, be lowered by calibrating over a lower concentration range, 0.05—1.00 μg/ml, and adding IPH as internal standard at 0.5 μg/ml.

Reproducibility. The intra-assay reproducibility was determined by extracting and analysing ten replicate human plasma samples spiked with CPH at 2.5, 15.0, 25.0 and 50.0 μg/ml. It is expressed by the coefficient of variation (%) in Table I. The inter-assay reproducibility was determined by analysing two series of ten replicates containing 2.5 and 25.0 μg/ml CPH. It is expressed by the coefficient of variation (%) between the two series, i.e. 7.5% at 2.5 μg/ml and 8.3% at 25.0 μg/ml.

TABLE I

REPRODUCIBILITY AND ACCURACY OF THE ASSAY

$n = 10$.

Theoretical (μg/ml)	Calculated (μg/ml ± S.D.)	Coefficient of variation (%)	Mean error (%)
2.5	2.56 ± 0.11	4.4	4.0
15.0	15.45 ± 0.50	3.2	3.7
25.0	24.80 ± 1.70	6.8	5.4
50.0	52.50 ± 2.70	5.1	6.4

Accuracy. Accuracy was assessed in terms of mean relative errors for the concentrations studied (Table I).

Linearity. Linearity is satisfactory over the range 0.2—50.0 μg/ml as shown by linear regression analysis of four calibration curves plotted on different days over a period of one month for both the ion at $m/z = 307$ ($Y = 0.110X + 0.041$, $r = 0.9993$) and that at $m/z = 309$ ($Y = 0.109X + 0.039$, $r = 0.9991$).

Specificity. Two ions characteristic of CPH were monitored, namely $m/z = 307$ and $m/z = 309$. Identity of the 307 CPH/307 IPH and 309 CPH/309 IPH ratios is consistent with absence of interference. In addition, we tested under the same analytical conditions various drugs likely to be administered along with CPH. No interference was observed in different control plasma samples spiked to maximum in vivo concentrations with adriblastin, teniposide, methotrexate, hydrocortisone, dexamethasone, ketoprofen, amphotericin B, allopurinol, metopimazine or clonidine, or after administration of 5-fluorouracil, adriamycin or vincristine to patients.

Sample stability

Jardine et al. [9] in a previous study of CPH and its metabolites reported the decomposition of CPH (11%), 4-keto-CPH (19%) and carboxyphosphamide (58%) into nornitrogen mustard in the course of work-up of biological samples. The extraction and chromatography procedures used by these authors were different from ours. We tested for the presence of this breakdown product in our plasma extracts and in CPH pure solutions (0.2, 1, 2, 5 and 10 μg) after trifluoroacetylation. Assay of nornitrogen mustard can be performed under the same conditions as that described for CPH if the chromatography is carried out at 130°C. The mass spectrum of the N-trifluoroacetyl derivative of nornitrogen mustard is given in Fig. 1. The ions monitored for quantitative analysis were those with $m/z = 188$ and $m/z = 190$. Under the conditions described, decomposition of CPH to nornitrogen mustard stayed below 0.1%.

Jardine et al. [9] report that, though breakdown of CPH and its metabolites occurs mainly during extraction, some decomposition also occurs during freezing. Analysis of our plasma samples after storage for one month at −20°C and comparison of results with those obtained from fresh plasma samples (Table II) failed to reveal any significant differences in assay values. Hence freezing apparently does not account to any great extent for the breakdown of CPH, though it may affect its metabolites, which were not assayed here.

TABLE II

STABILITY OF CYCLOPHOSPHAMIDE IN DEEP-FROZEN PLASMA SAMPLES AFTER STORAGE FOR ONE MONTH AT −20°C

Patient (dose injected)	Time of sampling after administration	Concentration of cyclophosphamide (μg/ml)		Coefficient of variation (%)
		Analysis after sampling	Analysis after one month at −20°C	
No. 1	5 min	6.1	5.8	4.9
(200 mg i.v.*)	1 h	5.0	4.5	10.0
	6 h	2.8	2.4	10.0
No. 2	5 min	6.7	7.1	6.0
(200 mg i.v.)	1 h	4.8	4.4	8.3
	6 h	2.4	2.6	8.3
No. 3	5 min	12.3	12.4	0.8
(300 mg i.v.)	1 h	4.4	4.6	4.6
	6 h	3.2	3.1	3.1
No. 4	5 min	38.9	37.0	4.9
(400 mg i.v.)	1 h	10.4	9.4	9.6
	6 h	5.5	5.4	1.8

*i.v. = intravenously.

To conclude, the method described here should provide a means of reliably monitoring plasma levels of CPH in patients, and thereby allow the influence of coadministered drugs on its pharmacokinetics to be accurately studied.

REFERENCES

1 J.L. Cohen, J.Y. Jao and W.J. Jusko, Brit. J. Pharmacol., 43 (1971) 677.
2 C. Pantarotto, A. Bossi, G. Belvedere, A. Martini, M.G. Donelli and A. Frigerio, J. Pharm. Sci., 63 (1974) 1554.
3 M. Jarman, E.D. Gilby, A.B. Foster and P.K. Bondy, Clin. Chim. Acta, 58 (1975) 61.
4 I. Jardine, R.B. Brundrett, M. Colvin and C. Fenselau, Cancer Treat. Rep., 60 (1976) 403.
5 U. Bahr and H.R. Schulten, Biomed. Mass Spectrom., 8 (1981) 553.
6 J.Y. Jao, W.J. Jusko and J.L. Cohen, Cancer Res., 32 (1972) 2761.
7 O.K. Faber, H.T. Mouridsen and L. Skovsted, Acta Pharmacol. Toxicol., 35 (1974) 195.
8 N. van den Bosch and D. de Vos, J. Chromatogr., 183 (1980) 49.
9 I. Jardine, C. Fenselau, M. Appler, M.N. Kan, R.B. Brundrett and M. Colvin, Cancer Res., 38 (1978) 408.

Journal of Chromatography, 310 (1984) 412—417
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2218

Note

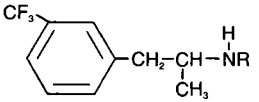
Determination of fenfluramine and norfenfluramine in plasma using a nitrogen-sensitive detector

HERMAN A. KREBS, LAWRENCE K. CHENG* and GEORGE J. WRIGHT

A.H. Robins Company, Drug Metabolism Department, 1211 Sherwood Avenue, Richmond, VA 23220 (U.S.A.)

(First received February 16th, 1984; revised manuscript received May 17th, 1984)

Fenfluramine (I, Fig. 1) is currently used as an appetite suppressant and is used experimentally to produce hyperthermia in laboratory animals [1]. A recent work [2] indicates the possible use of fenfluramine in the treatment of autism. Research is being carried out to determine the effects of fenfluramine on improvement of intelligence of autistic children [3]. It is thought that the action of fenfluramine is through reduction of brain serotonin levels [4]; and fenfluramine has been used to study the effects of serotonin depletion on release of prolactin and growth hormone [5] and on stimulation of adrenocorticotropin secretion in man [6].



I R– C_2H_5

II R– H

III R– C_3H_7

Fig. 1. Structures of compounds cited in the text. I, Fenfluramine; II, norfenfluramine; III, internal standard.

With increases in the clinical applications of fenfluramine, it is necessary to have a quick and reliable method to determine fenfluramine plasma levels. Previous gas—liquid chromatographic (GLC) methods have involved the use of an electron-capture detector and a time-consuming derivatization of the amino group to improve sensitivity and chromatography. Heptafluorobutyric

anhydride [7] and pentafluorobenzoyl chloride [8] derivatization processes require at least a 30-min reaction time and additional sample clean-up steps. Another gas chromatographic method reports a rapid extractive benzoylation of fenfluramine with electron-capture detection, but that method requires a larger sample volume (2 ml) and a higher limit of detection (20 ng/ml) than the method reported here [9]. A recent method assays fenfluramine and norfenfluramine by GLC without derivatization. This method also uses 2-ml sample volumes, requires time-consuming solvent clean-up procedures, and reports lower recoveries for both compounds than our method [10].

Our method uses ethyl chloroformate to derivatize the compounds during the extraction procedure. The elimination of extra derivatization and sample clean-up steps, the use of smaller plasma volumes and the use of the more stable but equally sensitive nitrogen—phosphorus detector are improvements over previous methods.

EXPERIMENTAL

Reagents and materials

Cyclohexane, dichloromethane and methanol were all nanograde solvents (Mallinckrodt, Paris, KY, U.S.A.); ethyl chloroformate (Pfaltz and Bauer, Stamford, CT, U.S.A.) was redistilled before use. Sodium hydroxide, sulfuric acid and ammonium hydroxide (Baker-analysed reagent; J.T. Baker, Phillipsburg, NJ, U.S.A.) solutions were prepared in deionized water.

The internal standard (III, Fig. 1) [N-propyl-α-methyl-3-(trifluoromethyl)-phenethylamine] used was synthesised in our laboratory (A.H. Robins, Richmond, VA, U.S.A.).

Aqueous solutions of fenfluramine and norfenfluramine were diluted in drug-free human plasma to concentrations of 2—100 ng/ml. Aliquots of these plasma standards and all samples were frozen at −20°C until needed for analysis.

Instrumentation

Analyses were performed on a Hewlett-Packard gas chromatograph, Model 5730, equipped with a nitrogen—phosphorus detector. Glass columns, 1.8 m × 4 mm I.D., packed with 3% OV-1 on GCQ, 100—120 mesh (Applied Science Labs., State College, PA, U.S.A.) were conditioned at 200°C overnight with a helium gas flow-rate of 20 ml/min. Chromatographic conditions for the analyses were: column oven temperature, 155°C; inlet temperature, 250°C; helium carrier gas flow-rate, 40 ml/min. Detector temperature was 250°C. Gas flow-rates to the detector were 3.0 ml/min for hydrogen and 50 ml/min for air. Voltage applied to the collector was approximately 19 V.

Assay procedure

Into a 15-ml centrifuge tube, with Teflon® stopper, 0.5 ml of aqueous internal standard solution (200 ng/ml) was placed. A 1-ml aliquot of plasma (standards or unknown samples), 0.5 ml absolute ethanol and 0.5 ml of 5% ammonium hydroxide were added to the tube, then 8 ml of cyclohexane—dichloromethane (3:2) were added and the tube was shaken vigorously for 15 min. The

tube was centrifuged for 10 min at 500 *g* and then the organic layer was transferred to a clean tube containing 1.0 ml of 0.05 *M* sulfuric acid. After the sample was shaken for 10 min, the tube was centrifuged. The organic layer was removed and discarded. An additional 5 ml of the extraction solvent was added and the tube vortexed for 10 sec. The organic layer was again removed and discarded. To the acidic layer, 0.5 ml of 2 *M* sodium hydroxide and 0.3 ml of 5% ethyl chloroformate in cyclohexane—dichloromethane (3:2) were added and the tube was vortexed for 15 sec. A 5-μl aliquot of the reaction mixture was injected into the gas chromatograph.

Chromatographic data were collected and processed electronically (Computer Inquiry Systems, Waldwick, NJ, U.S.A). Peak height ratios of standards were used to calculate unknown concentrations in the samples. There was a 3-min delay in the peak integrations of each chromatogram in order to minimize solvent front effects on data processing.

RESULTS AND DISCUSSION

The extraction efficiency of this method was determined by comparison of peak height ratios of extracted compound to peak height ratios of unextracted standards. It was found the extraction efficiency for fenfluramine (I) was 88% and for norfenfluramine (II) was 91%.

Typical chromatograms of plasma spiked with fenfluramine and norfenfluramine are shown in Fig. 2. There were no interfering chromatographic peaks at the retention time of norfenfluramine (4.1 min), fenfluramine (5.2 min), or the internal standard (6.7 min).

The method was validated to a lower quantitative limit of 2 ng/ml for fenfluramine and 5 ng/ml for norfenfluramine. This validation involved the assay of thirty samples spiked with fenfluramine and norfenfluramine within the concentration range of 0—100 ng/ml (Tables I and II). The concentration of the samples were unknown to the analyst at the time of the assay. All samples were assayed in duplicate.

Six calibration curves were assayed for each compound over a two-week period. These standard curves were linear over the concentration range of 2—100 ng for fenfluramine (r = 0.9997) and 5—100 ng for norfenfluramine (r = 0.9996). The coefficients of variation (C.V.) for points of the standard curve were 3.9% at 2 ng/ml and 1.9% at 100 ng/ml for fenfluramine; and 7.0% at 5 ng/ml and 4.3% at 100 ng/ml for norfenfluramine. The average C.V. for the five points of the standard curve was 3.4% for fenfluramine and 6.8% for norfenfluramine.

Plasma samples from patients dosed with fenfluramine (1.5 mg/kg, per os) were assayed. Fig. 3 shows typical chromatograms from the assay of plasma samples drawn (A) before dosing, (B) 90 min and (C) 180 min after dosing. The concentrations in these samples are 42.5 ng/ml fenfluramine and 6.7 ng/ml norfenfluramine after 90 min and 83.0 ng/ml fenfluramine and 19.2 ng/ml norfenfluramine after 180 min. The pre-treatment sample showed no interfering peaks.

Using chromatographic conditions listed, the structurally related compound *d*-amphetamine does not have baseline separation from norfenfluramine.

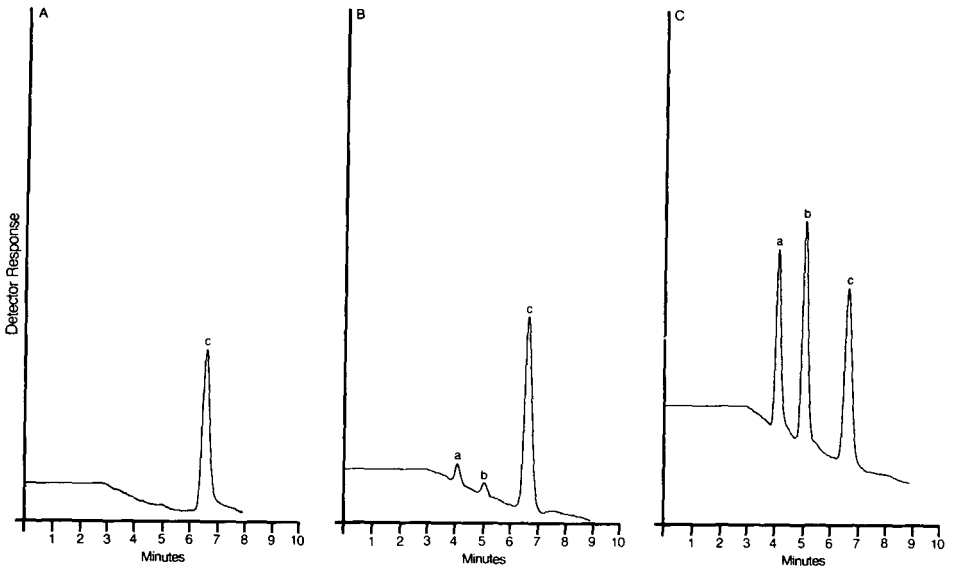


Fig. 2. Chromatograms of 1.0 ml of control plasma spiked with (A) internal stnadard (100 ng); (B) norfenfluramine (5 ng), fenfluramine (2 ng) and internal standard (100 ng); (C) norfenfluramine (50 ng), fenfluramine (50 ng) and internal standard (100 ng). Peaks: a = norfenfluramine; b = fenfluramine; c = internal standard.

TABLE I

DETERMINATION OF ADDED CONCENTRATION OF FENFLURAMINE TO PLASMA

Fenfluramine added as base (ng/ml)	*n*	Mean	S.D.	C.V. (%)	Percentage found
0	6	0.9	0.12	—	—
2	4	2.2	0.21	9.7	110
5	4	5.3	0.12	2.3	106
10	4	10.5	0.22	2.1	105
50	4	53.7	1.31	2.4	107
75	4	79.9	1.98	2.5	107
100	4	106	4.96	4.7	106

TABLE II

DETERMINATION OF ADDED CONCENTRATION OF NORFENFLURAMINE TO PLASMA

Norfenfluramine added as base (ng/ml)	*n*	Mean	S.D.	C.V. (%)	Percentage found
0	6	0.3	0.46	—	—
5	4	5.6	0.49	8.8	113
10	4	11.1	0.18	1.6	111
25	4	25.5	1.47	5.7	102
50	4	56.8	2.05	3.6	114
75	4	81.5	1.46	1.8	109
100	4	105	2.75	2.6	105

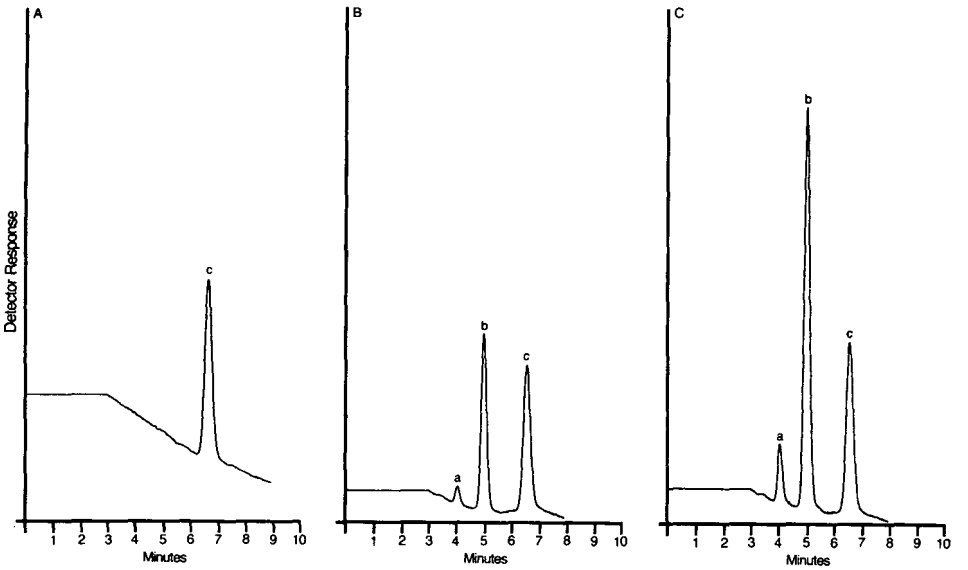


Fig. 3. Chromatograms of patient samples 0 (A), 90 (B) and 180 min (C) after administration of fenfluramine. Peaks: a = norfenfluramine; b = fenfluramine; c = internal standard. See text for explanation.

