VOL. 309 NO.1 JULY 13, 1984

(Biomedical Applications, Vol. 34, No. 1)

JURNAL OF CHROMATOGRAPHY BIOMEDICAL APPLICATIONS

ELSEVIER

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

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 Submission of an article is understood to imply that the article is original and unpublished and is not being considered for publication elsewhere.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

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

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

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

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

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

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

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

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

0378-4347/84/$03.00

Printed in The Netherlands

(Biomedical Applications, Vol. 34, No. 1)

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)

Contents (continued)

Notes

JOURNAL OF CHROMATOGRAPHY

VOL. 309 (1984)

(Biomedical Applications, Vol. 34)

JOURNAL *of* CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

BIOMEDICAL APPLICATIONS

Vol. 34 1984

ELSEVIER
AMSTERDAM — OXFORD — NEW YORK — TOKYO

0378-4347/84/$03.00

Printed in The Netherlands

Journal of Chromatography, 309 (1984) 1—15
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2112

REVIEW

SEPARATION OF OESTROGEN CONJUGATES IN URINE AND SYNTHETIC MIXTURES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS

R.P. AGER and R.W.A. OLIVER*

Department of Biochemistry, University of Salford, Salford M5 4WT (U.K.)

(First received October 11th, 1983; revised manuscript received February 17th, 1984)

CONTENTS

1. INTRODUCTION

The assay of oestrogens in biological fluids has a number of clinical applications, the chief one being the determination of oestrogen levels in maternal urine (or blood) in order to aid the assessment of the intrauterine viability of the foetus [1]. As a result of the importance of these applications, there now exists such a considerable literature on the subject of the methodologies available for this assay that it is being reviewed annually by one of us [2]. The most commonly determined analyte is the 24-h urinary total oestrogen excretion level and this has been statistically correlated with week of gestation for "normal" pregnancies [3] leading to the production of reference ranges

0378-4347/84/$03.00

suitable for clinical use. In our latest paper [4] on the subject of this assay we listed a number of the analytical difficulties experienced with it. Now, it should be noted that whilst the oestrogens are excreted almost totally as conjugates, they are normally assayed as free oestrogens (produced by a suitable hydrolysis procedure) which assists the analyst by reducing the molecular complexity of the oestrogens in the biological matrix.

There have been a number of detailed studies of the contributions made by the various oestrogens to 24-h urinary total oestrogen excretion rates. As a result of these investigations it is generally agreed that, for the three classical oestrogens (oestrone, oestradiol and oestriol), oestriol accounts for more than 90% of their total excretion rate in late human pregnancy [5]. Moreover, a recent Belgian study [6] has shown that this total oestriol excretion level is made up of contributions from the following four conjugates whose approximate percentages (in oestriol equivalents) are given in parentheses: oestriol-16-glucuronide (67%); oestriol-3-glucuronide (21%); oestriol-3-sulphate-16-glucuronide (8%) and oestriol-3-sulphate (3%)*. It should perhaps be noted that less than 1% of the total oestriol excretion rate was ascribed to the free steroid. It is logical to predict that analysis of the individual intact oestrogen conjugates instead of total oestrogens would give results having a higher clinical efficiency (i.e. fewer false positive and false negative results) for present applications of oestrogen assays. Further, such multicomponent analysis (*complete* oestrogen conjugate profiling) might also prove of value in the diagnosis of additional abnormal conditions. At present the only published approach to this problem of multicomponent analysis of complex biological fluids has involved the use of the sophisticated, expensive and time-consuming technique of combined gas chromatography—mass spectrometry. Before this analytical technique can be employed, the conjugates have first to be chromatographically separated, then hydrolysed to form free oestrogens which are then silylated in order to make suitable volatile derivatives [7]. This type of method is clearly unsuitable for general routine clinical use.

In contrast, the modern technique of high-performance liquid chromatography (HPLC) has the ability to separate and quantitate complex aqueous mixtures in short periods of time (min), with little or no pre-treatment of sample, and is, therefore, easily automated for routine use. Theoretically, it should be possible to devise an HPLC system which can separate the four major oestriol conjugates of pregnancy urine listed above. If this *partial* oestrogen conjugate profiling and individual assay could be achieved, then the routine assay of the total oestriol content would follow and the problems of poor precision encountered with the present common methods (for total oestrogens) [8] would be obviated. For these reasons, we have closely examined the published literature [2] on the subject of HPLC analysis of the oestrogen conjugates in general and of the above four oestriol conjugates in particular in order to determine if a system suitable for routine use has yet been published. Further, we have abstracted the key experimental details in a unified manner (Tables 1—3) to aid assessment of the present state of the

---

*These four oestriol conjugates are abbreviated to E_3-16-G, E_3-3-G, E_3-3-S-16-G and E_3-3-S, respectively, for the remainder of the paper.

art and hence to identify possible areas for improvement. The results of these combined bibliographic—abstracting studies will now be presented and discussed.

2. SURVEY OF REPORTED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATIONS OF OESTROGEN CONJUGATES

Tables 1—3 contain the key experimental details for a total of eighteen HPLC separations of one or more of the four oestriol conjugates discussed above (and of certain other oestrogen conjugates) abstracted from a total of fourteen papers. The elution order data shown in the tables is taken from chromatograms presented by the various authors. The separations are divided into three tables so as to group them according to the apparent mechanism of separation. The papers are arranged in chronological order within each table and each of these will now be discussed in turn.

2.1. Anion-exchange chromatography

Table 1 summarizes the experimental details, abstracted from four publications [9—12], of six separations of mixtures of oestrogen conjugates obtained using columns of appropriate support materials. The latter may be divided into two types: the first being formed of chemically modified celluloses (Table 1, refs. 9, 10 and 11); the second is Partisil SAX (Table 1, ref. 12), which is a silica gel bonded with a quaternary ammonium functional group.

A study of the elution order data given in Table 1 shows that all of these anion-exchange columns can indeed separate mixtures of oestrogen conjugates. Each of the synthetic mixtures studied contained at least one of the three oestriol mono-conjugates of interest, but unfortunately none of them contained the mixed di-conjugate E_3-3-S-16-G, probably because it is not commercially available. However, theoretically there should be no difficulty in separating this di-conjugate from the three mono-conjugates by anion-exchange chromatography because it should elute much later than any of the latter. Experimental evidence indicating the validity of this statement is afforded by the numerical anion-exchange chromatographic data for the di-conjugate, oestradiol-3-sulphate-17-glucuronide (see Table II of ref. 11).

We will now consider the separations achieved with these two types of anion-exchange material starting with the most extensively studied, the modified celluloses. Of the five separations of oestrogen conjugates shown in Table 1 using columns of these support materials [Table 1, refs. 9, 10 and 11], all were operated at relatively low eluent pressures because of the non-rigid nature of the support and this led to the generally long elution times listed in the table. The highest elution pressure employed with these columns (36 bar) was achieved by the expedient of mixing a (rigid) diatomite powder with the cellulose. A study of the separations reported in ref. 11 leads to the conclusion that all four oestriol conjugates under discussion should be separable from aqueous synthetic mixtures using anion-exchange cellulose columns operated under HPLC conditions. Indeed, a chromatogram presented in this paper, and abstracted in Table 1, probably does show separation of these four oestriol

TABLE 1

SEPARATION OF OESTROGEN CONJUGATES BY ANION-EXCHANGE CHROMATOGRAPHY

	Ref. 9	Ref. 10
Sample	Synthetic	Synthetic
Column		
Support	ECTEOLA-cellulose (Baker 300)	ECTEOLA-cellulose (Baker 300)—diatomite (5:1, v/v)
Particle diameter (μm)	13	7 for Baker 300
Length (cm)	25	10
I.D. (mm)	3	4
Temp. (°C)	25	70
Elution		
Pressure (bar)	NG*	36
Flow-rate (ml/min)	NG	NG
Mobile phase	0.125 *M* sodium chloride + 0.05 *M* sodium acetate, pH 5.0	0.025 *M* perchlorate + 0.01 *M* phosphate, pH 6.8
Detection	UV, 220 nm	UV, 220 nm
Elution time** (min)	14	28
Elution order***	E_3-3-G, E_1-3-G, E_2-3-G, E_3-16-G, E_2-17-G	E_1-3-G, E_3-3-S, E_1-3-S, 17α-E_2-3-S, 17α-Eq-3-S, Eqe-3-S, 17α-Eqe-3-S

*NG = not given.
**Elution time is the time taken for the last oestrogen to elute off the column.
***E_1 = oestrone, E_2 = oestradiol, E_3 = oestriol, Eq = equilin, Eqe = equilenin, G = glucuronide, S = sulphate.

conjugates since the sample applied was an Amberlite XAD-2 methanol extract of pregnancy urine. Six Kober-positive (i.e. oestrogenic steroids) peaks were obtained after an elution time of 75 min, four of which may be ascribed to the four oestriol conjugates. The two additional peaks could possibly be due to oestrone-3-glucuronide and 16-hydroxyoestrone-3(?16α)-glucuronide as these are excreted at comparable rates to the oestriol sulphate-glucuronide [13]. The finding of fourteen low ultraviolet- (UV)-absorbing peaks (220 nm) from the same urine sample in the same time period indicates that the modified cellulose columns are capable of considerable separation of multicomponent mixtures such as urine.

Interestingly, from a combined study of the abstracted elution order data in Table 1 and retention data for similar systems reported in the corresponding references, it is found that the elution order of oestrogen mono-glucuronides from cellulose anion-exchangers is primarily dependent on the site of conjugation since the steroid A-ring conjugated oestrogens tend to elute before those conjugated at the steroid D-ring. (It should perhaps be noted that the same

Ref. 11 (i)	Ref. 11 (ii)	Ref. 11 (iii)		Ref. 12
Synthetic	Synthetic	Pregnancy urine XAD-2 extract		Synthetic
ECTEOLA-cellulose (Baker 300)	ECTEOLA-cellulose (Whatman ET 41)	ECTEOLA-cellulose (Baker 300)		μPartisil 10 SAX
13	11	11		10
25	25	25		25 × 2
3	3	3		4.6
25	70	70		NG
16	30	20		83
NG	NG	NG		0.8
0.025 *M* perchlorate + 0.01 *M* phosphate, pH 7.0	0.25 *M* perchlorate + 0.01 *M* phosphate, pH 8.5	0.025 *M* perchlorate + 0.01 *M* phosphate, pH 6.8		0.1 *M* sodium chloride, pH 4.8
UV, 220 nm	UV, 220 nm	UV, 220 nm	Kober, fluorimetric	UV, 254 nm
25	31	75	75	23
E_3-3-G, E_1-3-G, E_2-3-G, E_3-16-G, E_2-17-G	E_3-3-S, E_1-3-S, Eq-3-S, 17α-Eq-3-S, Eqe-3-S, 17α-Eqe-3-S	14 peaks None of the peaks identified	6 peaks	(E_3-16-G, E_3-17-G), E_3-3-G, E_2-17-G, E_2-3-G, E_1-3-G, E_2-3-S, E_1-3-S

order of elution for these conjugates has been reported for a non-HPLC separation using a modified cross-linked dextran as the anion exchanger [7].) Further, within the group of oestrogen ring-A glucuronides the elution order is dependent upon the exact nature of the oestrogen moiety and is as follows: oestriol, oestrone, oestradiol. In addition, both of these types of relationship between structure and elution order are also found to hold for the series of classical oestrogen mono-sulphates [11]. Oestrogens conjugated with sulphate groups are invariably eluted after the corresponding glucuronide conjugates. To summarize, the expected elution order of the four oestriol conjugates under discussion from the modified cellulose anion-exchangers given in Table 1 is E_3-3-G, E_3-16-G, E_3-3-S, E_3-3-S-16-G.

The second type of anion-exchange chromatographic system abstracted in Table 1 consists of a single separation of a synthetic mixture of oestrogen conjugates obtained by the use of two columns of Partisil SAX arranged in series [12]. The mixture separated only included two of the four oestriol conjugates of interest (both glucuronides). Interestingly, a study of the

TABLE 2

SEPARATION OF OESTROGEN CONJUGATES BY ION-PAIR CHROMATOGRAPHY

	Ref. 14	Ref. 15 (i)	Ref. 15 (ii)	Ref. 16	Ref. 17
Sample	Synthetic	Synthetic	Synthetic	Synthetic	Pregnancy urine XAD-2 extract
Column					
Support	LiChrospher Si 100 (coated with 0.1 *M* TEABr*, pH 7.4)	LiChrosorb RP-18	LiChrosorb RP-18	LiChrosorb RP-18 (coated with *n*-pentanol)	Hypersil ODS
Particle diameter (μm)	10	5	5	10	5
Length (cm)	NG**	15	15	15	12.5
I.D. (mm)	NG	3	3	3.2	4.5
Temp. (°C)	25	70	70	25	NG
Elution					
Pressure (bar)	30	250	250	23	NG
Flow-rate (ml/min)	NG	NG	NG	0.27	1
Mobile phase	Dichloromethane—*n*-pentanol (9:1, v/v) saturated with TEABr	0.05 *M* phosphate pH 8.0, + 0.1% (w/v) CTMABr***—acetonitrile (6:4, v/v)	0.02 *M* phosphate pH 5.0, + 0.1% (w/v) CTMABr—methanol (3:7, v/v)	Sodium phosphate buffer, ionic strength 0.1, pH 6.4, saturated with *n*-pentanol + 0.041 *M* TPABr§	0.05 *M* phosphate pH 8.0, + 0.1% (w/v) CTMABr—methanol (5:5, v/v)
Detection	UV, 254 nm	UV, 223 nm	UV, 223 nm	UV, 254 nm	UV, 220 nm
Elution time§§ (min)	6	2	4	20	13 (for E_3-16-G)
Elution order§§§	E_2-3-S, E_2-17-S, E_3-17-S, E_3-3-S	E_3-3-G, E_3-17-G, E_2-17-G, E_1-3-G, E_3-3-S	E_3-3-S, E_2-17-S, E_2-3-S, 17α-Eqe-3-S, E_1-3-S	E_3-17-G, E_1-3-G, E_2-3-G, E_2-17-G	E_3-3-G, E_3-16-G, resolved from 7 other (unidentified) peaks

*TEABr = tetraethylammonium bromide.

**NG = not given.

***CTMABr = cetyltrimethylammonium bromide.

§TPABr = tetrapropylammonium bromide.

§§Elution time is the time taken for the last oestrogen to elute off the column.

§§§E_1 = oestrone, E_2 = oestradiol, E_3 = oestriol, G = glucuronide, S = sulphate.

tabulated chromatographic data for oestrogen mono-glucuronides shows that the specific nature of the oestrogen moiety is the major determinant of the elution order since these conjugates are eluted in the general order oestriol, oestradiol, oestrone. This is in contrast to the aforementioned finding for separations performed using columns of modified celluloses as for these the site of conjugation is of primary importance. Further, even the elution order of the two oestriol glucuronides of interest from Partisil SAX differs from that obtained using the modified cellulose anion exchangers; i.e. E_3-16-G is eluted before E_3-3-G. But in all of the anion-exchange systems it is found that glucuronides are eluted before the corresponding sulphates.

2.2. Ion-pair chromatography

Table 2 summarizes the experimental details, abstracted from four publications [14—17], of five separations of mixtures of oestrogen conjugates obtained using columns operated in ion-pair chromatographic modes. We shall first consider the three separations abstracted in Table 2 involving the use of columns of octadecyl-silica support materials and cetyltrimethylammonium bromide (CTMABr) as the ion-pair forming surfactant. The table of abstracted data shows that these systems separate synthetic and natural mixtures of oestrogen conjugates in much shorter periods of time (2—13 min) than that required for the previous group of separations carried out using modified celluloses. This finding is a direct consequence of the use of modern rigid support materials which can be operated at elution pressures about an order of magnitude greater than those compatible with the modified cellulose columns.

Considering now the application of these chromatographic systems to the problem of separating the four oestriol conjugates under discussion, a study of Table 2 shows that only synthetic mixtures containing a maximum of two of these conjugates have been studied. The first of these separations abstracted in the table shows that it is possible to separate E_3-3-G, oestriol-17-glucuronide (E_3-17-G) and E_3-3-S from a synthetic mixture within 2 min. The paper from which this is abstracted is ref. 15, and a detailed study of this paper showed that it contains numerical chromatographic data (Table III of ref. 15) which indicate that E_3-17-G co-elutes with E_3-16-G. Thus, it is predicted that an aqueous synthetic mixture of E_3-3-G, E_3-16-G and E_3-3-S would be separated, in this order, within 2 min by this chromatographic system. Interestingly, in the application of this type of high-speed separation to the analysis of pregnancy urine [17], two of the nine UV-absorbing chromatographic peaks eluted within 13 min were assigned to E_3-3-G and E_3-16-G (see Fig. 1). A comparative study of the elution order data for the chromatographic systems abstracted in Table 2 that employ CTMABr leads to the following three correlations. First, that the oestriol glucuronides are eluted before the oestriol sulphates and, secondly, that oestriol ring-A conjugates are eluted before the ring-D conjugated isomers. Additional support for these elution orders is afforded by the tabulated numerical data given in ref. 15. Thirdly, this same tabulated data also indicate that, within the two groups of classical oestrogen mono-sulphates and mono-glucuronides, the elution order is primarily

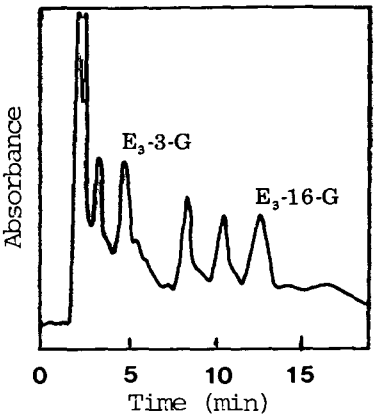


Fig. 1. HPLC chromatogram of a pregnancy urine extract. Re-drawn from Fig. 2. of ref. 17.

TABLE 3

SEPARATION OF OESTROGEN CONJUGATES BY REVERSED-PHASE CHROMATOGRAPHY

	Ref. 18	Ref. 15 (iii)	Ref. 19
Sample	Synthetic	Synthetic	Pregnancy urine
Column			
Support	μBondapak C_{18}	LiChrosorb RP-18	LiChrosorb RP-8 (coated with *n*-pentanol)
Particle diameter (μm)	10	5	5
Length (cm)	30	15	15
I.D. (mm)	6.4	3	4.5
Temp. (°C)	30.5	70	25
Elution			
Pressure (bar)	NG*	250	NG
Flow-rate (ml/min)	0.89	NG	NG
Mobile phase	H_2O—methanol (9.34:0.66, w/w)	0.05 *M* phosphate, pH 8.0—acetonitrile (8:2, v/v)	Phosphate buffer, ionic strength 0.1, pH 6.5—*n*-pentanol (9.81:0.19, v/v)
Detection	Refractive index	UV, 220 nm	UV, 280 nm
Elution time*** (min)	(34 or 54)?	3	13 (for E_3-16-G)
Elution order§	E_3-3-G, E_3-3-S, E_3-16-G	E_3-3-G, E_3-3-S, E_3-17-G, E_2-3-G, E_1-3-G	E_3-16-G resolved from 12 other (unidentified) peaks

*NG = not given.
**THF = tetrahydrofuran.
***Elution time is the time taken for the last oestrogen to elute off the column.
§E_1 = oestrone, E_2 = oestradiol, E_3 = oestriol, G = glucuronide, S = sulphate.

determined by the exact nature of the oestrogen moiety and is oestriol, oestradiol, oestrone.

The remaining two separations given in Table 2 [14, 16] are of synthetic mixtures of oestrogens reported in two separate papers and obtained using columns of 10-μm support materials and tetraethylammonium bromide and tetrapropylammonium bromide as the ion-pair reagents. The first paper reports a separation involving an oestriol conjugate of direct interest, namely E_3-3-S, and the second paper reports a separation of E_3-17-G. In view of the previous discussion of separations involving this type of chromatography, it would seem likely that this second separation would also have separated an oestriol conjugate of interest (E_3-16-G) had it been present in the test mixture.

2.3. Reversed-phase chromatography

Table 3 summarizes the experimental details, abstracted from seven publications [15, 18—23], of separations of mixtures of oestrogen conjugates obtained

Ref. 20	Ref. 21	Ref. 22	Ref. 23
Pregnancy urine (diluted)	Synthetic	Pregnancy urine (+ internal std., 16-epiE_3-17-G) extract	Synthetic
Spherisorb ODS	LiChrosorb RP-18	TSK gel LS -410 ODS-SIL	TSK gel LS-410 ODS-SIL
5	5	5	5
1.5	25	30	30
6.4?	9	4	4
21	NG	Ambient	Ambient
NG	172	NG	NG
0.4	2	1	1.5
Water—acetonitrile—acetic acid (26.5:6:2, w/w)	Convex gradient: 10% methanol in 0.01 *M* ammonium acetate, pH 6.9, to 100% methanol	0.05 *M* Na_2HPO_4, pH 3.0—THF** (6:1, v/v)	0.05 *M* Na_2HPO_4, pH 3.0—THF (6:1, v/v)
Kober, fluorimetric	UV, 280 nm	Electrochemical	UV, 280 nm
22	38	56 (16-epiE_3-17-G)	61
E_3-3-S?, E_3-3-G?, E_3-16-G	E_3-3-G, E_3-3-S, E_3-16-G, resolved from 12 other oestrogen peaks	E_3-16-G 16-epiE_3-17-G, resolved 6 other (unidentified) peaks	E_3-3-G, 16-epiE_3-3-G, E_3-17-G, E_3-16-G, 16-epiE_3-17-G, 16-epiE_3-16-G

using reversed-phase column chromatographic methods. As was found to be the case with all the previously discussed separations, none of the synthetic mixtures chromatographed included all four oestriol conjugates of interest. However, the first and the fifth of the separations listed in this section included all but the mixed oestriol di-conjugate. The two separations performed using columns of TSK gel at a pH of 3.0 [22, 23] are superior to those discussed previously in that they are able to separate E_3-16-G from E_3-17-G when both are present in a synthetic mixture or biological fluid, albeit after a long elution time (about 30—45 min). Indeed, the authors of one of these two abstracted papers [22] used this HPLC system to confirm that human pregnancy urine does not contain E_3-17-G, whilst bile from rats fed large amounts of oestriol was shown to contain significant quantities of both mono-conjugates. The resolution of the TSK gel column with respect to the separation of these isomeric oestriol glucuronides progressively decreased as the pH was raised [23]. Another separation of human urine performed on a reversed-phase column and abstracted in Table 3 (ref. 19) gave a complex UV chromatogram with a total of thirteen peaks resolved in 13 min, one of which was identified by the author as E_3-16-G (see Fig. 2). Again, the production of such a complex chromatogram indicates the high resolving power of these columns.

The second of the reversed-phase separations listed in Table 3 is a high-speed separation of three oestriol conjugates from a synthetic mixture of five oestrogen conjugates, achieved in approximately 1 min within a total elution time of only 3 min. This separation may be compared in terms of order of elution with the two rapid separations abstracted in Table 2 from the same paper and with the numerical chromatographic data which were also reported. When this was done it shows that the elution order of the conjugates off the reversed-phase column in the absence of CTMABr is E_3-3-G, E_3-3-S, E_3-17-G.

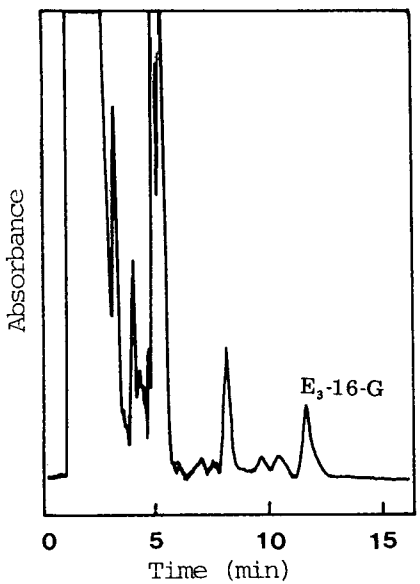


Fig. 2. HPLC chromatogram of pregnancy urine. Re-drawn from Fig. 4 of ref. 19.

This elution order differs from that found in the presence of the surfactant, which is E_3-3-G, E_3-17-G, E_3-3-S. (In both chromatographic systems the isomeric 16- and 17-glucuronides co-elute.) This difference in elution order of the oestriol conjugates means that the first of the correlations between elution order and molecular structure found for the previous type of chromatography does not apply to the present reversed-phase system. However, a study of the numerical elution order data for the reversed-phase system shows that the other two correlations are still applicable.

3. DISCUSSION ON THE APPLICATION OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY TO THE ROUTINE ASSAY OF OESTRIOL CONJUGATES

Although we are aware that there have been a number of reviews published recently which contain sections on the HPLC of oestrogen conjugates [24—26], the present account is considered distinctive because of the methodological details given in Tables 1—3 and for its delineation of the problem of routine analysis of the four major oestrogen conjugates present in human pregnancy urine (*partial* oestrogen conjugate profiling). To proceed, we shall first discuss the recent work of an American group which has devised a complex, multicolumn HPLC separation system for an extensive oestrogen conjugate profile (together with free oestrogens) of pregnant monkey urine [21]. The initial chromatographic stage in this system involves the use of a LiChrosorb RP-18 column operated in a convex gradient elution mode (Table 3, ref. 21). The resultant eluate was collected in six fractions which were evaporated to near dryness, reconstituted in the appropriate mobile phase and aliquots then subjected to further chromatography involving selective use of one of four HPLC systems. Even though synthetic mixtures of E_3-3-G, E_3-3-S and E_3-16-G can be separated by this method, we believe that such a complex analytical system is not suitable for routine clinical use and it will not therefore be considered further.

In addition to the above complex, multicolumn separation, another American research group published earlier (1978) a simple isocratic HPLC system identical to that given in Table 1 (ref. 12), in which seven oestrogen conjugates (from a total of eight) were separated from a spiked urine extract. Indeed, in the list of oestrogens separated were two of the oestriol conjugates of interest, namely E_3-16-G and E_3-3-G. Unfortunately, the authors did not demonstrate any application of this method to the analysis of human pregnancy urine. Consequently, it is not immediately clear whether this method would be suitable for adoption by clinical laboratories. However, upon further study of the paper it became apparent that the method possesses a certain practical disadvantage which would seem to rule out its routine application, namely corrosion of the steel column and tubing by the sodium chloride in the eluent.

In the discussion now to follow, attention will be concentrated on those five papers in which one (E_3-16-G) or more of the oestriol conjugates have been separated from human pregnancy urine, in order to ascertain if any of the published methods could be recommended as a candidate for adoption as a routine assay procedure. Complete details of the HPLC methods employed

have been abstracted and are given in Tables 1—3. These methods may be divided into two groups; namely, those in which the urine, either neat or diluted, is applied directly to the column (two methods), and those in which the urine is pre-treated before HPLC (three methods). If it is assumed that the various HPLC separations are equivalent, then, from the point of view of suitability as a routine assay procedure, the former methods are preferred and these will first be discussed in detail below.

The method to be initially considered is that published by Hermansson in 1980 [19]. In this procedure, 20 μl of untreated pregnancy urine were applied directly to a reversed-phase column (LiChrosorb RP-8) coated with *n*-pentanol by the expedient of using 1.9% (v/v) of this alcohol in phosphate buffer, pH 6.5, as the mobile phase. The resultant complex chromatogram (Fig. 2) obtained by using a UV detector operating at 280 nm included a well resolved peak, elution time about 13 min, which was assigned, on the basis of a co-chromatographic and mass spectroscopic study, to the most abundant oestrogen conjugate E_3-16-G. Apparently, therefore, the problem of measuring this conjugate routinely by HPLC would seem to be solved. However, when this method was further investigated in the authors' laboratory it became clear that the separation, in terms of elution time, is critically dependent upon the column temperature and is of limited use for studying pregnancy urines because of its low sensitivity [27]. Further, when an attempt was made to improve the latter by changing the monitoring wavelength from 280 to 215 nm, the previously clearly separated E_3-16-G peak became part of a new large non-resolved low UV-absorbing peak profile. For these reasons it is concluded that this method is not suitable for the routine assay of the E_3-16-G content of human pregnancy urine.

The second reported HPLC separation of oestriol conjugates present in human urine is that published in short note form by Keravis and Durand in 1980 [20]. In this method, a small aliquot of a diluted urine specimen is applied to a short column of Spherisorb ODS which is then eluted with a water—acetonitrile—acetic acid mixture. The eluate is then subjected to an on-line Kober fluorimetric procedure which leads to the production of a simple, completely resolved chromatogram containing a total of only three peaks which can be assigned to the following oestriol conjugates: E_3-16-G, E_3-3-S, E_3-3-G. Unfortunately, because of the brevity of the paper, this assignment of the peaks in the chromatogram and the exact chromatographic conditions are somewhat ambiguous (as indicated in Table 3). Further, the authors appear only to have measured E_3-16-G in the urine specimens studied even though they should have been able to measure all of the three conjugates listed above. Primarily, therefore, for these reasons this method cannot at present be recommended for routine HPLC assay of these oestriol conjugates in pregnancy urine.

The first of the HPLC studies of pre-treated pregnancy urines to be considered is that reported very recently by Shimada et al. [22]. In this paper the Japanese workers adopted the following complex pre-treatment procedure: first the conjugates present in samples of late-pregnancy urine are adsorbed onto a column of Amberlite XAD-2 resin which is then washed with water, eluted with methanol and the resultant eluate is evaporated to dryness and the

residue reconstituted in a small (?) volume of methanol, which is then subjected to ion-exchange chromatography using a column of modified Sephadex (PHP-LH-20). The oestrogen conjugates were removed from this column by eluting with 2.5% ammonium carbonate in 70% methanol and this fraction was evaporated to dryness before reconstitution in methanol. An aliquot of the resultant solution was then subjected to HPLC under the conditions tabulated in Table 3. The predominant oestriol conjugate, E_3-16-G, was separated after an elution period of some 45 min. When samples of the pure mono-glucuronide were added to non-pregnancy urines, which were then analysed by the above method, recovery figures of about 80% were found. It should be noted that this method involved the use of an electrochemical detector; it does not, therefore, detect any of the other oestriol conjugates of interest since these molecules do not contain the requisite free phenolic group. It is not possible to state if this method separates more of the oestriol conjugates from urine until the experiments have been repeated using a suitable UV detector. The present authors have not performed these repeat experiments primarily because we are of the opinion that the complex sample pre-treatment procedure, together with its long HPLC elution time, rules out the adoption of this method for routine use.

The next paper to be considered in which oestriol conjugates have been separated from pregnancy urine is that reported by Van der Wal and Huber [11]. This was published in 1977 and details of the separation are given in Table 1. A similar XAD-column—methanol extraction procedure was applied to the urine as in the previously discussed study. Now none of the peaks in the complex chromatograms, obtained using either a UV monitor or a Kober fluorimetric monitor, were identified. However, the authors indicated that they were continuing to work on the separation in order to develop a routine procedure for oestrogen conjugate urinary profiles and they were therefore contacted with a request for information on the progress of their studies. As a result of this request we were kindly sent a copy of Van der Wal's thesis [28] which contains a final 18-page chapter entitled "Rapid determination of estrogen conjugate profiles in human pregnancy urine. Preliminary report". A careful study of this section of the thesis unfortunately leads essentially to the same conclusion as before, namely, that none of the peaks in the complex chromatogram, obtained using an on-line Kober fluorimetric monitor, had been identified. It was also found that, in order to obtain these chromatograms reproducibly, the urine samples had first to be subjected to an XAD-column—methanol extraction pre-separation process. However, the chromatograms shown provide evidence that differing oestrogen profiles occur during a pregnancy and also that "various disorders" appear to affect these urinary steroid profiles. This finding supports the statement made in the Introduction that oestrogen conjugate profiling might prove of value in the diagnosis of abnormal clinical conditions. To conclude this discussion of these Dutch studies, it should be noted that it is now not possible to repeat these HPLC separations since the ECTEOLA-cellulose column support materials employed (Whatman ET-41 and Baker 300) are no longer commercially available [25].

The final paper to be considered is that published by Dixon et al. [17], details of which are given in Fig. 1 and the last column of Table 2. A study of

this paper showed that the urine specimens were subjected to an XAD-2 column—modified methanolic extraction procedure, before injection onto a Hypersil ODS column and subsequent ion-pair chromatography. The major modification of the extraction of the adsorbed conjugates is a final elution of the XAD-2 column with a 60% methanol aqueous mixture to produce a solution which is said to give a less complicated chromatogram than that obtained if pure methanol is employed as eluent. There are a number of favourable features of this published study which, incidentally, was stated by the authors to be a preliminary one. First, the two major oestriol conjugates, E_3-16-G and E_3-3-G, have been rapidly separated and identified by co-chromatography and disappearance following enzymatic hydrolysis. The concentration levels of the former conjugate were quantified using a UV monitor (at both 220 and 278 nm) and also using an electrochemical monitor. Further, unlike the previously discussed rapid HPLC method of Hermansson [19], this type of method is not markedly affected by temperature changes [15]. Because of these advantages, we are of the considered opinion that this method is indeed a promising candidate method for the rapid, routine assay of at least the oestriol mono-glucuronides. Clearly, further detailed studies have yet to be performed in order to validate this recommendation involving the assay of large numbers of samples and leading to statistical estimates of both the precision of the method and correlation with a currently widely accepted method. Such studies are currently underway in our laboratories and the results will, we hope, form the subject of a subsequent publication on the routine HPLC analysis of oestriol conjugates in human pregnancy urine.

4. ACKNOWLEDGEMENTS

These studies form part of a long-term research programme on the analysis of oestrogens in human pregnancy which has been generously supported by the Humane Research Trust, the Heinz and Anna Kroch Foundation and the North-west Branch of the Society for Mentally Handicapped Children.

5. SUMMARY

The analytical and clinical advantages that would be expected to follow the adoption by clinical laboratories of a routine HPLC method for the partial oestriol conjugate profiling of human pregnancy urine are outlined in the Introduction. In order to ascertain if a candidate method for this assay has yet been devised, a complete survey of the published HPLC separations of oestrogen conjugate mixtures is presented, in tabular form, and discussed. From this survey it is concluded that a number of good separations of these steroids from synthetic mixtures have already been published.

The third and final section of the paper contains the results of a detailed examination of those papers in which separation of oestriol conjugates present in pregnancy urine specimens have been reported. The paper is concluded with the recommendation that the method of Dixon, Lukha and Scott should be further investigated as a candidate method for adoption by clinical laboratories for the purpose of oestriol conjugate profiling.

REFERENCES

1 R.E. Oakey, J. Steroid Biochem., 11 (1979) 1057—1064.
2 C.T. Brooks, R.W.A. Oliver and J.E. Sugden, Fifty-two Years of Oestrogen Analysis: A Comprehensive Bibliography, University of Salford, Salford, 1983.
3 D.R. McDowell, Ph.D. Thesis, University of Salford, Salford, 1975.
4 R.P. Ager and R.W.A. Oliver, J. Chromatogr., 271 (1983) 325—340.
5 H. Adlercreutz and T. Luukainen, Ann. Clin. Res., 2 (1970) 365—380.
6 S. Alexander, V. Stavric, M. Smuk, J. Sugar and J. Schwers, J. Clin. Endocrinol. Metab., 49 (1979) 588—593.
7 T. Fotsis, H. Adlercreutz, P. Järvenpää, K.D.R. Setchell, M. Axelson and J. Sjövall, J. Steroid Biochem., 14 (1981) 457—463.
8 R.E. Oakey, Ann. Clin. Biochem., 17 (1980) 311—314.
9 Sj. van der Wal and J.F.K. Huber, J. Chromatogr., 102 (1974) 353—374.
10 Sj. van der Wal and J.F.K. Huber, J. Chromatogr., 135 (1977) 287—303.
11 Sj. van der Wal and J.F.K. Huber, J. Chromatogr., 135 (1977) 305—321.
12 P.I. Musey, D.C. Collins and J.R.K. Preedy, Steroids, 31 (1978) 583—592.
13 J. Ahmed and A.E. Kellie, J. Steroid Biochem., 3 (1972) 31—38.
14 B. Fransson, K.-G. Wahlund, I.M. Johansson and G. Schill, J. Chromatogr., 125 (1976) 327—344.
15 Sj. van der Wal and J.F.K. Huber, J. Chromatogr., 149 (1978) 431—453.
16 J. Hermansson, J. Chromatogr., 152 (1978) 437—445.
17 P.F. Dixon, P. Lukha and N.R. Scott, Proc. Anal. Div. Chem. Soc., 16 (1979) 302—305.
18 G. Keravis, M. Lafosse and M.H. Durand, Chromatographia, 10 (1977) 678—681.
19 J. Hermansson, J. Chromatogr., 194 (1980) 80—84.
20 G. Keravis and M.H. Durand, Pathol. Biol., 28 (1980) 351—352.
21 W. Slikker, Jr., G.W. Lipe and G.D. Newport, J. Chromatogr., 224 (1981) 205—219.
22 K. Shimada, F. Xie and T. Nambara, J. Chromatogr., 232 (1982) 13—18.
23 K. Shimada, N. Kaji, F. Xie and T. Nambara, J. Liquid Chromatogr., 5 (1982) 1763—1770.
24 G.J. Schmidt, in M.P. Kautsky (Editor), Steroid Analysis by HPLC. Recent Applications, Marcel Dekker, New York, 1981, pp. 145—172.
25 Sj. van der Wal and J.F.K. Huber, J. Chromatogr., 251 (1982) 289—310.
26 E. Heftmann and J.-T. Lin, J. Liquid Chromatogr., 5 (Suppl. 1) (1982) 121—173.
27 R.P. Ager, Ph.D. Thesis, University of Salford, Salford, 1984.
28 Sj. van der Wal, Thesis, University of Amsterdam, Amsterdam, 1977.

Journal of Chromatography, 309 (1984) 17—32
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2117

PROFILES OF STRONGLY POLAR AND LESS POLAR ACIDS OBTAINED FROM HUMAN BLOOD, PLASMA AND SERUM BY TWO-STEP ULTRAFILTRATION

HEINZ THOMA, JOSEF REINER and GERHARD SPITELLER*

Lehrstuhl Organische Chemie I der Universität Bayreuth, Universitätsstrasse 30, Postfach 3008, D-8580 Bayreuth (F.R.G.)

(First received December 28th, 1983; revised manuscript received February 21st, 1984)

SUMMARY

A two-step ultrafiltration method combined with anion-exchange chromatography is described for the separation of lipophilic and strongly lipophobic acids occurring in human blood, serum and plasma. After treatment with diazomethane, the acid fractions are separated further by gas chromatography. The acids were identified by their mass spectra. Profiles obtained from the strongly acidic fraction of blood samples of healthy individuals are characterized by main peaks corresponding to organic phosphoric acid esters. The peaks are absent in plasma and serum.

INTRODUCTION

Organic acids are obtained from biological fluids either by extraction [1—5] or anion-exchange chromatography [6—11]. Highly polar acids, e.g. amino acids or organic phosphoric acid esters, are lost due to their high water solubility if extraction methods are applied.

These polar acids can be adsorbed on anion-exchange columns. They are recovered by washing the columns with strong acids. This procedure requires the removal of accompanying high-molecular-weight compounds (mainly proteins) which easily clog the columns. The necessary preseparation can be done by precipitation [11]. This procedure is therefore more time-consuming than the extraction procedure and suffers sometimes by the fact that acids are coprecipitated. Therefore a better method for removing the interfering proteins is required.

Recently Issachar and co-workers [6, 7] described the profiling of organic acids obtained from human plasma using ultrafiltration. Although the

reproduced profiles are of excellent quality, only a limited number of compounds could be structurally identified by mass spectrometry. It seemed to us a challenging task to identify the remaining unknown compounds.

Structure elucidation of unknown compounds requires the separation of larger samples than used by Issachar and co-workers [6, 7]. When we repeated the separation technique used by Issachar and co-workers [6, 7], the microporous filter membrane became very clogged from the start of filtration. Almost no filtrate was obtained. Therefore we tried to remove the interfering cell wall components and proteins by ultrafiltration using a filter with rather large pores (pore size 0.45 μm) followed by a second ultrafiltration with a filter having small pores (exclusion volume MW 10,000). With this method no difficulties were encountered in obtaining an ultrafiltrate. The second ultrafiltrate was subjected to adsorption onto an anion-exchange column as described by Issachar and co-workers [6, 7].

To improve the filtration and to avoid the various rather time-consuming separation steps, ultrafiltration and anion-exchange chromatography were combined by using an integrated system. This system could separate acids not only from plasma and serum but also from whole blood, thus avoiding the preparation of plasma or serum.

Highly polar and weakly polar acids could easily be separated. They were identified after derivatization with diazomethane by mass spectrometry. The results of these investigations are reported in this paper.

EXPERIMENTAL

Materials

Freshly drawn human blood was heparinized and stored at −40°C until used. Blood samples of individuals who died by accident or suicide were drawn 1 h to two days after death. Human plasma and serum were obtained from the hospitals of Bayreuth and Marktredwitz.

Two-step ultrafiltration

A schematic diagram of the ultrafiltration system is shown in Fig. 1. The system comprised the following components: stock vessel (200 ml) (a), filter cassette (c) with a polysulphone membrane filter HVLP (pore size 0.45 μm; Millipore, Neu-Isenburg, F.R.G.), filter cassette (d) with a polysulphone

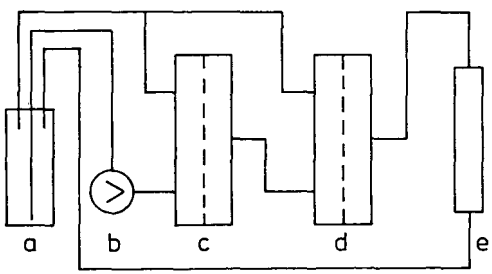


Fig. 1. Schematic diagram of the ultrafiltration system. a = stock vessel, b = toothed wheel pump, c and d = filter cassettes, e = glass column with Dowex 1-X8.

membrane filter PTGC (exclusion volume MW 10,000; Millipore), glass column (e) packed with 500 g of Dowex 1-X8 (mesh size 50—100, counter-ion Cl^-), toothed wheel pump (b) (2600 U/min; Multifix, Schwäbisch-Gmünd, F.R.G.). The components were connected by PTFE tubes (I.D. 0.8 cm).

Filtration conditions were as follows: entrance pressure at the HVLP filter, 3 bars, back-pressure at the HVLP filter, 2 bars; entrance pressure at the PTGC filter, 0.5 bar; back-pressure at the PTGC filter, 0 bar.

Procedure

To prepare the apparatus for filtration it was rinsed with 100 l of distilled water, activated with 10 l of physiological sodium chloride solution [12] and washed again with 50 l of distilled water (time: 30 min).

For filtration, 50 ml of the biological fluid (human blood, serum or plasma) are poured into the stock vessel; then 150 ml of distilled water are added to fill up the stock vessel (a). The stock vessel is connected to the filtration apparatus and to the outlet of the anion-exchange column (see Fig. 1). Then the toothed wheel pump is started and the filtration conditions are adjusted.

After the filtration (about 2 h) the anion-exchange column is disconnected and the remaining system rinsed with 800 ml of distilled water. These washings contain the less polar acids (acid fraction B).

The filtration apparatus can be cleaned by pumping through 20 l of physiological sodium chloride solution followed by 20 l of 0.1 *M* sodium hydroxide solution. After standing for 30 min with 0.1 *M* sodium hydroxide the apparatus is washed with 100 l of distilled water.

When not in use filters were stored in 0.1 *M* sodium azide solution [12].

Isolation of the fraction of polar acids

The anion-exchange column is washed with 1 l of distilled water. Then the adsorbed highly polar acids (fraction A) are eluted by treating the column with 2.5 l of 0.1 *M* methanolic hydrochloric acid. The eluate is evaporated on a water bath (40° C) to dryness. The residue is dissolved in 5 ml of methanol, ethereal diazomethane solution is added until the colour remains yellow and the solution is concentrated to 1 ml; 1 μl of this solution is used for gas chromatographic (GC) and gas chromatographic—mass spectrometric (GC—MS) analysis.

Isolation of the fraction of less polar acids

The washings of the filter apparatus containing the less polar acid fraction B are acidified to pH 1 by dropwise addition of concentrated hydrochloric acid. The solution is extracted three times with 600 ml of ethyl acetate each time. The combined ethyl acetate extracts are dried over sodium sulphate. The solvent is evaporated to dryness. The residue is treated as described for fraction A to prepare samples for GC and GC—MS analysis.

Instrumentation for GC—MS

The GC apparatus and conditions were as follows: Carlo-Erba gas chromatograph 4160; hydrogen flow-rate, 2 ml/min (OV-101); helium flow-rate, 3 ml/min (OV-1701); 30-m thin-film glass capillary coated with OV-101 or 50-m

thin-film quartz capillary Durabond OV-1701 (J & W Scientific); injector temperature, 270°C; oven temperature, 80—300°C (OV-101) or 70—270°C (OV-1701); temperature programme, 2°C/min; flame-ionization detector.

For MS measurements a Varian MAT 312 mass spectrometer was used with electron-impact ion source at an electron energy of 70 eV. The mass spectrometer was combined with a Varian 3700 gas chromatograph. The columns and temperature programme were identical to those described above. The instrument was combined with an SS 200 data system, using a PDP 11/34 computer.

High-resolution data were obtained with the same instrument.

^{1}H-NMR measurements were made with a Bruker WM 250 instrument.

Conditions for preparative GC were: Carlo-Erba Fractovap 2400 T; carrier gas, nitrogen; column, 1.5 m × 6 mm I.D., 3% OV-17 on Supelcoport 100—120; injector temperature, 250°C; oven temperature, 100—270°C; temperature programme, 2°C/min; detector temperature, 270°C; flame-ionization detector.

Reference compounds

The reference samples, phosphoenolpyruvic acid monopotassium salt, 2-phosphoglyceric acid sodium salt, 3-phosphoglyceric acid sodium salt and 2,3-diphosphoglyceric acid tris sodium salt, were obtained from Sigma (Munich, F.R.G.). These substances were transformed into the free acids by anion-exchange chromatography (see isolation of polar acids).

The free acids were transformed into their methyl esters by treatment with ethereal diazomethane solution.

Trimethylsilylated derivatives were obtained by evaporating the eluate from the anion-exchange column to dryness, treatment of the residue with an excess of MSTFA (N-methyl-N-trimethylsilyltrifluoroacetamide) and heating the mixture in a fused tube for 18 h at 60°C. Methyl esters and trimethylsilyl esters were analysed by GC and GC—MS.

^{1}H-NMR and MS data of the compounds isolated from blood samples by preparative gas chromatography

1-Methylpyrazol-3-carboxylic acid methyl ester. ^{1}H-NMR (C^2HCl_3): δ = 3.94 (s, 3H, $COOCH_3$), 4.00 (s, 3H, $N—CH_3$), 6.82 (d, J = 2 Hz, N—CH=CH), 7.4 (d, J = 2 Hz, N—CH=CH) ppm. MS: 140 (M^+, 25%), 110 (19), 109 (100), 82 (18).

Pyrazol-3-carboxylic acid methyl ester. ^{1}H-NMR (C^2HCl_3): δ = 3.94 (s, 3H, $COOCH_3$), 6.89 (d, J = 2 Hz, 1H, N—CH=CH), 7.65 (d, J = 2 Hz, 1H, N—CH=CH) ppm. MS: 126 (M^+, 80%), 95 (100), 81 (2), 71 (2), 68 (6), 66 (5), 59 (3).

2-Methylpyrazol-3-carboxylic acid methyl ester. ^{1}H-NMR (C^2HCl_3): δ = 3.89 (s, 3H, $COOCH_3$), 4.19 (s, 3H, $N—CH_3$), 6.84 (d, J = 2 Hz, 1H, N—CH=CH), 7.47 (d, J = 2 Hz, 1H, N—CH=CH) ppm. MS: 140 (M^+, 25%), 110 (19), 109 (100), 82 (18).

Phosphoenolpyruvic acid trimethyl ester. ^{1}H-NMR (C^2HCl_3): δ = 3.86 (d, J = 4 Hz, 6H, $P—OCH_3$), 3.92 (s, 3H, $COOCH_3$), 5.65 (dd, J = 2.2 Hz, J = 2.6 Hz, 1H, H_2C=), 5.98 (dd, J = 2.2 Hz, J = 2.6 Hz, 1H, H_2C=) ppm. MS: 210 (M^+, 18%), 179 (20), 151 (12), 150 (8), 141 (7), 139 (6), 127 (57), 109 (100), 96 (6), 79 (13).

1-Phosphocyclopropane-1-carboxylic acid trimethyl ester. ^{1}H-NMR (C^2HCl_3): δ = 3.80 (d, J = 4 Hz, 6H, P—OCH_3), 3.86 (s, 3H, COOCH_3), 1.50 (m, 4H, CH_2—CH_2) ppm. MS: 224 (M^+, 3%), 209 (4), 193 (19), 192 (84), 165 (19), 164 (10), 136 (16), 127 (18), 113 (9), 109 (100), 93 (11), 79 (16), 59 (8), 55 (76).

E,Z-3-Methylphosphoenolpyruvic acid trimethyl ester. ^{1}H-NMR (C^2HCl_3): δ = 1.90 (dd, J = 7.3 Hz, J = 2.8 Hz, 3H, CH_3—C=C), 2.08 (dd, J = 7.3 Hz, J = 2.8 Hz, 3H, CH_3—C=C), 3.83 (d, J = 4 Hz, 6H, P—OCH_3), 3.93 (s, 3H, COOCH_3), 6.26 (m, 1H, H—C=C), 6.57 (m, 1H, H—C=C) ppm. MS: 224 (M^+, 11%), 193 (15), 192 (84), 165 (6), 164 (8), 136 (19), 127 (25), 113 (8), 109 (82), 93 (11), 79 (15), 59 (5), 55 (100).

RESULTS

Two-step ultrafiltration

The difficulties encountered in the ultrafiltration of volumes larger than a few millilitres of biological liquid are caused by the presence of high-molecular-weight particles, e.g. proteins and also tissue components, clogging the microporous filter. Therefore we tried to remove proteins of high molecular weight first using a macroporous filter (pore size 0.45 μm). After that filtration step we removed proteins with a molecular weight larger than 10,000 in a second step. This stepwise filtration is rather time-consuming. Therefore we combined the two filtration processes and anion-exchange chromatography and enhanced the filtration procedure by applying a toothed wheel pump. An advantage of the method is the possibility of separating large quantities of biological liquids. The apparatus described in the experimental part is able to filter 20 l of human blood within 10 h if used in a continuous manner only by adjustment of the filtration conditions described in the experimental part. The filtration can be applied equally well to serum, plasma and blood.

A disadvantage of the method is the destruction of blood cells in the apparatus, rendering the addition of physiological sodium chloride solution useless. Therefore we used water to dilute the blood samples. This allowed the analysis of blood cell components as well.

The polar acids in ultrafiltrates from patients suffering from uraemia can also be isolated with this method.

The efficiency of the two-step ultrafiltration method using polysulphone membrane filters was checked by filtration of synthetic mixtures of organic acids with different polarities. The amounts of acids retained and passed were determined by preparing methyl derivatives of the acids followed by analysis of the mixture by GC. The results of these measurements are given in Table I.

Monocarboxylic acids with more than seven carbon atoms are not able to pass through the filters, nor are dicarboxylic acids with more than twelve carbon atoms. Dicarboxylic acids up to a carbon number of 12 are partly able to pass through the filters, the percentage decreasing with increasing carbon number. Aromatic acids like benzoic acid may pass through the filters, while the less polar urofuranic acids are mainly retained. Thus separation into a fraction A of highly polar acids and a less polar acid fraction B is achieved.

TABLE I

AMOUNTS OF ACIDS PASSED THROUGH HVLP AND PTGC MEMBRANE FILTERS

Acid	Percentage of acid passed through filters
Heptanoic acid up to undecanoic acid	0
Palmitic acid	0
Stearic acid	0
Oleic acid	0
Tetracosanoic acid	0
Undecenoic acid	0
Succinic acid	71
2-Methylsuccinic acid	67
Glutaric acid	65
3,3-Dimethylglutaric acid	63
Adipic acid	63
3-Methyladipic acid	59
Heptanedioic acid	59
Octanedioic acid	55
Nonanedioic acid	44
Decanedioic acid	27
Dodecanedioic acid	<1
Benzoic acid	100
Phenylacetic acid	100
Phenylpropionic acid	50
Phenylbutanoic acid	23
Propionylurofuranoic acid	65
Valeroylurofuranoic acid	45
Pentylurofuranoic acid	<1
Pentenylurofuranoic acid	2
Propenylurofuranoic acid	7

Separation of fractions A and B

The gas chromatograms of polar and less polar acid fractions of blood, serum and plasma, after derivatisation with diazomethane, are reproduced in Figs. 2—5. The compounds identified in the chromatograms are numbered and listed in Tables II and III. Saturated hydrocarbons were added to determine retention indices [13]. They are not numbered and not listed in the tables. Mass spectral data are given only if they have not previously been published, otherwise the literature is cited.

Since we were not able to identify many of the compounds of the highly polar acid fraction from their mass spectra, 2 l of pig's blood were worked up as described for human blood. The profiles looked rather similar to those of human blood. Peaks 13, 23, 27, 32, 37, 40 and 41 could be collected by preparative GC.

High-resolution mass measurements (peak matching) and the analysis of the ^{1}H-NMR spectra allowed structure determination. A commercial sample of phosphoenolpyruvic acid available as sodium salt was converted to the free acid by anion-exchange chromatography and treated with diazomethane to prepare the methylate. The gas chromatogram of the reaction product showed several peaks. The mass spectra of these peaks were identical with those of compounds

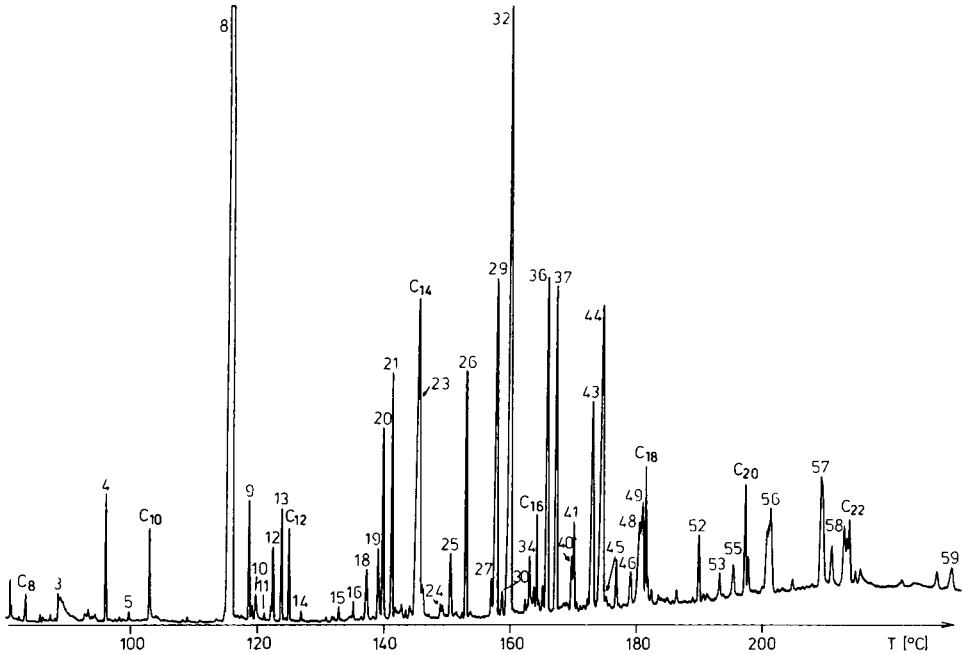


Fig. 2. Gas chromatogram of the strongly polar methylated acids (fraction A) obtained from the blood of a healthy male individual aged 20 years.

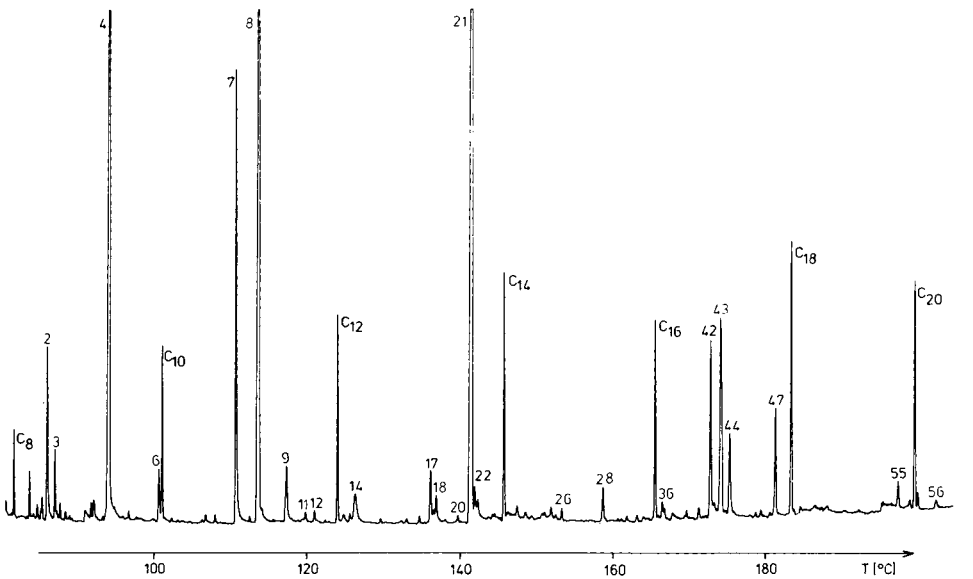


Fig. 3. Gas chromatogram of fraction A obtained from blood plasma.

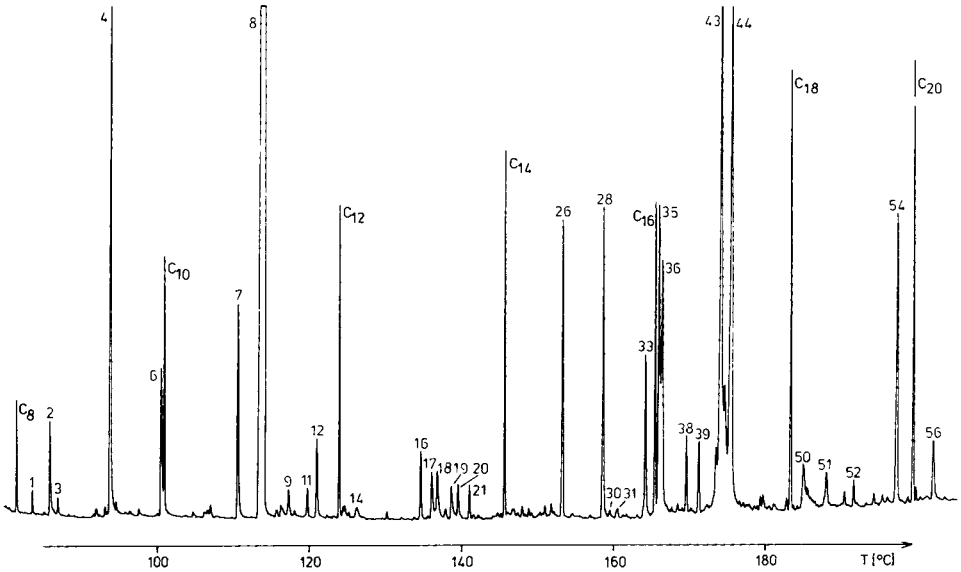


Fig. 4. Gas chromatogram of fraction A obtained from blood serum.

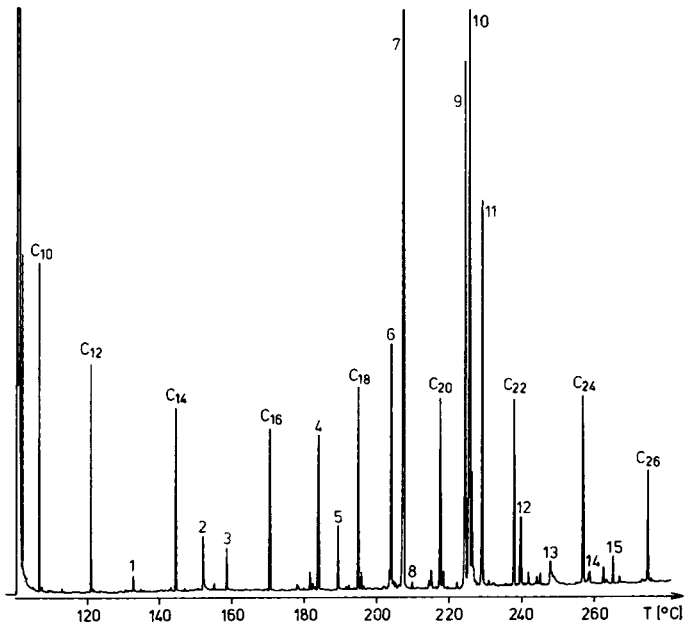


Fig. 5. Gas chromatogram of the less polar methylated acids of blood fraction B of a healthy individual.

13, 23, 27, 32, 37, 40 and 41, isolated by preparative GC. Therefore they were obviously produced by reaction of diazomethane with phosphoenolpyruvic acid in the way shown in Fig. 6 [24—30].

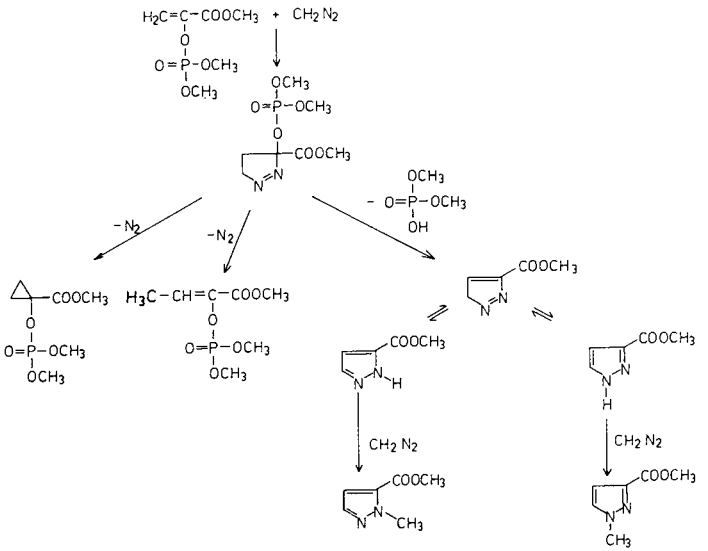
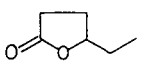
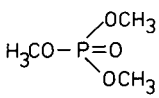


Fig. 6. Reaction of diazomethane with phosphoenolpyruvic acid.

TABLE II

STRONGLY POLAR METHYLATED ACIDS IDENTIFIED IN FIGS. 2–4

Compound No.*	Retention index	Structure	Key ions (*m/z*)	Remarks
1	835	$H_3C-CH=CH-COOCH_3$	[14]**	
2	845	$H_3C-\underset{\underset{OH}{|}}{CH}-COOCH_3$	[15]	
3	853	$H_3C-\overset{\overset{O}{\|\|}}{C}-COOCH_3$	[16]	
4	927	Unknown	89 (70%), 74 (33), 59 (24) 58 (100), 44 (25)	
5	967	$H_3COOC-COOCH_3$	[15]	
6	996	$H_3C-\underset{\underset{OH}{|}}{CH}-CH_2-COOCH_3$	[17]	
7	1084		[18]	
8	1118		[16]	
9	1144	$H_3COOC-CH=CH-COOCH_3$ (trans)	[15]	
10	1153	Unknown	104 (52%), 89 (14), 75 (100), 72 (8), 71 (4), 59 (7), 55 (6), 47 (2), 45 (38), 43 (42)	
11	1167	$H_3COOC-CH_2-CH_2-COOCH_3$	[16]	
12	1177	Unknown	153 (4%), 139 (6), 127 (100), 110 (22), 109 (44), 103 (2), 96 (18), 95 (20), 79 (8), 66 (4), 65 (4), 47 (4), 43 (5)	

(Continued on p. 26)

TABLE II (continued)

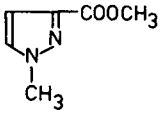
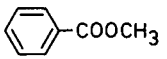
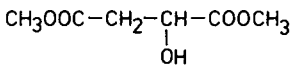
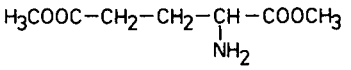
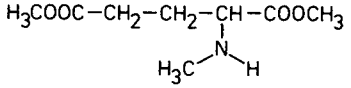
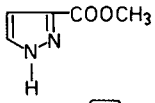
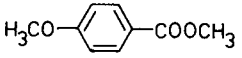
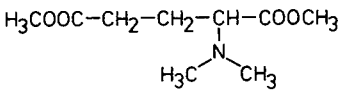
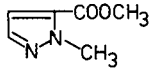
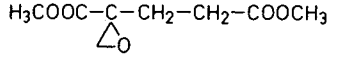
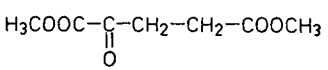
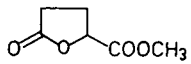
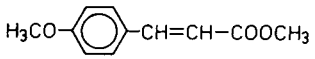
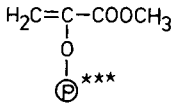
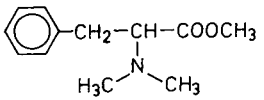
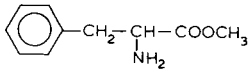
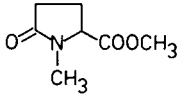
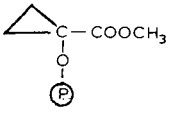
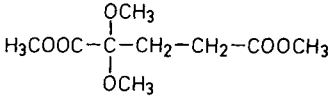
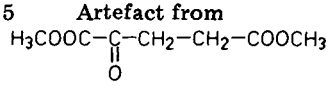
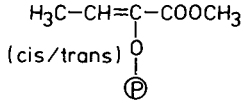
Compound No.*	Retention index	Structure	Key ions (*m/z*)	Remarks
13	1191		140 (M^+, 25%), 110 (19), 109 (100), 82 (18)	Artefact from compound no. 32
14	1220		[16]	
15	1269	Unknown	139 (2%), 129 (3), 117 (64), 110 (6), 101 (5), 85 (44), 75 (8), 74 (5), 64 (3), 59 (10), 58 (6), 43 (100)	
16	1290	Unknown	157 (3%), 98 (100), 70 (5), 59 (3), 55 (3), 42 (8), 41 (17)	
17	1308	Unknown	150 (2%), 117 (3), 102 (58), 91 (10), 87 (24), 59 (12), 58 (68), 55 (12), 45 (100), 43 (11)	
18	1321		[16]	
19	1336	Unknown	147 (8%), 103 (60), 100 (21), 88 (100), 87 (46), 74 (26), 71 (86), 61 (28), 59 (42), 57 (22), 44 (56), 43 (62)	
20	1347		[19]	
21	1362		[19]	Artefact from compound No. 20
22	1368	Unknown	175 (1%), 161 (5), 129 (7), 116 (12), 102 (84), 88 (6), 74 (42), 58 (4), 45 (22), 44 (21), 43 (18), 42 (100)	
23	1400		126 (M^+, 80%), 110 (2), 95 (100), 81 (2), 71 (2), 68 (6), 66 (5), 59 (3)	Artefact from compound No. 32
24	1437		[16]	
25	1456	Unknown	128 (62%), 113 (12), 100 (10), 85 (54), 69 (8), 59 (12), 57 (32), 43 (100)	
26	1480		[19]	Artefact from compound No. 20
27	1521		140 (M^+, 25%), 110 (19), 109 (100), 82 (18)	Artefact from compound No. 32
28	1531		188 (M^+, 2%), 173 (3), 157 (5), 141 (10), 129 (40), 128 (22), 125 (30), 115 (98), 113 (10), 101 (12), 97 (20), 87 (20), 69 (22), 59 (55), 45 (100)	Artefact from 

TABLE II (continued)

Compound No.*	Retention index	Structure	Key ions (m/z)	Remarks
29	1533	Unknown	198 (12%), 167 (20), 166 (28), 154 (7), 139 (82), 127 (20), 124 (98), 109 (100), 93 (14), 87 (10), 79 (32), 59 (7), 47 (10), 45 (22), 42 (11)	
30	1540		144 (M^+, 4%), 85 (100), 59 (3), 57 (5), 55 (2), 41 (2)	
31	1550		[20]	
32	1553		210 (M^+, 18%), 179 (20), 151 (12), 150 (8), 141 (7), 139 (6), 127 (57), 109 (100), 96 (6), 79 (13)	
33	1590	Unknown	134 (36%), 119 (8), 102 (100), 91 (6), 77 (3), 67 (4), 42 (36)	
34	1592	Unknown	168 (2%), 140 (10), 112 (11), 111 (17), 97 (40), 84 (39), 83 (66), 70 (68), 69 (76), 57 (64), 56 (80), 55 (99), 43 (100), 41 (70)	
35	1605		207 (M^+, 1%), 148 (38), 133 (10), 116 (100), 105 (5), 91 (9), 88 (11), 84 (6), 77 (5), 56 (10), 42 (16)	Artefact from 
36	1620		[21]	Artefact from compound No. 44
37	1635		224 (M^+, 3%), 209 (4), 193 (19), 192 (85), 165 (19), 164 (10), 136 (16), 127 (18), 113 (9), 109 (100), 93 (11), 79 (16), 59 (8), 55 (76)	Artefact from compound No. 32
38	1646		202 (M^+, 1%), 171 (5), 155 (16), 152 (18), 143 (40), 129 (26), 128 (32), 115 (54), 111 (33), 101 (26), 97 (22), 83 (50), 59 (88), 55 (100), 45 (94), 42 (60)	Artefact from 
39	1664	Unknown	174 (10%), 163 (20), 160 (12), 131 (60), 128 (100), 127 (59), 115 (12), 105 (20), 103 (22), 91 (96), 77 (22), 65 (26), 59 (6), 55 (12), 51 (21), 42 (82)	
40/41	1665/70		224 (M^+, 11%), 193 (15), 192 (84), 165 (6), 164 (8), 136 (19), 127 (25), 113 (8), 109 (82), 93 (11), 79 (15), 59 (5), 55 (100)	Artefact from compound No. 32

(Continued on p. 28)

TABLE II (continued)

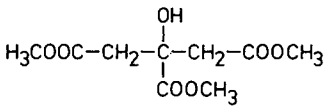
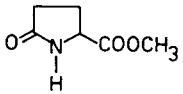
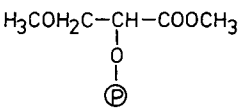
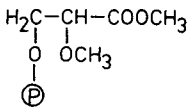
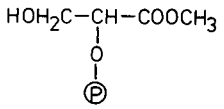
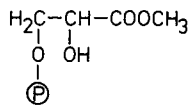
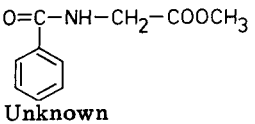
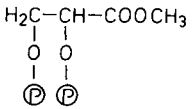
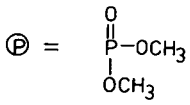
Compound No.*	Retention index	Structure	Key ions (*m/z*)	Remarks
42	1682	Unknown	223 (3%), 187 (10), 174 (10), 166 (24), 164 (80), 138 (6), 136 (23), 128 (11), 106 (23), 102 (10), 74 (5), 63 (8), 59 (7), 45 (100), 42 (50)	
43	1702		[16]	
44	1719		[21]	
45	1730		212 ($M^+ - 30$, 8%), 183 (19), 166 (4), 151 (4), 141 (22), 127 (35), 116 (11), 109 (30), 87 (28), 85 (25), 79 (8), 59 (3), 55 (8), 45 (100)	Artefact from compound No. 48
46	1776		212 ($M^+ - 30$, 4%), 183 (100), 151 (12), 141 (8), 139 (4), 127 (34), 116 (8), 109 (30), 87 (4), 79 (5), 75 (4), 58 (8), 57 (4), 45 (3)	Artefact from compound No. 49
47	1777	Unknown	233 (6), 202 (5), 174 (76), 146 (16), 143 (18), 116 (100), 103 (40), 88 (32), 84 (8), 75 (20), 58 (18), 57 (19), 45 (56), 42 (44)	
48	1791		198 ($M^+ - 30$, 5%), 169 (59), 166 (10), 140 (23), 137 (100), 127 (45), 113 (15), 110 (17), 109 (79), 102 (15), 96 (27), 95 (13), 79 (46)	
49	1779		198 ($M^+ - 30$, 4%), 169 (47), 166 (8), 140 (16), 137 (100), 127 (30), 113 (10), 110 (11), 109 (58), 102 (8), 96 (18), 95 (8), 79 (44)	
50	1818	Unknown	146 (2%), 128 (4), 114 (6), 89 (60), 74 (24), 59 (22), 58 (100), 57 (26), 44 (14), 42 (16)	
51	1857	Unknown	200 (18%), 169 (5), 168 (3), 143 (5), 141 (12), 140 (44), 127 (31), 115 (6), 83 (5), 72 (4), 59 (5), 56 (28), 55 (31), 43 (8), 42 (100)	
52	1908	Unknown	209 (2%), 207 (7), 201 (5), 199 (6), 161 (8), 133 (6), 119 (100), 105 (2), 83 (3), 74 (8), 67 (5), 59 (45), 53 (4), 47 (10), 43 (67)	
53	1951	Unknown	197 (12%), 176 (28), 174 (96), 165 (6), 157 (8), 153 (10), 138 (7), 126 (9), 119 (20), 117 (66), 109 (36), 91 (8), 89 (30), 76 (12), 74 (7), 58 (8), 52 (100)	

TABLE II (continued)

Compound No.*	Retention index	Structure	Key ions (*m/z*)	Remarks
54	1972	Unknown	214 (10%), 183 (17), 182 (47), 154 (10), 141 (24), 140 (62), 127 (21), 114 (4), 98 (24), 69 (12), 56 (22), 55 (15), 42 (100)	
55	1977	Unknown	269 (1%), 149 (100), 122 (6), 95 (4), 85 (4), 75 (5), 68 (6), 53 (3), 42 (9)	
56	2049		[16]	
57	2145	Unknown	260 (2%), 229 (2), 218 (2), 196 (7), 169 (22), 140 (12), 127 (100), 117 (7), 110 (10), 109 (20), 100 (38), 97 (26), 87 (27), 75 (22), 71 (56), 68 (23), 45 (14), 41 (12)	
58	2188	Unknown	248 (6%), 246 (10), 177 (8), 169 (9), 140 (6), 127 (27), 116 (100), 111 (10), 100 (22), 88 (8), 87 (6), 71 (14), 68 (7), 56 (5), 45 (6), 42 (9)	
59	2348		336 (M^+, 1%), 306 (6), 305 (8), 277 (58), 249 (18), 235 (39), 210 (29), 203 (16), 179 (34), 167 (14), 166 (16), 152 (25), 151 (46), 141 (22), 139 (16), 127 (37), 113 (16), 109 (100), 96 (24), 93 (9), 79 (18)	

*Refers to numbered peaks in Figs. 2—4.
**Literature references are quoted in square brackets.
***

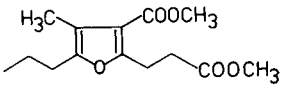
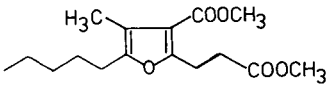
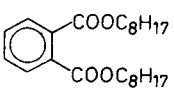


Comparison of the mass spectra and retention indices obtained after diazomethane treatment of other commercially available glycolysis products (see experimental part) allowed the identification of compounds 45, 46, 48, 49 and 59. Phosphoenolpyruvate, 2-phosphoglycerate, 3-phosphoglycerate and 2,3-diphosphoglycerate are therefore the main compounds found in the acid fraction A of human blood. They are absent in plasma and serum profiles as expected, since they are typical glycolysis products which occur in the erythrocytes.

