ISSN 0378–4347

VOL. 231 NO. 2 SEPTEMBER 10, 1982
(Biomedical Applications, Vol. 20, No. 2)
THIS ISSUE COMPLETES VOL. 231

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

JOURNAL OF CHROMATOGRAPHY

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

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 Submission of an article is understood to imply that the article is original and unpublished and is not being considered for publication elsewhere.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

Subscription Orders. Subscription orders should be sent to: Elsevier Scientific Publishing Company, 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. 221–230, 231–240* and *241–250*) has 25 volumes in 1982. The subscription prices for 1982 are:

J. Chromatogr. (incl. *Chromatogr. Rev.* and *Cum. Indexes Vols. 221–230, 231–240* and *241–250*) + *Biomed. Appl.* (Vols. 227–251):
Dfl. 3625.00 plus Dfl. 500.00 (postage) (total *ca.* US$ 1650.00)

J. Chromatogr. (incl. *Chromatogr. Rev.* and *Cum. Indexes Vols. 231–240* and *241–250*) only (Vols. 234–251):
Dfl. 2826.00 plus Dfl. 360.00 (postage) (total *ca.* US$ 1274.50)

Biomed. Appl. (incl. *Cum. Indexes Vols. 221–230*) only (Vols. 227–233):
Dfl. 1050.00 plus Dfl. 140.00 (postage) (total *ca.* US$ 476.00).

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 226) are available at Dfl. 173.00 (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. For customers in the U.S.A. and Canada, wishing additional bibliographic information on this and other Elsevier journals, please contact Elsevier Science Publishing Company Inc., Journal Information Center, 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, Index Medicus, Mass Spectrometry Bulletin, and Science Citation Index.

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

Printed in The Netherlands

VOL. 231, NO. 2 JOURNAL OF CHROMATOGRAPHY SEPTEMBER 10, 1982
(Biomedical Applications, Vol. 20, No. 2)

CONTENTS

(Abstracts/Contents Lists published in Analytical Abstracts, Biomedical Abstracts, Biological Abstracts, Chemical Abstracts, Chemical Titles, Current Contents/Physical, Chemical & Earth Sciences, Current Contents/Life Sciences, Index Medicus, Mass Spectrometry Bulletin, and Science Citation Index)

(Continued overleaf)

Contents (continued)

Notes

(Continued overleaf)

Contents (continued)

Journal of Chromatography, 231 (1982) 225—235
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1330

GLASS CAPILLARY OR FUSED-SILICA GAS CHROMATOGRAPHY—MASS SPECTROMETRY OF SEVERAL MONOSACCHARIDES AND RELATED SUGARS: IMPROVED RESOLUTION

OMER PELLETIER* and SERGE CADIEUX

Bureau of Medical Biochemistry, Laboratory Centre for Disease Control, Health Protection Branch, Tunney's Pasture, Ottawa, Ontario, K1A 0L2 (Canada)

(First received January 29th, 1982; revised manuscript received March 26th, 1982)

SUMMARY

The gas chromatographic separation of several monosaccharides and related sugars derivatized by methoxylation and trimethylsilylation reactions was optimized with glass capillary (SP-2250) and fused silica (SP-2100) columns. Individual sugars included aldoses, ketoses, polyols, acidic forms and N-acetylated amino sugars. Peaks were detected by selected ion monitoring (SIM). The fused silica column gave complete resolution of all peaks (two per hexose and one per hexitol) arising from glucose, galactose, mannose, fructose, sorbitol, mannitol and dulcitol. The resolution of these sugars with the glass capillary column was not as good, but full differentiation was possible on the basis of SIM. Because the fused silica column gave a better resolution of 33 sugars tested and was more easily installed than the glass capillary column, it was utilized for quantitative analysis. A deuterated algal sugar mixture used for quantitation by isotope dilution was found to contain glucose, galactose, mannose, xylose, arabinose, ribose and rhamnose. Full recoveries were obtained of various amounts of glucose, galactose, mannose, fructose and xylose added to human serum.