Relative retention times to fenfluramine are 0.79 for norfenfluramine and 0.84 for *d*-amphetamine. Although not tested, it is thought that methylamphetamine would separate from norfenfluramine and could also be used as the internal standard for this assay.

Ethyl chloroformate reacts with the amine group of the compound to form an amide derivative. This reaction occurs under alkaline conditions during the back-extraction step. The final product has been confirmed by mass spectrometry. This assay procedure and derivatization give chromatograms which are free of any interferences from endogenous plasma constituents.

The demonstrated reliability of this method, the rapid assay procedure and the use of a selective, sensitive detector, make this method applicable to the determination of fenfluramine and norfenfluramine in clinical studies.

ACKNOWLEDGEMENTS

The authors thank David Lewis, M.D., University of Iowa Hospital, Iowa City, IA, U.S.A. for patient plasma samples which were assayed, and Robert E. Lemieux, A.H. Robins Co., Richmond, VA, U.S.A. for synthesis of the internal standard.

REFERENCES

1 K. Shimonura, H. Satoh, O. Hirai, J. Mori, M. Tomoi, T. Terai, S. Katsuki, Y. Motoyama and T. Ono, Jap. J. Pharmacol., 32 (1982) 405.
2 E. Geller, E.R. Ritvo, B.J. Freeman and A.Y. Yuwiler, N. Engl. J. Med., 307 (1982) 165.
3 E. Geller, E.R. Ritvo, B.J. Freeman and A.Y. Yuwiler, J. Neurochem., 41 (1983) 557.

4 R.D. Ciaranello, N. Engl. J. Med., 307 (1982) 181.
5 J.O. Willoughby, M. Menadue and P. Jervois, Brain Res., 249 (1982) 291.
6 D.A. Lewis and B.M. Sherman, J. Clin. Endrocrinol. Metab., 58 (1984) 458.
7 R.B. Bruce and W.R. Maynard, Jr., Anal. Chem., 41 (1969) 977.
8 K.K. Midha, I.J. McGilveray and J.K. Cooper, Can. J. Pharm. Sci., 14 (1979) 18.
9 F.T. Delbeke, M. Debackere, J.A.A. Jonckheere and A.P. De Leenheer, J. Chromatogr., 273 (1983) 141.
10 R.G. Morris and P.A. Reece, J. Chromatogr., 278 (1983) 434.

Journal of Chromatography, 310 (1984) 418—423
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2196

Note

Analysis of *p*-hydroxyphenytoin in microsomal reactions by high-performance liquid chromatography with electrochemical detection

I.M. KAPETANOVIC* and H.J. KUPFERBERG

Preclinical Pharmacology Section, Epilepsy Branch, National Institute of Neurological and Communicative Disorders and Stroke, Bethesda, MD 20205 (U.S.A.)

(First received December 28th, 1983; revised manuscript received April 28th, 1984)

5,5-Diphenylhydantoin (5,5-diphenyl-2,4-imidazolidinedione; phenytoin; Dilantin®) is an important and widely used drug [1]. Phenytoin is almost entirely metabolized (> 95%), and *p*-hydroxylation to 5-(*p*-hydroxyphenyl)-5-phenylhydantoin [5-(4-hydroxyphenyl)-5-phenyl-2,4-imidazolidinedione; *p*-hydroxyphenytoin] is the major pathway for its biotransformation in humans [1, 2]. Under clinical conditions, phenytoin exhibits a saturation (Michaelis-Menten) kinetic profile, and the excretion of *p*-hydroxyphenytoin is dose-dependent [2]. Because phenytoin elimination is subject to inhibition by other drugs [3], plasma phenytoin levels may become elevated, leading to various side effects, including nystagmus, ataxia, and mental changes [4]. Therefore, the use of phenytoin may present a clinical management problem.

Various analytical methods have been reported for determination of *p*-hydroxyphenytoin, including gas chromatography [5, 6], high-performance liquid chromatography (HPLC) using ultraviolet absorbance detection [7, 8], and gas chromatography—mass spectrometry (GC—MS) [9—11]. All of these methods have some drawbacks, and all were designed primarily for analyses of concentrations considerably greater than those generated in microsomal reactions. These methods are relatively laborious, requiring derivatization, sophisticated instrumentation, or the use of stable isotopes. As an alternative to the previously used radiometric methods employing ^{14}C [12—15], we describe a simple, rapid, and sensitive method for the quantitation of *p*-hydroxyphenytoin from in vitro metabolic reactions.

0378-4347/84/$03.00

EXPERIMENTAL

Materials

The reference standards, 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (HPPH) and 5-(*m*-hydroxyphenyl)-5-phenylhydantoin, and the internal standard, 5-(*p*-hydroxyphenyl)-5-(*p*-tolyl)hydantoin (HPTH), were obtained from Aldrich (Milwaukee, WI, U.S.A.). Phenytoin was obtained from United States Pharmacopeial Convention (Rockville, MD, U.S.A.) and was used as a substrate. Sodium phenytoin, which was used for animal pretreatment, came from Parke-Davis (Ann Arbor, MI, U.S.A.). Water and diammonium hydrogen phosphate (both HPLC-grade) were obtained from J.T. Baker (Phillipsburgh, NJ, U.S.A.). Nicotinamide adenine dinucleotide phosphate, reduced form (NADPH, Type X), and polysorbate 20 were purchased from Sigma (St. Louis, MO, U.S.A.). Baker analyzed reagents were obtained from J.T. Baker and organic solvents came from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Microsomal isolation

Male Sprague—Dawley rats (200—225 g) were pretreated with an aqueous sodium phenytoin suspension [50 mg/kg/day intraperitoneally in 0.5% (v/v) polysorbate 20] for three days to approximate the situation in epileptic patients undergoing long-term phenytoin therapy. The rats were then decapitated and their livers were excised quickly and washed sequentially in 0.25 *M* sucrose, 0.05 *M* Tris buffer, pH 7.50, containing 1.15% potassium chloride, and finally 0.25 *M* sucrose. Washings were performed at 4° C by gentle stirring with a magnetic stirrer for 10 min. All subsequent procedures were also performed at 4° C. The livers were then homogenized in four volumes of 0.25 *M* sucrose for 1—2 min, using a Waring blender. The homogenate was centrifuged at 9000 *g* for 25 min. The supernatant was poured into clean centrifuge tubes through two layers of cheesecloth to filter out lipid and large debris and was centrifuged at 105,000 *g* for 90 min. The resultant microsomal pellet was resuspended in 0.05 *M* Tris buffer, pH 7.5, containing 1.15% potassium chloride, using a hand-driven Potter-Elvehjem homogenizer. The resuspended pellet was again centrifuged at 105,000 *g* for 90 min. The resultant washed microsomal pellet was resuspended in 0.25 *M* sucrose as before and divided into 0.5—1.0 ml aliquots. The aliquots were stored under nitrogen at about —65° C. Protein determinations were made by minor modifications of the colorimetric assay of Lowry et al. [16].

Microsomal reactions

The reaction mixture contained 0.78 mg microsomal protein, 0.40 m*M* EDTA, 0.40 m*M* magnesium chloride, 1.0 m*M* potassium chloride, 0.05 *M* potassium phosphate buffer pH 7.50, 1.0 m*M* NADPH, and varying amounts of phenytoin (0.79—79.4 μM), the substrate. Methanolic stock solution of phenytoin was used, and the solvent was evaporated before the other reaction mixture components were added. The total reaction volume was 2.5 ml. Reactions were carried out at 37° C in air for 10 min; they were initiated by adding NADPH after a 1-min equilibration period at 37° C and were terminated by adding 5 ml of methyl *tert.*-butyl ether and vortex mixing. All reactions were run in duplicate.

HPLC

After the reaction was terminated, the internal standard (HPTH, 250 ng) was added to the microsomal extracts. The samples were then mixed for about 15 sec and centrifuged for 10 min at about 2000 *g* at 4° C. The methyl *tert.*-butyl ether phase was transferred to clean tubes and evaporated to dryness under nitrogen at 30—40° C. The residue was redissolved in 200 μl of mobile phase, and 5—25 μl of this mixture were injected into the chromatograph.

The chromatographic system consisted of a pump (Model 110A, Beckman, Palo Alto, CA, U.S.A.), an injector with a 50-μl loop (Model 7125, Rheodyne, Cotati, CA, U.S.A.), an amperometric detector with a TL-5 glassy carbon electrode (Model LC-4A, Bioanalytical Systems, West Lafayette, IN, U.S.A.), a standard strip-chart recorder, and a 30 cm × 3.9 mm I.D., 10-μm particle size μBondapak C_{18} column (Waters Assoc., Milford, MA, U.S.A.). A guard column (MPLC 5-μm C_{18} guard cartridge OD-GU, Brownlee Labs., Santa Clara, CA, U.S.A.) was also employed. The column was kept at ambient temperature. The mobile phase was acetonitrile—0.05 *M* diammonium hydrogen phosphate (30:70, v/v) and the flow-rate was 2.0 ml/min. The electrochemical detector was set at a potential of 1.05 V and a range of 10 or 20 nA.

For each analysis, a standard curve was generated by adding known, varying amounts of HPPH and a constant amount of the internal standard and the substrate to non-incubated reaction mixture. These standards bracketed the range of experimental values. Quantitation was achieved by using peak height ratios of HPPH to HPTH. As an additional cross-verification of the HPLC method, samples remaining after HPLC analysis were analyzed by selected ion monitoring (SIM), using a GC—MS—computer (GC—MS—COMP) system.

RESULTS AND DISCUSSION

This relatively simple method for the quantitation of in vitro metabolically generated *p*-hydroxyphenytoin involves a single extraction step, isocratic reversed-phase HPLC separation, and electrochemical detection. The large excess of substrate (phenytoin) does not create a problem since the electrochemical detector does not respond to phenytoin. Under the HPLC conditions described, the relative retention time of phenytoin to metabolite (HPPH) is about 2.10, as determined by interposing an ultraviolet detector between the column and the electrochemical detector. Since the electrochemical detector is transparent to phenytoin, it is not necessary to wait for phenytoin elution, and the analysis time is shortened considerably.

Fig. 1 shows representative chromatograms of extracts of a microsomal blank, a microsomal reaction, and two standards. The blank was run in the same manner as the actual reaction, but NADPH was added only after the reaction was terminated, and internal standard was omitted. An additional peak (I), which eluted in front of the internal standard, was present in the extract of the microsomal reaction but not in those of the standards or the blank. This peak was suggestive of a metabolite, especially since its peak height relative to that of HPTH increased with increased substrate concentration. The identity of this peak is unclear, but it is not the *m*-hydroxy isomer; *m*-hydroxyphenytoin was not generated under these conditions since the peak

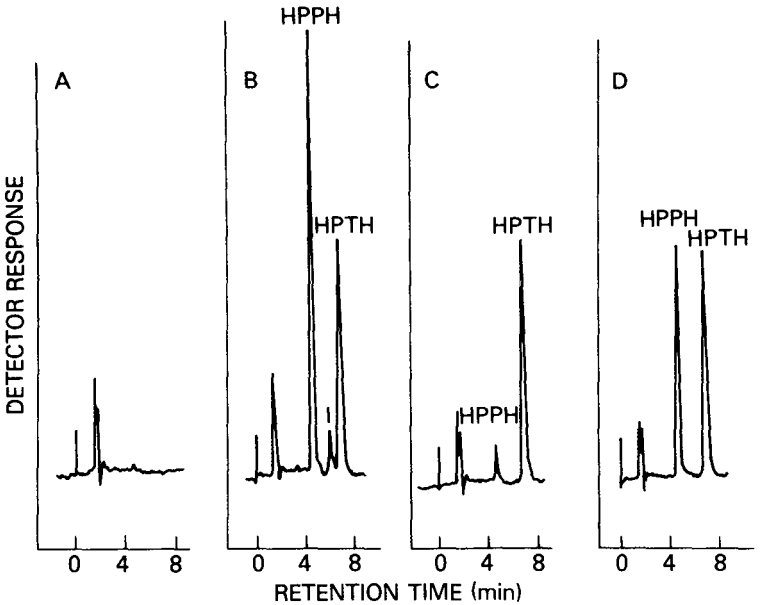


Fig. 1. High-performance liquid chromatogram of extracts from microsomal blank (A), microsomal reaction (B), standard containing 25 ng of HPPH (C), and standard containing 200 ng of HPPH (D). All samples initially contained 23.8 μM phenytoin. In all instances, 25 μl of each extract was injected into the chromatograph and the electrochemical detector range was set at 20 nA. See text for details.

corresponding to it (using a reference compound) was not detected by HPLC or SIM analysis. Further analysis of samples by GC—MS—COMP confirmed that phenytoin was not present in microsomes and that HPPH was not present and was not generated unless phenytoin was added to the reaction mixture.

The extraction efficiency was determined by comparing peak height ratios of HPPH to HPTH for samples spiked with HPPH and extracted relative to non-extracted samples. The recovery of HPPH was about 98%.

Standard curves were generated routinely by using eight standards covering a range of 25—1000 ng HPPH. Good linearity and acceptable Y-intercept were found routinely. By least-squares linear regression analysis, representative equation of the line and the regression coefficient (r^2) were $Y = 0.00537X - 0.00008$, $r^2 = 0.998$. The r^2 value was always at least 0.998.

The HPLC method was independently validated by SIM analysis of the same microsomal reaction extracts. The comparison of findings from 47 samples tested by both methods gave a correlation coefficient (r) of 0.998 and the equation of the least-squares linear regression was $Y = 0.994X - 0.79$, showing a good correspondence between the methods.

In the microsomal reactions, the substrate (phenytoin) concentration ranged from 0.79 to 79.4 μM. Fig. 2 shows a representative plot of the reaction velocity (v) versus substrate concentration (s). The data were replotted as the standard double reciprocal (Lineweaver-Burk) plot ($1/v$ versus $1/s$) (Fig. 3). A biphasic kinetic profile was observed, suggesting high- and low-affinity metabolic sites. This finding is consistent with recent findings of Tsuru et al. [15].

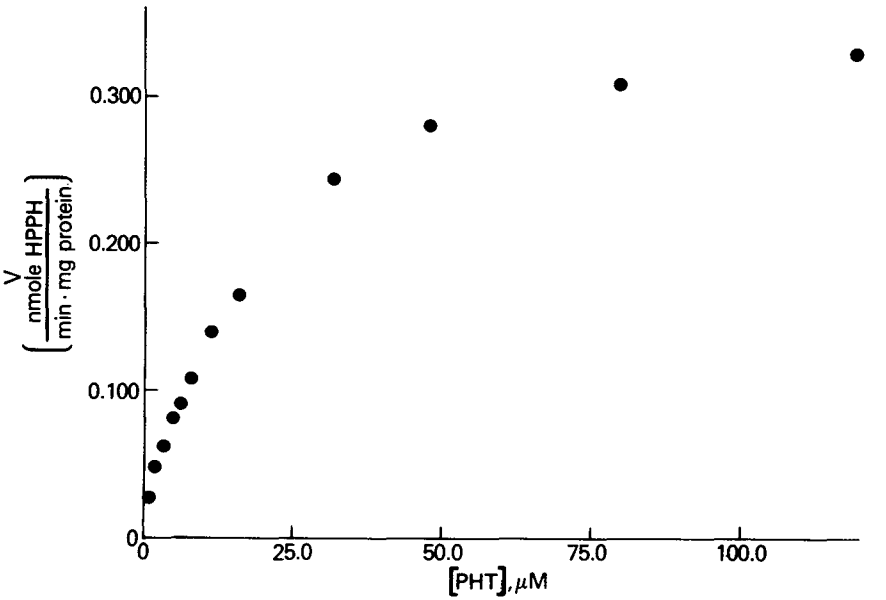


Fig. 2. Reaction velocity—substrate concentration profile for microsomal *p*-hydroxylation of phenytoin. See text for details.