The composition of the less polar acid fraction B was nearly identical for blood, serum and plasma (see Fig. 5).

TABLE III

LESS POLAR METHYLATED ACIDS IDENTIFIED IN FIG. 5.

Compound No.*	Retention index	Structure	Key ions (*m/z*)
1	1310	$H_3C-(CH_2)_8-COOCH_3$	[22]**
2	1459	Unknown	140 (12%), 125 (6), 112 (13), 111 (26), 98 (27), 97 (48), 84 (45), 83 (80), 82 (34), 70 (82), 69 (88), 68 (34), 57 (72), 56 (90), 55 (100), 45 (7), 43 (97), 41 (67)
3	1510	$H_3C-(CH_2)_{10}-COOCH_3$	[22]
4	1710	$H_3C-(CH_2)_{12}-COOCH_3$	[22]
5	1755		[23]
6	1884	$C_{15}H_{29}-COOCH_3$	[22]
7	1910	$H_3C-(CH_2)_{14}-COOCH_3$	[22]
8	1937		[23]
9	2072	$C_{17}H_{31}-COOCH_3$	[22]
10	2081	$C_{17}H_{33}-COOCH_3$	[22]
11	2110	$H_3C-(CH_2)_{16}-COOCH_3$	[22]
12	2227	$C_{19}H_{31}-COOCH_3$	[22]
13	2308	Unknown	154 (1%), 140 (2), 128 (2), 126 (6), 114 (4), 112 (5), 98 (6), 97 (5), 86 (5), 83 (7), 72 (58), 69 (10), 67 (5), 59 (100), 55 (21), 44 (8), 43 (18), 41 (20)
14	2418	Unknown	239 (8%), 221 (4), 166 (5), 161 (3), 159 (5), 148 (10), 147 (10), 145 (10), 133 (16), 131 (14), 119 (34), 117 (24), 108 (40), 105 (40), 93 (44), 91 (70), 79 (100), 77 (24), 74 (20), 67 (61), 57 (31), 45 (21), 43 (36), 41 (69)
15	2494		[22]

*Refers to the numbered peaks in Fig. 5.

**Literature references are given in square brackets.

DISCUSSION

Clogging of filters is one of the main difficulties encountered in the one-step ultrafiltration of serum and plasma introduced by Issachar and co-workers [6, 7]. This can be circumvented by use of the two-step ultrafiltration described in this paper. Filters and anion-exchange column can be combined into an almost automatical system able to work up serum and plasma on a scale between 5 ml and 20 l, in one day if commercially available filter cassettes are used with the appropriate volumes.

Issachar and co-workers [6, 7] recommended the addition of oleic acid to serum before ultrafiltration to replace carboxylic acids, particularly fatty acids, bound to albumin. Since albumin and consequently also albumin-bound carboxylic acids can not pass through our filters, as well as fatty acids (see Table I), the addition of oleic acid does not improve the yield of less polar acids. To determine if the yield of polar acids could be increased by addition of oleic acid, we added oleic acid to serum and blood in different amounts. The results of the analysis demonstrated no effect at all. Therefore the addition of oleic acid is useless and even causes interference if later the less polar acid fraction is extracted, since in large amounts it renders the determination of low amounts of other less polar acids more difficult. Addition of oleic acid is therefore not advisable.

The two-step ultrafiltration offers the possibility of analysing blood without the time-consuming and costly preparation of plasma or serum. The separation of blood compounds into polar and less polar acid fractions is a major advantage in the analysis of acids occurring in blood, not previously possible.

But the method still suffers from the difficulty of analysing the highly polar acid fraction quantitatively. The fraction obtained requires further separation. This is usually achieved by GC after appropriate derivatization, allowing the quantitative determination of single components. In the case of phosphoric acid derivatives, the necessary derivatization by methylation or trimethylsilylation [31] causes the production of several products, rendering quantitative measurement by summation of the peak heights of the corresponding peaks difficult.

Since the phosphoric acid derivatives do not show ultraviolet absorption their detection is also difficult. A possibility just for analysis of phosphoric acid derivatives may be hydrolysis after separation of the components and determination of phosphoric acid by reaction with molybdate, as shown by Bessman et al. [11]. But this method would exclude the analysis of all other polar components (e.g. amino acids).

In spite of these difficulties at least a semiquantitative analysis can be achieved by glass capillary GC. Since very often great changes in the composition of biological fluids are observed, if the metabolism of the individual is changed this semiquantitative analysis is sufficient to detect such events.

ACKNOWLEDGEMENTS

We gratefully acknowledge financial support by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie. We thank Prof. Dr.

Weidinger for taking the blood and Günther Möller and Georg Dietel for excellent technical assistance.

REFERENCES

1 E.C. Horning and M.G. Horning, Methods Med. Res., 12 (1970) 385—395.
2 M.C. Elphick and J.P. Lawor, J. Chromatogr., 130 (1977) 139.
3 T. Niwa, T. Ohki, K. Maeda, A. Suito and K. Kobayashi, Clin. Chim. Acta, 99 (1979) 71.
4 J. Pfordt, H. Thoma and G. Spiteller, Justus Liebigs Ann. Chem., (1981) 2298.
5 J. Pfordt and G. Spiteller, Justus Liebigs Ann. Chem., (1980) 175.
6 D. Issachar and C.C. Sweeley, Anal. Biochem., 113 (1981) 43.
7 D. Issachar, J.F. Holland and C.C. Sweeley, Anal. Chem., 54 (1982) 29.
8 K. Murayma and N. Shindo, J. Chromatogr., 143 (1977) 137.
9 A.M. Lewis, C. Waterhouse and L.S. Jacobs, Clin. Chem., 26 (1980) 271.
10 C.A. Burtis, G. Goldstein and C.D. Scott, Clin. Chem., 16 (1970) 201.
11 S.P. Bessman, P.J. Geiger, Lu Tsung-Cho and E.R.B. McCabe, Anal. Biochem., 59 (1974) 533.
12 Pellicon® Cassette System, Cat. No. 011 029, Part. No. 11 041, Rev. 3/81, Millipore Corporation, Bedford, MA, Copyright 1981.
13 E. Kovats, Helv. Chim. Acta, 41 (1958) 1915.
14 H. Hommes and J.K. Torlouw, Org. Mass Spectrom., 14 (1979) 51.
15 M. Bajardi, L. Boniforti, R. Dommarco and F. Merli, Riv. Soc. Ital. Sci. Aliment., 6 (1977) 249.
16 M. Spiteller and G. Spiteller, J. Chromatogr., 164 (1979) 253.
17 S. Blomberg and T. Gronholm, Eur. J. Biochem., 101 (1979) 111.
18 E. Honkanen, T. Moisio and P. Carvonen, Acta Chem. Scand., 19 (1965) 370.
19 G.R. Waller, Biomedical Applications of Mass Spectrometry, Wiley, New York, 1972, pp. 392—397.
20 V.T. Khvastenko, V.S. Fal'ko, G.P. Leplyanin, V.V. Takhistov and O.S. Sokolova, Izv. Akad. Nauk SSR Ser. Khim., (1978) 592.
21 G.R. Waller and O.C. Dermer, Biomedical Applications of Mass Spectrometry, First Supplementary Volume, Wiley, New York, 1980, p. 1071.
22 J. Pfordt, Dissertation, Bayreuth, 1980.
23 M. Spiteller, G. Spiteller and G.-A. Hoyer, Chem. Ber., 113 (1980) 699.
24 J. Bastide, O.H. Rousseau and L. Aspart-Pascot, Tetrahedron, 30 (1974) 3355.
25 O. Mlejnek, J. Chromatogr., 70 (1972) 59.
26 K. v. Auwers and F. König, Justus Liebigs Ann. Chem., 496 (1932) 252.
27 K. v. Auwers and E. Cauer, Justus Liebigs Ann. Chem., 470 (1929) 284.
28 D.D. McGreer, J. Org. Chem., 25 (1960) 852.
29 C. Wentrup, Reaktive Zwischenstufen I, G. Thieme, Stuttgart, 1979.
30 P. Karrer, Lehrbuch der Organische Chemie, G. Thieme, Stuttgart, 1950, p. 707.
31 D.J. Harvey and M.G. Horning, J. Chromatogr., 76 (1973) 51.

Journal of Chromatography, 309 (1984) 33—42
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2136

A NEW PROCEDURE TO ANALYZE FREE FATTY ACIDS

APPLICATION TO 20-mg BRAIN TISSUE SAMPLES

KENNETH G. ALLEN

Basic Science Laboratory, Veterans Administration Medical Center, Medical Research Service, Cincinnati, OH 45220 (U.S.A.)

JOSEPH MacGEE

Basic Science Laboratory, Veterans Administration Medical Center, Medical Research Service, Cincinnati, OH 45220 (U.S.A.) and Departments of Biological Chemistry and Internal Medicine, University of Cincinnati College of Medicine, Cincinnati, OH 45267 (U.S.A.)

McCLELLAN E. FELLOWS and PATRICIA A. TORNHEIM

Department of Anatomy and Cell biology, University of Cincinnati College of Medicine, Cincinnati, OH 45267 (U.S.A.)

and

KENNETH R. WAGNER*

**Veterans Administration Medical Center, Medical Research Service (151), 3200 Vine Street, Cincinnati, OH 45220 (U.S.A.) and Department of Neurology, University of Cincinnati College of Medicine, Cincinnati, OH 45267 (U.S.A.)*

(First received November 29th, 1983; revised manuscript received February 23rd, 1984)

SUMMARY

Fatty acids were analyzed by a new method which involved their isolation from hexane extracts of serum or brain tissue in aqueous potassium hydroxide (10 μl) and methylation directly in this solution with methyl iodide. The resulting fatty acid methyl esters were partitioned into ethylene chloride (25 μl) and were quantitated by gas—liquid chromato-

0378-4347/84/$03.00

graphy. The procedure was documented by comparison with conventional methylation reactions on serum fatty acids. This method, which avoids thin-layer chromatography and which measures individual free fatty acid concentrations in 20-mg brain tissue samples, should be of particular value for examining regional free fatty acids in brain following ischemia and trauma.

INTRODUCTION

The conventional method for separation of free fatty acids (FFA) from other lipid components in brain tissue extracts is thin-layer chromatography (TLC) [1–4]. However, TLC procedures usually require sizable quantities of tissue, prolonged isolation time, and can result in a low recovery of FFA (e.g. see ref. 5). Furthermore, the variability in the quality of separation of FFA from di- and triglycerides on TLC plates from different suppliers may result in erroneously high FFA concentrations [5]. In order to measure FFA concentrations in small brain tissue samples we developed an alternative method for their separation and methylation.

The present procedure integrates previously developed techniques [6, 7] for extraction of FFA and methyl esters with a new method for FFA methylation. We separated FFA from other lipids in a hexane extract by concentrating them in a small volume of an aqueous base [6]. To avoid the back-extraction of FFA into a non-aqueous medium, which is required for some methylation reactions, we utilized methyl iodide to methylate FFA directly in this basic solution. The resulting fatty acid methyl esters were partitioned into a small volume of ethylene chloride [7] and were quantitated by gas–liquid chromatography (GLC).

In the present report we compare our methyl iodide methylation procedure with: (1) a conventional method using boron trifluoride (BF_3) [8]; and (2) a previous method developed by our laboratory using trimethyl (α,α,α-trifluoro-*m*-tolyl)ammonium hydroxide (TMTFTH) [6] on total saponifiable serum fatty acids. Documentation of the methyl iodide procedure was initially obtained with serum because its relatively high concentration of polyunsaturated fatty acids enabled a test of whether our methylation procedure altered double bonds. Furthermore, the greater availability of serum versus brain tissue permitted macrochemical analyses thus avoiding the inherent difficulties associated with trace measurements. Having demonstrated the quality of our method with serum, we then successfully carried out a "trace analysis" of FFA concentrations on 20-mg samples of cortical gray matter of cat brain.

EXPERIMENTAL

Materials

Organic solvents and reagents were of reagent grade quality and were purchased from Fischer Scientific (Pittsburgh, PA, U.S.A.) with the following exceptions: BF_3–methanol (14%, w/v), and standards pentadeconic acid and Fat and Oil Reference Mix No. 6 from Applied Sciences Labs. (State College, PA, U.S.A.) and PUFA 1 and PUFA 2 from Supelco (Bellefonte, PA, U.S.A.);

TMTFTH from Regis (Morton Grove, IL, U.S.A.); hexane was HPLC grade quality. Glassware was soaked in methanolic potassium hydroxide (15%, w/v) and rinsed well to remove lipid contamination. All glass stoppers and glassware were handled carefully to prevent skin lipid contamination.

Saponification and extraction of fatty acids in serum

Comparison of boron trifluoride and methyl iodide methylations. Fatty acids in fresh pooled human serum (0.8 ml) were saponified with 4.0 ml of 15% methanolic potassium hydroxide by incubation in a heating block for 45 min at 65°C. The solution was cooled, and 4.0 ml of 1.5 *M* phosphoric acid were added. Following mixing, 1.6 ml of water and 40 ml of hexane were added, and the mixture was shaken vigorously by hand for 2 min to extract the fatty acids. Following centrifugation (500 *g*), most of the hexane phase was removed to a conical centrifuge tube, and the fatty acids were extracted with 1.0 ml of 0.5 *M* potassium hydroxide in water. Following centrifugation (500 *g*) for 1 min most of the lower potassium hydroxide—water phase was removed to another tube. The solution was then acidified with 1.5 *M* phosphoric acid, and 2.0 ml of water and 5.0 ml of ethylene chloride were added. The fatty acids were extracted by shaking vigorously for 2 min. Following a 1-min centrifugation (500 *g*) most of the ethylene chloride layer was removed to another tube that served as the common source of fatty acids for the methylations described below. Multiple aliquots (0.1 ml) of this solution were flushed with nitrogen and stored at −10°C. On the day of assay, six tubes (for each assay) were removed from the freezer and carefully evaporated to dryness under nitrogen at 65°C. Ethylene chloride (45 μl) was added to those tubes that were carried through the boron trifluoride methylation procedure described below. To the tubes that were carried through the methyl iodide methylation procedure described below, 10 μl of 0.5 *M* aqueous potassium hydroxide were added and the tubes vortexed to dissolve the fatty acids.

i. Boron trifluoride methylation. Following vortex mixing, boron trifluoride (0.5 ml of 14% BF_3 in methanol) was added. The solution was mixed by manual shaking for 15 sec and then incubated for 10 min at 65°C in a stoppered tube. Following cooling, 1.5 ml of water were added, the tube was vortexed for 30 sec and centrifuged for 2 min at 500 *g*. A 10-μl syringe was primed with ethylene chloride and 5 μl of the ethylene chloride layer were injected into the gas chromatograph.

ii. Methyl iodide methylation. N,N-Dimethylacetamide (DMA) (50 μl) and 30 μl of methyl iodide were added with mixing, and the mixture was incubated in a hood for 10 min at 65°C. Pyridine (60 μl) was then added with mixing, and the mixture was again incubated for 10 min at 65°C. Following cooling, 0.5 ml of 0.1 *M* phosphoric acid and 25 μl of ethylene chloride were added and the fatty acid methyl esters were extracted by vortexing for 30 sec. The sample was centrifuged for 2 min (500 *g*), and most of the upper phase was removed. Water (4 ml) equilibrated with ethylene chloride was added. The upper walls of the tube and lower phase were washed by suspending the lower phase six times with a vortex mixer. Following centrifugation for 2 min (500 *g*), most of the upper phase was removed. This wash step was repeated. A 10-μl syringe was primed with ethylene chloride, and the lower phase was drawn up.

All of the lower phase (5—10 μl) can be removed for injection into the gas chromatograph. Removal of a small amount of the upper phase did not affect the analysis.

Comparison of TMTFTH and methyl iodide methylations. Fresh pooled human serum (1.0 ml) (different batch than described in *Comparison of boron trifluoride and methyl iodide methylations*) was saponified as described above with adjustments in the reagents for the larger volume of serum. Following extraction of fatty acids into hexane, six 5.0-ml aliquots for the TMTFTH procedure described below and six 1.7-ml aliquots for the methyl iodide procedure were removed to 5-ml conical centrifuge tubes, flushed with nitrogen, and stored at −10° C.

i. TMTFTH methylation. The tubes containing fatty acids in 5.0 ml of hexane were extracted with 10 μl of TMTFTH according to the procedure of MacGee and Allen [6]. A 2.5-μl sample—methyl acetate (treated with anhydrous sodium carbonate) sandwich was injected into the gas chromatograph.

ii. Methyl iodide methylation. The tubes containing fatty acids in 1.7 ml of hexane were extracted with 10 μl of 0.5 *M* potassium hydroxide in water. The tubes were shaken hard for 1 min, centrifuged (500 *g*) for 1 min, and all of the hexane layer was removed using a Pasteur pipet and vacuum (drawing off liquid followed by air drying of the interface). The methylation was carried out as described above (*ii. Methyl iodide methylation* under *Comparison of boron trifluoride and methyl iodide methylations*).

Brain tissue analysis

Animal preparation. An adult conditioned cat was anesthetized with pentobarbital, 30 mg/kg, intravenously. The right femoral artery was catheterized with P.E. No. 90 polyethylene tubing, and the free end was attached to a Statham strain gauge transducer to record blood pressure and drive a cardiotachometer. Blood pressure and heart rate were recorded on a Brush Gould polygraph. The cat was intubated and ventilated with a Harvard respirator. The rectal temperature was continuously monitored, and body temperature was maintained at 38.0 ± 1° C with a heating pad.

Periodic blood samples were withdrawn and analyzed for pH, pO_2, and pCO_2 (Corning Model 168 blood gas analyzer). The adequacy of the animal's physiologic status throughout a 30-min control period and during in situ head freezing was verified by the recording of cardiovascular function and analysis of blood samples.

Brain tissue fixation by in situ freezing and tissue sampling. A bottomless plastic cup was attached to the surgically exposed calvarium. The cup was filled with liquid nitrogen, rapidly freezing the underlying skull and brain. Brain metabolites of rats [9] and cats [10] fixed by this method fall well within their normal ranges as defined by other methods. We have confirmed the validity of this method for the study of stable metabolic states in brains of monkeys [11] and cats [12].

The entire cat brain was frozen after 15 min of treatment with liquid nitrogen. After head freezing, the cat was decapitated and the frozen head stored at −80° C. The head was subsequently cut into 5—7 mm thick coronal

blocks using a band saw in a —20°C walk-in freezer. Tissue sampling and weighing procedures were accomplished in a —20°C glove box cryostat. A 2-mm stainless-steel trochar was used to sample cortical gray matter from the crowns of the gyri lateralis, suprasylvius, and ectosylvius. The tissue plugs were weighed on a Roller-Smith precision balance.

Extraction of FFA from brain tissue

i. Precision study. For the precision study on brain tissue (Table V), a tissue sample (159.6 mg) was homogenized by sonication in 3.2 ml of normal saline (0.9%) in a 50-ml centrifuge tube. Immediately, 12.0 ml of methanol, 25 ml of hexane, 4.0 ml of 1 *M* phosphoric acid and a known amount (9.068 μg) of the internal standard, pentadecanoic acid, were added to the homogenate. The mixture was shaken vigorously for 1 min and centrifuged (500 *g*). The hexane phase was then washed twice with 16.0 ml of 0.1 *M* phosphoric acid. Most of the hexane phase was removed to a volumetric flask and the volume was adjusted to 25 ml with hexane (2 ml). Then five 3.13-ml aliquots of the hexane extract (each equivalent to 19.98 mg brain tissue) were pipeted into individual tubes and 10 μl of aqueous 0.5 *M* potassium hydroxide were added. The tubes were shaken hard for 1 min, centrifuged (500 *g*) for 1 min, and all of the hexane layer was removed using a Pasteur pipet and vacuum (drawing off liquid followed by air drying of the interface). The tubes were carried through the methyl iodide procedure as described above (*ii. Methyl iodide methylation* under *Comparison of boron trifluoride and methyl iodide methylations*).

ii. General procedure. The general procedure for extraction of FFA from brain tissue involved homogenization of 20 mg of tissue by sonication in 400 μl normal saline in a glass centrifuge tube. Immediately 1.5 ml methanol, 4.0 ml hexane, 0.5 ml of 1 *M* phosphoric acid and a known amount of internal standard (1.136 μg pentadecanoic acid) were added to the homogenate. The mixture was shaken vigorously for 1 min and centrifuged (500 *g*). Most of the lower phase was removed and 2.0 ml of 0.1 *M* phosphoric acid were added and the hexane phase was washed for 1 min and centrifuged. This wash step was repeated. Most of the hexane phase was removed to another tube and extracted with 10 μl of 0.5 *M* potassium hydroxide in water. The tubes were then shaken hard for 1 min, centrifuged (500 *g*) for 1 min, and all of the hexane layer was removed as previously described. The methyl iodide methylation was then carried out as described above (*ii. Methyl iodide methylation* under *Comparison of boron trifluoride and methyl iodide methylations*).

Blanks

Blanks for the methyl iodide procedure were obtained on 400 μl of 0.9% saline using pentadecanoic acid as the internal standard and the procedure described above (*Extraction of FFA from brain tissue, General procedure*).

Gas—liquid chromatography

GLC was performed with either a Bendix 2600 gas chromatograph (Bendix, Ronceverte, WV, U.S.A.) or a Perking-Elmer Sigma 1 microprocessor-controlled computing gas chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.). Both instruments have flame ionization detectors and temperature programming. Two 182.9 cm × 6.35 mm (4 mm I.D.) glass columns were packed with 10% Silar 10 C on 100—120 mesh Gas-Chrom Q (Applied Science Labs.). The

temperatures of the injector and detector were 280° C and 250° C, respectively, for both instruments. The temperature programme for both instruments was 130° C to 220° C at 2° C/min. Peak areas and retention times for the Bendix instrument were determined with a Hewlett-Packard 3309A reporting integrator. Fatty acid methyl esters were identified on the basis of their retention times as compared to standards of known composition (Fat and Oil No. 6, PUFA 1, PUFA 2).

Calculations

The individual fatty acid concentrations for brain tissue were corrected by subtracting the corresponding blank values. The paired *t*-test was used to determine statistically significant differences between serum fatty acids analyzed by different methods.

RESULTS

Serum studies

There were no significant differences in serum fatty acid concentrations or in the precision of the measurements analyzed by the methyl iodide procedure as compared with the conventional BF_3 or TMTFTH methods (Tables I and II).

TABLE I

COMPARISON OF BORON TRIFLUORIDE AND METHYL IODIDE METHYLATION PROCEDURES FOR ANALYSIS OF FATTY ACID CONCENTRATIONS OF POOLED HUMAN BLOOD SERUM

Values are means of normalized peak areas ± standard deviation (S.D.) from six samples each for BF_3 and methyl iodide procedures. Different batches of pooled human blood serum were used for the analyses in Table I and Table II.

Fatty acid	BF_3	Methyl iodide
16:0	25.62 ± 0.26	25.34 ± 0.24
16:1	2.73 ± 0.22	2.54 ± 0.20
18:0	4.79 ± 0.26	4.64 ± 0.22
18:1	33.58 ± 0.23	33.75 ± 0.37
18:2	27.49 ± 0.25	28.05 ± 0.47
20:4	5.36 ± 0.29	5.69 ± 0.28

TABLE II

COMPARISON OF TMTFTH AND METHYL IODIDE METHYLATION PROCEDURES FOR ANALYSIS OF FATTY ACID CONCENTRATIONS OF POOLED HUMAN BLOOD SERUM

Values are means of normalized peak areas ± S.D. from six samples each for TMTFTH and methyl iodide procedures. Different batches of pooled human blood serum were used for the analyses in Table I and Table II.

Fatty acid	TMTFTH	Methyl iodide
16:0	24.15 ± 0.21	24.46 ± 0.22
16:1	4.36 ± 0.23	4.47 ± 0.32
18:0	5.38 ± 0.20	5.38 ± 0.20
18:1	32.17 ± 0.21	31.99 ± 0.28
18:2	27.20 ± 0.23	27.13 ± 0.25
20:4	6.74 ± 0.20	6.58 ± 0.20

A reaction time study determined that the methylation with methyl iodide was 90.0%, 94.7%, and 100% complete after 2.5, 5, and 10 min of incubation, respectively, at 65°C (data not shown). Further incubation for 15 or 30 min did not alter the results. In addition, a negative test with moist starch paper indicated that the pyridine destruction of excess methyl iodide was complete after 10 min of incubation at 65°C. FFA methyl esters were stable in ethylene chloride extracts for as long as seven days (Table III).

TABLE III

STABILITY OF FFA METHYL ESTERS FROM POOLED HUMAN BLOOD SERUM PREPARED BY THE METHYL IODIDE PROCEDURE

Values are normalized peak areas. The pooled human serum used for the data in this table is different from that presented in Tables I and II.

Fatty acid	Day 0	Day 1	Day 2	Day 3	Day 7
16:0	24.60	24.26	24.54	24.35	24.37
16:1	4.76	5.17	4.41	4.66	4.52
18:0	4.66	4.44	4.75	4.60	4.80
18:1	31.86	32.16	31.91	31.79	31.76
18:2	27.41	27.47	27.52	27.85	27.75
20:4	6.70	6.50	6.88	6.74	6.80

Brain tissue

The blank concentrations of palmitic, oleic, stearic, arachidonic and docosahexaenoic acids are presented in Table IV. These blank values were subtracted from the corresponding brain tissue fatty acid concentrations.

The free fatty acid concentrations in cortical gray matter of cat brain tissue as determined by the methyl iodide procedure are presented in Table V. A typical chromatogram of FFA in a 19.98-mg sample of cat brain cortex is presented in Fig. 1.

TABLE IV

METHYL IODIDE PROCEDURE BLANKS

Values are means ± S.D. expressed as nmol for six blanks.

Fatty acid	Blank value
16:0	0.936 ± 0.139
18:0	0.533 ± 0.092
18:1	0.249 ± 0.055
20:4	0.052 ± 0.015
22:6	0.112 ± 0.056

DISCUSSION

The results of the present study demonstrate that fatty acids can be successfully methylated with methyl iodide following their extraction from serum with aqueous potassium hydroxide. This new method uses non-adsorbing techniques, small-size glassware, and small amounts of reagents and solvents for

TABLE V

FREE FATTY ACID CONCENTRATIONS IN CORTICAL GRAY MATTER OF CAT BRAIN DETERMINED BY THE METHYL IODIDE PROCEDURE

Values are means ± S.D. expressed as nmol/g of five aliquots (3.13 ml per aliquot) of a hexane extract of brain tissue (19.98 mg per aliquot). See Experimental for description of sample handling.

Fatty acid	Concentration (nmol/g wet weight)
16:0	115.90 ± 10.97
18:0	86.83 ± 6.54
18:1	116.12 ± 8.07
20:4	28.29 ± 3.86
22:6	56.74 ± 8.80
Total	403.87 ± 31.54

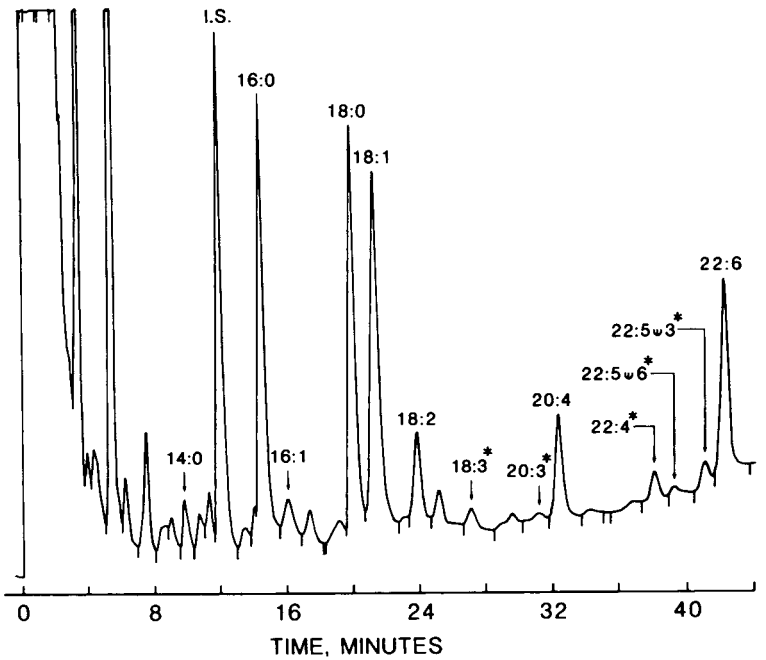


Fig. 1. Chromatogram of fatty acid methyl esters derived from a 19.98-mg sample of anterior cortical gyri of the cat brain. Fatty acids were extracted and methylated with methyl iodide as described in Experimental. FFA were identified on the basis of their retention times as compared with standards (see *Materials*). I.S. is the internal standard (pentadecanoic acid). The concentration of the 22:6 FFA appears to be variable between brain regions in the cat [13]. * = Tentative identification.

isolating FFA and preparing their methyl esters. Loss of FFA by irreversible adsorption, numerous steps utilizing different glassware, transfer of solutions, and evaporation or drying is thus eliminated.

Fatty acid methylation with methyl iodide yields results comparable to a conventional methylation method using BF_3 (Table I). In addition, similar results were obtained with the methyl iodide procedure as with our previously

described TMTFTH procedure (Table II). FFA concentrations can be successfully determined on 20 mg of brain tissue with this new method. Furthermore, the analysis time is short, about 1 h from homogenization of brain tissue to injection of the concentrated fatty acid methyl esters into the gas chromatograph. Recovery of most or all of the methyl esters is a necessary requirement when measuring FFA in small tissue samples. In addition, polyunsaturated fatty acid concentrations are not altered by the methyl iodide reaction. This method will enable an examination of FFA alterations in specific brain loci following ischemia, head trauma, and other insults which produce focal injury.

Other methods for esterification of FFA have certain disadvantages. Diazomethane has been used to esterify fatty acids [14]. However, this compound's explosive and toxic characteristics have discouraged its use [15]. In addition, diazomethane has occasionally produced artifacts and it appears to have a slower rate of esterification [15]. The commonly used BF_3—methanol reagent [8] does not yield quantitative recoveries of fatty acid methyl esters with small samples (50—150 mg) [15].

Our method is a modification of methods described by Grunert and Bassler [16], Gehrke and Goerlitz [17] and Johnson and Wong [18]. Grunert and Bassler [16] used methyl iodide over solid potassium carbonate at 90°C for fatty acid esterification which, when compared with classical procedures, was their method of choice. Gehrke and Goerlitz [17] described a silver salt—methyl iodide procedure which requires preparation of the silver salts of fatty acids with silver nitrate, evaporation of water, followed by an 8-h incubation with methyl iodide. Our method, which is considerably less complex and time-consuming than either of these procedures, completely methylates fatty acids in 10 min with methyl iodide in 0.5 *M* aqueous potassium hydroxide at 65°C with N,N-dimethylacetamide as a catalyst. Excess methyl iodide is then destroyed with pyridine.

We previously described that aqueous bases could be used for the quantitative extraction of free fatty acids from other lipids in the hexane phase including TMTFTH, trimethylphenylammonium hydroxide, tetramethylammonium hydroxide, potassium carbonate and potassium hydroxide [6]. In the present study, the TMTFTH methylation procedure, which is a more straight-forward method, was initially our method of choice. However, 7 or 8 μl of the TMTFTH extract of the hexane phase had to be injected into the gas chromatograph to obtain adequate methyl ester peaks. This resulted in a large solvent response that obscured resolution of the internal standard and made quantitation difficult. In addition, this large volume of TMTFTH led to a bothersome blank with a complex of peaks at the same retention times as palmitic acid and other fatty acids of interest. Thus, this method was unsatisfactory for the "trace analysis" of FFA concentrations in small quantities of brain tissue.

For quantitation of FFA in 20 mg of tissue, blanks have to be analyzed with the methyl iodide procedure. These blanks contain significant amounts of the fatty acids of interest (Table IV). A small amount of these fatty acids may arise from desorption from the GLC system, i.e., "memory effects" [6], etc. However, the probable source is trace impurities in the reagents. Fatty acid con-

tamination from glassware was likely eliminated by soaking with methanolic potassium hydroxide as described under *Materials.* This blank is the factor which limits the size of the tissue sample which can be used for the analysis.

A stability study demonstrated no changes in the concentrations of methyl esters extracted into ethylene chloride for at least one week at room temperature (Table III) in agreement with our previous findings [7]. Thus, a large number of samples can be prepared on one day and the chromatography performed on subsequent days.

In summary, the present new method utilizes a non-adsorbing technique for isolation of FFA from other lipids in a hexane extract of brain tissue, i.e., extraction with aqueous base. The FFA are then methylated with methyl iodide directly in this medium, and their methyl esters are partitioned into ethylene chloride and quantitated by GLC. This method, in which FFA concentrations in 20-mg brain tissue can be analyzed will enable a measurement of regional FFA in brain following such insults as ischemia and trauma.

ACKNOWLEDGEMENTS

The work was supported by NIH Grant R01 NS 17975 and the Veterans Administration. The authors gratefully acknowledge the excellent secretarial assistance of Ms. Judith Konerman and Ms. Janet Combs.

REFERENCES

1 N.G. Bazan and H.E.P. Bazan, in N. Marks and R. Rodnight (Editors), Research Methods in Neurochemistry, Vol. 3, Plenum Press, New York, 1975, pp. 309—324.
2 J. Marion and L.S. Wolfe, Biochim. Biophys. Acta, 574 (1979) 25—32.
3 G. Trovarelli, G.E. De Medio, R.V. Dorman, G.L. Piccinin, L.A. Horrocks and G. Porcellati, Neurochem. Res., 6 (1981) 821—833.
4 S. Rehncrona, E. Westerberg, B. Akesson and B.K. Siesjo, J. Neurochem., 38 (1982) 84—93.
5 G.K. Shiu, J.P. Nemmer and E.M. Nemoto, J. Neurochem., 40 (1983) 880—884.
6 J. MacGee and K.G. Allen, J. Chromatogr., 100 (1974) 35—42.
7 J. MacGee and K.G. Allen, J. Amer. Oil Chem. Soc., 54 (1977) 375—379.
8 L.D. Metcalfe and A.A. Schmitz, Anal. Chem., 33 (1961) 363—364.
9 U. Ponten, R.A. Ratcheson, L.G. Salford and B.K. Siesjo, J. Neurochem., 21 (1973) 1127—1138.
10 F.A. Welsh and W. Rieder, J. Neurochem., 31 (1978) 299—309.
11 K.R. Wagner, M.V. Williams, S. Yamaguchi and R.E. Myers, Soc. Neurosci. Abstr., 6 (1980) 130.
12 K.R. Wagner and P.A. Tornheim, Soc. Neurosci. Abs., 9 (1983) 599.
13 K.R. Wagner, K.G. Allen, M.E. Fellows and P.A. Tornheim, unpublished results.
14 H. Schlenk and J.L. Gellerman, Anal. Chem., 32 (1960) 1412—1414.
15 A.J. Sheppard and J.L. Iverson, J. Chromatogr. Sci., 13 (1975) 448—452.
16 A. Grunert and K.H. Bassler, Z. Anal. Chem., 267 (1973) 342—346.
17 C.W. Gehrke and D.F. Goerlitz, Anal. Chem., 35 (1963) 76—80.
18 C.B. Johnson and E. Wong, J. Chromatogr., 109 (1975) 403—408.

Journal of Chromatography, 309 (1984) 43—52
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2120

SIMULTANEOUS SEPARATION AND SENSITIVE DETERMINATION OF FREE FATTY ACIDS IN BLOOD PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

HIRONORI TSUCHIYA*

Department of Dental Pharmacology, Gifu College of Dentistry, Hozumi, Hozumi-Cho, Motosu-Gun, Gifu (Japan)

TOKISHI HAYASHI

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

MASARU SATO

Department of Oral Microbiology, Gifu College of Dentistry, Hozumi, Hozumi-Cho, Motosu-Gun, Gifu (Japan)

and

MIKIO TATSUMI and NOBUHIKO TAKAGI

Department of Dental Pharmacology, Gifu College of Dentistry, Hozumi, Hozumi-Cho, Motosu-Gun, Gifu (Japan)

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

SUMMARY

Fatty acids are separated by reversed-phase high-performance liquid chromatography after derivatization with a fluorescence reagent, 4-bromomethyl-7-acetoxycoumarin. Each derivative eluted from a column is successively hydrolysed by mixing it with an alkaline solution, and the produced fluorescence is detected. The derivatives of series of both saturated and unsaturated fatty acids ($C_{6:0}$—$C_{20:4}$) are simultaneously separated by a continuous gradient elution method using a methanol-based solvent containing acetonitrile.

The quantitative detection of fatty acids is over a range of 5—1000 pmol per derivatization mixture. This method is applicable to the quantitative analysis of free fatty acids in normal human blood samples and blood samples from diabetic patients. Ten microlitres of

0378-4347/84/$03.00

blood plasma are sufficient to carry out the determination. The analytical results show good recovery and good reproducibility. This sensitive method is very useful for the analysis of fatty acids in very low concentrations.

INTRODUCTION

Free fatty acids in blood are derived mainly from the decomposition of triglycerides in adipose tissues or from the action of lipoprotein lipase. In spite of their concentration being lower than those of other lipids, free fatty acids are the most actively metabolized of the lipids. Thus, their analysis in biological samples is clinically of much importance. Various methods using high-performance liquid chromatography (HPLC) have been reported for this purpose [1, 2]. Although HPLC offers high resolution for the separation of a complicated mixture of fatty acids, their detection is not so easy because they generally show neither strong absorption nor fluorescence in the ultraviolet or visible region, which is widely used as the sensitive detection system for HPLC. The absorption near 200 nm has been adopted for the detection of non-derivatized fatty acids [2, 3]. However, such detection is neither sensitive nor selective. Furthermore, this method is markedly influenced by the properties of the mobile phase or impurities in it [3]. This is a disadvantage for effective application of a gradient elution technique to HPLC separation.

In order to increase sensitivity and selectivity, a number of derivatization methods for fatty acids prior to HPLC separation were developed [4—9]. In pre-column derivatizations, the use of fluorescence reagents can generally be expected to provide higher sensitivity. In this respect, 4-bromomethyl-7-methoxycoumarin [10—13], 9,10-diaminophenanthrene [14], and 9-anthryldiazomethane [15, 16], for example, were reported for the fluorometric detection of fatty acids. However, the methods using them were not very successful in the simultaneous separation of series of fatty acid derivatives. In addition, one characteristic of fluorometric detection, i.e. sensitive detection, was not sufficiently utilized for quantitative analyses such as the determination of free fatty acids in small amounts of blood samples.

A sensitive detection system for HPLC of carboxylic acids was recently developed using the fluorescence reagent 4-bromomethyl-7-acetoxycoumarin (Br-Mac) [17]. Thus, the simultaneous separation of fatty acid derivatives by this system and its application to the determination of free fatty acids in blood plasma were investigated in the present study.

EXPERIMENTAL

Reagents and apparatus

Standard fatty acids of even carbon numbers were purchased from P-L Biochemicals (Milwaukee, WI, U.S.A.) and Wako Pure Chemicals (Osaka, Japan), and those of odd carbon numbers from Nakarai Chemicals (Kyoto, Japan). Dibenzo-18-*crown*-6 was obtained from Aldrich (Milwaukee, WI, U.S.A.) and used without further purification. Br-Mac was prepared according to the method reported previously [17]. Methanol, acetonitrile, acetone, and water of

liquid chromatographic quality were used. All other reagents and solvents were of analytical grade.

The HPLC system was constructed in a manner basically similar to the one used in the previous work [17], except that fatty acids labelled with Br-Mac (FA-Mac) were successively mixed with 0.2 *M* sodium hydroxide in 80% methanol after they were eluted from the column, and the hydrolysis of FA-Mac was performed in a mixing coil (stainless-steel tube 3.5 m × 0.5 mm I.D.). Detailed chromatographic conditions are shown in Fig. 3.

Determination of free fatty acids in human blood plasma

Ten microlitres of plasma were placed in a stoppered glass vessel of 10 ml volume, and 200 μl of 0.5 *M* phosphate buffer (pH 6.5), 50 μl of a methanol solution of $C_{17:0}$ (1 nmol per 50 μl) as an internal standard, and 2.0 ml of an extraction solution (chloroform—*n*-heptane, 1:1, v/v) were added to the vessel. The mixture was vortexed for 2 min and centrifuged (1000 *g*, 10 min). The lower organic phase was evaporated to dryness. The residue was dissolved with 200 μl of acetone (100 μl × 2), and the acetone solution was transferred into a glass ampoule. After evaporating the solvent, 2—3 mg of a finely powdered mixture of potassium bicarbonate and sodium sulphate (1:1, w/w), 50 μl of dibenzo-18-*crown*-6 acetone solution (40 nmol per 50 μl), and 50 μl of Br-Mac acetone solution (100 nmol per 50 μl) were added sequentially to the ampoule. The stock solution of Br-Mac was protected from light, stored at 4°C, and renewed every three days. The ampoule was sealed and covered with aluminium foil. The reaction mixture was warmed for 30 min at 50°C in the dark. About 50 μl of the resulting solution were injected onto the column.

Determinations were carried out based on the calibration graph obtained from a standard mixture of fatty acids treated in the same manner as plasma samples.

When the concentration of free fatty acids in plasma was higher than the quantitative range, the plasma was appropriately diluted with water and then treated. On the other hand, more than 10 μl of plasma were used in the case of very low concentrations of free fatty acids.

RESULTS AND DISCUSSION

The simultaneous separation of FA-Mac was tried using continuous gradient elution according to the elution methods used by other workers [12, 13, 17]. When using a mobile phase composed of acetonitrile and water, the derivatives of fatty acids not only of the same chain length and different degrees of unsaturation but also of different chain lengths and the same degree of unsaturation could be readily separated. However, it was not easy to separate the derivatives of fatty acids having different chain lengths and different degrees of unsaturation, such as the separation between $C_{18:2}$ and $C_{14:0}$, and between $C_{20:4}$ and $C_{16:1}$.

It was reported that the concentration ratios of methanol and acetonitrile in the mobile phase significantly influenced the resolution in the isocratic separation of fatty acid phenacyl esters [18, 19]. So, the conditions to separate series of FA-Mac simultaneously were investigated by the gradient elution

method using a mobile phase of various compositions of methanol and acetonitrile. The second solvent of 90% aqueous methanol solution was continuously added to the first solvent containing different concentrations of methanol and acetonitrile, in which the water content was fixed at 30%. Fig. 1 shows the variation in retention times (t_R) of FA-Mac. Although t_R decreased with increasing acetonitrile concentration, the slopes of the curves differed among the fatty acids, as is apparent for between $C_{18:3}$ and $C_{14:0}$, and between $C_{20:4}$ and $C_{16:1}$. The separation of $C_{18:3}$ and $C_{14:0}$ was better with decreasing methanol concentration, whereas the separation of $C_{20:4}$ and $C_{16:1}$ was better with increasing methanol concentration. From these results, the first solvent of 35% methanol and 35% acetonitrile was adopted.

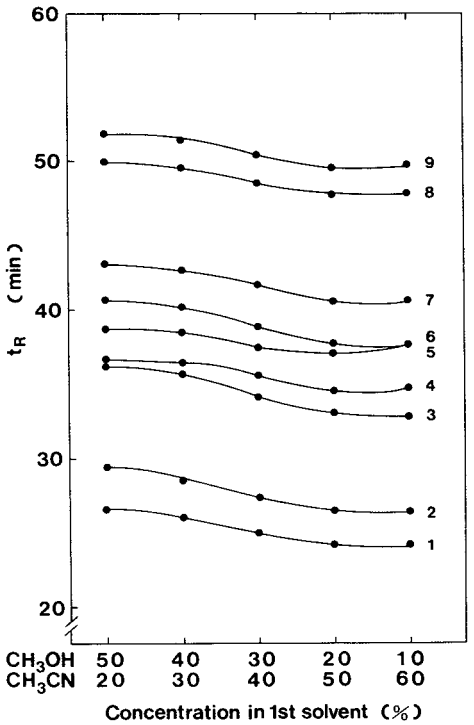


Fig. 1. Effect of methanol and acetonitrile concentrations in the first solvent on retention times (t_R) of FA-Mac. A standard mixture of FA-Mac was separated by the gradient elution method using a gradient prepared by adding the second solvent (90% aqueous methanol solution) to the first solvent (water content was fixed at 30%). The concentrations of both organic solvents are indicated. 1 = $C_{12:0}$; 2 = $C_{14:1}$; 3 = $C_{18:3}$; 4 = $C_{14:0}$; 5 = $C_{20:4}$; 6 = $C_{16:1}$; 7 = $C_{18:2}$; 8 = $C_{16:0}$; 9 = $C_{18:1}$.

As shown in Fig. 2A, t_R decreased linearly with increasing column temperature. By increasing the temperature, t_R decreased more for $C_{16:1}$ than it did for $C_{20:4}$. A similar phenomenon tended to be observed between $C_{18:3}$ and $C_{14:0}$. Fig. 2B shows typical resolution factors between adjacent peaks. A decrease of the column temperature was important for the separation of FA-Mac, especially between $C_{18:3}$ and $C_{14:0}$, and between $C_{20:4}$ and $C_{16:1}$. A column temperature of 40°C was used to shorten the analysis time and to keep the column pressure as low as possible.

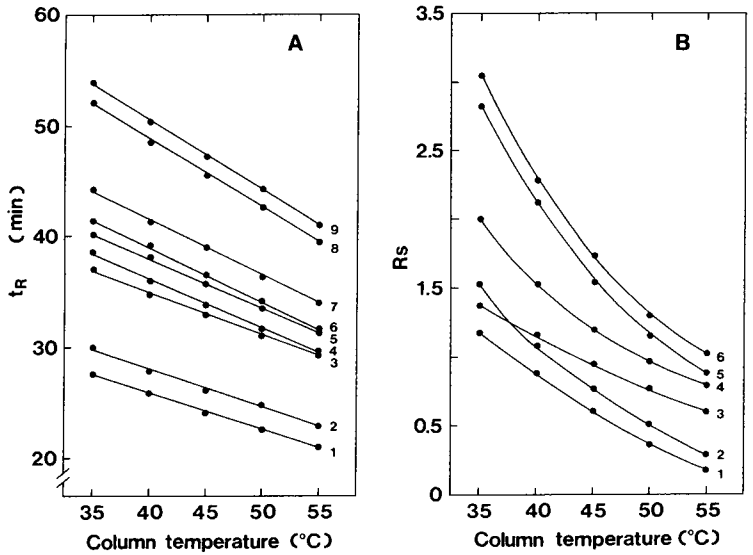


Fig. 2. Effect of column temperature on retention times (t_R) of FA-Mac (A) and on resolution factors (Rs) between adjacent peaks (B). Chromatographic conditions were the same as in Fig. 3, except for the column temperature. (A) 1 = $C_{12:0}$; 2 = $C_{14:1}$; 3 = $C_{18:3}$; 4 = $C_{14:0}$; 5 = $C_{20:4}$; 6 = $C_{16:1}$; 7 = $C_{18:2}$; 8 = $C_{16:0}$; 9 = $C_{18:1}$. (B) 1 = $C_{20:4}/C_{16:1}$; 2 = $C_{18:3}/C_{14:0}$; 3 = $C_{16:0}/C_{18:1}$; 4 = $C_{14:0}/C_{20:4}$; 5 = $C_{16:1}/C_{18:2}$; 6 = $C_{12:0}/C_{14:1}$.

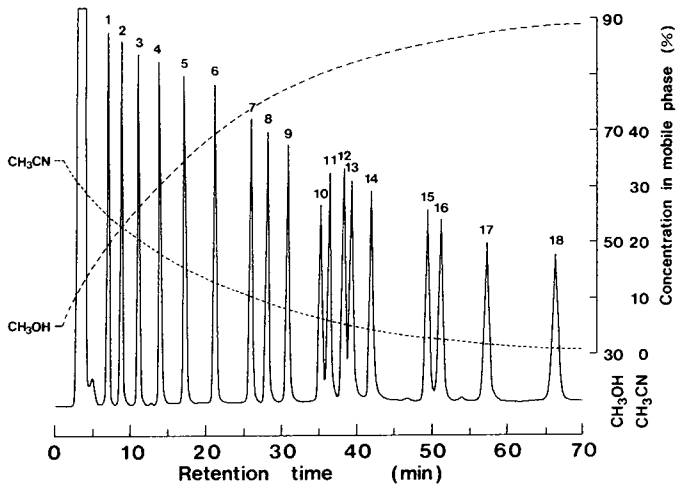


Fig. 3. High-performance liquid chromatogram obtained from a standard mixture of FA-Mac. Peaks: 1 = $C_{6:0}$; 2 = $C_{7:0}$; 3 = $C_{8:0}$; 4 = $C_{9:0}$; 5 = $C_{10:0}$; 6 = $C_{11:0}$; 7 = $C_{12:0}$; 8 = $C_{14:1}$; 9 = $C_{13:0}$; 10 = $C_{18:3}$; 11 = $C_{14:0}$; 12 = $C_{20:4}$; 13 = $C_{16:1}$; 14 = $C_{18:2}$; 15 = $C_{16:0}$; 16 = $C_{18:1}$; 17 = $C_{17:0}$; 18 = $C_{18:0}$. Chromatographic conditions: column, 250 × 4.0 mm I.D., LiChrosorb RP-18 (5 μm); column temperature, 40°C; mixing coil temperature, 50°C; mobile phase first solvent, methanol—acetonitrile—water, (35:35:30), second solvent 90% aqueous methanol solution (the gradient was prepared by adding the second solvent to the first solvent; the dotted lines show the concentrations of organic solvents in the mobile phase); mobile phase flow-rate, 1.2 ml/min; flow-rate of alkaline solution for hydrolysis, 0.4 ml/min; detector, spectrofluorometer (excitation 365 nm, emission 460 nm).

The chromatogram shown in Fig. 3 was obtained from a standard mixture of fatty acids. A methanol solution of standard fatty acids (1 nmol of each) was placed in a glass ampoule and subjected to derivatization after evaporation of the solvent. There were few significant peaks on the chromatogram other than those of fatty acids, except for the presence of the front peaks derived from an excess of Br-Mac and its decomposition products.

Fig. 4 shows the amount of FA-Mac formed (relative units) versus the reaction time at different reaction temperatures. The amount of derivative formed is proportional to the peak height ratio, i.e. the height of the FA-Mac peak relative to that of internal standard (anthracene). Even at 30°C, the reaction was completed within 40 min. The reaction was completed within 10 min at 70°C, but several unknown peaks tended to appear. In order to shorten the reaction time and minimize the production of unknown peaks, a reaction temperature of 50°C and a reaction time of 30 min were chosen in this study.

The effect of a catalyst on the reaction yield of FA-Mac is shown in Fig. 5. Although dibenzo-18-*crown*-6 is widely used as a catalyst for the derivatization of fatty acids, the effect of this reagent was not so significant in comparison with other derivatizing reagents [11, 20]. Even in the absence of the catalyst, the reaction proceeded smoothly.

A typical result of the quantitative investigation is shown in Fig. 6, which is

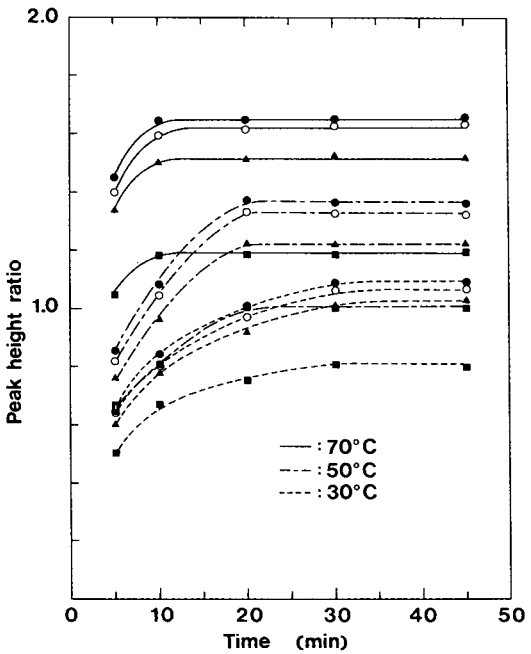


Fig. 4. Effects of reaction time and temperature on fluorescence intensity. A mixture of fatty acids (1 nmol of each) was subjected to derivatization. Chromatographic conditions were the same as in Fig. 3, except that the gradient was prepared by adding 80% aqueous acetonitrile solution to 65% aqueous acetonitrile solution. Peak height ratios were determined by dividing the peak heights of FA-Mac by the peak height of anthracene added in the reaction mixture. (●), $C_{14:0}$; (○), $C_{16:1}$; (▲), $C_{18:2}$; (■), $C_{18:3}$.

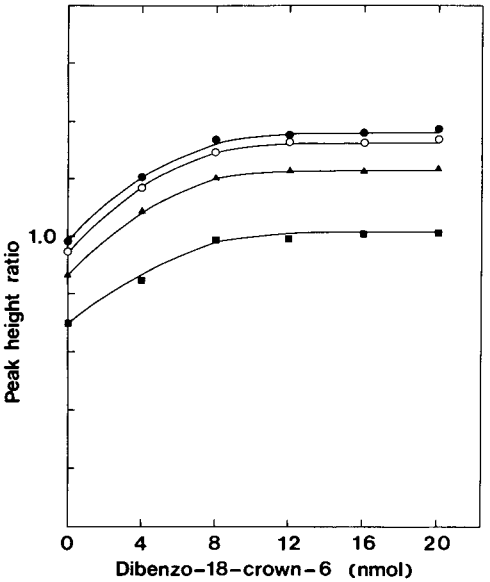


Fig. 5. Effect of dibenzo-18-*crown*-6 concentration in a reaction mixture on fluorescence intensity. A mixture of fatty acids (1 nmol of each) was subjected to derivatization for 30 min at 50°C. Chromatographic conditions as in Fig. 4. Peak height ratios were determined in the same manner as in Fig. 4. (●), $C_{14:0}$; (○), $C_{16:1}$; (▲), $C_{18:2}$; (■), $C_{18:3}$.

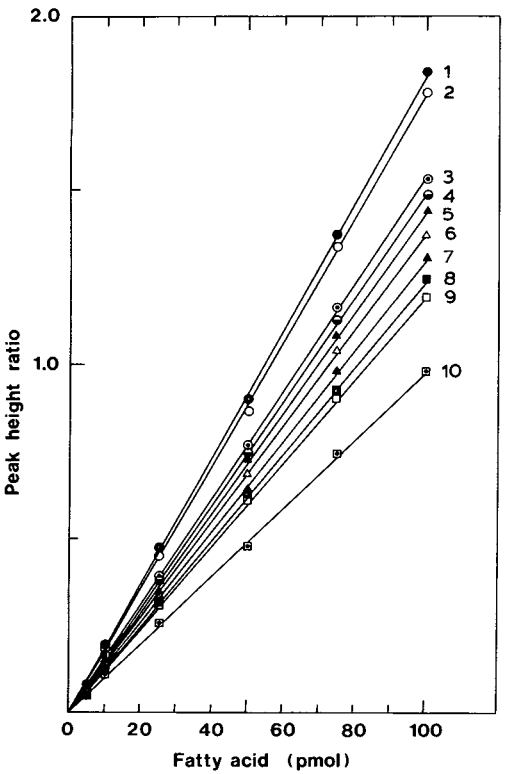


Fig. 6. Calibration graph of fatty acids. The separation was performed under chromatographic conditions shown in Fig. 3. Graphs: 1 = $C_{12:0}$; 2 = $C_{14:1}$; 3 = $C_{20:4}$; 4 = $C_{14:0}$; 5 = $C_{16:1}$; 6 = $C_{18:2}$; 7 = $C_{18:3}$; 8 = $C_{16:0}$; 9 = $C_{18:1}$; 10 = $C_{18:0}$.

a plot of the ratio of the peak height of each FA-Mac to that of internal standard ($C_{17:0}$). The plot gave linearity in a range from at least 1000 to 5 pmol (only less than 100 pmol are shown). This result indicates that the derivatization method described here can be used to quantitate fatty acids.

The method was applied to the determination of free fatty acids in human blood plasma samples. Solvent extraction was chosen for pre-purification of fatty acids in the samples because of its simplicity. The chromatograms obtained from normal human plasma and from plasma of a diabetic patient are shown in Figs. 7 and 8, respectively. There were few interfering peaks other

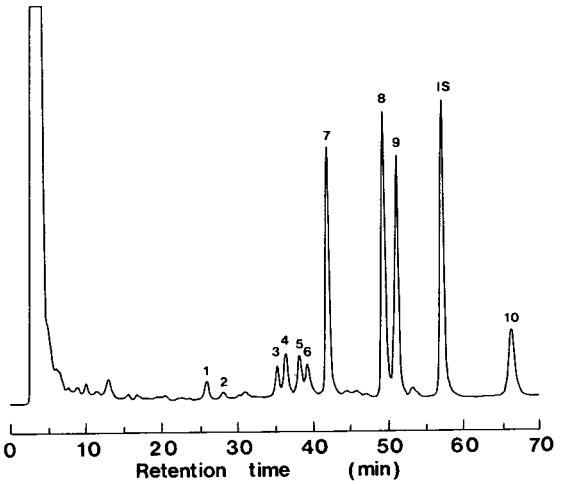


Fig. 7. High-performance liquid chromatogram obtained from a normal human plasma sample. Peaks: 1 = $C_{12:0}$; 2 = $C_{14:1}$; 3 = $C_{18:3}$; 4 = $C_{14:0}$; 5 = $C_{20:4}$; 6 = $C_{16:1}$; 7 = $C_{18:2}$; 8 = $C_{16:0}$; 9 = $C_{18:1}$; 10 = $C_{18:0}$; IS (internal standard) = $C_{17:0}$. Chromatographic conditions as in Fig. 3.

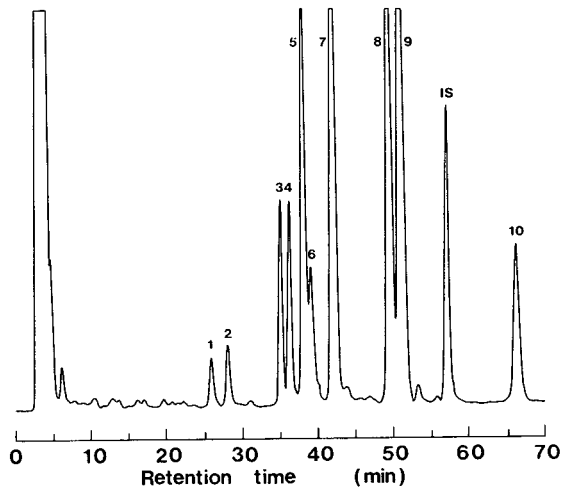


Fig. 8. High-performance liquid chromatogram obtained from the plasma sample of a diabetic patient. Peak numbers as in Fig. 7. Chromatographic conditions as in Fig. 3.

than free fatty acid derivatives, which were identified by comparing their retention times with those of standard derivatives.

Analytical recovery and reproducibility were estimated by adding a standard mixture of fatty acids ($C_{18:2}$, $C_{16:0}$, and $C_{18:1}$ = 1.00 nmol; others = 0.30 nmol) to 10 μl of plasma (n = 7). The percentage recoveries and coefficients of variation (C.V., %) were as follows: $C_{12:0}$ = 100 (2.7); $C_{14:1}$ = 97 (3.7); $C_{18:3}$ = 97 (3.7); $C_{14:0}$ = 93 (4.3); $C_{20:4}$ = 93 (3.0); $C_{16:1}$ = 97 (3.4); $C_{18:2}$ = 99 (2.0); $C_{16:0}$ = 96 (1.8); $C_{18:1}$ = 97 (2.4); $C_{18:0}$ = 90 (4.5).

The results of determinations of free fatty acids in plasma samples are summarized in Table I. They agreed with the report of other workers [9]. The concentrations of certain kinds of free fatty acids such as $C_{20:4}$, $C_{18:2}$, $C_{16:0}$, and $C_{18:1}$ significantly increased in the samples of diabetic patients. Their composition ratios in free fatty acids also differed from those of normal samples.

TABLE I

FREE FATTY ACIDS IN HUMAN PLASMA

Results are expressed as nmol/ml; — indicates that the peak was detectable but the determination was impossible.

Sample	$C_{12:0}$	$C_{14:1}$	$C_{18:3}$	$C_{14:0}$	$C_{20:4}$	$C_{16:1}$	$C_{18:2}$	$C_{16:0}$	$C_{18:1}$	$C_{18:0}$
Normal										
1	5.6	2.7	6.4	14.9	15.6	6.3	57.5	118	145	38.4
2	1.6	—	2.5	5.3	4.6	4.2	18.7	46.6	48.5	15.3
3	2.2	—	2.3	7.0	3.2	8.7	30.5	49.2	45.7	15.2
4	3.7	—	5.1	8.2	12.0	8.8	73.3	68.3	80.3	26.7
5	1.9	—	6.4	5.7	4.2	5.9	42.7	46.7	44.3	18.1
6	2.2	—	5.5	7.0	5.2	4.5	39.0	61.4	54.6	21.9
7	1.5	—	12.8	5.7	4.8	5.6	141	61.2	221	27.7
8	3.0	—	2.2	9.4	3.3	5.2	16.6	54.8	46.8	17.6
9	2.3	—	5.1	14.5	5.4	6.5	32.7	61.7	69.3	27.8
10	2.2	—	3.9	7.0	7.0	5.2	27.5	56.2	55.4	20.4
11	3.0	1.7	5.6	10.8	4.0	13.1	56.2	95.9	135	32.4
12	2.6	1.8	7.3	8.4	13.2	7.0	60.3	74.0	129	22.3
13	2.7	2.5	6.2	12.6	7.4	21.8	36.7	71.3	91.8	20.4
14	1.8	—	2.8	6.1	2.5	7.1	24.1	41.8	46.8	13.9
15	1.6	—	3.4	7.9	5.0	8.7	25.3	70.6	59.8	22.3
Diabetic patient										
1	6.3	4.9	22.6	22.2	75.5	12.6	218	258	750<	71.8
2	4.6	4.6	23.1	23.8	74.5	20.9	300	289	750<	66.7
3	5.2	4.1	38.2	38.8	60.0	15.3	240	396	506	61.0
4	9.3	13.2	26.4	42.4	172	21.9	239	454	750<	83.8
5	5.9	6.9	36.0	29.4	102	16.0	380	321	527	54.3
6	8.1	8.4	10.4	23.2	32.6	70.9	307	426	437	114

Although HPLC separation of fatty acids has been widely attempted using ultraviolet-absorbing reagents [4—10, 19], the sensitivity was not sufficient for treating very small amounts of blood samples. With such methods, at least 0.5—1.0 ml of plasma or serum was necessary for the determination [9, 19]. In this study, a plasma sample volume of 10 μl was used for precision of sampling. If precise quantitation is not required, the determination can be performed using plasma sample volumes of less than 10 μl.

CONCLUSIONS

A sensitive determination method for fatty acids was developed by employing fluorometric detection. It is considered that the high reactivity of Br-Mac and the high sensitivity are useful for treating samples containing very small amounts of fatty acids.

The simultaneous separation of series of fatty acids was achieved, which was difficult for HPLC methods using other fluorescence reagents. The analysis time per sample, however, became relatively long to obtain better resolution. It might be reduced by using another gradient elution mode or a different reversed-phase column.

REFERENCES

1 M.J. Cooper and M.W. Anders, J. Chromatogr. Sci., 13 (1975) 407.
2 M. VanRollins, M.I. Aveldano, H.W. Sprecher and L.A. Horrocks, Methods Enzymol., 89 (1982) 618.
3 M.I. Aveldano, M. VanRollins and L.A. Horrocks, J. Lipid Res., 24 (1983) 83.
4 R.F. Borch, Anal. Chem., 47 (1975) 2437.
5 P.-T.S. Pei, W.C. Kossa, S. Ramachandran and R.H. Henly, Lipids, 11 (1976) 814.
6 H.C. Jordi, J. Liquid Chromatogr., 1 (1978) 215.
7 D.P. Matthees and M.C. Purdy, Anal. Chim. Acta, 109 (1979) 61.
8 J.F. Lawrence, in R.W. Frei and J.F. Lawrence (Editors), Chemical Derivatization in Analytical Chemistry, Vol. 2, Plenum, New York, 1982, p. 191.
9 M. Ikeda, K. Shimada and T. Sakaguchi, J. Chromatogr., 272 (1983) 251.
10 W. Dünges and N. Seiler, J. Chromatogr., 145 (1978) 483.
11 S. Lam and E. Grushka, J. Chromatogr., 158 (1978) 207.
12 S.G. Zelenski and J.W. Huber, III, Chromatographia, 11 (1978) 645.
13 W. Voelter, R. Huber and K. Zech, J. Chromatogr., 217 (1981) 491.
14 J.B.F. Lloyd, J. Chromatogr., 189 (1980) 359.
15 N. Nimura and T. Kinoshita, Anal. Lett., 13 (1980) 191.
16 S.A. Barker, J.A. Monti, S.T. Christian, F. Benington and R.D. Morin, Anal. Biochem., 107 (1980) 116.
17 H. Tsuchiya, T. Hayashi, H. Naruse and N. Takagi, J. Chromatogr., 234 (1982) 121.
18 J. Halgunset, E.W. Lund and A. Sunde, J. Chromatogr., 237 (1982) 496.
19 M. D'Amboise and M. Gendreau, Anal. Lett., 12 (1979) 381.
20 G. Gübitz, J. Chromatogr., 187 (1980) 208.

Journal of Chromatography, 309 (1984) 53—61
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2131

DETERMINATION OF NEUROTRANSMITTER SYSTEMS IN HUMAN CEREBROSPINAL FLUID AND RAT NERVOUS TISSUE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ON-LINE DATA EVALUATION

C.G. HONEGGER*, R. BURRI, HELEN LANGEMANN and A. KEMPF

Section of Neurobiology, Department of Research, Cantonal Hospital, CH-4031 Basel (Switzerland)

(First received December 7th, 1983; revised manuscript received February 27th, 1984)

SUMMARY

We describe an improved high-performance liquid chromatographic method for the determination of tyrosine, 5-hydroxytryptamine, 5-hydroxyindoleacetic acid, tryptophan and homovanillic acid in cerebrospinal fluid and nerve tissue, using the new microbore cartridges with 5 μm average particle size. The first four substances are quantified fluorometrically and the last two electrochemically. Both detectors are connected to the same integrator through a relay which can be switched as required. Data are collected in an on-line personal computer and evaluated statistically. An improvement in the method for extraction and separation of catecholamines is also reported.

INTRODUCTION

In the course of studies on central nervous system function we needed a high-performance liquid chromatographic (HPLC) method for detecting changes in the catecholamine and indoleamine systems, i.e. one able to measure not only the neurotransmitters themselves but also their precursors and metabolites. Finding a satisfactory single system presented a difficult problem, for two reasons. Firstly, the polarities of the compounds differ widely. Secondly, baseline separation and sharp peaks are required for maximum accuracy, so that elution times should not be too short or too long. Most of the systems described in the literature are either not suitable for all the substances which interested us or do not fulfill this second requirement (e.g. refs. 1—7). Gradient elution with increasing proportions of organic solvent should be the ideal system; however, in contrast to Krstulovic et al. [8], we found this to be

0378-4347/84/$03.00

incompatible with amperometric electrochemical detection and even to a lesser extent with fluorometric detection. We therefore decided to use two systems. Catecholamines were determined with a modification of the usual alumina extraction method (e.g. refs. 9—11). For the other compounds (5-hydroxytryptamine, 5-hydroxyindoleacetic acid, tryptophan, tyrosine, homovanillic acid) we developed a direct injection method with the following innovations:

(1) Microbore cartridges were used (internal diameter 2.1 mm, 5 μm average particle size), which operate with a low flow-rate, requiring greatly reduced amounts of mobile phase. The advantage of the cartridge system is that the column length can be tailored without difficulty to suit the required separation.

(2) The first cut from the column was diverted into the waste before the electrochemical detector, thus preventing an overload and disturbance of the baseline when extracts were injected.

(3) An electrochemical detector and a fluorometric detector were connected to the same low-price one-channel integrator through a relay, so that all the peaks, including the amino acids tryptophan and tyrosine, could be quantified as accurately as possible.

(4) The chromatographic data were stored on discs on-line with the aid of an Apple IIe computer. Software was developed for transferring data to statistical programs either in the personal computer, or in the Univac 1100 computer for more advanced evaluation.

MATERIALS

Chemicals were obtained from the following sources: 5-hydroxytryptamine creatinine sulphate (5-HT) from Serva (Heidelberg, F.R.G.); 5-hydroxyindoleacetic acid (5-HIAA), L-tyrosine (Tyr), *n*-octyl sodium sulphate, ethylenediaminetetraacetic acid disodium salt (EDTA, Titriplex III) and aluminium oxide 90 (active neutral for column chromatography) from E. Merck (Darmstadt, F.R.G.). L-Tryptophan (Trp), homovanillic acid (HVA), dopamine hydrochloride (DA), L-adrenaline-D-hydrogentartrate (A), and L-noradrenaline hydrochloride (NA) from Fluka (Buchs, Switzerland); 3,4-dihydroxybenzylamine hydrobromide (DHBA) from Aldrich Europe (Beerse, Belgium). Acetonitrile and methanol (both chromatographic grade, Merck) were used as supplied. All solutions were prepared in water that had been double-distilled in glass.

Chromatographic system A (for Tyr, 5-HT, 5-HIAA, Trp and HVA)

A solvent delivery pump (LC 414, Kontron Analytic, Zurich, Switzerland) was used in conjunction with an automatic sampler (MSI 660, Kontron) equipped with a 10-μl injection loop. Pulseless solvent delivery was achieved with a membrane-type damper (Portmann, Therwil, Switzerland). The column system, which was at room temperature, consisted of three commercially available reversed-phase cartridges in series, all with internal diameter 2.1 mm. The total length was 350 mm (RP-18 Spheri 5, 5 μm spherical particle size, 30 mm + 100 mm + 220 mm; Brownlee Labs., Santa Clara, CA, U.S.A.). Two detectors were used; upstream a fluorescence detector (LC fluorometer FS 970,

Schoeffel Instruments, Kratos, Trappenkamp, F.R.G.) with a wavelength of 225 nm for excitation and a band pass filter 7-54 for emission; downstream an electrochemical detector (656 VA, Metrohm, Herisau, Switzerland) with a glassy carbon working electrode set at a potential of 0.75 V versus the Ag/AgCl reference electrode. The output of the fluorometer was changed from 10 mV to 1 V using an FSA 987 computer output, and both detectors were connected via a relay to the same integrator (HP 3390 A, Hewlett-Packard, Avondale, PA, U.S.A.). The relay was controlled by the integrator through an intermediate 19400 A sampler/event control module (Hewlett-Packard). By programming the integrator the relay could be switched at any desired time so that either the output of the electrochemical detector or that of the fluorometer could be recorded by one integrator. Between the two detectors an automatic Rheodyne 7040 valve was inserted, which allowed the automatic cut-off of the effluent after the fluorescence detector. This was also time-controlled by the integrator. Mobile phase A contained sodium dihydrogen phosphate (0.1 *M*), EDTA (0.08 m*M*) and *n*-octyl sodium sulphate (0.025 m*M*) brought to pH 3.5 with phosphoric acid. It was filtered through a 0.4-μm filter and acetonitrile was added to a final concentration of 8%. It was degassed by stirring under vacuum (water pump) for 10 min. The flow-rate was 0.3 ml/min, at a pressure of about 175 bars.

Chromatographic system B (for catecholamines)

This was essentially as described above, the Kontron sampler being replaced by a Wisp 710 B (Waters Assoc., Milford, MA, U.S.A.). The electrochemical detector was set at 0.65 V and the fluorometer and valve were omitted. The stationary phase was an RP-18 Spheri 5 column, 100 mm × 4.6 mm (Brownlee Labs.), average particle size 5 μm, and the mobile phase was disodium hydrogen phosphate 6.7 m*M*, citric acid 13.3 m*M*, *n*-octyl sodium sulphate 2.5 m*M*, EDTA 0.05 m*M*, methanol 13—15% depending on column condition, pH 3.3 [10]. The flow-rate was 1.3 ml/min. The mobile phase was recycled. This had two advantages: firstly, the stability of the electrochemical detector was improved; and secondly, the amount of mobile phase used was greatly reduced (to 2 l in three weeks; even less than would be required with a microbore system).