INTRODUCTION

The International Federation of Clinical Chemistry has recommended the definitive-reference method system for achieving the ultimate in accuracy for reference materials in clinical chemistry [1]. Thus, in order to assign exact glucose values to human sera for evaluating the accuracy of a wide variety of routine glucose methods used in clinical laboratories, it was necessary to first set up a gas chromatographic—mass spectrometric (GC—MS) method capable not only of differentiating glucose but also other monosaccharides and related sugars.

Cohen et al. [2] have proposed two candidate definitive isotope dilution MS methods for serum glucose by converting glucose to 1,2:5,6 di-O-iso-

0378-4347/82/0000—0000/$02.75

propylidine-D-glucofuranose or to D-glucofuranose cyclic 1,2:3,5-bis(butylborate) following intensive preparation and purification steps designed expressly for glucose determination.

Jankowski and Gaudin [3] identified several sugars by MS following trimethylsilylation (TMS) of 5-mg amounts under rigorously controlled silylation conditions and GC on a 91-cm 3% SE-30 packed glass column. In this laboratory, when their procedure was applied to lower glucose concentrations normally encountered in serum, the glucose derivatives gave two peaks with relative intensities of 37% and 63% representing normal proportions of α- and β-anomers instead of the reported values of 3% and 97% [3].

The derivatization method of Laine and Sweeley [4], consisting of methyloximation (MO) followed by TMS avoids the variation in the ratios of α- and β-anomers of the pyranose and furanose forms that yield the same ratios of *syn*- and *anti*-isomers from both α- and β-anomers [5].

Bjorkhem et al. [5] have chromatographed MO-TMS glucose derivatives on a packed 3% SE-30 column but reported no data on the resolution of other sugars such as galactose, mannose and fructose that may be present in human serum. A 50-m glass capillary column coated with SE-30 has been used by Eldjarn et al. [6] for MO-TMS glucose derivatives but they gave no account of the resolution of other sugars. Using a similar column but a somewhat more complex procedure for MO-TMS derivatization, Zegota [7] demonstrated a satisfactory separation of glucose, galactose and fructose but did not report on mannose. According to data obtained by Størset et al. [8] for a 25-m capillary column coated with SE-30, the major peak of mannose had a retention time very close to those of the second peak of fructose and the major peak of galactose; consequently, it appears that even with a 50-m column under the conditions utilized by Zegota [7] there would be insufficient time for a well resolved mannose major peak to fit between the peaks for these two other sugars.

The primary objective of this study was to improve the GC resolution of glucose, galactose, mannose and fructose and a variety of monosaccharides and related sugars derivatized according to the MO-TMS procedure of Laine and Sweeley [4], in conjunction with selected ion monitoring (SIM). The applicability of a high-resolution GC procedure to quantitative measurements of glucose and a few other sugars by isotope dilution with a deuterated algal sugar mixture was also evaluated.

EXPERIMENTAL

Reagents

All of the sugars were obtained from commercial sources. Other reagents were obtained as follows: methoxylamine hydrochloride from Eastman Kodak (Rochester, NY, U.S.A.), N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Pierce (Rockford, IL, U.S.A.) and ACS certified pyridine from Fisher Scientific (Fair Lawn, NJ, U.S.A.). The deuterated algal sugar mixture solution of unspecified composition was obtained from Merck Sharp & Dohme Canada (Montreal, Canada).

Sample preparation

The method of derivatization was essentially that of Laine and Sweeley [4] as described by Bjorkhem et al. [5]: a 100-μl volume of serum or of 0.05% benzoic acid containing one or several sugars ($5 \cdot 10^{-3}$ mol/l) was added to 2-ml screw-top vials with Teflon-lined caps and made up to a final volume of 400 μl with 0.05% benzoic acid. After lyophilization, 500 μl of a solution of methoxylamine hydrochloride (1%, w/v) in dry pyridine were added and the sealed vials were heated for 2 h at 80°C with occasional mixing. Subsequently, 150 μl of BSTFA were added and allowed to react for 15 min at 80°C with occasional mixing.