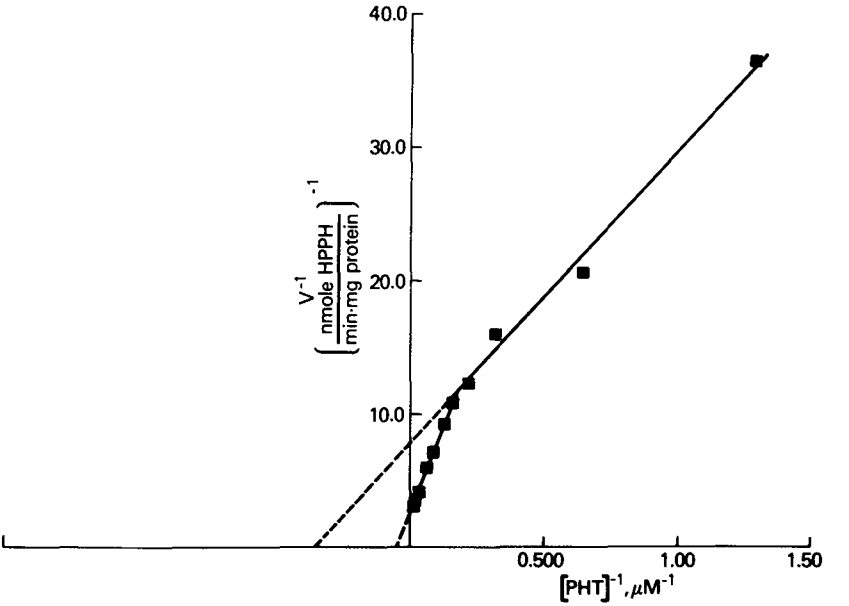


Fig. 3. A double reciprocal plot for microsomal *p*-hydroxylation of phenytoin. The lines were drawn based on least-squares linear regression. See text for details.

Therefore, the method is well suited for kinetic studies of in vitro *p*-hydroxylation of phenytoin and is currently being employed to study drug—drug interactions. Microsomes from untreated rats were used in subsequent studies, and the results were comparable to those described here for phenytoin-treated rats.

The method provides good sensitivity. The smallest amount of HPPH injected was about 3 ng. A further increase in sensitivity can be achieved by injecting a large fraction of the sample and/or increasing the sensitivity setting on the detector. Specificity is offered by the electrochemical detector, which preferentially detects the compound of interest, in this case the metabolite and not the substrate. The method should be suitable for in vitro studies of other *p*-hydroxylation reactions since the detector is selective for and relatively sensitive to phenolic structures.

ACKNOWLEDGEMENT

We are grateful to B.J. Hessie for editorial assistance.

REFERENCES

1 T. Chang and A.J. Glazko, in D.M. Woodbury, J.K. Penry and C.E. Pippenger (Editors), Antiepileptic Drugs, Raven Press, New York, 2nd ed., 1982, p. 209.
2 D.M. Woodbury, in D.M. Woodbury, J.K. Penry and C.E. Pippenger (Editors), Antiepileptic Drugs, Raven Press, New York, 2nd ed., 1982, p. 191.
3 H. Kutt, in D.M. Woodbury, J.K. Penry and C.E. Pippenger (Editors), Antiepileptic Drugs, Raven Press, New York, 2nd ed., 1982, p. 227.
4 M. Dam, in D.M. Woodbury, J.K. Penry and C.E. Pippenger (Editors), Antiepileptic Drugs, Raven Press, New York, 2nd ed., 1982, p. 247.
5 K.K. Midha, C. Charette, H.S. Buttar and I. Dupuis, J. Chromatogr., 157 (1978) 416.
6 F. Sarhan, J.M. Ziegler, A. Nicolas and G. Siest, J. Chromatogr., 183 (1980) 505.
7 J. Hermansson and B. Karlén, J. Chromatogr., 130 (1977) 422.
8 R.W. Dykeman and D.J. Ecobichon, J. Chromatogr., 162 (1979) 104.
9 C. Hoppel, M. Garle and M. Elander, J. Chromatogr., 116 (1976) 53.
10 J.D. Baty and P.R. Robinson, Biomed. Mass Spectrom., 4 (1977) 36.
11 A. van Langenhove, C.E. Costello, J.E. Biller, K. Biemann and T.R. Browne, Clin. Chim. Acta, 115 (1981) 263.
12 H. Kutt and J.R. Fouts, J. Pharmacol. Exp. Ther., 176 (1971) 11.
13 D.A. Blake, F. Cohen and B.C. Miyasaki, Drug Metab. Dispos., 6 (1978) 240.
14 S.A. Atlas, J.L. Zweier and D.W. Nebert, Dev. Pharmacol. Ther., 1 (1980) 281.
15 M. Tsuru, R.R. Erickson and J.L. Holtzman, J. Pharmacol. Exp. Ther., 222 (1982) 658.
16 O.H. Lowry, N.J. Rosenbrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.

Journal of Chromatography, 310 (1984) 424—430
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2199

Note

Silica gel high-performance liquid chromatography for the simultaneous determination of propranolol and 4-hydroxypropranolol enantiomers after chiral derivatization

MICHAEL J. WILSON and THOMAS WALLE*

Department of Pharmacology, Medical University of South Carolina, Charleston, SC 29425 (U.S.A.)

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

Propranolol (Inderal®) is used clinically as a racemate of (+)- and (—)-propranolol with the (—)-enantiomer being responsible for most of the drug's antihypertensive and other cardiovascular actions [1, 2]. The kinetics and metabolism of the enantiomers differ considerably in both animals [3—6] and man [7—12]. It is therefore essential to be able to analyze individually the enantiomers of both parent drug and its main metabolites.

Practically useful approaches for separation of the enantiomers of propranolol include stable isotope-labeled pseudoracemates with gas chromatographic—mass spectrometric (GC—MS) analysis [3—6, 10—12] and high-performance liquid chromatography (HPLC) after chiral derivatization [7—9, 13—16]. The chiral reagent first used in the HPLC approach was N-trifluoroacetyl-(—)-prolyl chloride [13, 14]. The prolyl reagent can, however, racemize during storage [14, 17, 18]. An alternative, more stable, reagent appears to be (+)-1-phenylethyl isocyanate [(+)-PEI] [15]. Although all of the HPLC methods give good separation of the enantiomers of propranolol and appear to be useful for analysis of biological samples, these methods have not been used for simultaneous separation of the enantiomers of propranolol metabolites. This would be of clinical importance in particular for 4-hydroxypropranolol (4-HOP). This metabolite has biological actions similar to those of propranolol [19, 20], actions that should be dependent on the stereochemical composition of this metabolite.

In the present communication we describe the simultaneous determination of the enantiomers of both propranolol and 4-HOP by HPLC after derivatization with (+)-PEI. The method was applied to determinations of the stereochemical composition of conjugates of propranolol and 4-HOP in human urine.

0378-4347/84/$03.00

MATERIALS AND METHODS

Reagents

R-(+)-1-Phenylethyl isocyanate [(+)-PEI] was purchased from Aldrich (Milwaukee, WI, U.S.A.) and propranolol hydrochloride from Sigma (St. Louis, MO, U.S.A.). 4-Hydroxypropranolol (4-HOP) hydrochloride was prepared as previously described [20] as were the pure enantiomers of propranolol [6, 21] and 1-(α-naphthoxy)-2,3-propylene glycol [22]. Glusulase was obtained from Endo Labs. (Garden City, NY, U.S.A.). All solvents were glass-distilled from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Glass-distilled water was used for all aqueous reagents.

Chiral derivatization

The free bases of 4-HOP and propranolol (10 to 60 μg of each) were isolated by extraction with 10 ml ethyl acetate from 1 ml of a pH 9.6 carbonate buffer. After transfer to a 15-ml silanized conical centrifuge tube the ethyl acetate was evaporated to dryness and the residue dissolved in 0.5 ml of chloroform. (+)-PEI (5 μl) was added and the tube left for 15 min at room temperature tightly capped. 0.1 *M* Hydrochloric acid (10 ml) was then added and the tube was shaken on a reciprocating shaker for 10 min. After centrifugation the aqueous layer was aspirated off. The chloroform was evaporated to dryness by a stream of nitrogen. The residue was redissolved in 50 μl of mobile phase. A 15-μl sample was injected into the HPLC instrument.

Human urinary metabolites

24-h Urine collections were made from normal volunteers, each receiving a single oral 80-mg dose of propranolol (Inderal).

Glucuronic acid conjugates of propranolol and 4-HOP. Samples (2 ml) were treated with 100 μl of glusulase at pH 4.7 (90 min at 50°C) [23, 24]. After extraction at pH 9.6 with ethyl acetate [25], the extract was derivatized with (+)-PEI as above.

Sulfate conjugate of 4-HOP. Samples (2 ml) were extracted by an ion-pair procedure (chloroform and tetrabutylammonium at pH 12) [26]. The extracted sulfate conjugate was purified by reversed-phase HPLC. Following treatment with glusulase and extraction at pH 9.6 with ethyl acetate, 4-HOP was derivatized as above.

Instrumentation

HPLC. The HPLC system consisted of a Model 6000 high-pressure pump, a Model U6K injector, and a Model 440 UV detector with a 313-nm filter from Waters Assoc. (Milford, MA, U.S.A.). HPLC columns (25 cm × 4.6 mm) were either a 5-μm C_{18} or a 10-μm silica from Alltech Assoc. (Deerfield, IL, U.S.A.). The mobile phases were methanol—water (70:30) and methanol—chloroform (1.2:100), respectively. All solvents were filtered and degassed. The flow-rate was 1 ml/min.

Fast atom bombardment mass spectrometry (FAB-MS). A Finnigan/MAT 212 mass spectrometer with an SS-200 data system was modified for FAB-MS utilizing an Ion-Tek fast atom gun [27]. Samples were analyzed in a glycerol

matrix on a 316 stainless-steel probe tip at ambient temperature using argon bombardment at approximately 8 keV.

RESULTS AND DISCUSSION

The chiral derivatization of 4-HOP with (+)-PEI was performed in chloroform at room temperature as previously described for propranolol [15]. Analysis of the reaction mixture by reversed-phase HPLC after evaporation of the solvent revealed two sets of equal size peaks (Fig. 1A). The first set of peaks, at 13—14 min, was the diastereomer derivatives of 4-HOP after reaction at the secondary amino nitrogen only (mono-PEI derivative, see below), whereas the second set of peaks, at 45—52 min, was assumed to be the di-derivatives after reaction at both the amino and phenolic groups (di-PEI derivative). Very short reaction times produced mainly the mono-PEI derivative, whereas longer reaction times at elevated temperature mainly gave rise to the di-PEI derivative. Under no conditions was it possible to produce a single set of peaks. A complicating factor was the observation that the diastereomers resulting from the PEI derivative of propranolol appeared at 41—46 min, thus overlapping with the di-PEI derivative of 4-HOP. However, when the reaction mixture was shaken with 0.1 *M* hydrochloric acid, the derivative at the phenolic group (carbamate derivative) was hydrolyzed, producing the mono-PEI derivative only (Fig. 1B).

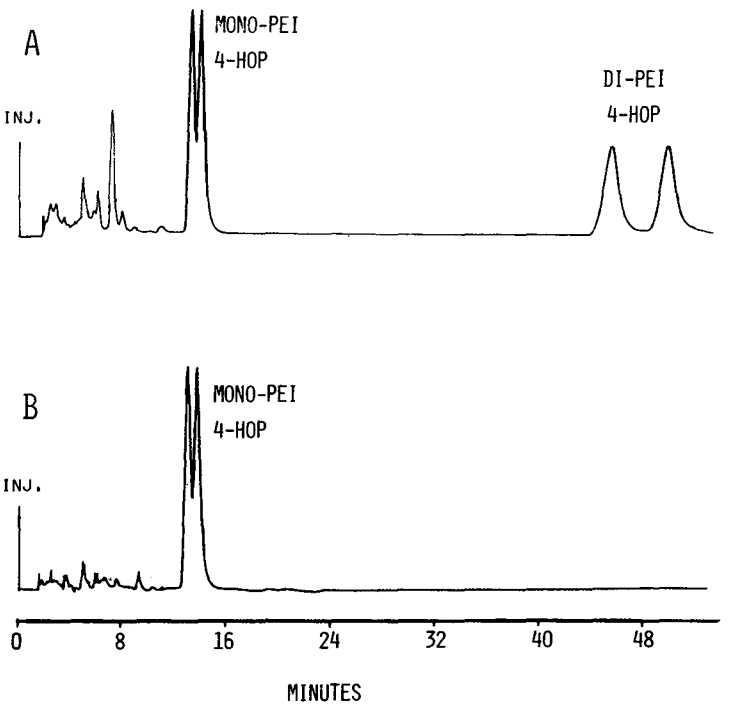


Fig. 1. Reversed-phase HPLC of (+)-PEI diastereomer derivatives of 4-HOP before (A) and after (B) acid-wash. About 5 μg injected at 0.1 a.u.f.s.

The structure of the mono-PEI derivative was confirmed by subjecting the peaks in Fig. 1B to positive-ion FAB-MS. The spectrum in Fig. 2 is consistent with the assigned structure, i.e. a urea-type of derivative. Characteristic ions are the quasimolecular ion at *m/z* 423 $(M+H)^+$ and the fragment ions at *m/z* 276 (loss of the derivatizing group; fragmentation I in Fig. 2) and at *m/z* 263 and 159 (fragmentations II and III). Characteristic of the derivatizing group is the base peak at *m/z* 105 ($[C_6H_5CHCH_3]^+$; fragmentation IV). The derivative is thus analogous to that previously established for propranolol [15].

The identification of the PEI derivatives of the β-receptor blocking drugs by electron-impact MS, rather than by FAB-MS, may be complex. For example, the propranolol derivative gave no molecular ion by this mode of ionization, even after trimethylsilylation [15]. Similarly, the mono-derivative of 4-HOP gave no interpretable spectrum either by direct probe MS or GC—MS after various derivatization techniques and electron-impact conditions.

Although the peak symmetry of the mono-PEI derivative of 4-HOP in Fig. 1 was quite good, the resolution of the diastereomers (R = 0.62) was inadequate, in particular when one enantiomer was present in considerably lower concentrations than the other. The resolution of the corresponding diastereomers of propranolol at a similar retention time was found to be considerably better (R = 1.5), which is similar to previous observations [15].

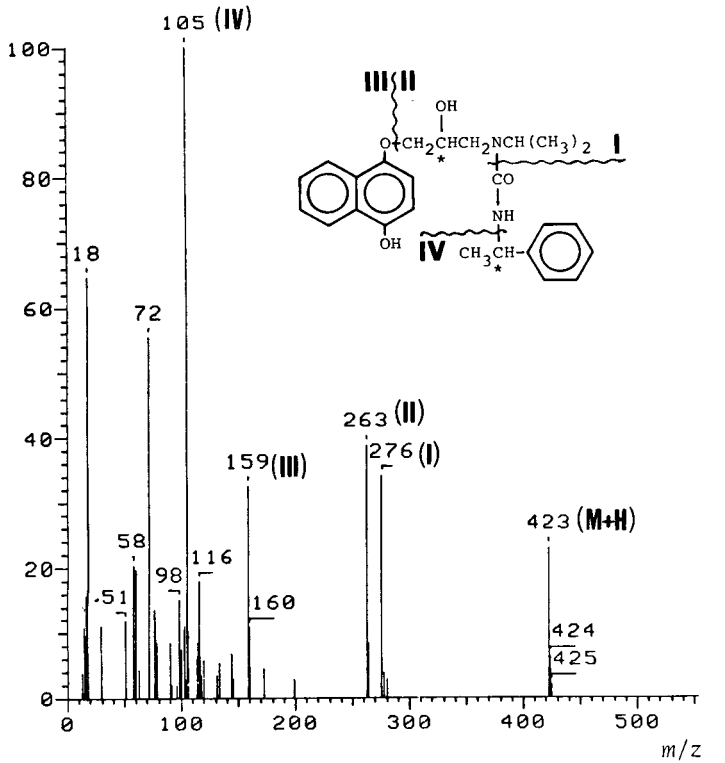
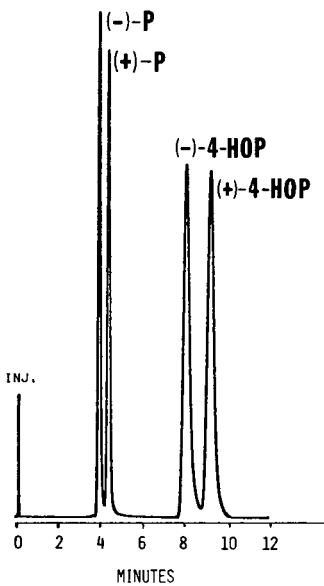


Fig. 2. Positive-ion FAB-MS of the mono-(+)-PEI derivative of 4-HOP. The derivative was collected from the HPLC effluent in Fig. 1, retention time 13—14 min. Roman numerals I to IV indicate sites of fragmentation for ions at *m/z* 276, 263, 159 and 105, respectively.

Manipulation of the mobile phase gave some improvement in the resolution of the 4-HOP diastereomers but only at the expense of a marked prolongation of the retention time, which at 13—14 min already was rather long.

In attempts to improve the resolution normal-phase silica column HPLC was examined. Using this mode of separation the diastereomers of both propranolol and 4-HOP appeared well resolved (see Fig. 3). The resolution of the 4-HOP diastereomers showed a dramatic improvement (R = 1.9) compared to reversed-phase chromatography. The resolution of the propranolol diastereomers (R = 1.5) remained the same. This high degree of resolution was seen in spite of considerably shorter retention times on the silica as compared to the reversed-phase column. The peak symmetry was excellent.