Preparation of extracts

Human cerebrospinal fluid (CSF) samples were obtained from the Neurological Clinic, Kantonsspital, Basel, and cooled in ice until they reached the laboratory. They were treated with 1 *M* perchloric acid (10:1, v/v) to precipitate protein, then centrifuged for 25 min at 4°C and 9000 *g* in a Sorvall RC-5 Superspeed refrigerated centrifuge (Dupont, Newton, CT, U.S.A.). The supernatant was either injected directly (system A, 10 μl) or stored at −80°C for not longer than one week. Samples stored before perchloric acid treatment could be kept for up to three months. In both cases the thawed samples were recentrifuged for 25 min as above before injection (10 μl).

Lewis rat brain or spinal cord regions (30 mg to 1.3 g) were isolated and stored at −80°C until used. They were homogenized with cooling in ice in 0.1 *M* perchloric acid (1:10, w/v) with an ultrasonic cell disrupter, either a micro-

model from Kontes (Vineland, NJ, U.S.A.) or a Sonicator W 375 (Heat Systems—Ultrasonics, as supplied by Kontron) depending on the amount. For catecholamine determinations DHBA (final concentration 10^{-6} *M*) was added as internal standard and 0.01% sodium bisulphite as antioxidant. The homogenates were centrifuged as described above. A portion of the supernatant was removed for catecholamine isolation and determination in system B, and the rest stored at −80°C for not longer than a month. After thawing it was recentrifuged as above before direct injection (10 µl) into system A.

Analysis and quantification of Trp, 5-HT, 5-HIAA, HVA, Tyr

Direct injection of CSF and homogenates into system A caused no problem with the fluorometer. However, when the electrochemical detector was set to a sensitivity sufficient for our measurements (usually 5 nA/V) there was an overload as the initial peak reached the detector, due to water-soluble, easily oxidizable cell constituents, such as ascorbic acid. To prevent this, the first part of the efflux was diverted as described above before reaching the electrochemical detector, which greatly improved the baseline characteristics. In the concentration ranges present in our extracts, 5-HT can be determined either electrochemically or fluorometrically. 5-HIAA and HVA must be determined electrochemically if any degree of accuracy is required, whereas with 0.75 V potential Trp and Tyr must be determined fluorometrically. (They can also be determined electrochemically when the detector potential is higher than 0.9 V; in our experience, however, the system is then more unstable and much less specific.)

Retention times are in the order Tyr < 5-HT < Trp < 5-HIAA < HVA. Therefore, before injection, the relay was set so that the integrator was connected to the fluorometer until the three peaks Tyr, 5-HT and Trp had been recorded (about 14 min). The relay was then switched so that the integrator recorded the output from the electrochemical detector for the rest of the chromatogram (peaks 5-HIAA and HVA). Because the substances are present in the extracts in greatly varying concentrations, the integration attenuation was sometimes changed according to the peak size, which improved the reproducibility of the determination. Quantitative determinations were made by comparing the peak areas of the samples with those of standard solutions using the external standard calibration program of the integrator. Stock solutions in water (10^{-3} *M*) were made fresh every week, stored at 4°C and diluted as required. Because no cooled sampler was available, all solutions and extracts were brought to room temperature only a short time before injection. The detectors were allowed to stabilize for 1 h each morning before use, and were re-calibrated if necessary with two or three standard solutions. The relationship between peak area and amount injected was found to be linear for each substance in the concentration range in which it is present in nervous tissue and CSF, except for Tyr which showed a plateau at higher concentrations.

Extraction and quantification of catecholamines

A suspension of aluminium oxide (15 mg/ml) in Tris—HCl buffer [tris(hydroxymethyl)aminomethane 0.6 *M*, EDTA 0.05 *M*, pH 8.6] was stirred

magnetically for 15 min. Portions (1 ml) were pipetted during stirring into plastic tubes, to which the supernatants (200 µl) prepared as above were added (according to a private communication of A. Enz, Sandoz, Basel). The mixture was shaken for 15 min and centrifuged. The aluminium oxide was washed with water (3 × 1 ml) and as much as possible of the final washing water was removed. The catecholamines were eluted by agitation for 5 min in 200 µl of mobile phase B, brought to pH 2 with phosphoric acid. After centrifugation the supernatant, usually 20 µl, was injected directly into system B.

Standard solutions containing DHBA (10^{-6} *M*) and the other catecholamines ($2.5 \cdot 10^{-7}$ *M*, $5 \cdot 10^{-7}$ *M* or $7.5 \cdot 10^{-7}$ *M*) in 0.1 *M* perchloric acid were made and stored in portions at −80°C for up to six months. Every time tissue samples were extracted as described above, these three solutions (180 µl each) were taken through the same procedure. The aluminium oxide was eluted with mobile phase (200 µl) and 20 µl of the eluate were injected. The internal standard program of the integrator calculated the ratios of the peak areas of the catecholamines to those of DHBA and by comparison with these calculated the catecholamine content of the extracts.

Catecholamines are more stable than indoles at room temperature and the automatic sampler could be loaded and run overnight.

Computer evaluation

Hardware. This comprised the following components: Apple IIe computer with screen and optional printer; two floppy disc drives; 7710-A serial interface (from CCS) for the Apple; cable to connect the two RS 232-C in Apple and HP integrator.

Software. Our "Multi"-program was written in Applesoft Basic, apart from a subroutine to transfer the data coming from the integrator to a temporary store in the Apple's memory. This had to be written in Assembler (the machine language) because Basic is not fast enough to handle all the in-coming data. Where possible, Basic is preferable as it is easier than Assembler to write, read and modify. Using the Multi-program, the HPLC data can be manipulated in the following ways:

(1) Chromatograms can be stored in files (Chro-files) on floppy discs. Using two floppy discs, up to 260 chromatograms with seven calibrated peaks are available on-line. Each chromatogram is first stored temporarily in the Apple as described above, then sent to the floppy disc during the next chromatographic run.

(2) Any Chro-file or series of Chro-files can be called up on the screen or printed out.

(3) Data needed for statistical calculations can be sorted out from a series of Chro-files and sent to a single file, together with extra information if required. This new data file has a previously defined and programmed structure; e.g. an array (*A* matrix with *N* Chro-files and *M* defined variables). These data files can be evaluated using private statistics programs or commercial software packages. One of our Multi-program options creates data files which can be read by Micro-SPSS 4.1 [12]. These matrix data files can be enlarged vertically, i.e. the data from other chromatograms can be added at a later date without difficulty. Faulty or missing data which could be added later are replaced with

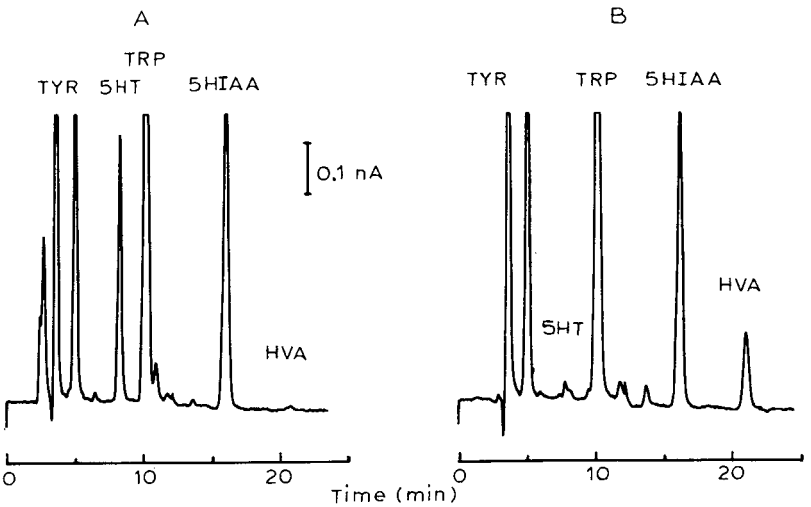


Fig. 1. Chromatograms of perchloric acid extracts prepared as described in text. Injection volume 10 μl; chromatographic conditions, system A. (A) Lewis rat spinal cord, (B) human cerebrospinal fluid.

TABLE I

RETENTION TIMES OF SOME CATECHOLAMINES, INDOLEAMINES AND THEIR METABOLITES AND PRECURSORS

Retention times are given in min.

Compound*	System A	System B
Tyr	3.47	
NA	3.50	6.24**
DOMA	3.51	4.44**
DOPA	3.54	4.03**
A	3.65	8.90**
DOPEG	3.71	1.19**
DHBA	3.84	12.50**
VMA	4.05	1.36
DA	4.47	19.69**
5-HTP	4.88	11.51
NM	5.17	12.19
MHPG	5.25	1.83
5-HT	8.45	ca. 50
DOPAC	9.12	3.30**
Trp	10.19	
5-HIAA	16.59	5.11
HVA	20.96	6.90

*DOMA = 3,4-dihydroxymandelic acid, DOPA = 3,4-dihydroxyphenylalanine, DOPEG = 3,4-dihydroxyphenylethylene glycol, VMA = vanillylmandelic acid, 5-HTP = 5-hydroxytryptophan, NM = 3-O-methylnoradrenaline, MHPG = 3-methoxy-4-hydroxyphenylethylene glycol, DOPAC = 3,4-dihydroxyphenylacetic acid.

**Can be extracted from homogenates by Al_2O_3.

a selected "missing value". Another option of the Multi-program enables data to be arranged in a format that can be read by BMDP statistical software (on a Univac 1100) for sophisticated statistical evaluation of large amounts of data. With a special program and a corresponding hardware connection developed by Dr. Christen (URZ, Basel), data from the Apple can be sent to the Univac.

RESULTS AND DISCUSSION

System A

Fig. 1A and B shows typical chromatograms obtained by direct injection of rat spinal cord extract and human CSF into system A. The first part of the chromatogram shows the peaks recorded by the fluorometer, the second part those recorded electrochemically. As can be seen, the switching of the relay took place without greatly affecting the baseline. In developing our method we considered baseline separation to be of primary importance. As can be seen from the figures, we have obtained good separations of Tyr, 5-HT, Trp, 5-HIAA and HVA, even with extracts. The identification of peaks with the direct injection method always causes problems. We approached this in the same way as other authors [4, 12, 13]: multiple detectors (electrochemical, fluorometric), variable detector potential (0.65—0.95 V), extraction with alumina (specific for catechol derivatives) and injection into a different chromatographic system (without octyl sulphate, results not shown). The five peaks quantified can be considered to be unambiguously identified. Table I lists typical retention times of the catecholamines, indoleamines and some of their common metabolites in the two systems. In system A, catecholamines and their metabolites were eluted in the first 5.5 min, well before 5-HT. An exception was 3,4-dihydroxyphenylacetic acid (DOPAC), which eluted between 5-HT and Trp; however, as it has practically no fluorescence under our conditions, it caused no interference. The electrochemical method would be more sensitive for 5-HT [4] but as it is present in relatively large quantities in nerve extracts this is not of great importance. We have tried to obtain improved separations of CSF using other chromatographic systems, including that of Gattaz et al. [14], and columns with smaller particle size. However, our latest results with the column RP-18 Spheri 3 μm, length 100 mm (Brownlee) show that (a) the pressure is so high (160 bars at a flow-rate of 0.3 ml/min) that the column could not be much lengthened, and (b) the separation on this column is not good enough to replace our system A. As already reported by others [15], we found that Tyr, Trp, 5-HT, HVA and 5-HIAA were not stable for more than a few hours at room temperature, even in the presence of anti-oxidants, in standard solutions and to a lesser extent also in extracts. All samples were therefore brought to room temperature only a short time before injection to prevent errors. Recoveries, determined by the standard addition method, were as follows: Tyr 83%, Trp 100%, 5-HIAA 100% and HVA 100% from CSF; and Tyr 98%, 5-HT 99%, Trp 100%, 5-HIAA 100% and HVA 98% from Lewis rat spinal cord.

System B

Fig. 2 shows a chromatogram of an alumina extract from rat spinal cord

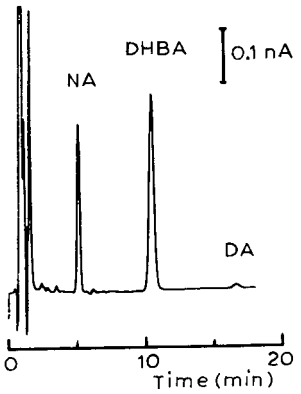


Fig. 2. Chromatogram of catecholamines extracted by alumina from Lewis rat spinal cord as described in text. Injection volume 20 μl; chromatographic conditions, system B.

TABLE II

CONTENTS OF SUBSTANCES IN RAT BRAIN AND HUMAN CSF

Results are measured in ng/g fresh weight (rat spinal cord) or ng/ml (human CSF) and expressed as mean ± S.E.M.

	Tyr	5-HT	Trp	5-HIAA	HVA	NA	DA
Rat spinal cord (n = 10)	12,978 ± 712	823.2 ± 14.9	3830 ± 129	406.6 ± 6.9	0.66 ± 0.66	559.9 ± 13.6	33.3 ± 2.02
Human CSF (n = 8)	n.d.*		372 ± 18	23.3 ± 2.2	31.8 ± 2.6	—	—

*n.d. = not determined.

homogenate injected into system B. The catecholamines NA, A, DHBA (internal standard) and DA were separated in 20 min. The separation required only a 100-mm column (5 μm) compared with 250 mm using a Bondapak RP-18 with 10 μm average particle size. Alumina extraction is specific for catecholamines, so peaks could be readily identified. Recovery rates from standard solutions were 84.7 ± 0.4% for NA, 88.9 ± 1.3% for A, 84.4 ± 2.1% for DHBA and 84.9 ± 2.2% for DA (n = 4). These high rates were only obtained when the alumina was stirred in Tris buffer prior to extraction and may be the result of better contact between the buffer and the alumina particles. By eluting the catecholamines from the alumina with mobile phase, instead of perchloric acid, the initial peak was reduced and the baseline improved without affecting the yield.

Table II shows the contents of the substances which we found in rat spinal cord and human CSF. For CSF they are comparable with those found in the literature [14, 16—18], the main difficulty here being the enormous variation found in normal persons [17]. For rat spinal cord the values for 5-HT, NA and DA are similar to those obtained by Zivin et al. [19]. In a more recent publication [20] values are expressed as pg/mg protein and are therefore not directly comparable.

Our present method, like other chromatographic methods, is a compromise. For instance 3-methoxy-4-hydroxyphenylethylene glycol (MHPG), a substance of great interest in CSF, can only be quantified with two-channel integration. Other authors [21—24] have used direct injection combined with electrochemical detection for both catecholaminergic and indoleaminergic substances. However, we chose the alumina extraction method for catecholamines because, when extracts are directly injected, large amounts of interfering non-catecholaminergic substances co-elute with the catecholamines. We found that this made baseline separation and reproducible results very difficult to obtain. In a recent publication Tjaden and de Jong [25] suggest that dual coulometric electrochemical detection may solve the problem of gradient-induced baseline shift. This could lead to an improved separation of substances in one run, which would offer an alternative to isocratic methods such as ours.

ACKNOWLEDGEMENTS

We thank the Swiss Multiple Sclerosis Society and the Swiss National Science Foundation (Grant 3.603.80) for financial support.

REFERENCES

1 I.N. Mefford, J. Neurosci. Methods, 3 (1981) 207.
2 J. Wagner, P. Vitali, M.G. Palfreyman, M. Zraika and S. Huot, J. Neurochem., 38 (1982) 1241.
3 W.H. Lyness, Life Sci., 31 (1982) 1435.
4 G.M. Anderson, J.G. Young, D.K. Batter, S.N. Young, D.J. Cohen and B.A. Shaywitz, J. Chromatogr., 223 (1981) 315.
5 I.N. Mefford and J.D. Barchas, J. Chromatogr., 181 (1980) 187.
6 G.M. Anderson, J.G. Young and D.J. Cohen, J. Chromatogr., 164 (1979) 501.
7 W.E. Wilson, S.W. Mietling and J.-S. Hong, J. Liquid Chromatogr., 6 (1983) 871.
8 A.M. Krstulovic, L. Bertani-Dziedzic, S. Bautista-Cerqueira and S.E. Gitlow, J. Chromatogr., 227 (1982) 379.
9 C.R. Freed and P.A. Asmus, J. Neurochem., 32 (1979) 163.
10 J. Wagner, M. Palfreyman and M. Zraika, J. Chromatogr., 164 (1979) 41.
11 L.J. Felice, J.D. Felice and P.T. Kissinger, J. Neurochem., 31 (1978) 1461.
12 R. Rose, Angewandte Statistik mit Micro-SPSS, R. Rose, Hauptstrasse, 2341 Mohrkirch, F.R.G., 1982.
13 D.E. Mais, P.D. Lahr and T.R. Bosin, J. Chromatogr., 225 (1981) 27.
14 W.F. Gattaz, P. Waldmeier and H. Beckmann, Acta Psychiat. Scand., 66 (1982) 350.
15 R. Zaczek and J.T. Coyle, J. Neural Transm., 53 (1982) 1.
16 J. Montplaisir, J. de Champlain, S.N. Young, K. Missala, T.L. Sourkes, J. Walsh and G. Rémillard, Neurology, 32 (1982) 1299.
17 J.C. Ballenger, R.M. Post, F.K. Goodwin, in J.H. Wood (Editor), Neurobiology of Cerebrospinal Fluid, Vol. 2, Plenum, New York, 1983, p. 143.
18 E.H.F. McGale, I.F. Pye, C. Stonier, E.C. Hutchinson and G.M. Aber, J. Neurochem., 29 (1977) 291.
19 J.A. Zivin, J.L. Reid, J.M. Saavedra and I.J. Kopin, Brain Res., 99 (1975) 293.
20 S.R. White, R.K. Bhatnagar and M.T. Bardo, J. Neurochem., 40 (1983) 1771.
21 E. Morier and R. Rips, J. Liquid Chromatogr., 5 (1982) 151.
22 G.S. Mayer and R.E. Shoup, J. Chromatogr., 255 (1983) 533.
23 R.B. Taylor, R. Reid, K.E. Kendle, C. Geddes and P.F. Curle, J. Chromatogr., 277 (1983) 101.
24 R.J. Martin, B.A. Bailey and R.G.H. Downer, J. Chromatogr., 278 (1983) 265.
25 U.R. Tjaden and J. de Jong, J. Liquid Chromatogr., 6 (1983) 2255.

Journal of Chromatography, 309 (1984) 63—71
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2113

SENSITIVE FLUORIMETRY OF ADENINE-CONTAINING COMPOUNDS WITH HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

MASANORI YOSHIOKA*

Faculty of Pharmaceutical Sciences, Setsunan University, 45-1, Nagaotogecho, Hirakata, Osaka 573-01 (Japan)

KAZUYOSHI NISHIDATE, HIDEAKI IIZUKA, ATSUSHI NAKAMURA, MAHMOUD M. EL-MERZABANI and ZENZO TAMURA

Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113 (Japan)

and

TADASHI MIYAZAKI

Japan Spectroscopic Co., Ltd., 2967-5, Ishikawacho, Hachioji, Tokyo 192 (Japan)

(First received October 31st, 1983; revised manuscript received February 8th, 1984)

SUMMARY

A definitive method to determine adenine compounds simultaneously was established by introducing a new fluorescent reagent into high-performance liquid chromatography. Bromoacetaldehyde was the best reagent among the haloacetaldehydes examined. A quantitative reaction was obtained even for unstable ADP and ATP. A high resolution of adenine nucleotides was obtained using a column of Hitachi gel No. 3012-N. The method was applied to the measurement of cyclic AMP in urine, and ADP and ATP in brain and blood. Further, the sensitivity of the method was increased by a new fluorescence spectrophotometer constructed for micro-HPLC. Femtomole amounts of the adenine nucleotides were clearly separated.

*Presented at the 8th Symposium on Nucleic Acids Chemistry, Sapporo, August 21—23, 1980 (see ref. 18).

0378-4347/84/$03.00

INTRODUCTION

There are a number of adenine-containing compounds such as adenine nucleosides and nucleotides in natural and synthetic compounds, and development of a highly sensitive and selective method of determination is fundamental to progress in medical, biochemical and chemical studies.

Specific radioimmunoassay (RIA), enzymatic and protein binding assays for adenine compounds such as cyclic AMP (cAMP) [1] and adenosine [2] have been developed, if appropriate binding proteins are available.

Adenine compounds are not suitable for conversion into volatile derivatives for gas chromatography. A liquid-chromatographic method is useful for their systematic analysis. Recently, high-performance liquid chromatography (HPLC) has been applied to determine adenine compounds. Detection, however, is based on ultraviolet absorption of the adenine base and is not selective [3–6].

It is relevant to use a fluorescence reaction specific for the adenine base. Kochetkov et al. [7] found that chloroacetaldehyde reacted with adenine base to produce a 1-N^6-ethenoadenine derivative. Secrist et al. [8] examined the fluorescence characteristics of the derivatives.

Previously, we developed a method in which adenine compounds were quantitatively converted with chloroacetaldehyde into their etheno derivatives, which were separated by HPLC [9]. Kuttesch et al. [10] modified the method to determine adenosine and deoxyadenosine in patients with immunodeficiency diseases. Preston [11] also extended the method to separate adenine and related nucleotides in an extract of phytoplankton.

In this paper, we shall describe in detail the improved method which was communicated in brief [18]. A new fluorescence reagent, separation system and fluorescence detector were adopted. The method was applied to determine adenine compounds in biological samples.

EXPERIMENTAL

Materials

Bromoacetaldehyde was prepared and crystallized according to the method of Schukovskaya et al. [12]. Iodoacetaldehyde was prepared as described by Glinsky [13]. The other chemicals, of reagent grade, were commercially obtained.

Fluorescence reactions of haloacetaldehydes with adenine compounds

To 500 μl of 10 μ*M* cAMP or a mixture of the adenine compounds (10 μ*M* each) were added 200 μl of buffer (1 *M* citrate buffer pH 3, 1 *M* acetate buffer pH 3.5–5.5, or 1 *M* phosphate buffer pH 6.0–7.0) and 40 μl of 1.2 *M* bromoacetaldehyde in a Reacti-Vial (Pierce, Rockford, IL, U.S.A.). The vial was tightly closed and the mixed solution was heated at various temperatures (80°C or 100°C) for various times (0–40 min). In place of bromoacetaldehyde, 25 μl of 8 *M* iodoacetaldehyde dissolved in dioxane warmed at 60°C, or 10 μl of 4 *M* chloroacetaldehyde, were added. The reacted solution was analysed by HPLC to compare the reactivities of the haloacetaldehydes.

Quantitative reaction of the adenine compounds with bromoacetaldehyde

To 100 μl of the solution of adenine compounds in 0.1 *M* phosphate buffer (pH 7) or 1 *M* acetate buffer (pH 5) at various concentrations were added 10 μl (for pH 7) or 5 μl (for pH 5) of 1.9 *M* bromoacetaldehyde. The Reacti-Vial containing the mixed solution was heated at 80° C for 15 min and stored at 4° C until HPLC analysis.

High-performance liquid chromatography

A column (10 cm × 4.6 mm) of Hitachi gel No. 3012-N (porous polystyrene polymer beads for anion exchange, mean diameter of 7 μm, Hitachi, Tokyo, Japan) was maintained at 45° C. An eluent consisted of 0.025 *M* citric acid—0.05 *M* disodium hydrogen phosphate—0.4 *M* sodium chloride buffer (pH 5.0) and methanol (1:1, v/v). The flow-rate of the eluent was set at 0.2 ml/min with a Twincle pump (Jasco). The column inlet pressure was 10 kg/cm^2. The column was connected to a fluorescence detector FLD-1 (Shimadzu, Kyoto, Japan) equippped with a low-pressure mercury lamp of maximum energy at 253.7 nm, a Shimadzu EX-2 primary filter that transmitted radiation in the range 250—400 nm, a quartz flow-through cell of 10-μl capacity and an EM3 secondary filter that transmitted radiation of wavelengths above 400 nm.

Micro-HPLC

A PTFE tube (130 mm × 0.5 mm) was packed with Hitachi gel No. 3012-N and maintained at 12° C The flow-rate of the eluent mentioned above was set at 4 μl/min by a microsyringe-type pump Familic 100 N (Jasco).

The eluent was monitored by a fluorescence spectrophotometer.

Fluorescence spectrophotometer for micro-HPLC

The spectrometer was newly constructed and later named FP-110 (Jasco) (Fig. 1). The light from a low-pressure mercury lamp is filtered to cut off visible emissions, and is then introduced into an excitation monochromator through an entrance slit. The light beam is dispersed by an excitation concave grating (EXCG) of *F* 2.5 and focused on a flow-through cell of 1.5-μl capacity through an exit slit. The fluorescence is collected by the emission concave grating (EMCG), dispersed and focused on an exit slit of an emission monochromator. The emission photomultiplier (EMPM) converts the fluorescence

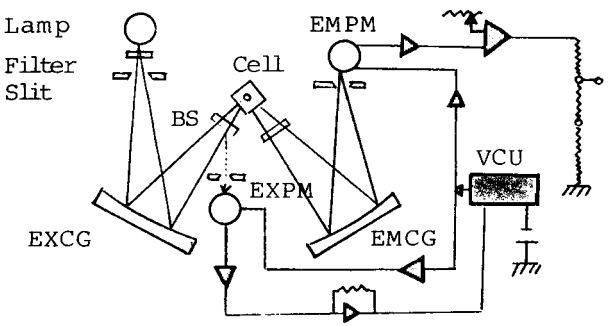


Fig. 1. A fluorescence spectrophotometer for micro-HPLC. For explanation of abbreviations, see text.

intensity to electrical signal. The fluctuation of the emission from the light source is compensated by monitoring a portion of the emission, which is split by the beam splitter (BS). The excitation signal level was maintained constant with the excitation photomultiplier (EXPM) for which high-tension voltage was regulated with a voltage control unit (VCU).

Determination of cAMP in human urine

Urine samples from healthy men were collected between 15.00 and 17.00 hours. To 0.5 ml of each urine were added 5 μl of 1 m*M* 2-deoxy cAMP as internal standard and 0.5 ml of water. Then 200 μl of the diluted urine were poured on to a column (5 mm × 4.5 mm) of Dowex 50W-X4 (H^+). The column was washed with 800 μl of water. To 50 μl of effluent were added 50 μl of 1 *M* acetate buffer (pH 5) and 5 μl of 1.9 *M* bromoacetaldehyde. The fluorescence reaction was carried out as described under Quantitative reaction.

Determination of adenine nucleotides in brain

A brain weighing 1.6 g was taken from a Wistar rat (210 g body weight) into 3 ml of 0.4 *M* perchloric acid chilled on ice. The brain was homogenized in a glass homogenizer. The homogenate was centrifuged at 15,000 *g* for 20 min. To 100 μl of the supernatant was added an equal volume of 1 *M* potassium bicarbonate and the mixture was centrifuged at 3500 *g* for 5 min. To 50 μl of the supernatant were added an equal volume of 0.2 *M* phosphate buffer (pH 7) and 10 μl of 1.9 *M* bromoacetaldehyde. The fluorescence reaction was carried out as described above. A 1-μl aliquot of the reacted solution was injected into the chromatograph.

Determination of adenine nucleotides in blood

Blood from human brachial vein was drawn into a tube containing disodium EDTA (100 mg/ml of blood). The tube was centrifuged at 3500 *g* for 5 min. To 100 μl of the plasma were added 25 μl of 4 *M* perchloric acid and the mixture was centrifuged at 15,000 *g* for 20 min. A 100-μl volume of the supernatant was reacted as described under Determination of adenine nucleotides in brain.

RESULTS

The adenine nucleotides were well separated without the aid of gradient elution, as shown in Fig. 2. The column (10 cm × 4.6 mm) used in this experiment had a higher resolution than the one (50 cm × 2 mm) previously reported [18], although the other conditions were the same. The column of Hitachi gel No. 3012-N was very stable and maintained constant resolution after one year of use. The deoxyadenine nucleotides were separated from the corresponding adenine nucleotides and eluted just in front of them as indicated for deoxy cAMP in Fig. 2.

It was demonstrated that each adenine nucleotide was converted with any haloacetaldehyde into the corresponding 1-N^6-ethenoadenine nucleotide, which was eluted at the same retention time, as shown in Fig. 2.

In Fig. 3, the pH optima of the reactions with bromoacetaldehyde and iodoacetaldehyde were 5.0 and 4.0, respectively, whereas with chloroacetaldehyde

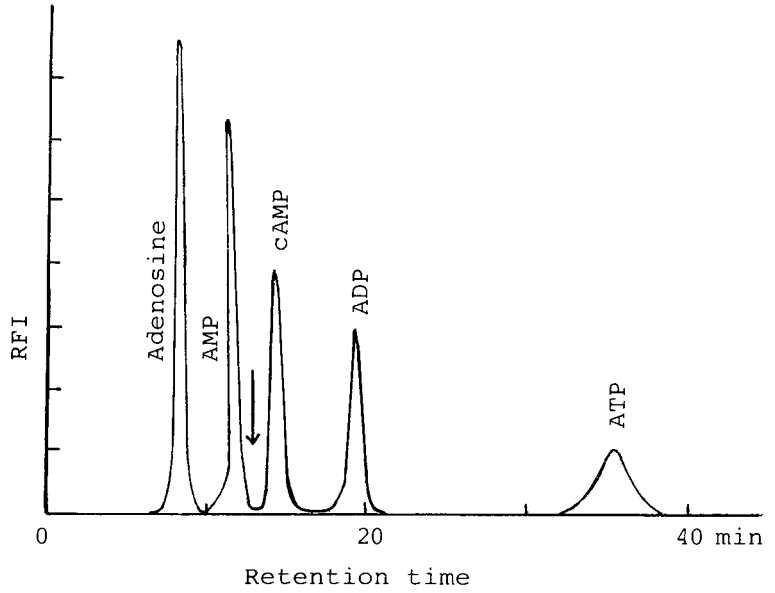


Fig. 2. Chromatogram of authentic compounds: 10 pmol of each were injected. Relative fluorescence intensity (RFI) of the peak was drawn. The arrow shows the location of the deoxy cAMP peak.

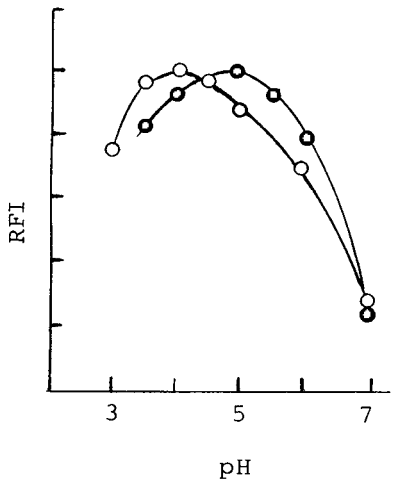


Fig. 3. Reactivities of haloacetaldehydes with cAMP at various pH values. •, Bromoacetaldehyde; ○, iodoacetaldehyde.

it was 4.5 as described before. The reactivity of bromoacetaldehyde was the strongest, as shown in Fig. 4. The reaction of bromoacetaldehyde reached a plateau at 100°C for 8 min or at 80°C for 15 min. Iodoacetaldehyde showed decreased fluorescence after the reaction maximum. Compared with standard synthesized 1-N^6-etheno cAMP, the yield of the reaction with bromoacetaldehyde at the plateau was 94%, while the reaction yield with chloroacetaldehyde was 80% at 100°C for 30 min. Thus, bromoacetaldehyde was adopted as the fluorescence reagent.

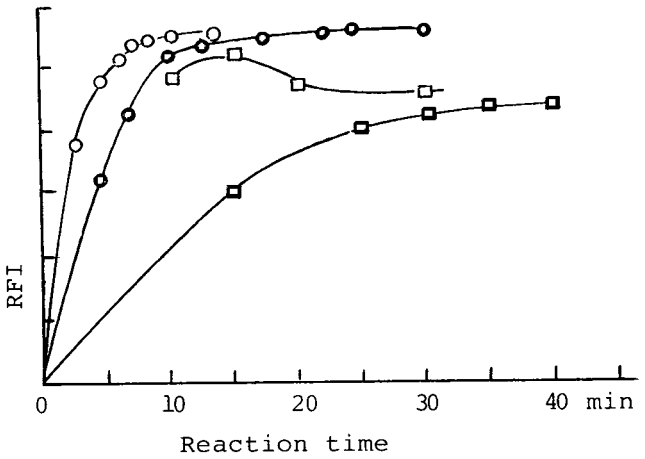


Fig. 4. Time courses of the reactions of haloacetaldehydes with cAMP. Each haloacetaldehyde was reacted at its own pH optimum as described in the text. ○, Bromoacetaldehyde at 100°C; ●, bromoacetaldehyde at 80°C; □, iodoacetaldehyde at 80°C; ■, chloroacetaldehyde at 100°C.

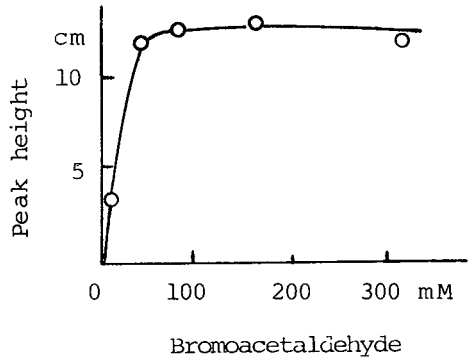


Fig. 5. Reactions of various concentrations of bromoacetaldehyde with cAMP at pH 5.

A dose-dependent reaction with bromoacetaldehyde showed a plateau at 90 m*M* at pH 5 as shown in Fig. 5, or at 170 m*M* at pH 5. ATP and ADP must be reacted at pH 7, since the pyrophosphate linkage was hydrolysed to some extent at pH 5. Two standard reaction conditions were made as described under Quantitative reaction. The reaction products were stable for as long as fifteen days when stored at 4° C (data not shown).

Working curves of the adenine nucleotides were linear for injected amounts between 1 and 160 pmol (data not shown).

The established method was applied to the measurement of cAMP in human urine after clean-up. In the chromatograms, deoxy cAMP (as internal standard) and cAMP appeared and were followed by low peaks of unknown compounds as shown in Fig. 6. To separate deoxy cAMP from cAMP completely, the concentration of sodium chloride in the eluent had to be decreased to 0.3 *M*. The values obtained with HPLC were compared with those obtained by RIA. The good correlation is shown in Fig. 7.

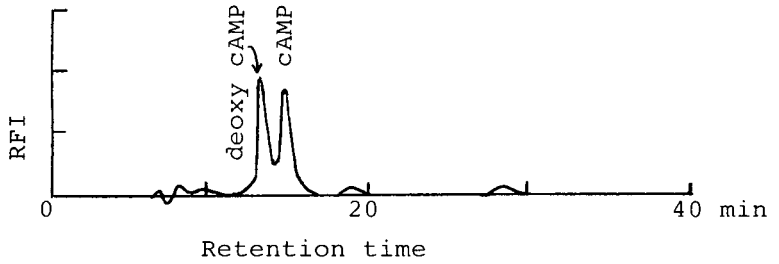


Fig. 6. Chromatogram of human urine. The amount is in the range as shown in Fig. 7.

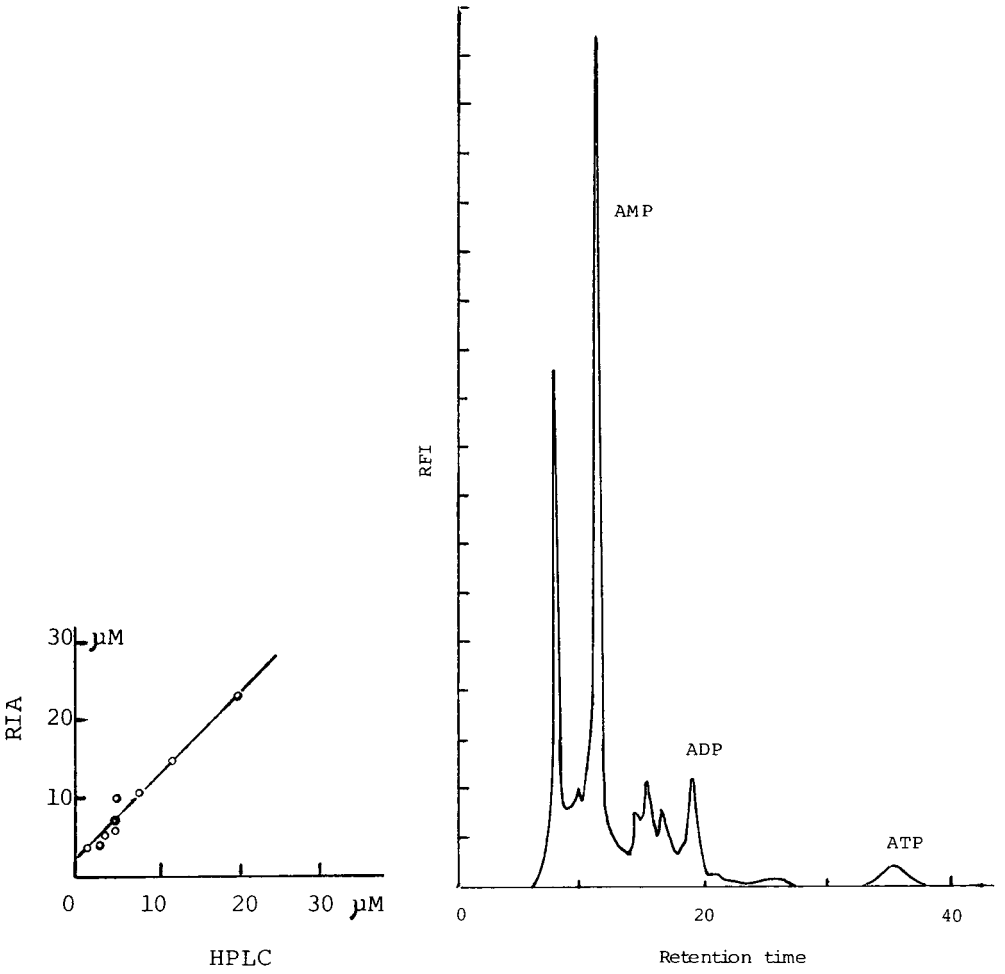


Fig. 7. Correlation between concentrations of cAMP in urine determined by HPLC and RIA. The concentration of cAMP was measured by RIA [1]. $Y = 1.07X + 2.25$ ($r = 0.98$).

Fig. 8. Chromatogram of rat brain. The amounts are described in the text.

The adenine nucleotides in rat brain were determined as shown in Fig. 8. The amounts of AMP, ADP and ATP were 68, 115 and 419 nmol/g wet weight, respectively. The recovery of the adenine nucleotides, each added with 0.4 *M* perchloric acid, was 100% from brain or blood.

ADP and ATP in plasma are shown in Fig. 9. Their concentrations were 13 and 28 μM, respectively. Their concentration in serum was very low, the concentration of ATP being 2—3 μM.

To obtain a higher sensitivity, the fluorescence spectrophotometer shown in Fig. 1 was constructed. It was more efficient to make the light of 253.7 nm from the low-pressure mercury lamp monochromatic with the concave grating than with the regular interference filter. The concave grating of F 2.5 used was the brightest in this kind. The lamp and flow-through cell could be used with other fluorescence systems. The sensitivity was extremely high, as shown in Fig. 10. Femtomole amounts of adenine nucleotides were quantitatively separated by micro-HPLC with the spectrophotometer.

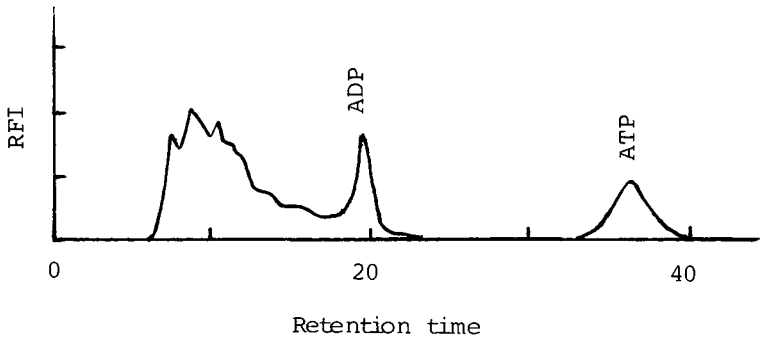


Fig. 9. Chromatogram of human plasma. The amounts are described in the text.

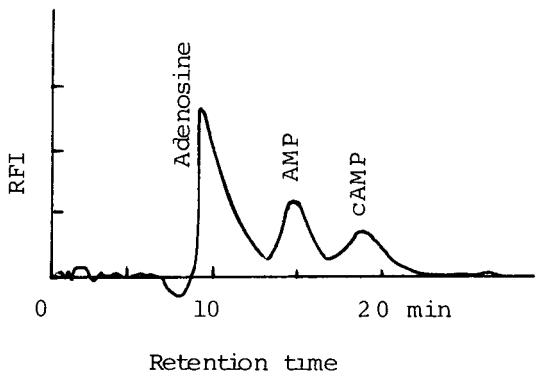


Fig. 10. Chromatogram by micro-HPLC. A mixture of the compounds (20 fmol of each was injected.

DISCUSSION

It was established that the adenine compounds could be determined simultaneously by HPLC with the new fluorescence reagent. The reaction of bromoacetaldehyde with adenine compounds was completed in a short time, and was reproducible because of the high yield and stability of the fluorescence products. The reaction of bromoacetaldehyde with nucleic acid bases was as selective as with chloroacetaldehyde except the reaction with guanine base, which will be described elsewhere. Taking the high resolution of HPLC into consideration, the method was made selective for the adenine compounds. It

was relevant for reliable determination to see the peaks of ADP and ATP in biological samples as shown in Figs. 8 and 9.

These compounds have been separated by inconvenient gradient elution HPLC assays [3–6, 14] which are not selective and sensitive. They have also been determined by an enzymatic method with luciferase [15], which is troubled with a high background. The present method is sensitive enough to measure cAMP in urine. The values obtained were compatible with those obtained by RIA as shown in Fig. 7, but the present method does not involve the care required in handling radioisotopes.

Further, the method is widely applicable to the measurement of adenine compounds in various samples. We have already described substantial findings such as adenosine in synaptosomes from guinea pig cerebral cortex [16] and adenine nucleotide release with catecholamines from adrenal chromaffin cells [17].

The applicability of the method to other adenine compounds can be widened by changing the separation system. Micro-HPLC as shown in Fig. 10 is also promising to cope with minute volumes of important biological samples.

Thus, the method described is useful for the exploration of adenine compounds in basic research and clinical fields.

ACKNOWLEDGEMENTS

This work was supported by a Grant-in-Aid for Scientific Research No. 487145 from the Ministry of Education, Science and Culture, and a grant from the Iatrochemical Foundation, for which the authors' thanks are due. We are deeply grateful to Mr. K. Suga of Daiichi Pure Chemicals Co. for measuring cAMP by RIA. We appreciate that Hitachi Ltd., Tokyo, supplied us with the Hitachi gel No. 3012-N under investigation.

REFERENCES

1 M. Honda, T. Satoh, J. Takezawa and M. Ui, Biochem. Med., 18 (1977) 257.
2 T. Satoh, A. Kuninaka, H. Yoshino and M. Ui, Anal. Biochem., 121 (1982) 409.
3 P.R. Brown, R.E. Parks, Jr. and J. Herod, Clin. Chem., 19 (1973) 919.
4 R.A. Hartwick and P.R. Brown, J. Chromatogr., 112 (1975) 651.
5 C.D. Lothrop, Jr. and M. Uziel, Clin. Chem., 26 (1980) 1430.
6 I. Kleinmann and V. Svoboda, J. Chromatogr., 201 (1980) 265.
7 N.K. Kochetkov, V.N. Shibaev and A.A. Kost, Tetrahedron Lett., (1971) 1993.
8 J.A. Secrist, III, J.R. Barrio, N.J. Leonard and G. Weber, Biochem., 11 (1972) 3499.
9 M. Yoshioka and Z. Tamura, J. Chromatogr., 123 (1976) 220.
10 J.F. Kuttesch, F.C. Schmalstieg and J.A. Nelson, J. Liquid Chromatogr., 1 (1978) 97.
11 M.R. Preston, J. Chromatogr., 275 (1983) 178.
12 L.L. Schukovskaya, S.N. Ushakov and N.K. Galania, Izv. Akad. Nauk SSSR Otd. Khim. Nauk, (1962) 1692.
13 G. Glinksy, Z. Chem., (1868) 618.
14 K. Yoshida, H. Haraguchi and K. Fuwa, Anal. Chem., 55 (1983) 1009.
15 M.A. Deluca and W.D. McElroy, Methods Enzymol., 57 (1978) 3.
16 K. Kobayashi, Y. Kuroda and M. Yoshioka, J. Neurochem., 36 (1981) 86.
17 Y. Kuroda, M. Yoshioka, K. Kumakura, K. Kobayashi and T. Nakajima, Proc. Jap. Acad., 56B (1980) 660.
18 M. Yoshioka, Nucleic Acids Res., Symp. Ser., 8 (1980) S61.

Journal of Chromatography, 309 (1984) 73—80
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2115

DETERMINATION OF MORPHINE IN BIOLOGICAL SAMPLES BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

EVIDENCE FOR PERSISTENT TISSUE BINDING IN RATS TWENTY-TWO DAYS POST-WITHDRAWAL

A.W. JONES*

Department of Alcohol and Drug Addiction Research, Karolinska Institutet, Stockholm (Sweden)

YLVA BLOM and ULF BONDESSON

Psychiatric Research Center, Ullerâker Hospital, Uppsala (Sweden)

and

ERIK ÄNGGÅRD

Department of Alcohol and Drug Addiction Research, Karolinska Institutet, Stockholm (Sweden)

(First received October 20th, 1983; revised manuscript received February 16th, 1984)

SUMMARY

Combined gas chromatography—mass spectrometry with capillary and packed column gas chromatography and a deuterium-labelled internal standard was used to determine morphine in biological specimens from rats 22 days after abrupt withdrawal. Morphine was extracted from urine and body organs at pH 9 and the pentafluoropropionyl derivatives were made for analysis by gas chromatography—mass spectrometry. The stationary phase was OV-17 and the mass spectrometer was focused on *m/z* 414 for morphine and *m/z* 417 for the internal standard, [NC^2H_3]morphine. With fused-silica capillary columns, the sensitivity of the assay was increased about ten-fold over packed columns. Urinary excretion of total morphine (free + conjugated) was 22 ng/h (range 11—51 ng/h, n = 8) at 22 days post-withdrawal. Free morphine was mainly detected in the lung (1.8—6.5 ng/g, n = 7), kidney (1.5—4.0 ng/g, n = 7) and liver (1.8—4.6 ng/g, n = 4). Traces of morphine were also detected in brain of some rats. Treatment with the opiate antagonist naltrexone, 10 mg/kg on four consecutive days before death, failed to change the urinary excretion pattern or the

0378-4347/84/$03.00

concentration of free morphine in body organs. The biological significance of the residual morphine, if any, remains to be determined.

INTRODUCTION

A host of analytical methods exist for the determination of morphine and its congeners in biological material [1—3]. The combination of gas chromatography and mass spectrometry (GC—MS) with selected ion monitoring furnishes a method with high sensitivity and unsurpassed specificity. This technique has been widely used for the quantitative analysis of drugs of abuse in body fluids and tissue and has emerged as a reference method [4].

We recently showed that rats chronically treated with morphine continued to excrete the drug for several weeks after abrupt withdrawal [5]. This persistence of morphine in the body may have important implications in the withdrawal process and the duration of the abstinence phase. Moreover, if the residual morphine retains its pharmacological activity it could account for the physiological disturbances seen in man and animals during protracted abstinence [6, 7]. The unequivocal identification of free morphine in body organs or tissue from late abstinent rats would support the notion of an in vivo depot of opiate associated with tolerance and dependence.

This paper describes a GC—MS method for the quantitative analysis of morphine by multiple ion detection with a deuterium-labelled analogue as internal standard. This technique was used to detect morphine in the urine and body organs of rats killed 22 days after abrupt withdrawal.

MATERIALS AND METHODS

Drugs and chemicals

Morphine hydrochloride was obtained from the pharmacy at Karolinska Hospital and a deuterium-labelled analogue [NC^2H_3] morphine was synthesised by Mass Analysis (Stockholm, Sweden). Naltrexone hydrochloride was a gift from Endo Labs. (New York, NY, U.S.A.). Pentafluoropropionic anhydride (PFPA) was bought from Mass Analysis and all other chemicals and solvents were of the purest grade obtainable through local suppliers (Kebo Grave, Stockholm, Sweden).

Morphine and natrexone were dissolved in 0.9% (w/v) sodium chloride to make solutions in the concentration range 0.5—3.0% (w/v) of the opiate. For constructing calibration curves and for use as internal standard, morphine and its deuterium-labelled analogue were dissolved in ethanol and calculated as nanogram of the free base.

Animals and treatment

Male Sprague—Dawley rats (Anticimex, Sollentuna, Sweden) were used as animal model of morphine addiction. They were chronically treated with morphine by twice daily (9.00 and 18.00 hours) intraperitoneal injections with gradually increasing doses. The first dose was 10 mg/kg body weight, which was maintained for two days, then 20 mg/kg (two days) and thereafter 20 mg/kg

increments every second day to a maximum of 200 mg/kg. Rats in a control group were injected with 0.9% sodium chloride.

Morphine withdrawal and abstinence

Rats were abruptly withdrawn from morphine after 22 days of chronic treatment (10—200 mg/kg). After seven days of abstinence the animals were moved to metabolic cages and their daily urine samples were collected for the following fourteen days. The samples were frozen at —20° C until analysed. At 18, 19, 20 and 21 days post-withdrawal one group of abstinent rats (n = 7) was injected with 10 mg naltrexone per kg body weight. Another group of morphine-abstinent rats received injections of 0.9% (w/v) sodium chloride.

At 22 days post-withdrawal all the animals were killed by decapitation after a few seconds of anaesthesia with chloroform. Body organs (brain, lung and kidney) were rapidly dissected out, washed free of blood and extraneous tissue fragments and frozen at —20° C pending analysis. The liver from some of the rats was also analysed for morphine.

Extraction and analysis of morphine

Total morphine (free + conjugated) was determined in urine whereas only free morphine was determined in body organs. Two slightly different procedures were employed for the clean-up of biological samples.

Samples of urine (0.1—1 ml) were transferred into polypropylene test tubes and diluted to 2 ml with water and/or hydrochloric acid to give a final acidity of 1 *M*. Thereafter, 50—200 ng of [NC^2H_3]morphine were added as internal standard; the exact amount depended on the concentration of morphine expected from the results of previous determinations. After mixing, the samples were autoclaved at 110° C for 30 min to hydrolyse morphine glucuronide. The samples of urine were allowed to cool and the pH was adjusted to 9 with ammonium hydroxide—ammonium chloride buffer to give a total volume of about 4 ml.

The buffered aliquots of hydrolysed urine were extracted with organic solvent (chloroform—isopropanol, 3:1) by mixing in a shaking table device for 15 min. After centrifuging at 1400 *g* for 10 min, the organic solvent phase was transferred to a new polypropylene tube containing 1 ml of 1 *M* hydrochloric acid. The morphine was back-extracted into the aqueous phase and the solvent was discarded. Finally, the aqueous sample was adjusted to pH 9 and the morphine was once again taken into the organic solvent as described above. The solvent phase was moved to a clean glass tube with tapered bottom and evaporated to dryness under a stream of nitrogen at 50° C.

Body organs were thawed at 4° C and quickly homogenized in 5 ml of ice-cold perchloric acid (0.2 *M*) with an Ultra-Turrax blender fitted with a PTFE pestle. To a 3-ml aliquot of the homogenate, 6 ng of [NC^2H_3]morphine were added as internal standard. The pH was then adjusted to 9 with ammonium hydroxide and bicarbonate buffer and the free morphine was extracted by mechanically shaking for 15 min with a mixture of toluene—2-butanol (80:20). After centrifuging, the organic solvent phase was transferred to another tube containing 0.5 ml of 0.05 *M* sulphuric acid and mixed by shaking for 15 min; thereafter the organic phase was discarded. The

acid aqueous phase was buffered to pH 9 and shaken with 4 ml of dichloromethane—2-butanol (80:20) for 10 min. The organic phase was finally transferred to a small glass test tube and evaporated to dryness under nitrogen.

These two somewhat different extraction procedures for the clean-up of biological samples reflect the techniques currently used in our respective laboratories. Neither method is new; and the triple extraction procedure — first into organic solvent then into an aqueous phase and back into solvent — recovers about 60—80% of morphine [8]. The actual recoveries with [^{3}H]-morphine as marker were 64.8 ± 3.8% (± S.D., n = 5) with tissue homogenates and 64.9 ± 2.4% with specimens of urine. Because we added deuterated morphine as an internal standard no corrections were made to allow for less than 100% recovery.

Gas chromatography

For the GC analysis we prepared pentafluoropropionyl derivatives of morphine. PFPA (100 μl) was added to the residue after evaporation of solvent. The tubes were sealed with tight-fitting stoppers and placed in a heating cabinet at 60° C for about 30 min. The remaining PFPA was evaporated under a stream of nitrogen and the residue was dissolved in ethyl acetate (50 μl) for GC analysis. These PFPA derivatives of morphine were stable for about one week when kept in a refrigerator at 4° C.

The packed column was made from a silanized glass tube (130 cm × 3 mm I.D.) with OV-17 as the stationary phase. The oven temperature was 250° C, injection port 280° C; helium was the carrier gas at 20 ml/min. A 1—2 μl sample was injected and the retention time of morphine was about 2.8 min. The capillary column was made of fused silica (25 m × 0.3 mm I.D.) with OV-17 as the stationary phase. Column temperature was 270° C, and helium was used as carrier gas. A droplet (1—4 μl) of the sample was transferred to the tip of a movable glass needle, and after evaporation of the solvent the needle together with the sample was moved down to the column. The retention time of morphine under these conditions was about 1.5 min. Westerling et al. [9] recently reported a GC—MS assay for morphine in plasma samples employing glass capillary columns.

Mass spectrometry

An LKB 2091 combined gas chromatograph—mass spectrometer was used for the assay of morphine in specimens of urine. The operating conditions were separator 240° C, ion source 270° C, trap current 50 μA, and energy of the electrons 22.5 eV. The concentration of free morphine in body organs was determined with a Finnigan 4000 GC—MS unit and an Incos 2300 data system for data collection. The instrument was operated in the electron impact mode. The other operating conditions were ion source 250° C, electron emission current 0.3 A, and energy of the electrons 40 eV. The electron multiplier voltage was set at 2500 V.

The electron impact mass spectrum of the PFPA derivatives of morphine has a base peak at m/z 414 and a molecular ion with 30% relative intensity at m/z 577. For quantitative analysis the multiple ion detector was focused on m/z 414 for morphine and m/z 417 for the [NC^2H_3]morphine internal standard. In

some runs, the molecular ions at *m/z* 577 and *m/z* 580 were monitored in addition to the base peaks.

RESULTS

Validity of the GC—MS assay of morphine

Linear calibration lines were obtained when the peak height ratio *m/z* 414/417 was plotted against concentration of morphine in spiked standard samples. At the high concentration range (0—200 ng/ml) with a packed column, the regression equation was $Y = 0.025 + 0.005X$ (correlation coefficient, $r = 0.99$) and with the capillary column (0—20 ng/ml) $Y = 0.027 + 0.056X$ ($r = 0.99$). The intercepts on the ordinate can be attributed to the presence of about 2% of non-deuterated morphine contaminating the internal standard. Even at the zero level, when the [NC^2H_3]morphine was added as internal standard a small background peak appeared at *m/z* 414.

Typical mass fragmentograms are shown in Fig. 1, representing a run made with the Finnigan GC—MS with data collecting system. The biological specimen was kidney and the *m/z* 414 peak corresponds to about 4.5 ng of morphine in this organ. The occurrence of mass fragments at *m/z* 414 and *m/z* 417 with very close retention times on this relatively clean chromatogram strongly supports the presence of morphine. The deuterated compound elutes slightly earlier than the unlabelled species.

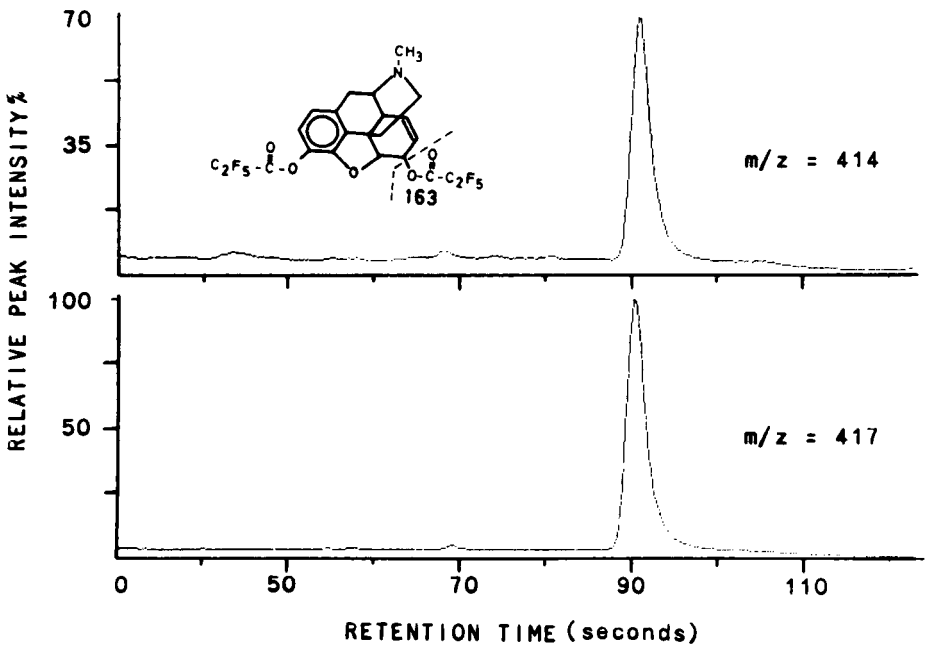


Fig. 1. Typical mass fragmentograms obtained from the assay of morphine by GC—MS (Finnigan Model 4000) with data system. The mass fragments at *m/z* 414 (free morphine) and *m/z* 417 (deuterium-labelled internal standard) show almost identical retention times. The structure of the pentafluoropropionic anhydride derivative of morphine (molecular weight = 577) used for the analysis is shown.

The precision of this method of analysis was assessed (A) from the variance of differences between duplicate determinations and (B) from spiked tissue homogenates. From duplicate determinations at 100 ng/ml urine, the coefficient of variation (C.V.) was 5% and at 10 ng/ml it was nearer 10%, implying a lower precision. Spiked tissue homogenates with 1 ng and 20 ng of morphine resulted in coefficients of variation of 10.8% and 2.1%, respectively (n = 5). At these low concentrations of morphine, the risk of loss of sample during extraction and GC—MS analysis is diminished by the presence of the deuterated internal standard.

Urinary excretion of morphine

Fig. 2 shows the excretion profiles of morphine in individual rats for samples of urine collected between 7 and 22 days post-withdrawal. Total morphine is plotted on the ordinate because samples were hydrolysed before the assay. In spite of fairly wide variations among the rats, a similar excretion time course is evident. The peaked character of some of the curves in the early stage of sampling is difficult to explain. At 22 days, the median excretion of morphine was 22 ng/h (range 11—51 ng/h, n = 8). In a preliminary kinetic analysis of these curves the half-life of morphine excretion for this terminal period, at 18—22 days post-withdrawal, was estimated at ten days [5]. In a group of rats treated with naltrexone a smilar pattern of morphine excretion was noted (data not shown).

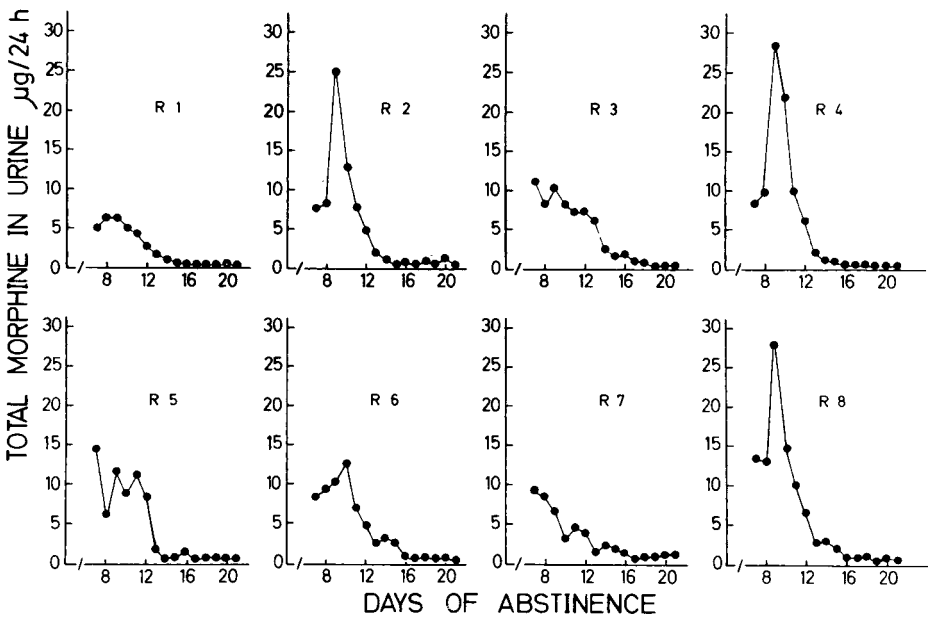


Fig. 2. Urinary excretion profiles of morphine in eight individual rats between 7 and 22 days after withdrawal. Total morphine (free + conjugated) was assayed.

Morphine concentrations in body organs

Table I gives the concentrations of morphine determined in kidney and lung of rats killed 22 days after abrupt withdrawal. The levels were well above the

TABLE I

CONCENTRATIONS OF FREE MORPHINE IN KIDNEY AND LUNG OF RATS KILLED 22 DAYS AFTER ABRUPT WITHDRAWAL

One group of morphine-abstinent rats was injected with 10 mg/kg naltrexone on four consecutive days before they were killed. A control group received injections of saline.

Body organ	Treatment	Concentration of morphine (ng/g)		
		Median	Range	*n*
Kidney	Saline	2.5	1.5—4.0	7
	Naltrexone	3.6	0.6—8.2	7
Lung	Saline	2.6	1.8—6.5	7
	Naltrexone	3.0	1.8—18.8	4

background detection limits (0.2 ng/g). Injections (intraperitoneal) of naltrexone did not change this distribution pattern of morphine. The liver contained 1.8—4.6 ng/g morphine (n = 4); the brain contained 1.0—4.6 ng/g (n = 6), but the levels were below detection limits in the other rats tested.

DISCUSSION

In our previous study on morphine excretion in abstinent rats the concentrations of free morphine in tissue and body organs were near the borderline of the limits of detection. This early study was hampered by the limited sensitivity associated with the use of packed columns for GC—MS assay, as well as background from the deuterium-labelled analogue used as internal standard. In the present method, fused-silica capillary columns furnished a roughly ten-fold increase in sensitivity and even trace quantities of morphine were readily detectable in the body organs.

The distribution pattern of residual morphine in late abstinent rats fits with the results from acute administration of morphine [10]. The kidney contained most free morphine, as might be expected, because rats excrete substantial amounts of morphine as the base [11]. Free morphine in the liver could have been the result of enterohepatic circulation as suggested by others [12]. Even the lung can take part in metabolism of morphine to its glucuronide, and free morphine might therefore accumulate in lung tissue.

The excretion of morphine in urine was assessed as total morphine (free + conjugated) making it difficult to compare results with the levels of free morphine determined in kidney. The peaks seen in Fig. 2 might have resulted from a higher muscular activity of the rats on these days because they were regrouped together for the purpose of cleaning the metabolic cages. They had previously been housed individually for the first week of abstinence. On mixing, rats were highly active and ran around their cages; vigorous fighting broke out among some animals.

With the use of highly specific GC—MS methodology and deuterium-labelled internal standard it seems that the presence of morphine in abstinent rats is definitely established. But what is its biological significance? This is the crucial question that remains unanswered in this study. It seems unlikely that the

residual morphine remains pharmacologically active because injections of naltrexone, a specific opiate antagonist, failed to precipitate any of the well known signs of abstinence. A non-specific binding to tissue components and anionic groups is the most likely explanation.

Both humans and rats have a protracted abstinence phase associated with opiate withdrawal that can last for several months. During this period of withdrawal subtle physiological disturbances persist and addicts undergoing rehabilitation often tend to relapse to using narcotics [13]. Speeding up the excretion of residual morphine might prove a worthwhile treatment but naltrexone showed no clear-cut effects in this connection. Other opioid antagonists with affinity for subpopulations of receptors were not tested.

This study, making use of rats as an animal model of morphine dependence and abstinence, further documents the long wash-out constant of morphine in protracted abstinence [14, 15]. Additional investigations are needed to establish the significance of the residual morphine, if any, in the opiate withdrawal syndrome.

ACKNOWLEDGEMENTS

This study was supported by grants from the Swedish Medical Research Council, the Swedish Delegation for Social Research and the Karolinska Institute Fund.

REFERENCES

1 S.J. Mulé, J. Chromatogr. Sci., 12 (1974) 245.
2 J.-P.G. Thenôt and K.D. Haegele, Methods Biochem. Anal., 24 (1977) 1.
3 P.O. Edlund, J. Chromatogr., 206 (1981) 109.
4 R.L. Foltz, A.F. Fentiman and R.B. Foltz, GC/MS Assays for Abused Drugs in Body Fluids, NIDA Research Monograph No. 32, U.S. Government Printing Office, Washington, 1980, pp. 1—202.
5 A.W. Jones, A. Neri and E. Änggård, J. Pharmacol. Exp. Ther., 224 (1983) 419.
6 C.K. Himmelsbach, Arch. Intern. Med., 69 (1942) 766.
7 W.R. Martin, A. Wikler, C.G. Eades and F.T. Pescor, Psychopharmacology, 4 (1963) 247.
8 Ch.W. Gorodetzky; in W.R. Martin (Editor), Handbook of Experimental Pharmacology, Vol. 45/1, Springer, Berlin, 1977, pp. 319—409.
9 D. Westerling, S. Lindahl, K.-E. Andersson and A. Andersson, Eur. J. Clin. Pharmacol., 23 (1982) 59.
10 L.A. Woods, J. Pharmacol., 112 (1954) 158.
11 E.L. Way and T.K. Adler, The Biological Disposition of Morphine and its Surrogates, World Health Organization, Geneva, 1962, pp. 1—58.
12 C.T. Walsh and R.R. Levine, J. Pharmacol. Exp. Ther., 195 (1975) 303.
13 W.R. Martin and D.R. Jasinski, J. Psychiat. Res., 7 (1969) 9.
14 A.L. Misra, C.L. Mitchell and L.A. Woods, Nature, 232 (1971) 48.
15 K.B. Mullis, D.C. Perry, A.M. Finn, B. Stafford and W. Sadee, J. Pharmacol. Exp. Ther., 208 (1979) 228.

Journal of Chromatography, 309 (1984) 81—93
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2109

RELIABLE ROUTINE METHOD FOR THE DETERMINATION OF ANTIDEPRESSANT DRUGS IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

TIM VISSER*,*, MARC C.J.M. OOSTELBOS and PETER J.M.M. TOLL

Laboratory of Clinical Pharmacology and Toxicology, Pharmacy of the Groot Ziekengasthuis, Nieuwstraat 34, 5211 NL 's-Hertogenbosch (The Netherlands)

(First received September 15th, 1983; revised manuscript received February 13th, 1984)

SUMMARY

We describe a rapid and reliable method using high-performance liquid chromatography for the simultaneous measurement of plasma concentrations of currently prescribed antidepressants and their main metabolites (amitriptyline, nortriptyline, *trans*-10-hydroxynortriptyline, clomipramine, desmethylclomipramine, imipramine, desipramine, zimeldine, norzimeldine, doxepin, desmethyldoxepin, trimipramine and mianserin). The method involves a single extraction of plasma at pH 10.1 with hexane—acetonitrile (98:2), solvent transfer to and evaporation in a disposable glass tube and subsequent chromatography of the residue on a CN bonded-phase column using acetonitrile—methanol—phosphate buffer (pH 7.0) as mobile phase. Protriptyline is used as the internal standard. Calibration curves remain linear up to at least 200 μg/l, detection limits are 5 μg/l, absolute recoveries are over 92%, and precision (coefficient of variation) is 6.9%. Norzimeldine and 10-hydroxynortriptyline show lower recoveries, protriptyline and 10-hydroxynortriptyline higher detection limits. Adsorption to glassware and chemical decomposition during analysis are shown to be negligible. Psychoactive and other drugs frequently prescribed in combination with antidepressants have been tested for their chromatographic properties under the same conditions.

INTRODUCTION

Antidepressant drugs are frequently used in psychiatric clinics for treatment of major depression. Large interindividual variations in plasma concentrations among patients receiving the same dosage of these drugs have been shown in several reports [1]. There is no consensus concerning the relationship between

*Present address: Laboratory of Clinical Pharmacology and Toxicology, Central Hospital Pharmacy, Boerhaavelaan 24, 2035 AC Haarlem, The Netherlands

plasma concentration and clinical response [2, 3]. However, measurement of serum concentrations of antidepressants and their metabolites is justified by the pharmacokinetic variability, and in cases of intoxication or patient non-compliance. Reliable and sensitive methods should be available. A review of methodology has recently been published [4].

For application in the clinical laboratory, a large number of gas chromatographic methods (GLC) have been reported, especially for amitriptyline and its metabolite nortriptyline. The earliest methods used flame-ionization detection [5, 6] or electron-capture detection [7]. In more recent studies the introduction of nitrogen-sensitive detectors, which leads to improved sensitivity, has been described [8, 9]. Some papers report analytical methods that have been worked out for several of the currently prescribed antidepressants [6, 8]. High-performance liquid chromatography (HPLC) has also been used successfully for quantifying antidepressant drugs in plasma [10—13]. Techniques like ion-pair partition, adsorption and reversed-phase liquid chromatography have all been applied with a number of different types of column packing and elution solvents. Methods described in the literature vary widely with regard to sample treatment. Recoveries and detection limits of the drugs may differ as well. Some investigators have advocated elaborate sample preparation techniques [11, 12].

Other techniques that have been used for the determination of antidepressants include GLC combined with mass spectrometry and immunoassays. Gas chromatography in combination with mass spectrometry is sensitive and specific but not easily adaptable to the clinical laboratory [14]. Immunoassays are prone to interference [15]. We report a procedure for determining amitriptyline, nortriptyline, *trans*-10-hydroxynortriptyline, clomipramine, desmethylclomipramine, imipramine, desipramine, zimeldine, norzimeldine, doxepin, desmethyldoxepin, trimipramine and mianserin in plasma samples by HPLC. All but two of these antidepressants can be measured simultaneously. Sample pretreatment is easy and fairly rapid, while recovery and detection limit of the method are excellent.

MATERIALS AND METHODS

Instrumentation

A high-pressure liquid chromatograph (Waters Assoc., Etten-Leur, The Netherlands), Model 6000 A, in conjunction with an ultraviolet detector (Waters Assoc.), Lambda Max Model 480, was used. Extinction was measured at 254 nm, the sensitivity being 0.02 a.u.f.s. The column (stainless steel, 30 cm × 3.9 mm I.D.) was packed with 10 μm diameter cyanopropylsilane-coated silica beads (μBondapak-CN®, reversed phase). The column was obtained commercially (Waters Assoc.).

The mobile phase was prepared by mixing 625 ml of acetonitrile, 155 ml of methanol and 220 ml of phosphate buffer pH 7.0. The mixture was filtered (0.45 μm) before use. Chromatography was performed at room temperature, the flow-rate being 1.5 ml/min.

Chemicals and reagents

Aqueous solutions were prepared using glass-distilled water. Acetonitrile, hexane and methanol were analytical reagent (p.a.) grade (Merck, Darmstadt, F.R.G.).

Sodium carbonate (Ph. Eur.) was used for preparation of the 1.0 *M* sodium carbonate solution. The phosphate buffer used for the elution solvent was made by dissolving $Na_2HPO_4 \cdot 2H_2O$ (Merck) in water (5 mmol/l) and adjusting the pH to 7.0 with 0.1 *M* H_3PO_4 (Ph. Eur.). Higher pH values result in decreased stability of the column packing materials.

The antidepressants were obtained as hydrochloride salts except for trimipramine (maleate). Amitriptyline, nortriptyline and *trans*-10-hydroxynortriptyline were obtained from Lundbeck (Copenhagen, Denmark). Desipramine, imipramine, clomipramine and desmethylclomipramine were from Ciba-Geigy (Basel, Switzerland). Doxepin and desmethyldoxepin were from Pfizer (New York, NY, U.S.A.). Zimeldine and norzimeldine were from Astra (Södertälje, Sweden). Trimipramine was from Rhône-Poulenc (Paris, France), mianserin from Organon (Oss, The Netherlands) and protriptyline from Merck Sharp and Dohme (Rahway, NJ, U.S.A.). Methadone hydrochloride was Ph. Eur. grade.

Materials

Tubes used for extraction were glass, 100 × 16 mm round-bottomed (Renes, Zeist, The Netherlands). The tubes were stoppered with polyethylene stoppers and used once only.

Serum and internal standard were pipetted using disposable tips. The standard solutions were dispensed by an analytical syringe.

Procedures

Preparation of standard solutions. The experiments were carried out with two working standard solutions. One working standard (I) was a mixture of amitriptyline, nortriptyline, *trans*-10-hydroxynortriptyline, imipramine, desipramine, doxepin, desmethyldoxepin, mianserin and trimipramine. The other working standard solution (II) was a mixture of zimeldine, norzimeldine, clomipramine and desmethylclomipramine. Both working standards contained 4.0 mg/l of each antidepressant in 0.005 *M* hydrochloric acid in methanol and were prepared by 50-fold dilution of stock standards in 0.005 *M* hydrochloric acid in methanol. The working internal standard was a 4.0 mg/l solution of protriptyline, prepared in the same way. An external standard solution, containing methadone 32 mg/l in 0.005 *M* hydrochloric acid in methanol was used for determination of the recoveries. All standard solutions were stored in the dark at 4°C. No decomposition could be detected after one month of storage.