Gas chromatography—mass spectrometry

The derivatized solutions were analysed with a Finnigan GCMS Model 4000 instrument consisting of a quadrupole spectrometer, a Model 9610 gas chromatograph and a 6110 data system. The ionizer was normally set at 70 eV, except for quantitation at 40 eV, and was operated at 250°C. The temperature of the transfer oven was also set at 250°C. The glass capillary column (30 m × 0.245 mm I.D.), coated with SP-2250 (methylphenylsilicone) was supplied by J & W Scientific (Rancho Cordova, CA, U.S.A.). The fused silica column (50 m × 0.2 mm I.D.), deactivated with Carbowax 20M and coated with SP-2100 (methylsilicone), was supplied by Hewlett-Packard (Canada) (Mississauga, Canada). The columns were adjusted to a height of about 10 mm from the bottom of the glass insert tube into the injector. The other end of the fused silica column was inserted directly into the GC—MS transfer line at a distance of about 1 mm from the ion source block. The temperature of the injector was set at 250°C.

With the glass capillary column, a volume of 1 μl was injected in the split mode (20:1) at a column flow-rate of helium of 2 ml/min, a sweep of 10 ml/min and an oven temperature of 75°C; after 30 sec the oven temperature was programmed to rise to 150°C at 10°C/min, where it was held for 23 min, and finally at full capacity to 180°C.

With the fused silica column, a volume of 3 μl was injected in the split mode (10:1) with a column flow-rate of helium of 1 ml/min, a sweep of 10 ml/min and an oven temperature of 180°C.

For the analysis of pentoses and pentitols, additional experiments were done with the fused silica column at an oven temperature of 140°C and with the glass capillary column utilizing a programme where the oven temperature was raised from 75°C to 100°C at 2°C/min and from 100°C to 180°C at 10°C/min.

RESULTS AND DISCUSSION

Selected EI ion pattern of sugars and derivatives

Similarities and differences in the ion patterns of various sugars derivatized by MO-TMS reactions and chromatographed on the SP-2100 fused silica column are summarized in Table I. A similar ion pattern (not shown) with differences in elution order was obtained with the SP-2250 glass capillary column. Characteristic ions in Table I were selected on the basis of the mass