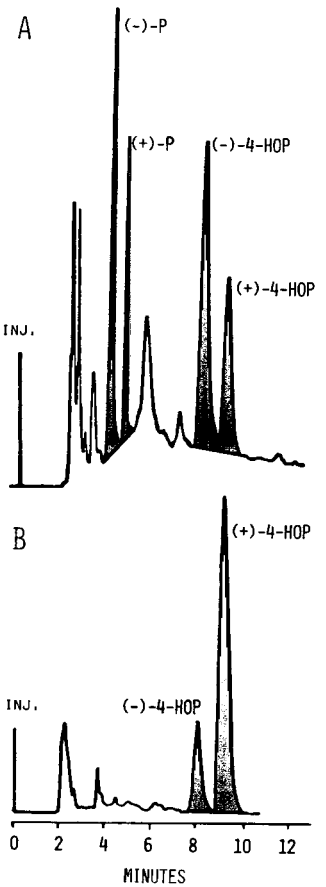


Fig. 3. Normal-phase HPLC of (+)-PEI diastereomer derivatives of propranolol (P) and 4-HOP, respectively, after acid-wash. Peak assignments were made as described in the text.

Fig. 4. Normal-phase HPLC of (+)-PEI diastereomer derivatives of (A) hydrolyzed glucuronic acid conjugates of propranolol (P) and 4-HOP and (B) hydrolyzed sulfate conjugate of 4-HOP. The conjugates were isolated from urine of normal subjects administered 80-mg single oral doses of propranolol. Chromatographic conditions were identical to those in Fig. 3.

The order of elution of the individual diastereomers of propranolol was established by separate derivatization of each optically pure enantiomer. For 4-HOP this was established by derivatization of 4-HOP isolated from urine following separate administration of each optically pure propranolol enantiomer. Both for propranolol and 4-HOP the (—)-enantiomer eluted first (Fig. 3).

The derivatization reaction was then tested for reproducibility. The concentrations of 4-HOP used ranged from 10 to 60 μg per sample. This was the amount of total 4-HOP anticipated to be present in a 2-ml human urine sample after a daily dose of 40—240 mg propranolol. A standard curve for this concentration range, using 1-(α-naphthoxy)-2,3-propylene glycol as internal standard (not derivatized), was linear (r = 0.997). The standard deviation at the midpoint of the curve was 5.9% (n = 6). The ratio of (—)/(+)-4-HOP was 1.00 ± 0.01 (mean ± S.D.; n = 6) using peak area measurements. In addition to making possible a reproducible derivatization, the acid-wash also effectively hydrolyzed and eliminated excess reagent, which could prove important in order to prolong the life of the column. Furthermore, the acid-wash removed most of the peaks early in the chromatogram (cf. Fig. 1). The derivatives were stable for at least several days following the acid-wash.

The method was applied to determinations of the stereochemical composition of the glucuronic acid conjugates of propranolol and 4-HOP as well as the sulfate conjugate of 4-HOP in human urine after 80-mg single oral doses of propranolol. For the glucuronides, urine was enzymatically hydrolyzed with glusulase. This procedure is quantitative for the two glucuronides [23, 24] and does not hydrolyze sulfate conjugates [28]. The aglycons were then extracted at pH 9.6. Following derivatization with (+)-PEI silica column HPLC produced the chromatogram in Fig. 4A. The diastereomers of both propranolol and 4-HOP were well separated from normal biological constituents. The (—)/(+)-enantiomer ratio of 1.47 for propranolol glucuronide and 1.80 for 4-HOP glucuronide, suggesting stereoselective glucuronidation of both propranolol and 4-HOP, agreed well with data recently obtained by administration of a stable isotope-labeled pseudoracemate and GC—MS separation of the enantiomers [12]. The acid-wash following the derivatization as well as the use of a high-wavelength ultraviolet detection (313 nm) both aided considerably in minimizing interferences in the chromatogram. The sulfate conjugate of 4-HOP was isolated intact from urine by HPLC [26, 28], hydrolyzed to 4-HOP by glusulase, extracted at pH 9.6 and derivatized with (+)-PEI as above. Silica column HPLC then produced the very clean chromatogram in Fig. 4B. The (—)/(+)-enantiomer ratio of 0.29 for this metabolite suggests a very large preference for the (+)-enantiomer in sulfate conjugation of 4-HOP. This also agreed well with a stable isotope-labeled approach [12].

Although the sensitivity of this method at present is adequate for determinations in urine, application to nanogram levels in plasma using fluorometric detection is under investigation.

CONCLUSIONS

A previously published method for chiral derivatization and HPLC separation of the enantiomers of propranolol has been extended to include its main

pharmacologically active metabolite, 4-HOP. New features are an acid-wash of the (+)-PEI derivative for consistent derivatization and as a clean-up step and also silica column HPLC for improved separation. Applications to metabolic studies of propranolol in man suggest stereoselectivity in both glucuronidation and sulfation of 4-HOP and in the glucuronidation of propranolol.

ACKNOWLEDGEMENTS

This work was supported by NIH Grant HL 29566 and a MUSC institutional grant. The authors thank U.K. Walle for excellent technical assistance and for her help in preparing the manuscript. K.D. Ballard and Dr. D.R. Knapp are greatly acknowledged for making possible the FAB-MS spectrum. Nita Pike is thanked for typing the manuscript.

REFERENCES

1 K.H. Rahn, A. Hawlina, F. Kersting and G. Planz, Naunyn-Schmiedeberg's Arch. Pharmacol., 286 (1974) 319.
2 A.S. Nies and J.G. Gernber, in L.E. Hollister (Editor), Year Book of Drug Therapy, Year Book Medical Publishers, 1980, p. 9.
3 T. Walle and U.K. Walle, Res. Commun. Chem. Pathol. Pharmacol., 23 (1979) 453.
4 S.A. Bai, U.K. Walle, M.J. Wilson and T. Walle, Drug Metab. Dispos., 11 (1983) 394.
5 S.A. Bai, M.J. Wilson, U.K. Walle and T. Walle, J. Pharmacol. Exp. Ther., 227 (1983) 360.
6 T. Walle, M.J. Wilson, U.K. Walle and S.A. Bai, Drug Metab. Dispos., 11 (1983) 544.
7 B. Silber, N.H.G. Holford and S. Riegelman, J. Pharm. Sci., 71 (1982) 699.
8 C. von Bahr, J. Hermansson and K. Tawara, Brit. J. Clin. Pharmacol., 14 (1982) 79.
9 C. von Bahr, J. Hermansson and M. Lind, J. Pharmacol. Exp. Ther., 222 (1982) 458.
10 U.K. Walle, T. Walle, S.A. Bai and L.S. Olanoff, Clin. Pharmacol. Ther., 34 (1983) 718.
11 L.S. Olanoff, T. Walle, U.K. Walle, T.D. Cowart and T.E. Gaffney, Clin. Pharmacol. Ther., 35 (1984) 755.
12 T. Walle, U.K. Walle, M.J. Wilson, T.C. Fagan and T.E. Gaffney, Brit. J. Clin. Pharmacol., in press.
13 J. Hermansson and C. von Bahr, J. Chromatogr., 221 (1980) 109.
14 B. Silber and S. Riegelman, J. Pharmacol. Exp. Ther., 215 (1980) 643.
15 J.A. Thompson, J.L. Holtzman, M. Tsuru, C.L. Lerman and J.L. Holtzman, J. Chromatogr., 238 (1982) 470.
16 A.J. Sedman and J. Gal, J. Chromatogr., 278 (1983) 199.
17 G. Manius and R. Tscherne, J. Chromatogr. Sci., 17 (1979) 322.
18 J. Gal, J. Pharm. Sci., 66 (1977) 169.
19 J.D. Fitzgerald and S.R. O'Donnell, Brit. J. Pharmacol., 43 (1971) 222.
20 J.E. Oatis, Jr., M.P. Russell, D.R. Knapp and T. Walle, J. Med. Chem., 24 (1981) 309.
21 Y. Yost and J.L. Holtzman, J. Pharm. Sci., 68 (1979) 1181.
22 D.A. Saelens, T. Walle, P.J. Privitera, D.R. Knapp and T.E. Gaffney, J. Pharmacol. Exp. Ther., 188 (1974) 86.
23 T. Walle, T.C. Fagan, E.C. Conradi, U.K. Walle and T.E. Gaffney, Clin. Pharmacol. Ther., 26 (1979) 167.
24 T. Walle, E.C. Conradi, U.K. Walle, T.C. Fagan and T.E. Gaffney, Clin. Pharmacol. Ther., 27 (1980) 22.
25 T. Walle, J. Morrison, K. Walle and E. Conradi, J. Chromatogr., 114 (1975) 351.
26 K.H. Wingstrand and T. Walle, J. Chromatogr., 305 (1984) 250.
27 K.D. Ballard and D.R. Knapp, unpublished results.
28 T. Walle, U.K. Walle, D.R. Knapp, E.C. Conradi and E.M. Bargar, Drug Metab. Dispos., 11 (1983) 344.

Journal of Chromatography, 310 (1984) 431—437
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2201

Note

Determination of aminoglutethimide and N-acetylaminoglutethimide in human plasma by high-performance liquid chromatography

G. MENGE* and J.P. DUBOIS

Ciba-Geigy Limited, Pharma Research and Development, Basle (Switzerland)

(First received January 31st, 1984; revised manuscript received May 10th, 1984)

Aminoglutethimide (AG) was shown to inhibit steroidgenesis, and has therefore found use as an alternative in the therapy of breast carcinoma [1].

Since the acetylation of AG is acetylator phenotype dependent [2] and N-acetylaminoglutethimide (N-AcAG) is the major metabolite excreted in urine, we developed a high-performance liquid chromatographic (HPLC) assay for the simultaneous measurement of AG and N-AcAG in human plasma using 2-(*p*-N-acetylaminophenyl)-2-methylglutarimide as internal standard (Fig. 1).

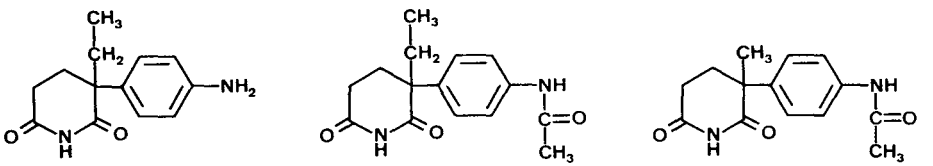


Fig. 1. Structures of AG [aminoglutethimide, 2-(*p*-aminophenyl)-2-ethylglutarimide], N-AcAG [N-acetylaminoglutethimide, 2-(*p*-N-acetylaminophenyl)-2-ethylglutarimide], and internal standard [2-(*p*-N-acetylaminophenyl)-2-methylglutarimide].

The HPLC methods available for the determination of AG in plasma [3] or for AG and metabolites in urine [2] worked without an internal standard. Only recently did we learn of a method [4] offering the possibilities we were looking for during the development of our method.

EXPERIMENTAL

Chemicals

All solvents and reagents were of analytical grade (Fluka, Buchs, Switzer-

0378-4347/84/$03.00

land, and E. Merck, Darmstadt, F.R.G.) and were used without further purification. ^{14}C-Labelled AG and N-AcAG, as well as the internal standard 2-(*p*-N-acetylaminophenyl)-2-methylglutarimide, originated from Ciba-Geigy, Basle, Switzerland (Fig. 1). Water was deionized, distilled in a glass apparatus and filtered through a 4.5-μm Millipore® (Waters Assoc., Milford, MA, U.S.A.) filter before use.

Apparatus

The chromatographic system consisted of a Vista 5040 ternary liquid chromatograph and the Vista 401 data system (both from Varian, Palo Alto, CA, U.S.A.). The WISP (Waters Assoc.) was used for automatic sample injection and a variable-wavelength detector Spectroflow SF 773 (Kratos, Ramsey, NJ, U.S.A.) served to monitor the eluent at 235 nm. The column (100 mm × 4.8 mm I.D.) was packed with Nucleosil C_{18}, 5 μm (Macherey-Nagel, Düren, F.R.G.). The mobile phase (acetonitrile—methanol—water, 5:20:75, v/v/v) was used at a flow-rate of 1 ml/min and a column temperature of 30°C.

Preparation of standard solutions

Stock solutions of AG and N-AcAG were prepared by dissolving 10 mg of each compound in 100 g of water containing 10% ethanol. Aliquots of these stock solutions were combined and diluted with water to yield a working solution containing 1 μg/g of each of the two compounds. This solution served to prepare spiked plasma samples for calibration curves and recovery analysis.

A stock solution of internal standard was prepared by dissolving 10 mg of 2-(*p*-N-acetylaminophenyl)-2-methylglutarimide in 100 ml of water containing 10% ethanol. An aliquot was diluted with water to yield a working solution containing 1 μg/ml. To each analytical plasma sample 0.5 μg of the internal standard was added using a Repipette® (Labindustries, Berkeley, CA, U.S.A.) sampler.

Both working solutions, if kept at 5°C, were found to be stable for at least four weeks.

Procedure

Weigh 0.5 g of plasma (AC 100 balance, Mettler, Greifensee, Switzerland) into a ground-glass stoppered centrifuge tube and dilute with 1 g of water. Weighing of plasma aliquots was preferred because of higher precision and better documentation. (For calibration curves and recovery analysis add 1 g of aqueous solution containing known amounts of the AG and N-AcAG working solution instead of 1 g of water.) Add 0.5 ml (Repipette sampler) of the internal standard working solution and shake for 5 min (DSG 304 vertical mixer; Heidolph, Kelheim, F.R.G.) to mix homogeneously the internal standard and analytical sample. Then add 7 ml of extraction solvent (diethyl ether—dichloromethane, 2:1, v/v). Seal the extraction tube with a stopper and shake for 12 min (HT horizontal shaker at 200 rpm; Infors, Basle, Switzerland). Centrifuge for 5 min (Multex centrifuge at 940 *g*; MSE, Crawley, U.K.). For easy separation freeze the aqueous phase by dipping the lower part of the tube into a dry ice—ethanol mixture. Transfer the organic layer into a 16 × 40 mm disposable glass ampoule and evaporate the organic solvents by

gently blowing nitrogen into the ampoule at 40°C. Reconstitute the residue in 0.2 ml of the mobile phase and transfer the solution into a micro injection vial.

A ternary solvent system was used since adding 5% acetonitrile improved the resolution by selectively lowering the retention of the biological background. Retention times of AG and N-AcAG were 8.9 and 12.1 min, respectively. The retention time of the internal standard was 6.1 min. Typical chromatograms are shown in Fig. 2.

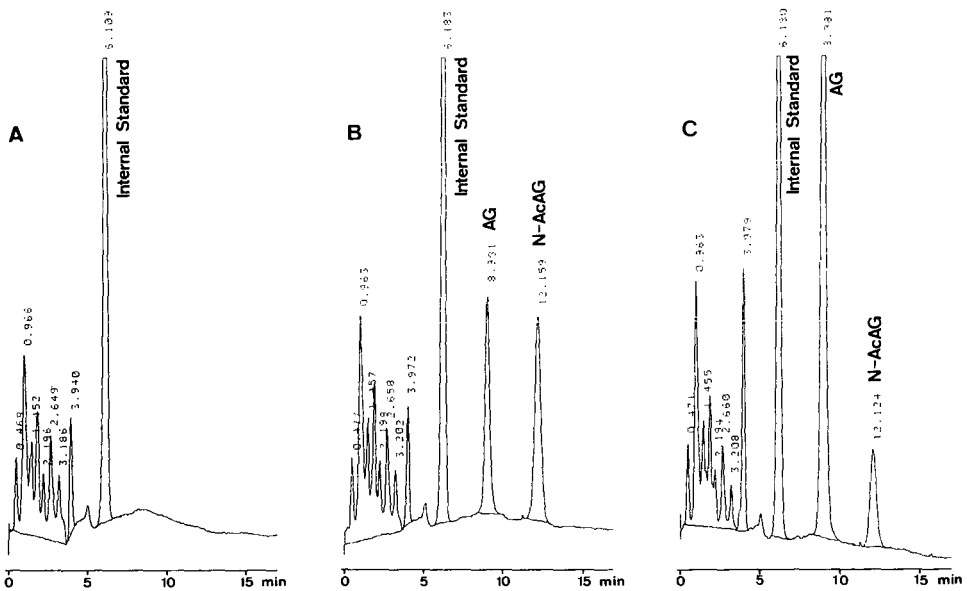


Fig. 2. (A) Chromatogram obtained from analysis of a blank plasma sample containing internal standard. (B) Chromatogram obtained from analysis of a spiked plasma sample containing 0.4 μg/g each of aminoglutethimide (AG) and N-acetylaminoglutethimide (N-AcAG). (C) Chromatogram obtained from analysis of a plasma sample obtained from a healthy volunteer 1 h after administration of a 250-mg tablet of aminoglutethimide.

Calibration

To establish calibration curves, plasma samples with known concentrations were prepared by adding working solution of AG and N-AcAG to 0.5 g of blank human plasma. After addition of 0.5 μg of the internal standard, the samples were processed as described above.