Calibration curve measurements. Of the working standard solutions 12.5, 25, 50 and 100 µl were measured into test tubes and 2 ml of plasma were added. Equilibration took place overnight. These solutions, like patient plasmas, were treated as described under *Plasma sample handling.* The ratios between the peak areas of each drug and the internal standard were plotted against the

plasma concentration of the drug. Slopes and correlation coefficients were calculated using a least-squares procedure.

Plasma sample handling. Mix 2.0 ml of plasma, 200 µl of working internal standard solution (protriptyline) and 200 µl of 1.0 *M* sodium carbonate solution in a test tube. Add 8 ml of hexane—acetonitrile (98:2, v/v). Close the tube with a polyethylene stopper and extract for 10 min by mixing on a rotator rack. Centrifuge for about 2 min to separate the layers. Put the organic layer into a second test tube and evaporate *almost* to dryness under a stream of nitrogen while warming the tube in a water bath (40° C). Add 200 µl of mobile phase, mix on a Vortex mixer and keep the test tube in an ultrasonic bath for 10 sec. Inject about 100 µl into the liquid chromatograph.

For routine measurement, patient blood samples are centrifuged immediately after collection. The plasma is removed and placed in tubes used for the assay. If not analysed promptly plasmas are frozen at −20° C and not permitted to thaw until assay.

Procedure for determination of coefficient of variation. Of the working standard solutions 125 µl and 250 µl were mixed with 10.0 ml of plasma. In each of the four spiked plasmas drugs were measured three times, using each time 2.0 ml of plasma.

Recovery studies. Recoveries of the antidepressants at the 25 µg/l and 100 µg/l levels were determined by adding after extraction 50 µl of an external standard solution (methadone) to the extract while evaporating the organic layer. At both concentrations the peak areas relative to this external standard (Q_1) were calculated. These values were compared with the ratios (Q_2) obtained by injecting a comparative reference solution, prepared without extraction. The recovery is the ratio between Q_1 and Q_2.

The reference solutions were prepared by pipetting appropriate quantities of the methanolic working standards in extraction tubes, evaporating the methanol and adding mobile phase to the residue. For determining the recovery at 25 µg/l, the reference solution consisted of 50 ng of the drugs, 800 ng of internal standard (protriptyline) and 1600 ng of methadone as external standard in 200 µl of mobile phase. The reference solution for determining the recovery at 100 µg/l contained 200 ng of the drugs, 800 ng of protriptyline and 1600 ng of methadone.

Adsorption studies. In order to find out whether our reference solutions could be interpreted as real references, a comparison was made between the reference solutions and so-called control solutions. Reference and control solutions were prepared at both the 25 µg/l and 100 µg/l levels. The control solutions contained 40-fold quantities of antidepressant, protriptyline (internal standard) and methadone (external standard) compared to the reference solutions, in a volume of 8 ml of mobile phase (40-fold volume). On preparing the control solutions, factors critical for adsorption onto glass, like evaporation of the methanolic standards and exposure of a small volume of liquid to a large glass surface area (when dissolving the residue), were avoided. Preparation took place by pipetting into a test tube appropriate volumes of the methanolic working and stock standard solutions (to keep the quantity of methanol minimal) and diluting to volume without evaporating the methanol. The peak

areas relative to methadone after injecting about 100 μl of the control solutions and reference solutions were compared.

Extraction studies. Norzimeldine and *trans*-10-hydroxynortriptyline were investigated for incomplete extraction. At the 100 μg/l level the recovery of both metabolites was determined as described under *Recovery studies.* After extraction, the pH of the remaining plasma was measured for control. The plasma was extracted for a second time after addition of 200 μl of the protriptyline internal standard solution. Peak areas were determined relative to methadone. As a control amitriptyline was run through the same procedure.

RESULTS

Drug determination

The resolution of both standard mixtures of antidepressants after chromatography is shown in Fig. 1. If the antidepressants were injected as a single mixture, clomipramine would not be completely separated from amitriptyline and zimeldine would interfere with trimipramine. There is no interference with possible peaks from blank plasma.

Calibration curves of some of the drugs are shown in Fig. 2. For all antidepressants a good linear relationship between peak area ratios and plasma levels over a large concentration range is found.

Retention times, coefficients of correlation and variation, recoveries and analytical detection limits at 254 nm are summarized in Table I. The detection limit in plasma, defined as twice the noise level by 0.001 a.u.f.s., for most antidepressants is 2 μg/l and for their desmethyl metabolites 5 μg/l. Incomplete extraction causes the detection limit of 10-hydroxynortriptyline to be a little higher. For protriptyline, the internal standard, the detection limit has not been determined. However, because this compound has a minimum extinction at 250 nm, the detection limit will be high and can be lowered by measuring nearer to the extinction maximum at 292 nm [16]. As shown in Table I, recoveries of norzimeldine and *trans*-10-hydroxynortriptyline are rather low. In order to find out to what extent adsorption onto glass or incomplete extraction influenced our results, adsorption and extraction studies were carried out.

The results of the adsorption studies are shown in Table II. Between the 200-μl reference solutions and the 8-ml control solutions no significant differences were found when peak area ratios of antidepressant relative to methadone were calculated for either method.

Table III shows the results of the extraction studies. Equal fractions of norzimeldine and *trans*-10-hydroxynortriptyline are extracted at the first and second extraction step. Incomplete extraction appears to be the cause of the low recovery (Table I) of both forenamed antidepressant metabolites. The relating chromatograms are shown in Fig. 3.

Selectivity of the method

Several drugs were tested for potential interference with our procedure by comparing the retention times of these drugs with those of the antidepressants. We did not determine the extraction of these materials from plasma. Table IV shows the absolute and relative retention times of the investigated drugs.

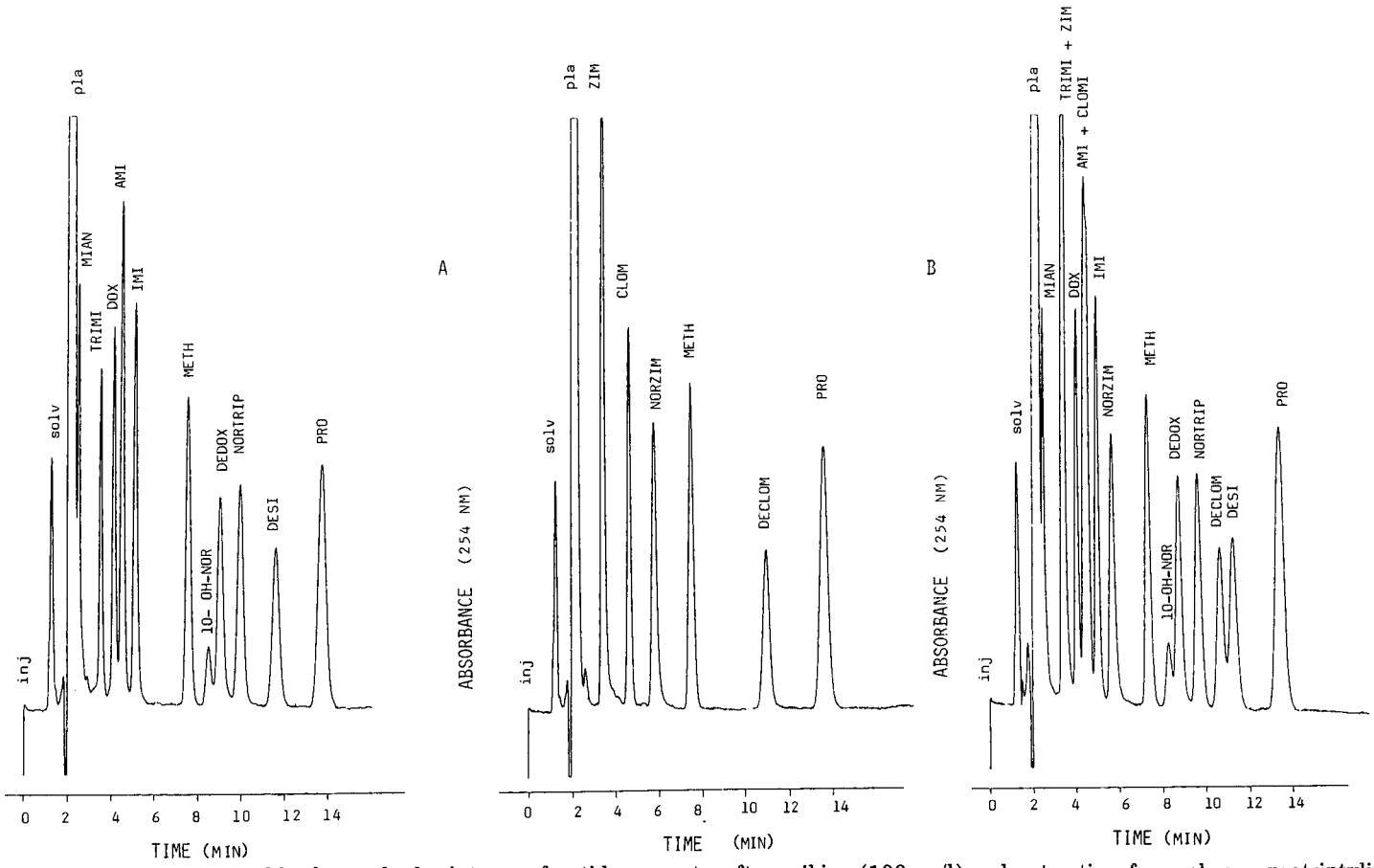


Fig. 1. Chromatograms of both standard mixtures of antidepressants, after spiking (100 μg/l) and extraction from plasma; protriptyline (400 μg/l) and methadone were added as internal and external standards. Method as described in the text. A, standard mixture I; B, standard mixture II; C, a combination of both standard mixtures. Abbreviations: inj = injection, solv = solvent peak, pla = plasma peak, MIAN = mianserin, TRIMI = trimipramine, DOX = doxepin, AMI = amitriptyline, IMI = imipramine, METH = methadone, 10-OH-NOR = *trans*-10-hydroxy-nortriptyline, DEDOX = desmethyldoxepin, NORTRIP = nortriptyline, DESI = desipramine, PRO = protriptyline, ZIM = zimeldine, CLOM = clomipramine, NORZIM = norzimeldine, DECLOM = desmethylclomipramine.

TABLE I

RESULTS FOR THE DETERMINATION OF ANTIDEPRESSANTS

Generic name	Brand name	Retention time		Correlation coefficient*	Coefficient of variation (%) (n = 3)		Recovery (n = 3)		Detection limit (254 nm) (μg/l plasma)
		Absolute (min)	Relative to protriptyline		50 μg/l	100 μg/l	25 μg/l	100 μg/l	
Mianserin	Tolvon	2.56	0.185	0.9995	5.7	6.5	92	92	3
Zimeldine	Zelmid	3.50	0.253	0.9998	2.3	3.6	96	92	2
Trimipramine	Surmontil	3.59	0.260	0.9990	4.2	4.8	106	96	2
Doxepin	Sinequan, Quitaxon	4.20	0.304	0.9992	1.7	5.7	107	100	2
Amitriptyline	Sarotex, Tryptizol	4.58	0.331	0.9998	3.7	4.7	107	98	2
Clomipramine	Anafranil	4.73	0.342	0.9996	1.3	3.0	98	94	3
Imipramine	Tofranil	5.19	0.376	0.9997	3.8	3.9	111	101	2
Norzimeldine		5.89	0.426	0.9999	4.6	4.4	51	50	5
trans-10-Hydroxynortriptyline		8.61	0.623	0.9998	6.9	5.9	40	43	12
Desmethyldoxepin		9.14	0.661	0.9999	4.6	2.2	99	93	5
Nortriptyline		10.07	0.729	0.9998	1.8	6.6	111	96	4
Desmethylclomipramine		11.10	0.803	0.9999	0.9	2.6	99	93	5
Desipramine	Pertofran	11.72	0.848	0.9999	1.5	3.6	102	95	4
Protriptyline	Concordin	13.82	1.000	—	—	—	97	92	—

*Calibration curve: 25, 50, 100, 200 μg/l plasma.

TABLE II

RESULTS OF THE ADSORPTION STUDIES

Data represent peak area ratios of antidepressant to external standard (methadone).

Generic name	Corresponding plasma level = 25 μg/l		Corresponding plasma level = 100 μg/l	
	Ratio for reference solution (n = 3)	Ratio for control solution (n = 3)	Ratio for reference solution (n = 3)	Ratio for control solution (n = 3)
Mianserin	0.130 ± 0.006	0.136 ± 0.005	0.658 ± 0.017	0.704 ± 0.031
Zimeldine	0.532 ± 0.024	0.576 ± 0.009	2.107 ± 0.036	2.236 ± 0.030
Trimipramine	0.218 ± 0.005	0.238 ± 0.006	0.866 ± 0.021	0.867 ± 0.010
Doxepin	0.278 ± 0.008	0.292 ± 0.007	1.075 ± 0.024	1.069 ± 0.009
Amitriptyline	0.379 ± 0.009	0.409 ± 0.011	1.480 ± 0.040	1.457 ± 0.009
Clomipramine	0.288 ± 0.004	0.305 ± 0.007	1.118 ± 0.009	1.188 ± 0.017
Imipramine	0.347 ± 0.008	0.377 ± 0.012	1.370 ± 0.039	1.357 ± 0.009
Norzimeldine	0.435 ± 0.019	0.445 ± 0.011	1.575 ± 0.045	1.672 ± 0.012
trans-10-Hydroxynortriptyline	0.181 ± 0.009	0.199 ± 0.004	0.731 ± 0.015	0.731 ± 0.008
Desmethyldoxepin	0.284 ± 0.013	0.328 ± 0.005	1.146 ± 0.014	1.109 ± 0.007
Nortriptyline	0.290 ± 0.010	0.326 ± 0.014	1.161 ± 0.017	1.135 ± 0.006
Desmethylclomipramine	0.246 ± 0.002	0.241 ± 0.013	0.886 ± 0.031	0.937 ± 0.014
Desipramine	0.256 ± 0.006	0.287 ± 0.005	1.003 ± 0.021	0.988 ± 0.018
Protriptyline	1.901 ± 0.043	1.842 ± 0.031	1.830 ± 0.075	1.707 ± 0.041

TABLE III

RESULTS OF THE EXTRACTION STUDIES

Spiked quantity = 200 ng of each antidepressant in 2 ml of plasma (n = 3).

Generic name	First extraction			Second extraction			Total recovery	
	Absolute recovery (ng)	Relative recovery (%)	C.V. (%)	Absolute recovery (ng)	Relative* recovery (%)	C.V. (%)	Absolute (ng)	Relative (%)
Amitriptyline	189.2	94.6	4.6	11.0	102	13.3	200.2	100.1
trans-10-Hydroxynortriptyline	79.2	39.6	6.0	47.8	39.6	16.2	127.0	63.5
Norzimeldine	98.2	49.1	4.1	48.6	47.7	15.2	146.8	73.4

*Relative to the quantity present after the first extraction.

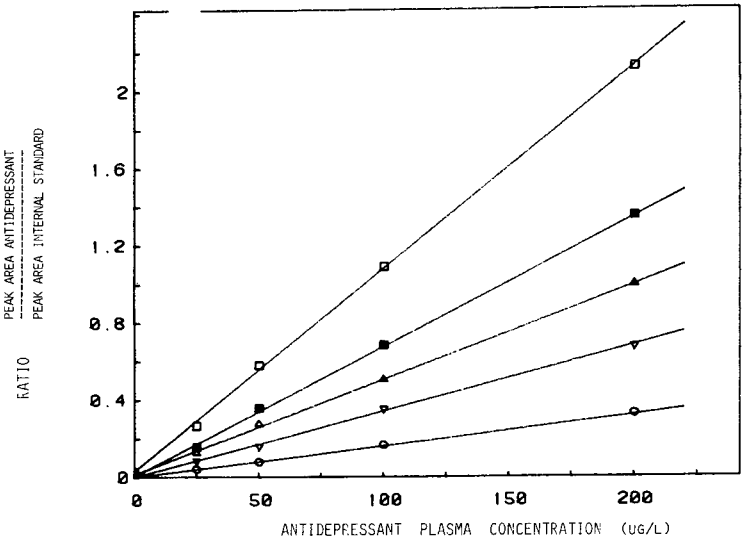


Fig. 2. Calibration curves of spiked plasma. □, ZIM; ■, AMI; △, CLOMI; ▽, MIAN; ○, 10-OH-NOR (abbreviations as in Fig. 1).

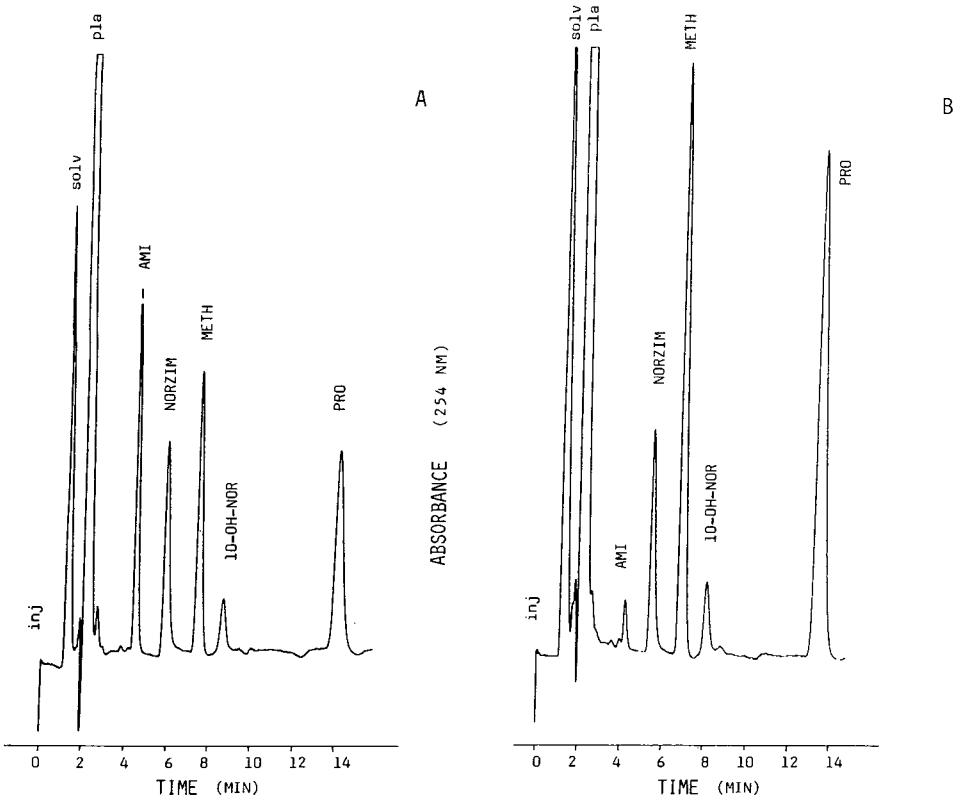


Fig. 3. Chromatograms of a mixture of amitriptyline, norzimeldine and *trans*-10-hydroxy-nortriptyline (each 100 μg/l plasma) after the first (A) and after the second (B) extraction from plasma; protriptyline and methadone were added as internal and external standards. Method as described in the text. Same abbreviations as used in Fig. 1. Each figure is presented on a different scale. Note the relative ratios of the peak heights.

TABLE IV

RETENTION TIMES FOR SOME COMMON DRUGS TESTED WITH THE HPLC PROCEDURE

Generic name	Retention time	
	Absolute (min)	Relative to protryptyline
Phenobarbitone	1.83	0.13
Theophylline	2.07	0.15
Acetaminophen	2.21	0.16
Flupentixol	2.21	0.16
Caffeine	2.25	0.16
Carbamazepine	2.30	0.17
Chlordiazepoxide	2.37	0.17
Nitrazepam	2.37	0.17
Diazepam	2.38	0.17
Oxazepam	2.38	0.17
n-Desalkylflurazepam	2.38	0.17
Fluphenazine	2.49	0.18
Clopentixol	2.54	0.18
Perphenazine	2.56	0.19
Opipramol	2.58	0.19
Penfluridol	2.89	0.21
Promethazine	3.07	0.22
Sulpiride	3.17	0.23
Levomepromazine	3.24	0.23
Desmethylmianserin	3.26	0.24
Prochlorperazine	3.47	0.25
Chlorpromazine	3.81	0.28
Sulforidazine	4.24	0.31
Orphenadrine	4.28	0.31
Promazine	4.59	0.33
Phenytoin	5.25	0.38
Thioridazine	5.25	0.38
Propanolol	5.26	0.38
Mesoridazine	5.46	0.40
2-Hydroxyimipramine	6.31	0.46
Methadone	7.66	0.55
2-Hydroxydesipramine	10.51	0.76
Disopyramide	11.35	0.82
Maprotiline	13.32	0.96
Protriptyline	13.82	1.00

DISCUSSION

With the described assay many commonly used antidepressants and their major therapeutically active metabolites can be measured in plasma. The method is highly selective within the group of the tricyclics. Antidepressant therapy in psychiatric patients can be monitored and therapy can be evaluated in the light of the acquired data.

Since protriptyline itself is only rarely administered as a drug, while having about the same solubility properties as the other antidepressants, we selected it as the internal standard for the assay. In clinical situations where knowledge of protriptyline or maprotiline concentration (Table IV) is required, an alternative internal standard should be used. Favourable results will be obtained with one of the other tricyclic antidepressants. In rare cases, when clomipramine and amitriptyline or trimipramine and zimeldine are prescribed together, modification of the eluent will be necessary to achieve peak separation.

Our assay is well suited for routine application in the clinical laboratory because the extraction procedure is simple (one-step extraction) and fairly rapid. Extraction procedure and chromatography take about 15 min each. The assay provides adequate sensitivity and precision for monitoring steady-

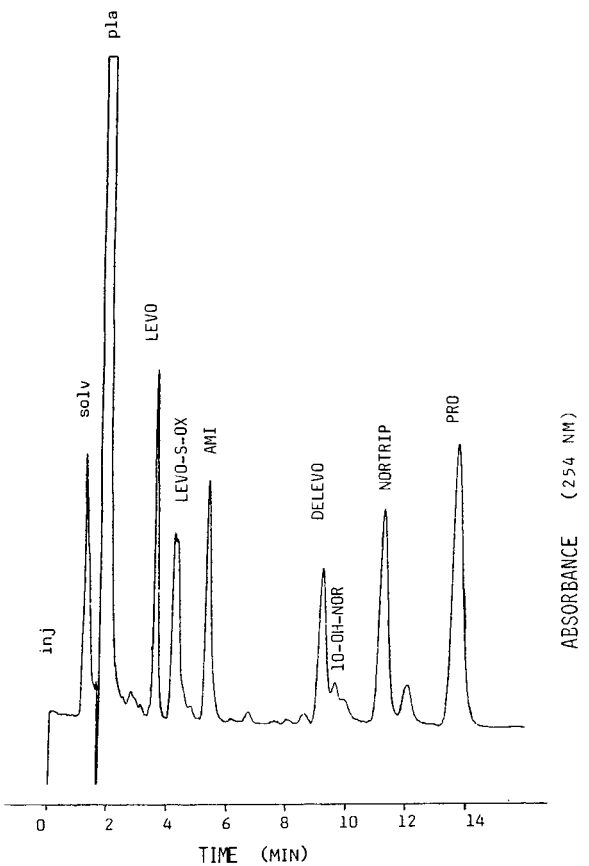


Fig. 4. Chromatogram of a patient's plasma after extraction. Patient was treated daily with 2 × 75 mg of Tryptizol® and 3 × 50 mg of Nozinan®. Method as described in text. Abbreviations: inj = injection, solv = solvent peak, pla = plasma peak, LEVO = levomepromazine, LEVO-S-OX = compound peak, one of which is levomepromazine sulfoxide, AMI = amitriptyline (85 μg/l plasma), DELEVO = desmethyllevomepromazine, 10-OH-NOR = *trans*-10-hydroxynortriptyline, NORTRIP = nortriptyline (117 μg/l plasma), PRO = protriptyline (internal standard).

state concentrations, as well as subtherapeutic and toxic concentrations of antidepressants. Recoveries of the antidepressants were determined at concentrations of 25 and 100 μg/l. We found high recoveries at both concentrations (Table I). Low recoveries were found only for norzimeldine and 10-hydroxynortriptyline. Irregular losses of norzimeldine when concentrated by gentle evaporation have been noted before [17]. However, in our case the low recovery is explained by incomplete extraction. The incomplete extraction of 10-hydroxynortriptyline compared to nortriptyline can be understood because of the introduction of a polar hydroxyl group. No reasonable explanation, however, has been found for the large difference in extraction properties between norzimeldine and zimeldine. Koteel et al. [13], though working with similar chromatographic conditions, report very low recoveries of antidepressant drugs. Our results suggest that their extraction procedure is not suitable.

Several authors report loss of antidepressant during determination, most likely through adsorption to the glassware [18, 19]. According to some, recovery decreases with decreasing amounts of drug [12, 20]. Evaporation conditions seem to be critical [18, 21, 22]. We investigated whether interaction with the glass surface influenced our results. As shown in Table II, neither glass surface area nor our way of evaporating significantly influences the results. So our reference solutions used for the recovery studies can be interpreted as real references. Lack of adsorption is also suggested by the high correlation coefficients of the calibration curves. This information underlines the value of the described assay method.

Since patients treated with antidepressants frequently receive other psychoactive drugs, among them sedatives and tranquillizers that are structurally related to the antidepressants, the clinical usefulness of any method for monitoring antidepressants will largely be determined by the degree of freedom from interferences by these drugs. Of the drugs tested some interfere with the described assay of antidepressants, the relevance of which is determined by the individual drug and by the individual antidepressant.

The described method has been successfully used in our laboratory for monitoring clinical and forensic cases. As an illustration, a chromatogram obtained by working up plasma from a patient undergoing antidepressant therapy is shown in Fig. 4. Also, participation in quality control schemes for antidepressant drugs has yielded good results.

REFERENCES

1 L.F. Gram, Clin. Pharmacokin., 2 (1977) 237.
2 S.C. Risch, L.Y. Huey and D.S. Janowsky, J. Clin. Psychiat., 40 (1979) 4.
3 S.C. Risch, L.Y. Huey and D.S. Janowsky, J. Clin. Psychiat., 40 (1979) 58.
4 B.A. Scoggins, K.P. Maguire, T.R. Norman and G.D. Burrows, Clin. Chem., 26 (1980) 5.
5 H.B. Hucker and S. Stauffer, J. Pharm. Sci., 63 (1974) 296.
6 G. Nyberg and E. Mårtensson, J. Chromatogr., 143 (1977) 491.
7 O. Borgå and M. Garle, J. Chromatogr., 68 (1972) 77.
8 S. Dawling and R.A. Braithwaite, J. Chromatogr., 146 (1978) 449.
9 D.N. Bailey and P.I. Jatlow, Clin. Chem., 22 (1976) 777.

10 D.R.A. Uges and P. Bouma, Pharm. Weekbl. Sci. Ed., 1 (1979) 45.
11 F.L. Vandermark, R.F. Adams and G.J. Schmidt, Clin. Chem., 24 (1978) 87.
12 H.F. Proelss, H.J. Lohmann and D.G. Miles, Clin. Chem., 24 (1978) 1948.
13 P. Koteel, R.E. Mullins and R.H. Gadsen, Clin. Chem., 28 (1982) 462.
14 R.G. Jenkins and R.O. Friedal, J. Pharm. Sci., 67 (1978) 17.
15 K.P. Maguire, G.D. Burrows, T.R. Norman and B.A. Scoggins, Clin. Chem., 24 (1978) 549.
16 R.B. Moyes and I.C.A. Moyes, Postgraduate Med. J., 53 (Suppl. 4) (1977) 117.
17 B. Emanuelsson and R.G. Moore, J. Chromatogr., 146 (1978) 113.
18 P.K. Sonsalla, T.A. Jennison and B.S. Finkle, Clin. Chem., 28 (1982) 1401.
19 P.M. Edelbroek, E.J.M. de Haas and F.A. de Wolff, Clin. Chem., 28 (1982) 2143.
20 A. Jørgensen, Acta Pharm. Toxicol., 36 (1975) 79.
21 E. Antal, S. Mercik and P.A. Kramer, J. Chromatogr., 183 (1980) 149.
22 J.J. Thoma, P.B. Bondo and C.M. Kozak, Ther. Drug Monit., 1 (1979) 335.

Journal of Chromatography, 309 (1984) 95—105
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2116

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF PIRLIMYCIN IN HUMAN SERUM AND URINE USING 9-FLUORENYLMETHYLCHLOROFORMATE

JYOTI A. SHAH* and DENNIS J. WEBER

Drug Metabolism Research, The Upjohn Company, Kalamazoo, MI 49001 (U.S.A.)

(First received November 2nd, 1983; revised manuscript received February 21st, 1984)

SUMMARY

A reversed-phase high-performance liquid chromatographic (HPLC) assay method has been developed for determining pirlimycin in human serum and urine. The method involves chloroform extraction of pirlimycin free base followed by derivatization with 9-fluorenylmethylchloroformate to form a carbamate ester. The reaction is rapid, reproducible, and quantitative. 9-Fluorenylmethylchloroformate reacts with amines to form derivatives sensitive to both ultraviolet and fluorescence detection. Human serum and urine samples following 50-mg and 500-mg single oral doses of pirlimycin were analyzed. The samples were chromatographed on an RP-18 Spherisorb 5-μm, 250 × 4.6 mm I.D. reversed-phase HPLC column. The eluent for the serum assay was acetonitrile—water (58:42) containing 0.02% acetic acid, and for the urine assay was acetonitrile—methanol—tetrahydrofuran—water (48:2:1:49). Fluoranthene was used as an internal standard. The assay sensitivity by ultraviolet detection (λ_{max} = 264) was about 5 ng/ml and by fluorescence detection ($\lambda_{excitation}$ = 270 nm, $\lambda_{emission}$ = 300 nm) was 0.1 ng/ml. Statistical analysis indicates an average drug recovery of 101 ± 4.2% from serum and 102.0 ± 2.62% from urine.

INTRODUCTION

The development of pirlimycin (P), an analogue of clindamycin, required a sensitive analytical method for its measurement in biological fluids.

A major problem in developing a sensitive high-performance liquid chromatographic (HPLC) assay was the lack of useful ultraviolet absorption by the lincosaminide family of antibiotics of which pirlimycin is a member. Although some work has been reported [1] on an HPLC assay for pirlimycin using ultraviolet end-absorption at 214 nm, the procedure is not sensitive for measuring nanogram levels of drug in biological fluids.

Alkylchloroformate esters have been used for the gas chromatographic

0378-4347/84/$03.00

analysis [2—6] and for the HPLC analysis [7] of many amines. However, the HPLC procedure required heating at 100° C for 1 h which seemed excessive.

Recently, a new chloroformate reagent was developed as an amino-protective group in the solid phase synthesis of peptides [8—12]. This reagent, 9-fluorenylmethylchloroformate (9-FMClF), was found to react quantitatively and rapidly at ambient conditions with the amine group of the piperidine moiety of pirlimycin. This report describes the development of an HPLC assay procedure for pirlimycin in human serum and urine using pre-column derivatization of the drug with 9-FMClF.

EXPERIMENTAL

Reagents and materials

Pirlimycin · HCl and pirlimycin free base were synthesized within the Research Division of the Upjohn Company. Fluoranthene and 9-fluorenylmethylchloroformate were obtained from Eastman Kodak (Rochester, NY, U.S.A.) and from Aldrich (Milwaukee, WI, U.S.A.), respectively, and used as received. Acetonitrile, chloroform, dioxane, and methanol were distilled-in-glass UV grade from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) All other chemicals were analytical reagent grade.

Synthesis of pirlimycin derivative

Method 1. Pirlimycin free base ($3.84 \cdot 10^{-3}$ mol) was dissolved in 25 ml dioxane and added to 10 ml of 10% (w/v) sodium carbonate. The solution was cooled in an ice-bath. An equimolar amount of 9-FMClF was dissolved in dioxane and then added drop-wise to the above solution with continuous stirring for about 20 min. The mixture was allowed to stir for an additional 45 min, and then poured into water; the formed derivative was extracted into chloroform. The extract was evaporated to dryness to yield an oily residue; the residue was dissolved in diethyl ether and crystalline material was obtained upon room temperature drying.

Method 2. Equimolar solutions of pirlimycin free base and 9-FMClF were prepared in 25 ml and 5 ml acetonitrile, respectively, then mixed. The mixture was allowed to stand at room temperature for about 45 min. The derivative was precipitated by adding 50 ml water to the reaction mixture and stirring for about 20 min. The filtered precipitate was dried under vacuum.

Chromatography instruments

An Altex Model 110A pump, a Rheodyne Model 7125 low-dead-volume injector, and an LDC Spectromonitor III variable-wavelength UV detector or Perkin-Elmer LS-5 variable-wavelength fluorescence detector were used. Data were recorded on a linear dual-channel strip-chart recorder. The column was a Spherisorb, 5-μm, 25 cm × 4.6 mm RP-18 column from Brownlee Labs. fitted with a Spherisorb, 5-μm, 3 cm × 4.6 mm RP-18 guard column, also from Brownlee Labs.

Operating parameters for serum and urine analysis

Serum samples were analyzed using acetonitrile—water (58:42) containing

0.02% acetic acid as the mobile phase at a flow-rate of 1.2 ml/min. For UV detection the absorbance was set at 264 nm, and for fluorescence detection excitation was set at 270 nm and emission at 300 nm. Under these conditions using UV detection, the retention times of pirlimycin derivative and fluoranthene (internal standard) were 14.5 and 37.5 min, respectively.

For the urine analysis, the mobile phase consisted of acetonitrile–methanol–tetrahydrofuran–water (48:2:1:49). With the exception of the flow-rate of 1.5 ml/min, all other conditions were identical to the conditions employed for serum analysis. Under these conditions using UV detection, retention times of pirlimycin derivative and internal standard were 28 and 60 min, respectively.

Thin-layer chromatographic (TLC) conditions

Thin-layer chromatography was performed on C_{18} reversed-phase TLC plates with a 200-μm layer (Whatman $MKC_{18}F$). The developing solution was acetonitrile–water (80:20) and detection was at 254 nm. Under these conditions the R_F for the pirlimycin carbamate derivative was 0.5.

Human study protocol

Pirlimycin · HCl capsules were administered orally at 50-, 125-, 250- and 500-mg dose levels. Blood and urine samples were taken at recorded time intervals. Blood samples were taken into non-heparinized tubes, allowed to clot, and then centrifuged at 1000 *g* for 10 min. The harvested serum was put into appropriately labelled vials and immediately frozen. The frozen samples were stored at −20° C until assayed.

Total urine volume was recorded for each interval before an aliquot was withdrawn, immediately frozen, and stored at −20° C until assayed.

ASSAY PROCEDURE

Preparation of serum samples

Stock solutions of pirlimycin · HCl (2.088 μg/ml) and fluoranthene internal standard (9.3 μg/ml) were prepared separately in methanol, while 9-FMClF derivatizing reagent, a stock solution (63.8 μg/ml), was prepared in acetonitrile. A series of standard serum samples of pirlimycin · HCl was prepared by pipetting pirlimycin · HCl stock solutions into 15-ml centrifuge tubes. The solvent was evaporated to dryness then reevaporated after adding 50 μl of internal standard solution; 1 ml of human serum and 0.3 ml of 0.1 *M* sodium hydroxide were added to each tube. The samples were extracted twice, each time with 4 ml of chloroform, then mixed and centrifuged; the chloroform extract was evaporated to dryness. Similarly, a blank serum sample was prepared from the same serum specimen. The 10 μl of $2.5 \cdot 10^{-4}$ *M* sodium hydroxide and 50 μl of 9-FMClF were added to the dry residue. The mixture was kept at room temperature for 2.5 h, and then 20–40 μl were injected for analysis into HPLC.

Test serum samples were prepared by pipetting 50 μl internal standard solution into a series of centrifuge tubes. After evaporating to dryness, 1 ml of serum and 0.3 ml of 0.1 *M* sodium hydroxide were added; subsequent steps

involving chloroform extraction and derivatization were carried out as described for the standard serum samples.

Preparation of urine samples

Stock solutions of pirlimycin · HCl (10.33 μg/ml) and internal standard (9.3 μg/ml) were prepared in methanol. A series of standard samples was prepared by pipetting pirlimycin · HCl stock solutions into 15-ml centrifuge tubes; 100 μl of internal standard were added to each tube, then evaporated to dryness. Blank human urine (1 ml), 0.5 ml of 0.1 *M* sodium hydroxide and 5 ml of chloroform were added; the samples were mixed and centrifuged and the choroform extract was evaporated to dryness. Similarly, a blank urine sample was prepared from the same human urine. The 10 μl of $2.5 \cdot 10^{-4}$ *M* sodium hydroxide and 80 μl of 150 μg/ml 9-FMClF were added to the dry residue. The mixture was kept at room temperature for about 2.5 h, then 20—70 μl were injected for analysis into HPLC.

Test urine samples were prepared by pipetting 100 μl of internal standard into series of centrifuge tubes. After evaporation to dryness, 1 ml urine for low-level doses and 0.1 ml urine for high-level doses were added to each centrifuge tube. Subsequent steps of chloroform extraction and derivatization were carried out as described for the standard urine samples.

Calculations

Peak height ratios were calculated by dividing the peak height of the pirlimycin derivative by the peak height of the internal standard. The peak height ratio was corrected by taking into account the corresponding blank serum samples. A calibration curve was constructed by plotting peak height ratios versus the concentrations of the pirlimycin · HCl standards. The concentration of pirlimycin · HCl in the unknowns was calculated from the peak height ratios using the slope and intercept obtained by linear-regression analysis of the calibration curve data. The amount of drug excreted in urine was calculated by multiplying the urine volumes by the analytical concentration.

RESULTS AND DISCUSSION

Synthesis and characterization of 9-FMClF derivative of pirlimycin

Pirlimycin free base reacts with 9-FMClF to form a pirlimycin carbamate derivative and an equimolar amount of hydrochloric acid (see Fig. 1). Hydrochloric acid can rapidly convert the unreacted pirlimycin free base into its hydrochloride salt. Since the salt does not react with 9-FMClF, only about 50% of the free base can be converted into its carbamate derivative. Sodium carbonate was used to neutralize the acid in the Method 1 synthesis. This method is similar to the one reported previously [8]. Since the Method 2 synthesis was carried out without the addition of a base, about 50% yield was obtained by this procedure. However, Method 2 is much simpler than Method 1.

TLC and HPLC analysis showed that the synthesized material was a single component. Elemental analysis, IR, and mass spectrometric analysis supported the proposed structure.

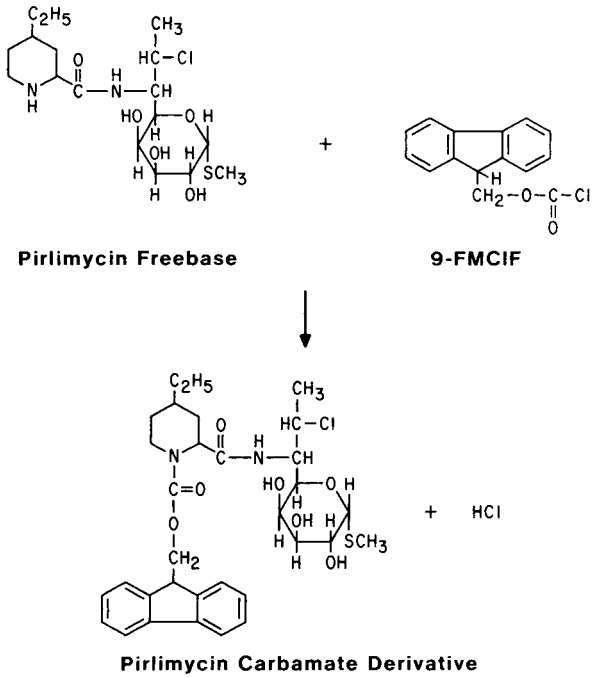


Fig. 1. Reaction scheme for formation of carbamate derivative of pirlimycin.

Chromatographic analysis

Serum and urine samples containing pirlimycin free base were extracted with chloroform, evaporated and reacted with 9-FMClF in an organic solvent (acetonitrile). In order to achieve complete derivatization of all the drug, the reaction had to be carried out in the presence of an inorganic base which could neutralize hydrochloric acid from the reaction mixture. The use of 18-Crown-6 ether is common for dissolving inorganic ions in organic solvents [13]. Several trials were made using potassium hydroxide and other inorganic bases in 18-Crown-6 ether to derivatize pirlimycin free base in acetonitrile. However, these efforts were not fully successful due to the interfering effects of 18-Crown-6 ether and the partial decomposition of the drug derivative. The use of a very small volume of sodium hydroxide solution in water yielded 100% derivatization of drug with reproducible results.

Chromatographic experiments were performed using C_8 and C_{18}, 5-μm and 10-μm particle size, reversed-phase columns, and various mobile-phase compositions consisting of acetonitrile—water, acetonitrile—tetrahydrofuran—methanol—water, acetonitrile—water—acetic acid, and methanol—water—acetic acid. Among these, a C_{18} reversed-phase column, having a 5-μm particle size and a mobile phase composed of acetonitrile—water (58:42) containing 0.02% acetic acid, provided optimum resolution for serum analysis. Fluoranthene was chosen as an internal standard because of its non-reactivity to the reagent, complete recovery from biological fluids and high UV-fluorescence detector response.

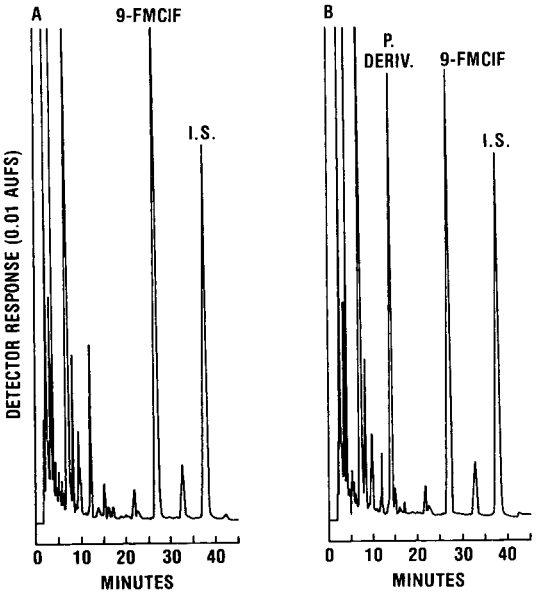


Fig. 2. Chromatogram of human serum extract of (A) blank serum containing internal standard (I.S.) and derivatizing reagent (9-FMClF); and (B) serum containing pirlimycin carbamate derivative (P. Deriv.), internal standard (I.S.) and derivatizing reagent (9-FMClF).

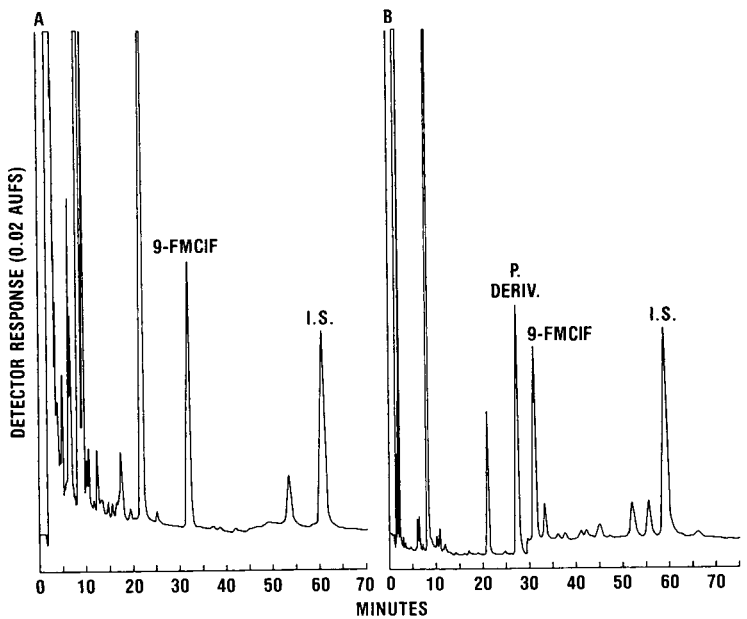


Fig. 3. Chromatogram of human urine extract of (A) blank urine containing internal standard (I.S.) and derivatizing reagent (9-FMClF); and (B) urine containing pirlimycin carbamate derivative (P. Deriv.), internal standard (I.S.) and derivatizing reagent (9-FMClF).

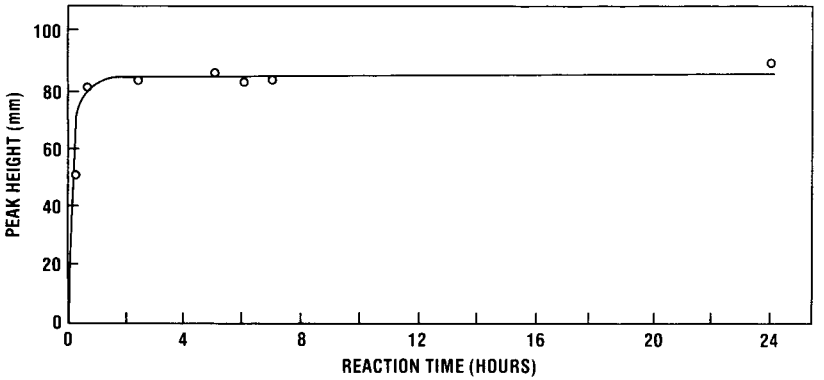


Fig. 4. Effect of reaction time on formation of pirlimycin derivative.

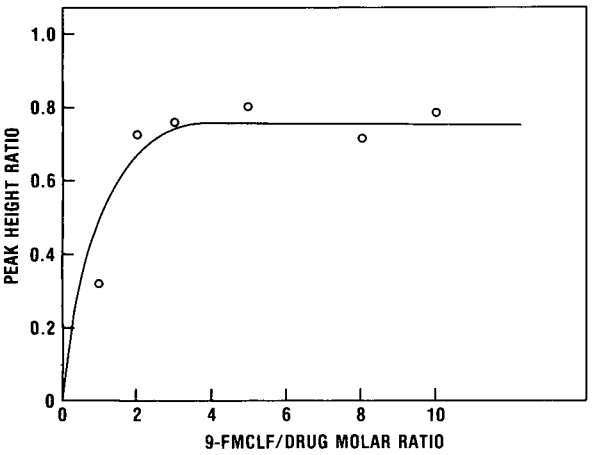


Fig. 5. Effect of 9-fluorenylmethylchoroformate on pirlimycin derivative formation. Reaction time = 2.30 h at room temperature.

Fig. 2A shows a typical HPLC chromatogram of an extract of blank human serum containing internal standard and derivatizing reagent (9-FMClF). Fig. 2B shows a chromatogram of serum containing pirlimycin carbamate derivative, internal standard, and 9-FMClF. Fig. 3A and Fig. 3B show similar chromatograms for blank urine and spiked urine extracts, respectively.

Fig. 4 shows the effect of reaction time on the formation of pirlimycin derivative at room temperature. The reaction is essentially completed within 1 h. The derivative is stable for at least 24 h. Fig. 5 shows the effect of derivatizing reagent concentration upon the extent of formation of pirlimycin derivative. A molar ratio of drug to reagent of 1:3 gives complete formation of the derivative.

Extraction efficiency

Serum or urine specimens (1 ml) were spiked with known concentrations of pirlimycin · HCl and the drug was extracted according to the procedure described in Experimental. These samples were assayed at 214 nm using chromatographic conditions reported by Asmus and Landis [1]. The extraction

TABLE I

EXTRACTION EFFICIENCY OF PIRLIMYCIN · HCl FROM HUMAN SERUM AND URINE

Trial	Serum extraction efficiency (%)	Urine extraction efficiency (%)
1	94.16	98.28
2	94.50	101.50
3	103.33	—
Mean (%)	97.33	99.89
Coefficient of variation (%)	5.3	2.3

efficiency of pirlimycin from serum and urine was calculated by comparing absorbance with the known standard drug solution at 214 nm. The results, listed in Table I, show an average extraction efficiency of 97.33% from serum an 99.89% from urine.

Reaction efficiency

Blank serum or urine (1 ml) was spiked with a known concentration of pirlimycin · HCl. The sample was extracted and reacted with the derivatizing reagent (9-FMClF) in acetonitrile in the presence of a small amount of base as described in Experimental. The sample was chromatographed along with a standard solution of the pure derivative in acetonitrile. The results confirmed stoichiometric conversion of drug into its carbamate derivative.

Assay precision and accuracy

Linear-regression analysis of the calibration curve data indicated no significant deviations from linearity for pirlimycin hydrochloride concentrations up to 2 μg/ml serum and up to 20 μg/ml urine. Correlation coefficients were better than 0.993 for the standard curves prepared at three different times.

Assay precision and accuracy were established by adding a known concentration (417.6 ng/ml serum) of pirlimycin hydrochloride to several 1-ml blank serum samples. These replicate samples were extracted, derivatized and assayed

TABLE II

ACCURACY AND PRECISION DATA FOR THE RECOVERY OF PIRLIMYCIN · HCl FROM HUMAN SERUM

Added (ng/ml)	Found (ng/ml)	Recovery (%)
417.6	439.11	105.15
	421.89	101.03
	407.95	97.69
	426.51	102.13
	436.51	104.53
	436.76	104.58
	386.81	92.63
	Average = 422.22	Mean = 101 ± 4.2%
Coefficient of variation = 4.5%		

TABLE III

ACCURACY AND PRECISION DATA FOR THE RECOVERY OF PIRLIMYCIN·HCl FROM HUMAN URINE

Added (μg/ml)	Found (μg/ml)	Recovery (%)
5.165	5.346	103.51
	5.279	102.22
	5.071	98.18
	5.051	97.80
	5.439	105.31
	5.309	102.69
	5.373	104.02
	Average = 5.26	Mean = 102 ± 2.62%
Coefficient of variation = 2.8%		

as described previously. The average interday recovery of pirlimycin · HCl obtained was 101 ± 4.2% (Table II).

Table III gives precision and accuracy data for the replicate analysis of urine samples spiked with a known concentration (5.165 μg/ml urine) of drug. An average interday drug recovery of 102.0 ± 2.62% was obtained.

Applicability of the methodology

The utility of the analytical method for pharmacokinetic studies was demonstrated by monitoring serum and urine levels of pirlimycin · HCl in humans. A typical serum profile is shown in Fig. 6 for a dose of 500 mg of pirlimycin · HCl. A typical cumulative urinary elimination profile is shown in Fig. 7 for a dose of 50 mg of pirlimycin · HCl. A summary of the pharmacokinetic parameters is given in Table IV. The area under the serum curve (AUC) data may indicate a non-linear dose response since the AUC for the 50-mg dose is much less than expected based on the 500-mg dose results. The values of U_∞

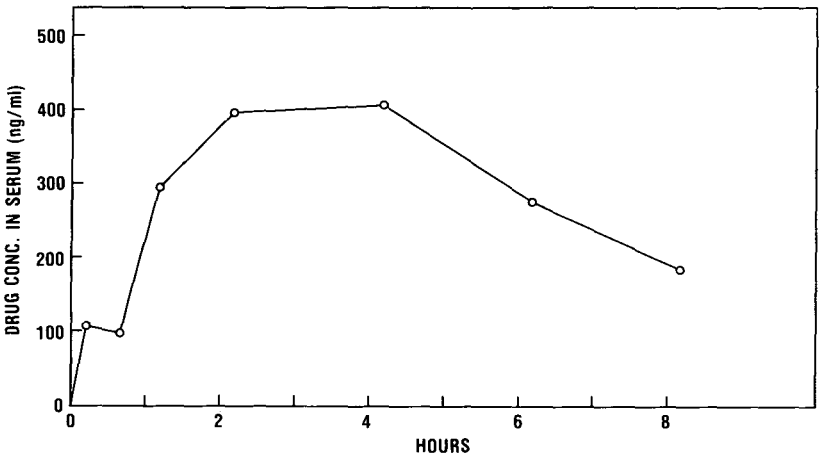


Fig. 6. Pirlimycin · HCl concentration in human serum following a 500-mg oral dose of pirlimycin · HCl to a human male.

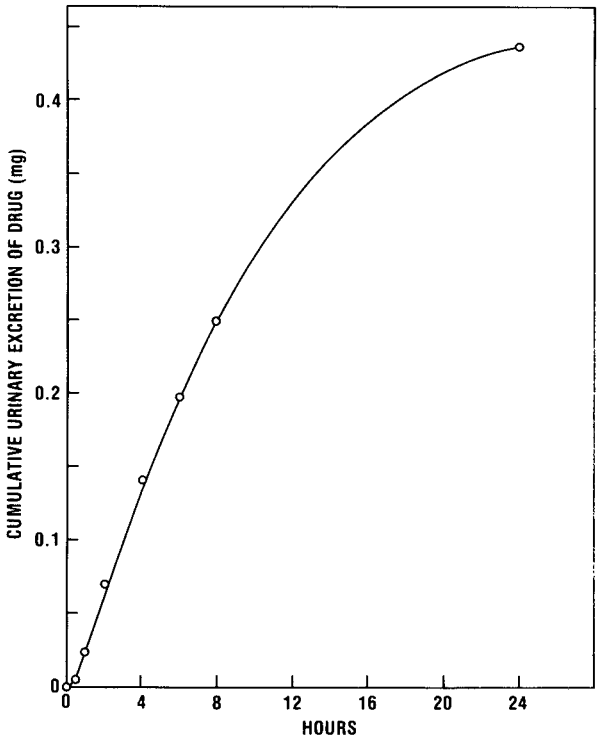


Fig. 7. Cumulative urinary elimination of pirlimycin ◦ HCl following an oral dose of 50 mg pirlimycin ◦ HCl to a human male.

TABLE IV

PHARMACOKINETIC PARAMETERS IN MALE HUMANS FOLLOWING ORAL DOSES OF PIRLIMYCIN · HCl

	500-mg Dose		50-mg Dose
	Subject 1	Subject 2	Subject 1
AUC_0 8 h (ng h ml^{-1})	2721	2462	44.5
k_e* (h^{-1})	0.097		0.101

* The value of U_∞ used to calculate these rate constants were estimated assuming first-order kinetics.

were estimated assuming first-order kinetics for the calculation of the renal elimination rate constants. Therefore, these rate constants should be considered only as approximations.

Fluoresence detection

Derivatives prepared from 9-FMClF are highly fluorescent and may be useful in increasing the assay sensitivity to sub-nanogram levels. A preliminary evaluation of the fluorescence sensitivity using blank serum samples spiked with the 9-FMClF derivative of pirlimycin indicated an analytical sensitivity of 0.1 ng/ml.

ACKNOWLEDGEMENT

Helpful suggestions by Dr. Walter Morozowich are gratefully acknowledged.

REFERENCES

1 P.A. Asmus and J.B. Landis, The Upjohn Company, personal communication.
2 N. Ahnfelt, Acta Pharm. Suecica, 19 (1982) 471.
3 N. Ahnfelt and P. Hartwig, Acta Pharm. Suecica, 19 (1982) 367.
4 K.-E. Karlsson and P. Hartwig, Acta Pharm. Suecica, 18 (1981) 337.
5 K.-E. Karlsson, J. Chromatogr., 219 (1981) 373.
6 K.-E. Karlsson, Acta Pharm. Suecica, 19 (1982) 49.
7 G. Gübitz, R. Wintersteiger and A. Hartinger, J. Chromatogr., 218 (1981) 51.
8 J. Meienhofer, M. Waki, E.P. Heimer, T.J. Lambros. R.C. Makafske and C.-D. Chang, Int. J. Pept. Protein Res., 13 (1979) 35.
9 E. Atherton, H. Fox, D. Harkiss, C.J. Logan, R.C. Sheppard and B.J. Williams, J. Chem. Soc., Chem. Commun., 13 (1978) 537.
10 E. Atherton, H. Fox, D. Harkiss and R.C. Sheppard, J. Chem. Soc., Chem. Commun., 13 (1978) 539.
11 C. Chang and J. Meienhofer, Int. J. Pept. Protein Res., 11 (1978) 246.
12 L.A. Carpino and G.Y. Han, J. Org. Chem., 37 (1972) 3404.
13 Crown-Ethers, Technical Bulletin, PCR, Gainesville, FL.

Journal of Chromatography, 309 (1984) 107—114
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2119

MEASUREMENT OF MAPROTILINE AND OXAPROTILINE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF FLUORESCENT DERIVATIVES

U. BREYER-PFAFF*, R. WIATR and K. NILL

Department of Toxicology, University of Tübingen, Wilhelmstrasse 56, D-7400 Tübingen (F.R.G.)

(First received November 29th, 1983; revised manuscript received February 21st, 1984)

SUMMARY

The antidepressant maprotiline and its hydroxylated analogue oxaprotiline were assayed in plasma by solvent extraction and formation of fluorescent derivatives, which were purified by thin-layer chromatography and quantitated by high-performance liquid chromatography with fluorescence monitoring. The procedure possesses a high sensitivity, accuracy and reproducibility, and metabolites of the drugs did not interfere.

INTRODUCTION

Maprotiline (Ludiomil®) is a tetracyclic antidepressant with well documented clinical efficacy [1]. Introduction of an alcoholic hydroxy group into its side-chain leads to oxaprotiline (Fig. 1), a compound currently being tested for its therapeutic activity in depressive disorders [2, 3]. In the course of clinical studies, it is desirable to obtain information on plasma drug levels, since unsatisfactory response may be a consequence of very low as well as of extremely high levels [4].

The double radioisotope derivative method of Riess [5] does not seem suited for routine laboratory use unless very large series have to be analysed.

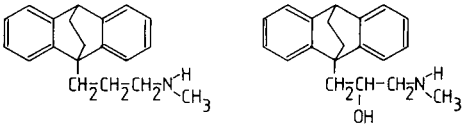


Fig. 1. Structures of maprotiline and oxaprotiline.

Whereas several gas chromatographic procedures have been described for the analysis of maprotiline in plasma [6—9], the application to oxaprotiline has only been realized using a mass spectrometer as detector [10]. The only published procedure for the determination of both compounds [11] used high-performance liquid chromatography (HPLC) of the unchanged drugs with absorbance monitoring at 214 nm. This necessitated extraction with a very unpolar solvent and implicated low recoveries. Higher percentages were recovered in a similar HPLC assay of maprotiline and desmethylmaprotiline [12], but the applicability of the method to oxaprotiline analysis was not tested. Since the latter drug produces low plasma levels, the formation of fluorescent derivatives in toluene extracts appeared promising.

EXPERIMENTAL

Materials

Blood was obtained from patients under treatment with divided doses of maprotiline (125 mg/day Ludiomil) or oxaprotiline (150 mg/day C-49802 B-Ba; Ciba-Geigy, Basel, Switzerland). Samples were drawn in the morning before ingestion of the first drug dose and placed into heparinized tubes. After centrifugation, plasma was stored at −20° C for up to eighteen months without apparent loss of drug. Blank plasma was obtained from drug-free healthy volunteers.

Reference compounds were donated by the following companies: maprotiline hydrochloride, desmethylmaprotiline methanesulphonate, oxaprotiline hydrochloride, desmethyloxaprotiline hydrochloride, desmethyl- and didesmethylimipramine hydrochloride, desmethylclomipramine hydrochloride by Ciba-Geigy; nortriptyline hydrochloride by Tropon (Cologne, F.R.G.); and *E*-10-hydroxynortriptyline by A. Jørgensen (Lundbeck, Copenhagen, Denmark). Hydroxylated maprotiline metabolites were isolated from patient urine and their structures elucidated by nuclear magnetic resonance and mass spectrometry [13].

Fluorescent derivatives of maprotiline and oxaprotiline were obtained on a preparative scale by reacting 0.2 mmol of drug hydrochloride in 1 ml of water with 100 mg of potassium bicarbonate and 0.4 mmol of 5-dimethylamino-naphthalene-1-sulphonyl chloride (dansyl chloride, Merck, Darmstadt, F.R.G.) in 2 ml of acetone. The mixture was stirred for 30 min at 45° C. The oily precipitate dissolved upon extraction with three 2-ml portions of chloroform. The residue of the chloroform extract was subjected to thin-layer chromatography (TLC) on five sheets precoated with silica gel (Polygram Sil G/UV_{254}, Macherey-Nagel, Düren, F.R.G.) in hexane—acetone—toluene (21:2.5:1 for dansyl maprotiline, 21:4:0.7 for dansyl oxaprotiline). The compounds were eluted from the gel using chloroform and recrystallized from chloroform—hexane. Dansyl derivatives of other reference compounds were prepared on a small scale by the procedure used for analytical work.

Plasma level assay

Principle. In the determination of maprotiline oxaprotiline (400 ng/ml) served as an internal standard, while didesmethylimipramine (30 ng/ml) was

the internal standard in the measurement of oxaprotiline. Plasma extracts were treated with dansyl chloride to form the dansyl derivatives of drug and internal standard. These were extracted and pre-purified by TLC, the conditions being chosen in a way that the two compounds had identical R_F values. Eluates of the spots were subjected to HPLC with fluorescence detection.

Procedure. To plasma (1 ml) the internal standard was added in water: 40 µl of oxaprotiline solution (10 µg/ml) for maprotiline analysis or 10 µl of didesmethylimipramine solution (3 µg/ml) for oxaprotiline analysis. After addition of 250 mg of sodium chloride, 35 µl of 10% sodium deoxycholate solution and 100 µl of sodium hydroxide (0.2 mol/l), the sample was extracted twice with 1.5 ml of toluene. In maprotiline analyses, the organic phase was reduced to about 1 ml in a rotary evaporator and extracted with 0.5 ml of sulphuric acid (5 mmol/l). In assays of oxaprotiline, the toluene phase was extracted without evaporation. The aqueous phase was washed with 1 ml of hexane, residual hexane being removed under a stream of nitrogen. Then it was mixed with 0.1 ml of potassium bicarbonate (0.6 mol/l) and 0.6 ml of dansyl chloride solution (1.5 mg/ml in acetone) and incubated in open tubes for 20 min at 45°C. Following addition of 0.3 ml of L-proline solution (2.5 mg/ml in water), the incubation was continued for 20 min. Residual acetone was removed by blowing a stream of nitrogen for 5—10 min at 30°C and the aqueous solution was twice extracted with 1 ml of hexane. The extract was brought to dryness under a stream of nitrogen in tapered tubes, the residue being concentrated in the tip by rinsing the tube with 0.3 ml of hexane. The extracts were sensitive to light and had to be stored frozen unless processed immediately.

For TLC the dansyl derivatives were spotted at 1.5-cm distances on 20 × 20 cm sheets coated with silica gel (see above). In maprotiline analyses, chromatograms were run to a height of 16.5 cm above the starting line in hexane—acetone—toluene (21:4:0.7), dried for 2 min and subsequently run in ethanol—water (15:10) to a height of 7.5 cm. For the determination of oxaprotiline, running distances were 12 and 6 cm in the first and second solvent, respectively. Chromatograms had to be run and dried in the dark. The spot containing the dansyl derivatives of drug and internal standard (for R_F values see Table I) was visualized under light of 365 nm and removed after lightly spraying with water. The gel was extracted twice with 0.5 ml of a freshly prepared solution of 0.1% diethylamine in methanol. The eluate was evaporated in tapered tubes and the residue concentrated in the tip as above. For HPLC it was dissolved in 50 µl of hexane—ethanol (4:1) and a 10-µl aliquot was injected.

The column (300 × 4.5 mm) contained 7-µm silica gel particles (Nucleosil 50-7, Macherey-Nagel). The eluent was hexane—absolute ethanol (95:5, v/v) at a flow-rate of 1.8 ml/min. Fluorimetric detection was done at excitation and emission wavelengths of 365 and 420 nm, respectively, with the fluorometer (FFM 32, Kontron, Munich, F.R.G.) set at medium sensitivity for maprotiline determinations and at high sensitivity for oxaprotiline analyses. For quantitation, peak height ratios of dansylated drug and internal standard were read on a calibration curve produced by processing blank plasma samples with additions of known quantities of maprotiline or oxaprotiline.

Between-series reproducibility was examined by running control samples with each series of patient plasma. These contained 200—400 ng/ml (721—1442 nmol/l) of maprotiline or 20—50 ng/ml (68—170 nmol/l) of oxaprotiline. Absolute recovery in maprotiline analysis could be derived from a comparison of peak heights of substances obtained from plasma with peak heights of standards containing known quantities of dansyl derivatives.

A series of twelve plasma samples can be processed by one person within two days.

RESULTS

Selectivity and sensitivity

Table I presents R_F values in TLC and retention times in HPLC of dansyl derivatives of secondary and primary amines that are administered as antidepressants or produced as antidepressant drug metabolites. It can be seen that in the modification used for maprotiline analysis, secondary amines devoid of a hydroxy group (nortriptyline, desmethylimipramine) would interfere with the determination of maprotiline, whereas all its metabolites are separated from it. Similarly, oxaprotiline differs from its demethylated metabolite in its behaviour on HPLC.

TABLE I

CHROMATOGRAPHIC BEHAVIOUR OF DANSYL DERIVATIVES OF DRUGS AND DRUG METABOLITES

Dansyl derivative of	Running distance in TLC (cm) under conditions for		Retention time in HPLC (min)
	Maprotiline	Oxaprotiline	
Maprotiline	4.1	3.3	3.8
Desmethylmaprotiline	3.0	2.5	5.2
3-Hydroxymaprotiline	1.8	1.4	7.6
3-Hydroxydesmethylmaprotiline	1.2	1.0	11.3
2-Hydroxymaprotiline	2.0	1.6	7.0
Oxaprotiline	3.5	2.9	6.8
Desmethyloxaprotiline	3.4	2.8	8.7
Desmethylimipramine	3.9	3.0	3.8
Didesmethylimipramine	3.2	2.6	5.4
Desmethylclomipramine	4.2	3.3	3.9
Nortriptyline	4.5	3.6	3.8
E-10-Hydroxynortriptyline	4.5	3.6	10.3

Examples of chromatographic traces are presented in Figs. 2 and 3. No definite peaks appeared at the retention times of dansyl oxaprotiline and dansyl didesmethylimipramine when blank plasma had been subjected to the analytical procedure. A small peak was, however, present at the retention time of dansyl maprotiline, which corresponded to about 2 ng of the drug (Fig. 2A). It could be disregarded in assays of clinically relevant maprotiline concentrations, but it was a reason not to use maprotiline as an internal standard for oxaprotiline analyses which had to be more sensitive by a factor of 5.

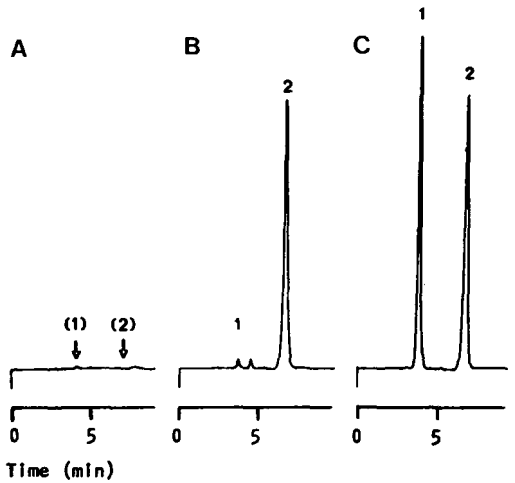


Fig. 2. HPLC fluorescence records obtained upon maprotiline analysis in plasma (1 ml). (A) Blank plasma. (B) Blank plasma with additions of 10 ng of maprotiline and 400 ng of oxaprotiline as internal standard. (C) Plasma from a patient ingesting maprotiline 150 mg per day; it was found to contain 290 ng/ml (1045 nmol/l) maprotiline. 1 = dansyl maprotiline, 2 = dansyl oxaprotiline.

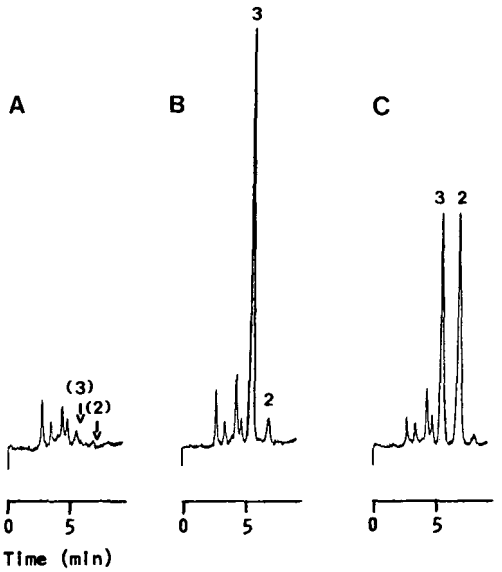


Fig. 3. HPLC fluorescence records obtained upon oxaprotiline analysis in plasma (1 ml). (A) Blank plasma. (B) Blank plasma with additions of 2 ng of oxaprotiline and 30 ng of didesmethylimipramine as internal standard; in this case a 40% aliquot was injected. (C) Plasma from a patient treated with oxaprotiline 150 mg per day; it was found to contain 32 ng/ml (109 nmol/l) oxaprotiline. 2 = dansyl oxaprotiline, 3 = dansyl didesmethylimipramine.

The detection limit of maprotiline was about 10 ng/ml (Fig. 2B) and reproducible determinations could be made at 20 ng/ml (72 nmol/l) and higher. Oxaprotiline was detected at minimal levels of 2 ng/ml (Fig. 3B) and could be quantitated at 5 ng/ml (17 nmol/l).

Accuracy and reproducibility

Calibration curves revealed a strictly linear dependence of the peak height ratio of dansylated drug and internal standard on the drug quantity added (r = 0.994, n = 43 for maprotiline 20—400 ng/ml, and r = 0.998, n = 30 for oxaprotiline 10—80 ng/ml).

Control samples analysed along with each series of patient plasma gave the results listed in Table II. Duplicate determinations of maprotiline in 48 samples from patients resulted in a mean difference of 6.1%.

The procedure for oxaprotiline determination was further controlled by a blind analysis of seven spiked plasma samples prepared by a laboratory of Ciba-Geigy at Basel. A very good accuracy becomes apparent from the results presented in Table III. At levels of 10 ng/ml (34 nmol/l) or above, the values found did not deviate by more than 4% from the theoretical ones. In addition, a series of seven pooled patient plasma samples was analysed by the present procedure and at Ciba-Geigy by gas chromatography—mass spectrometry. Maximal differences amounted to 4 ng/ml or 14% at concentrations of 4—32 ng/ml (14—109 nmol/l).

TABLE II

RESULTS OF ANALYSES OF CONTROL SAMPLES RUN IN DIFFERENT SERIES OF MAPROTILINE AND OXAPROTILINE ASSAYS

Compound	Concentration (ng/ml free base)		Percentage of theoretical value	n
	Added	Found		
Maprotiline	200	193 ± 15*	96 ± 7*	8
	300	312 ± 27	104 ± 9	9
	400	405 ± 27	101 ± 7	9
Oxaprotiline	20	18.5 ± 1.5	92 ± 7	6
	40	38.2 ± 5.5	95 ± 14	8
	50	49.6 ± 2.7	99 ± 5	11

*Mean ± S.D.

TABLE III

THEORETICAL AND EXPERIMENTAL VALUES IN A BLIND ANALYSIS OF PLASMA SAMPLES SPIKED WITH OXAPROTILINE

Sample No.	Oxaprotiline concentration (ng/ml free base)	
	Added	Found
1	1.1	0
2	10.7	10.5
3	21.7	22.5
4	32.3	33.5
5	32.3	33
6	42.4	44
7	53.1	52

Recovery

In the entire analytical procedure for maprotiline the recovery of dansyl maprotiline amounted to 58% and that of dansyl oxaprotiline to 60%.

Values in patient plasma

Maprotiline plasma levels in twenty patients ingesting 125 mg per day slowly increased during weeks 2—4 of treatment. After four weeks they varied from 34 to 225 ng/ml (128 ± 56 ng/ml or 461 ± 202 nmol/l, mean ± S.D.) [14]. Higher concentrations are observed in single cases at a daily dose of 150 mg (Fig. 2C).

Twenty-five depressed patients ingesting oxaprotiline 150 mg per day mostly had constant plasma drug levels from two to four weeks after initiation of treatment. Interindividual variation was considerable, with steady-state values ranging from 9.5 to 74 ng/ml (34 ± 15 ng/ml or 116 ± 51 nmol/l).

DISCUSSION

The analytical procedure described here could be shown to satisfy the usual quality criteria. Its sensitivity is sufficient for measuring plasma maprotiline and oxaprotiline concentrations well below the range of values occurring upon treatment with clinically effective drug doses. While it does not allow separate determination of several antidepressants with secondary amino groups, no disturbance is caused by the corresponding primary amines and other known drug metabolites. A similar procedure not involving a purification by TLC has recently been published for the assay of metapramine [15].

As has been documented earlier [14], the range of values measured in plasma of maprotiline-treated patients agreed well with that reported by other authors. Also oxaprotiline levels are similar to those obtained by a HPLC procedure without derivatization [11].

Though the present method is somewhat laborious, this draw-back seems to be compensated for by avoiding the use of radioactive reagents or expensive instrumentation. In comparison with an alternative HPLC method [11] it has the advantage that the first extraction step can be carried out with toluene; this results in a higher recovery and thus improves the reliability of the assay.

ACKNOWLEDGEMENTS

The authors wish to thank Mr. R. Ackermann, Ciba-Geigy, Basel, for the preparation of spiked plasma samples. They are grateful to Dr. H.J. Gaertner and Dr. H. Giedke, Department of Psychiatry, University of Tübingen, for supplying patient plasma. The work was financially supported by the Deutsche Forschungsgemeinschaft.