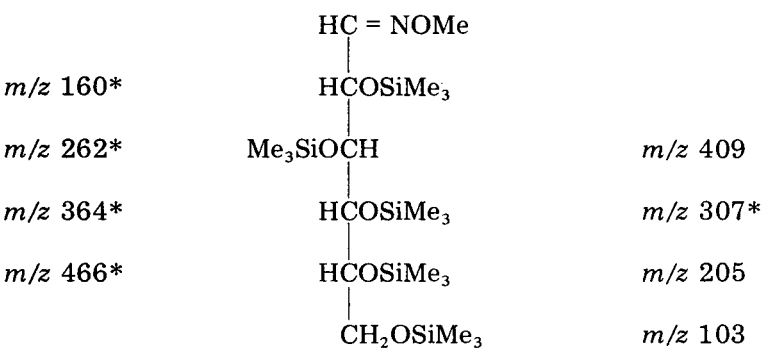
TABLE I

SELECTED ION PATTERN OF SUGARS DERIVATIZED WITH MO-TMS AND CHROMATOGRAPHED ON SP-2100 FUSED SILICA COLUMN

Sugar*	Selected ions (numerically decreasing intensities)								
	160	173	229	231	262	277	307	319	Various
Erythrol (A, 1)				2		4	1		293/3 320/5
Xylose (B, 2)	2	5	5	5	7	4	1		232/3
Lyxose (C, 2)	2	5		5	7	3	1		233/4
Arabinose (D, 1)	2	5		6	6	3	1		233/3
Ribulose (E, 2)		1		4					232/3 263/2 333/6 364/5
Xylulose (F, 1)		1		5					232/4 263/2 333/6 364/3
Ribose (G, 1)	2	5		5	5	3	1		233/3
Rhamnose (H, 2)	1	6		4		2			233/3 321/5
Fucose (I, 2)	1	6		3		2			233/4 321/5
Xylitol (J, 1)			5			4	1	2	243/3 332/6 422/7
Arabitol (K, 1)			5			4	1	2	243/3 332/6
Adonitol (L, 1)			5			4	2	1	243/3 332/6 422/7
Deoxyglucose (M, 1)							4	3	174/1 244/6 276/2 288/5
Gluconic acid (N, 2)			2	5				1	174/3 243/4 333/5 361/7
Glucuronic acid (O, 1)	1								174/2 244/4 274/3 288/4
Tagatose (P, 3)		2			4	2	1		364/4
Sorbose (Q, 2)		2			2	2	1		364/5
Fructose (R, 2)		3		4		2	1		263/4 364/4
Gluconic acid (N, 1)				5					244/1 305/4 332/2 334/3
Allose (S, 2)	2	5	3	4	5	5	5	1	233/5
Mannose (T, 2)	1	5	3	4	8	8	5	2	233/5 364/10
Talose (U, 3)	1	7	3	4	8	8	6	2	233/5
Galactose (V, 2)	1	6	3	4	6	6	5	2	233/6
Glucose (W, 2)	1	7	3	4	7	7	5	2	233/5 364/7
Sedoheptulose (X, 1)			5	4					243/2 317/3 333/1 375/5
Glucuronic acid (O, 2)	1								305/3 333/2 364/4 423/5
Mannitol (1, 1)			3	4		5	2	1	331/7 345/6 421/8
Sorbitol (2, 1)			3	4		5	2	1	331/6 345/6 421/8
Dulcitol (3, 1)			4	3		5	2	1	331/5 345/7 421/7
N-Acetyl-									
glucosamine (4, 2)		1	7	4	4			2	274/4 333/3
mannosamine (5, 2)		1	4	3	5			2	274/5
galactosamine (6, 1)		1	6	3	4			2	274/4 333/6
Myoinositol (7, 1)									265/3 305/1 318/2 432/4
Mannoheptulose (8, 2)		4	3		2		5	1	
Glucoheptulose (9, 2)	1			6	6		2	3	259/4 331/4

*In order of t_R of major or single peak. First character within parentheses identifies peaks in chromatograms and is followed by the number of peaks for each sugar. Gluconic and glucuronic acid each gave two distinct ion patterns.

spectrum of the peaks from individual sugars, and the relative intensities at each characteristic m/z were evaluated from each sugar. Laine and Sweeley [4] have explained the origin of some ions from glucose according to the following scheme, where Me represents methyl groups:



The ions at *m/z* 160, 262, 364, and 307 marked with an asterisk (see above scheme) are listed in Table I. As reported by Laine and Sweeley [4], ions at *m/z* 409 were not detected, but in contrast no ion was observed at *m/z* 466, possibly because we injected smaller amounts. Ions at *m/z* 205 and 103 were deleted from Table I because they were considered to be non-specific. According to Laine and Sweeley [4], the presence of other ions can be explained as follows:

1. Ions at *m/z* 409 − 90 (Me_3SiOH) = *m/z* 319.
2. Ions at *m/z* 409 − 180 (2 Me_3SiOH) = *m/z* 229.

In addition, *m/z* 277 could be explained as for TMS derivatives of hexitols [9] by expulsion of CH_2O from the ions of *m/z* 307. The intensities at *m/z* 173 produced by glucose and other aldoses, although weak, were listed because of the importance of *m/z* 173 for identifying ketoses and N-acetylated hexosamines. Similarly, a relatively low intensity at *m/z* 231 for aldoses, ketopentoses and the first peak of fructose (the second showing none) were listed because the intensity at *m/z* 231 for fucose, dulcitol, N-acetylmannosamine and N-acetylgalactosamine ranked third in decreasing order among the ions selected for these sugars. Although intensities at *m/z* 233 were not important for the identification of all aldoses, ketopentoses and 6-deoxysugars, their intensities were listed because intensities at *m/z* 233 were not negligible (ranking third) for xylose, arabinose, ribose and rhamnose. A thorough explanation of the origin of all specific masses for the various sugars, including those at *m/z* 173, 231 and 233, is outside the scope of this paper.