A 50-μl volume of the reconstituted extract of each sample was injected into the chromatographic system. The peak area values of the compounds were divided by the peak area value of the internal standard and the resulting ratios (F_X) plotted against initial concentrations given. By regression analysis the following terms for calibration curves in the range 0—2.0 μg/g were obtained: $F_X = 1.004 \cdot X$, $s_Y = 0.023$, $r = 0.9996$ for AG; and $F_X = 1.073 \cdot X$; $s_Y = 0.006$, $r = 0.9999$ for N-AcAG. X denotes the independent variable, i.e. the concentrations of AG and N-AcAG in plasma in units of μg/g. F_X denotes the dependent variable, i.e. the ratio of the peak area values; s_Y and r denote the estimated standard deviation and the coefficient of correlation [5].

In routine analyses of large series of plasma samples single-point calibration was used to calculate the concentrations of AG and N-AcAG.

RESULTS

Extractability

To isolate the compounds to be analysed from plasma, solvent extraction after dilution of the plasma samples was found to be suitable. Besides the high purification power the single-step solvent extraction offers the possibility of concentrating the extract prior to injection.

The partition of 1 μg of ^{14}C-labelled aminoglutethimide between buffered plasma samples (0.5 ml of plasma and 1.5 ml of buffer) and organic extraction phase (7 ml of diethyl ether—dichloromethane, 2:1, v/v) was measured radiometrically in dependence on the pH (Fig. 3). In the pH range 4—10 the amount of AG in the organic phase was not dependent on the pH (pK_{a1} of AG = 4.2, pK_{a2} of AG = 11.9).

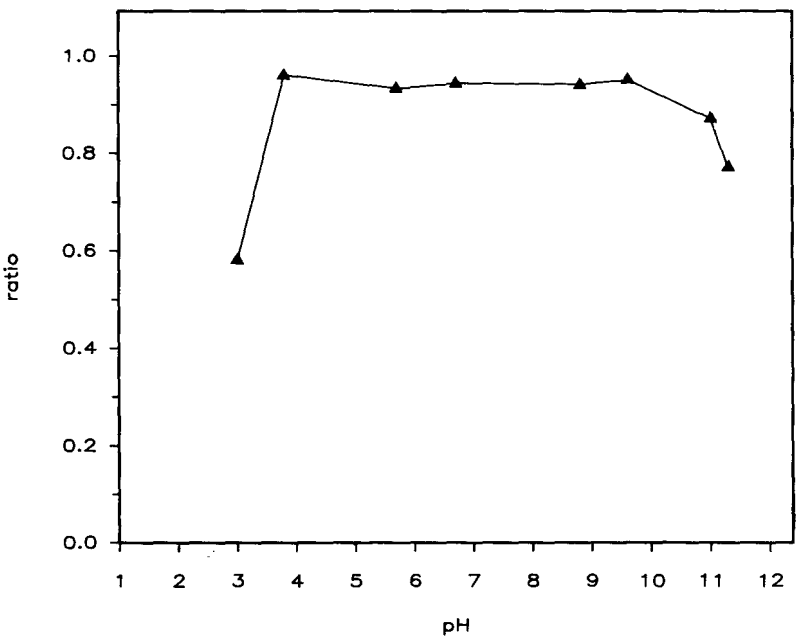


Fig. 3. Partition of 1 μg of ^{14}C-labelled aminoglutethimide between buffered plasma samples (0.5 ml plasma and 1.5 ml buffer) and organic extraction phase (7 ml of diethyl ether—dichloromethane, 2:1, v/v) in dependence on the pH. Aminoglutethimide: pK_{a1} = 4.2, pK_{a2} = 11.9.

Since the extractable biological background could not be drastically decreased at any pH, the plasma samples were extracted at their physiological pH.

The extent of the extractabilities of AG, N-AcAG and the internal standard from plasma at physiological pH was determined by comparison of the peak area after direct injection of known amounts with the peak area resulting after injection of the reconstituted extracts of spiked plasma samples that underwent

the whole work-up procedure. The extractabilities (mean ± S.D., n = 20) measured at ten different concentration levels covering the range 0.004—2.0 μg/g were 71 ± 5.4% for AG, 91 ± 3.3% for N-AcAG and 75 ± 6.7% for the internal standard.

Precision and accuracy

Recovery of spiked plasma samples was always analysed together with series of analytical samples. Four samples each containing 0.4 and 0.8 μg/g of both AG and N-AcAG were analysed in four independent analytical series. The results of these recovery analyses are given in Table I.

The standard deviations expressed in per cent of the total mean were 8.7% and 5.6% for AG and N-AcAG, respectively, at the concentration level 0.4 μg/g. The respective values were 5.3% and 3.1% at the 0.8 μg/g concentration level.

The mean deviations from given concentrations were between —8.7% and +10.1% for AG and between —7.2% and +1.9% for N-AcAG when a concentration of 0.4 μg/g was given. At the concentration level of 0.8 μg/g the mean

TABLE I

RESULTS OF RECOVERY ANALYSES USING SPIKED PLASMA SAMPLES

The analyses were performed on four different days with a single-point calibration procedure.

	AG found (ng/g)				N-AcAG found (ng/g)			
	Day 1	Day 2	Day 3	Day 4	Day 1	Day 2	Day 3	Day 4
	Given 400 ng/g							
	403.4	373.0	453.4	393.2	390.6	402.0	401.8	402.0
	381.6	344.4	438.0	376.2	393.8	365.4	390.8	402.6
	349.6	342.8	440.4	386.4	349.4	377.4	407.2	388.8
	373.8	400.4	429.4	385.8	351.4	378.2	430.8	414.2
Mean	377.1	365.2	440.3	385.4	371.3	380.8	407.7	401.9
± R.S.D.*	± 5.9	± 7.5	± 2.3	± 1.8	± 6.5	± 4.0	± 4.1	± 2.6
Accuracy**	—5.7%	—8.7%	+10.1%	—3.7%	—7.2%	—4.8%	+1.9%	+0.5%
	Given 800 ng/g							
	843.6	794.9	893.8	808.8	750.8	763.8	809.6	776.4
	836.4	869.2	879.8	802.6	762.8	784.4	815.4	779.2
	848.8	798.4	861.2	878.8	756.4	750.0	806.6	824.4
	825.8	717.2	860.0	819.8	766.2	759.6	800.0	793.6
Mean	838.7	794.9	873.7	827.5	759.1	764.5	807.9	793.4
± R.S.D.	± 1.2	± 7.8	± 1.9	± 4.2	± 0.9	± 1.9	± 0.8	± 2.8
Accuracy	+4.8%	—0.6%	+9.2%	+3.4%	—5.1%	—4.4%	+1.0%	—0.8%

*R.S.D. = $\frac{\text{S.D.}}{\text{Mean}} \times 100\%$.

**Accuracy = $\frac{\text{Found} - \text{given}}{\text{Given}} \times 100\%$.

deviations were between —0.6% and +9.2% for AG and —5.1% and +1.0% for N-AcAG.

The limit of quantitation was at least 0.2 μg/g. At this concentration level the relative standard deviations (R.S.D.) were 6.2% for AG and 4.6% for N-AcAG after analysis of four spiked plasma samples. The accuracy was +1.2% for AG and —1.1% for N-AcAG.

The detection limit was found at a concentration level of 0.02 μg/g. With parameters adjusted for the range 0—2.0 μg/g the integrator failed to integrate the area of the still visible peak at 0.02 μg/g.

Plasma concentration of aminoglutethimide and of its N-acetyl metabolite

After administration of one 250-mg tablet of aminoglutethimide to a healthy volunteer, the plasma concentrations of unchanged AG reached a maximum level of 2.4 μg/g 2 h after intake of the dose and dropped to 0.5 μg/g within 24 h (Fig. 4).

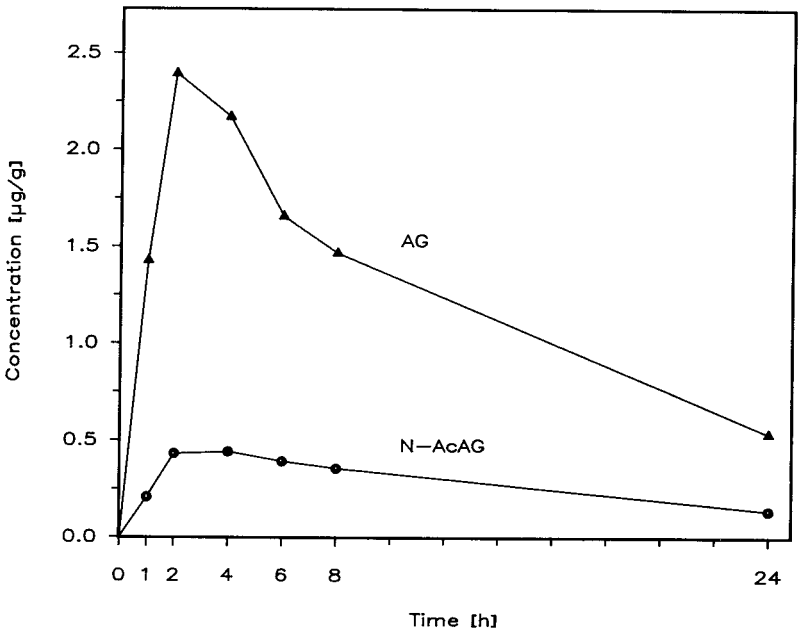


Fig. 4. Concentrations of aminoglutethimide (AG) and of N-acetylaminoglutethimide (N-AcAG) in plasma after administration of 250 mg of aminoglutethimide to a healthy volunteer, typed as slow acetylator using sulfadimidine.

The elimination half-life of aminoglutethimide was 10.4 h. This value was calculated by semilogarithmic—linear regression analysis using the concentrations of AG measured between 4 and 24 h after administration of the dose.

The N-acetyl metabolite showed a plateau concentration of about 0.4 μg/g between 2 and 4 h after administration; 24 h after ingestion of the dose 0.14 μg/g was measured.

Since the therapeutic dose is 1 g of AG per day, higher plasma levels of AG and of N-AcAG will have to be measured in patients.

CONCLUSION

The HPLC assay described for simultaneous analysis of aminoglutethimide (AG) and N-acetylaminoglutethimide (N-AcAG) using 2-(*p*-N-acetylaminophenyl)-2-methylglutarimide as internal standard permits the quantitation of plasma levels as they emerge after a single oral dose of 250 mg of aminoglutethimide.

ACKNOWLEDGEMENT

We thank Mr. G. Musslin for excellent technical assistance.

REFERENCES

1 R.W. Elsdon-Dew, I.M. Jackson and G.F.B. Birdwood (Editors), Aminoglutethimide — An Alternative Endocrine Therapy for Breast Carcinoma (International Congress & Symposium Series, No. 53), The Royal Society of Medicine, London, 1982.
2 R.C. Coombes, A.B. Foster, S.J. Harland, M. Jarman and E.C. Nice, Brit. J. Cancer, 46 (1982) 340.
3 B.A. Robinson and F.N. Cornell, Clin. Chem., 29 (1983) 1104.
4 C.O. Ruud, J.S. Kovach, M.E. Sanders and M.M. Ames, Proc. Amer. Ass. Cancer Res., 24 (1983) 288.
5 R.B. Davis, J.E. Thompson and H.L. Pardue, Clin. Chem., 24 (1978) 611.

Journal of Chromatography, 310 (1984) 438—444
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2203

Note

Simple and rapid high-performance liquid chromatographic method for the analysis of sulfinpyrazone and four of its metabolites in human plasma

Y.K. TAM*, S.M. FERGUSON and M.L. YAU

Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta T6G 2N8 (Canada)

and

D.G. WYSE

Electrophysiological Unit, University of Calgary, Calgary, Alberta (Canada)

(First received February 8th, 1984; revised manuscript received May 14th, 1984)

Sulfinpyrazone (SO), a derivative of phenylbutazone with pronounced uricosuric properties [1, 2], has been mainly used in the treatment of gout. Following the demonstration of its antiplatelet action [3], it has been shown that SO has probable beneficial effects in thromboembolic disorders [4]. Recently, in two major clinical trials, it has been demonstrated that SO reduces the recurrence of myocardial infarction [5] and sudden cardiac deaths [6]. Since the antiplatelet action of SO could be attributed to the active circulating metabolites [7], new interest in studying the pharmacokinetics of SO has arisen.

In the investigation of the cardiac electrophysiological effects of SO in human, it is necessary to have a sensitive and specific assay method to quantitate SO and its metabolites in plasma. There are nine high-performance liquid chromatographic (HPLC) methods [8—16] and two gas chromatographic methods [17, 18] published to date on SO and its metabolites. All of the published methods required extensive and time-consuming sample treatment ranging from single extraction to multiple extractions. There are only two methods reported [15, 16] which are capable of quantitating SO and most of its metabolites. Baseline resolution of all the components has not been achieved [15] using isocratic solvent systems and a gradient solvent system is required

0378-4347/84/$03.00

for total separation [16]. The purpose of this manuscript is to report a simple and rapid chromatographic method to quantitate SO and its metabolites in plasma.

MATERIALS AND METHODS

Reagents

Sulfinpyrazone {SO, 1,2,-diphenyl-4-[-2-(phenylsulfinyl)ethyl]-3,5-pyrazolidinedione} and its metabolites, the sulfone (G31442, SO_2), the *p*-hydroxylated sulfinpyrazone (G-32642, SOOH), the sulfide (G-25671, S) and the *p*-hydroxylated sulfide (G33378, SOH) were kindly supplied by Ciba-Geigy (Basel, Switzerland). The internal standard, naproxen, was obtained from Astra Pharmaceuticals. Acetonitrile (HPLC grade) was purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.).

Chromatography

A Waters Assoc. chromatographic system which consists of two M-45 pumps, an M-480 variable-wavelength UV detector, automatic sampler (WISP), integrator (Model 730) and system controller (Model 721) was used. Separation of SO and its metabolites was achieved using a 5-μm C_{18} reversed-phase Radial-Pak cartridge (11.5 cm × 8 mm I.D.). A precolumn packed with C_{18} packings (Waters Bondapak C_{18}/Corasil) was connected in front of the column. The absorbance of individual components was measured at 254 nm. The solvent system was acetonitrile—0.02 *M* phosphate buffer pH 7.0 (26:74). The solvent flow-rate was 2 ml/min.

Plasma sample treatment

To a 0.5-ml plasma sample containing SO and its metabolites were added 0.2 ml of naproxen (42 μg/ml) and 0.5 ml of acetonitrile. The mixture was gently shaken and centrifuged at 10,000 *g* for 2 min. The supernatant was removed and an equal volume of water was added. The sample (50—200 μl) was injected into the HPLC system.

Human studies

Two-stage infusion. To test the hypothesis that SO has cardiac electrophysiologic effects, SO was administered intravenously to subjects undergoing invasive electrophysiological testing. SO was given by bolus (4 or 8 mg/kg over 2—3 min) and constant intravenous infusion (0.015—0.045 mg/kg/min) in two stages 45 min apart. Blood samples were taken at 10-min intervals 15 min into the infusion. The blood samples were collected into heparinized vacutainers and were immediately centrifuged; plasma was separated and stored at −20° C until analysis.

Single oral dose study. A subject was fasted overnight and two 100-mg anturan tablets were administered with 150 ml water. Food was not allowed until 2 h after drug administration. Venous blood (5 ml) was collected at appropriate time intervals. The blood samples were immediately centrifuged; plasma was separated and stored at −20° C until analysis.

RESULTS

Fig. 1a shows a chromatogram of blank human plasma. There is no interference observed. Fig. 1b is a representative chromatogram of blank human plasma spiked with SO and its metabolites. All the peaks are baseline-resolved. Fig. 1c is the chromatogram of the plasma sample collected from a subject after oral route of administration. The peak at 8.13 min, which did not interfere with the analysis, was inherent in the subject's plasma (Fig. 1c). S was not detected after a single oral dose of SO. The minor *p*-hydroxy sulfone metabolite was separated but not observed in any of the patient samples; therefore, attempt to quantitate this species was abandoned.

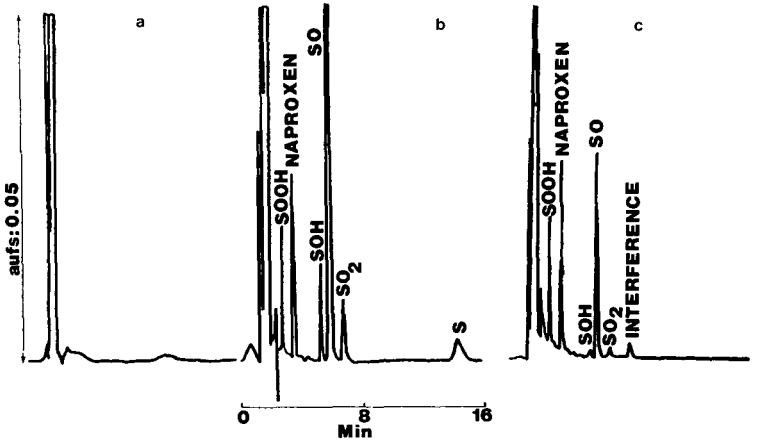


Fig. 1. a, Blank plasma. b, Blank plasma spiked with sulfinpyrazone (SO, 27.2 μg/ml) and metabolites (SOOH, 3.87 μg/ml; SOH, 4.5 μg/ml; SO_2, 5 μg/ml; S, 3.92 μg/ml), with naproxen (4 μg/ml) as internal standard. An 85-μl sample was injected. c, Plasma sample collected 4.0 h after 200 mg of anturan were administered orally. The peak with a retention time at 8.13 min was an inherent peak which was present in the blank plasma sample. This peak did not interfere with the assay.