REFERENCES

1 R.M. Pinder, R.N. Brogden, T.M. Speight and G.S. Avery, Drugs, 13 (1977) 321.
2 J.P. Feighner, M. Roffman and R.B. Dixon, Curr. Ther. Res., 29 (1981) 363.
3 M. Roffman, E.E. Gould, S.J. Brewer, H. Lau, B. Sachais, R.B. Dixon, L. Kaczmarek and A. Lesher, Curr. Ther. Res., 32 (1982) 247.

4 U. Breyer-Pfaff, H.J. Gaertner, F. Kreuter, G. Scharek, M. Brinkschulte and R. Wiatr, Psychopharmacologia, 76 (1982) 240.
5 W. Riess, Anal. Chim. Acta, 68 (1974) 363.
6 U.P. Geiger, T.G. Rajagopalan and W. Riess, J. Chromatogr., 114 (1975) 167.
7 R.N. Gupta, G. Molnar and M.L. Gupta, Clin. Chem., 23 (1977) 1849.
8 S. Kärkkäinen and F. Seppälä, J. Chromatogr., 221 (1980) 319.
9 C. Charette, T.J. McGilveray and K.K. Midha, J. Chromatogr., 224 (1981) 128.
10 J.P. Dubois, personal communication.
11 H.J. Kuss and E. Feistenauer, J. Chromatogr., 204 (1981) 349.
12 S.H.Y. Wong and S.W. Waugh, Clin. Chem., 29 (1983) 314.
13 U. Breyer-Pfaff, M. Kroeker, T. Winkler and P. Kriemler, Xenobiotica, submitted for publication.
14 H.J. Gaertner, G. Golfinopoulos and U. Breyer-Pfaff, Pharmacopsychiatria, 15 (1982) 170.
15 J.P. Sommadossi, M. Lemar, J. Necciari, Y. Sumirtapura, J.P. Cano and J. Gaillot, J. Chromatogr., 228 (1982) 205.

Journal of Chromatography, 309 (1984) 115—123
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2122

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

J. OSTROWSKI*, J. THEUMER, W. GÄRTNER and K. RESAG

Pharmaforschung, Cassella AG, Hanauer Landstrasse 526, 6000 Frankfurt/Main-61 (F.R.G.)

and

H. PASSING

Praktische Mathematik, Hoechst AG, Frankfurt/Main (F.R.G.)

(First received December 2nd, 1983; revised manuscript received February 17th, 1984)

SUMMARY

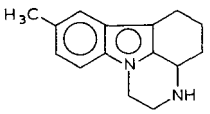
A high-performance liquid chromatographic method is described for the analysis of pirlindole [2,3,3a,4,5,6-hexahydro-8-methyl-1H-pyrazino(3,2,1-*jk*)carbazole hydrochloride], a new antidepressive drug. The drug was extracted from plasma into dichloromethane, and the analysis was carried out on a reversed-phase column, the effluent being monitored by fluorescence detection. The method is selective and sensitive (limit of detection 1—2 ng/ml plasma). Urine analysis was done by direct injection of the diluted sample. The method was applied to the analysis of plasma and urine samples of eight healthy male volunteers who received a 75-mg oral dose of a tablet formulation of pirlindole. The method was also applied to a study in three beagle dogs which received pirlindole (1 mg/kg) by infusion (0.1 mg/kg/min) and orally (10 mg/kg) to estimate the absolute bioavailability of the drug.

INTRODUCTION

Pirlindole* [2,3,3a,4,5,6-hexahydro-8-methyl-1H-pyrazino(3,2,1-*jk*)carbazole hydrochloride] is a new antidepressive drug. Its clinical efficacy is comparable to amitriptyline. Imipramine, desipramine and maprotiline, but the mechanism of action is different. Pirlindole has in vitro and in vivo monoamine oxidase A inhibitory activity which is reversible and short-lasting. The drug also inhibits

*Euthym®, manufactured by Cassella Riedel Pharma GmbH, Frankfurt/Main, F.R.G.;

0378-4347/84/$03.00



Pirlindole

the neuronal re-uptake of norepinephrine and serotinin into presynaptic vesicles. It does not possess anticholinergic activity [1, 2].

Up to now no selective analytical method has been available to measure the pharmacokinetics of the unchanged drug in body fluids. This paper describes a selective and sensitive high-performance liquid chromatographic (HPLC) method for the determination of pirlindole in plasma and urine and its application to man and dog.

EXPERIMENTAL

Apparatus and materials

The chromatograph consisted of a pump (M6000, Waters, Eschborn, F.R.G.), an automatic injector (WISP 710, Waters), and a fluorescence detector (650-10LC variable-wavelength monitor, Perkin Elmer, Offenbach, F.R.G.) operated at excitation 295 nm and emission 340 nm. The separation column was a 300 × 3.9 mm I.D. stainless-steel tube packed with μBondapak C_{18} reversed phase (particle size 10 μm, Waters).

The mobile phase consisted of the components acetonitrile (HPLC grade S, Rathburn Chemicals, Walkerburn, U.K.), methanol (Riedel, Seelze, F.R.G., No. 32 213), and 0.05 *M* phosphoric acid (Riedel, No. 30 417), buffered at pH 3.5 with aqueous tetraethylammonium hydroxide (Riedel, No. 16 262). All chemicals and solvents were of analytical reagent grade at least and used without further pretreatment. Stock solutions of pirlindole were freshly prepared in distilled water every day.

Analysis of plasma and urine samples

To 1.0 ml of plasma were added 0.1 ml of an aqueous solution of 0.1 *M* Na_2—EDTA (Riedel, No. 34 549, Idranal III) and 5 ml of dichloromethane (Riedel, No. 32 222) saturated with 0.1 *M* aqueous Na_2-EDTA. The mixture was agitated in stoppered 10-ml glass tubes for 20 sec. After centrifuging (6000 *g*, 5 min, 5° C) to separate the layers, 4 ml of the organic phase were transferred to a tapered tube and mixed with 0.1 ml of a freshly prepared methanolic solution of 0.01 *M* Na_2—EDTA. This solution was evaporated to dryness in a stream of nitrogen at 55° C in about 25 min. The dry residue was taken up in 0.2 ml of the mobile phase, acetonitrile—methanol—phosphoric acid (40:1:60, v/v), agitated using a Vortex mixer for 10 sec and centrifuged for 1 min before injection into the chromatograph. The above procedure was duplicated for each plasma sample.

Pirlindole peak height was measured and plasma concentration calculated by reference to a calibration curve. Urine samples (50 μl diluted in 500 μl of mobile phase, acetonitrile—methanol—phosphoric acid—PIC B-7, 35:1:70:1.3, v/v) were analysed by direct injection into the chromatograph. Plasma and urine samples are stable in the frozen state and dark at —20° C for at least one year.

The HPLC method was applied to the analysis of pirlindole in plasma and urine samples of eight healthy male volunteers (27—57 years, body weight 63—88 kg), who had given informed consent. All subjects received the drug as a 75-mg tablet. The method was also applied to a study in three fasted beagle dogs, two male and one female, body weight 14—18 kg, which received 1.0—1.1 mg/kg pirlindole by infusion (0.1 mg/kg/min) and 10 mg/kg pirlindole per os (aqueous solution).

Calculation of pharmacokinetic parameters

Calculation of the area under the plasma concentration—time curve, AUC_∞, the terminal half-life of elimination, $t_{½}$, total plasma clearance, Cl_{tot}, and the absolute bioavailability, f, were based on the RIP-fitting procedure [3] using a desk computer (WANG 2200). When analysing human data, areas were calculated by means of the trapezoidal rule. Mean transit times of pirlindole after intravenous (T_{vss}) and oral (T_{sys}) administration were estimated to obtain information about the steady-state volume of distribution V_{ss} ($V_{ss} = Cl_{tot} \times T_{vss}$) and the rate constant k_a of the drug's entrance ("invasion") into the central compartment from the gastrointestinal tract. k_a does not relate to absorptive processes alone, but rather reflects the joint effects of several subprocesses including most often distribution into readily accessible side compartments [4]. The concept of moment analysis from plasma data is valid if the drug was cleared exclusively from the central compartment, and if invasion and elimination were strictly first-order processes.

Statistics

Mean values are given as arithmetical means together with their standard deviations (S.D.). Statistical analysis of calibration curves was done by linear-regression routine on a desk computer (WANG 2200). A new non-parametric regression procedure [5] was used for comparison of two analytical methods for determination of pirlindole in plasma.

RESULTS AND DISCUSSION

Chromatography and recovery from plasma

Pirlindole was added to human plasma and extracted at physiological pH. Good separation of the drug from the endogenous matrix was obtained on an octadecylsilyl reversed-phase column, eluting with an acetonitrile—methanol—phosphoric acid mobile phase as described above (Fig. 1).

This chromatographic procedure is also suitable for dog and rat plasma. The recovery of pirlindole from plasma was determined by referring the peak height to a calibration curve obtained by analysing aqueous solutions of the drug, and amounted to 78 ± 3%.

Calibration

Pirlindole was added to plasma to give six replicate samples at five concentrations between 5 and 100 ng/ml (C_{added}). This is the concentration range typically found in the plasma of human subjects following an oral dose of 75 mg. The samples were processed as described and the peak height of the

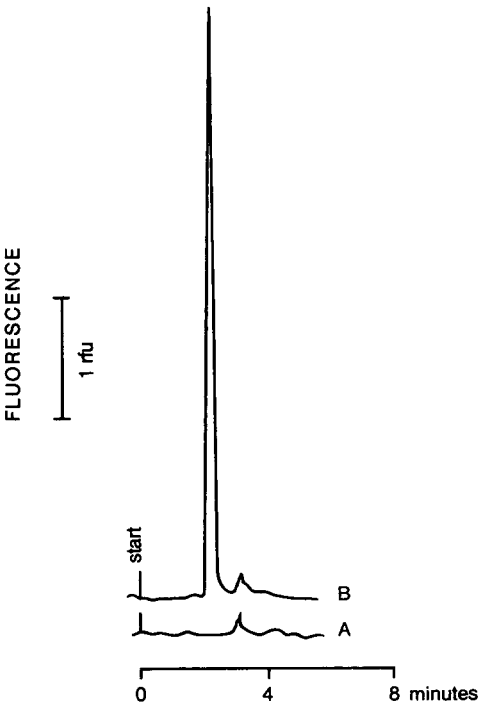


Fig. 1. (A) Chromatogram of an extract of human plasma (blank). (B) Chromatogram of an extract of human plasma to which had been added pirlindole to give a plasma concentration of 50 ng/ml (rfu = relative fluorescence units).

fluorescence signal (h) depends linearly on the concentration of pirlindole. The linearity of the calibration curve holds up to 500 ng/ml as the analysis of supplementary samples has shown.

Precision

The precision of the method [8] was expressed as standard deviation (S.D.) or relative standard deviation (C.V.) of the measured concentration values C_{found}. The within-run precision changed with C_{found} in an approximately

TABLE I

PRECISION (C.V.) OF THE HPLC METHOD FOR DETERMINATION OF THE CONCENTRATION OF PIRLINDOLE IN PLASMA (C_{found})

$C_{found} \approx (0.99 \pm 0.02) \times C_{added}$.

C_{added} (ng/ml)	C_{found} ± S.D. (ng/ml)	Coefficient of variation (C.V., %)
5	5.1 ± 1.1	21.6
10	9.0 ± 0.9	10.0
20	19.4 ± 1.3	6.7
50	45.2 ± 2.3	5.1
100	99.7 ± 5.1	5.1

linear relationship S.D. $\approx 0.4 + 0.047 \times C_{found}$ in the concentration range of therapeutic relevance (Table I). The between-run precision was 6.9% for a spiked plasma sample.

Accuracy

Accuracy [6] — referred to as bias of ($C_{added} - C_{found}$) of each of the concentrations — was tested from the same samples that were used for testing precision (Table I). Regression of C_{added} versus C_{found} shows that C_{found} is very similar to C_{added} [$C_{found} \approx (0.99 \pm 0.02) \times C_{added}$].

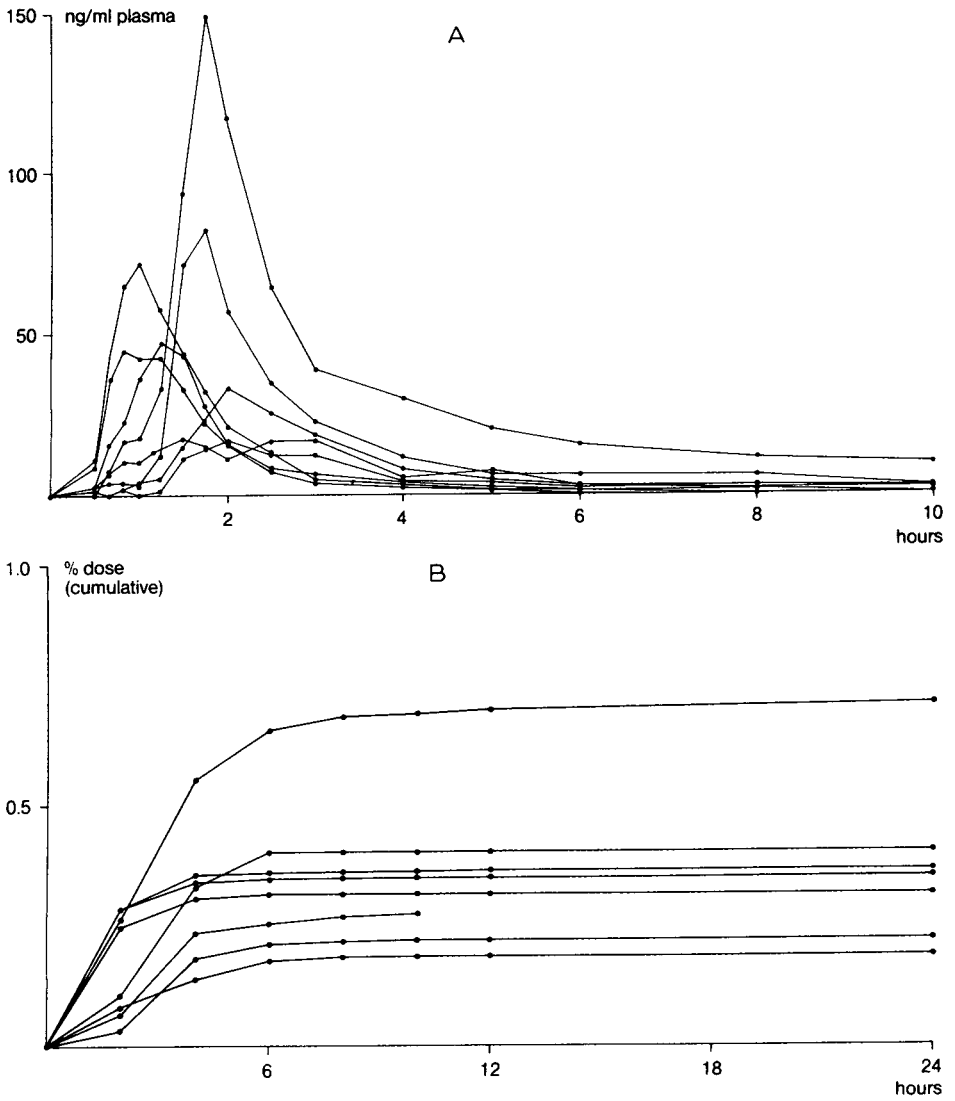


Fig. 2. Plasma levels (A) and urine excretion (B) of pirlindole in eight fasted healthy male volunteers after a single oral dose of 75 mg of pirlindole (tablet).

Sensitivity

As the standard deviations of six blank samples and of six replicates in the low concentration range are nearly equal (S.D. $\approx$ 0.4 ng/ml), the limit of detection (L.D.) was taken as 3.0 $\times$ S.D. This means the L.D. is in the range of 1—2 ng/ml pirlindole when extracting 1 ml plasma for analysis.

Selectivity

The selectivity of the method was imparted by the chromatographic system and the fluorescence detection wavelength. Additional evidence for the selectivity is that the fluorescence of the main metabolites in plasma of man, dog and rat do not interfere in the chromatographic analysis of pirlindole. The HPLC procedure has been validated by reference to a second analytical technique measuring fluorescence [7]. Human plasma samples were prepared by admixing known amounts of pirlindole. Equivalent results were obtained from both analytical methods measuring the plasma concentration of pirlindole (slope B = 0.987), as could be shown by non-parametric linear-regression analysis [5].

Application of the HPLC method

In routine practice, plasma and urine samples are analysed as independent duplicates. Calibration of the instrument is adjusted daily. By processing five freshly prepared external plasma or urine standards containing a known amount of pirlindole (C_{stand}) in the upper expected concentration range, the calibration line $h_{stand} = f \times C_{stand}$ is constructed by plotting the peak heights of the blank-corrected fluorescence signals of the five samples against C_{stand} and joining the mean peak height at C_{stand} with the zero coordinates. For purposes of quality control, another series of deep-frozen plasma or urine samples with a lower but known content of pirlindole ($C_{control}$) between zero

TABLE II

PHARMACOKINETIC PARAMETERS OF PIRLINDOLE CALCULATED FROM CONCENTRATIONS IN PLASMA AND URINE OF FASTED HEALTHY MALE VOLUNTEERS AFTER A SINGLE DOSE OF A 75-mg TABLET

Volunteer No.	C_{pmax} (ng ml^{-1})	t_{max} (h)	$t_{½,\ terminal}$ (h)	AUC_∞ (ng ml^{-1} h)	AUC_{ex} (%)	Cl_{tot}/f (l h^{-1})	Cl_r (l h^{-1})
1	17.1	2.00	0.98	37.2	4.3	2016.1	4.5
2	47.4	1.25	1.07	69.1	2.9	1085.4	3.4
3	82.2	1.75	3.10	148.8	5.7	503.9	1.4
4	72.3	1.00	1.90	98.2	5.9	763.1	2.7
5	42.8	1.25	2.00	67.1	0	1117.7	4.1
6	148.8	1.75	6.51	383.4	4.9	195.5	1.4
7	33.7	2.00	5.19	87.5	14.5	856.6	3.4
8	17.5	1.50	2.06	67.2	5.3	1115.9	2.1
Median	45.1		2.03	78.3		971.0	3.1
Minimum	17.1	1.00	0.98	37.2		195.5	1.4
Maximum	148.8	2.00	6.51	383.4		2016.1	4.5
$\overline{X}$	57.7		2.85	119.8		956.8	2.9
S.D.	43.6		1.99	111.3		537.5	1.2

and C_{stand} are measured. Whenever there is a tendency of exceeding 2 S.D. of $C_{control}$, routine analysis is stopped in favour of a trouble-shooting programme.

The HPLC method has been applied to the analysis of pirlindole in plasma and urine following a 75-mg oral dose as a tablet to eight healthy male volunteers. The plasma concentration—time course (Fig. 2) could be measured in all cases, and pharmacokinetic parameters were calculated (Table II). Only 0.4 ± 0.2% of the administered drug was excreted unchanged in the urine (Fig. 2 and Table II). The mean value of renal clearance was low (Cl_r = 2.9 ± 1.21 l/h). The drug was also administered intravenously (1 mg/kg) and orally (10 mg/kg)

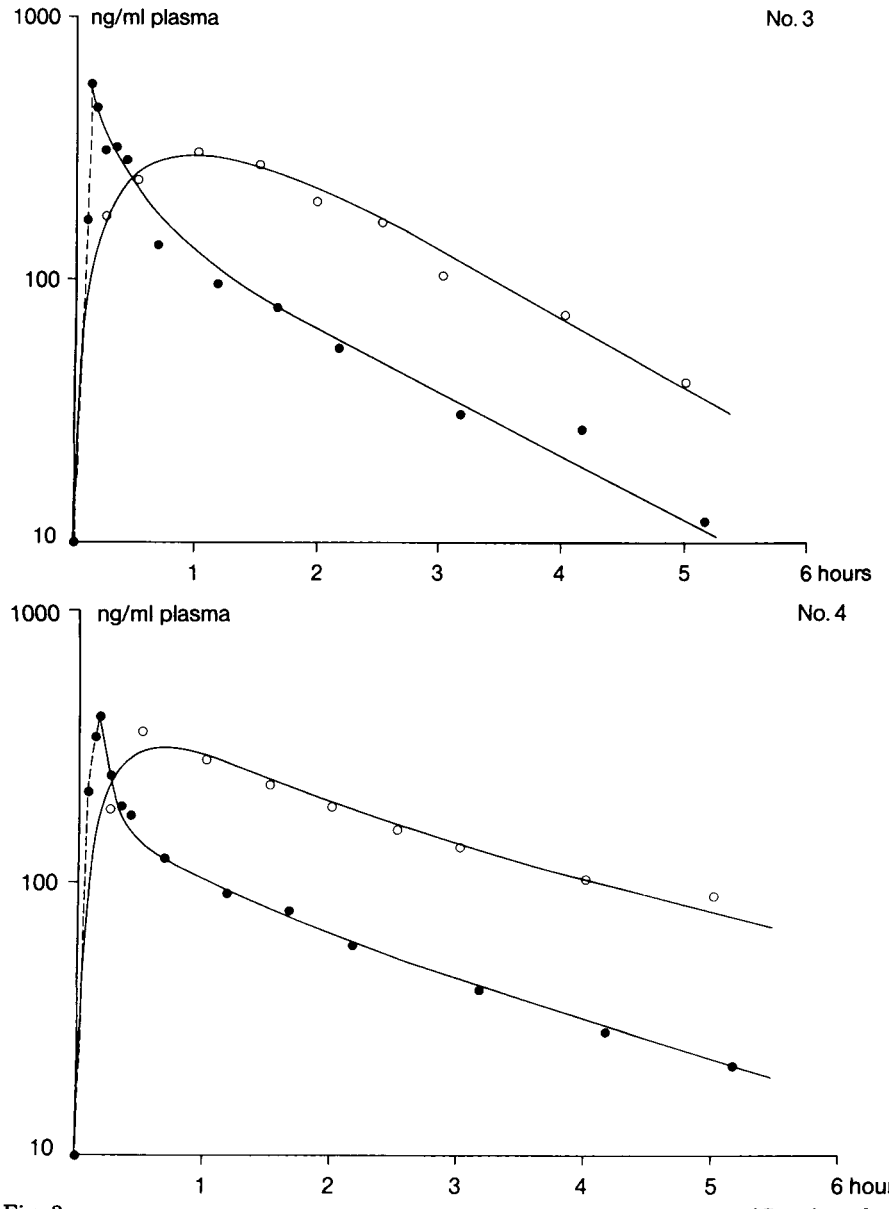


Fig. 3.

(Continued on p. 122)

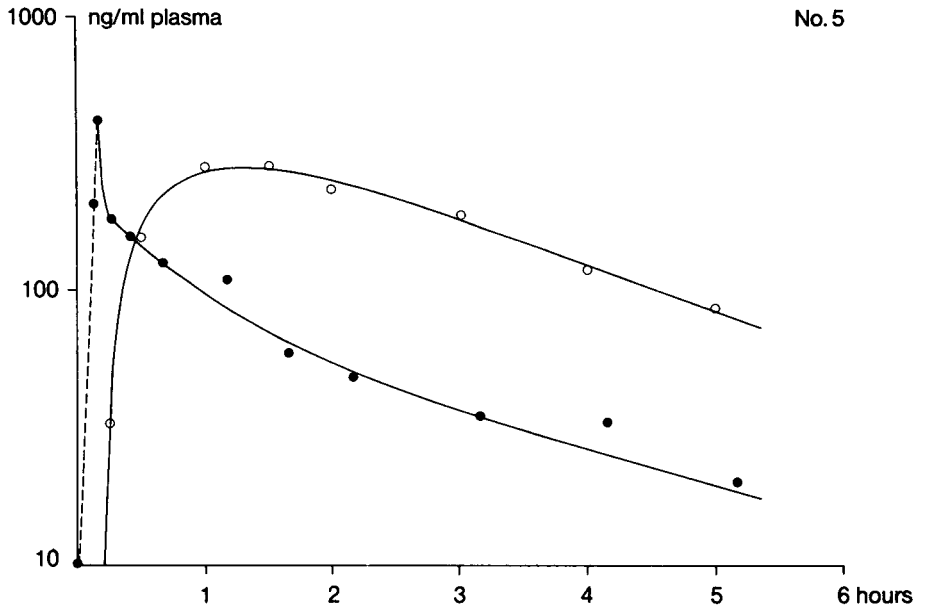


Fig. 3. Pirlindole plasma levels in three beagle dogs which received a single dose of 1 mg/kg by infusion (0.1 mg/kg/min) (•) and 10 mg/kg (aqueous solution) orally (○). (—), RIP-fitting [3].

TABLE III

PHARMACOKINETIC PARAMETERS OF PIRLINDOLE CALCULATED FROM PLASMA CONCENTRATIONS IN FASTED BEAGLE DOGS WHICH RECEIVED THE DRUG (1 mg/kg) BY INFUSION (0.1 mg/kg/min) AND ORALLY (10 mg/kg AS AQUEOUS SOLUTION)

T_0 = lag-time, $T_{abs} = T_{sys} - T_{vss}$.

Dog No.	Dose (mg/kg)	C_{pmax} (ng/ml) (infusion end)	AUC_∞ (ng/ml h)	AUC_{ex} (%)	Cl_{tot} (l/h)	T_{vss} (h)	V_{ss} (l)	$t_{½}$ terminal (h)
Infusion								
3 M	1.14	400.2	434.2	15.0	36.82	2.71	99.7	2.12
4 M	1.11	446.6	433.4	4.9	46.08	1.61	74.2	1.26
5 F	1.03	420.0	409.2	15.0	39.06	2.76	107.8	2.27
$\bar{X}$		422.3	425.6		40.63	2.36	93.9	1.88
S.D.		19.0	14.2		4.83	0.65	17.5	0.55

	C_{pmax} (ng/ml)	t_{max} (h)	AUC_∞ (ng/ml h)	AUC_{ex} (%)	T_{sys} (h)	T_{abs} (h)	k_a (l/h)	T_0 (h)	$t_{½}$ terminal (h)	f (%)
Oral administration										
3 M	355.7	0.5	1154.3	25.7	3.74	1.03	0.9709	0.01	2.69	30.3
4 M	308.2	1.0	876.0	7.2	2.22	0.61	1.6393	0.00	1.12	22.4
5 F	285.1	1.0	1100.6	19.2	3.19	0.53	1.8868	0.21	1.75	27.7
$\bar{X}$	316.3		1043.6		3.05	0.72	1.4990	0.07	1.85	26.8
S.D.	36.0		147.6		0.77	0.26	0.4737	0.12	0.79	4.0
Range		0.5—1.0								

to three beagle dogs to estimate the absolute bioavailability of pirlindole (26.8 ± 4.0%). The plasma levels after single oral dosing were characterized by the absorption constant k_a = 1.5 ± 0.5 h^{-1}, C_{pmax} = 316.3 ± 36.0 ng/ml, t_{max} = 0.5—1.0 h, AUC_∞ = 1043 ± 148 ng/ml h^{-1}, and the terminal half-life of elimination, $t_{½}$ = 1.85 ± 0.79 h. Total plasma clearance (Cl_{tot} = 40.7 ± 4.8 l/h) and steady-state volume of distribution (V_{ss} = 93.9 ± 17.5 l) were estimated from the corresponding intravenous data (Fig. 3 and Table III).

REFERENCES

1 E. Schraven and R. Reibert, Arzneim.-Forsch., in press.
2 M.D. Mashkovsky and N.I. Andrejeva, Arzneim.-Forsch., 31 (1981) 75.
3 H.M. von Hattingberg, D. Brockmeier and G. Kreuter, Eur. J. Clin. Pharmacol., 11 (1977) 381.
4 H.M. von Hattingberg and D. Brockmeier, in G. Bozler and J.M. Van Rossum (Editors), Pharmacokinetics during Drug Development; Data Analysis and Evaluation Techniques, Fischer, Stuttgart, 1982, pp. 315—323.
5 H. Passing and W. Bablok, J. Clin. Chem. Clin. Biochem., 21 (1983) 709.
6 N.-E. Saris, J. Clin. Chem. Clin. Biochem., 14 (1976) 265.
7 P. Hajdu, Hoechst AG, internal report.

Journal of Chromatography, 309 (1984) 125—131
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2127

DETERMINATION OF BEPRIDIL IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

KUNG-TAT NG*, JANET A. PLUTTE and LEONARD J. GALANTE

McNeil Pharmaceutical, Spring House, PA 19477 (U.S.A.)

(Received January 27th, 1984)

SUMMARY

A rapid, selective and sensitive assay has been developed for the determination of the anti-anginal drug, bepridil, in biological samples. The lowest concentration of bepridil which can be measured accurately and precisely in a 2-ml plasma or urine sample is 10 ng/ml. The standard curve is linear in the concentration range 10—2000 ng/ml. Accuracy and precision of the assay, expressed as relative deviation and coefficient of variation (inter-run) are < 6.5% at all concentrations in the linear range. No interfering peaks are observed. Using an automatic injector and a laboratory computer system, 48 samples are analyzed routinely in an 8-h day.

INTRODUCTION

Bepridil hydrochloride, β-[(2-methylpropoxy)methyl]-N-phenyl-N-(phenylmethyl)-1-pyrrolidineethanamine monohydrochloride monohydrate, is a new cardiovascular drug currently undergoing clinical evaluation for the treatment of angina pectoris. Its anti-anginal and anti-arrhythmic properties have been demonstrated in animal studies [1—7] and in clinical studies [8, 9].

Recently a gas chromatographic (GC) assay for bepridil was reported [10] which uses nitrogen-specific detection. This GC assay has a detection limit of 5 ng/ml using 2—3 ml of plasma. However, some clinical plasma samples may require reanalysis with adjusted amounts of added internal standard because they fall outside the anticipated concentration range. A gas chromatographic—mass spectrometric (GC—MS) assay for the determination of both stable-isotope-labelled and unlabelled bepridil in human plasma was also reported [11]. Accuracy and precision for the GC—MS assay were only 30% at 20 ng/ml or below.

In the present paper, a new high-performance liquid chromatographic

0378-4347/84/$03.00

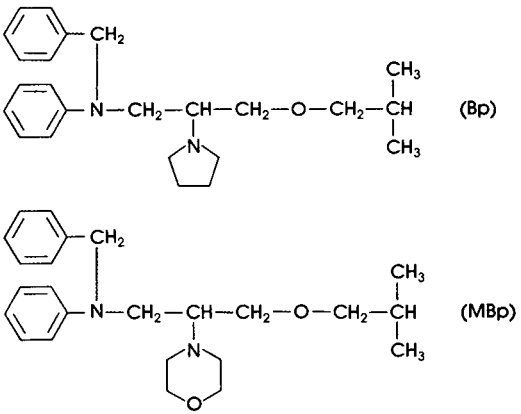


Fig. 1. Chemical structures of bepridil (Bp) and the morpholine analogue of bepridil (MBp).

(HPLC) assay for the determination of bepridil in biological fluids is described. The detection limit is 10 ng/ml. The standard curve is linear between 10 and 2000 ng/ml. The method is highly reproducible (inter-run coefficient of variation < 6.5%) and accurate (relative deviation < 3%). The assay has been automated to analyze 48 or more samples during an 8-h working day.

EXPERIMENTAL

Reagents

Hexane and methanol were of HPLC grade (Fisher Scientific, Fair Lawn, NJ, U.S.A.). Ammonium hydroxide (58%) and orthophosphoric acid (85%) were of ACS grade (Mallinckrodt, Paris, KY, U.S.A.). Monobasic potassium phosphate and dibasic sodium phosphate used in the preparation of the pH 7.4 buffer were of certified ACS grade (Fisher Scientific, Fair Lawn, NJ, U.S.A.). The 10% triethylamine (TEA) solution used for the mobile phase was prepared by diluting 100 ml of 99% TEA (Eastman Kodak, Rochester, NY, U.S.A.) to 1 l with distilled water followed by titration to pH 3.3 with orthophosphoric acid (85%).

A morpholine analogue of bepridil (Fig. 1, MBp) was used as the internal standard. Bepridil (Fig. 1, Bp) was obtained as the hydrochloride salt (McNeil Pharmaceutical, Spring House, PA, U.S.A.).

Liquid chromatography

The HPLC system consisted of a Beckman Model 112 solvent delivery system and a Beckman Model 160 UV absorbance detector equipped with a 254-nm wavelength filter. The column was 10 cm × 4.6 mm I.D. packed with 5-μm RP-18, Spheri-5 sorbent (Brownlee Labs., Santa Clara, CA, U.S.A.). A 3 cm × 4.6 mm I.D. 10-μm RP-18 guard column from Brownlee Labs. was also used. The mobile phase was methanol—water—10% TEA solution (68:22:10, v/v). The mobile phase was prepared fresh daily and filtered through a 0.45-μm Millipore® filter (Millipore, Bedford, MA, U.S.A.). The column was conditioned with approximately 30 ml of the mobile phase prior to use. After conditioning the column, the flow-rate was maintained at 2.0 ml/min. The

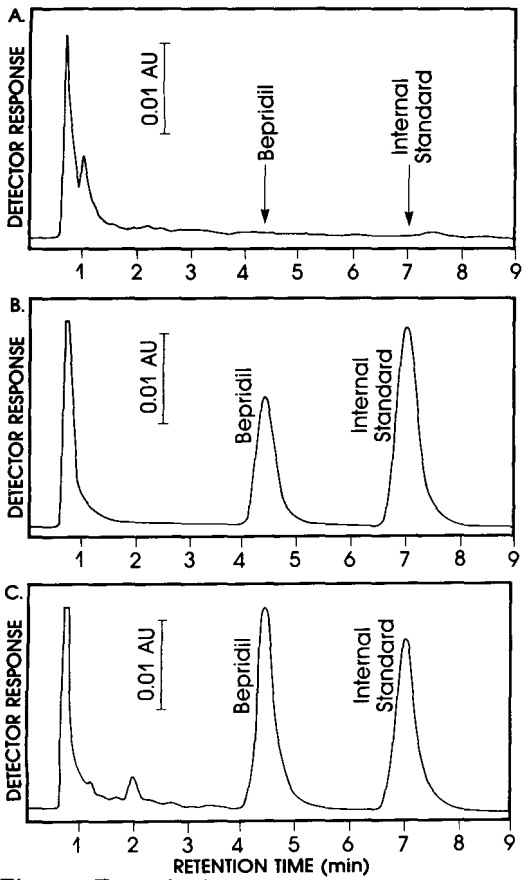


Fig. 2. Typical chromatograms from extracted human plasma samples. (A) Blank. (B) Sample seeded with 400 ng/ml of bepridil and 800 ng/ml of the internal standard. (C) A clinical sample obtained 2 h following a 400-mg oral dose; the bepridil concentration was calculated to be 719 ng/ml.

retention times were 4.4 min for bepridil and 7.0 min for the internal standard (Fig. 2). Samples were injected using a Waters Intelligent Sample Processor (WISP-710B, Waters Assoc., Milford, MA, U.S.A.).

Equipment

Disposable screw-top bottles (volume, 14.5 ml) with polyethylene-lined caps and 12-ml centrifuge tubes (conical bottom) were used for extraction. Prior to use, all glassware was soaked in detergent for 2 h, rinsed thoroughly with distilled water and heat-treated for 3 h at 270°C. Polyethylene-lined screw caps were soaked in *n*-heptane for 1 h and dried at 60°C before use.

Plasma standard solutions

Plasma standards (volume, 10.0 ml) containing 10—1000 ng of bepridil per ml of plasma were prepared as follows: 0.5 ml of a methanolic solution of bepridil hydrochloride (conversion factor to bepridil free base = 1.149), containing the appropriate amount (200—20,000 ng equivalent) of the bepridil free base, was added to 9.5 ml of drug-free plasma.

Extraction procedure

An aliquot of plasma or urine (1.0—2.0 ml), containing bepridil as a standard or an unknown was placed in a 14.5-ml disposable screw-top bottle. To this were added 2.0 ml of pH 7.4 phosphate buffer, 0.4 ml of a methanolic internal standard solution containing 1600 ng of internal standard (MBp) and 9.5 ml of hexane. The capped bottle was shaken for 10 min on a table-top shaker (Eberbach) at 240 oscillations per minute and centrifuged at 1000 *g* for 10 min. An 8-ml aliquot of the supernatant hexane layer was pipetted into another 14.5-ml screw-top bottle containing 4.0 ml of methanol and 0.2 ml of 0.58% ammonium hydroxide solution. The bottle was capped, shaken and centrifuged as before. A 7.0-ml aliquot of the supernatant hexane layer was transferred to a 12-ml centrifuge tube and evaporated to dryness under a stream of dry nitrogen at room temperature. The residue was reconstituted in 200 μl of the HPLC mobile phase. This was mixed on a Vortex Genie® (Scientific Instruments, Springfield, MA, U.S.A.) at a speed setting of 6 for 10 sec. All of the resulting solution was transferred to a limited-volume insert tube (Waters Assoc.) which was placed onto the sample carousel of the Waters Intelligent Sample Processor (WISP-710B). A 100-μl aliquot of this solution was subsequently injected into the HPLC system.

Quantitation and data handling

Standard curve data were generated by analyzing a series of plasma standards (10—2000 ng/ml). Data were analyzed by linear-regression analysis (peak height ratios versus plasma concentration) using the reciprocal of the variance of the peak height ratios as the weighting factor. Concentrations of bepridil in unknown plasma samples were determined using the calculated peak height ratios and the linear-regression equation.

A Hewlett-Packard 3354 Lab Automation System was used for automatic data acquisition, temporary data storage, data analysis and report generation. Calibration functions, calculated bepridil concentrations and final reports were generated using internally developed application software.

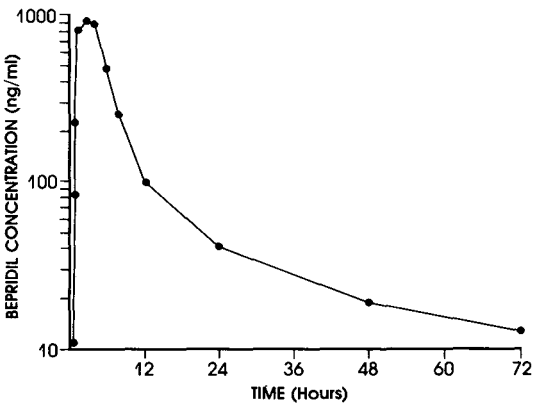


Fig. 3. Bepridil plasma concentration versus time following oral administration of a single 400-mg dose of bepridil hydrochloride to a fasted healthy human volunteer.

RESULTS AND DISCUSSION

Sensitivity

Bepridil and the internal standard (MBp) absorb ultraviolet strongly at 254 nm in the acidic mobile phase (for bepridil $\epsilon_{254\,nm}$ = 12,500). When 1 ng of bepridil was injected into the liquid chromatograph under the above conditions, a peak with a signal-to-noise ratio of 33 was obtained. The lowest concentration of bepridil that has been determined quantitatively in 2-ml plasma/urine samples is 10 ng/ml (relative standard deviation $\leqslant$ 6.5% for precision). This detection limit is adequate for therapeutic drug monitoring since the average plasma concentration of bepridil at 72 h following oral administration of a single therapeutic dose (400 mg) is approximately 15 ng/ml (Fig. 3).

Stability

Freshly prepared plasma/urine standard solutions were compared to plasma/urine standard solutions frozen at −5° C for one month. The variations in peak height ratios at each drug concentration between 10 and 2000 ng/ml were insignificant. Furthermore, bepridil and the internal standard were found to be stable in dried plasma/urine extract or in mobile phase at room temperature overnight. Therefore, injection of samples can be performed on the day following extraction without observable changes in peak height ratios.

Recovery

Recovery of bepridil from plasma/urine was calculated by comparing the slope of the detector response curve to that of the standard curve (peak height for internal standard normalized to 1.0). This ratio was found to be 51.6 ± 0.2%. After correction for volume losses, the intrinsic extraction efficiency for bepridil from plasma/urine was determined to be 66.3 ± 0.3%. The extraction efficiency for the internal standard (at 800 ng/ml) was 54.2 ± 2.6% (mean ± S.D. from twelve determinations).

Selectivity

The selectivityof the assay is shown in Fig. 2. No interfering peaks due to endogenous materials or metabolites of bepridil were observed in plasma/urine samples from various studies involving mouse, rat, rabbit, dog, monkey and man. The extraction procedure is highly selective for bepridil. An experiment was conducted in which plasma samples from eight rats were pooled. These samples were obtained 3 h following oral administration of a 100 mg/kg dose of [^{14}C]bepridil. Samples from this pool were assayed for total radioactivity and extracted using the present procedure.

Total radioactivity and unchanged bepridil content in the resultant plasma extracts were determined. The results, when expressed as percentages of the total radioactivity in the samples before extraction, were 5.1 ± 0.2% and 3.2 ± 0.2%, respectively (six determinations each). Because the extraction efficiency for bepridil from plasma is 66%, it can be calculated that the overall extraction efficiency for bepridil metabolites is only 2.0 ± 0.2%. The 33-fold difference in selectivity is attributed to the hexane—methanol partitioning step.

TABLE I

SUMMARY OF STANDARD CURVE DATA GENERATED ON THREE CONSECUTIVE DAYS OF ANALYSIS OF BEPRIDIL IN PLASMA (n = 6)

Actual concentration (ng/ml)	Calculated concentration* (ng/ml)	Standard deviation (ng/ml)	Precision (%)	Accuracy (%)
10	10.0	0.2	2.4	0.0
25	25.0	1.3	5.2	0.0
50	49.1	3.1	6.3	−1.8
100	101.2	5.2	5.1	1.2
200	204.4	11.2	5.5	2.2
400	396.2	7.9	2.0	−1.0
600	611.0	27.5	4.5	1.8
800	793.3	30.9	3.9	−0.8
1000	1025.9	43.1	4.2	2.6

*Calculated from the equation: $[\text{Bepridil}] = \frac{(\text{peak height ratio} - 0.004)}{2.35 \cdot 10^{-3}}$ where $2.35 \cdot 10^{-3}$ and 0.004 are the slope and intercept of the regression equation, respectively. The regression equation was obtained by method of least squares with data weighted by 1/variance.

Standard curve

Standard curve data generated by analyzing plasma standard solutions are presented in Table I. Linear-regression analysis (peak height ratios versus bepridil plasma concentrations) using reciprocals of variance as weighting factors gave a slope of $2.35 \cdot 10^{-3} \pm 0.01 \cdot 10^{-3}$ (mean ± S.D.), a y-intercept of 0.004 ± 0.003 and a correlation coefficient of 0.999 with a Student's t of 171.

Similar standard curve data have also been generated for spiked urine samples. Excellent accuracy and precision were obtained. For smaller sample volumes, assay sensitivity was reduced proportionally. Using 1-ml plasma samples, the standard curve was found to be linear between 20 and 4000 ng/ml.

TABLE II

DATA SUMMARY OF SEEDED CONTROL SAMPLES TO ILLUSTRATE THE LONG-TERM STABILITY IN INTER-RUN ASSAY PRECISION (n = 36)

Actual bepridil concentration (ng/ml)	Calculated bepridil concentration* (ng/ml)	Inter-run assay precision** (%)
100	98.8	6.4
400	394.4	5.6
800	805.4	5.3

*Mean of 36 values derived from 18 daily calibration curves.
**Relative standard deviation.

Accuracy and precision

Accuracy and precision of the assay were measured by the relative difference between the mean experimental bepridil concentration and the theoretical value, and the relative standard deviation, respectively (Table I). Duplicate frozen seeded control samples at three different concentrations were blind-coded and analyzed with plasma samples from each clinical study. The data for one such study are presented in Table II (inter-run precision $\leqslant$ 7% at all three concentrations over a period of one month).

Application of the procedure to plasma samples

To date, the procedure has been employed successfully in analyzing over 5000 biological samples from mouse, rat, rabbit, dog, monkey and man. Fig. 3 shows a typical plasma concentration versus time profile from a subject following oral administration of a single 400-mg capsule dose of bepridil hydrochloride. Serial blood samples were drawn up to 72 h after dosing. The 13 ng/ml plasma concentration observed at the final time point is above the detection limit of the assay (10 ng/ml).

ACKNOWLEDGEMENT

We thank Dr. R.K. Nayak for permission to use the human plasma data.

REFERENCES

1 D. Cosnier, P. Duchene-Marullaz, G. Rispat and G. Streichenberger, Arch. Int. Pharmacodyn. Ther., 225 (1977) 133.
2 M.T. Michelin, M. Cheucle and P. Duchene-Marullaz, Therapie, 32 (1977) 485.
3 P. Piris, M. Beaughard, D. Cosnier and C. Labrid, Arch. Int. Pharmacodyn. Ther., 235 (1978) 147.
4 C. Chassaing, N. Moins, J. Lavarenne and P. Duchene-Marullaz, J. Pharmacol., 8 (1977) 503.
5 M. Boucher and P. Duchene-Marullaz, Arch. Int. Pharmacodyn. Ther., 233 (1978) 65.
6 D.R. Harder and N. Sperelakis, J. Cardiovasc. Pharmacol., 3 (1981) 906.
7 N.F. Reifart, M. Zierler, A. Taylor and S. Khuri, Clin. Res., 30 (1982) 215A.
8 J.C. Canicave, J. Deu and F.X. Lesbre, Lyon Med., 243 (1980) 107.
9 D. Granatelli, D. Germa and L. Tatibouet, Quest. Med., 15 (1981) 1041.
10 J. Vink, H.J.M. van Hal, J.-F. Pognat and J.-L. Bouquet Des Chaux, J. Chromatogr., 272 (1983) 87.
11 J. Vink, H.J.M. van Hal, F.M. Kaspersen and H.P. Wijnand, Int. J. Mass Spectrom. Ion Phys., 48 (1983) 217.

Journal of Chromatography, 309 (1984) 132–138
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2130

Note

Gas chromatographic—mass spectrometric characterization of N-methylated basic amino acids in human urine

F. MARCUCCI*, L. COLOMBO, G. DE PONTE, D. CANI, L. COBELLI, A. FRIGERIO and E. MUSSINI

Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan (Italy)

(First received December 30th, 1983; revised manuscript received February 23rd, 1984)

Methylamino acids such as 3-methylhistidine (3-MeHis), N^{ϵ}-monomethyllysine (MML), N^{ϵ},N^{ϵ}-dimethyllysine (DML), N^{ϵ},N^{ϵ},N^{ϵ}-trimethyllysine (TML), N^{G},N^{G}-dimethylarginine (N^{G},N^{G}-DMA) and N^{G},N'^{G}-dimethylarginine (N^{G},N'^{G}-DMA) are normal constituents of human urine [1]. These are also the constituents of several tissue proteins of various organs [2]. Specific sites of histidine, lysine and arginine residues of the proteins are methylated after protein synthesis. The methylated amino acids are released upon protein degradation and excreted into urine without being reutilized for protein synthesis [3]. Muscle and connective tissue proteins constitute the predominant proportions of the structural proteins of the animal body. Connective tissue proteins are not methylated. Of muscle proteins, actin contains 3-MeHis residue [4], and myosin contains 3-MeHis, DML, TML and N^{G},N^{G}-DMA residues [5]. 3-MeHis in urine originates only from these two proteins [2, 6, 7] and large amounts of DML, TML and N^{G},N^{G}-DMA in urine originate from muscle proteins.

Any gross change in the amounts or turnover rates of the muscle proteins may therefore be reflected in a change in the amounts of methylamino acids in urine in the presence of pathological changes of muscle tissue. Accurate determination of the concentrations of methylated amino acids in biological samples is essential because their concentrations in some instances are very low, but no specific assay has been reported. Methylated amino acids have been determined mostly by ion-exchange chromatography and a number of methods have been published specifically for their analysis [2, 8—12]. Recently we developed a method for the isolation of 3-MeHis from biological specimens and its characterization by glass capillary gas chromatography (GC) [13].

0378-4347/84/$03.00

This present report describes a method for the derivatization and detection of N^G-MMA, N^G,N^G-DMA, N^G,N'^G-DMA, MML, DML and TML using a glass capillary GC technique and gas chromatography—mass spectrometry (GC—MS).

EXPERIMENTAL

Materials

N^G-Monomethyl-L-arginine (N^G-MMA) [di-(*p*-hydroxyazobenzene-*p'*-sulphonate)], N^G,N^G-dimethyl-L-arginine [di-(*p*-hydroxyazobenzene-*p'*-sulphonate)], N^G,N'^G-dimethyl-L-arginine [di-(*p*-hydroxyazobenzene-*p'*-sulphonate)], N-ε-methyl-L-lysine · HCl and N-ε-trimethyl-L-lysine, bis(*p*-hydroxyazobenzene-*p'*-sulphonate) were obtained from Calbiochem-Behring (La Jolla, CA, U.S.A.). N-ε-Dimethyl-L-lysine · HCl was synthesized according to the method described by Benoiton [14]. The arginine and lysine bis(*p*-hydroxyazobenzene-*p'*-sulphonate) were converted to the free base form after esterification, as reported below, and MML and DML crystallized as hydrochlorides were used as such.

Derivatization

The preparation of methylarginine and methyllysine derivatives is a two-step process, initially requiring esterification of the carboxyl groups. As esterification agent, the mixture of dry acetyl chloride 5% in *n*-propanol was chosen; 5 ml of the esterification mixture were added to 0.2 μmol of MML, DML and 25 mg of TML, N^G-MMA, N^G,N^G-DMA and N^G,N'^G-DMA. Each vial was sealed, mixed and left to react overnight at 90°C. Samples were evaporated to dryness under vacuum, then MML and DML were N-acylated with 250 μl of trifluoroacetic anhydride and 750 μl of ethyl acetate in sealed vials at 110°C for 30 min.

In order to remove *p*-hydroxyazobenzene-*p'*-sulphonic acid, N^G-MMA, N^G,N^G-DMA, N^G,N'^G-DMA and TML propyl ester, each dissolved in 0.2 ml of *n*-propanol were passed through a column (7 × 1 cm) of silica gel 60 extra pure (70—230 mesh ASTM) (E. Merck, Darmstadt, F.R.G.) packed with chloroform and equilibrated with 20 ml of a mixture of *n*-propanol—acetic acid— water (4:1:1). The column was eluted with 25 ml of the equilibration mixture; the first 15 ml of intensely yellow effluent, containing *p*-hydroxyazobenzene-*p'*-sulphonic acid, were discarded and the next 10 ml of effluent were evaporated to dryness under nitrogen. TML, N^G-MMA, N^G,N^G-DMA and N^G,N'^G-DMA propyl ester in amounts of 0.2 μmol each were subsequently trifluoroacetylated as previously described. The completeness of the derivatization steps was checked by thin-layer chromatography. After evaporation, the residue was dissolved in 1 ml of ethyl acetate to give a final concentration of 0.2 m*M* of each amino acid; 1 μl was sufficient for analysis (0.2 nmol).

Apparatus and instrumental conditions

N^G-MMA, N^G,N^G-DMA, N^G,N'^G-DMA, MML, DML and TML derivatives give sharp, symmetrical peaks on common stationary phases such as pretested QF-1, SE-30, OV-1, OV-17 and OV-101 (Applied Science Labs., State College, PA, U.S.A.) in packed columns, but when biological samples are analysed,

resolution is not as good as that of the standard pool because of interfering peaks. For this reason glass capillary columns were chosen.

A high-resolution dedicated gas chromatograph 3900-B (Dani, Monza, Italy) equipped with a flame ionization detector was used. The glass capillary column (20 m × 0.85 mm O.D., 0.30 mm I.D., Duran 50) was prepared according to the barium carbonate procedure described by Grob et al. [15] and given a 0.15-μm thick Pluronic F-68 coat using the static procedure. The split injection mode was used. Temperatures were as follows: oven programmed from 60 to 200°C, detector 280°C, injector 300°C. Carrier gas was hydrogen (oxygen-free) with a flow-rate of 0.7 ml/min. Splitter flow was 15 ml/min. The chromatograph was equipped with an Infotronic CR S-124 digital integrator for determination of peak areas for quantitative work. For quantitative studies, internal standardization with 1,2,4,5-tetramethylbenzene (TMB) (B.D.H., Poole, U.K.) dissolved in ethyl acetate (0.2 m*M*) was used.

RESULTS AND DISCUSSION

A typical gas chromatogram of synthetic N^G-MMA, N^G,N^G-DMA, N^G,N'^G-DMA, MML, DML and TML derivatives is illustrated in Fig. 1. The separation is quite good and preliminary experiments using the isolation procedure of Kakimoto and Azakawa [1] from rat and human urine and the derivatization procedure described for synthetic substances indicated there were no interfering peaks in these biological samples (Fig. 2).

Briefly the isolation procedure was carried out as follows: an amount of urine containing 10 mg of creatinine was passed through a column (0.9 × 5 cm)

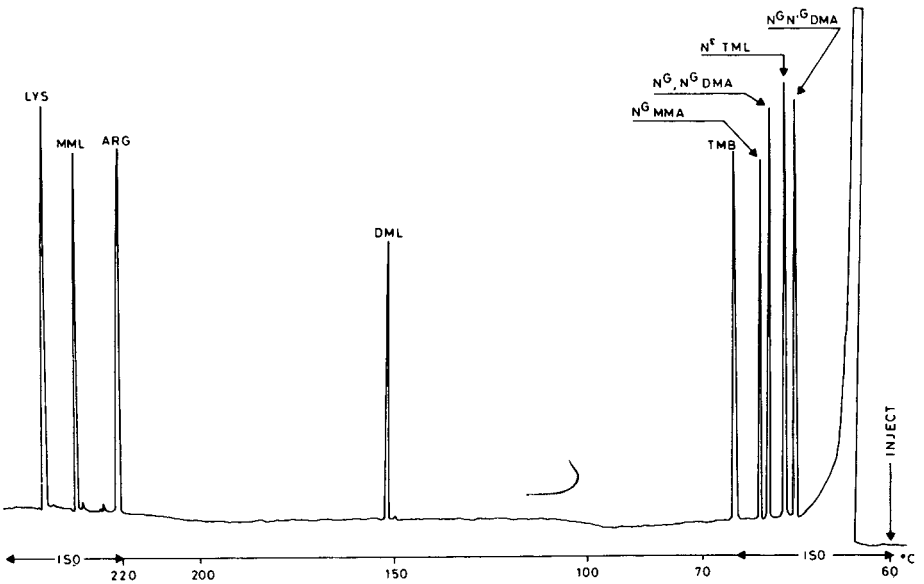


Fig. 1. Typical chromatogram of synthetic methylated basic amino acid derivatives. A Duran 50 glass capillary column (20 m × 0.85 mm O.D., 0.30 mm I.D.) was used with a 0.15-μm thick Pluronic F-68 coat. The split injection mode was used. Injector temperature was 220°C. Flame ionization detector was set to 250°C. Oven temperature programme: 60°C for 5 min, raised at 5°C/min to 220°C, held constant for 5 min.

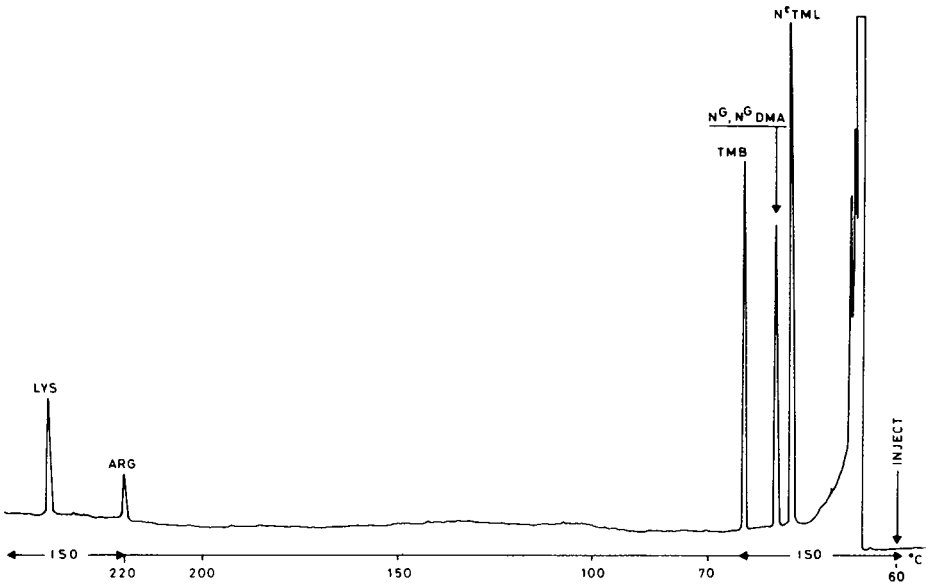


Fig. 2. Typical chromatogram obtained from the assay of urinary human methylated basic amino acids measured in μmol/g creatinine. N^{ϵ}TML = 55, N^{G},N^{G}-DMA = 49. GLC conditions for separation as in Fig. 1.

of AG 50 W × 4 resin, H^+ form, 100—200 mesh (Bio-Rad Labs., Richmond, CA, U.S.A.). The column was washed with 20 ml each of water and 1 *M* pyridine, and the adsorbed basic amino acids were eluted with 20 ml of 3 *M* ammonium hydroxide. The eluate was evaporated to dryness under vacuum. The residue was dissolved in 2 ml of water and the solution was passed through a column (0.9 × 5 cm) of Bio-Rex 70 resin NH_4^+ form, 100—200 mesh (Bio-Rad Labs.). The resin was washed with 20 ml of water, and the retained amino acids were eluted with 20 ml of 2 *M* ammonium hydroxide. The eluate was evaporated to dryness under vacuum and the residue was submitted to the derivatization procedure described for synthetic substances.

Recovery of these methylated basic amino acids added to human and rat urine in amounts of 100 μg each was always 90—95% (S.D. = 3%). The limit of sensitivity of the method was 2.0 nmol/ml. The reproducibility was determined by repeated analyses of the synthetic mixture of the six methylated basic amino acids. The intra-assay coefficient of variation, calculated from ten chromatograms in sequence, was 0.10% . The inter-assay coefficient of variation (n = 10) was 0.15%. For each of the methylated basic amino acids the sample concentration injected was plotted against the ratio between the area of each amino acid and that of the internal standard. With concentrations up to 10 pmol/ml of each amino acid a linear relation (regression coefficient = 0.99) was found. The identity of GC peaks of the methylated basic amino acids tested was elucidated by GC—MS (Figs. 3—5).

With the exception of TML, all the derivatives tested gave fragmentation patterns consistent with the structure of propyltrifluoroacetyl derivatives (mass spectra of methylarginines are unreported because quite expected). Figs. 3 and 4 show the mass spectra of MML and DML. Both show a very

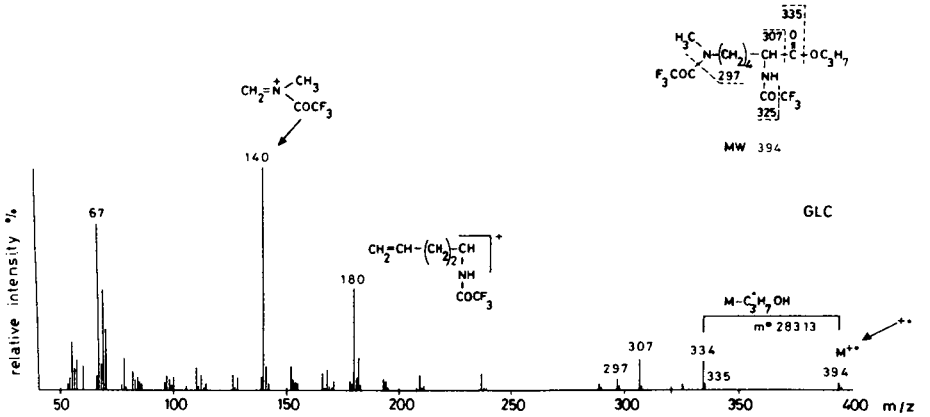


Fig. 3. Mass spectrum of MML. GLC—MS conditions and fragment ions as given in the text.

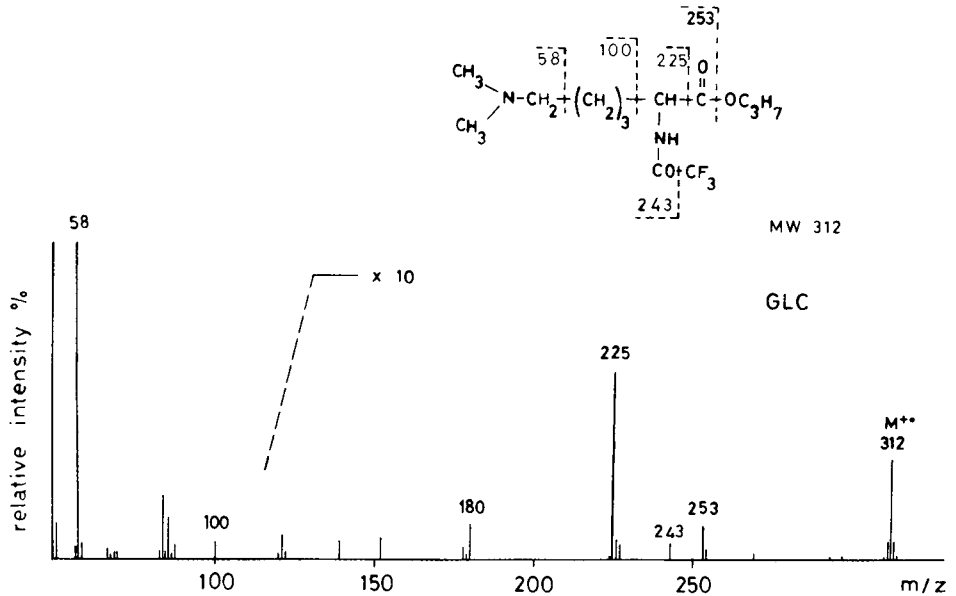


Fig. 4. Mass spectrum of DML. GLC—MS conditions and fragment ions as given in text.

intense peak at *m/z* 180 and the molecular ions are *m/z* 394 and 312, respectively. The mass spectrum of TML is shown in Fig. 5. The spectrum consists chiefly of one very intense peak at *m/z* 180, common to all the methyllysine derivatives tested. The molecular ion at *m/z* 267 is an artefact probably resulting from the extremely easy thermal degradation of TML; in fact the molecular weight of the TML derivative is 327. At the bottom of Fig. 5, the mass spectrum analysed by direct inlet system MS shows the molecular ion at *m/z* 312, indicating thermal degradation of the TML derivative. For this reason, gas—liquid chromatography (GLC) of the TML derivative gives no warranty of reproducible results and further studies are required to quantify the thermal phenomenon. In order to make easier quantitative GLC determination of those methylated basic amino acids at present available as

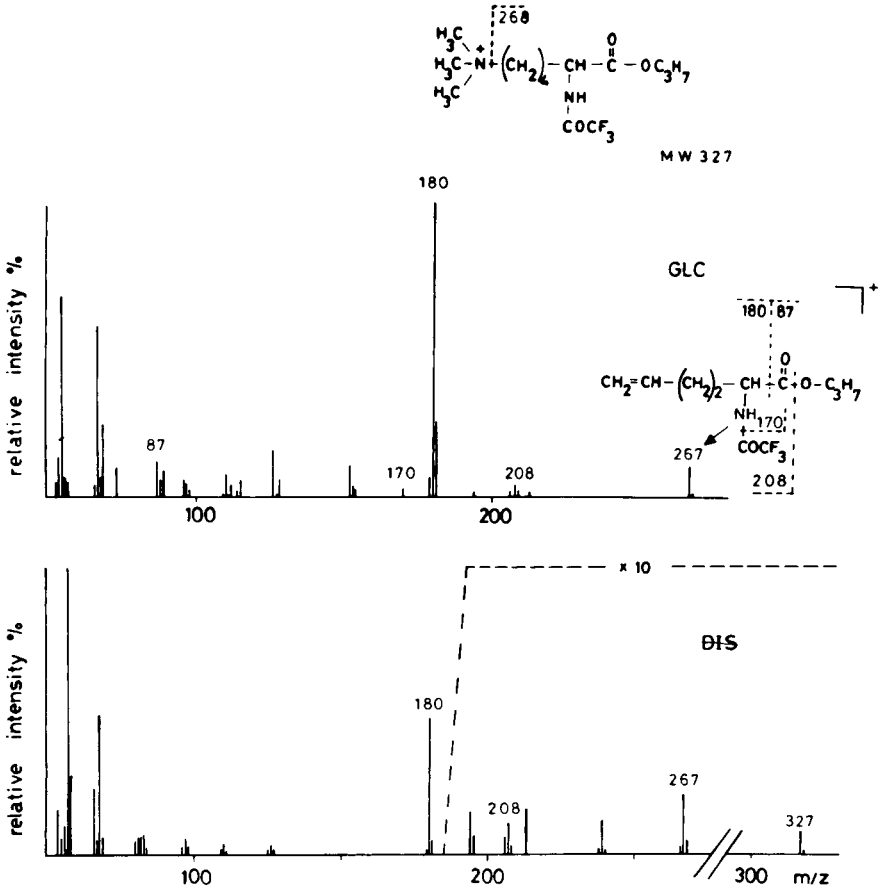


Fig. 5. Mass spectrum of TML. GLC—MS conditions and fragment ions as given in text. At the bottom mass spectrum analysed by direct inlet system MS.

di-(*p*-hydroxyazobenzene-*p'*-sulphonate) salts, we are preparing them according to the methods of Benoiton [14] and Kakimoto and Azakawa [1].

The present method could be useful for the quantitative determination of methylated basic amino acids in human urine in conditions involving pathological changes of muscle tissue.

ACKNOWLEDGEMENTS

This work was partially supported by C.N.R. Contract No. 82.02054.56. The generous contribution of the Gustavus and Louise Pfeiffer Research Foundation, Los Angeles, CA, U.S.A., is gratefully acknowledged.

REFERENCES

1 Y. Kakimoto and S. Akazawa, J. Biol. Chem., 245 (1970) 5751.
2 Y. Kakimoto, Y. Matsuoka and M. Miyake, J. Neurochem., 24 (1975) 893.
3 M. Miyake and Y. Kakimoto, Metabolism, 25 (1976) 885.

4 A.M. Asatoor and M.D. Armstrong, Biochem. Biophys. Res. Commun., 26 (1967) 168.
5 M. Reporter and J.L. Corbin, Biochem. Biophys. Res. Commun., 43 (1971) 644.
6 L.N. Haverberg, P.T. Omstedt, H.N. Munro and V.R. Young, Biochim. Biophys. Acta, 405 (1975) 67.
7 C. Bilmazes, R. Uauy, L.N. Haverberg, H.N. Munro and V.R. Young, Metabolism, 27 (1978) 525.
8 L.N. Haverberg, H.N. Munro and V.R. Young, Biochim. Biophys. Acta, 371 (1974) 226.
9 G.E. Deibler and R.E. Martenson, J. Biol. Chem., 248 (1973) 2387.
10 C.L. Long and J.W. Geiger, Biochem. Med., 12 (1975) 267.
11 R.T. Markiw, Biochem. Med., 13 (1975) 23.
12 C.G. Zarkadas, Can. J. Biochem., 53 (1975) 96.
13 L. Cotellessa, F. Marcucci, D. Cani, P. Sfrondrini, L. Colombo, E. Mussini and F. Poy, J. Chromatogr., 221 (1980) 149.
14 L. Benoiton, Can. J. Chem., 42 (1964) 2043.
15 K. Grob, G. Grob and K. Grob, Jr., Chromatographia, 10 (1977) 181.

Journal of Chromatography, 309 (1984)139—144
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2111

Note

Determination of γ-aminobutyric acid in physiological samples by a simple, rapid high-performance liquid chromatographic method

A.S. HERRANZ, J. LERMA and R. MARTIN DEL RIO*

Departamento de Investigacion, Centro "Ramon y Cajal", Ctra. Colmenar Viejo Km 9, Madrid 34 (Spain)

(First received December 1st, 1983; revised manuscript received February 13th, 1984)

Since the discovery of γ-aminobutyric acid (GABA) in the nervous system, various methods for its quantitation have been developed [1—11]. Most of them, however, display low sensitivity [2—4], require expensive equipment [5—9] or are difficult to handle [10, 11].

The introduction of high-performance liquid chromatography (HPLC) represented a considerable improvement in analytical techniques and it has been used for the determination of a great number of compounds. HPLC has also been applied to the measurement of amino acids [12, 13], and some HPLC methods for GABA determination have recently been reported [14—16]. The purpose of the present work is to describe a new rapid method for GABA analysis in physiological samples using a simple reversed-phase HPLC procedure with *o*-phthalaldehyde (OPA) pre-column derivatization.

MATERIALS AND METHODS

Chromatographic equipment

A Beckman HPLC apparatus Model 344 was used. The chromatographic system included a controller Model 421 CRT, a pump Model 112, an injection valve Model 340 with a 20-μl loop, and a data processor/recorder Chromatopac C-R1B (Shimadzu) for peak integration. Analyses were performed on a 150 × 4.6 mm I.D. column, pre-packed with 5-μm Ultrasphere-ODS (Beckman). The detector consisted of a Gilson Spectra/Glo fluorometer with a 15-μl micro-flow cell, a quartz-halogen lamp, and the combination of 360-nm excitation with 455-nm emission filters. The sensitivity dial of the fluorometer was usually set at 100 range units.

0378-4347/84/$03.00

A Beckman 121/MB automatic amino acid analyser was used for free GABA determination.

Reagents

The following chemicals were purchased: amino acid standard solution from Beckman; GABA from Sigma (St. Louis, MO, U.S.A.); methanol (HPLC grade), concentrated hydrochloric acid and 2-ethanethiol from Scharlau (Barcelona, Spain); acetic and boric acids from E. Merck (Darmstadt, F.R.G.); and OPA from Fluka (Buchs, Switzerland). Distilled, deionized water was used, further purified by passage through a filter for adsorption of organic substances (Norganic, Millipore) and a 0.4-μm Millipore filter.

Chromatographic conditions

Optimum chromatographic conditions were as follows: isocratic solvent of water—methanol (20:80) with a flow-rate of 1.0 ml/min. All analyses were performed at room temperature (22—25° C).

Esterification of GABA and amino acid standard mixture

To obtain the corresponding methyl ester derivatives, an amino acid standard mixture solution containing eighteen α-amino acids (2.5 m*M*) and the GABA solution (1.7 m*M*), were diluted (× 100, by vol.) with absolute methanol containing hydrochloric acid at a final concentration of 0.1 *M*. The esterification reaction was maintained overnight at room temperature.

Saponification of the GABA methyl ester derivative

The saponification reaction of GABA methyl ester was obtained by diluting an aliquot of the esterified sample five times with 0.2 *M* sodium hydroxide and heating the resultant solution at 60° C overnight in a sealed tube. For quantitation of free GABA, the sample was acidified by adding concentrated hydrochloric acid immediately before analysis.

Tissue GABA extraction and esterification

Sprague—Dawley female rats were killed by decapitation and their brains and oviducts were rapidly dissected and frozen in liquid nitrogen. Tissues were then weighed and resuspended in 25 vols. (w/v) of a solution of absolute methanol containing 0.1 *M* hydrochloric acid, and ground with a glass homogenizer. The tissue GABA extraction and the esterification reaction were left overnight at room temperature. Samples were centrifuged at 5000 *g* for 10 min and the clear supernatant was used for analysis.

Derivatization procedure

The method used is essentially that of Hill et al. [17] with minor modifications. Two hundred microlitres of sample were mixed with 50 μl of 0.5 *M* potassium borate buffer (pH 10.5); 50 μl of methanol and 100 μl of 2-ethanethiol solution (10 μl/ml in absolute methanol) were then added to this mixture. The amino acid derivatization was initiated by adding 100 μl of OPA solution (10 mg/ml in methanol) and the reaction was acidified 1 min later with 100 μl of acetic acid (5% in methanol) in order to lower the pH to 5—6. Then 20 μl of

the final solution were injected into the chromatographic system. The 2-ethanethiol and OPA solutions were kept in light-protected tubes and prepared weekly.

RESULTS

The carboxyl group of GABA was esterified as described in Materials and methods and the progress of the reaction was monitored at different times by measuring the remaining unmethylated GABA. Determinations were performed with an automatic amino acid analyser until the non-reacting GABA was below the detection limit ($\simeq$ 100 pmol.). Fig. 1 shows the gradual decline in the reaction mixture of free (unmethylated) GABA with time. Conversion to the methyl derivative is complete after 4 h. Recovery of the free form of GABA following saponification with sodium hydroxide ranged between 98 and 100%, demonstrating that the amino acid had previously been totally converted to the methyl ester form.

When the GABA methyl ester was combined with OPA, it produced a fluorescent compound which, in our optimum chromatographic conditions, eluted in a well defined peak within a short time (4.9 $\pm$ 0.12 min), as shown in Fig. 2A.

In order to ascertain the resolution of GABA from α-amino acids, a mixture of eighteen α-amino acid methyl esters was added to the GABA standard. Fig. 2A depicts a typical chromatogram obtained. It can be seen that the α-amino acid mixture eluted in a broad peak well ahead of GABA. Moreover, the methyl ester of two β-amino acids (β-alanine and β-aminoisobutyric acid), which might be present in physiological samples, also eluted far away from the α-amino acids and were well separated from the GABA peak (Fig. 2B).

Flow-rates higher than 1.0 ml/min and a methanol content greater than 80% shortened the analysis time but diminished the resolution of the GABA peak. The converse conditions improved the resolution, but a longer analysis time was required. The optimum derivatization reaction time was 1 min. A shorter reaction time diminished the fluorescence response but longer periods, up to 10 min, did not improve it.

The fluorescence response of GABA was examined in the 1—100 pmol range.

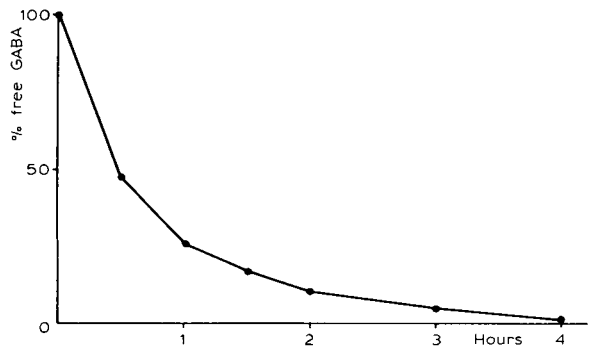


Fig. 1. Declining concentration of free GABA with increasing esterification time. Each point, represented as a percentage of the initial amount (100%), is the mean of two determinations.