Laine and Sweeley [10] have discussed the mass spectra of the MO-TMS derivatives of xylose, ribose, fucose, fructose and glucoheptulose. The fragmentation pattern of MO-TMS derivatives of N-acetylated hexosamines has been covered by Orme et al. [11]. Discussions of mass spectra for TMS derivatives for pentitols and hexitols by Petersson [9] and for inositol by Sherman et al. [12] also apply to the derivatives in the present study.

In general, the spectral similarities for stereoisomers within each of various groups such as aldopentoses, ketopentoses, pentitols, 6-deoxyhexoses, hexitols and N-acetylated hexosamines demonstrate that separation of the stereoisomers is a prerequisite for their identification.

Glass capillary column gas chromatography

In our experience, it was not possible to separate galactose and mannose

with a glass capillary column coated with SE-30, and none of the authors reviewed [5—8] using this type of column with MO-TMS derivatives have reported the separation of these two sugars together with glucose. Fig. 1 (central portion) demonstrates a satisfactory separation of these three sugars with a glass capillary column coated with a more polar phase (SP-2250). Each of these sugars in a mixture produced two peaks (representing *syn* and *anti* forms) at *m/z* 319 and 160; these peaks were well resolved, except for the minor peak of mannose, which shouldered the major peak of galactose but not to the extent that would produce interference in their peak height measurements. Tagatose and fructose contained in the same mixture gave no intensity at *m/z* 319 and 160 and were well resolved at *m/z* 307. Injection of another mixture containing allose, sorbose and talose showed that the minor peak of talose eluted at the same time as the major peak of glucose and the major peak of talose would interfere with peak-area and not peak-height measurements of the major peak of galactose; in practice, talose would not be expected to occur naturally in biological samples. Allose would not present any problem in the resolution of the other aldoses, but it produced some intensities coincident with the first fructose peak at *m/z* 307. The first and second peak of sorbose coincided with the second peak of tagatose and fructose, respectively. A separate injection of a mixture of hexitols showed that these would interfere with some of the aldose peaks at *m/z* 319 but not 160; the three hexitols were completely resolved at *m/z* 307.

Deoxy-D-glucose gave two adjacent peaks that eluted before the hexoses, and was preceded (in the first segment of Fig. 1) by the 6-deoxysugars: fucose