Some of the subjects recruited into the study were on nitroglycerin and isosorbide dinitrate. These medications were stopped at least twelve days prior to the SO study. Plasma samples collected just before the study did not show any interfering peaks. Since the study was designed to measure the electrophysiological effects of SO and its metabolites, the assay method has not been subjected to an exhaustive test of cross-contamination by drugs which may be given during SO therapy. However, it has been found that lidocaine and its metabolites, propranolol and metoprolol did not interfere with the assay. Ibuprofen interfered with SOOH indicating the quantitation of this metabolite in subjects on ibuprofen has to be treated with caution.

Two sets of calibration curves were prepared. One has SO concentrations ranging from 0.2 to 100 μg/ml and another has SO concentrations from 0.2 to 25 μg/ml. The metabolite concentrations range from 0.2 to 10 μg/ml in both cases. Linearity has been observed in all cases and the results are summarized in Table I. Each standard curve has five points and each point was determined in triplicate. The coefficient of variation in each point was less than 9%. The

TABLE I

EQUATIONS OBTAINED FROM LINEAR REGRESSION ANALYSIS AND PRECISION OF SAMPLE ANALYSIS FOR SO AND METABOLITES

Compound	Linear regression equation	C.V. (%)	
		At 0.5 μg/ml	At 10 μg/ml
SO	$Y = 0.0610X - 0.0089$	5.0*	1.9
SOOH	$Y = 0.0649X - 0.0087$	6.2	1.6
SO_2	$Y = 0.0641X - 0.00942$	8.8	1.2
SOH	$Y = 0.0466X - 0.00424$	1.7	3.7
S	$Y = 00.806X - 0.0143$	2.7	2.2

*At 1 μg/ml.

sensitivity for all the species were 0.1 μg/ml except for S which has a sensitivity of 0.2 μg/ml. Since the plasma samples were not extracted, recovery of the drug and metabolites after protein precipitation was evaluated by comparing the peak area ratios between the spiked plasma samples and an aqueous solution which contained the same concentration (5 μg/ml) of each species. Recovery was found to be higher than 99% in all cases. The stability of SO and its metabolites in plasma after treatment with acetonitrile was observed to be more than 24 h. This was substantiated by the fact that the peak area ratios of the drug and metabolites to internal standard were changed no more than 1%.

DISCUSSION

There are a number of HPLC assays reported in the literature [8—16] to quantitate SO and its metabolites in biological fluids. In the earlier studies [8—15], the biological samples have to be treated with extensive extraction procedures to isolate SO and its metabolites. When a single extraction step was used, the recovery of one or more metabolites would be significantly diminished [12, 14]. Recently, De Vries et al. [16] reported a solvent system which was capable of extracting all of the components with high yield (> 90%) except SOOH (60%) in plasma and urine. The present method required no extraction. The only sample treatment was deproteinization of plasma protein with acetonitrile. The yield of all the components was 100%. It was observed that if water was not added to the supernatant of the deproteinized plasma samples, injection of more than 50 μl of the sample would result in skewed peaks and loss of separation. This phenomenon could be attributed to the high percentage of acetonitrile present in the sample [19]. The addition of equal volume of water to the sample allowed higher volume of sample (> 200 μl) to be introduced into the HPLC system without any loss of separation efficiency and sensitivity.

Chromatographically, SO and its metabolites have not been completely separated (base-line separation) using an isocratic solvent system and a suitable stationary phase. De Vries et al. [16] have recently separated SO and its metabolites with a gradient solvent system and a reversed-phase (C_{18}) column.

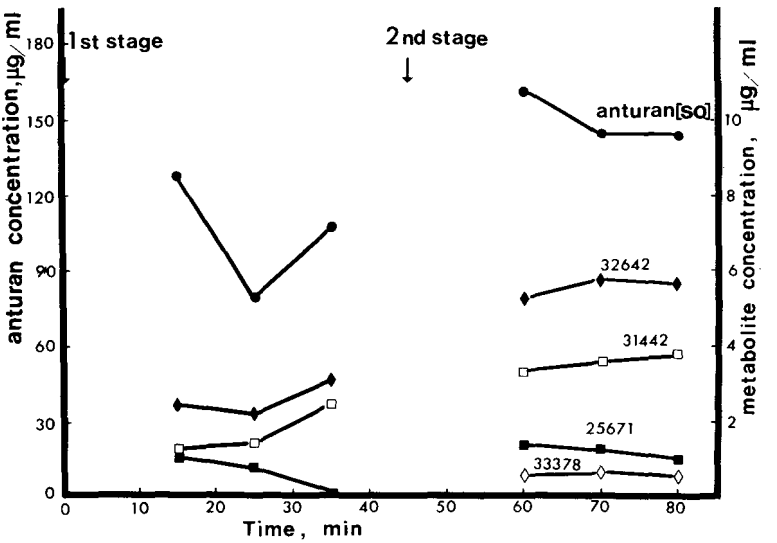


Fig. 2. Representative plasma profiles of sulfinpyrazone SO (•) and its metabolites [SOOH (♦), SOH (◇), SO_2 (□) and S (■)] for a patient who was on a two-stage intravenous study. The first loading dose was 8 mg/kg and the infusion-rate was 0.03 mg/kg/min. The loading dose at the second stage was 4 mg/kg and the infusion-rate was 0.045 mg/kg/min.

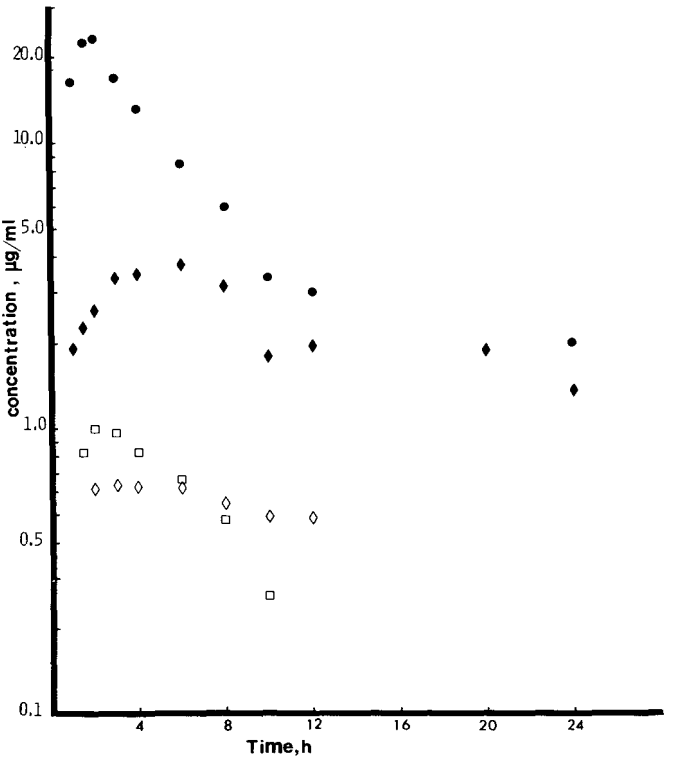


Fig. 3. Plasma profile of SO (•), SOOH (♦), SOH (◇) and SO_2 (■) after a subject has taken 200 mg sulfinpyrazone orally.

The present method has the advantage that SO and four of its metabolites could be separated by an isocratic solvent system and a reversed-phase (C_{18}) column. The analysis time was approximately the same as reported in the method of De Vries et al. [16]. The sensitivity of this assay method was compatible with the published methods except that the procedures were highly simplified. The applicability of this method was demonstrated by measuring SO and its metabolites in human plasma after different routes of administration.

Figs. 2 and 3 are plasma profiles of SO and its metabolites during a two-stage intravenous infusion and after 200 mg oral administration of anturan, respectively. The *p*-hydroxy (SOOH) and the sulfone (SO_2) metabolites were found to be the major species in plasma besides the drug itself. The low level of S observed during intravenous infusion and the absence of S after oral administration were consistent in all the experiments (n = 24). The relatively high SO level at 24 h after oral administration of anturan seemed to be unique in this study. No SO could be detected at 24 h in another study. It is realized that SOOH and SOH have been reported as minor metabolites and S as major metabolite after single and multiple oral doses [16, 20]. The reason for the quantitative difference in the metabolic profiles between this study and the literature is not readily apparent.

With our experience, approximately eighty samples can be processed in a day. Since a pre-column was employed, the column can tolerate a high number of injections (> 500 to date) into the HPLC system.

ACKNOWLEDGEMENTS

This work was supported in part by Ciba-Geigy, Alberta Heritage Foundation for Medical Research and Alberta Heart Foundation.

REFERENCES

1 B.B. Brodie, T.F. Yu, J.J. Burns, T. Chenkin, B.C. Paton, J.M. Steele and A.B. Gutman, Proc. Soc. Exp. Biol. Med., 86 (1954) 884.
2 J.J. Burns, T.F. Yu, A. Ritterband, J.M. Perel, A.B. Gutman and B.B. Brodie, J. Pharmacol. Exp. Ther., 119 (1957) 418.
3 H.A. Smythe, M.A. Ogryzlo, E.A. Murphy and J.F. Mustard, Can. Med. Assoc. J., 92 (1965) 818.
4 E.H. Margulies, A.M. White and S. Sherry, Drugs, 20 (1980) 179.
5 The Anturan Reinfarction Italian Study Group: Sulphinpyrazone in postmyocardial infarction. Lancet, i (1982) 237.
6 The Anturan Reinfarction Trial Research Group: Sulfinpyrazone in the prevention of cardiac death after myocardial infarction. N. Engl. J. Med., 298 (1978) 289.
7 M.P. Buchanan, R. Rosenfeld and J. Hirsch, Thromb. Res., 13 (1978) 883.
8 T. Inaba, M.E. Basley and E.J. Chow, J. Chromatogr., 104 (1975) 103.
9 L.T. Wong, G. Solomonraj and B.H. Thomas, J. Chromatogr., 150 (1978) 521.
10 W. Dieterle, J.W. Faigle, H. Mary, W.J. Richter and W. Theobald, Eur. J. Clin. Pharmacol., 9 (1975) 135.
11 W. Dieterle, J.W. Faigle and J. Moppert, Arzneim.-Forsch., 30 (1980) 989.
12 J. Godbillon, S. Gauron and G. Gosset, J. Chromatogr., 227 (1982) 516.
13 J.B. Lecaillon and C. Souppart, J. Chromatogr., 121 (1976) 227.

14 T.D. Bjornsson, K.M. Wolfam, P.A. Routledge and D.G. Shand, J. Chromatogr., 181 (1980) 417.
15 P. Jakobsen and A. Kirstein Pederson, J. Chromatogr., 223 (1981) 460.
16 J.X. de Vries, C. Staiger, N.S. Wang and F. Schlicht. J. Chromatogr., 277 (1983) 408.
17 P. Jakobsen and A. Kirstein Pedersen, J. Chromatogr., 163 (1979) 259.
18 P. Jakobsen and A. Kirstein Pedersen, J. Pharm. Pharmacol., 33 (1981) 89.
19 M. Broquaire and P.R. Guinebault, J. Liquid Chromatogr., 4 (1981) 2039.
20 A. Kirstein Pedersen, P. Jakobsen, J.P. Kampmann and J. Molhelm Hansen, Clin. Pharmacokin., 7 (1982) 42.

Journal of Chromatography, 310 (1984) 445—449
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2205

Note

Rapid high-performance liquid chromatographic analysis of oxaprozin, a non-steroidal anti-inflammatory agent

RITA MATLIS and DAVID J. GREENBLATT*

Division of Clinical Pharmacology, Departments of Psychiatry and Medicine, Tufts-New England Medical Center, 171 Harrison Avenue, Boston, MA 02111 (U.S.A.)

(First received February 16th, 1984; revised manuscript received April 28th, 1984)

Oxaprozin (Fig. 1) is a non-steroidal anti-inflammatory agent of the proprionic acid class which is under investigation for the treatment of pain and inflammation [1, 2]. Similar to other drugs in this category [3], high-performance liquid chromatography (HPLC) has been successfully used for quantitation of oxaprozin in biological fluids [4]. The present report describes a rapid and sensitive HPLC technique for analysis of oxaprozin in plasma. Analytic cost is greatly reduced with the use of the radial compression separation system [3, 5]. With the use of an automatic sampler, large numbers of samples can be quantitated with minimal technical time.

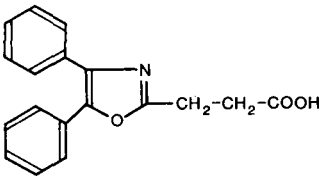


Fig. 1. Structural formula of oxaprozin.

METHODS

Reagents and standards

Pure samples of oxaprozin (Wyeth Labs., Radnor, PA, U.S.A.) and ketoprofen (Ives Labs., New York, NY, U.S.A.) were kindly provided by their pharmaceutical manufacturers. All other reagents, analytical grade or better, were purchased from commercial sources and used without further purification.

0378-4347/84/$03.00

Standard solutions were prepared by dissolving 100 mg of oxaprozin or ketoprofen each in 100 ml of methanol. Further dilutions with methanol were made as necessary.

Instrumentation

Analyses were performed using a Waters Assoc. (Milford, MA, U.S.A.) HPLC system, consisting of a solvent delivery system (M6000), ultraviolet absorbence detector (M440 or M480), data processor (M730), and automatic sampling system (WISP 710B). The column was a reversed-phase C_{18} radial compression cartridge (spherical 10-μm particles, non-postsilanized), 10 cm × 5 mm I.D., which was housed in an RCM-100 radial compression module. Column effluent was quantitated with the absorbance detector set at 280 nm. The mobile phase consisted of acetonitrile—water (45:55), to which were added 2.5 ml of acetic acid per liter. The flow-rate was 1 ml/min, and analyses were performed at room temperature.

Preparation of samples

Ketoprofen (50 μg) was added to a series of 13-ml round-bottom glass culture tubes with PTFE-lined screw top caps. Calibration standards were prepared by adding variable amounts of oxaprozin, ranging from 5 through 100 μg, to a series of these tubes. All other tubes contained only the internal standard. To each of the calibration tubes is added 1.0 ml of drug-free control plasma; 1 ml of "unknown" plasma is added to all other tubes. The samples are acidified with 0.2 ml of 1.0 *M* hydrochloric acid, and 4—5 ml of ethyl acetate are added. The samples are agitated in the upright position on a vortex-type mixer for 90—120 sec. After centrifugation for 10 min at 400 *g*, an aliquot of the upper organic layer is transferred to a tapered centrifuge tube and evaporated to dryness under mildly reduced pressure at 40—50°C. The extracts are redissolved in 0.2 ml of methanol and transferred to an automatic sampling vial equipped with a limited volume insert. The automatic sampler is programmed to deliver 20 μl per sample.

Clinical pharmacokinetic study

A healthy male volunteer participated after giving written informed consent. After an overnight fast, he received a single 1200-mg dose (two 600-mg tablets) of oxaprozin with 100—200 ml of tap water. He remained fasting until 3 h after dosage. Venous blood samples were drawn into heparinized tubes at multiple points in time over the next fourteen days. Blood samples were centrifuged, and the plasma separated and frozen until the time of assay. Concentrations of oxaprozin in all samples were determined by the method described above.

RESULTS

Evaluation of the method

Oxaprozin and the internal standard ketoprofen gave two well resolved chromatographic peaks (Fig. 2). Extracts of drug-free plasma samples were consistently free of endogenous contaminants at the retention times

corresponding to these two compounds. Oxaprozin plasma concentration was linearly related to the peak height ratio of oxaprozin versus ketoprofen. The equation of a typical calibration line is: $Y = 0.0273X + 0.023$ ($r = 0.999$), where X is plasma oxaproxin concentration and Y is peak height ratio. Coefficients of variation for identical samples ($n = 6$ at each concentration) were: at 1 μg/ml, 6.8%; at 10 μg/ml, 7.4%; at 50 μg/ml, 2.4%; and at 100 μg/ml, 4.4%. The sensitivity limits are approximately 0.5 μg/ml of plasma. This can be improved by minor modifications such as injection of larger aliquots of the final reconstituted extract.

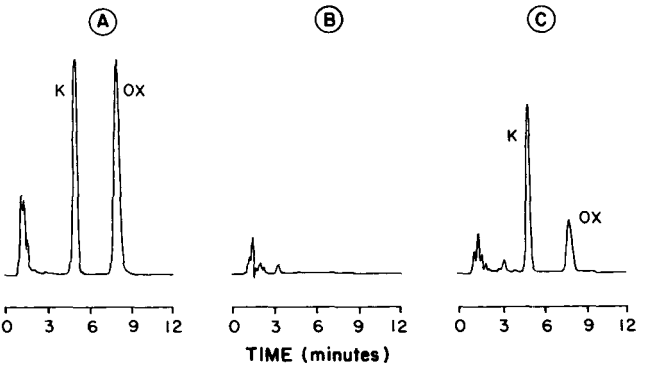


Fig. 2. Chromatograms of plasma extracts. (A) Calibration standard containing 50 μg/ml ketoprofen (K) and 50 μg/ml oxaprozin (OX). (B) Plasma sample from a subject prior to ingestion of oxaprozin. (C) Plasma sample (to which was also added the internal standard) from a subject taken 96 h after a single 1200-mg dose of oxaprozin.