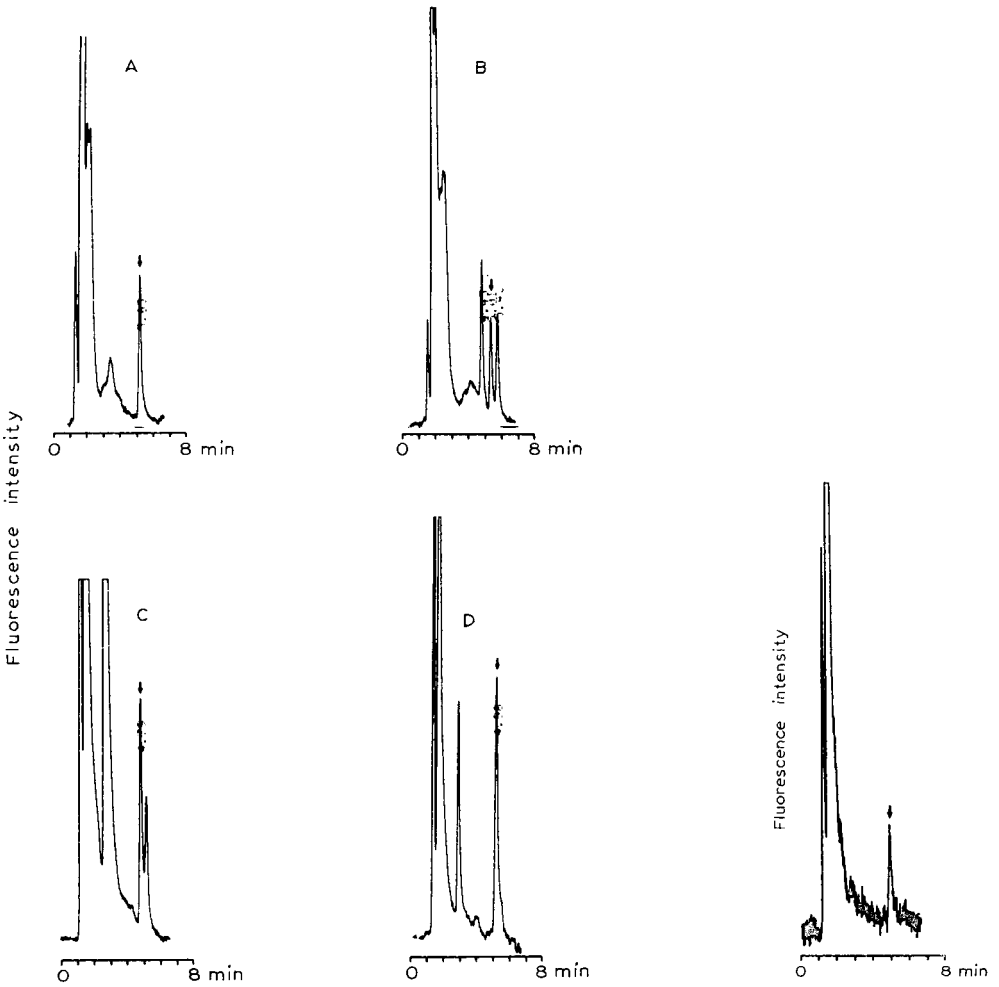


Fig. 2. Arrows indicate the GABA methyl ester peaks in four different situations: (A) in a mixture of eighteen α-amino acids; (B) as in A with β-alanine (peak ahead of GABA) and β-aminoisobutyric acid (peak slower than GABA) included; (C) in a chromatogram of brain extract representing 13.3 μg of tissue; (D) in a chromatogram of oviduct extract representing 6.6 μg of tissue. In all cases the recorder attenuation was set at 2^4.

Fig. 3. Typical chromatogram showing the analysis of 1 pmol of GABA (arrow). Recorder attenuation was set at 2^1.

The analysis of 1 pmol gave peak heights with a signal-to-noise ratio higher than 2 (Fig. 3), demonstrating the high sensitivity of this method. The regression line between GABA amount and fluorescence response was calculated with data obtained from assays performed on three different days, and was linear over the concentration range studied ($r = 0.99, P < 0.05$).

Tissue GABA determination

Brains and oviducts of five female rats were processed as described in Materials and methods and the free GABA content in pellets (non-extracted),

supernatants (non-esterified GABA), and in saponified supernatants (extracted and esterified GABA) was measured with the aid of an amino acid analyser. In addition, GABA from five other animals was extracted and measured as previously described [4]. Brain and oviduct GABA values were considered to be the total GABA content in these tissues.

TABLE I

FREE GABA CONTENT IN TOTAL, PELLET, SUPERNATANT AND SAPONIFIED SUPERNATANT OF RAT BRAIN AND OVIDUCT

Data are expressed as mean ± S.D. and percentage of the total amount.

	Total		Pellet		Supernatant		Saponified supernatant	
	μmol/g	%	μmol/g	%	μmol/g	%	μmol/g	%
Brain	2.01 ± 0.19	100	<0.032	<1.6	<0.095	<4.7	1.96 ± 0.15	97.5
Oviduct	4.65 ± 0.31	100	<0.087	<1.8	<0.024	<4.4	4.45 ± 0.32	95.7

Table I shows the free GABA content found in pellet, supernatant, and saponified supernatant, expressed in μmol/g original tissue and also as a percentage of total GABA. It can be seen that the non-extracted and non-esterified GABA are less than 2% and 5%, respectively, and that the GABA ester obtained in the supernatant, measured as free GABA after saponification, is 95—98% of the total GABA.

The GABA derivative was further analysed with the HPLC method. Fig. 2C and D shows typical chromatograms obtained from samples of both structures, brain and oviduct. The GABA peak was well resolved and the coefficients of variation obtained from the five samples analysed were 2.2% and 4.0%, respectively, for brain and oviduct.

The accuracy of the described method was assessed by comparing GABA values obtained from brain and oviduct with those measured by the amino acid analyser. The results obtained by these techniques differed by less than 5%.

DISCUSSION

Various methods have been developed [1—11] for the quantification of GABA since its discovery in the nervous system more than 30 years ago [18, 19]. Recently, GABA—OPA [12, 13], dansyl-GABA [14, 15] and trinitrobenzene sulphonic acid—GABA derivatives [16] have been measured by HPLC. The present study describes a new HPLC method for GABA determination which quantifies its methyl ester after derivatization with OPA.

Although our method is based on obtaining the methyl ester of the amino acid, the derivative is actually formed during the tissue extraction step; hence there is no complicated handling procedure and this is one of several advantages over other OPA methods. The mobile phase needed is less harmful to the stationary phase than that of Lindroth and Mopper [12], Jones et al. [13] or Caudill et al. [16], thus increasing the life of the chromatographic system. The run time is quite short (less than 7 min) and the sensitivity (less than 1 pmol)

is satisfactory. The method has adequate precision (coefficient of variation = 4%), and, because of the isocratic procedure, the equipment needed is relatively inexpensive. Dansyl derivative methods [14, 15] also offer all of these advantages but the preparation of the sample is difficult and time-consuming, and the recovery of the derivative is relatively low, e.g. 87% [14]. Finally, the method described by Caudill et al. [16] is suitable, although the retention time of GABA is almost three times longer than in the present method.

Although it is not known why the β- and γ-amino acid methyl esters elute later than the α-amino acids, it may be hypothesized that the absence of polar lateral chains in β- and γ-amino acids, the blocked carboxyl group and their capability to adopt a more extended conformation after derivatization than the α-amino acids, make them more hydrophobic [12] thus allowing greater interaction with the stationary phase and subsequently increasing retention times.

Measurements of GABA levels in human cerebrospinal fluid may be helpful for diagnosis and therapy of brain disorders, thus the reported method provides with a rapid, sensitive, selective and relatively inexpensive assay for this purpose.

ACKNOWLEDGEMENTS

The authors are indebted to Dr. J.M.R. Delgado for research facilities, Dr. E. Herrera for critical evaluation, C.S. Delgado and D. Muñoz for editorial help, and A. Latorre for technical assistance.

REFERENCES

1 N. Seiler, in N. Marks and R. Rodnight (Editors), Research Methods in Neurochemistry, Vol. 3, Plenum, New York, 1975, p. 409.
2 M.K. Gaitonde, in N. Marks and R. Rodnight (Editors), Research Methods in Neurochemistry, Vol. 2, Plenum, New York, 1974, p. 321.
3 F.W. Wagner and R.L. Liliedahl, J. Chromatogr., 71 (1972) 567.
4 R. Martin del Rio and A. Latorre Caballero, J. Neurochem., 34 (1980) 1584.
5 P. Bõlen, P.J. Schechter, W. Van Damme, G. Coquillat, J.C. Dosch and J. Koch-Weser, Clin. Chem., 24 (1978) 256.
6 T.A. Hare and N.V. Bala Manyam, Anal. Biochem., 101 (1980) 349.
7 J.A.M. Van der Heyden, K. Venema and J. Korf, J. Neurochem., 32 (1979) 469.
8 J.D. Huizinga, A.W. Teelken, F.A.J. Muskiet, J.V.D. Meulen and B.G. Wolthers, N. Engl. J. Med., 296 (1977) 692.
9 P.H. Wu, D.A. Durden and L. Hertz, J. Neurochem., 32 (1979) 379.
10 M. Otsuka, K. Obata, Y. Miyata and Y. Tanaka, J. Neurochem., 18 (1971) 287.
11 S.J. Enna, J.H. Wood and S.H. Snyder, J. Neurochem., 28 (1977) 1121.
12 P. Lindroth and K. Mopper, Anal. Chem., 51 (1979) 1667.
13 B.N. Jones, S. Pääbo and S. Stein, J. Liquid Chromatogr., 4 (1981) 565.
14 G.E. Griesmann, W. Chan and O.M. Rennert, J. Chromatogr., 230 (1982) 121.
15 L. Zecca, F. Zambotti, N. Zonta and P. Mantegazza, J. Chromatogr., 233 (1982) 307.
16 W.L. Caudill, G.P. Houck and R.M. Wightman, J. Chromatogr., 227 (1982) 331.
17 D.W. Hill, F.H. Walters, T.D. Wilson and J.D. Stuart, Anal. Chem., 51 (1979) 1338.
18 E. Roberts and S. Frankel J. Biol. Chem., 187 (1950) 55.
19 J. Awapara, A.J. Landau, R. Fuerst and B. Seale, J. Biol. Chem., 187 (1950) 35.

Journal of Chromatography, 309 (1984)145—150
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2134

Note

Determination of urinary tryptophan, 5-hydroxytryptamine and 5-hydroxyindoleacetic acid in neonatal hyperbilirubinaemic infants using reversed-phase high-performance liquid chromatography with fluorescence detection

ATSUKO IWATANI* and HAJIME NAKAMURA

Department of Pediatrics, Kobe University School of Medicine, Chuo-ku, Kusunoki-cho, 7-5-1, Kobe, 650 (Japan)

(First received January 24th, 1984; revised manuscript received February 29th, 1984)

5-Hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) are related as neuroregulators to physiological function (sleep regulation and sexual behaviour) [1, 2]. Tryptophan regulates 5-HT synthesis in the brain through a rate-limiting enzyme, tryptophan hydroxylase [3—7]. Tryptophan is known to be photosensitive [8—13]. We demonstrated that tryptophan promotes the photodecomposition of bilirubin in the presence of riboflavin [14]. Rubaltelli et al. [15] and Antoni et al. [16] also demonstrated that the urinary excretion of tryptophan metabolites of the kynurenic pathway decreased in hyperbilirubinaemic infants treated with phototherapy.

Recently several methods have been reported for the simultaneous separation of tryptophan, 5-HT and 5-HIAA in brain [17—24], cerebrospinal fluid [23], serum [19, 23, 25, 26] and lung tissue [20]. Some reports have described an analysis for tryptophan metabolites in urine [27—33]. We propose a method for the simultaneous quantitative assessment of tryptophan, 5-HT and 5-HIAA in urine. We measured urinary tryptophan and its metabolites in hyperbilirubinaemic infants treated with phototherapy.

EXPERIMENTAL

Reagents

The reagents used were all of the highest purity. L-Tryptophan and 5-hydroxytryptamine were purchased from Wako (Tokyo, Japan), 5-hydroxyindoleacetic acid was from Aldrich and 5-hydroxytryptophan (5-HTRP)

0378-4347/84/$03.00

from Sigma (St. Louis, MO, U.S.A.). Disodium hydrogen phosphate, potassium dihydrogen phosphate, acetic acid, sodium acetate, sodium sulphate and anhydrous methyl alcohol were obtained from Wako. A creatinine kit was purchased from Daiichi Chemical Co. (Tokyo, Japan).

Apparatus

Chromatography was performed with a Hitachi 635 S pump, and fluorescence detection was accomplished using a Hitachi 650-60 fluorescence spectrophotometer with an 18-μl flow cell (excitation 285 nm, emission 340 nm). Monitor outputs were recorded on a Hitachi 050 recorder. The column was a stainless-steel Unicil C_{18} (25 cm × 4 mm I.D., 10 μm particle size) obtained from Gaskuro (Tokyo, Japan). Sample injections were made with a 100-μl Hamilton syringe.

Chromatographic conditions

Stock solution consisted of 4.8 g of acetic acid, 1.64 g of sodium acetate and 28 g of sodium sulphate in 1000 ml of distilled water. The low concentration eluent (solvent 1) was made by diluting the stock solution with distilled water (1:1, v/v). The high concentration eluent (solvent 2) was a mixture of stock solution—water—anhydrous methyl alcohol (4:3:1, v/v). The column was equilibrated to solvent 1 for at least 10 min before injection. Solvent 1 flowed for just 10 min after sample injection and was followed by solvent 2 for 20 min. The flow-rate was 1.0 ml/min.

The column was stored in 50% methyl alcohol when not in use.

Sample preparation

Standard solutions of each compound were prepared by dissolving the respective compounds in 0.2 *M* phosphate buffer pH 5.6. Urine samples from fourteen hyperbilirubinaemic infants treated with phototherapy were collected before treatment, after 48 h of phototherapy and 24 h after the treatment was brought to an end. The urine was stored at −18° C. Samples were centrifuged at 300 *g* for 10 min and filtered through a Millipore membrane, pore size 0.45 μm (Millipore, Bedford, MA, U.S.A.); 20 μl of each sample were injected on the column. These procedures were performed in dimmed light. The concentration of each compound in urine was determined by its peak height and expressed in μmol/g of creatinine.

Photoirradiation in vitro

Urine was put in a petri dish with 10 μg/ml riboflavin, and was illuminated under a blue white light (intensity 3 mW/cm^2/sec, FL 20BW-NU, National Co., Osaka, Japan), which cuts off ultraviolet rays.

Identification of peaks

Initial identification of the peaks of interest was based on retention time and co-chromatography with the reference compounds. The peaks of samples were compared with stopped-flow spectra of the reference compounds.

RESULTS

The separation of a test mixture of tryptophan, 5-HT and 5-HIAA by two-step gradient with low and high eluents is shown in Fig. 1a. Analysis of these compounds was complete about 30 min after injection.

The reproducibilities of the peak heights were 5.2% (tryptophan), 5.1% (5-HT) and 3.4% (5-HIAA) as shown in Table I. The system was routine tested with a standard mixture before analysis of the samples. Retention times of all compounds showed daily variations of up to 1.0%.

Detection by fluorescence was found to be sensitive at excitation 285 nm and emission 340 nm for these substances. Standard curves of peak height versus amount injected were linear in the range of at least 15—300 pmol for tryptophan, 3—75 pmol for 5-HT and 25—750 pmol for 5-HIAA.

Chromatograms of urine of the newborn infants are shown in Fig. 1b. Tryptophan, 5-HT and 5-HIAA were all detected in basal urine. The peak of 5-HTRP overlapped an unknown peak. The identification of the compounds was performed by spiking the urine samples with the standards. The stopped-flow spectra of each peak corresponded to those of the standards as shown in Fig. 2.

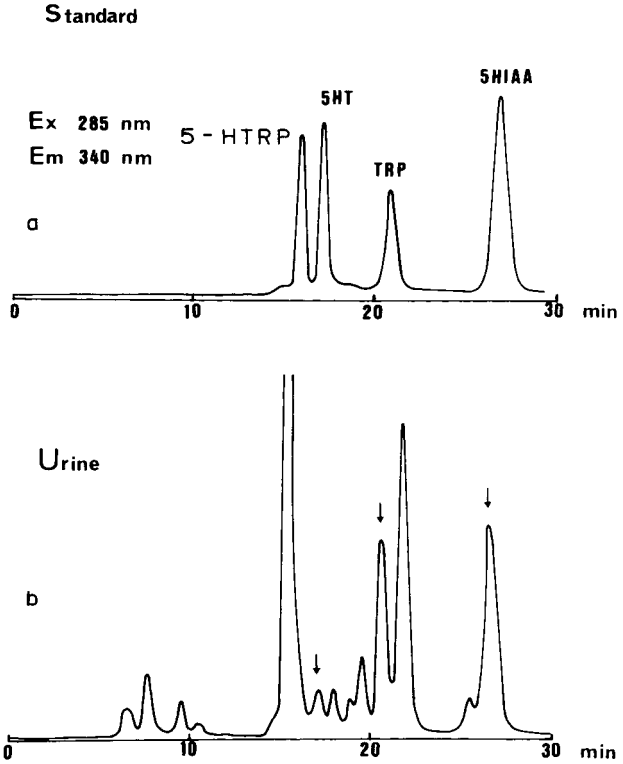


Fig. 1. (a) Chromatogram illustrating the separation of reference compounds detected by native fluorescence with an excitation wavelength of 285 nm and emission cut-off filter of 340 nm. The amounts of 5-HTRP, 5-HT, tryptophan (TRP) and 5-HIAA are 50 pmol, 50 pmol, 200 pmol and 500 pmol, respectively. (b) Separation of the urine sample.

TABLE I

REPRODUCIBILITY

	Peak height (mm)			Retention time (min)		
	Mean	S.D.	C.V. (%)	Mean	S.D.	C.V. (%)
5-HT	76.0	3.9	5.1	8.6	0.1	0.8
TRP*	60.2	3.1	5.2	10.3	0.1	0.8
5-HIAA	146.5	5.6	3.8	13.2	0.1	1.0

*TRP = tryptophan.

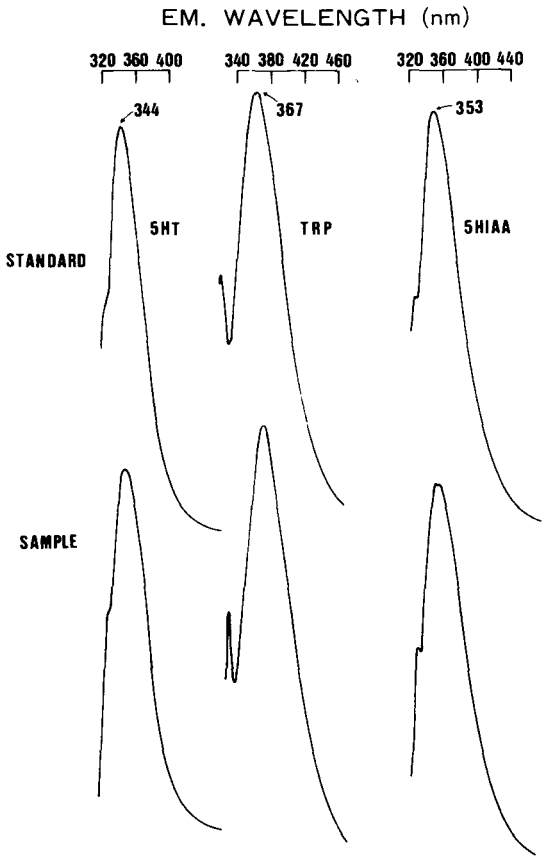


Fig. 2. Stopped-flow spectra of the reference compounds and the urine sample.

The concentrations of urinary tryptophan, 5-HT and 5-HIAA before phototherapy were 43.5, 1.4 and 49.4 μmol/g of creatinine, respectively. In in vitro study, the amounts of tryptophan, 5-HT and 5-HIAA in the urine decreased markedly by photoirradiation (Fig. 3). However, the urinary excretion of these compounds was not influenced by phototherapy in the infants with hyperbilirubinaemia (Table II).

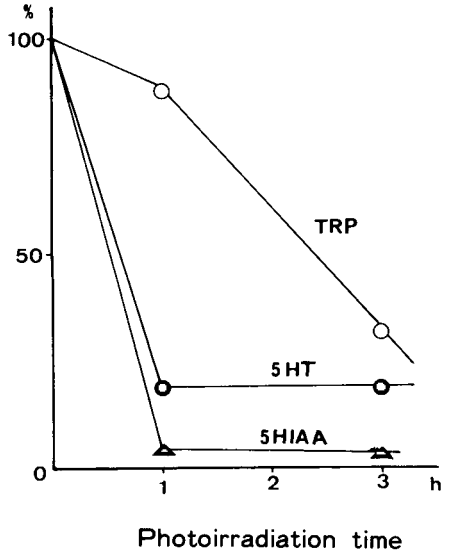


Fig. 3. Photodecomposition of tryptophan metabolites in vitro.

TABLE II

EFFECTS OF PHOTOTHERAPY ON TRYPTOPHAN METABOLITES IN URINE

Results are given as μmol/g of creatinine (mean ± S.D.).

	Before therapy	After 48 h of therapy	24 h after end of therapy
TRP	43.5 ± 8.1	46.1 ± 10.4	50.9 ± 9.6
5-HT	1.4 ± 0.2	1.4 ± 0.3	1.5 ± 0.3
5-HIAA	49.4 ± 4.6	50.9 ± 7.9	47.4 ± 9.4

DISCUSSION

Several methods have been employed for the separation of urinary tryptophan and its metabolites: Denkla's method, ion-exchange [29, 32, 33], and straight-phase [28] and reversed-phase high-performance liquid chromatography [27, 30, 31]. The method described in the present report is suitable for analysis of very small samples with minimum quantities of tryptophan and its metabolites. The sensitivity of the determination was found to be suitable for the analysis of urinary tryptophan and its metabolites in neonatal urine without tryptophan loading. This method guarantees a quantitative determination of tryptophan and its metabolites via 5-HT.

It has been shown that, in jaundiced infants treated with phototherapy, the excretion of tryptophan metabolites of the kynurenine pathway (kynurenine, 3-hydroxykynurenine and 3-hydroxyanthranilic acid) was lower in comparison with untreated infants [15, 16]. When the urine of jaundiced newborn infants was exposed to light in vitro, tryptophan and its metabolites via 5-HT decreased significantly. However, the excretory pattern of tryptophan metabolites via 5-HT in jaundiced newborn infants after phototherapy did not differ from that before phototherapy.

There is no evidence that the photoirradiation directly induces the alterations in vivo. We stress the importance of defining the dose—effect relationship in phototherapy to avoid overexposure of newborn infants.

ACKNOWLEDGEMENTS

We wish to thank Professor Tamotsu Matsuo and Professor Kimiaki Sumino, who suggested and encouraged us to conduct this study, for their continuous support and helpful comments.

REFERENCES

1 E. Garelis, Adv. Exp. Med. Biol., 133 (1981) 717.
2 G.A. Kennett and M.H. Joseph, Neuropharmacology, 20 (1981) 39.
3 M. Hamon, S. Bourgoin, F. Artaud and S.E. Mestikawy, J. Physiol. (Paris), 77 (1981) 269.
4 S.N. Young, Brit. J. Pharmacol., 74 (1981) 695.
5 G. Curzon, Adv. Exp. Med. Biol., 133 (1981) 207.
6 S.N. Young and S. Gauthier, Adv. Exp. Med. Biol., 133 (1981) 221.
7 S.N. Young, G. Chouinard and L. Aunable, Adv. Exp. Med. Biol., 133 (1981) 727.
8 R.S. Asquith and D.E. Rivett, Biochim. Biophys. Acta, 252 (1971) 111.
9 D.V. Bent and E. Hayon, J. Amer. Chem. Soc., 97 (1975) 2612.
10 H. Templer and P.J. Thistlethwaite, Photochem. Photobiol., 23 (1976) 79.
11 L.A. Holt, B. Milligan, D.E. Rivett and F.H.C. Stewart, Biochim. Biophys. Acta, 499 (1977) 131.
12 C. Jacobsen and J. Jacobsen, Biochem. J., 181 (1979) 251.
13 P. Cavatorta, R. Favilla and A. Mazzini, Biochim. Biophys. Acta, 578 (1979) 541.
14 Y. Uetani, Y. Kitsunezuka, R. Shimabuku, H. Nakamura, T. Matsuo, M. Ichihashi and Y. Fujiwara, Photomed. Photobiol., 2 (1980) 91.
15 F.F. Rubaltelli, G. Allegri, C. Costa and A. Antoni, J. Pediatr., 85 (1974) 865.
16 A. Antoni, F.F. Rubaltelli, C. Costa and G. Allegri, Acta Vitaminol. Enzymol., 29 (1975) 145.
17 C.J. Earley, J. Pharmacol. Methods, 5 (1981) 185.
18 G.M. Anderson, J.G. Young, D.K. Batter, S.N. Young, D.J. Cohen and B.A. Shaywitz, J. Chromatogr., 223 (1981) 315.
19 A.M. Krstulovic and A.M. Powell, J. Chromatogr., 171 (1979) 345.
20 D.E. Mais, P.D. Lahr and T.R. Bosin, J. Chromatogr., 225 (1981) 27.
21 A.J. Falkowsky and R. Wei, Anal. Biochem., 115 (1981) 311.
22 I.N. Mefford and J.D. Barchas, J. Chromatogr., 181 (1980) 187.
23 L. Semerdjian-Rouquier, L. Bossi and B. Scatton, J. Chromatogr., 218 (1981) 663.
24 S.M. Lasley, I.A. Michaelson, R.D. Greenland and P.M. McGinnis, J. Chromatogr., 305 (1984) 27.
25 A.M. Krstulovic and C. Matzura, J. Chromatogr., 163 (1979) 72.
26 G.M. Anderson, J.G. Young, D.J. Cohen, K.R. Schlicht and N. Patel, Clin. Chem., 27 (1981) 775.
27 F. Geeraerts, L. Schimpfessel and R. Crokaert, Clin. Chim. Acta, 102 (1980) 247.
28 M. Ghebregzabher, S. Rufini, M.G. Castellucci and M. Lato, J. Chromatogr., 222 (1981) 191.
29 K. Seta, M. Washitake, T. Anmo, N. Takai and T. Okuyama, J. Chromatogr., 181 (1980) 311.
30 E. Grushka, E.J. Kikta, Jr. and E.W. Naylor, J. Chromatogr., 143 (1977) 51.
31 J.B. Tarr, Biochem. Med., 26 (1981) 330.
32 C.D. Scott, Clin. Chem., 14 (1968) 521.
33 S. Katz and C.A. Burtis, J. Chromatogr., 40 (1969) 270.

Journal of Chromatography, 309 (1984)151—155
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2110

Note

Sensitive assay for diamine oxidase activity using high-performance liquid chromatography

P.A. BIONDI*, T. SIMONIC, C. SECCHI and S. RONCHI

Istituto di Fisiologia Veterinaria e Biochimica, Università di Milano, via Celoria 10, I-20133 Milan (Italy)

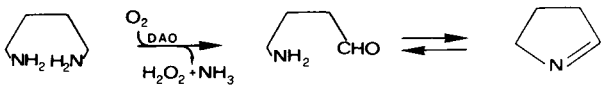
and

A. MANZOCCHI

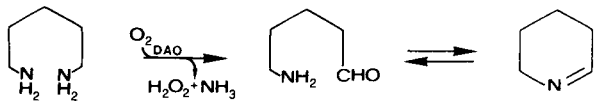
Dipartimento di Chimica e Biochimica Medica, via Saldini 50, I-20133 Milan (Italy)

(First received November 4th, 1983; revised manuscript received February 13th, 1984)

Diamine oxidases [diamine:oxygen oxidoreductase (deaminating), EC 1.4.3.6] (DAO) catalyse the oxidation by molecular oxygen of various diamines, such as putrescine and cadaverine, to the corresponding aminoaldehydes, hydrogen peroxide and ammonia. The aminoaldehydes are in equilibrium with their cyclic condensation products, Δ^1-pyrroline and Δ^1-piperideine, for putrescine and cadaverine, respectively (Fig. 1).



Pu



Ca

Fig. 1. Scheme of DAO-catalysed oxidation of putrescine (Pu) and cadaverine (Ca).

0378-4347/84/$03.00

Since DAO activity has been considered a useful parameter in various clinical studies [1, 2], many different assays for DAO activity have been proposed. Methods using radioactive substrate [3, 4] achieve maximum sensitivity, but often only simple instrumentation is available in analytical laboratories and alternative procedures are demanded. In this connection, the potentials of high-performance liquid chromatography (HPLC) have been recently exploited by Pietta et al. [5]. Their method estimates hydrogen peroxide using coupled peroxidase-catalysed oxidation of a fluorogenic compound.

Because of the criticism by Neufeld and Chayen [6] of coupled peroxidase assays we avoided this approach. It was our aim to reach the sensitivity of the method of Pietta et al. [5] by direct evaluation of a stable derivative of the reaction product. For this purpose the classical method of Holmsted et al. [7] was followed. This method is based on the treatment of Δ^1-pyrroline with *o*-aminobenzaldehyde (OAB) to give a condensation product (2,3-trimethylene-1,2-dihydroquinazolinium ion) which is spectrophotometrically analysed. Sakamoto and Samejina [8] modified this procedure by converting the condensation product to the 2,3-trimethylene-4-quinazolone (triMQ, Fig. 2) which can easily be estimated by gas chromatography with a higher sensitivity than that attained by spectrophotometry.

By exploiting the still higher sensitivity of HPLC we expected to obtain at the same time a more rapid method than that described by Sakamoto and Samejina [8]. In order to demonstrate the suitability of the described procedure, enzymatic activity in pea seedling extracts and bovine plasma samples was measured.

EXPERIMENTAL

Materials

Putrescine dihydrochloride and cadaverine dihydrochloride as well as ethyl acetate for HPLC were purchased from E. Merck (Darmstadt, F.R.G.). OAB was prepared by reducing *o*-nitrobenzaldehyde [9]. A solution of Δ^1-pyrroline was obtained by treating γ-aminobutyraldhyde diethyl acetal (Janssen, Beerse, Belgium) with 50 m*M* hydrochloric acid.

DAO from pea seedlings was prepared according to the method of Hill [10] up to Step 4, giving an enzymatic preparation with a protein content of 1.87

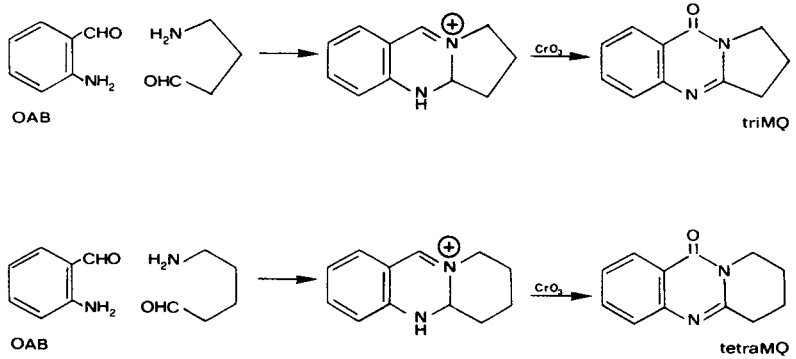


Fig. 2. Scheme of post-enzymatic reactions used for derivatization of aminoaldehydes which are produced by reaction of DAO with putrescine and cadaverine.

mg/ml. Bovine plasma samples were obtained in heparinized tubes by routine venous puncture from a normal healthy adult animal and stored frozen until analysis.

Pure triMQ was obtained on a preparative scale from Δ^1-pyrroline according to the procedure described below. 2,3-Tetramethylene-4-quinazolone (tetraMQ, Fig. 2) was obtained in the same manner from Δ^1-piperideine. This was produced by reaction of pea seedling DAO with cadaverine.

Chromatographic conditions

The HPLC unit consisted of a Twincle pump, an injector and a Uvidec 100 III ultraviolet detector (Jasco, Japan). Analyses were performed using a LiChrosorb Si 100 column (250 × 4.6 mm, particle size 7 μm) (Merck). Ethyl acetate was used as an eluent at a flow-rate of 2 ml/min. Absorbance was measured at 268 nm.

Post-enzymatic reaction conditions

In a set of screw-capped test tubes, samples containing 0.1 ml of 10% trichloroacetic acid and 20 nmol of Δ^1-pyrroline in 1 ml of aqueous solution were treated with 0.1 ml of 10 m*M* OAB for different times at various temperatures (room temperature, 50° C and 80° C). Then 0.5 ml of 1 *M* CrO_3 in 2 *M* sulphuric acid was added and the mixtures were kept for different times at the temperatures mentioned above. The solutions were made alkaline with 5 *M* sodium hydroxide and extracted with 3 ml of diethyl ether containing 20 nmol of tetraMQ, which served as internal standard. The organic phase was removed and a second extraction with 3 ml of diethyl ether was performed. The combined organic phases were evaporated to dryness; the residue was redissolved in 50 μl of ethyl acetate and 5-μl portions were analysed by HPLC. By comparing ratios between peak heights of triMQ derived from Δ^1-pyrroline and tetraMQ, the yield of the reaction was evaluated. Maximum yields were obtained either at room temperature after 2-h treatment with OAB and 4-h treatment with CrO_3 or by two treatments of 15 min at 80° C. These latter conditions were used in the subsequent experiments.

Calibration curve

In 1 ml of 25 m*M* phosphate buffer pH 6.5, amounts of cadaverine varying between 1 and 20 nmol were enzymatically converted to Δ^1-piperideine by addition of pea seedling DAO and OAB in a large excess and warming the solution at 37° C for 20 min. The reaction was stopped by the addition of 10% trichloroacetic acid. Then 10 nmol of Δ^1-pyrroline were added as internal standard and the mixtures were subjected to the procedure described above except that tetraMQ was not added before the extraction step. TetraMQ and triMQ obtained from cadaverine by enzymatic oxidation and from Δ^1-pyrroline, respectively, were analysed by HPLC and the ratios between their peak heights were calculated and plotted against the amounts of cadaverine.

DAO essay

Various aliquots (2—5 μl) of the enzyme preparation from pea seedlings or different volumes of bovine plasma (0.2—0.5 ml) were diluted to 1 ml with

phosphate buffer (pH 6.5 for pea seedling extracts, pH 8.0 for plasma). After the addition of 1 μmol of cadaverine and 1 μmol of OAB the mixtures were kept for 10 min at 37°C. The reaction was stopped with 10% trichloroacetic acid and the mixtures were treated as described above. DAO activity was calculated from the amount of tetraMQ formed by enzymatic oxidation of cadaverine:

$$\text{Activity (mU/ml)} = \frac{\text{nmoles tetraMQ}}{\text{10 (min)} \times \text{sample volume (ml)}}$$

One U is defined as the amount of enzyme which catalyses the oxidation of 1 μmol of substrate per min under the described conditions.

RESULTS

In Fig. 3 the HPLC profile of an enzymatic assay is shown. Under the conditions described the retention times of tetraMQ and triMQ were 3.9 min and 6.8 min, respectively.

A linear relationship between the ratios of the peak heights (R_h) of tetraMQ and triMQ and the amount of oxidized substrate was found in the range 1—20 nmol according to the equation

$$R_h = 0.532 \times \text{oxidized cadaverine (nmol)} + 0.091 \quad (r = 0.997)$$

By this method tetraMQ could be determined in amounts at least as low as 0.2 nmol, and therefore 0.02 mU of DAO could be detected. The reproducibility of the method was determined by repeated analyses of the same DAO preparation from pea seedlings. Enzyme activity and reproducibility were 21.5 ± 0.82 mU/ml (mean ± S.D., $n = 5$).

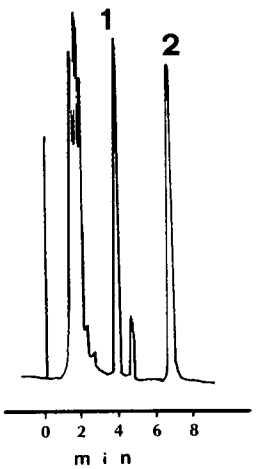


Fig. 3. HPLC profile of a sample assayed for DAO activity. Peaks: 1 = tetraMQ; 2 = triMQ.

Five bovine plasma samples obtained from the same animal on different days during two weeks were tested. The DAO activity was found to be between 0.92 and 1.32 mU/ml with a mean of 1.14 mU/ml.

DISCUSSION

The reactions shown in Fig. 2, which were described by Sakamoto and Samejina [8], were shown to be a reliable and simple basis for a method to trap short-chain aliphatic aminoaldehydes and allow the determination of the DAO-catalysed oxidation product of cadaverine and putrescine. By increasing the temperature, the previously reported reaction times can be decreased. Because of the stability of triMQ and tetraMQ, samples can be stored for several hours at room temperature without loss.

Cadaverine was preferred to putrescine as substrate because of the commercial availability of γ-aminobutyraldehyde diethyl acetal. Thus Δ^1-pyrroline could be used as internal standard.

The extraction of triMQ and tetraMQ from aqueous solution in an organic phase eliminates possible interferences deriving from biological material. Moreover, the convenient evaporation of organic solvents allows highly concentrated samples to be obtained, thus increasing the detection sensitivity. The separation of triMQ and tetraMQ by HPLC on silica is very effective and the short retention times are suitable for routine analysis.

The present method does not reach the sensitivity of the radiochemical method, which is still the procedure most used in putrescine metabolism studies [11]. We can state, however, that, because of its high reliability, the described method appears to be a useful alternative procedure for DAO activity assay in plant extracts and animal plasma samples. Additional experiments are required to apply this method to tissue homogenates.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Ministero della Pubblica Istruzione. The authors wish to thank Miss Anna Berrini for her helpful technical assistance.

REFERENCES

1 S.B. Baylin, Proc. Nat. Acad. Sci. U.S., 74 (1977) 883.
2 G.D. Luk, T.M. Bayless and S.B. Baylin, J. Clin. Invest., 66 (1980) 66.
3 T. Okuyama and Y. Kobayashi, Arch. Biochem. Biophys., 95 (1961) 242.
4 J. Kusche, H. Richter, R. Hesterberg, J. Schmidt and W. Lorenz, Agents Actions, 3 (1973) 148.
5 P.G. Pietta, A. Calatroni and R. Colombo, J. Chromatogr., 243 (1982) 123.
6 E. Neufeld and R. Chayen, Anal. Biochem., 96 (1979) 411.
7 B. Holmsted, L. Larsson and R. Tham, Biochim. Biophys. Acta, 48 (1961) 182.
8 S. Sakamoto and K. Samejina, Chem. Pharm. Bull., 27 (1979) 2220.
9 L.I. Smith and J.W. Opie, in H.C. Horning (Editor), Organic Syntheses, Vol. 3, Wiley, New York, 1955, p. 56.
10 J.M. Hill, Methods Enzymol., 17 B (1971) 730.
11 W.A. Fogel, T. Bieganski, R. Shayer and C. Maslinski, Agents Actions, 11 (1981) 679.

Journal of Chromatography, 309 (1984) 156—159
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2121

Note

Determination of monoamine oxidase B activity by high-performance liquid chromatography

ERKKI NISSINEN

Orion Pharmaceutical Co., Research Center, P.O. Box 8, SF-02101 Espoo 10 (Finland)

(First received December 6th, 1983; revised manuscript received February 13th, 1984)

Monoamine oxidase (MAO; monoamine O_2:oxidoreductase, EC 1.4.3.4) is the enzyme which catalyses the oxidative deamination of monoamines. It has a broad substrate specificity and it plays a major role in the catabolism of monoamines in the central nervous system. Two forms of MAO, designated MAO A and MAO B, have been identified both in vivo and in vitro in animal brain [1]. They are demonstrable by their preference for different amines as substrates and by their sensitivity to specific inhibitors. MAO A preferentially deaminates norepinephrine and 5-hydroxytryptamine and is selectively inhibited by clorgyline. MAO B deaminates benzylamine and β-phenylethylamine and is sensitive to inhibition by deprenyl and pargyline [2].

MAO activity can be determined by a large number of different techniques. These include spectrophotometry [3, 4], fluorometry [5, 6], polarographic monitoring of oxygen consumption [7], and measurement of ammonia [8] or hydrogen peroxide [9] formed in the reaction. The most frequently used are radiochemical assays because of their specificity and sensitivity [10—12]. However, radiochemical assays require time-consuming extractions and use of rather expensive isotopes.

High-performance liquid chromatography (HPLC) is an accurate and a sensitive method to assay many of the catecholamine-metabolizing enzymes [13]. MAO A activity and non-specific MAO activity have been determined using HPLC with fluorescence detection [14]. This communication describes a fast, simple assay for MAO B activity using benzylamine as substrate, deproteinization with perchloric acid and detection of the product benzaldehyde at 254 nm.

0378-4347/84/$03.00

EXPERIMENTAL

Reagents

Benzylamine hydrochloride and benzaldehyde were obtained from EGA-Chemie (Steinheim, F.R.G.). Pargyline was from Sigma (St. Louis, MO, U.S.A.). Clorgyline was purchased from May & Baker (Dagenham, U.K.). Methanol, HPLC grade, was from Orion Pharmaceutical Co. (Espoo, Finland). Sodium heptane sulphonic acid was from Eastman-Kodak (Rochester, NY, U.S.A.). All other reagents were of analytical grade and purchased from commercial sources.

Sample preparation

Female rats weighing about 200 g were killed with carbon monoxide. Brains were quickly removed and homogenized in four volumes of cold 0.9% potassium chloride. Homogenates were kept in small aliquots at −20°C until assayed. Protein determinations were carried out using a Bio-Rad protein assay kit (Bio-Rad Labs., Richmond, CA, U.S.A.).

Assay

The enzyme incubation mixture contained the following components in a total volume of 0.5 ml: 0.35 ml of 0.2 *M* sodium phosphate buffer pH 7.2, 0.1 ml of brain homogenate and 0.05 ml of 2 m*M* benzylamine. The mixture was incubated for 30 min at 37°C except for the study of time course. The reaction was stopped by the addition of 50 μl of 4 *M* perchloric acid. Protein was removed by centrifugation. A 20-μl aliquot was injected into the liquid chromatograph.

Chromatography

The modular liquid chromatographic system consisted of a Waters Model 6000A pump, a Waters Intelligent Sample Processor (WISP) Model 710 B (Waters Assoc., Milford, MA, U.S.A.), a 150 × 4.6 mm, 5-μm Ultrasphere-ODS column fitted with a 45 × 4.6 mm precolumn (Beckman Instruments, Fullerton, CA, U.S.A.). The eluted components were detected by ultraviolet (UV) absorption at 254 nm with a Waters Model 441 fixed-wavelength detector. The elution was carried out isocratically at ambient temperature using 40% methanol containing 50 m*M* sodium phosphate and 1 m*M* heptane-sulphonic acid. The pH was adjusted to 3.2 with sodium hydroxide. The flow-rate was 1.5 ml/min. The enzyme activity was calculated as nmoles benzaldehyde formed per min per mg protein.

RESULTS AND DISCUSSION

Fig. 1 shows typical chromatograms obtained with crude rat brain homogenate. The blank (Fig. 1A), i.e. homogenate without substrate, shows that no interfering peaks are present in the described assay system. The retention time for the product benzylaldehyde is 5.2 min (Fig. 1B). It can be further decreased down to 3 min by increasing methanol and phosphate concentrations or omitting the ion-pairing reagent. The assay system was developed

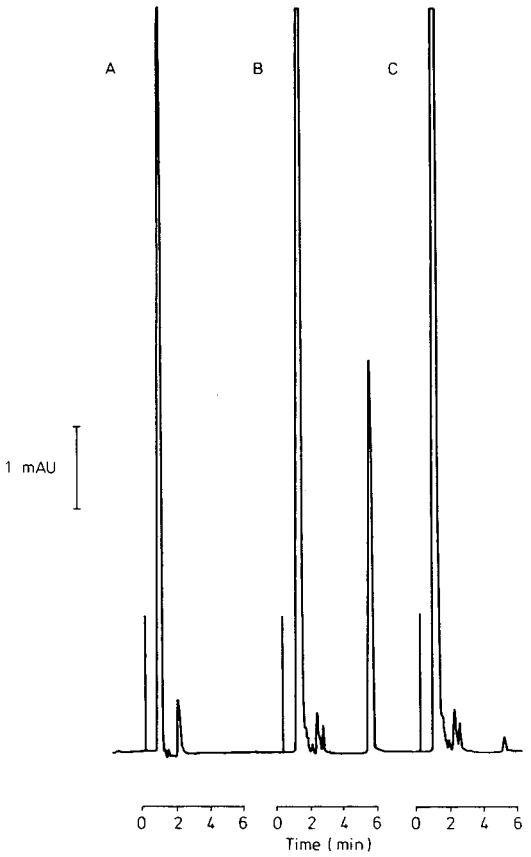


Fig. 1. Chromatograms obtained with MAO preparations: (A) blank, i.e. rat brain homogenate incubated without benzylamine; (B) homogenate incubated with 0.2 m*M* benzylamine; (C) homogenate incubated with 0.2 m*M* benzylamine and 10 n*M* pargyline. Chromatographic conditions: 5-μm Ultrasphere-ODS column (4.5 × 150 mm); mobile phase, 40% methanol in 50 m*M* sodium phosphate buffer, pH 3.2, containing 1 m*M* sodium heptanesulphonic acid; flow-rate, 1.5 ml/min; detection at 254 nm; sensitivity, 0.01 a.u.f.s., injection volume, 20 μl.

mainly for rapid screening of MAO B inhibitors, which would have interfered with the product in other elution systems.

Since the inhibition of MAO activity with pargyline decreases the size of the peak with retention time corresponding to benzaldehyde (Fig. 1C), it can be concluded that no endogenous compounds are present in the system. It also confirms the specific and sensitive inhibition of MAO B activity by pargyline. The activity was not diminished in the presence of 10 n*M* clorgyline which selectively inhibits MAO A [10].

The calibration curve indicates a linear relationship between the peak height and the amount of benzaldehyde from 0.2 to 20 nmol/ml. The detection limit with signal-to-noise ratio of 5:1 was 150 pmol/ml, making it possible to detect very low MAO B activities.

The rate of aldehyde formation expressed as growing height of the benzaldehyde peak on the liquid chromatogram showed a linear relationship Y =

$9.27X + 0.47$, $r = 0.996$ ($n = 3$), with up to 60 min of incubation time. The standard error between data points was less than 3.0%. Linearity was also demonstrable between the amount of enzyme preparation for 10—250 μg protein and enzymatically formed benzaldehyde during 30 min of incubation at 37°C. It is described by the equation $Y = 0.13X + 0.33$ with $r = 0.994$ and C.V. < 5.0% between data points ($n = 3$).

The reproducibility of the assay using the standard procedure ($n = 6$) was 2.5%, expressed as coefficient of variation. This precision was achieved without the use of an internal standard and illustrates the excellent properties of HPLC techniques for accurate enzyme activity determinations. Since the sample recovery is 100%, no internal standard is required.

The MAO B activity in whole rat brain ($n = 6$) with 0.2 m*M* benzylamine as substrate was 1.28 ± 0.03 nmol/min/mg protein. This value is in close agreement with the values obtained by radiochemical assay [15].

MAO B activity has recently been assayed also using gas chromatography (GC). However, these techniques require derivatization procedures before the products of MAO reaction can be determined. The derivatization of hydrogen peroxide for GC is fast but produces high blanks [16], while the derivatization of aldehydes for GC is slow [17].

The described HPLC method is precise, sensitive and rapid, allowing the handling of about sixty samples per day and thus making it useful for rapid screening of MAO B activity in different tissues.

REFERENCES

1 J.P. Johnston, Biochem. Pharmacol., 17 (1968) 1285.
2 J. Knoll and K. Magyar, Adv. Biochem. Psychopharmacol., 5 (1972) 393.
3 C.W. Tabor, H. Tabor and S.M. Rosenthal, J. Biol. Chem., 208 (1954) 645.
4 H. Weissbach, T.E. Smith, J.W. Daley, B. Witkop and S. Udenfriend, J. Biol. Chem., 235 (1960) 1160.
5 K.F. Tipton, Anal. Biochem., 28 (1969) 318.
6 K. Zaitsu, H. Nagai, K. Kohashi and Y. Ohkura, Chem. Pharm. Bull., 25 (1977) 918.
7 D.R. Weetman and A.J. Sweetman, Anal. Biochem., 41 (1971) 517.
8 H. Yamada, H. Suzuki and Y. Ogura, Adv. Biochem. Psychopharmacol., 5 (1972) 185.
9 H.Y. Meltzer and S.M. Stahl, Res. Commun. Chem. Pathol. Pharmacol., 7 (1974) 419.
10 J.A. Roth and K. Feor, Biochem. Pharmacol., 27 (1978) 1606.
11 C. Mitra and S.R. Guha, Biochem. Pharmacol., 29 (1980) 1213.
12 R. Lewinsohn, V. Glover and M. Sandler, Biochem. Pharmacol., 29 (1980) 777.
13 P.T. Kissinger, C.S. Bruntlett and R.E. Shoup, Life Sci., 28 (1981) 455.
14 R.D. Kobes, R.J. Wyatt and L.M. Neckers, J. Pharmacol. Methods, 3 (1980) 305.
15 C.J. Fowler, L. Oreland, J. Marcusson and B. Winblad, Naunyn-Schmiedeberg's Arch. Pharmacol., 311 (1980) 263.
16 K. Kobayashi, S. Kawai, T. Nakana and T. Nagatsu, J. Chromatogr., 274 (1983) 313.
17 A. Uji, S. Kawai and T. Nagatsu, J. Chromatogr., 221 (1980) 155.

Journal of Chromatography, 309 (1984)160—164
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2132

Note

Rapid isocratic high-performance liquid chromatographic purification of platelet activating factor (PAF) and *lyso*-PAF from human skin

A.I. MALLET*, F.M. CUNNINGHAM and R. DANIEL

The Wellcome Laboratories for Skin Pharmacology, Institute of Dermatology, Homerton Grove, London E9 6BX (U.K.)

(First received January 9th, 1984; revised manuscript received February 29th, 1984)

Platelet activating factor (PAF) is a mixture of at least two 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphorylcholines in which the length of the alkyl substituent can vary. It is reported that the natural product is a mixture of straight chain C_{16} and C_{18} homologues in 9:1 mol/mol proportions [1]. Although PAF was first recognised by virtue of its potent effect on the aggregation and degranulation of platelets [2], more recent research has demonstrated a wider spectrum of biological activity including induction of leukocyte aggregation, chemotaxis and chemokinesis, generation of superoxide and lysosomal enzyme release [3]. A variety of cell types synthesise and release PAF following stimulation [4].

Psoriasis is an inflammatory skin disorder characterised by an epidermal neutrophil infiltrate. Biologically active concentrations of arachidonic acid metabolites have been shown to be present in the involved skin of psoriatics [5] and may, by virtue of their chemotactic properties, be relevant to the pathogenesis of the neutrophil infiltrate. As PAF is also chemotactic and chemokinetic for human neutrophils [6], we were interested in measuring PAF in the skin of psoriatic patients and have developed a rapid, isocratic high-performance liquid chromatographic (HPLC) procedure for the recovery of substantial amounts of PAF-like biologically active material from psoriatic plaque.

PAF itself does not absorb in the UV above 200 nm and, in order to permit the use of phosphorylcholines as markers for the elution of PAF, solvents which permit the eluent to be monitored at 210 nm or lower must be employed. To be able to process a reasonably large number of samples an isocratic HPLC method is preferred, and it is essential that the column provides

0378-4347/84/$03.00

reproducible retention volumes for the analyte. In addition, the method should ensure the separation of PAF from other lipids which have similar biological activity, and from the metabolite, *lyso*-PAF, which is biologically inactive [7].

The HPLC separation of phospholipids on a variety of stationary phases has been reviewed [8]. The majority of systems described have employed silica gel phases eluted with hexane—alcohol mixtures containing varying proportions of water often with the admixture of acid or base [9]. In 1981 Briand et al. [10] described gradient elution HPLC using a DIOL-bonded LiChrosorb packing material, eluted with acetonitrile—water solvent mixtures, for the separation of phosphorylcholines and reported the improved peak shapes obtained using this material. Several HPLC separation methods have been reported for PAF itself [11—13], but none of these has, to date, satisfied all of the criteria mentioned above.

MATERIALS AND METHODS

Psoriatic plaque was obtained from volunteer patients by gentle scraping of the lesional areas, a technique yielding 250—1000 mg scale. Scale from separate individuals was used, none of whom had received any treatment for at least two weeks.

Lipids were extracted by disintegrating (Polytron, Kinematica, Luzern, Switzerland) in chloroform—methanol (2:1, v/v) (100 ml/g scale). After centrifugation (2000 *g*, 10 min) and decanting, 20% of its volume of water was added to the supernatant, the mixture was shaken and allowed to separate. The lower layer was collected and evaporated to dryness. The extract was then applied, in solution in chloroform—methanol (2:1), as a streak to a 20 × 20 cm thin-layer chromatographic (TLC) plate (Silica gel G, 500 μm; Anachem, Luton, U.K.) and developed in chloroform—methanol—acetic acid—water (50:25:10:4, v/v), in a filter-paper-lined, pre-saturated glass tank at room temperature. A second TLC plate was developed simultaneously, with two marker spots of lecithin and *lyso*-lecithin, which were visualised using iodine vapour. PAF runs on this system between the two markers (typical R_F 0.26) and this region of the plates was scraped off and extracted with chloroform—methanol—water (1:2:0.8, v/v). After centrifugation (2000 *g*, 10 min), 25% by volume each of water and chloroform were added to the supernatant and the mixture was shaken. The lower layer was recovered and evaporated to dryness. This procedure obviates the carry-over of silica gel into the organic extract [14].

HPLC was performed on a 10-μm LiChrosorb DIOL (Technicol, Stockport, U.K.) 25 cm × 4.6 mm column using a solvent mixture of *tert.*-butylmethyl ether—methanol—water—ammonia (200:100:10:0.02, v/v). The relative amounts of the first two solvents may be varied over a wide range in order to achieve convenient retention volumes for specific compounds. Using the solvent mixture described above, the UV monitor could be operated down to a limit of 207 nm. The chromatography was performed on a Waters Model 6000 pump with a Rheodyne valve injector and a Pye Unicam PU 4020 variable-wavelength detector. Solvent flow-rates between 1 and 2 ml/min were used

throughout. Radioactivity eluting from the column was monitored, either on a Reeve Analytical (Glasgow, U.K.) radioactivity monitor, or by fraction collection and scintillation counting.

The chemokinetic activity of the fractions collected from the HPLC system was determined by an agarose micro-droplet assay [15].

[^{14}C]PAF, [^{14}C]*lyso*-PAF and [^{3}H]PAF were obtained from Amersham International (Amersham, U.K.) and all phospholipids were supplied by Sigma (Poole, U.K.). The chromatographic solvents were of HPLC grade (Fisons, Loughborough, U.K.). Synthetic PAF and *lyso*-PAF were obtained from Cambridge Research Biochemicals (Cambridge, U.K.). A 9:1 w/w mixture of C_{18} and C_{16} PAF has been used to represent "authentic" PAF.

RESULTS AND DISCUSSION

The majority of published methods for the HPLC of phosphorylcholines which have used solvents with sufficient UV transparency to permit monitoring at 210 nm or lower, employed silicic acid columns with aqueous organic solvents. In our hands these methods failed to give reproducible retention times for the two phosphorylcholines, lecithin and lysolecithin, the major sources of difficulty lying in the varying activity of the column and in the near saturating quantities of water required to provide reasonable retention volumes for these very polar substances.

One publication has reported [10] the use of DIOL LiChrosorb packing material in this field, and its bonded nature might be expected to provide a packing material with more controlled activity than silicic acid itself. Polar solvents are required to elute the cholines from this column and we have used a mixture of *tert.*-butylmethyl ether, methanol and water with the addition of a trace of ammonia. The latter improves the peak shape considerably over the use of a neutral solvent mixture. This solvent can be used down to 207 nm and is sufficiently volatile to be removed easily by blowing dry with nitrogen at 45° C.

Fig. 1 shows a chromatogram obtained from the injection of a mixture of phospholipids onto this system. The profile of recovered radioactivity from coinjected [^{14}C]PAF and [^{14}C]*lyso*-PAF is also shown. PAF is separated from lecithin, *lyso*-lecithin and from *lyso*-PAF. Sphingomyelin, with which PAF comigrates on TLC, is also separated from PAF.

We have applied this system to the extraction of PAF from human psoriatic scale. The final analysis for PAF depends upon chemokinetic activity and it has been important to employ a procedure which would separate PAF from other biologically active lipids, such as arachidonic acid metabolites [5] and from complement-derived peptides [16]. The lesional psoriatic scale obtained from volunteers was first extracted using the method of Folch et al. [17]. The total lipid fraction produced was then separated on a TLC system in which PAF ran at a low R_F between lecithin and *lyso*-lecithin. All the arachidonic acid metabolites were eluted to the top of the plate. The region of the TLC plate containing the PAF was then extracted and the material thus obtained applied to the HPLC system described above. One-minute fractions (1.5 ml) of the eluting material were collected, evaporated to dryness and assayed for

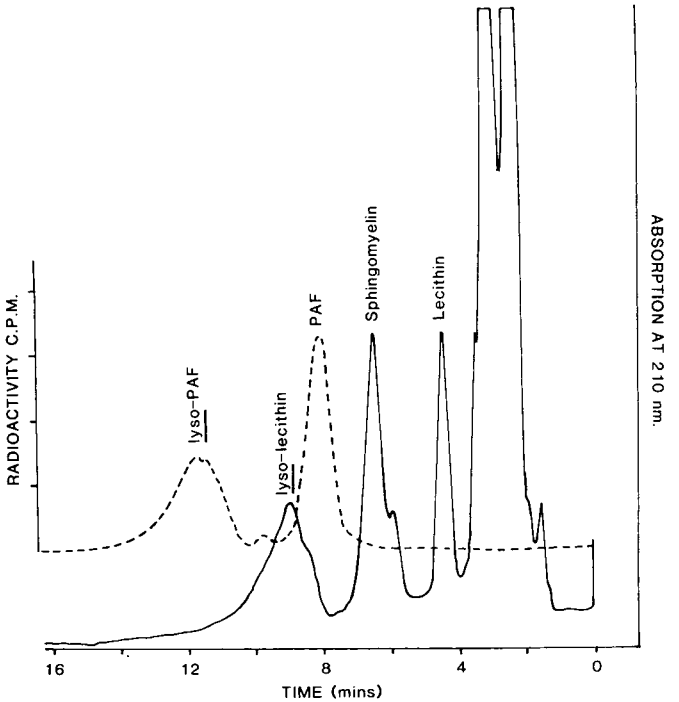


Fig. 1. HPLC traces for the separation of phosphorylcholines at 210 nm (solid line) and of [^{14}C]PAF and [^{14}C]*lyso*-PAF using an on-line radioactivity monitor (dashed line).

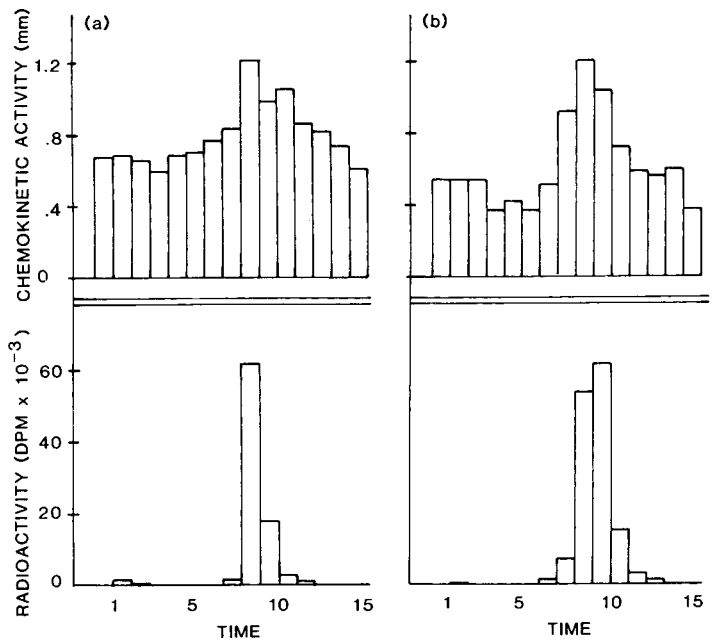


Fig. 2. Profiles of chemokinetic activity (upper part) and radioactivity (lower part) obtained from successive 1-min fractions collected from an HPLC column are shown for 100 ng authentic PAF (a) and for extracted psoriatic scale (b).

leukocyte chemokinetic activity. Samples of authentic PAF and [^{3}H]PAF were also run on the same system and the fractions were collected as before. The profiles of the biological activity and radioactivity so obtained are shown in Fig. 2. Here the excellent coincidence of the maxima of biological activity and radioactivity for a sample of authentic PAF (Fig. 2a) and a sample of extracted psoriatic scale (Fig. 2b), can be seen. This result has been confirmed in three patients and it seems clear that PAF-like material can be extracted from this source. Comparison of the levels of biological activity obtained from psoriatic scale with a dose response curve for authentic PAF indicates that some 20—100 ng of PAF-like material are extractable from 1 g of scale.

Whether the PAF-like material is released from polymorphonuclear leukocytes (PMNs) already present in psoriatic lesions, or is necessary for the initial migration of PMNs into the affected site, remains to be established, as does the structural identity of the PAF-like material we have isolated. We are now preparing mass spectrometric identification and assay procedures firstly to confirm these findings and, secondly, to enable more precise quantitation. The chromatographic methods described here should also prove to be of use in the separation of other classes of phospholipids.

ACKNOWLEDGEMENTS

The work described here was supported in part by the Medical Research Council, the Wellcome Trust and the Nuffield Foundation.

REFERENCES

1 D.J. Hanahan, C.A. Demopoulos, J. Liehr and R.N. Pinckard, J. Biol. Chem., 255 (1980) 5514.
2 J. Benveniste, P.M. Henson and C.G. Cochrane, J. Exp. Med., 136 (1972) 1356.
3 E. Jouvin-Marche, B. Poitevin and J. Benveniste, Agents Actions, 12 (1982) 716.
4 J. Benveniste, R. Roubin, M. Chignard, E. Jouvin-Marche and J.P. Le Couedic, Agents Actions, 12 (1982) 711.
5 S.D. Brain, R.D. Camp, P.M. Dowd, A.K. Black, P.M. Woollard, A.I. Mallet and M.W. Greaves, Lancet, ii (1983) 762.
6 E.J. Goetzl, C.K. Derian, A.I. Tauber and F.H. Valone, Biochem. Biophys. Res. Commun., 94 (1980) 881.
7 J. Polonsky, M. Tence, P. Varenne, B.C. Das, J. Lunel and J. Benveniste, Proc. Nat. Acad. Sci. U.S., 77 (1980) 7019.
8 A. Nasner and Lj. Kraus, J. Chromatogr., 216 (1981) 389.
9 J.R. Yandrasitz, G. Berry and S. Segal, J. Chromatogr., 225 (1981) 319.
10 R.L. Briand, S. Harold and K.G. Blass, J. Chromatogr., 223 (1981) 277.
11 F.H. Chilton, J.T. O'Flaherty, J. Marshall Ellis, C.L. Swendsen and R.L. Wykle, J. Biol. Chem., 258 (1983) 6357.
12 J. Benveniste, J.P. LeCouedic, J. Polonsky and M. Tence, Nature, 269 (1977) 170.
13 M.L. Blank and F. Snyder, J. Chromatogr., 273 (1983) 415.
14 M.M. Billah and J.M. Johnston, Biochem. Biophys. Res. Commun., 113 (1983) 51.
15 M.J.H. Smith and J.R. Walker, Brit. J. Pharmacol., 69 (1980) 473.
16 H. Tagami and S. Ofuji, Brit. J. Dermatology, 97 (1977) 509.
17 J. Folch, M. Lees and G.H. Sloane-Stanley, J. Biol. Chem., 226 (1957) 497.

Journal of Chromatography, 309 (1984) 165—169
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2128

Note

Gas chromatographic method for the routine serum monitoring of mexiletine

ODETTE GRECH-BÉLANGER

School of Pharmacy, Laval University, Ste-Foy, Quebec G1K 7P4 (Canada)

(First received December 2nd, 1983; revised manuscript received February 23rd, 1984)

Mexiletine [1-(2,6-dimethylphenoxy)-2-aminopropane] is a class 1 antiarrhythmic drug effective in the management of ventricular arrhythmias [1, 2]. Its electrophysiological properties are similar to those of lidocaine, but it differs from the latter in that it is active orally and that it has a long elimination half-life. Effective plasma concentrations fall in the range 0.75—2.0 mg/l [3, 4]; an unacceptable frequency of serious side effects occurs at plasma concentrations higher than 2.0 mg/l [5].

Various gas chromatographic (GC) methods for the determination of mexiletine in serum or plasma have been published. In addition to flame-ionization detection [6, 7], both nitrogen-sensitive [8—12] and electron-capture [13—17] detection have been used. Some procedures [11—13] require a back-extraction of the drug in order to obtain a clean sample and all except one method [7] require 1.0 ml or more of serum or plasma. So far, only one of these methods has been adapted to monitor serum mexiletine levels during therapy [7]. A disadvantage of this method is that 2,7-dimethylquinoline, the external standard used, besides being very different in structure from mexiletine, is kept in solution in chloroform, a solvent that is known to react with amino compounds [18, 19]. Also due to the volatility of this solvent, the concentration of the reference standard may vary on storage. A modification of this method has recently been reported [20]. In this case, 2,7-dimethylquinoline is kept in acidic aqueous solution and used as internal standard and chloroform is replaced by dichloromethane as extracting solvent.

A simple GC method using flame-ionization detection for the routine monitoring of mexiletine serum levels is described herein. Only 500 μl of serum or plasma are required and the internal standard used, besides being a primary aliphatic amine like mexiletine, is stable for several months when kept in aqueous solution.

0378-4347/84/$03.00

EXPERIMENTAL

Reagents and materials

All chemicals used were analytical reagent grade: isopropanol, *n*-pentane and diethyl ether were distilled before use. Mexiletine hydrochloride and its two metabolites, 1-(2,6-dimethyl-4-hydroxyphenoxy)-2-aminopropane hydrochloride and 1-(2-hydroxymethyl-6-methylphenoxy)-2-aminopropane oxalate were gifts from Boehringer Ingelheim Canada (Burlington, Canada), whilst rimantadine hydrochloride (1-methyl-1-adamantylmethylamine) was given by Du Pont de Nemours (Wilmington, DE, U.S.A.).

Synthesis of N-acetylmexiletine

To 500 mg of mexiletine base dissolved in sodium-dried diethyl ether, 1 ml of acetic anhydride was added and the resulting solution was stirred for 3 h at room temperature. The solvent in the reaction mixture was then evaporated under vacuum and the residue was redissolved in 100 ml of diethyl ether. This ethereal solution was washed successively with 15 ml of 2 mol/l hydrochloric acid, 15 ml of 2 mol/l sodium hydroxide and water, dried over anhydrous sodium sulphate, filtered and evaporated to dryness to give an oily residue which was crystallized from methanol as a white solid; m.p. 70.5°C (uncorrected); IR (KBr disc) 3250 (NH), 1615 (CO), 1530 (NH) cm^{-1}; electron-impact mass spectrum (solid inlet, 70 eV) *m/z* 100 (100%), 58 (80%), 44 (37%), 43 (35%), 77 (14%), 91 (11%), 122 (7%).

Instrumentation

A Hewlett-Packard gas chromatograph Model 5711A equipped with a flame-ionization detector and an integrator was used. The coiled glass column (1.2 m × 4 mm I.D.) which was packed with 100—120 mesh Gas Chrom Q, coated with OV-17 3% was preconditioned at 250°C for 16 h and treated with hexamethyldisilazane before use. The injection and detector temperatures were set at 250°C and 300°C, respectively, while the column temperature was kept at 180°C. The nitrogen (carrier gas) flow-rate was 60 ml/min and the hydrogen and air flow-rates were 60 and 240 ml/min, respectively.

Extraction procedure

A 0.5-ml aliquot of serum sample was rendered alkaline by the addition of 1.0 ml of sodium hydroxide (2 mol/l); 0.5 ml of rimantadine hydrochloride solution containing 2.5 mg/l of the base was added as internal standard and the mixture was extracted twice with *n*-pentane containing 3% isopropanol. The organic extracts were combined and evaporated to dryness in a water-bath at 44°C. The residue was dissolved in 100 μl of diethyl ether, 2 μl of acetic anhydride was added and the resulting solution was returned to the water-bath for 10 min and afterwards analyzed by gas—liquid chromatography (GLC).

Calibration curves

Aqueous solutions of mexiletine hydrochloride and of rimantadine hydrochloride were added to serum or plasma. The concentration range of mexiletine used was 0.1—5.0 mg/l and the concentration or rimantadine hydrochloride

was 2.5 mg/l. All the samples were extracted and analyzed using the procedure described above. Calibration curves based on the area ratios of mexiletine to the internal standard were constructed using six different concentrations of mexiletine analyzed in duplicate for each sample. The data were subjected to linear-regression analysis to give the appropriate calibration factor.

RESULTS AND DISCUSSION

The structure of synthesized acetylmexiletine was confirmed by solid-inlet mass spectrometry. A sample of this compound gave a single peak having the same retention time as that of the derivatized mexiletine in the serum samples. Analysis of mexiletine as its acetyl derivative was necessary in order to eliminate interference from the serum or plasma constituents. Rimantadine is also a primary amine and it reacts with acetic anhydride under the same conditions as mexiletine. Derivatization of both compounds was optimal and reproducible using the conditions described herein.

Acetic anhydride was used by Kelly [6] to form the acetyl derivative of mexiletine prior to GLC analysis. The reaction was carried out using a large volume of the reagent (20 μl) and excess reagent was evaporated at 60°C under nitrogen. The present method uses only 2 μl of acetic anhydride and therefore no evaporation of excess reagent is necessary. The reaction is complete within 10 min.

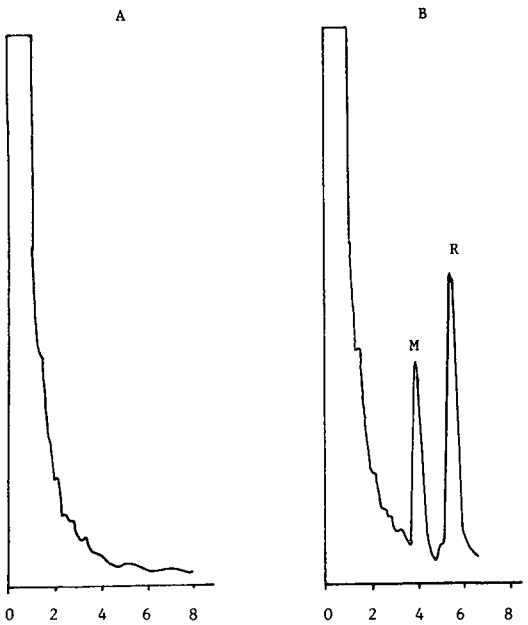


Fig. 1. Representative gas—liquid chromatograms of (A) blank human serum and (B) serum sample containing 0.8 mg/l mexiletine obtained from a patient stabilized on 200 mg of mexiletine four times a day. Peaks: M = mexiletine; R = rimantadine, internal standard.

n-Pentane containing 3% isopropanol was preferred to other low-boiling-point extracting solvents such as diethyl ether or dichloromethane since it gave a cleaner extract. When serum samples were spiked with mexiletine to give final concentrations of 0.5, 2.0 and 5.0 mg/l and extracted as described except that rimantadine was used as an external standard, the recoveries were 94%, 96% and 99%, respectively. The same procedure was repeated for rimantadine, with similar results. In this case, mexiletine was used as the external standard.

Fig. 1A is a chromatogram of an extract of blank serum treated with acetic anhydride while Fig. 1B is a chromatogram of a similarly treated sample containing 0.8 mg/l mexiletine; the internal standard was added prior to extraction. The retention times of the acetyl derivatives of mexiletine and rimantadine were 4.0 and 5.7 min, respectively.

The calibration curve was linear between 0.1 and 0.5 mg/l. The mean concentration, standard deviation and coefficient of variation for intra- and inter-day analysis of spiked serum samples containing 0.2, 1.0 and 5.0 mg/l mexiletine are given in Table I. The selectivity of the assay was checked against the parahydroxy and the ring monohydroxymethyl derivatives of mexiletine which have been shown to be the two major metabolites of mexiletine [21, 22] as well as against the following cardiovascular drugs, hypnotics and tranquillizers: disopyramide, lidocaine, procainamide, diltiazem, nifedipine, verapamil, isosorbide nitrate, prazosin, hydralazine, amiloride, flurazepam, diazepam, lorazepam, triazolam, chlorpromazine and theophylline. Interference from the above mentioned drugs was checked by analyzing the serum of several cardiac patients taking one or more of these compounds thus indicating selectivity of the mexiletine assay against the metabolites of such drugs as well.

TABLE I

INTRA- AND INTER-ASSAY VARIATIONS IN GC MEASUREMENT OF SERUM MEXILETINE (n = 4)

Concentration (mg/l)	Intra-assay		Inter-assay	
	Mean ± S.D. (mg/l)	C.V. (%)	Mean ± S.D. (mg/l)	C.V. (%)
0.2	0.21 ± 0.01	4.76	0.21 ± 0.01	4.76
1.0	0.95 ± 0.03	3.16	0.99 ± 0.04	4.04
5.0	5.01 ± 0.08	1.60	4.93 ± 0.19	3.85

ACKNOWLEDGEMENTS

The author acknowledges the technical assistance of Mr. Michel Blouin and is grateful to Miss Carole Murphy for typing the manuscript. Boehringer Ingelheim, Burlington, Canada and Du Pont de Nemours, (Wilmington, DE, U.S.A.) are also thanked for supplying pure samples of mexiletine and rimantadine, respectively.