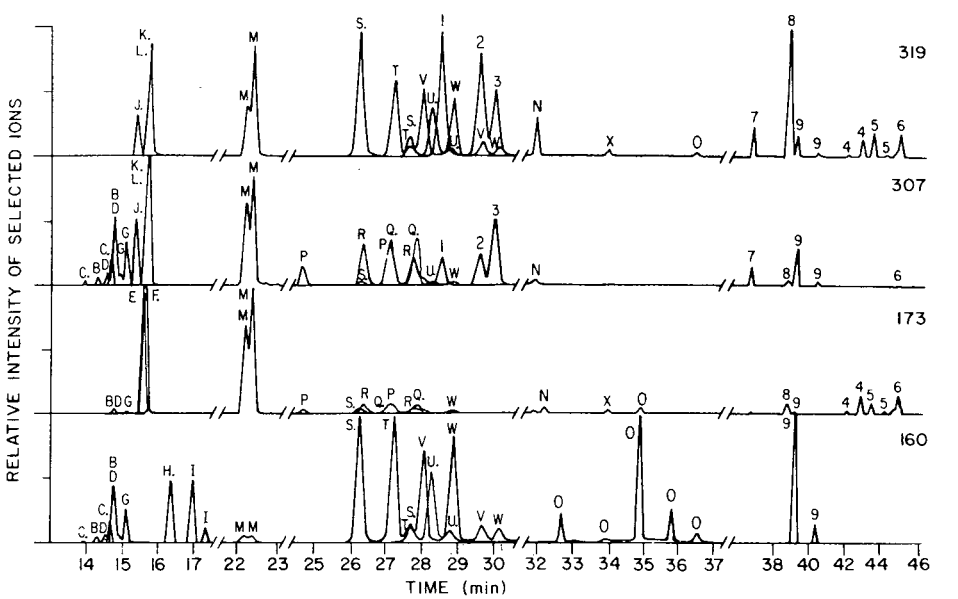


Fig. 1. Glass capillary (SP-2250) gas chromatography of various sugars derivatized by methoxylation and trimethylsilylation reactions, at *m/z* 319, 307, 173 and 160. See Table I for peak identification. The various segments divided by vertical bars include one or more mixtures where the tracing of each mixture is identified by letters, by letters followed by points or by numbers.

(two peaks) and rhamnose (one peak). Except for ribose, the resolution of peaks for individual pentoses and pentitols in mixtures was unsatisfactory under the conditions used for the chromatograms in Fig. 1. A less rapid increase in temperature differentiated (Table II) the minor peaks of xylose, lyxose and arabinose, the major peaks of ribose and the single peaks of xylulose, ribulose and xylitol, but the peaks of arabitol and adonitol had coincident retentions.

TABLE II

DIFFERENTIATION OF MONOSACCHARIDES WITH LESS THAN SIX CARBONS BY RELATIVE RETENTION TO XYLOSE MAJOR PEAK WITH SP-2250 GLASS CAPILLARY COLUMN* AND SP-2100 FUSED SILICA COLUMN**

Sugar	Relative retention of peaks***			
	SP-2250		SP-2100	
	1	2	1	2
Erythrol	0.775		0.512	
Xylose	(0.977)	1.000	(0.954)	1.000
Lyxose	(0.963)	0.998	(0.965)	1.029
Arabinose	(0.990)	0.999	(1.030)	1.042
Ribose	(1.007)	1.016	1.114	1.130
Xylulose	1.060		1.095	
Ribulose	1.036		1.107	1.118
Xylitol	1.027		1.333	
Arabitol	1.043		1.428	
Adonitol	1.043		1.467	

*Temperature programme: 75 to 100°C at 2°C/min and to 150°C at 10°C/min; t_R of xylose major peak: 24.4 min.
**Isothermally at 140°C; t_R of xylose major peak: 41.2 min.
***Underlined values identify unresolved peaks; values in parentheses identify minor peaks (intensity about 20—30% of major peak).

As shown in the fourth segment of Fig. 1, gluconic acid eluted after hexoses and hexitols and was followed by several peaks from glucuronic acid; sedoheptulose eluted after the first peak of glucuronic acid according to measurements (not shown) at *m/z* 243 and 317.

Finally, as shown in the last segment of Fig. 1, inositol was followed by mannoheptulose (one peak), glucoheptulose (two peaks), N-acetylglucosamine (two peaks), N-acetylmannosamine (two peaks) and N-acetylgalactosamine (shouldered peak).

Fused-silica column gas chromatography

The major peak of glucose (central portion of Fig. 2) was completely separated from all other sugars while its minor peak only lacked baseline resolution adjacent to the minor peak of galactose. The major peaks of galactose and mannose were well resolved. Tagatose and fructose showed no intensity at *m/z* 319 and 160, but each produced two major peaks that were well resolved; at *m/z* 307 and 173, a third very minor peak arising from the tagatose sample might be a sorbose contaminant. Sorbose from a separate mixture