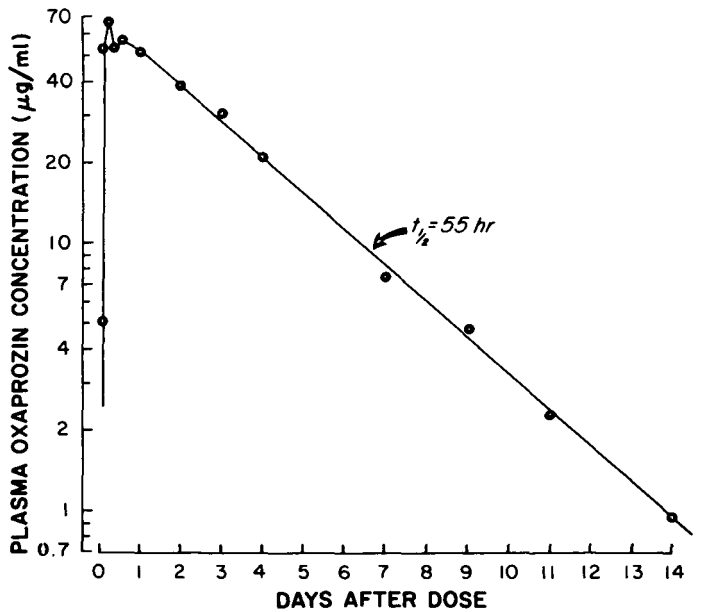


Fig. 3. Plasma concentrations of oxaprozin in the volunteer subject who participated in the pharmacokinetic study.

Potential assay interferences by other analgesic agents were evaluated for those compounds of which pure reference standards were available. Salicylic acid and acetaminophen had considerably shorter retention times than the internal standard ketoprofen, while indomethacin and phenybutazone had longer retention times than oxaprozin. Ibuprofen and piroxicam did not yield detectable chromatographic peaks under the described conditions. Therefore the above compounds do not interfere with the assay. Flurbiprofen and fenoprofen had retention times similar to oxaprozin and therefore are potential assay interferences.

Pharmacokinetic results

A peak plasma oxaprozin concentration of 67 μg/ml was reached at 6 h after dosage. Thereafter, plasma concentrations declined exponentially with an apparent half-life of 55 h (Fig. 3). Under the assumption that the entire 1200-mg dose of oxaprozin was available to the systemic circulation, apparent volume of distribution and total metabolic clearance were calculated as described previously [6]. The calculated values were: volume of distribution, 18 l; total clearance, 3.8 ml/min.

DISCUSSION

The present report describes a rapid and sensitive HPLC method for quantitation of oxaprozin in plasma. After addition of a structurally related internal standard, acidified plasma samples are directly extracted into ethyl acetate. The extract is separated, evaporated to dryness, and chromatographed on a reversed-phase C_{18} radial compression separation system. The overall approach is analogous to that previously developed by McHugh et al. [4]. However, the radial compression system has advantages in that the solvent consumption is low, and the cartridges themselves are considerably less expensive and have a longer average life than stainless-steel columns [3, 5]. Furthermore the automatic injection system allows quantitation of large numbers of plasma samples. One technical person can easily prepare up to 100 samples for analysis in a single working day, while the actual chromatography can proceed overnight using the automatic sampling system.

The applicability of the method to pharmacokinetic studies of oxaprozin in humans is demonstrated. Due in part to its extensive binding to plasma protein, the extent of oxaprozin distribution is very limited [7—9]. This, together with its low metabolic clearance, leads oxaprozin to have a very long elimination half-life [7—9]. Thus, oxaprozin has the potential to be effective as a non-steroidal anti-inflammatory agent with a once daily or once every other day dosing schedule.

ACKNOWLEDGEMENTS

We are grateful for the assistance of the Research Division of Wyeth Laboratories, Radnor, PA, U.S.A. This work was supported in part by Grant MH-34223 from the United States Public Health Service.

REFERENCES

1 J.A. Hubsher, R.S. Northington and B.R. Walker, Arthritis Rheum., 26 (1982) S117.
2 W.J. Reynolds, S.F. Shaar, A. Buik and W.J. Lancee, J. Rheumatol., 6 (1979) 345—350.
3 D.J. Greenblatt, R.M. Arendt and A. Locniskar, Arzneim.-Forsch., 33 (1983) 1671—1673.
4 S.L. McHugh, S.K. Kirkman and J.A. Knowles, J. Pharm. Sci., 69 (1980) 794—796.
5 D.J. Greenblatt, R. Matlis, D.R. Abernethy and H.R. Ochs, J. Chromatogr., 275 (1983) 450—457.
6 D.J. Greenblatt, M. Divoll, J.S. Harmatz and R.I. Shader, J. Pharmacol. Exp. Ther., 215 (1980) 86—91.
7 F.W. Janssen,, W.J. Jusko, S.T. Chiang, S.K. Kirkman, P.J. Southgate, A.J. Colemen and H.W. Ruelius, Clin. Pharmacol. Ther., 27 (1980) 352—362.
8 C.A. Homon, E.R. Fluck, F.W. Janssen and H.R. Ruelius, Agents Actions, 12 (1982) 211—215.
9 S.T. Chiang, G. Morrison, J.A. Knowles, H.W. Ruelius and B.R. Walker, Clin. Pharmacol. Ther., 31 (1982) 509—515.

Journal of Chromatography, 310 (1984) 450—454
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2206

Note

Improved procedure for the high-performance liquid chromatographic determination of valproic acid in serum as its phenacyl ester

MIKIO NAKAMURA*

San-Ei Chemical Industries, Ltd., Sanwa-cho, Toyonaka, 561 (Japan)

KAZUKO KONDO

Kyoto Children Welfare Center, Senbon-Takeya-machi, Kamigyo-ku, Kyoto 602 (Japan)

and

RYOTA NISHIOKA and SATOSHI KAWAI

Gifu College of Pharmacy, Mitahora-higashi, Gifu 502 (Japan)

(First received February 16th, 1984; revised manuscript received May 7th, 1984)

Valproic acid (VPA) is clinically used as an anticonvulsant in the treatment of epilepsy, and the serum levels are frequently monitored for efficient control of seizures. In recent years, high-performance liquid chromatography has been increasingly employed for the analysis of drugs in serum. A sensitive assay of VPA by liquid chromatography is difficult because of the poor ultraviolet absorbance. In order to overcome this problem, derivatives by reaction with aryl bromomethyl ketones have been used as ideal derivatives having high ultraviolet absorbance properties for high-performance liquid chromatographic determination of VPA, such as phenacyl [1], 4-bromophenacyl [2] and 2-naphthacyl [3] esters.

Usually the derivatization was carried out under non-aqueous conditions after extraction of the acidified sample with organic solvent [1, 3]. The extraction technique was troublesome and the recovery of VPA from aqueous solution by solvent extraction was irreproducible, so a procedure for the derivative formation of VPA was proposed in aqueous conditions using 4-bromo-

0378-4347/84/$03.00

phenacyl bromide [2] and phenacyl bromide [4]. However, insufficient investigations on conditions for phenacylation of VPA were made.

We have chosen to use phenacyl bromide as derivatizing reagent since it was easier to obtain in this country, and triethylamine instead of crown-ether catalysis. The present paper describes evaluations of the factors affecting the yield in phenacylation under aqueous conditions, and a simple, effective procedure for phenacylation of VPA in serum has been established without recourse to a solvent extraction step.

EXPERIMENTAL

Reagents and standard

Sodium valproate (VPA) was obtained from Kyowa Hakko (Tokyo, Japan). Phenytoin, phenobarbital, hexobarbital, cyclohexane carboxylic acid (CCA), α-bromoacetophenone (phenacyl bromide) and triethylamine (TEA) were from Tokyo Kasei (Tokyo, Japan). Carbamazepine was purchased from Nippon Ciba-Geigy (Hyogo, Japan). All other chemicals were of analytical grade.

The esterification agent was a solution containing α-bromoacetophenone, 3 g/l in acetonitrile. The internal standard was CCA, 4 μg/ml in acetonitrile. The VPA standards were 1—100 μg (as valproic acid) per ml in drug-free serum. Aliquots of 10—1000 μg as valproic acid per ml in distilled water were diluted ten-fold with drug-free serum.

Procedure

Serum or VPA standard (50 μl) was added to a 1.5-ml tube containing 1.0 ml of internal standard. The tube was stoppered, mixed on a Vortex mixer (10 sec) and centrifuged. An 800-μl aliquot of the supernatant was transferred to a glass tube containing 200 μl of esterification agent and 100 μl of TEA. The mixture was heated in a dry heating block for 30 min at 80° C, keeping the tube open; on cooling 10-μl aliquots were injected into the liquid chromatograph.

High-performance liquid chromatography

Analyses were performed using a Japan Spectroscopic liquid chromatograph equipped with a Twincle pump and a Model Uvidec 100-IV UV (ultraviolet) variable-wavelength spectrophotometric detector, capable of monitoring at 245 nm. Chromatography was performed at 30°C on a 25 cm × 4.6 mm I.D. stainless-steel column packed with Sil $C_{18\text{-}5}$ (particle size 5 μm) (from Japan Spectroscopic, Tokyo, Japan) with acetonitrile—water (60:40, v/v) as mobile phase. The flow-rate was 1.0 ml/min.

Calculations

VPA concentrations were determined as free acid from graphs of peak height ratios against known concentrations constructed using VPA standards.

RESULTS AND DISCUSSION

The following factors affecting phenacylation of VPA in aqueous solution were investigated in detail. All examinations were performed through the entire

procedure described in *Procedure*, except that 1.0 ml of acetonitrile was added instead of internal standard and the reaction tube was heated keeping it tightly stoppered. The results were followed by measuring the average of the peak heights of phenacyl valproate obtained by duplicate injections.

Esterification agent and TEA concentrations

Using 50 μl of VPA standard (100 μg/ml), the necessary concentrations of esterification agent and TEA were examined. As shown in Fig. 1, the concentrations of α-bromoacetophenone and TEA sufficient to obtain a constant phenacylation were 0.2% (w/v) and 50 μl, respectively. As previously reported, phenacylation was carried out in aqueous solution with crown-ether catalysis [2, 4, 5]. However, it was found that the phenacyl ester of VPA was easy to prepare in aqueous conditions using TEA instead of crown ethers.

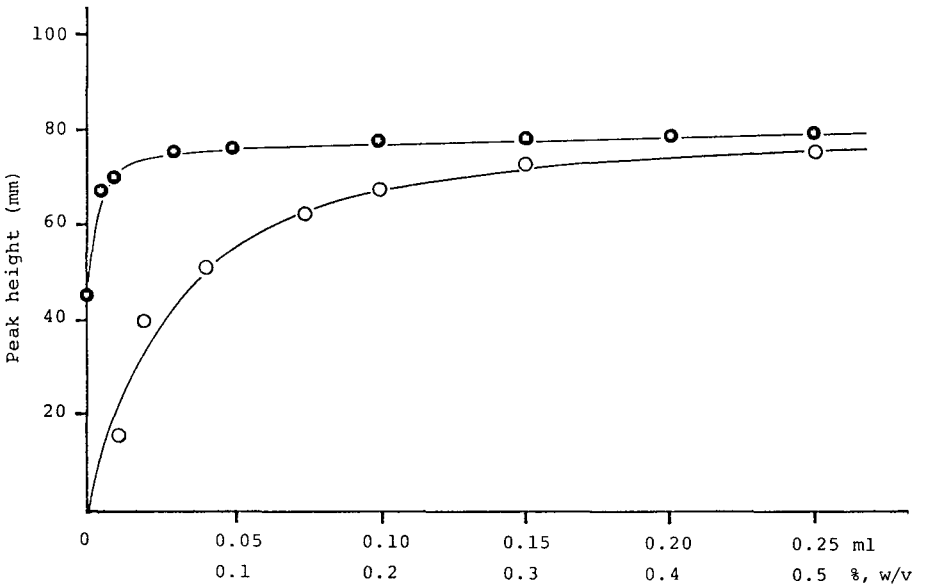


Fig. 1. Effect of α-bromoacetophenone and triethylamine concentrations on phenacylation. •, Quantity of added triethylamine (1 ml); ○, concentration of α-bromoacetophenone (%, w/v).

Reaction temperature and period

Using 50 μl of VPA standard (100 μg/ml), the effects of the reaction temperature and period were investigated through the entire procedure. The effects of these two factors were not so large. As regards the time course of derivatization, the reaction was sufficient in 30 min at 80°C to obtain a constant peak height of phenacyl valproate. The yield of phenacylation in these conditions was about 90% of that obtained in 72 h at 80°C.

Influence of water content in the reaction mixture on phenacylation

The water content of the reaction mixture seriously affected phenacylation. Using 50 μl of VPA standard (100 μg/ml), the influence of various water contents on phenacylation was investigated and the results are shown in Fig.

2. The phenacylation yield increased with decreasing water content and rose rapidly in the range of less than 10%. The same result was also obtained in the case of 4-bromophenacylation (Fig. 2). In the procedure, a relatively large volume of acetonitrile was added to the sample solution for the purpose of complete deproteinization and of obtaining a better phenacylation yield. The tubes were kept open during the phenacylation reaction. The sample solution diluted by the addition of this large amount of acetonitrile could be concentrated as phenacylation proceeded by heating the reaction mixture at 80°C for 30 min. The open system made the detection limit decrease markedly. Addition of CCA, which has properties similar to those of VPA, as an internal standard obviated the need for accurate estimation of reagent amounts, reaction temperature, reaction period and water content of the reaction mixtures which gave a slight influence on the phenacylation yield.

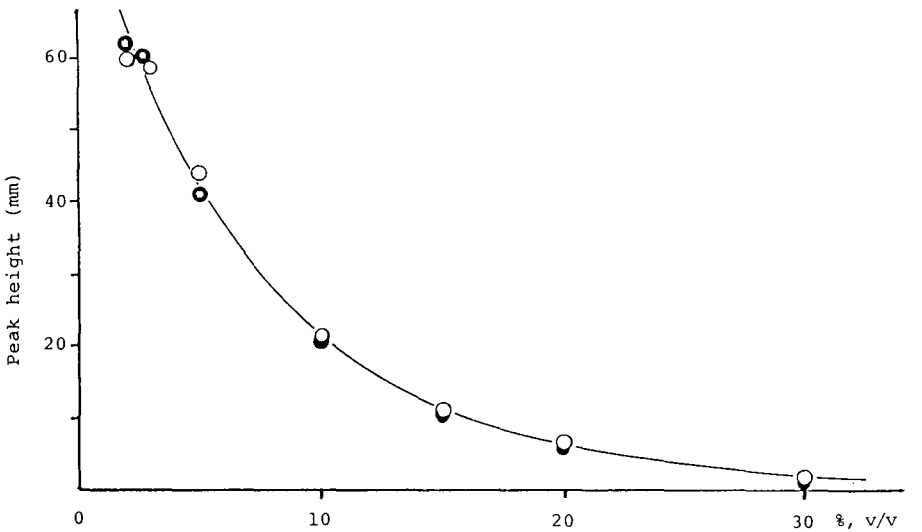


Fig. 2. Effect of water content of the reaction mixtures on phenacylation. ○, Phenacylation; ●, 4-bromophenacylation.

The calibration curve was linear from 1 to 100 μg/ml VPA in serum. The precision of the method was examined by following the procedure at three different concentrations: 20, 40 and 80 μg/ml VPA in serum. The coefficients of variation for three concentrations of VPA (n = 6 each) were 3.4% for 80 μg/ml, 2.7% for 40 μg/ml and 5.3% for 20 μg/ml. The procedure was found to be satisfactorily reproducible. The minimum concentration for quantitation of VPA was 0.5 μg/ml for a 50-μl specimen.

Fig. 3 shows a chromatogram of serum from a patient who was receiving VPA. The peak corresponded to about 50 μg/ml in serum. We selected an ODS column as the stationary phase for the analysis of phenacyl esters because it is commonly used in the assay of drugs. Peaks of coexisting substances in serum and of reagents were eluted early, while there was excellent separation of phenacyl valproate and internal standard from each other and from reagent peaks.