REFERENCES

1 N.P.S. Campbell, V.C. Chaturvedi, J.G. Kelly, J.E. Strong, R.G. Shanks and J.F. Pantridge, Lancet, ii (1973) 404.
2 R.G. Talbot, R.A. Clark, J. Nimmo, J.M.M. Neilson, D.G. Julian and L.F. Prescott, Lancet, ii (1973) 399.
3 N.P.S. Campbell, J.G. Kelly, A.A.J. Adgey and R.G. Shanks, Brit. J. Clin. Pharmacol., 6 (1978) 103.
4 F. Follath, V. Ganzinger, E. Schuetz, Clin. Pharmacokin., 8 (1982) 63.
5 R.G. Talbot, D.G. Julian and L.F. Prescott, Amer. Heart J., 91 (1976) 58.
6 J.G. Kelly, Postgrad. Med. J., 53, Suppl. 1 (1977) 48.
7 D.W. Holt, R.J. Flanagan, A.M. Hayler and M. Loizou, J. Chromatogr., 169 (1979) 295.
8 J.G. Kelly, J. Nimmo, R.G. Rae and L.F. Prescott, J. Pharm. Pharmacol., 25 (1973) 550.
9 I.D. Bradbrook, C. James and H.J. Rogers, Brit. J. Clin. Pharmacol., 4 (1977) 380.
10 K.J. Smith and P.J. Meffin, J. Chromatogr., 181 (1980) 469.
11 S.M. Elfing, E.H. Svens and E.E.A. Leskinen, J. Clin. Chem. Clin. Biochem., 19 (1981) 1189.
12 M.A. Pilling, J. Tse and K. Chan, Meth. Find. Exp. Clin. Pharmacol., 4 (1982) 243.
13 R.J. Perchalski, B.J. Wilder and R.H. Hammer, J. Pharm. Sci., 63 (1976) 1489.
14 J.J. Cereghino, B.J. Wilder, H.J. Kupperberg, W.D. Yonekawa, R.J. Perchalski, R.E. Ramsey, B.G. White, J.K. Penry and L.D. Smith, Epilepsia, 16 (1975) 665.
15 S. Willox and B.N. Singh, J. Chromatogr., 128 (1976) 196.
16 A. Frydman, J.-P. Lafrage, F. Vial, R. Rullière and J.-M. Alexandre, J. Chromatogr., 145 (1978) 401.
17 M.A. Kiddie, R.B. Boyds and T.R.D. Shaw, Brit. J. Pharmacol., 47 (1973) 676.
18 E.J. Cone, W.F. Buchwald and W.D. Darwin, Drug Metab. Dispos., 10 (1982) 561.
19 H. Badad and A.G. Zeiler, Chem. Rev., 73 (1973) 75.
20 A.T. Kacprowicz, Clin. Chem., 28 (1982) 245.
21 K.N. Scott, M.N. Couch, B.J. Wilder, C.M. Williams, Drug Metab. Dispos., 1 (1973) 506.
22 A.H. Beckett and E.C. Chodimere, Postgrad. Med. J., 53, Suppl. 1 (1977) 60.

Journal of Chromatography, 309 (1984) 170—176
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2108

Note

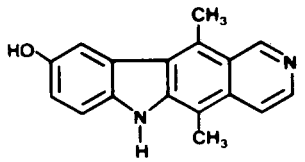
Use of electrochemical detection in the high-performance liquid chromatographic determination of hydroxylated ellipticine derivatives

P. BELLON, P. CANAL*, J. BERNADOU and G. SOULA

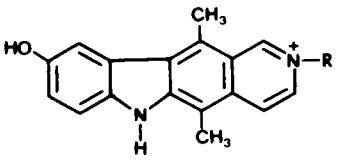
Groupe de Recherches, Centre Claudius Regaud, 20—24, rue du Pont St. Pierre, 31052 Toulouse (France)

(First received November 29th, 1983; revised manuscript received February 14th, 1984)

Ellipticine derivatives were first described as natural antitumour alkaloids [1, 2]. It was later shown that hydroxylation in position 9 (9-hydroxyellipticine, 9-OH-E) and quaternization of the pyridine nitrogen (N_2-methyl-9-hydroxyellipticinium acetate, 9-OH-NME) increase the antitumour activity (Fig. 1). This last compound (Celiptium®) is actually one of the most efficient anticancer drugs in this series and has been retained for clinical use in man [3, 4].



9-OH-E (1)



R = $-CH_3$: 9-OH-NME (2)

R = $-CH_2-CH_2-CH_3$: 9-OH-NPE (3)

Fig. 1. Chemical structure of ellipticines: 1 = 9-hydroxyellipticine (9-OH-E); 2 = N^2-methyl-9-hydroxyellipticinium acetate (9-OH-NME); 3 = N^2-propyl-9-hydroxyellipticinium acetate (9-OH-NPE).

0378-4347/84/$03.00

Up to now, a reversed-phase high-performance liquid chromatographic method using an internal standard (N^2-propyl-9-hydroxyellipticinium acetate, 9-OH-NPE) has been described [5]; detection was by ultraviolet (low sensitivity) or fluorescence (high sensitivity but requires extraction plus derivatization) measurement. It seemed of interest to develop a sensitive and specific detection method directly utilizable for biological samples without derivatization.

The 9-hydroxylated ellipticine derivatives are easily oxidized in a two electron process in *p*-quinone-imine structures [6, 7], for example by a peroxidase such as horseradish peroxidase in the presence of hydrogen peroxide. On the other hand, the one-electron electrochemical oxidation of 9-OH-E and 9-OH-NME has been described by Moiroux and Armbruster [8]. These two reactions are summarized in Fig. 2. We took advantage of this property to develop the electrochemical detection of the 9-hydroxylated ellipticines. Experimental conditions for the two compounds, 9-OH-E and 9-OH-NME, are discussed.

The proposed technique was used to carry out a pharmacokinetic investigation of Celiptium after intravenous infusion and compared with fluorescence detection.

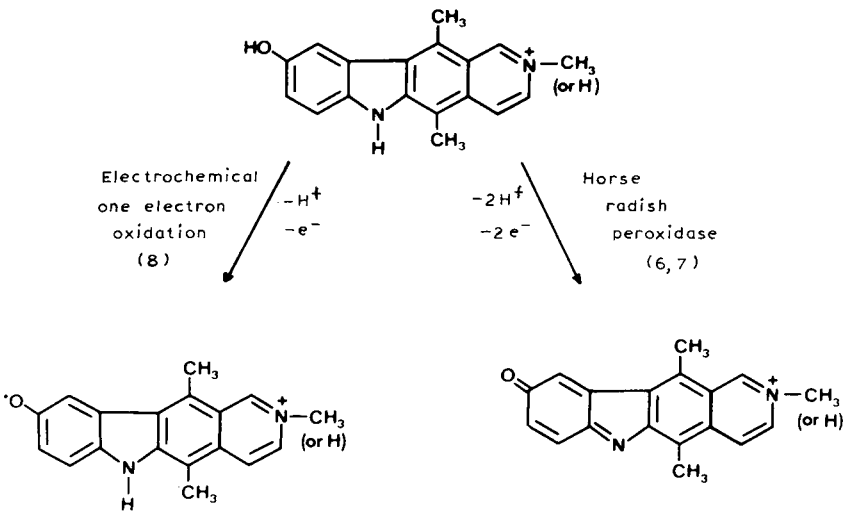


Fig. 2. Two reaction schemes for oxidation of 9-hydroxylated ellipticine.

MATERIALS AND METHODS

Chemicals and reagents

9-Hydroxyellipticine (9-OH-E), N^2-methyl-9-hydroxyellipticinium acetate (9-OH-NME) and N^2-propyl-9-hydroxyellipticinium acetate (9-OH-NPE) were provided by LABAZ Labs. (Sanofi Group, Sisteron, France). Aqueous solutions of the compounds were kept at 4°C for one month without any degradation.

9-OH-NPE was used as internal standard for the determination of 9-OH-NME.

Methanol was chromatographic grade (Merck, Darmstadt, F.R.G.); sodium tetraphenylborate, ammonium acetate and glacial acetic acid were obtained from Merck.

All mobile phases were filtered through a type FH 0.5-μm Millipore filter, and renewed every week.

Apparatus

The chromatographic system consisted of a high-pressure pump (solvent delivery system, Model 6000A, Waters Assoc., Milford, MA, U.S.A.), and an injection device (Model U6K, Waters Assoc.). The electrochemical detector comprised a Faraday cage enclosing a TL-5 glassy carbon electrode and an LC-4 controller, both from Bioanalytical Systems (West Lafayette, IN, U.S.A.). A Waters Assoc. μBondapak C_{18} reversed-phase column (30 X 3.9 mm I.D.; particle size 10 μm) was eluted isocratically at room temperature. Chromatograms were traced on a PE Servotrace recorder at a speed of 0.5 cm/min.

Procedure

The study of electrochemical detection raised two analytical problems: determination of the oxidative potential and optimization of the mobile phase.

The influence of the oxidative potential on the peak heights of 9-OH-NME, 9-OH-NPE and 9-OH-E was investigated by 0.05-V stepwise change of the electrode potential from 0.1 to 1.1 V. This study was performed using the mobile phase methanol—water (60:40) with 100 mmol/l ammonium acetate adjusted to pH 6 with glacial acetic acid, at a flow-rate of 1.5 ml/min.

In order to study the effects of pH, the mobile phase was adjusted to various pH values between 4.7 and 7.0 by decreasing the ratio of acetic acid. The influence of counter-ion concentration was investigated using increasing amounts of ammonium acetate from 10 to 250 mmol/l.

Preparation of biological samples

Blood samples were taken for a period of 7 h after administration of 160 mg of 9-OH-NME. They were collected in heparinized tubes and centrifuged at 1000 *g* for 5 min. Aliquots (200 μl) of plasma were spiked with 10 μl of 9-OH-NPE as internal standard (final concentration 100 ng/ml). Both drug and internal standard were extracted three times with ethyl acetate (3 X 1 ml) after addition of 5 μl of sodium tetraphenylborate (0.5%, w/v, final concentration) as counter-ion. For electrochemical determination, the combined upper organic phases were evaporated to dryness under a gentle stream of nitrogen and the residue was redissolved in 200 μl of mobile phase. For fluorimetric determination, we used the method described by Muzard and Le Pecq [5].

RESULTS AND DISCUSSION

Determination of oxidative potential

It is known from the literature [6] that 9-OH-NME is polarographically active. In Fig. 3, the relation is given between the potential applied to the glassy carbon electrode and the peak heights of 9-OH-NME, 9-OH-NPE and 9-OH-E. It can be seen that at a potential of about 600 mV, an adequate signal for the three compounds is obtained using the mobile phase methanol—water (60:40), with 100 mmol/l ammonium acetate, adjusted to pH 6 with acetic acid.

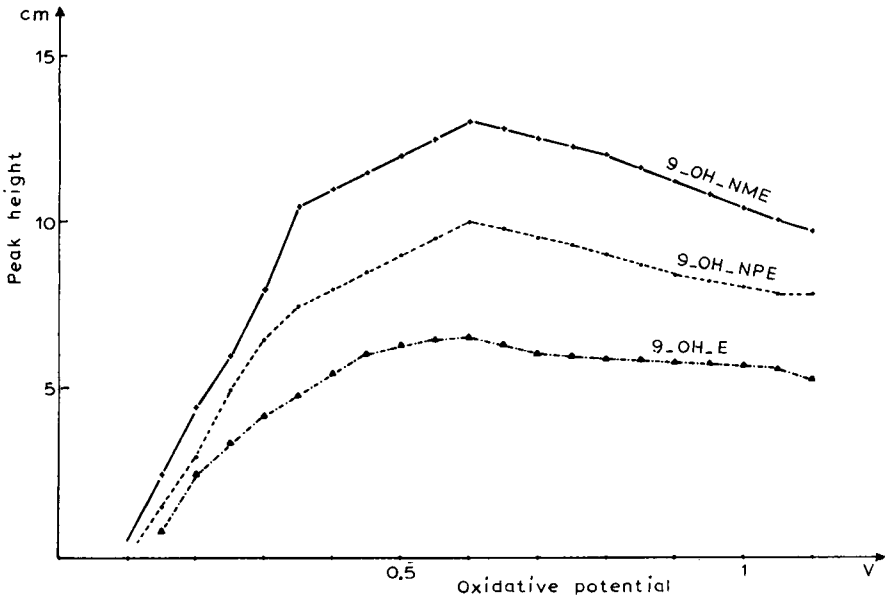


Fig. 3. Effect of oxidative potential on peak heights of different compounds.

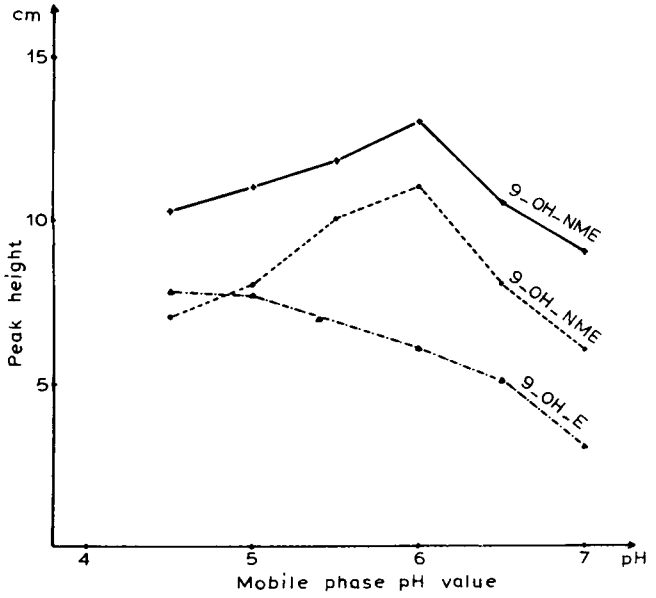


Fig. 4. Influence of pH of mobile phase on peak heights of different compounds.

Mobile phase composition

The peak heights of the three compounds were measured as a function of pH and ammonium acetate content of the mobile phase (Figs. 4 and 5). In the pH range 4.7—7.0, detection efficiencies for 9-OH-NME and 9-OH-NPE were maximum between 5.7 and 6.2, while that of 9-OH-E was between 4.5 and 5.

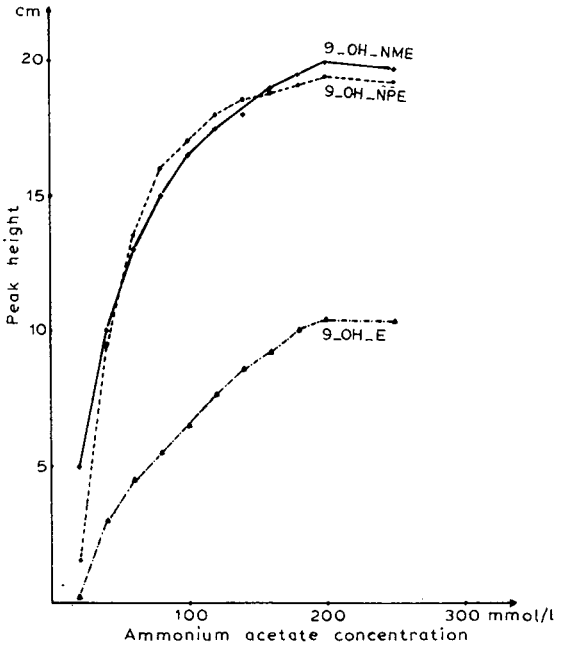


Fig. 5. Influence of counter-ion concentration on peak height of the three compounds.

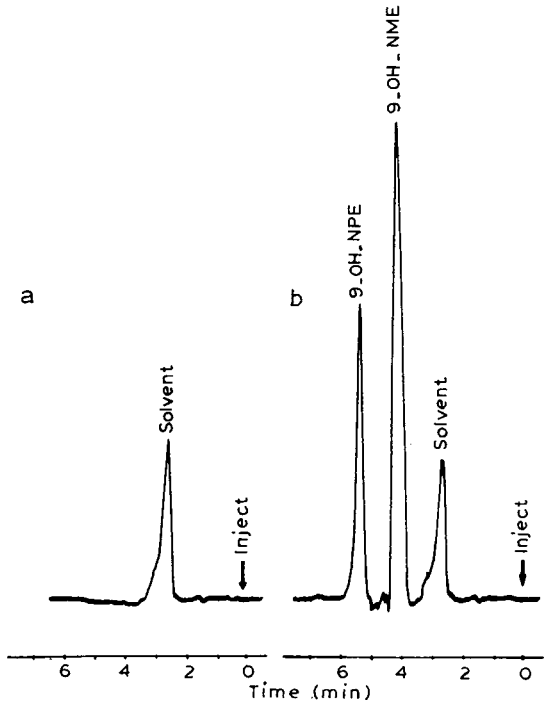


Fig. 6. Representative chromatograms of (a) a blank plasma extract, (b) an extract of plasma from a patient (adding 9-OH-NPE at 100 ng/ml of plasma) 1 h after administration of 160 mg of 9-OH-NME. Calculation from this chromatogram gave the original plasma concentration as 187 ng/ml.

At these pH values, addition of ammonium acetate has a beneficial effect on the efficiency of detection. It seems that a concentration of 200 mmol/l optimized the peak heights of the three compounds.

For determination of 9-OH-NME with use of 9-OH-NPE as internal standard, the optimal composition of the mobile phase was found to be methanol—water (60:40), 200 mmol/l ammonium acetate, adjusted to pH 6.0 with glacial acetic acid. In these conditions, the retention times of 9-OH-NME and 9-OH-NPE were 3.6 ± 0.2 min and 5.5 ± 0.2 min, (n = 50), respectively, Fig. 6a shows the chromatogram of a control plasma sample while Fig. 6b represents a chromatogram of a plasma sample containing 187 ng/ml 9-OH-NME. There was an absence of interfering peaks in control plasma for all subjects examined.

Calibration, linearity, sensitivity, reproducibility

Calibration curves, determined every day, were obtained by analysing spiked plasma from healthy volunteers with various concentrations of 9-OH-NME. They were linear over the range 30 ng/ml to 1 μg/ml. These calibration curves were used for calculating the 9-OH-NME concentration in samples from patients by the internal standard method.

The lowest amount of 9-OH-NME detectable, defined as five times the noise level, was 250 pg. This amount corresponds, for the 10 μl injected into the loop column, to a concentration of 25 ng/ml.

A serum spiked with 100 ng/ml 9-OH-NME was always assayed, along with plasma extracts, to check the status of the chromatographic system, after addition of 100 ng/ml 9-OH-NPE as internal standard. Injections of 10 μl were

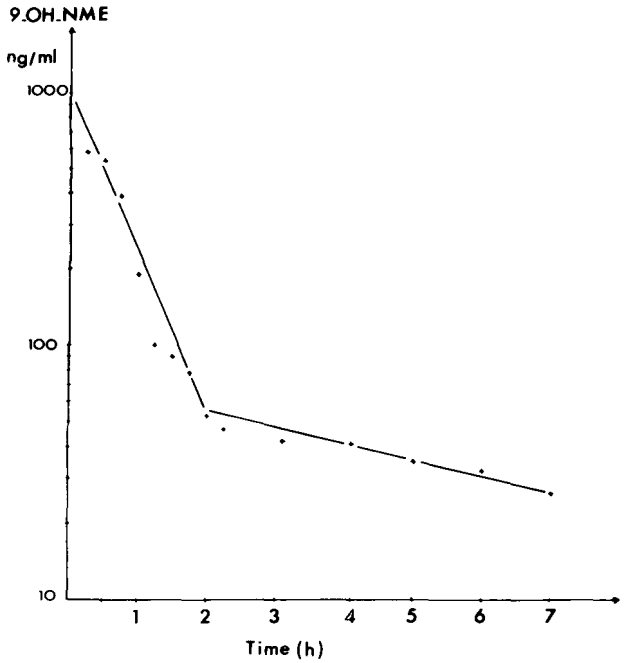


Fig. 7. Concentration of 9-OH-NME as a function of time obtained by analysis of plasma samples from a patient who had received 160 mg of 9-OH-NME.

made three times a day and over a period of ten days. The coefficients of variation were 1.5% and 4.8%, respectively.

Preliminary clinical application

The method described was tested and compared with fluorimetric detection on blood samples from a patient suffering from a metastatic breast carcinoma and receiving a 60-min infusion of 100 mg/m^2 9-OH-NME. Fig. 7 shows the plasma concentration—time course. The decline of blood level appears to be biphasic as described by Gouyette et al. [9]. The results obtained for fluorimetric and electrochemical detection were well correlated (r = 0.997).

The principle of the method could be potentially applicable to all ellipticine derivatives bearing a preserved phenolic function (i.e. glutathione or cysteine adducts recently observed in human and animal bile and urine [10, 11]).

ACKNOWLEDGEMENT

The authors are indebted to Doctor J.P. Armand (Centre Claudius-Regaud, Toulouse, France) who made possible the clinical application of 9-OH-NME.

REFERENCES

1 L.K. Dalton, S. Demerac, B.C. Elmes, J.W. Loder, J.M. Swan and T. Teitei, Austr. J. Chem., 20 (1967) 2715.
2 J.B. Le Pecq, N. Dat-Xuong, C. Gosse and C. Paoletti, Proc. Nat. Acad. Sci. U.S., 71 (1974) 5078.
3 C. Paoletti, J.B. Le Pecq, N. Dat-Xuong, P. Juret, H. Garnier, J.L. Amiel and J. Rouesse, Recent Results Cancer Res., 74 (1980) 107.
4 P. Juret, J.F. Heron, J.E. Couette, T. Delozier and J.Y. Le Talaer, Cancer Treat. Rep., 66 (1982) 1909.
5 G. Muzard and J.B. Le Pecq, J. Chromatogr., 169 (1979) 446.
6 C. Auclair and C. Paoletti, J. Med. Chem., 24 (1981) 289.
7 J. Bernadou, G. Meunier, C. Paoletti and B. Meunier, J. Med. Chem., 26 (1983) 574.
8 J. Moiroux and A.M. Armbruster, J. Electroanal. Chem., 114 (1980) 139.
9 A. Gouyette, D. Huertas, J.P. Droz, J. Rouesse and J.L. Amiel, Eur. J. Cancer Clin. Oncol., 18 (1982) 1285.
10 M. Maftouh, G. Meunier, B. Dugue, B. Monsarrat, B. Meunier and C. Paoletti, Xenobiotica, 13 (1983) 303.
11 B. Monsarrat, M. Maftouh, G. Meunier, B. Dugue, J. Bernadou, J.P. Armand, C. Fraire, B. Meunier and C. Paoletti, Biochem. Pharmacol., 32 (1983) 3887.

Journal of Chromatography, 309 (1984) 177—182
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2123

Note

High-performance liquid chromatographic procedure for the quantitation of norfloxacin in urine, serum and tissues

CONCETTA FORCHETTI

Istituto di Discipline Mediche, Facoltà di Medicina, Università degli Studi dell'Aquila, 67100 l'Aquila (Italy)

DOMENICA FLAMMINI, GIUSEPPE CARLUCCI and GIANCARLO CAVICCHIO

Istituto di Chimica, Facoltà di Scienze, Università degli Studi dell'Aquila, 67100 l'Aquila (Italy)

LODOVICO VAGGI

Divisione di Urologia, Ospedale Civile S. Salvatore, 67100 l'Aquila (Italy)

and

MAURO BOLOGNA*

Cattedra di Patologia Generale, Istituto di Discipline Biologiche, Facoltà di Medicina, Università degli Studi dell'Aquila, 67100 l'Aquila (Italy)

(First received December 29th, 1983; revised manuscript received February 18th, 1984)

Norfloxacin [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid; MK-366] is a new quinolone antibacterial agent. Because of its pharmacokinetic properties [1] and broad spectrum of activity, norfloxacin is considered one of the most effective chemotherapeutic agents in urinary tract infections. Experimental studies performed on animals demonstrated that the distribution of the drug in both tissues and organic fluids is rapid, and the concentrations reached are higher than the corresponding serum levels [2]. A recent clinical study [3] demonstrated that norfloxacin eradicates relapsing prostatic infections.

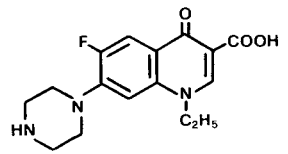
0378-4347/84/$03.00

To our knowledge, no previous study has been attempted to correlate the pharmacological effects of norfloxacin with its tissue concentrations. In the present communication, we describe an extraction and quantitation procedure for measuring norfloxacin concentrations in human prostate and kidney after oral administration of the drug. Serum, urine and tissue levels were determined by a high-performance liquid chromatographic (HPLC) method modified from the procedure of Boppana and Swanson [4].

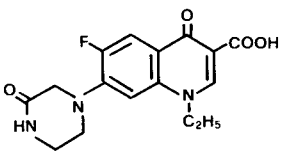
EXPERIMENTAL

Chemicals

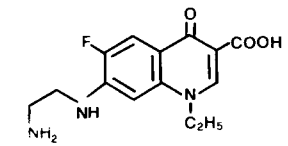
Norfloxacin and three of its metabolites (Fig. 1) with modifications in the 7 position {7-(3-oxo-1-piperazinyl), M-1; 7-[(2-aminoethyl)amino], M-2; and 7-[(2-acetylaminoethyl)amino], M-3} were provided by Merck Sharp & Dohme (West Point, PA, U.S.A.). Acetonitrile (HPLC grade), methylene chloride, and mono- and dibasic phosphates were all from Merck (Darmstadt, F.R.G.)



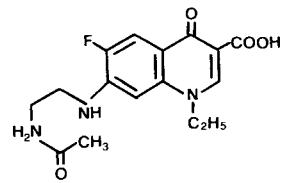
NORFLOXACIN



M-1



M-2



M-3

Fig. 1. Chemical formulae of norfloxacin and of its three major metabolites.

Samples

Specimens were obtained from patients scheduled for either prostatectomy or nephrectomy because of neoplastic and/or chronic infectious pathologies. All patients were below the age of 70 years, had no drug allergies and had serum creatinine concentrations below 2.0 mg/dl. Norfloxacin (400 mg) was given orally at time 0 the evening before surgery and again 11 h later (8.00 a.m.); at the same times urine specimens were collected. The first blood sample was taken before anaesthesia at 12—13 h; a second blood sample and a tissue fragment (either prostate or kidney) was taken during surgery at 14—15 h. All specimens were kept at —20° C until extraction.

Extraction procedure

The tissues were homogenized in 10 vols. of cold 0.4 *M* perchloric acid with an Ultra-Turrax TP 10-N homogenizer (Janke Kunkel, Staufen, F.R.G.) running for two cycles of 30 sec at 20,000 rpm, with a pause of 1 min. The homogenate was centrifuged for 15 min at 1500 *g* (TJ-6 centrifuge, Beckman, Ireland), the pellet discarded and the supernatant adjusted to pH 7.5. A 4-ml fraction was lyophilized and resuspended to 1 ml with 0.05 *M* sodium hydroxide. Serum (1 ml) and urine (200 μl) samples were mixed with 100 μl of 0.05 *M* sodium hydroxide in a 50-ml plastic centrifuge tube (Falcon Plastics, Oxnard, CA, U.S.A.). Methylene chloride (8 ml) and 0.5 *M* sodium phosphate buffer pH 7.5 (0.5 ml) were added to all the samples and the tubes were shaken for 10 min in a Dubnoff mechanical shaker (150 cycles/min). Separation of the two phases was achieved by centrifugation at 1500 *g* for 10 min and 7 ml of the organic phase (lower layer) were transferred to a second tube. Fresh methylene chloride (8 ml) was added to the first tube and the same extraction procedure was repeated twice. The organic phases collected from the three extractions of the same sample were pooled (21 ml). Sodium hydroxide (0.3 *M*) was then added to the serum (250 μl), to the tissue extract (250 μl) and to the urine (500 μl) samples. The tubes were shaken for 10 min and then centrifuged at 1500 *g* for 10 min. The aqueous phase (upper layer) was collected in Eppendorf tubes and frozen (—20° C) until the HPLC assay; 25-μl aliquots were used for chemical analysis.

Chromatography

The equipment used was a Waters HPLC apparatus with a Waters M 6000A pump (Waters Assoc., Milford, MA, U.S.A.), a U6K injector, a Model 440 absorbance ultraviolet detector (wavelength 280 nm), a Leeds Northrup Speedomax XL-682 linear recorder (paper-speed 0.2 cm/min) and a Hewlett-Packard HP 18850A integrator. The chromatographic separation was achieved in a Vydac 10-μm anion-exchange column (25 cm × 4.5 mm; Separations Group, Hesperia, CA, U.S.A.), connected to an AXGU 10-μm anion-exchange precolumn (Rainin Instruments, Woburn, MA, U.S.A.). The mobile phase used was a mixture of acetonitrile and 0.05 *M* phosphate buffer pH 7 in HPLC grade water (20:80, v/v) at a flow-rate of 1.2 ml/min. The mobile phase was prepared daily. The phosphate buffer was filtered through an HA 0.45-μm filter, while the acetonitrile was filtered through an FA 0.5-μm filter (Millipore, Bedford, MA, U.S.A.). The mixture was continuously stirred under vacuum for 30 min

before use. For our control, known amounts of norfloxacin were added to tissue, serum and urine specimens from untreated patients to measure the efficiency of the extraction procedure and to assess inter-assay variations. Solutions of norfloxacin and of its metabolites, prepared in 0.5 *M* sodium hydroxide and maintained at 4°C, were used as the inter-assay standardization. For the mathematical data processing a Texas Instruments TI-59 programmable calculator and a PC-100A printer were used, with a specifically developed computing program.

RESULTS AND DISCUSSION

Fig. 2A—D shows the HPLC separations of norfloxacin from human prostate, kidney, urine and serum, respectively. Quantitation of the compound was obtained by comparing the peak areas from the samples with the peak areas of known amounts of norfloxacin solutions injected directly into the HPLC system. The retention time of norfloxacin, both in the standards and in the extracted samples, was 7.10 min. No interference was observed during the assay between norfloxacin, its major metabolite (M-1 had a retention time of 4.20 min, unmarked peaks of Fig. 2) and the endogenous substances in the clinical samples.
standard solution in 0.3 *M* sodium hydroxide. Standard curves prepared by adding known amounts of norfloxacin to the samples were linear from 1.0 to 500 μg/ml in urine and from 0.01 to 2 μg/ml in kidney; an analogous linearity was found in both serum and prostate samples.

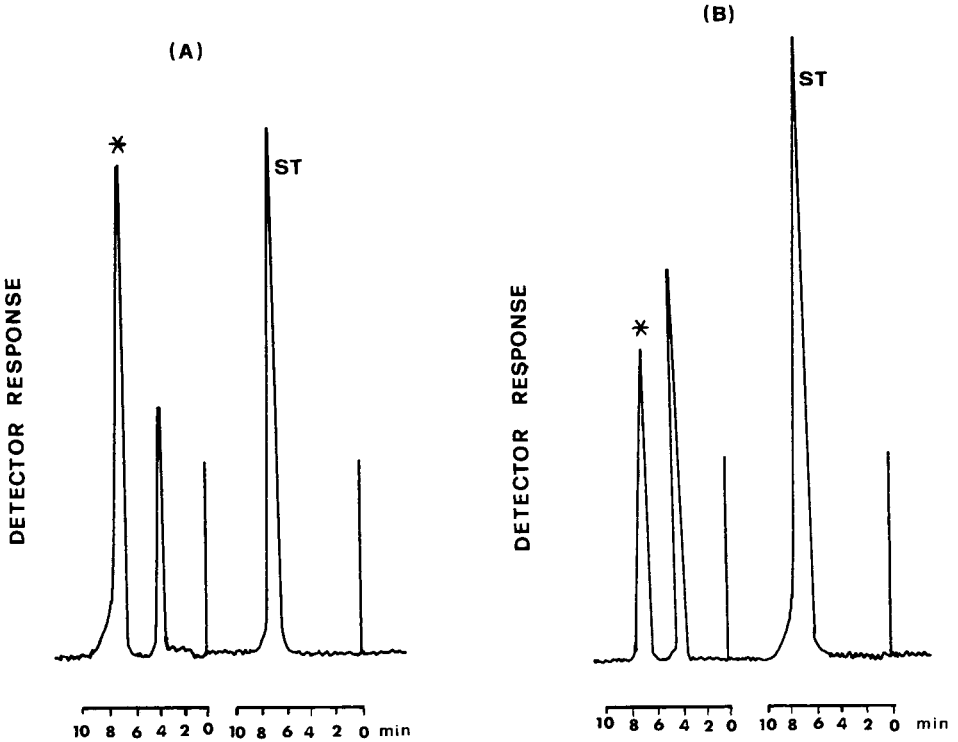


Fig. 2.

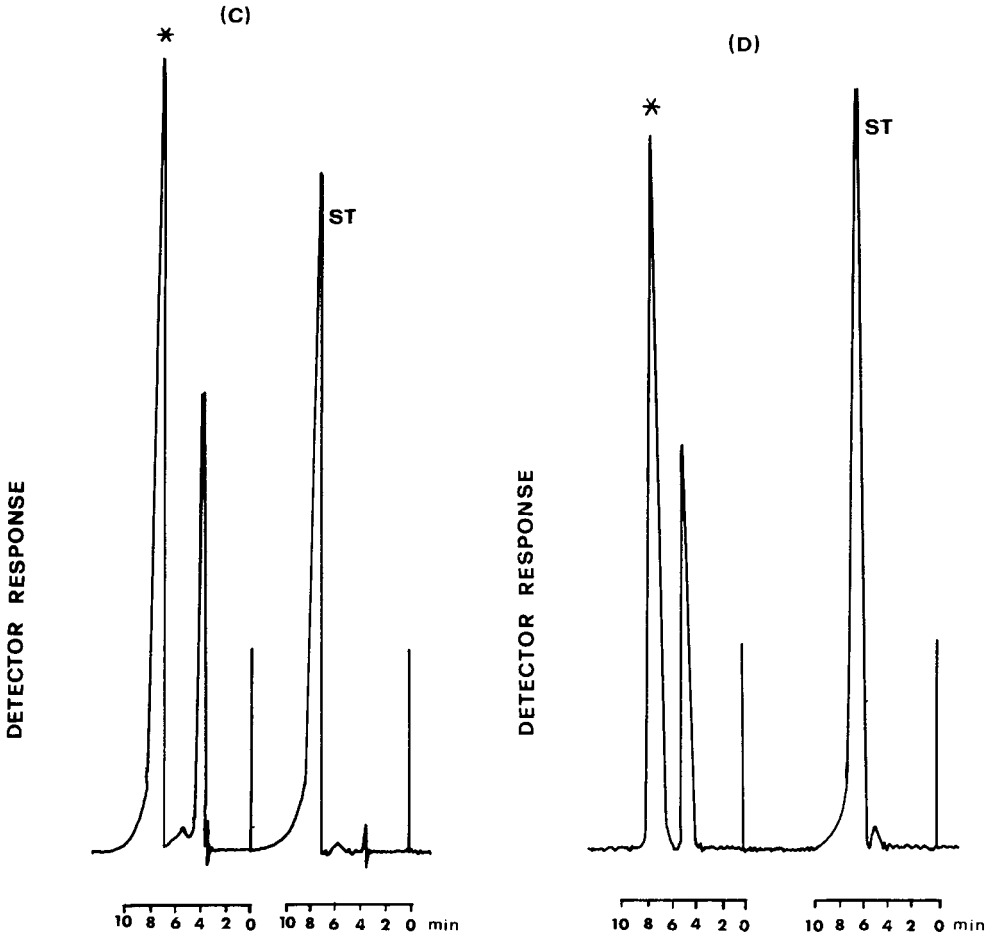


Fig. 2. HPLC chromatograms from human prostate (A), kidney (B), urine (C) and serum (D), after two 400-mg doses of norfloxacin. The norfloxacin peaks are marked with a star. The concentrations of the standard samples of norfloxacin (ST) were 5 μg/ml for A, B and D and 50 μg/ml for C; the calculated norfloxacin concentrations in these samples were 1.14 μg/g of prostate (A), 0.61 μg/g of kidney (B), 115.0 μg/ml of urine (C) and 0.60 μg/ml of serum (D). The unmarked peaks represent the M-1 metabolite of norfloxacin.

Extraction recoveries from prostate, kidney, urine and serum were 90%, 60%, 80% and 60%, respectively. The inter-assay coefficient of variation was 2.2% (eighteen assays) for a 1.25 μg/ml standard.

Table I shows the typical norfloxacin concentrations found in two out of twenty patients examined so far. The data indicate that after only two 400-mg administrations norfloxacin reaches concentrations in the prostate and kidney similar to those attained in the serum. The tissue levels of norfloxacin, compared with the in vitro minimal inhibitory concentrations (MIC, 90%) [5, 6], confirm that the drug attains concentrations in the prostate and kidney that are effective to control infections sustained by the most common pathogens involved in urinary tract infections. The very high concomitant urinary levels are described also by other authors [1, 4] and account for the high rate of success

TABLE I

NORFLOXACIN CONCENTRATIONS IN URINE, SERUM AND EITHER PROSTATE OR KIDNEY IN PATIENTS WHO INGESTED TWO 400-mg DOSES BEFORE SURGERY

Patient	Urine 1 Control (μg/ml)	Urine 2 11 h from 1st dose (μg/ml)	Serum 1 14 h from 1st dose, 30 min from 2nd dose (μg/ml)	Serum 2 15.5 h from 1st dose, 2 h from 2nd dose (μg/ml)	Prostate 15.5 h from 1st dose, 2 h from 2nd dose (μg/g)	Kidney 15.5 h from 1st dose, 2 h from 2nd dose (μg/g)
D.S.R.	0.00	146.25	0.60	1.10	1.14	
I.G.	0.00	115.00	0.40	0.55		0.61

in the treatment of lower urinary tract infections, even due to highly resistant bacterial strains [7].

In our study we have demonstrated a correlation between the clinical observations in complicated and relapsing urinary infections such as prostatitis [3] and the pharmacokinetic properties of norfloxacin, which is able to reach effective antibacterial levels in the examined tissues even after only two 400-mg oral doses.

REFERENCES

1 S.R. Norrby, Eur. J. Chemother. Antibiot., 3 (1983) 19—25.
2 S. Murayama, Chemotherapy (Tokyo), 29 (1981) 97—104.
3 L. Vaggi, L. Perilli, C.M. Forchetti and M. Bologna, Farmaci, Suppl. 2 (1984) in press.
4 V.K. Boppana and B.N. Swanson, Antimicrob. Agents Chemother., 21 (1982) 808—810.
5 S.W.B. Newsom, Eur. J. Chemother. Antibiot., 3 (1982) 9—14.
6 M.Y. Khan, R.P. Gruninger, S.M. Nelson and R.E. Klicker, Antimicrob. Agents Chemother., 21 (1982) 848—851.
7 M. Bologna, L. Vaggi, E. Martini, and E. Tomei, Eur. J. Chemother. Antibiot., 3 (1983) 41—46.

Journal of Chromatography, 309 (1984) 183—186
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2124

Note

Microdosage de deux antifibrinolytiques (acide ϵ-aminocaproïque et acide tranexamique) par chromatographie liquide et détection fluorimétrique

C. LACROIX*, P. LEVERT, G. LAINE et J.P. GOULLE

Laboratoire de Biochimie, Centre Hospitalier Général, 55 bis, rue Gustave Flaubert, B.P. 24, 76083 Le Havre Cedex (France)

(Reçu le 16 janvier 1984)

L'acide ϵ-aminocaproïque (AEAC, acide 6-aminohexanoïque) et l'acide tranexamique (AMCHA, acide *trans*-4-aminomethylcyclohexane carboxylique) sont essentiellement utilisés en thérapeutique comme agents antifibrinolytiques (inhibiteurs de la conversion du plasminogène en plasmine) dans différents processus hémorragiques [1, 2].

D'autres indications proposent leur emploi dans l'oedème angio-neurotique [3, 4], dans certaines réactions allergiques ou comme anti-inflammatoires.

Deux voies sont possibles pour l'évaluation de ces composés: (1) l'une indirecte faisant appel aux différentes méthodes d'exploration de la coagulation [5] ou de lyse du caillot [6]; (2) l'autre plus directe par détermination du taux sanguin de l'antifibrinolytique lui-même.

Dans ce cas, différentes techniques ont été développées jusqu'à présent et mettent en jeu: l'électrophorèse [7], la chromatographie sur papier [8], la chromatographie sur colonne échangeuse de cation [9], en phase gazeuse [10], en phase liquide [11]. Cette technique appliquée à l'AEAC met en oeuvre une détection fluorimétrique des dérivés obtenus après dansylation. L'emploi de la fluorescamine [12] nous a semblé représenter un gain de temps et une simplification du dosage de ces composés.

MATÉRIEL ET MÉTHODE

Appareillage

L'appareil utilisé comprend une pompe haute pression (Touzard et Matignon, Vitry-sur-Seine, France) Type E.C.-93 équipée d'une vanne d'injection à boucle de 20 μl (Rhéodyne 71-25, Berkeley, CA, E.U.), un spectrofluori-

0378-4347/84/$03.00

mètre (Shimadzu Type RF-530, Touzard et Matignon), comportant une cuve de 12 μl. La longueur d'onde d'excitation est de 390 nm, celle d'émission 475 nm. La colonne est une LiChrosorb RP 18 (10 μm, 25 cm × 4.6 mm I.D.) (E. Merck, Darmstadt, R.F.A.). L'appareil est raccordé (sortie 1 mV) à un enregistreur Omniscribe (Houston Instruments).

Prélèvements étalonnage

Les prélèvements sanguins (5 ml) sont recueillis dans des tubes de verre sans anticoagulant (Vacutainers) immédiatement centrifugés à 1000 *g* pendant 10 min. La fraction sérique est conservée jusqu'au dosage à 4° C. L'étalonnage est réalisé en surchargeant des sérums par des solutions d'AEAC ou d'AMCHA à différentes concentrations.

Réactifs

Acide ϵ-aminocaproïque et acide tranexamique (EGA-Chimie, Strasbourg, France), acétonitrile qualité HPLC (Carlo Erba, St Cloud, France), fluorescamine (Fluram, Laboratoires Roche, Neuilly-sur-Seine, France).

Méthode

À 20 μl de sérum, mélanger 2 μl de solution aqueuse d'étalon interne (3 g/l d'AMCHA pour le dosage sérique de l'AEAC et 1 g/l d'AEAC pour celui de l'AMCHA), et déprotéiniser par 20 μl d'acétonitrile. Après 3 min de centrifugation (10,000 *g*), prélever 5 μl de surnageant et le mélanger à 100 μl de tampon phosphate 0.025 mol/l ajusté à pH 8. La fluorescamine (100 μl d'une solution à 300 mg/l dans l'acétone) est ajoutée rapidement en maintenant le tube en agitation sur un vortex. Injecter 20 μl dans le chromatographe. La phase mobile est constituée par un mélange acétonitrile—eau—acide acétique—tetrahydrofuranne (300:690:5:5) contenant 40 mmol/l d'acétate de sodium. Avant usage, la phase mobile est passée sur filtre Millipore (0.45 μm). Le débit est de 2 ml/min soit une pression d'environ 14 MPa.

RÉSULTATS

La Fig. 1 montre le tracé obtenu avec le sérum d'un patient traité par l'AEAC ainsi qu'un chromatogramme d'un sérum exempt d'AEAC et AMCHA.

L'étude de la linéarité a été effectuée sur du sérum surchargé en AEAC ou en AMCHA aux concentrations suivantes: AEAC: 62.5, 125, 250, 500, 750 et 1000 mg/l; AMCHA: 25, 50, 100 et 200 mg/l. La relation entre les concentrations et le rapport de hauteur du pic du dosage et de l'étalon interne s'est avérée linéaire pour les deux composés dans l'intervalle des concentrations indiquées plus haut. Le recouvrement entre les gammes aqueuses et sériques traitées dans des conditions identiques a donné des valeurs de 94—103% pour l'AEAC et de 98—105% pour l'AMCHA.

Des limites de détection des produits, compte tenu des valeurs des blancs sériques sont de 6 mg/l (AEAC) et de 4 mg/l (AMCHA).

La reproductibilité a fait l'objet de dix déterminations pour chaque niveau et figure dans le Tableau I. Des dosages répétés pendant dix jours de deux pools de sérum pour chaque antifibrinolytique ont donné les résultats suivants:

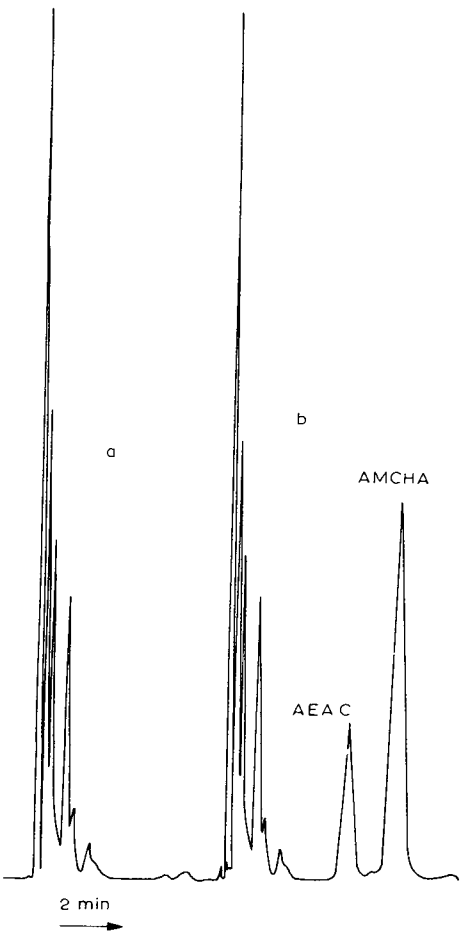


Fig. 1. Chromatogramme obtenu sur un blanc sérique (a) et lors d'un traitement par l'AEAC (b) (52 mg/l).

AEAC: 127.8 ± 2.6 mg/l (C.V. = 2.1%); 251.1 ± 9.2 mg/l (C.V. = 3.7%); AMCHA: 52.8 ± 1.3 mg/l (C.V. = 2.4%); 101.4 ± 1.9 mg/l (C.V. = 1.9%).

DISCUSSION

Ces deux antifibrinolytiques sont caractérisés par une clairance rénale élevée [13], ce qui implique des administrations répétées afin de rester dans des zones thérapeutiques (AEAC = 100—400 mg/l; AMCHA = 10—50 mg/l), et des risques d'accumulation lors d'atteintes rénales.

Dans ces conditions, l'évaluation des taux plasmatiques permet une meilleure adaptation de la posologie et d'anticiper éventuellement sur l'apparition des problèmes hématologiques. Mais, pour cela, il est nécessaire de disposer d'une méthode rapide de dosage et c'est pourquoi l'emploi de la fluorescamine comme agent de dérivation apporte une solution simple et rapide à ce problème, le temps de manipulation étant réduit à une déprotéinisation.

TABLEAU I

ÉTUDE DE LA REPRODUCTIBILITE

Chaque concentration a fait l'objet de dix déterminations.

	Concentration (mg/l)	Écart-type (mg/l)	C.V.* (%)
AEAC	62.5	6.1	1
	125	3	2.4
	250	3	1.1
	500	16	3.1
	1000	28	2.8
AMCHA	25	1	3.3
	50	1.5	2.8
	100	1.3	1.2
	200	2	1.1

*Coefficient de variation.

La technique décrite permet le dosage de l'un ou l'autre des produits en inversant l'étalon interne, ces antifibrinolytiques n'étant pas utilisés simultanément en thérapeutique. De plus, la prise d'essai de 20 μl de sérum peut être très facilement diminuée car seulement 5 μl du surnageant sont repris, le facteur limitant étant constitué par le matériel qui ne se prête pas toujours à un travail sur des microvolumes.

BIBLIOGRAPHIE

1 I.M. Nilsson, L. Anderson et S.E. Bjorkman, Acta Med. Scand. Suppl., 448 (1966) 5.
2 S. Okamoto, S. Sato, Y. Takada et N. Okamoto, Keio J. Med., 13 (1964) 177.
3 M.M. Franck, J.S. Sergent, M.A. Kane et D.W. Alling, New Engl. J. Med. 286 (1972) 808.
4 A.L. Sheffer, F. Austel et S. Rosen, New Engl. J. Med., 287 (1972) 452.
5 F. Markwardt, H.P. Klöcking et M. Richter, Pharmazie, 22 (1967) 83.
6 N. Wolosowicz, S. Niewiarowski et K. Czerepko, Thromb. Diath. Haemorrh., 10 (1964) 309.
7 A. Sjoerdsma et E.A. Hanson, Acta Chem. Scand., 13 (1959) 2150.
8 G.P. McNichol, A.P. Fletcher, N. Alkjaersig et S. Sherry, J. Clin. Lab. Med., 59 (1962) 7.
9 Y. Takada, A. Takada et U. Okamoto, Keio J. Med., 13 (1964) 115.
10 T.R. Keucher, E.B. Solow, J. Metaxas et R.L. Campbell, Clin. Chem., 22 (1976) 806.
11 R.F. Adams, G.J. Schmidt et F.L. Vondemark, Clin. Chem., 23 (1977) 4226.
12 M. Weigele, S.L. De Bernardo, J.P. Tengi et W. Leimgruber, J. Amer. Chem. Soc., 94 (1972) 5927.
13 G.P. McNichol, A.P. Fletcher, N. Alkjaersig et S. Sherry, J. Clin. Lab. Med., 59 (1962) 15.

Journal of Chromatography, 309 (1984) 187—192
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2125

Note

Determination of the beta-adrenoceptor blocking drug sotalol in plasma and tissues of the rat by high-performance liquid chromatography with ultraviolet detection

BJÖRN LEMMER*, THOMAS OHM and HORST WINKLER

Centre of Pharmacology, J.W. Goethe-University, Frankfurt/M. (F.R.G.)

(Received January 13th, 1984)

Sotalol hydrochloride {4-[1-hydroxy-2-(isopropylamino)ethyl]methanesulphonanilide hydrochloride, Fig. 1} is a β-adrenoceptor blocking drug which differs in molecular structure and ionic character from the other β-blockers in use, e.g. propranolol, metoprolol and atenolol, the high-performance liquid chromatographic (HPLC) analysis of which we described earlier [1]. Sotalol has an arylethanolamine structure similar to that of the β-receptor agonist isoprenaline, differing only by the aryl substituent, i.e. sotalol has a methanesulphonamide group instead of two hydroxyl groups. Furthermore, the acidic nitrogen of the metanesulphonamide group (pK_a = 8.3) and the basic nitrogen of the amino group in the side-chain (pK_a = 9.8) yield the zwitterionic character of the sotalol molecule. This property requires modifications in the extraction procedure as described for the other β-blockers mentioned above [1].

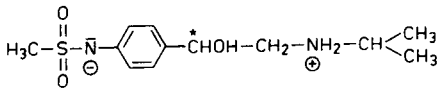


Fig. 1. The molecular structure of sotalol and the charges of the acidic (pK_a = 8.3) and the basic (pK_a = 9.8) centre at the isoelectric point (pH 9.0).

Different methods have been described for measuring sotalol concentrations in biological fluids. Garrett and Schnell [2] reported a fluorimetric assay, Walle [3] a gas—liquid chromatographic method, and Lefebvre and co-workers [4, 5] an HPLC method with fluorimetric detection. However, no method was described for the determination of sotalol levels in tissues. Since we are

0378-4347/84/$03.00

interested in the pharmacokinetic behaviour of different β-adrenoceptor blocking drugs in plasma and in tissues [6—8], which are target organs for their pharmacological effects, an HPLC method with ultraviolet (UV) detection for sotalol was developed in order to be able to quantify this hydrophilic compound in plasma as well as in organs.

EXPERIMENTAL

Standards and reagents

All reagents used were reagent grade and purchased from E. Merck (Darmstadt, F.R.G.). Only deionized glass-distilled water was used. The racemic mixture of sotalol · HCl was kindly supplied by Bristol-Myers (Bergisch-Gladbach, F.R.G.).

Standard solutions from 2 mg/ml to 10 ng/ml were obtained from an aqueous stock solution (2 mg/ml). This solution was stored at 4°C for up to one month.

HPLC instrumentation and conditions

The HPLC system consisted of a constant-flow pump (Gynkotek, 600/200), an autosampler (Waters, WISP 710 B), a reversed-phase column (Knauer, 10 cm × 4 mm; Shandon ODS 5 μm), a spectrophotometer with a deuterium lamp (Kratos, Spectroflow 773) and a computing integrator (Spectra Physics, SP 4100).

The mobile phase was methanol—water—acetonitrile (55:45:20) containing 1% acetic acid and 0.005 *M* of dodecyl sodium sulphate. The flow-rate was 1 ml/min and the monochromator of the spectrophotometer was set at 227 nm. The spectrophotometer was connected to the integrator; areas were measured. Chromatography was carried out at ambient temperature.

Sample preparation

Plasma and tissue samples of five organs (heart, muscle, lung, liver, kidney) from light—dark synchronized male Wistar rats of about 150—180 g body weight were used. The rats were sacrificed by decapitation, and blood was collected in 12-ml conical glass tubes containing 50 μl of heparin (250 I.U.). After centrifugation (900 *g*, 15 min), 1-ml plasma portions were pipetted into 12-ml screw-capped glass tubes and kept at −35°C. The organs were dissected out, rinsed in ice-cold 0.9% saline solution, blotted on filter paper, weighed, frozen in liquid nitrogen and finally stored at −35°C.

Plasma extraction

The extraction procedure is summarized in Fig. 2. The 1-ml plasma samples were thawed and 2 ml of acetonitrile were added. After vortexing, then centrifuging for 10 min at 800 *g*, 2.5 ml of each supernatant were transferred to 12-ml screw-capped glass tubes and evaporated to dryness under a stream of dry nitrogen. The residues were redissolved in 1 ml of buffer (0.5 *M* borate buffer, pH 9.0) by vortexing. Then 6 ml of extraction mixture (benzyl alcohol—chloroform, 60:40) were added, mixed by shaking for 10 min and separated by centrifugation for 10 min at 800 *g*. Aliquots of 5 ml of the organic layer

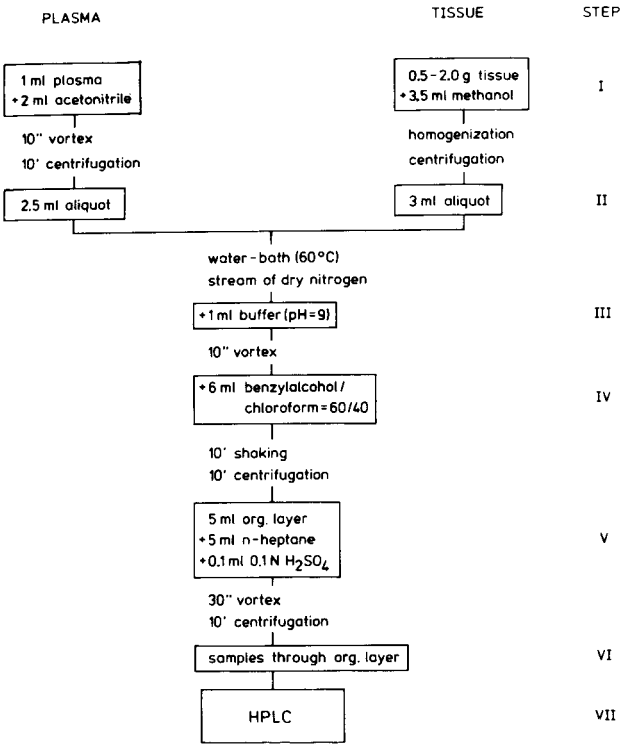


Fig. 2. Schematic outline of the extraction procedure.

were transferred to 12-ml screw-capped glass tubes; 5 ml of *n*-heptane and 0.1 ml of 0.05 *M* sulphuric acid were added, and the tubes were vortexed for 30 sec, then centrifuged for 10 min at 800 *g*. Finally, aliquots of the aqueous layer were injected into the HPLC system.

Tissue extraction

The extraction procedure is summarized in Fig. 2. To each sample of frozen tissue (0.5—2.0 g wet weight) 3.5 ml of methanol were added. The samples were homogenized with an Ultra-Turrax homogenizer in glass tubes at 4° C. After centrifugation at 7000 *g* for 10 min, 3 ml of each supernatant were transferred to 12-ml screw-capped glass tubes and evaporated to dryness under a stream of dry nitrogen. The further procedure was as described for the plasma samples (see Fig. 2).

Standard curves and recovery studies

Blank plasma and tissue samples from untreated rats were spiked with varying amounts of sotalol. These samples were treated as described above and standard curves of added concentrations versus peak area were calculated. From these data recovery in each organ was calculated over the whole concentration range. Sotalol plasma and tissue concentrations ex vivo were determined in the respective tissues after 60, 110, 160 and 210 min after intravenous injection of 6 μmol/kg sotalol · HCl in rats.

RESULTS AND DISCUSSION

The aim of the present investigation was to develop a method to be able to quantify sotalol concentrations in organs as well as in plasma, the determination of which is necessary for pharmacokinetic studies. The methods described by Garrett and Schnell [2] and Lefebvre and co-workers [4, 5] are similar as regards the extraction procedure. Using HPLC with fluorimetric detection, Lefebvre and co-workers [4, 5] succeeded in lowering the detection limit of sotalol to 20 ng/ml of plasma, whereas Garrett and Schnell [2] achieved a detection limit of 100 ng/ml ten years ago when using only a spectrofluorimetric method without HPLC. In preliminary experiments we were unable to reproduce a recovery of 60% (as calculated from refs. 4 and 5) from plasma with the extraction procedure described by Lefebvre et al. [4]; only a recovery of 38% for plasma and even less for tissue was obtained. Since, for the intended pharmacokinetic studies, extraction of sotalol from tissues is the most crucial point, a new HPLC method with UV detection for sotalol was developed by which low drug concentrations could be detected and reproducibly quantified in plasma as well as in various tissues.

In order to optimize the extraction procedure and to increase the recovery from tissues, various solvents were tested in preliminary studies. The extraction procedure finally adopted is depicted in Fig. 2. To precipitate the protein, methanol or acetonitrile was used (Step I), instead of perchloric acid as described for sotalol by Lefebvre et al. [4, 5] and Garrett and Schnell [2], since the samples had to be evaporated (Steps II and III). Methanol was a better solvent for homogenizing the tissue samples while acetonitrile yielded a better precipitate of the plasma proteins (Step I).

The mixture used for extraction of the redissolved sotalol from buffer (Step IV) was the result of a study on the extraction behaviour of 13 solvents and 48 binary mixtures of these solvents [9]. The best results concerning extraction (Steps IV and V) and back-extraction (Steps V and VI) into 0.05 *M* sulphuric acid were obtained by a mixture of 60 parts of benzyl alcohol and 40 parts of chloroform, with which about 69% of sotalol was extracted from the buffer solution (pH 9; Step III) into the aqueous layer (Step VI). The extraction ratio from buffer (pH 9) into the organic layer (Steps III and IV) was 74% and the ratio of the back-extraction from this layer into 0.05 *M* sulphuric acid

TABLE I

DATA FOR SOTALOL OBTAINED FROM SPIKED PLASMA AND TISSUE SAMPLES

Organ	No. of samples	Concentration* (ng/sample)	Correlation coefficient	Recovery (%) (mean ± S.E.M.)
Plasma	12	44— 880	0.999	58.4 ± 2.2
Heart	12	88—1760	0.999	41.5 ± 2.6
Muscle	12	88—1760	0.981	43.9 ± 1.7
Lung	12	88—1760	0.997	38.4 ± 1.7
Liver	12	88—1760	0.994	36.1 ± 0.8
Kidney	12	88—1760	0.999	50.5 ± 2.1

*Depending on the plasma and tissue concentrations expected in the pharmacokinetic studies.

(Steps V and VI) was 93%. Thus, the above-mentioned 69% of total recovery from buffer resulted. When plasma samples were spiked, an extraction ratio of about 60% was obtained (Table I). At Step V of the extraction procedure addition of an equivalent volume of *n*-heptane was useful to change the density of the organic layer; thus about 90% of the aqueous phase could be taken by a syringe through the organic layer (Step VI). Addition of *n*-heptane had no effect on the recovery. In accordance with Lefebvre et al. [5], sulphuric acid was superior to hydrochloric acid in the ratio of the back-extraction of sotalol.

The mobile phase used was a ternary mixture of methanol, water and acetonitrile containing acetic acid and counter-ion which proved to be the best of various mixtures studied. Addition of acetonitrile (20%) to the mobile phase decreased the retention time of sotalol.

The HPLC method described has been successfully used to study the kinetic behaviour of sotalol after a single application of the drug (6 μmol/kg, intravenously) in plasma and five organs of the light—dark synchronized rat. Fig. 3 shows chromatograms obtained from blank, spiked (44—440 ng/ml, depending on the plasma concentrations expected in the pharmacokinetic studies) and ex vivo plasma samples (60, 110, 160 and 210 min after application). As can be seen, the sotalol peak is well separated from the other peaks and at least 10 ng/ml could be detected. The correlation coefficients between drug concentrations and peak areas were greater than 0.99 for plasma and all organs except muscle for which a correlation coefficient of 0.98 was calculated (Table I). The recovery was 58% from plasma and 36—50% from the organs (Table I). Representative chromatograms of blank and ex vivo samples of all five organs are depicted in Fig. 4. In tissues sotalol concentrations of at least

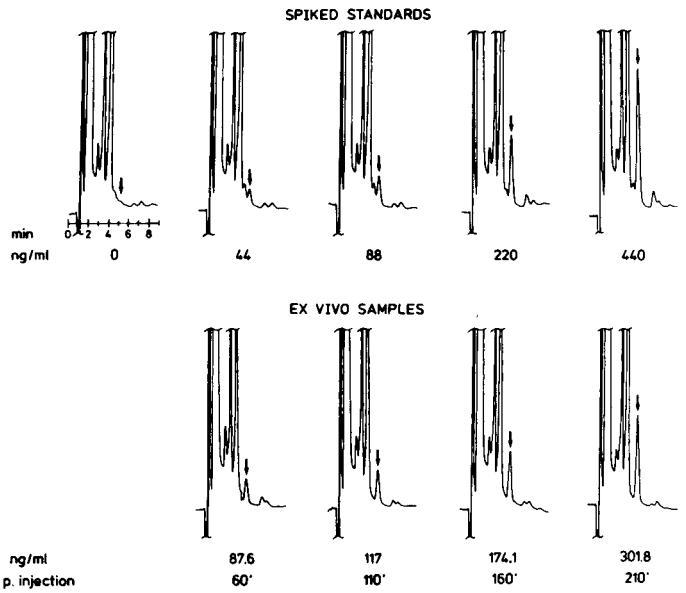


Fig. 3. Representative chromatograms of sotalol in plasma samples. Upper panel shows spiked plasma samples in which sotalol was added at 0—440 ng/ml. Lower panel shows chromatograms of ex vivo samples; sotalol was injected intravenously into rats (6 μmol/kg), and plasma samples were taken 60—210 min after drug application. The concentrations obtained are indicated.

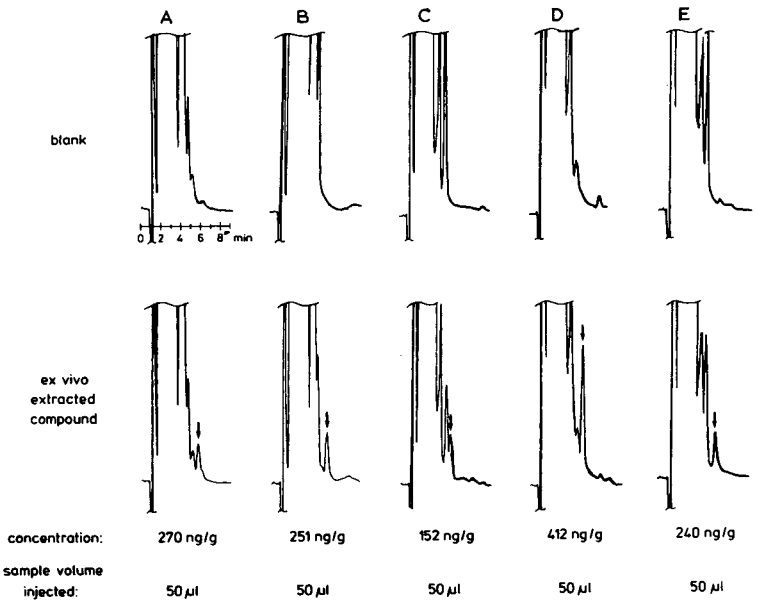


Fig. 4. Representative chromatograms of sotalol in heart (A), muscle (B), lung (C), liver (D) and kidney (E). Upper panel shows blank tissue samples of the respective organs from untreated rats. Lower panel shows the corresponding ex vivo samples (6 µmol/kg, intravenously) which were isolated 210 min after drug application. The concentrations obtained are indicated.

40 ng/g could be measured. As can be seen, there is no baseline separation of the sotalol peak in lung tissue, but the separation allowed sotalol concentrations to be determined in this organ even 210 min after drug application.

In conclusion, the HPLC method presented here provides the applicability of a liquid—liquid extraction of a zwitterionic compound and the short-wave UV detection of this compound within a pharmacokinetic study not only in plasma but also in such target organs as heart, muscle, lung, liver and kidney.

ACKNOWLEDGEMENTS

This work was supported by grants of the Deutsche Forschungsgemeinschaft (Le 318/8-2—Le 318/8-4). The technical assistance of U. Schmidt is gratefully acknowledged.

REFERENCES

1 H. Winkler, W. Ried and B. Lemmer, J. Chromatogr., 228 (1982) 223.
2 E.R. Garrett and K. Schnell, J. Pharm. Sci., 60 (1971) 833.
3 T. Walle, J. Pharm. Sci., 63 (1974) 1885.
4 M.A. Lefebvre, J. Girault, M.Cl. Saux and J.B. Fourtillan, J. Pharm. Sci., 69 (1980) 1216.
5 M.A. Lefebvre, J. Girault and J.B. Fourtillan, J. Liquid Chromatogr., 4 (1981) 483.
6 B. Lemmer, Naunyn-Schmiedeberg's Arch. Pharmacol., 316 (1981) R60.
7 B. Lemmer and K. Bathe, J. Cardiovasc. Pharmacol., 4 (1982) 635.
8 B. Lemmer, K. Bathe, P.H. Lang, G. Neumann and H. Winkler, J. Amer. Coll. Toxicol., 2 (1983) 347.
9 H. Winkler, T. Ohm and B. Lemmer, unpublished results.

Journal of Chromatography, 309 (1984) 193—197
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2126

Note

Determination of the beta-adrenoceptor blocking drug bupranolol in plasma and tissues of the rat by high-performance liquid chromatography with ultraviolet detection

HORST WINKLER and BJÖRN LEMMER*
Centre of Pharmacology, J.W. Goethe-University, Frankfurt/M. (F.R.G.)

(Received January 13th, 1984)

Bupranolol [1-chloro-2-(2-hydroxy-3-*tert.*-butylamino)propoxy-4-methylbenzene, Fig. 1] is the only β-adrenoceptor blocker with a chlorine substituent in the molecule. Until now, no methods have been available for the quantitative determination of bupranolol in plasma and tissues. On account of the chlorine atom, bupranolol does not fluoresce when activated at about 220 or 280 nm, as demonstrated for other β-blockers and which can easily be measured and quantified by spectrofluorimetric detection following high-performance liquid chromatographic (HPLC) separation (see, for example, refs. 1—3). Since we are interested in the structure—activity relationship of β-blockers of different polarities including their pharmacokinetic behaviour [4, 5] an HPLC method with ultraviolet (UV) detection at 200 nm for bupranolol was developed to be able to determine plasma and tissue levels of this lipophilic compound.

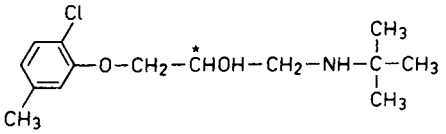


Fig. 1. The molecular structure of bupranolol.

EXPERIMENTAL

Standards and reagents

All reagents used were at least reagent grade and purchased from E. Merck (Darmstadt, F.R.G.). Only deionized glass-distilled water was used. The racemic mixture of bupranolol · HCl was kindly supplied by Sanol Schwarz (Monheim, F.R.G.). Standard solutions from 2 mg/ml to 10 ng/ml were obtained from an aqueous stock solution (2 mg/ml). This solution was stored at 4°C for up to one month.

0378-4347/84/$03.00

HPLC instrumentation and conditions

The HPLC system consisted of a constant-flow pump (Gynkotek, 600/200), an autosampler (Waters, WISP 710 B), a cyano column (Bischoff, 12.5 cm × 4.6 mm; Shandon CPS 5 μm), a spectrophotometer with a deuterium lamp (Kratos, Spectroflow 773) and a computing integrator (Spectra Physics, SP 4100).

The mobile phase was acetonitrile—water (70:30) containing 0.1% phosphoric acid. The flow-rate was 1.5 ml/min and the detection wavelength 200 nm. Chromatography was carried out at ambient temperature.

Sample preparation

Plasma and tissue samples of four organs (heart, muscle, brain, lung) from light—dark synchronized male Wistar rats of about 150—180 g body weight were used. The rats were sacrificed by decapitation, and blood was collected in 12-ml conical glass tubes containing 50 μl of heparin (250 I.U.). After centrifugation (900 *g*, 15 min) 1-ml plasma portions were pipetted into 12-ml screw-capped glass tubes and kept at −35° C. The organs were dissected out, rinsed in ice-cold 0.9% saline solution, blotted on filter paper, weighed, frozen in liquid nitrogen and finally stored at −35° C.

Plasma extraction

The extraction procedure is summarized in Fig. 2. The 1-ml plasma samples

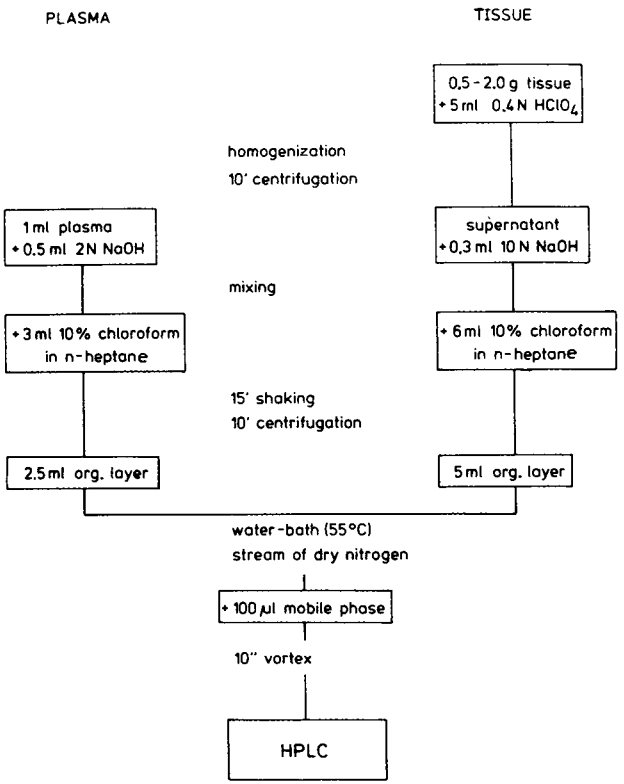


Fig. 2. A schematic outline of the extraction procedure.

were thawed and 0.5 ml of 2 *M* sodium hydroxide (saturated with sodium chloride) was added. Then 3 ml of a mixture of 10% chloroform in *n*-heptane were added; the tubes were shaken for 15 min and the layers separated by centrifugation. Aliquots of 2.5 ml of the organic layer were transferred to 12-ml screw-capped conical glass tubes and evaporated in a water bath at 55°C under a stream of dry nitrogen. After cooling the dry tubes in an ice bath, 100 μl of the mobile phase were added to redissolve the residue. The tubes were vortexed for 15 sec and aliquots (50—80 μl) were injected into the HPLC system.

Tissue extraction

The extraction procedure is summarized in Fig. 2. The frozen tissues (0.5—2.0 g wet weight) were homogenized in 5 ml of 0.4 *M* perchloric acid (saturated with sodium chloride) with an Ultra-Turrax homogenizer in glass tubes at 4°C. After centrifugation at 7000 *g* for 10 min, the supernatants were transferred to 12-ml screw-capped glass tubes containing 0.3 ml of 10 *M* sodium hydroxide and vortexed for 10 sec. Then 6 ml of the extraction mixture (10% chloroform in *n*-heptane) were added; the samples were shaken for 15 min and centrifuged for 10 min at 800 *g*. Aliquots of 5 ml of the organic layer were transferred to 12-ml screw-capped conical glass tubes. The further extraction was as described for the plasma samples (Fig. 2).

Standard curves and recovery studies

Blank plasma and tissue samples from untreated rats were spiked with varying amounts of bupranolol. These samples were treated as described above and standard curves of added concentrations versus peak area were calculated. From these data recovery in each organ was calculated over the whole concentration range.

Bupranolol plasma and tissue concentrations ex vivo were determined in the respective tissues 60, 90, 120 and 150 min after the intravenous injection of 6 μmol/kg bupranolol · HCl to rats.

RESULTS AND DISCUSSION

The HPLC system described provides for the first time a method of determining bupranolol concentrations not only in plasma but also in target organs such as heart, muscle, brain and lung, which are important when studying the kinetic behaviour of β-adrenoceptor blocking drugs [3—5].

Fig. 3 shows chromatograms of blank and spiked plasma samples from untreated rats and ex vivo plasma samples. As can be seen the extraction procedure employed yielded clean samples without interfering peaks in the chromatograms measured at 200 nm. Concentrations as low as 1 ng/ml plasma could be quantified. Chromatograms of blank and ex vivo samples of bupranolol in the four organs studied are depicted in Fig. 4. The chromatograms of the tissue samples also do not show any interfering peaks. The detection limit from tissue was about 10 ng/g.