When serum from a patient receiving various antiepileptics (hexobarbital,

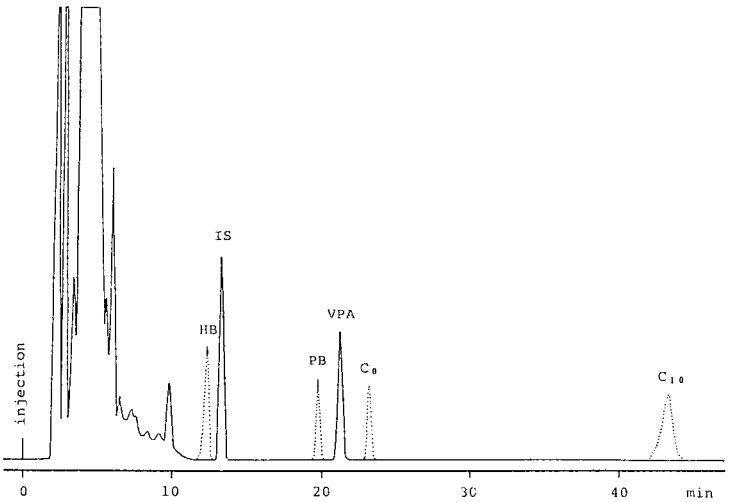


Fig. 3. Chromatogram of serum from a patient receiving valproate. HB = Hexobarbital; IS = internal standard (cyclohexane carboxylic acid); PB = phenobarbital; VPA = valproic acid; C_8 = octanoic acid; C_{10} = decanoic acid. (As their phenacyl derivatives.) Conditions: column, Finepak Sil C_{18-5}, 25 cm × 4.6 mm I.D.; eluent, acetonitrile—water (60:40, v/v); temperature, 30°C; flow-rate, 1.0 ml/min; detector, 245 nm.

phenobarbital, phenytoin, carbamazepine) is measured by the present method, serious trouble may arise. Retention values of some phenacylated drugs are shown by the dotted line in Fig. 3. Hexobarbital and phenobarbital gave no interference and produced sharp, symmetrical peaks. Alric et al. [3] reported an incomplete separation of 2-naphthacyl derivatives of phenobarbital and VPA using the mixture acetonitrile—water (83:17, v/v) as mobile phase, while a separation of phenacyl esters of these two drugs was complete using the present solvent mixture (acetonitrile—water, 60:40, v/v). Phenytoin and carbamazepine did not show any peak. These two drugs did not react with bromomethyl naphthyl ketones [3]. Probably the two drugs do not react with α-bromoacetophenone either. They themselves eluted early, close to reagent peaks, using the present mobile phase and no interference was encountered in specimens from patients receiving these drugs.

Some factors affecting phenacylation of VPA have been examined in detail. The phenacyl esters were easily prepared and proved to be satisfactory for analysis. The most important advantages of the present procedure are direct and effective derivatization in sample solution by adding a large volume of acetonitrile, concentration of the reaction mixtures with the open system during heating for phenacylation and excellent sensitivity permitting measurement of 0.5 μg/ml VPA using only 50 μl of serum.

REFERENCES

1 R.N. Gupta, P.M. Keaene and M.L. Gupta, Clin. Chem., 25 (1979) 1984.
2 J.P. Moody and S.M. Allan, Clin. Chim. Acta, 127 (1983) 263.
3 R. Alric, M. Cociglio, J.P. Blayac and R. Puech, J. Chromatogr., 224 (1981) 289.
4 S.C. Chan, Clin. Chem., 26 (1980) 1528.
5 H.D. Durst, M. Milano, E.J. Kikta, Jr., S.A. Connelly and E. Grushka, Anal. Chem., 47 (1975) 1797.

Journal of Chromatography, 310 (1984) 455—459
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2211

Note

Rapid method for the determination of either piroxicam or tenoxicam in plasma using high-performance liquid chromatography

J.S. DIXON* and J.R. LOWE

Clinical Pharmacology Unit, Royal Bath Hospital, Harrogate, North Yorkshire HG1 2PS (U.K.)

and

D.B. GALLOWAY

Roche Products Limited, Welwyn Garden City, Hertfordshire (U.K.)

(First received March 2nd, 1984; revised manuscript received May 4th, 1984)

At least two methods for the determination of piroxicam [4-hydroxy-2-methyl-N-(2-pyridyl)-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide] concentrations in plasma using a high-performance liquid chromatographic (HPLC) technique have already been described [1, 2]. Piroxicam is an established non-steroidal anti-inflammatory drug (NSAID), and a new NSAID of similar chemical structure, tenoxicam {4-hydroxy-2-methyl-N-(2-pyridyl)-2H-thieno-[2,3-*e*]-1,2-thiazine-3-carboxamide 1,1-dioxide}, is currently undergoing clinical trials. We have previously reported a method for the determination of plasma concentrations of tenoxicam (Ro 12-0068) [3] which we have now modified so that the method can be used to measure either piroxicam or tenoxicam in plasma using the other as internal marker.

EXPERIMENTAL

Reagents and solvents

Acetonitrile and methanol (both HPLC grade) were obtained from Rathburn (Peebleshire, U.K.); dichloromethane (AnalaR) and monosodium phosphate from BDH (Poole, U.K.) and disodium phosphate (AnalaR) from Fisons

0378-4347/84/$03.00

(Loughborough, U.K.). For the preparation of aqueous solutions, single-distilled water was used.

Standard solutions

A stock solution of 1 mg ml^{-1} piroxicam (supplied by Pfizer) was prepared in methanol and stored in the dark at 4°C. This solution was prepared freshly each week. An aqueous dilution of 1:100 (10 $\mu g\ ml^{-1}$) was prepared daily and used for calibration purposes. Solutions of equivalent concentrations were prepared for tenoxicam (supplied by Roche) for use as internal marker.

Calibration procedure

Blank plasma samples (1 ml) were spiked with aqueous piroxicam in the concentration range 0.2—2.5 $\mu g\ ml^{-1}$ for single-dose studies or 1—20 $\mu g\ ml^{-1}$ for multiple-dose studies. Appropriate volumes of water were also added to ensure an equivalent total volume (e.g. 0.5 ml) was added in each case. Aqueous internal marker solution (100 μl) was added to every calibration and unknown sample, and all tubes were whirlymixed before extraction.

Extraction procedure

Water (0.5 ml) was added to 1 ml plasma in addition to 100 μl of internal marker for all unknown samples. All samples were acidified with 1 ml of 1 *M* hydrochloric acid, the mixture was whirlymixed and 8 ml dichloromethane were added. The stoppered tube was shaken for 3 min and centrifuged at 1800 *g* for 10 min at 10°C. The upper aqueous layer was then discarded and the organic layer decanted into a clean tube, blown dry with nitrogen at a temperature $\leqslant$ 35°C and the residue taken up into 0.5 ml of mobile phase [acetonitrile—water—0.1 *M* phosphate buffer pH 5.0 (30:30:40)].

Chromatography

The chromatographic system consisted of a Constametric III pump (Laboratory Data Control), Rheodyne injection system (20-μl loop) and LiChrosorb RP-18 column (150 × 3.2 mm I.D., steel), 5-μm particle size (Magnus Scientific). Detection was by means of a Spectromonitor III variable-wavelength dual-cell ultraviolet detector operated at 361 nm, coupled to a 308 computing integrator (both from Laboratory Data Control). A guard column (5 cm × 4.6 mm) of Whatman Co:Pell ODS was used between injector and analytical column. Samples could be automatically injected at 8-min intervals using an autosampler (Laboratory Data Control).

The mobile phase was degassed before being used at a flow-rate of 0.75 ml min^{-1}. The retention times of tenoxicam and piroxicam were about 2.3 and 4.5 min, respectively. If required, resolution could be improved, at the expense of increased retention times, by using buffer at pH 4 or by reducing the amount of acetonitrile to 25%.

Recovery and reproducibility

The percentage recovery of piroxicam was determined by comparing the extraction from plasma spiked with piroxicam (5 $\mu g\ ml^{-1}$) with the equivalent

concentration of standard solution in methanol blown down and redissolved directly into mobile phase. In both cases internal marker was added and blown down after removal of the final organic layer prior to addition of mobile phase. Reproducibility of the method could also be determined from the six duplicate results.

Application to plasma samples after a single oral dose of piroxicam (20 mg)

Three normal male volunteers received an oral dose of piroxicam (20 mg) at 09.00 a.m. after fasting from 22.00 hours the previous evening. Venous blood samples (10 ml) were taken via a standard BD cannula at 0, 1, 1.5, 2, 3, 4, 6, 8, 24, 48, 72, 96 and 168 h following the dose. The blood samples were placed in lithium heparin tubes, centrifuged (1800 *g* for 10 min) and the plasma transferred to a plain tube for storage at −20° C to await analysis.

Assay for plasma tenoxicam

When tenoxicam is measured and piroxicam is used as internal standard, then 400 μl of the 10 μg ml^{-1} aqueous solution of the latter are added to 1 ml of plasma. A calibration range of 0.5—6.0 μg ml^{-1} for single-dose studies and 2—30 μg ml^{-1} for multiple-dose studies are recommended. Recovery of tenoxicam was determined using the same approach as that used to measure piroxicam recovery.

RESULTS

Extraction of plasma blanks demonstrated that neither piroxicam nor the internal marker were subject to interference from co-extracted endogenous substances. The two peaks were well separated as illustrated in Fig. 1.

A reproducible, linear calibration was obtained for plasma piroxicam concentrations ranging from 0.2 to 20 μg ml^{-1}. Peak height ratios for a 0.2—2.5 μg ml^{-1} calibration curve carried out on five separate occasions by three different analysts are shown in Table I. Mean recovery of piroxicam was found to be 81 ± 3.0% S.D. Reproducibility of the assay was acceptable giving a coefficient of variation of 3.6%. Accuracy of determined piroxicam concentrations $>$ 0.6 μg ml^{-1} was $\leqslant$ ± 2.0% but deteriorated at lower concentrations, with a probable limit of quantitation around 0.1 μg ml^{-1}.

Application of the assay to plasma samples obtained from a single-dose kinetic profile demonstrated the successful use of the assay, and confirmed that metabolites of piroxicam did not interfere. The mean data from the three subjects are shown in Fig. 2. The plasma half-life of piroxicam for each subject was calculated using linear least-squares regression analysis and the mean result was 46.7 h. These profiles also demonstrated that this analytical method is sufficiently sensitive to measure piroxicam levels in plasma for at least 96 h after a single dose. It would therefore be suitable for monitoring pharmacokinetics in individual patients after single or multiple doses.

When the method was used for the measurement of tenoxicam a limit of quantitation similar to that of piroxicam was found. Mean recovery of tenoxicam was found to be 81 ± 7.9% S.D.

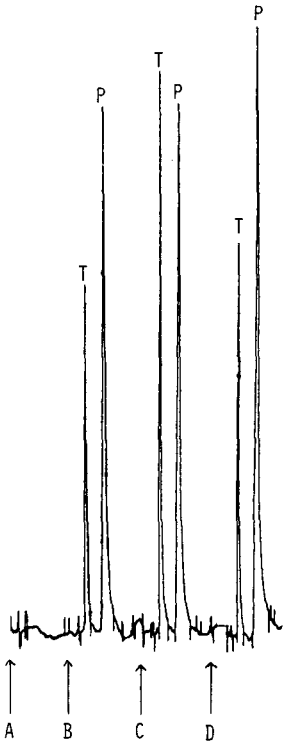


Fig. 1. Chromatograms showing: (A) blank plasma extract; (B) tenoxicam (1 μg ml^{-1}) and piroxicam (1 μg ml^{-1}) added to blank plasma; (C) sample from patient 4 h after receiving a single 20-mg oral dose of piroxicam, tenoxicam being used as internal marker; (D) sample from patient 3 h after receiving a single 20-mg oral dose of tenoxicam, piroxicam being used as internal marker. Peaks: T = tenoxicam, retention time 165 sec; P = piroxicam, retention time 370 sec.

TABLE I

PEAK HEIGHT RATIOS AND REGRESSION ANALYSIS FOR FIVE CALIBRATION CURVES

Calibration number	Analyst	Concentration of piroxicam (μg ml^{-1})								Correlation coefficient	Gradient	Intercept
		0.2	0.4	0.6	0.8	1.0	1.5	2.0	2.5			
1	A	0.095	0.241	0.379	0.563	—	1.014	1.415	1.720	0.999	0.711	0.038
2	A	—	0.241	0.322	0.478	0.632	0.978	1.369	1.697	0.999	0.709	—0.076
3	B	0.121	0.214	—	0.427	0.571	0.924	1.195	1.627	0.997	0.651	—0.055
4	C	0.137	0.273	0.436	0.584	0.678	1.070	1.421	1.830	0.999	0.728	—0.016
5	C	0.182	0.337	0.407	0.506	0.690	0.998	1.474	1.800	0.996	0.713	—0.003

DISCUSSION

This assay provides a rapid, sensitive and reproducible method for the quantitative analysis of either tenoxicam or piroxicam in plasma. The method allows for marginally more sensitive and more accurate measurement of piroxicam than the method of Riedel and Laufen [2]. The latter method involves sophisticated automation and hence rapid sample throughput, whereas our own laboratory uses readily available, standard HPLC equipment. Nevertheless seventy samples can readily be extracted and chromatographed in a 24-h

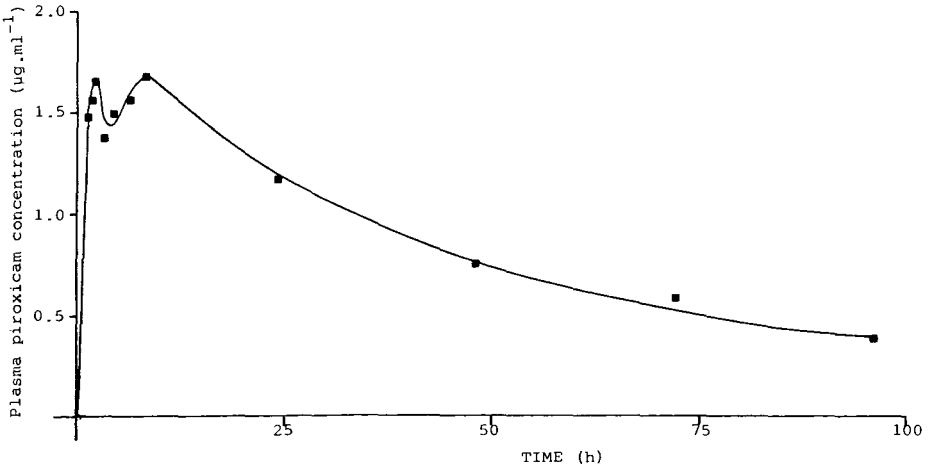


Fig. 2. Plasma concentrations of piroxicam after a 20-mg single oral dose (mean data for three subjects).

cycle. Our method also compares favourably with the previous HPLC method of Twomey et al. [1], though their lower limit of quantitation is 0.5 μg ml^{-1} compared with 0.2 μg ml^{-1}. A third HPLC method for the determination of piroxicam has been described [4], but full validation details were not given.

The principal advantage of this method is the use of tenoxicam as internal marker, readily allowing for the adaptation of the method for measuring either drug. The third oxicam non-steroidal anti-inflammatory drug undergoing clinical trials is isoxicam and this drug can also be detected by this assay technique, though the assay characteristics for this drug have not been fully validated. In conclusion this method provides an alternative assay for the determination of piroxicam and also illustrates the value of choosing an internal marker which is also of quantitative interest.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. J.G. Allen for helpful discussion, Mrs. K.E. Surrall and Mrs. C. Astbury for technical assistance, and Mrs. R.H. Schofield for secretarial assistance. We also wish to thank Pfizer for supplies of piroxicam and Roche Products for supplies of tenoxicam.

The Clinical Pharmacology Unit acknowledges the financial support of Roche Products.

REFERENCES

1 T.M. Twomey, S.R. Bartolucci and D.C. Hobbs, J. Chromatogr., 183 (1980) 104.
2 K.-D. Riedel and H. Laufen, J. Chromatogr., 276 (1983) 243.
3 M.E. Pickup, J.R. Lowe and D.B. Galloway, J. Chromatogr., 225 (1981) 493.
4 P. Schiantarelli, D. Acerbi and G. Bovis. Arzneim.-Forsch., 31 (1981) 92.

Related articles published in *Journal of Chromatography*, Vols. 295—298

Volume 295

Volume 296

Volume 297

Volume 298

Author Index

Subject Index

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	S	O	N
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	297 298/1 298/2 298/3	299/1 299/2 301/1	301/2 302 303/1	303/2
Chromatographic Reviews		300/1					300/2					300/3
Bibliography Section		304/1	304/2			304/3					304/4	304/5
Biomedical Applications		305/1	305/2	306	307/1	307/2	308	309/1	309/2	310/1	310/2	311/1 311/2

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 295, No. 2, pp. 555-558. 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.

EVALUATION OF ANALYTICAL METHODS IN BIOLOGICAL SYSTEMS

Part A: Analysis of Biogenic Amines

edited by GLEN B. BAKER *and* RONALD T. COUTTS, *Neurochemical Research Unit, Department of Psychiatry, and Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Canada*

TECHNIQUES AND INSTRUMENTATION IN ANALYTICAL CHEMISTRY, 14

This is the first volume of a new multi-volume work entitled "Evaluation of Analytical Methods in Biological Systems" that will evaluate the various analytical techniques and approaches that can be used in a particular bioanalytical field. This volume provides an overview of techniques for the analysis of catecholamines, 5-hydroxytryptamine (serotonin), 'trace amines' and histamine in biological systems.

The authors describe the basic principles underlying the various techniques and discuss their advantages and disadvantages relative to other available methods, thus making this a valuable reference both for those experienced in analysis of biogenic amines who are considering using a new technique, and to those entering the field for the first time.

1982 xvi + 308 pages
US $ 72.25 (In USA & Canada); Dfl. 170.00 (Rest of World)
ISBN 0-444-42110-6

ELSEVIER

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

P.O. Box 1663, Grand Central Station, New York, NY 101t

The Dutch guilder price is definitive. US $ prices are valid only in the USA & Canada.