The data obtained from spiked standard samples are shown in Table I. As can be seen, the correlation coefficients for plasma and all organs were greater

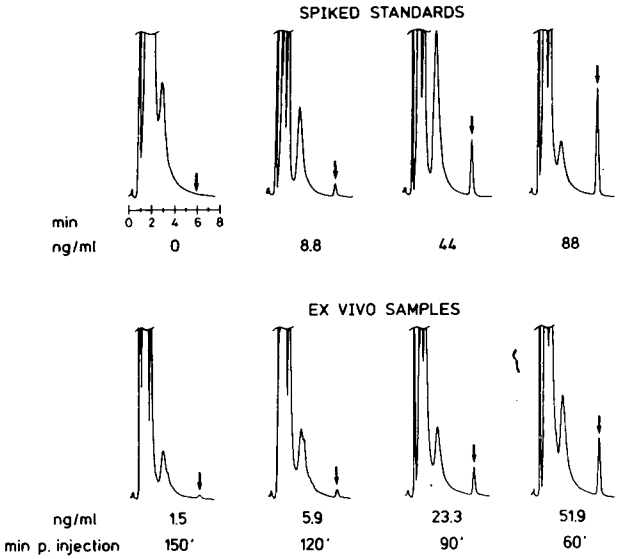


Fig. 3. Chromatograms of bupranolol plasma samples. Upper panel shows spiked plasma samples in which bupranolol was added at 0—88 ng/ml. Lower panel shows chromatograms of ex vivo samples; bupranolol was injected intravenously into rats (6 μmol/kg) and plasma samples were taken 60—150 min after drug application. The concentrations obtained are indicated.

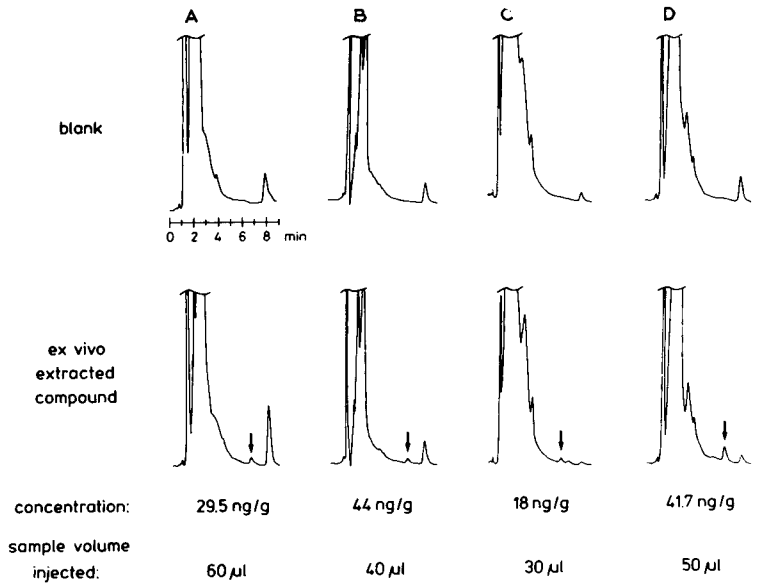


Fig. 4. Representative chromatograms of bupranolol in heart (A), muscle (B), brain (C) and lung (D). Upper panel shows blank tissue samples of the respective organ from untreated rats. Lower panel shows the corresponding ex vivo samples which were isolated 150 min after drug application (6 μmol/kg). The concentrations obtained are indicated.

TABLE I

DATA FOR BUPRANOLOL OBTAINED FROM SPIKED PLASMA AND TISSUE SAMPLES

Organ	No. of samples	Concentration* (ng/sample)	Correlation coefficient	Recovery (%) (mean ± S.E.M.)
Plasma	10	8.8—264.0	0.999	96.6 ± 0.8
Heart	10	17.6—52.8	0.988	26.8 ± 2.3
Muscle	10	8.8—264.0	0.992	21.5 ± 1.8
Lung	10	44.0—880.0	0.995	24.9 ± 1.7
Brain	10	88.0—1320.0	0.995	26.5 ± 2.4

*Depending on the plasma and tissue concentrations expected in the pharmacokinetic studies.

than 0.98. The recoveries achieved were 97% from plasma and 22—27% from the organs. The difference in the recoveries from plasma compared to those from the tissues is similar to the difference we found when studying the kinetic behaviour of propranolol [1, 2], which is a β-blocker of nearly the same polarity as bupranolol. These lipophilic compounds have a strong affinity for tissue membranes which results in a lower recovery from tissues than from plasma. A dependency of the bupranolol recovery upon the amount of tissue extracted as described for propranolol [1, 2] could not be observed.

In conclusion, the HPLC system presented for the determination of bupranolol concentrations has shown its applicability within a study on the kinetic behaviour of this drug (6 μmol/kg, intravenously) in plasma and four organs of the rat [4]. It has been shown that it is possible to quantify substances in biological material by short-wavelength UV detection without difficulty.

ACKNOWLEDGEMENTS

This work was supported by grants from the Deutsche Forschungsgemeinschaft (Le 318/8-2—318/8-4). The technical assistance of U. Schmidt is gratefully acknowledged.

REFERENCES

1 H. Winkler, W. Ried and B. Lemmer, J. Chromatogr., 228 (1982) 223.
2 H. Winkler, Thesis, Frankfurt, 1983.
3 B. Lemmer and K. Bathe, J. Cardiovasc. Pharmacol., 4 (1982) 635.
4 B. Lemmer, H. Winkler and G. Neumann, Naunyn-Schmiedeberg's Arch. Pharmacol., 321 (1982) R20.
5 B. Lemmer, K. Bathe, P.H. Lang, G. Neumann and H. Winkler, J. Amer. Coll. Toxicol., 2 (1983) 347.

Journal of Chromatography, 309 (1984) 198—202
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2129

Note

Determination of two metoprolol metabolites in human urine by high-performance liquid chromatography

J. GODBILLON* and M. DUVAL

Ciba-Geigy Biopharmaceutical Research Center, B.P. 308, 92506 Rueil-Malmaison Cedex (France)

(Received January 27th, 1984)

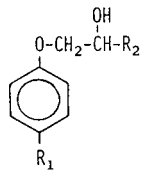
Pharmacokinetic studies in man have demonstrated that the β-blocker metoprolol is eliminated from the body mainly in the form of metabolites; only a minor fraction of the given dose is recovered as unchanged drug in urine [1]. Four urinary metabolites were identified (compounds I—IV in Fig. 1) and quantified after administration of a single dose of ^{3}H-labelled metoprolol [2]. Two of them, α-hydroxymetoprolol (I) and O-desmethylmetoprolol (II) possess β_1-receptor blocking activity in the cat [2] and dog [3] but are five to ten times less potent than the parent drug. Metabolite I, whose plasma concentrations are about 50% of those of metoprolol in healthy subjects [4, 5], accounts for 10% of the dose in urine. Metabolite II is rapidly oxidized to an inactive amino acid (III) and recovered in only minor amounts in human body fluids [2, 4, 5]. The other two metabolites, III and IV, have no pharmacological activity; they account, respectively, for about 65% and 10% of an oral dose [2].

Several methods have been described for the assay of the active metabolite (I) in plasma or urine [6—10].

Metabolite III is highly water-soluble so that extraction from biological fluids cannot be achieved. The only method available involves three evaporation steps and two derivatization stages before gas chromatography [5]. Its limit of quantitation is 0.5 μg/ml of urine.

We describe a simpler high-performance liquid chromatographic (HPLC) method for the major metabolite, III, involving direct injection of diluted urine. It allows the assay also of α-hydroxymetoprolol which is the major biotransformation product in plasma. The applicability of the method is demonstrated.

0378-4347/84/$03.00



COMPOUND	R_1	R_2	RENAL ELIMINATION (% OF DOSE) (2)
METOPROLOL	$CH_2-CH_2-O-CH_3$	$CH_2-NH-CH(CH_3)_2$	⩽ 5
METABOLITE I α-HYDROXY-METOPROLOL (H 119/66)	$CH(OH)-CH_2-O-CH_3$	$CH_2-NH-CH(CH_3)_2$	10 (ACTIVE)
METABOLITE II O-DESMETHYL-METOPROLOL (H 105/22)	CH_2-CH_2-OH	$CH_2-NH-CH(CH_3)_2$	< 0.4 (ACTIVE)
METABOLITE III (H 117/04)	CH_2-COOH	$CH_2-NH-CH(CH_3)_2$	60 - 65 (INACTIVE)
METABOLITE IV (H 104/83)	$CH_2-CH_2-O-CH_3$	COOH	10 - 15 (INACTIVE)

Fig. 1. Chemical structure of metoprolol and of its known metabolites.

EXPERIMENTAL

Chemicals and reagents

Metoprolol tartrate was supplied by Ciba-Geigy (Basle, Switzerland). Metabolites I—IV were supplied by Hässle (Mölndal, Sweden). All solvents and reagents were of analytical grade. Solution A for the mobile phase was made of 2.9 mmol (238 mg) of anhydrous sodium acetate and 40 mmol (2.3 ml) of acetic acid in 1 l of water (pH 3.5 buffer).

Reference solutions

These were prepared in water from the hydrochloride of III and the *p*-hydroxybenzoic acid salt of I.

Sample preparation

A 0.5-ml volume of urine is diluted to 20 ml with distilled water. A 1-ml aliquot of the diluted urine and 100 μl of water or of aqueous reference solutions are mixed in a glass tube; 60 μl are injected onto the column.

Chromatography

The chromatography was performed on a Hewlett-Packard instrument, Model 1081 B, equipped with a variable-wavelength detector (Schoeffel SF 770) set at 222 nm.

The column was a stainless-steel tube (25 cm × 4.7 mm I.D.) filled with LiChrosorb RP-8, 5 μm (E. Merck, Darmstadt, F.R.G.). The slurry, made of 3.6 g of LiChrosorb (preliminarily dried at 110°C for 2 h) dispersed in a mixture of 10.8 ml of *n*-heptane and 10.8 ml of isopropyl alcohol, was forced into the column with *n*-heptane. The column was rinsed with 50 ml of ethanol before use. The degassed mobile phase acetonitrile—solution A (70:30, v/v) was used at a flow-rate of 1 ml/min. The mobile phase and the column were at room temperature.

The retention times were about 10 and 13 min for metabolites III and I, respectively. The top pressure was about 90 bars.

Calibration curves

Calibration samples were prepared by adding 100 μl of reference solutions containing both metabolites I and III as described in the sample preparation procedure. The added amounts corresponded to concentrations ranging from 16.5 μmol/l (5 μg/ml) to 329.5 μmol/l (100 μg/ml) for metabolite III and from 11.9 μmol/l (5 μg/ml) to 237.5 μmol/l (100 μg/ml) for metabolite I.

The calibration curves were obtained by plotting the peak height of each metabolite versus the concentrations. Their equations were calculated by the least-squares method using linear regression. For routine analysis, a calibration curve is established every day from five to seven calibration samples, each sample being injected once.

RESULTS AND DISCUSSION

Urine interferences

Metabolites I and III are well separated from the urine components. Urine from several volunteers was tested. A typical chromatogram of blank urine and spiked urine is shown in Fig. 2.

Accuracy, precision, reproducibility, limit of quantitation

Metabolites I and III can be measured with good precision and accuracy at concentrations down to 5 μg/ml (11.9 μmol/l for I and 16.5 μmol/l for III) (Table I).

Selectivity

The parent drug metoprolol and the other known metabolites (Fig. 1) did not interfere in the assay of metabolites I and III.

The acidic metabolite (IV) was eluted in the solvent front. The relative retention times were 1, 1.19, 1.77 for III, I and metoprolol, respectively. Two peaks were observed for metabolite II (relative retention times 1 and 1.19). As this metabolite, which is an intermediate in the formation of metabolite III, accounts for less than 0.4% of the dose excreted in urine [4], it will not interfere significantly in the assay of I and III.

Provided the concentrations of metoprolol are higher than 14.6 μmol/l (5 μg/ml), the unchanged drug can be simultaneously assayed with the two metabolites, with comparable precision and accuracy (Table I).

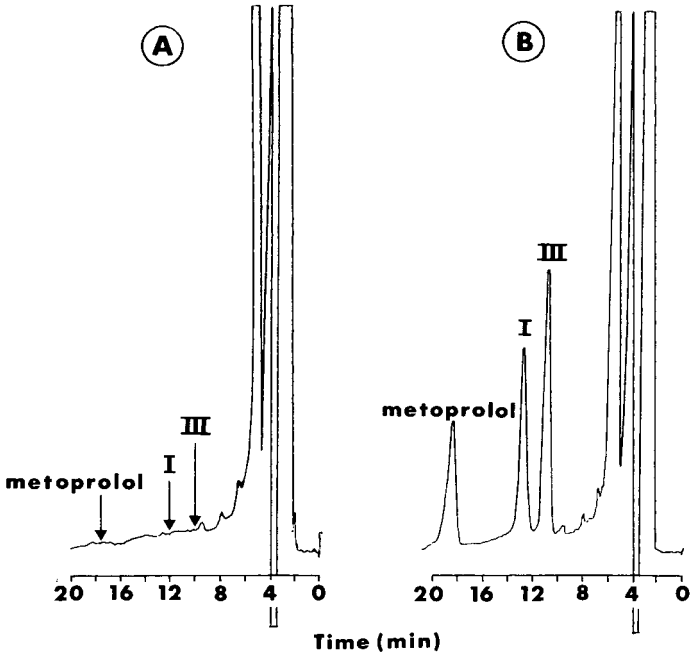


Fig. 2. Chromatograms corresponding to 1 ml of diluted blank urine (A) and to 1 ml of diluted urine spiked with 5 nmol of metabolite III, 3.6 nmol of metabolite I and 4.4 nmol of metoprolol (B).

TABLE I

ACCURACY, PRECISION, REPRODUCIBILITY AND QUANTITATION (SPIKED SAMPLES)

	Metabolite III					α-Hydroxymetoprolol (metabolite I)			Metoprolol		
Amount added (μmol/l)*	16.5	32.9	65.9	131.8	197.7	11.9	47.5	118.8	14.6	29.2	87.7
Amount found (μmol/l)*	17.5	33.3	65.5	134	198.3	12.1	46.3	116.5	12.7	28.9	86.2
Number of replicates	6	18	18	18	18	6	6	8	6	6	6
Coefficient of variation (%)	4.5	5.2	3.4	3.4	2.7	7.0	1.5	1.8	6.8	4.9	1.5
Mean recovery (%)	106	101	100	102	100	102	97	98	88	99	99

*The data are expressed in μmol of free base per l.

Application

The urinary excretion data given in Fig. 3 indicate that the present method is suitable for the assay of these metabolites after administration of metoprolol. The results given were in agreement with those already reported [2, 5]. The limit of quantitation for α-hydroxymetoprolol is higher than that obtained by Lennard and Silas [10] by HPLC with fluorimetric detection (200 ng/ml). However, it appeared sufficient for pharmacokinetic studies with metoprolol [11, 12].

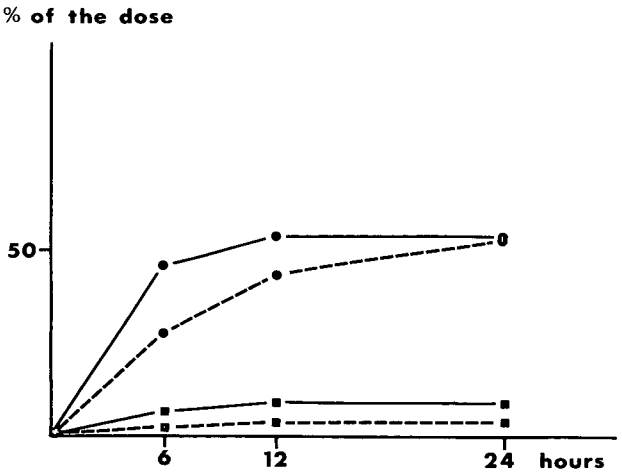


Fig. 3. Urinary elimination (24-h) of metoprolol metabolites I (▪) and III (•) after administration of a single oral dose of 100 mg of metoprolol tartrate (292 μmol of metoprolol base). (——), Subject 1; (- - -), subject 2.

REFERENCES

1 C.G. Regårdh, K.O. Borg, R. Johansson, G. Johnsson and L. Palmer, J. Pharmacokin. Biopharm., 2 (1974) 347.
2 K.O. Borg, E. Carlsson, K.J. Hoffmann, T.E. Jönsson, H. Thorin and B. Wallin, Acta Pharmacol. Toxicol., 36 (Suppl. V) (1975) 125.
3 C.G. Regårdh, L. Ek and K.J. Hoffmann, J. Pharmacokin. Biopharm., 7 (1979) 471.
4 K.J. Hoffmann, C.-G. Regårdh, M. Aurell, M. Ervik and L. Jordö, Clin. Pharmacokin., 5 (1980) 181.
5 C.P. Quaterman, M.J. Kendall and D.B. Jack, Brit. J. Clin. Pharmacol., 11 (1981) 287.
6 J.-B. Lecaillon, J. Godbillon, F. Abadie and G. Gosset, J. Chromatogr., 305 (1984) 411.
7 C.P. Quaterman, M.J. Kendall and D.B. Jack, J. Chromatogr., 183 (1980) 92.
8 M. Ervik, K.J. Hoffmann and K. Kylberg-Hanssen, Biomed. Mass Spectrom., 8 (1981) 322.
9 D.B. Pautler and W.J. Jusko, J. Chromatogr., 228 (1982) 215.
10 M.S. Lennard and J.H. Silas, J. Chromatogr., 272 (1983) 205.
11 J. Godbillon, D. Evard, N. Vidon, M. Duval, J.P. Schoeller, J.J. Bernier and J. Hirtz, Brit. J. Clin. Pharmacol., in press.
12 D. Evard, N. Vidon, J. Godbillon, M. Bovet, M. Duval, J.P. Schoeller, J.J. Bernier and J. Hirtz, Brit. J. Clin. Pharmacol., in press.

Journal of Chromatography, 309 (1984) 203—208
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2133

Note

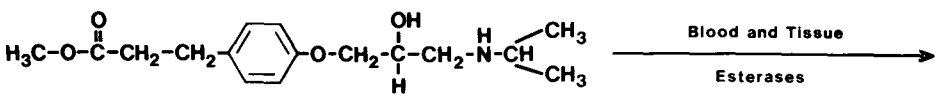
High-performance liquid chromatographic assay for the major blood metabolite of esmolol — an ultra short acting beta blocker

HERMAN F. STAMPFLI, CHII-MING LAI, AVRAHAM YACOBI and CHECK Y. SUM*

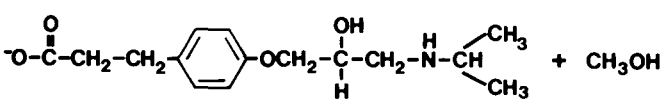
American Critical Care, Research and Development, 1600 Waukegan Road, McGaw Park, IL 60085 (U.S.A.)

(First received August 19th, 1983; revised manuscript received March 1st, 1984)

Esmolol, methyl 3-{4-[2-hydroxy-3-(isopropylamino)propoxy]phenyl}propionate · HCl is a novel, cardioselective ultra short acting beta-adrenergic receptor antagonist with a duration of action of 10—15 min in anesthetized dogs [1]. The key feature of esmolol as an ultra short acting beta blocker is the ester linkage in the compound [2]. Rapid hydrolysis of the ester functionality results in methanol and 3-{4-[2-hydroxy-3-(isopropylamino)propoxy]phenyl}-



ESMOLOL



Methanol

I

Fig. 1. Hydrolysis of esmolol resulting in methanol and I.

0378-4347/84/$03.00

propionic acid (I, Fig. 1). Based upon in vitro isolated tissue studies, I is a beta blocker, but is 200-fold less potent than esmolol [3]. With conventional drugs, metabolic transformation converts the parent compound into metabolites that have faster elimination rates, thus facilitating the removal of the drug from the body. In contrast, metabolism of ultra short acting therapeutic agents like chloroprocaine [4], succinylcholine [5], and esmolol [6] results in metabolites that have elimination half-lives much longer than the parent compound.

Since esmolol has an elimination half-life of less than 15 min in man [6], long term pharmacological and toxicological effects, if any, are more likely to be caused by I and not esmolol. This is especially true in chronic studies where the possibility of accumulation of I may occur. Thus, a good understanding of the pharmacokinetics of esmolol requires careful monitoring of blood levels of esmolol and I.

This paper describes an isocratic reversed-phase high-performance liquid chromatographic (HPLC) method for measuring I. In addition, application of the method to measure blood levels of I during a dose range-finding study of esmolol in man is also presented.

EXPERIMENTAL

Chemicals

I and its internal standard, 3-{1-amino-[3-(4-chlorophenoxy)]2-propanol}-propionic acid were synthesized at American Critical Care (McGaw Park, IL, U.S.A.). Methylene chloride (spectro grade) and acetonitrile (HPLC grade) were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.); perchloric acid (70%) and sodium acetate were purchased from Mallinckrodt (St. Louis, MO, U.S.A.). Sodium heparin was purchased from Organon (W. Orange, NJ, U.S.A.).

Sample preparation

Heparinized blood (1 ml) was transferred to 20 mm × 125 mm tubes (Scientific Products, McGaw Park, IL, U.S.A.) containing 10 ml of methylene chloride. After capping, the tubes were manually mixed vigorously for 15 sec before placing the tubes on a mechanical shaker (Eberbach, Ann Arbor, MI, U.S.A.) for 10 min. The tubes were then centrifuged at 1700 *g* using an IEC Centra 7-R centrifuge (International Equipment, Needham Heights, MA, U.S.A.) for 10 min. A 0.5-ml aliquot of the reddish aqueous phase was removed and transferred to 100 mm × 13 mm tubes (Scientific Products) containing a 100-μl aliquot of the internal standard solution (0.1 mg/ml). The samples were mixed for 15 sec using a Vortex Genie mixer (Scientific Products). A 500-μl aliquot of 14% perchloric acid was added to the tubes followed by mixing on a Vortex Genie mixer for 15 sec. The samples were centrifuged at 1700 *g* for 10 min and the resultant supernatants analyzed by HPLC.

Sample analysis

The samples were analyzed using a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A pump, Model 440 UV detector with a 280-nm filter, Model 710A automatic injection system, and a 30 cm × 3.9 mm μBondapak phenyl column packed with 10-μm particles. The mobile phase consisted of 0.01 *M* sodium

acetate—acetonitrile—glacial acetic acid, pH 3.7 (87:12:1), set at a flow-rate of 2.0 ml/min.

Recovery and reproducibility

Recovery of I from blood was examined by spiking varying amounts of the compound into blood and comparing these samples with results obtained with aqueous standards. Reproducibility of the assay was determined by preparing standard curves over a 500-fold range and examining the variability at each concentration.

Clinical protocol

Six healthy males, 20—30 years old (mean 23.2 years) and weighing 67.5—82.5 kg (mean 73.4 kg), participated in the study. All the subjects were paid and signed a written consent form. Each subject received constant increasing intravenous infusions of 10, 40, 100, 150, 200, 300, 450, and 650 μg/kg/min of esmolol for 60 min on eight consecutive days. The minimum period of time between treatments was 23 h. In each treatment, blood samples were collected into heparinized tubes before (—1 min), at 30 and 60 min after initiation of the infusion, and at 5 and 15 min after stopping the infusion. Selected samples from six of the doses were assayed for I.

Data analyses

Identification of the individual peaks was accomplished by referring to the elution time (R_t) of water standards. Quantitation of I was accomplished by using a Hewlett-Packard Model 3356 Laboratory Automation System (Palo Alto, CA, U.S.A.) which determined the ratio of the peak areas of I to that of the internal standard.

RESULTS

A representative chromatogram of I and the internal standard is shown in Fig. 2. Endogenous blood components did not interfere with the accurate quantitation of both compounds. By using water standards as references, the HPLC peaks with R_t of 7.3 and 9.7 min were identified as I and the internal standard, respectively. Under the conditions of analysis, esmolol had an R_t of 23.4 min (data not shown).

The recovery of I from whole blood averaged 96.9% and appeared to be independent of concentration (Table I). The high recovery of I at the detection limit of the assay (1.0 μg/ml blood) probably reflected a low signal-to-noise ratio, with endogenous blood components contributing to the observed detector response. Over the concentration range examined, the assay was highly reproducible with an average coefficient of determination of 5.8% (Table I) and linear with a correlation coefficient of 0.999 (data not shown).

The assay was used to measure blood levels of I during a Phase I dose-range finding study of esmolol in man. Increasing doses of esmolol were intravenously administered to normal volunteers. Blood levels of I were determined during and immediately after 1-h infusions of esmolol and the results are summarized in Table II. During the 1-h infusion period, blood levels of I did

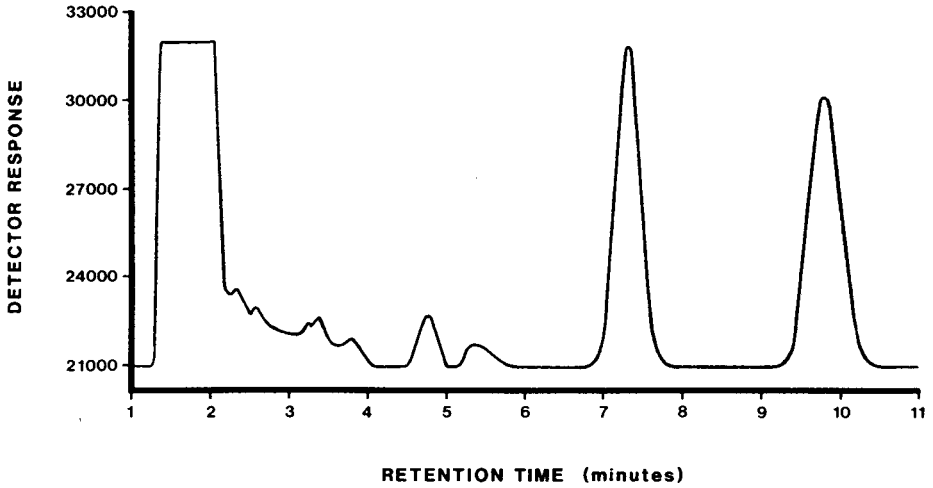


Fig. 2. HPLC chromatogram of whole blood spiked with I (7.3 min) and the internal standard (9.7 min). All other peaks are endogenous to blood.

TABLE I

RECOVERY AND REPRODUCIBILITY OF THE HPLC ASSAY FOR I IN BLOOD

Concentration (μg/ml)	Recovery* ± S.D. (%)	Coefficient of determination** (%)
1.0	127 ± 20.3	10.9
2.5	83.6 ± 10.0	8.3
5.0	90.2 ± 9.1	7.1
10	90.4 ± 1.4	2.6
25	96.0 ± 1.5	6.9
50	94.0 ± 3.2	7.0
100	98.4 ± 6.4	4.6
250	95.2 ± 10.0	1.4
500	97.0 ± 9.9	3.4
Mean ± S.D.	96.9 ± 12.2	5.8 ± 3.0

*n = 3 at each concentration.
**n = 6 at each concentration.

not reach steady-state after any of the doses. The metabolism of esmolol to I did, however, appear to follow linear kinetics since a plot of the average blood concentration of I versus the dose of esmolol at the end of 1-h infusion showed an excellent linear relationship (Fig. 3). The average blood concentrations at the end of the infusion period were 1.52, 4.18, 10.2, 17.0, 32.9, and 35.6 μg/ml blood after esmolol infusion rates of 40, 100, 200, 300, 450, and 650 μg/kg/min, respectively (Table II).

DISCUSSION

I is an amphoteric compound having two pK_a values such that it will be doubly charged at physiological pH. Therefore, the partition of I into non-polar

TABLE II

CONCENTRATION OF I (μg/ml) IN WHOLE BLOOD FROM THE DOSE-RANGE FINDING STUDY OF ESMOLOL IN NORMAL VOLUNTEERS

Values are expressed as mean ± S.D.

Dose of esmolol (μg/kg/min)	No. of subjects (*n*)	Concentration of I (μg/ml)			
		Sampling time* (min)			
		30	60	65	75
40	2	BDL**	1.52 ± 0.276	1.65 ± 0.587	1.52 ± 0.537
100	3	BDL	4.18 ± 0.718	5.34 ± 1.88	6.41 ± 1.65
200	6	2.96 ± 1.10	10.2 ± 2.90	11.8 ± 2.83	13.6 ± 2.11
300	1	4.32	17.0	19.0	25.5
450	3	11.2 ± 4.37	32.9 ± 9.04	35.7 ± 10.5	35.1 ± 12.8
650	1	13.1	35.6	43.7	43.6

*Sampling times are as follows: 30 and 60 min after start of infusion; 65 and 75 min are 5 and 15 min after termination of infusion, respectively.
**BDL = below detection limit (1 μg/ml).

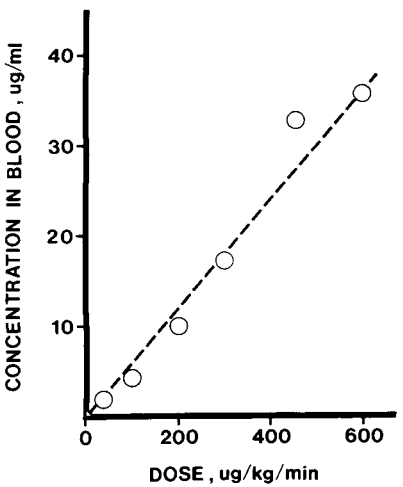


Fig. 3. Average blood concentration of I as a function of dose at the conclusion of 1-h infusion of esmolol in normal subjects (n = 1—6).

organic solvents is very low resulting in quantitative recovery of the compound in the aqueous layer. Under the conditions of the extraction, esmolol preferentially partitions into the methylene chloride layer resulting in < 5% remaining in the aqueous layer [7]. Deproteinization of the red aqueous layer with perchloric acid after extracting blood with methylene chloride results in a supernatant suitable for analysis by HPLC.

Data from the human dose range finding study demonstrated linear increases in blood concentrations of I as a function of esmolol doses indicating that saturation of metabolic and/or elimination process(es) during the 1-h infusions did not occur. Blood levels of I attained at the end of the infusion period were

10 to 15 times greater than corresponding levels of esmolol [7], but results from an efficacy study in healthy volunteers, however, demonstrated a good correlation between beta blocking effects and blood concentrations of esmolol [6].

REFERENCES

1 J. Zaroslinksi, R.J. Borgman, J.P. O'Donnell, W.G. Anderson, P.W. Erhardt, S.T. Kam, R.D. Reynolds, R.J. Lee and R.J. Gorczynski, Life Sci., 31 (1982) 899.
2 C.Y. Sum, H.F. Stampfli, C.M. Lai, S.T. Kam, P.W. Erhardt, C. Woo and A. Yacobi, Proc. 33rd Nat. Meet. APhA, 12 (1982) 177.
3 P.W. Erhardt, C.M. Woo, W.G. Anderson and R.J. Gorczynski, J. Med. Chem., 25 (1982) 1408.
4 J.E. O'Brien, V. Abbey, O. Hinsvark, J. Perel and M. Finster, J. Pharm. Sci., 68 (1979) 75.
5 L.B. Wingard and D.R. Cook, Clin. Pharmacokin., 2 (1977) 330.
6 C.Y. Sum, A. Yacobi, R. Kartzinel, H. Stampfli, C.S. Davis and C.M. Lai, Clin. Pharmacol. Ther., 34 (1983) 427.
7 C.Y. Sum and A. Yacobi, J. Pharm. Sci., in press.

Journal of Chromatography, 309 (1984) 209—213
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2135

Note

Reversed-phase high-performance liquid chromatographic method to determine ceftriaxone in biological fluids

DAVID B. BOWMAN*, M.K. ARAVIND, JOSEPH N. MICELI and RALPH E. KAUFFMAN

*Departments of Pediatrics and Pharmacology, Wayne State University School of Medicine and *Division of Clinical Pharmacology and Toxicology, Children's Hospital of Michigan, 3901 Beaubien Boulevard, Detroit, MI 48201 (U.S.A.)*

(First received October 12th, 1983; revised manuscript received February 20th, 1984)

Ceftriaxone is a member of the "third generation" of cephalosporin antibiotics characterized by a relatively broad antibacterial spectrum, including moderate activity against many strains of *Pseudomonas aeruginosa*. In addition to its broad antibacterial spectrum, ceftriaxone exhibits a uniquely long elimination half-life, is resistant to β-lactamase producing organisms, and diffuses well into extravascular spaces, including the cerebrospinal fluid (CSF). These characteristics offer potential clinical advantages which may lead to increasing use of this drug to treat bacterial meningitis and a variety of other serious infections.

Current published methods for determining ceftriaxone in biological fluids include bioassay of total antibacterial activity [1] and high-performance liquid chromatography (HPLC) [2—4]. The bioassays tend to be cumbersome, require a relatively long turnaround time, are susceptible to interference from concurrently administered antibiotics, and exhibit considerable random variation inherent in the assay system. Two of the previously described HPLC methods [2, 3] require a different mobile phase for determining the ceftriaxone concentration in each of these biological specimens: bile, serum, and urine. Another recent method uses normal-phase column chromatography [4].

We describe here a reversed-phase HPLC method using ion-pair chromatography to quantitate ceftriaxone in body fluids. This method utilizes the same chromatographic conditions and column for serum and urine. Additionally, by use of this same method, ceftriaxone can be measured in CSF as well. The

0378-4347/84/$03.00

method requires a small sample volume, provides sensitivity and precision, simplifies sample preparation and analysis, and allows rapid turnaround time.

EXPERIMENTAL

Materials and reagents

Ceftriaxone analytical standard was supplied by Hoffmann-LaRoche (Nutley, NJ, U.S.A.). Monobasic and dibasic potassium phosphate (Baker Analyzed) were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). Fisher Scientific (Fairlawn, NJ, U.S.A.) was the source of HPLC-grade acetonitrile, methylene chloride, and the paired-ion reagent, hexadecyltrimethylammonium bromide (HDTMAB).

A stock standard solution of 1.0 mg/ml ceftriaxone in deionized water was prepared monthly and stored at 5° C. The mobile phase was prepared in the following sequence to prevent the buffer salts from crystallizing out of solution. Acetonitrile (400 ml) was added to 2.73 g of HDTMAB followed by the addition of 200 ml of deionized water. HDTMAB was completely dissolved prior to the addition of 7.5 ml of a 1 *M* monobasic—dibasic potassium phosphate buffer (pH 7.0). The volume was made up to 1 l with deionized water and degassed under vacuum prior to use.

Chromatography

Chromatography was performed utilizing a Waters Assoc. (Milford, MA, U.S.A.) M-45 solvent delivery system in conjunction with a Perkin-Elmer (Norwalk, CT, U.S.A.) oven and LC-55 UV/VIS variable-wavelength spectrophotometer. The detector was interfaced with a Perkin-Elmer Sigma 10 data system which provided the retention times and the integrated area under the peaks. A 5-μm 25 × 0.45 cm LiChrosorb RP-8, reversed-phase column (Supelco, Bellefonte, PA, U.S.A.) was used for analysis. The effluent was monitored at 280 nm at a flow-rate of 1.6 ml/min, while the oven temperature was maintained at 40° C.

Procedure

Standard curves were prepared by adding ceftriaxone stock solutions (1 mg/ml) to drug-free serum or urine to yield final concentrations of 5, 20, 50, 100, 200, and 400 μg/ml. A 200-μl aliquot of sample or standard (serum or urine diluted 1:10 with deionized water) was added to 200 μl of acetonitrile in a 1.5-ml Eppendorf tube. The tubes were vortexed and centrifuged for 5 min at 21,000 *g* in an Eppendorf 3200 centrifuge. The supernatant was transferred into a clean tube and extracted with 500 μl of methylene chloride. Each tube was vortexed for 20 sec and centrifuged for 5 min. A 10-μl volume of the upper aqueous phase was injected onto the column.

The concentration of ceftriaxone in CSF was determined by calibrating with aqueous standards in the range 0.5—50 μg/ml. CSF specimens were only centrifuged and 10 μl was injected onto the column without prior sample preparation.

RESULTS

Typical chromatograms obtained from serum are shown in Fig. 1. The retention time of ceftriaxone was 7.0 min. Similar chromatograms were obtained from CSF and urine specimens. Concentrations and peak areas were linearly related over the respective ranges in serum, urine, and CSF. Least-squares regression of the ceftriaxone standard curve generated the line $Y = 0.98X - 0.15$ with a correlation coefficient of 0.99.

Day-to-day variation was established by analyzing freshly prepared serum ceftriaxone concentrations of 5, 10, 20, 50, 100, 200 and 400 μg/ml. The average coefficient of variation (C.V.) was 5.5% with a range of 2.5—11.3% (Table I).

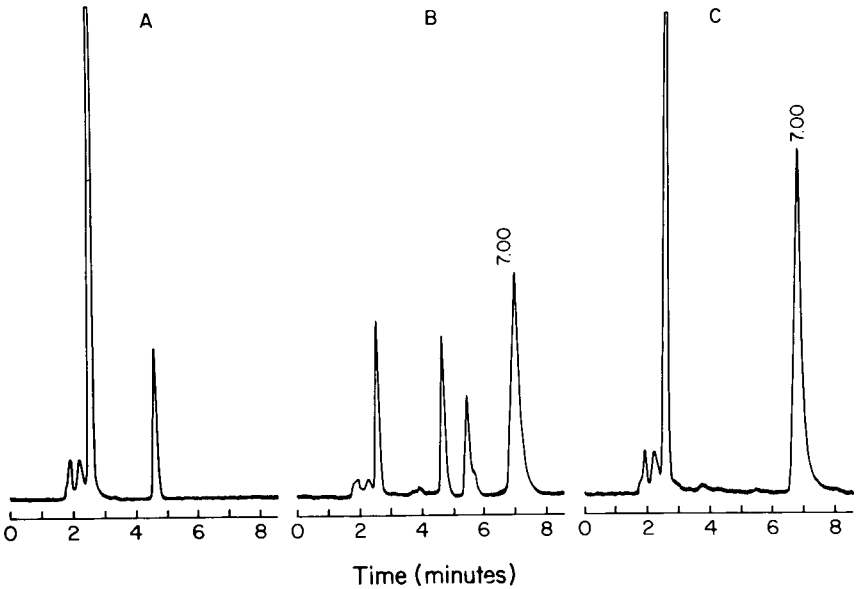


Fig. 1. Chromatograms of (A) drug-free serum; (B) a patient's serum sample in which the determined concentration of ceftriaxone was 29.4 μg/ml; (C) a control serum containing 50 μg/ml ceftriaxone. The retention time of ceftriaxone was 7.00 min.

TABLE I

DAY-TO-DAY VARIATION OF FRESHLY PREPARED SERUM CEFTRIAXONE STANDARDS

In all cases $n = 10$.

Ceftriaxone added (μg/ml)	Mean ± S.D. (μg/ml)	C.V. (%)
5	4.9 ± 0.6	11.3
20	19.5 ± 1.5	7.7
50	49.5 ± 1.5	3.0
100	100.5 ± 2.7	2.7
200	196.8 ± 11.4	5.8
400	395.0 ± 9.8	2.5

TABLE II

STABILITY OF CEFTRIAXONE IN SERUM AT −20°C OVER A FIVE-WEEK PERIOD

Week	Determined concentration of ceftriaxone (μg/ml)
1	52.1
2	59.8
3	57.6
4	54.8
5	53.3
Mean	55.5
± S.D.	3.2
C.V. (%)	5.7

The stability of ceftriaxone in serum was determined by freezing aliquots of a pooled serum containing ceftriaxone which were assayed over a five-week period. There was no significant loss of ceftriaxone during storage at −20°C (Table II). Analytical recovery of ceftriaxone from serum samples was determined to be 96% and 95% at concentrations of 50 and 400 μg/ml, respectively, when compared with aqueous standards.

Commonly co-administered antibiotics, such as methicillin, penicillin, ampicillin, sulfamethoxazole, gentamicin, tobramycin, and chloramphenicol, did not produce interfering peaks.

DISCUSSION

Ceftriaxone promises to be a very useful antibiotic to treat serious bacterial infections because of its broad spectrum of activity, prolonged half-life, and diffusion characteristics. The clinical efficacy of the drug can be further enhanced by the ability to accurately and rapidly determine concentrations in biological fluids thereby ensuring that bactericidal levels are achieved.

In the method presented here, we have taken advantage of using an RP-8 column, an ion-paired mobile phase, and a single, unique sample preparation step to obtain chromatograms free of peaks that interfere with the analysis of ceftriaxone. This clean chromatogram is obtained from serum, CSF, and, most importantly, urine. The method of Trautmann and Haefelfinger [2] used a separate, different mobile phase for serum and urine, while our method uses the same chromatographic conditions for serum, urine, and CSF. Using our method, specimens from CSF were injected directly onto the column, without prior sample processing. The use of one set of analytical conditions decreases the time and cost required for analysis and eliminates the change-over time from one set of chromatographic conditions to another. This method has a sample volume requirement of 200 μl which makes it suitable for use in pediatric patients as well as adults.

The range of pediatric ceftriaxone concentrations in our experience during 6 h after a dose was from 31 to 227 μg/ml in serum and from 0.7 to 20.6 μg/ml in CSF. If these values can be considered as an indication of the therapeutic range for these two fluids, then the method described here has the required

sensitivity and linearity (5.0—400 μg/ml in serum; 0.5—50 μg/ml in CSF) to be used in the routine therapeutic monitoring of ceftriaxone.

ACKNOWLEDGEMENTS

This study supported in part by a grant from Hoffmann-LaRoche, Nutley, NJ, U.S.A. The authors gratefully acknowledge the contributions of Adnan Dajani, Joyce Buckley and Denise Kobos.

REFERENCES

1 G.H. McCracken, Jr., J.D. Nelson and L. Grimm, Antimicrob. Agents Chemother., 21 (1982) 262.
2 K.H. Trautmann and P. Haefelfinger, J. High Resolut. Chromatogr. Chromatogr. Commun., 4 (1981) 54.
3 I.H. Patel, S. Chen, M. Parsonnet, M.R. Hackman, M.A. Brooks, J. Konikoff and S.A. Kaplan, Antimicrob. Agents Chemother., 20 (1981) 634.
4 V. Ascalone and L. Dal Bo, J. Chromatogr., 273 (1983) 357.

Journal of Chromatography, 309 (1984) 214—218
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2137

Note

High-performance liquid chromatographic determination of mepixanthone in serum

GABRIELE GROSSI*, AVELIA LIPPI, RITA BATTISTONI and ELENA MARTELLI

Laboratorio Centralizzato, Policlinico S. Orsola, Via Massarenti 9, 40138 Bologna (Italy)

and

CARLO STURANI

Servizio di Fisiopatologia Respiratoria, Policlinico S. Orsola, Bologna (Italy)

(First received December 29th, 1983; revised manuscript received February 23rd, 1984)

Mepixanthone (3-methoxy-4-piperidinomethylxanthone, Fig. 1) is an analeptic drug used in respiratory and cardiorespiratory insufficiency [1-4]. An antidoping screening procedure for mepixanthone is reported in the literature [5], but quantitative methods for the determination of this drug are not available. In this paper a simple and sensitive high-performance liquid chromatographic (HPLC) method is described which has also been used for the determination of mepixanthone after intravenous administration.

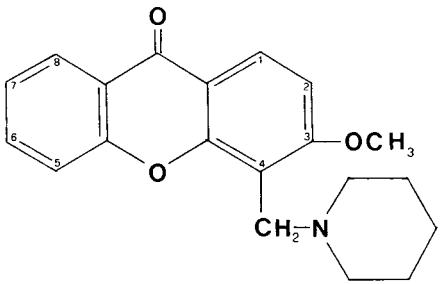


Fig. 1. Structure of mepixanthone.

0378-4347/84/$03.00

EXPERIMENTAL

Chemicals and reagents

Mepixanthone was supplied by Dott. Formenti, Milan, Italy; chlorpromazine [2-chloro-10-(3-dimethylaminopropyl)phenothiazine] (internal standard) was from Farmitalia Carlo Erba, Milan, Italy. Acetonitrile and isopropyl alcohol were HPLC grade (E. Merck, Darmstadt, F.R.G.). Diethylamine was analytical grade (Merck). The extraction tubes Toxi Tube A, active ingredients of which are sodium carbonate (3.5%), sodium bicarbonate (3.5%), dichloromethane (18%) and dichloroethane (17%), buffered at pH 9.0, were supplied by Analytical Systems (Laguna Hills, CA, U.S.A.).

Instrumentation

A Perkin-Elmer Series 3B liquid chromatograph equipped with a 5-μm silica Si 60 column 250 × 4 mm (Merck) was used. The mobile phase was acetonitrile–isopropyl alcohol–diethylamine (50:50:0.004) at a flow-rate of 1 ml/min. The column effluent was monitored with a Perkin-Elmer LC-75 variable-wavelength detector set at 237 nm.

The samples were injected using an automatic sampler (Perkin-Elmer Model LC-420) fitted with a 50-μl sample loop. The 2-ml vials for automatic sampling (Supelco, Bellefonte, PA, U.S.A.) were adapted by inserting 250-μl microtest tubes (LP Italiana, Milan, Italy) to allow the automatic injection from samples having a total volume of only 200 μl [6].

All the chromatographic data were processed with a Perkin-Elmer Model Sigma 10 data system.

Preparation of standards

Standard calibration solutions, containing 0, 5, 20, 50, 200, 400, 800, 2000 μg/l mepixanthone, were prepared in human serum by dilution of an aqueous solution of mepixanthone.

Chlorpromazine was used as a 0.9 mg/l solution in bidistilled water.

Extraction procedure

Serum or plasma samples (1 ml) and chlorpromazine solution (4 ml) were added to a previously mixed Toxi Tube A extraction tube and mixed for 5 min on a rotary mixer. After centrifugation (3000 *g* for 5 min) the organic layers (upper phase) were transferred to glass tubes and evaporated at room temperature under a stream of dry nitrogen. The residues were reconstituted in 200 μl of acetonitrile–diethylamine mixture (500:0.02) and transferred into the automatic sampler vials.

The sample concentrations were calculated by comparing the sample's peak area ratio of mepixanthone to chlorpromazine with those of calibration standards.

The Toxi Tube A extraction tubes are reproducible and time-saving. Care should be taken to continue the full extraction operation until the final rotary mixing, completing one sample extraction before the next one, thus avoiding the deposition of salts.

RESULTS AND DISCUSSION

Fig. 2 shows typical chromatograms of serum extracts from a patient receiving mepixanthone intravenously. For the system used, the number of theoretical plates was 7160 and the capacity ratio of mepixanthone was 2.66. The limit of sensitivity for mepixanthone is 1 μg/l.

The within-assay variation was measured by replicate analyses (n = 20) of mepixanthone standard solutions in human serum, containing 5, 50 and 400 μg/l. The coefficients of variation (C.V.) were 5.4%, 3.4% and 3.0%, respectively. Between-assay C.V. values were determined by comparing the results obtained on different days (n = 10) over a period of three months, for three control sera containing 50, 400 and 800 μg/l mepixanthone. The C.V. values were 6.3%, 5.9% and 5.8%, respectively.

The extraction recovery of mepixanthone from human serum (Table I) was

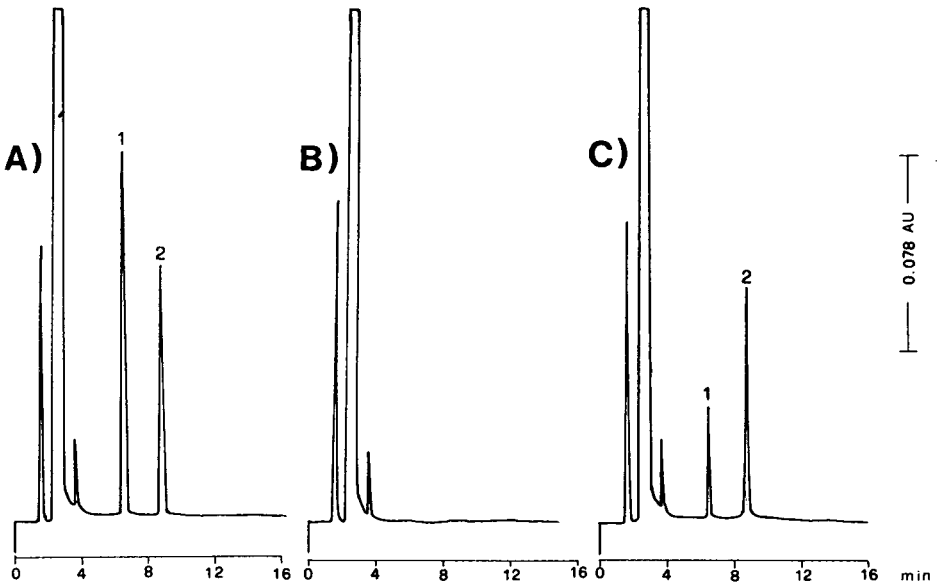


Fig. 2. Chromatograms of (A) an extract from a plasma sample spiked with 800 μg/l mepixanthone and 3.6 mg/l chlorpromazine, (B) plasma blank, and (C) plasma after the administration of mepixanthone to the patient. Peaks: 1 = mepixanthone, 2 = chlorpromazine. AU = absorbance unit.

TABLE I

EXTRACTION RECOVERY

Mepixanthone concentration (μg/l)	Mean recovery (n = 20)	
	%	S.D.
5	71.2	6.2
50	81.7	3.6
400	88.3	3.1

calculated at three concentrations by comparing the sample's peak area of mepixanthone with that of an unextracted equivalent concentration.

The correlation coefficient between the found (Y) and theoretical (X) concentration for serum spiked with 50, 200, 400, 800 and 2000 μg/l mepixanthone was $r = 0.99991$, and the correlation curve was described by the equation $Y = 1.069X - 16.18$.

The reliability of the proposed method was tested in a patient by determination of plasma mepixanthone following intravenous administration of 50 mg. Blood samples were taken at 0, 15 and 30 min, and 1, 3, 6, 12 and 24 h after administration of the drug. The pharmacokinetic behaviour with related parameters is shown in Fig. 3.

The method described has the advantages of a small sample requirement, sensitivity, selectivity and speed. No interferences from endogenous serum constituents were seen and it was therefore possible to operate at a wavelength of 237 nm, at which mepixanthone absorbs more than at the other analytical wavelength of 302 nm (absorbance ratio, 237/308 nm = 3.24).

Retention times of other drugs that were extracted under the assay conditions described and studied as potential sources of interference are shown in Table II. The only drug that has a retention time close to that of mepixanthone is dimefline [5], but the two peaks are separated with a resolution of 1.257 and a column selectivity of 1.087. Moreover, a patient who is treated with mepixanthone is not expected to be treated also with dimefline, since the two drugs belong to the same pharmacological class and are alternative.

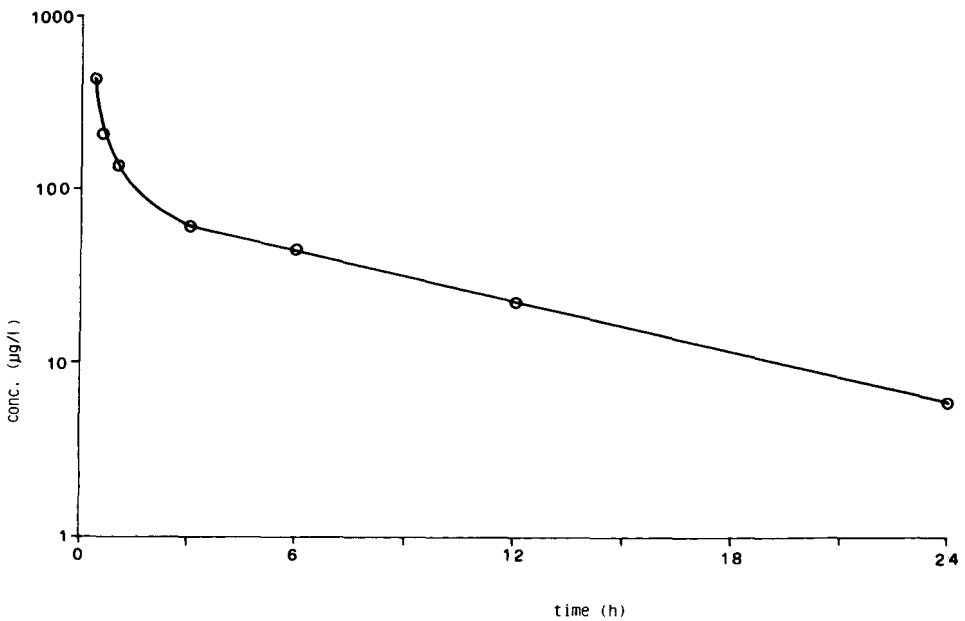


Fig. 3. Serum concentration curve following intravenous administration of mepixanthone (50 mg). Kinetic parameters: $K_e = 2.01\ h^{-1}$, $K_{21} = 0.81\ h^{-1}$, $K_{12} = 4.48\ h^{-1}$, $V_d = 234.71$ l, $V_1 = 26.99$ l, $V_{d\beta} = 317.22$ l, $t_{1/2} = 3.00$ h.

TABLE II

RETENTION TIMES OF MEPIXANTHONE AND OTHER POTENTIAL INTERFERING COMPOUNDS

The conditions are as described in the text. The resolution between mepixanthone and chlordiazepoxide, nikethamide and dimefline is 2.341, 2.020 and 1.257, respectively.

Compound	Retention time (min)	Relative retention time*
Fluphenazine	2.74	0.32
Levomepromazine	4.02	0.47
Doxapram	4.02	0.47
Chlordiazepoxide	5.39	0.63
Nikethamide	5.48	0.64
Mepixanthone	6.06	0.71
Dimefline	6.44	0.75
Promethazine	8.05	0.94
Chlorpromazine	8.56	1.00
Dipyrone	9.12	1.06
Thioridazine	11.98	1.40
Amitriptyline	12.24	1.43
Promazine	12.68	1.48
Imipramine	15.41	1.80

*Chlorpromazine = 1.00.

In agreement with other authors, the analytical column shows a long useful lifetime [7, 8] due to the predominancy of organic solvent in the mobile phase.

The method seems to be suitable for kinetic studies as it can detect very low concentrations found 24 h after administration.

ACKNOWLEDGEMENT

We wish to thank Dr. C. Benvenuti of Dott. Formenti for bibliography service.

REFERENCES

1 G. Barusco and M. Simone, Acta Anaesthesiol., 21 (1970) 277.
2 M. Vagliasindi, S. Caltagirone, G.U. Di Maria and N. Crimi, G. Med. Pneumol., 26 (1977) 217.
3 P. Capra Marzani, E. Piccolini and M. Zola, Gazz. Med. Ital., 139 (1980) 681.
4 D. Lauri, Gazz. Med. Ital., 130 (1971) 469.
5 G. Grossi, A. Lippi, R. Callivà, A. Ballardini, S. Piazzi, M. Paolini, R. Battistoni and E. Martelli, Atti Soc. Ital. Sci. Vet., 36 (1982) 232.
6 J.A. Donohue, Chromatogr. Newslett., 8 (1980) 59.
7 C. Sutheimer, Chromatogr. Newslett., 7 (1979) 38.
8 F.L. Vandemark, R.F. Adams and G.J. Schmidt, Clin. Chem., 24 (1978) 87.

Journal of Chromatography, 309 (1984) 219—221
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2114

Letter to the Editor

Simultaneous determination of antipyrine and metabolites in human plasma and urine by high-performance liquid chromatography

Sir,

Antipyrine (AP) is used as a model substance to measure the influence of environmental factors, drugs, and disease states on the activity of the microsomal oxidative degradation enzymes in in vivo systems [1]. After antipyrine administration (oral or intravenous), plasma (or saliva) [2] is analysed for the main substance at various time intervals and pharmacokinetic parameters may be calculated. However, more information may be gained by analysing additionally the metabolites 4-hydroxyantipyrine (OHA), noranti-pyrine (NAP) and 3-hydroxymethylantipyrine (HMA) in urine [3]. Clinical pharmacological work in this Department on enzyme induction (or inhibition) of drugs [4, 5] prompted us to develop a method for the analysis of antipyrine in blood and saliva and of antipyrine and metabolites in urine involving enzymatic hydrolysis, extraction and gradient elution with reversed-phase high-performance liquid chromatography (HPLC).

Recently, Teunissen et al. [6] described in this journal an HPLC assay for antipyrine and metabolites in blood, saliva and urine. Our procedure was developed independently and is comparable to their method; it differs mainly in the sample work-up and in the chromatographic system.

To 1 ml of plasma (or saliva), 10 μg of phenacetin, 200 μl of 2 *M* sodium hydroxide and 6 ml of dichloromethane were added and the mixture was shaken for 10 min. After centrifugation 5 ml of the organic phase were evaporated to dryness under a stream of nitrogen. Or, 1 ml of urine was hydrolysed for 4 h with 3 ml of 0.1 *M* acetate buffer containing 4 mg of sodium metabisulphite and 40 mg of sulphatase—glucuronidase at 37°C. Then 20 μg of phenacetin were added and the solution was extracted successively with 5 ml of dichloromethane—pentane (3:7, v/v) (A) and after the addition of 600 mg of anhydrous sodium sulphate and 400 μl of 4 *M* sodium hydroxide with 5 ml of dichloromethane (B). The mixture of the organic phases A and B was evaporated under nitrogen.

The HPLC system consisted of two pumps (6000A), an automatic injector (WISP Model 710B), a gradient programmer (M660), an ultraviolet (UV) filter detector (M440) (all from Waters, F.R.G.), a printer plotter (1RB,

0378-4347/84/$03.00

Shimadzu, F.R.G.) and a LiChrosorb RP-2 column (250 × 4 mm I.D., irregular particles, 10 μm; Merck, F.R.G.) preceded by a precolumn filled with Perisorb RP-2 (25 × 4 mm I.D., spherical particles 30—40 μm). For plasma and urine the residue was dissolved in 200 μl of methanol and 10 μl were injected for analysis. The detector was set at 254 nm and 0.1 a.u.f.s. The mobile phase for plasma was phosphate buffer (pH 6.5, 0.05 *M*)—methanol (60:40, v/v) at a flow-rate of 2 ml/min (k' values for antipyrine and phenacetin were 1.66 and 2.34, respectively). Urine was analysed using a linear gradient: solvent A was phosphate buffer (pH 6.5, 0.05 *M*)—methanol (28:72, v/v); solvent B was methanol. The gradient was run from 0% to 100% of solvent B in 15 min at a total flow-rate of 2 ml/min (k' values for HMA, NAP, AP, OHA and phenacetin were 1.81, 3.44, 4.33, 4.95 and 5.73, respectively).

Antipyrine in plasma was analysed in the range 0.1—20 mg/l, and antipyrine and metabolites in urine in the range 1—200 mg/l. Sensitivity was 0.1 mg/l for antipyrine in plasma and 1 mg/l for antipyrine and metabolites in urine. Precision was in the range 1.6—3.8%; the extraction recoveries were between 68% and 91%, depending on the substance. Most of the drugs and metabolites tested did not interfere with the assay. Excellent correlations were found with previously published procedures [7, 8].

The use of the extraction steps described above allowed the simultaneous determination of antipyrine and metabolites in urine, in place of the double-extraction procedure [8] with separate determinations of NAP with OHA, and AP with HMA, affording considerable economy in the hydrolysis, extraction and chromatographic steps. Teunissen et al. [6] have also described a single-step extraction with chloroform—ethanol (9:1, v/v), which in our hands leads to interference by several drugs and metabolites. The use of gradient elution for the analysis of urine produced sharp separations and peaks of antipyrine and metabolites without co-elution of many drugs and their metabolites. The total analysis time is 15 min and the use of an automatic injector permits a large number of assays.

Plasma extraction and chromatography were simple, with short analysis times (5 min) and absence of interference. The chromatographic system is very stable, and frequent changes of the precolumn filling allows a long column life.

The method is comparable to the one described by Teunissen et al. [6]. They used a short column filled with spherical reversed-phase particles and employed isocratic elution for plasma and urine with short analysis times. The analysis of antipyrine and metabolites in the urine of volunteers who had been treated with sulfinpyrazone [5, 9] lead to interference when analysed using a similar isocratic system; this was circumvented by the use of the gradient elution system described above. Theunissen et al. [6] also described the determination of 4,4′-dihydroxyantipyrine, which we did not assay as it is a minor antipyrine metabolite in humans.

The use of the modified conditions for extraction and the use of gradient elution for urine samples present a simple, fast, specific and sensitive method for the analysis of antipyrine in blood and saliva, and its metabolites in urine, adequate for drug-interaction, enzyme-induction or clinical studies where

interference from foreign compounds is to be expected. Similar metabolite profiles to those already published [3] were found with this method.

ACKNOWLEDGEMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (Bonn-Bad Godesberg). N.S. Wang was a fellow from the Deutschen Akademischen Austauschdienst (Bonn-Bad Godesberg).

Medizinische Klinik der Universität Heidelberg, Abteilung für Klinische Pharmakologie, Bergheimerstrasse 58, D-6900 Heidelberg (F.R.G.)

JAN XAVER DE VRIES*
CHRISTOPH STAIGER
NING SHENG WANG
FRITZ SCHLICHT
BÄRBEL KOROKNAY

1 E.S. Vessell, Clin. Pharmacol. Ther., 26 (1979) 275.
2 P.J. Meffin, R.L. Williams, T.F. Blaschke and M. Rowland, J. Pharm. Sci., 66 (1977) 135.
3 M. Danhof, A. van Zuilen, J.K. Boeijing and D.D. Breimer, Eur. J. Clin. Pharmacol., 21 (1982) 433.
4 E. Walter, C. Staiger, J. de Vries, R. Zimmermann and E. Weber, Eur. J. Clin. Pharmacol., 19 (1981) 353.
5 Ch. Staiger, F. Schlicht, E. Walter, U. Gundert-Remy, R. Hildebrandt, J. de Vries, N.S. Wang, J. Harenberg and E. Weber, Eur. J. Clin. Pharmacol., 25 (1983) 797.
6 M.W.E. Teunissen, J.E. Meerburg-van der Torren, N.P.E. Vermeulen and D.D. Breimer, J. Chromatogr., 278 (1983) 367.
7 Ch. Staiger, J. de Vries and E. Walter, J. Clin. Chem. Clin. Biochem., 18 (1980) 817.
8 M. Danhof, E. de Groot-van der Vis and D.D. Breimer, Pharmacology, 18 (1979) 210.
9 J.X. de Vries, Ch. Staiger, N.S. Wang and F. Schlicht, J. Chromatogr., 277 (1983) 408.

(First received October 28th, 1983; revised manuscript received February 8th, 1984)

Journal of Chromatography, 309 (1984) 222—224
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2118

Letter to the Editor

High-performance liquid chromatographic determination of 3,4-diaminopyridine in human plasma

Sir,

4-Aminopyridine (4-AP) has been used in clinical practice for the treatment of human neuromuscular diseases [1—4]. We have previously developed a method for determining 4-AP and 3,4-diaminopyridine (3,4-DAP) in rat cerebrospinal fluid and serum [5], but this method is not sensitive enough for pharmacokinetic studies in humans. High-performance liquid chromatographic (HPLC) methods for the determination of 4-AP in body fluids have been reported [6]. This paper describes a sensitive assay for the determination of 3,4-DAP in human plasma which is useful for its pharmacokinetic study in humans.

Methanol, acetonitrile and dichloromethane, HPLC grade, and potassium dihydrogen phosphate and potassium carbonate, analytical reagent grade, were purchased from E. Merck (Darmstadt, F.R.G.). 4-AP and 3,4-DAP were from Aldrich-Europe (Beerse, Belgium). Water was deionized and then double-distilled.

Stock solutions of 3,4-DAP and 4-AP (internal standard) were prepared in methanol at the concentration of 1 mg/ml and were stored at 0—5°C. These solutions were prepared every week.

A liquid chromatograph (Varian Model 5000) equipped with a variable-wavelength detector (Varichrom, Varian) was used in a reversed-phase system with a Micropak C_{18} column as the stationary phase (300 × 4 mm I.D., particle size 10 μm; MCH 10, Varian). The mobile phase was made up of acetonitrile—phosphate buffer (0.05 *M*, pH 7.4) + tetramethylammonium chloride (0.02 *M*) (23:77). The volume of sample injected was 20 μl (Valco valve). The effluent was monitored at 288 nm with a sensitivity of 0.02 a.u.f.s. The mobile phase flow-rate was 1.3 ml/min and the chart-speed 0.25 cm/min.

For the sample preparation, about 300 mg of potassium carbonate and 50 μl of the internal standard (solution of 4-AP, 10 mg/l in water) were added to 500 μl of plasma. The mixture was stirred (10 sec on a Vortex mixer), extracted twice with 5 ml of dichloromethane (1 min on Vortex) and centrifuged (2600 *g* for 1 min). The organic phase was transferred to another tube and re-extracted with 50 μl of 0.1 *M* hydrochloric acid (1 min on Vortex).

0378-4347/84/$03.00

After centrifugation (2600 *g* for 1 min), the supernatant was transferred into a cone-shaped tube and 20 μl of the aqueous phase were injected onto the column.

Calibration curves were constructed by spiking blank serum samples with an aqueous solution of 3,4-DAP giving plasma concentrations ranging from 6.25 to 400 μg/l. The ratios between the peak heights of 3,4-DAP and the internal standard versus the concentrations of 3,4-DAP were used to construct the calibration curves.

Typical chromatograms are shown in Fig. 1. The retention times of 3,4-DAP and 4-AP were 228 and 288 sec, respectively.

The recovery of 3,4-DAP was 75.6 ± 6.9% (mean ± S.D., n = 5) and 78.8 ± 5.8% (mean ± S.D., n = 5) at concentrations of 50 μg/l and 400 μg/l, respectively. For 4-AP the recovery was 86.7 ± 2.4% (mean ± S.D., n = 5) at a concentration of 500 μg/l.

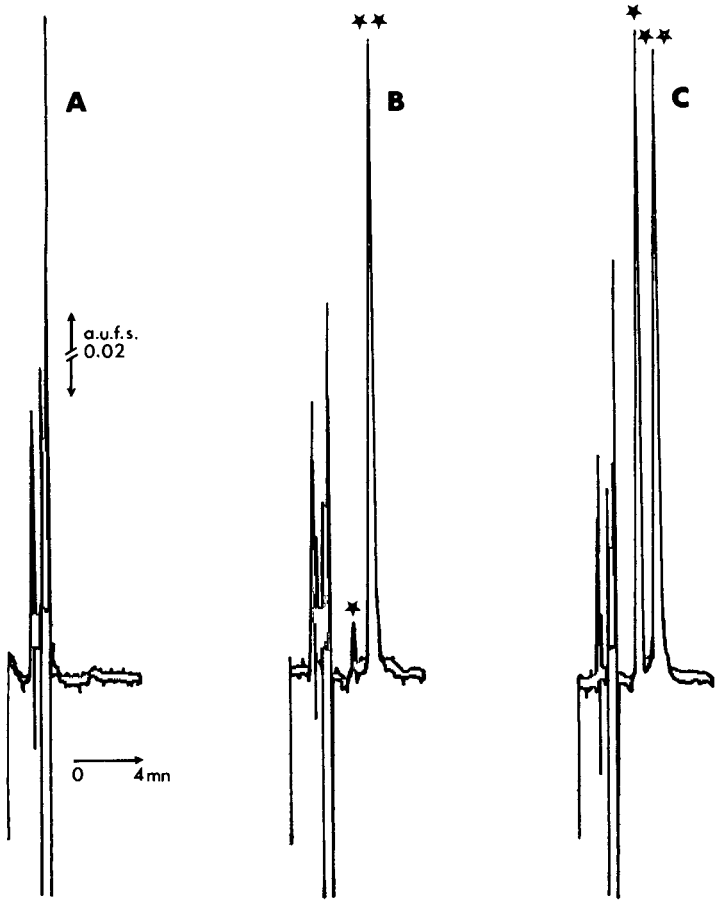


Fig. 1. (A) Chromatogram obtained from analysis of a blank human plasma sample. (B) Chromatogram obtained from analysis of a spiked human plasma sample containing 12.5 μg/l of 3,4-DAP (⋆) and 400 μg/l of 4-AP (⋆⋆) (internal standard). (C) Chromatogram obtained from analysis of a spiked human plasma sample containing 200 μg/l of 3,4-DAP (⋆) and 400 μg/l of 4-AP (⋆⋆) (internal standard).

The within-run precision was evaluated by analysing plasma samples (n = 5) spiked with known amounts of 3,4-DAP, and was found to be 10.8% and 6.8% at concentrations of 50 μg/l and 400 μg/l, respectively.

A linear relationship was observed between the peak height ratio of 3,4-DAP/4-AP (Y) and the amount of 3,4-DAP added to the plasma (X, μg/l): $Y = 0.0054X - 0.0018$; $r = 0.9983$; $n = 16$; concentration range 6.25—400 μg/l.

No interfering peaks with the same retention times as 3,4-DAP and 4-AP were present in blank plasma. The limit of detection in plasma allowing a signal-to-noise ratio of 2 was 3.4 μg/l.

The reported procedure was capable of quantitating 3,4-DAP in plasma to a concentration as low as 3 μg/l with a 0.5-ml plasma sample, and this was useful for its kinetic study in humans.

ACKNOWLEDGEMENT

The authors wish to thank Mr. J. Denis for technical assistance.

Laboratoire de Pharmacologie Médicale, Faculté de Médecine, 51, rue Cognacq-Jay, 51095 Reims Cedex (France)

D. LAMIABLE* and H. MILLART

Institut de Pharmacologie, 15, rue de l'École de Médecine, 75270 Paris Cedex (France)

M. LEMEIGNAN

and

Laboratoire de Pharmacologie, Faculté de Pharmacie, 51, rue Cognacq-Jay, 51095 Reims Cedex (France)

R. VISTELLE

1 H. Lundh, O. Nilsson and I. Rosen, J. Neurol. Neurosurg. Psychiat., 40 (1977) 1109.
2 S. Agoston, T. van Weerden, P. Westra and A. Broekert, Brit. J. Anaesth., 50 (1978) 383.
3 A.C. Ray, J.N. Dwyer, G.W. Fambro and J.C. Reagor, Amer. J. Vet. Res., 39 (1978) 329.
4 H. Lundh, O. Nilsson and I. Rosen, J. Neurol. Neurosurg. Psychiat., 42 (1979) 171.
5 D. Lamiable and H. Millart, J. Chromatogr., 272 (1983) 221.
6 D.R.A. Uges and P. Bouma, Clin. Chem., 27 (1981) 437.

(First received August 17th, 1983; revised manuscript received February 21st, 1984)

PUBLICATION SCHEDULE FOR 1984

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	D 1983	J	F	M	A	M	J	J	A	
Journal of Chromatography	282	283 284/1	284/2 285/1	285/2 285/3 286 287/1	287/2 288/1 288/2 289	290 291 292/1	292/2 293 294	295/1 295/2 296	297 298/1 298/2 298/3	The publication schedule for further issues will be published later
Chromatographic Reviews		300/1					300/2			
Bibliography Section		304/1	304/2			304/3				
Biomedical Applications		305/1	305/2	306	307/1	307/2	308	309/1	309/2	

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.

SPOT TESTS IN INORGANIC ANALYSIS

Sixth English Edition, Completely Revised and Enlarged.

By FRITZ FEIGL in collaboration with VINZENZ ANGER.

This standard work investigates the application of specific, selective, and sensitive reactions to research problems in qualitative inorganic analysis. It examines all pertinent information relating to each of the 742 tests cited, including details of operation, interferences and limits of detection. The book is an easy-to-use research tool for laboratory workers and researchers in analytic inorganic chemistry and related fields.

CONTENTS: Chapters.
1. Development, present state and prospects of inorganic spot test analysis.
2. Methodology of spot test analysis (completely revised and enlarged by Dr. G. Skalos).
3. Preliminary orientational tests. 4. Tests for the elements, their ions and compounds. 5. Application of spot reactions in tests of purity, examination of technical materials, studies of minerals. 6. Tabular summary.

"... will doubtlessly remain the authoritative book on the subject for a long time."

- Journal of the Electrochemical Society

"The book should be in chemistry libraries at all levels."

- Choice

"The volume contains a wealth of useful information and belongs on the reference shelf of every chemist who has occasion to carry out simple, rapid and selective detection tests without resorting to the use of a $40,000 instrument.

- Journal of Chemical Education

1972 1st Repr. 1982
xxix + 698 pages
US $ 117.00/Dfl. 275.00
ISBN 0-444-40929-7

SPOT TESTS IN ORGANIC ANALYSIS

Seventh English Edition, Completely Revised and Enlarged.

by FRITZ FEIGL in collaboration with VINZENZ ANGER.

This volume deals with all theoretical and practical aspects of the applications of spot tests to organic analysis. Information is given on more than 900 tests. This book continues to be of great value to organic and analytical chemists in academic as well as industrial laboratories.

CONTENTS: Chapters:
1. Development, present state and prospects of organic spot test analysis. 2. Preliminary (exploratory) tests.
3. Detection of characteristic functional groups in organic compounds. 4. Detection of structures and certain types of organic compounds.
5. Identification of individual organic compounds.
6. Applications of spot tests in the differentiation of isomers and homologous compounds. Determination of constitutions. 7. Applications of spot reactions in the testing of materials, examination of purity, characterization of pharmaceutical products etc. Appendix. Individual compounds and products examined. Author index. Subject Index.

"Even in these days of physical instrumentation there is ample room for the techniques described in this book which were originated and largely developed by Prof. Feigl."

- Laboratory Practice

"It should be classified as a 'must' for every laboratory, regardless of its field of specialization."

- Talanta

1966 3rd Repr. 1983
xxiii + 772 pages
US $ 117.00/Dfl. 275.00
ISBN 0-444-40209-8

ELSEVIER

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

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

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