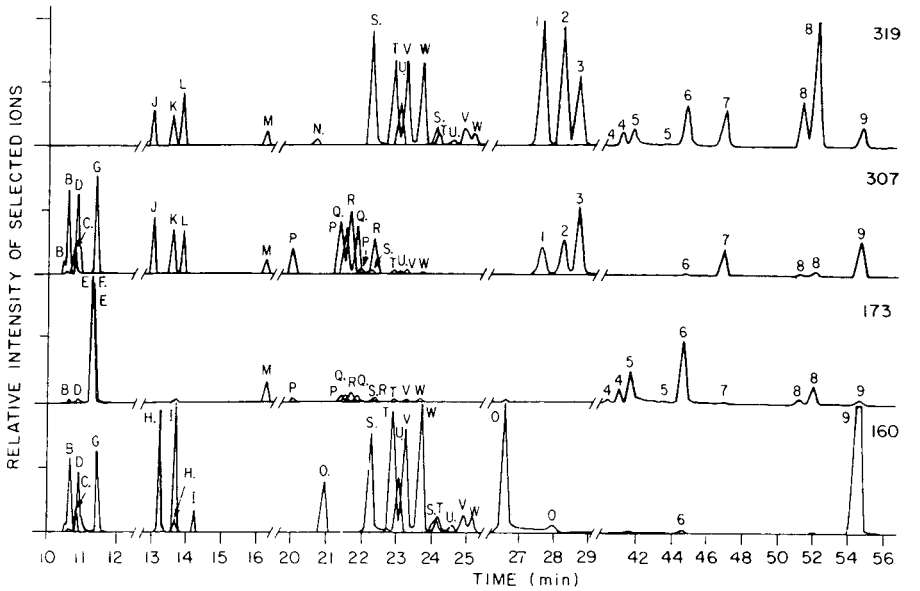


Fig. 2. Fused silica (SP-2100) gas chromatography of various sugars derivatized by methoxylation and trimethylsilylation reactions, at *m/z* 319, 307, 173 and 160. See Table I for peak identification. The various segments divided by vertical bars include one or more mixtures where the tracing of each mixture is identified by letters, by letters followed by points or by numbers.

containing a few other monosaccharides showed two peaks that would fuse on one side with a tagatose peak and on the other with a fructose peak. The major peak of allose was completely separated from all other peaks at *m/z* 319 and 160 but gave a little residual intensity coinciding with the second peak of fructose. Talose, which is unlikely to be found naturally in biological samples, would interfere with measurements of areas but not peak heights of the major peaks of mannose and galactose. Sedoheptulose gave no intensity in this chromatogram, but at *m/z* 333 (not shown) produced a peak coinciding with the second peak of allose. At *m/z* 160 one major peak from glucuronic acid was perfectly resolved from all others, and a minor peak eluted at the same time as the major peak of glucose. At *m/z* 319, a first peak from glucuronic acid preceded all the hexoses, a second peak without intensity at *m/z* 319, but monitored at *m/z* 244 (not shown) eluted at the same time as the first peak of tagatose; two other peaks from glucuronic acid were detected following hexoses but only at *m/z* 160.

In contrast to chromatography with the glass capillary column, the hexitol peaks seen at *m/z* 319 and 307 eluted well after the hexoses. On the other hand, hexoses were preceded by 2-deoxy-D-glucose (single peaks at *m/z* 319, 307 and 173) and the 6-deoxysugars which gave peaks only at *m/z* 160 where the major peak of fucose coincided with the minor peak of rhamnose. As with the glass capillary column, and except for ribose, unsatisfactory separations of individual peaks of pentoses and pentitols were obtained with the fused silica column at 180°C, but all could be differentiated at 140°C (Table II) although the major peak of lyxose coincided with the minor peak of arabinose.

The last segment of Fig. 2 shows well separated peaks: two from both N-acetylglucosamine and N-acetylmannosamine, one from both N-acetylgalactosamine and inositol, two from mannoheptulose and one from glucoheptulose.

In a different type of application where differentiation of monosaccharides and the corresponding alditols was not required, and the former were reduced to the latter, Bradbury et al. [13] reported a comparable resolution of TMS alditols with a Perkin-Elmer OV-101 fused silica column. This resolution had a considerably longer t_R than in the present study with a 50-m column, even at a temperature 10°C higher.

In general, compared with the SP-2250 glass capillary column, the SP-2100 fused silica column not only gave a better overall resolution of the various sugars but also had the advantage of being easy to install without the risk of breakage associated with glass capillary columns.

Quantitative analysis with SP-2100 fused silica capillary column

Analysis of the deuterium-labelled algal sugar mixture used for isotope ratios calibration identified the presence of various deuterated sugars (Fig. 3).

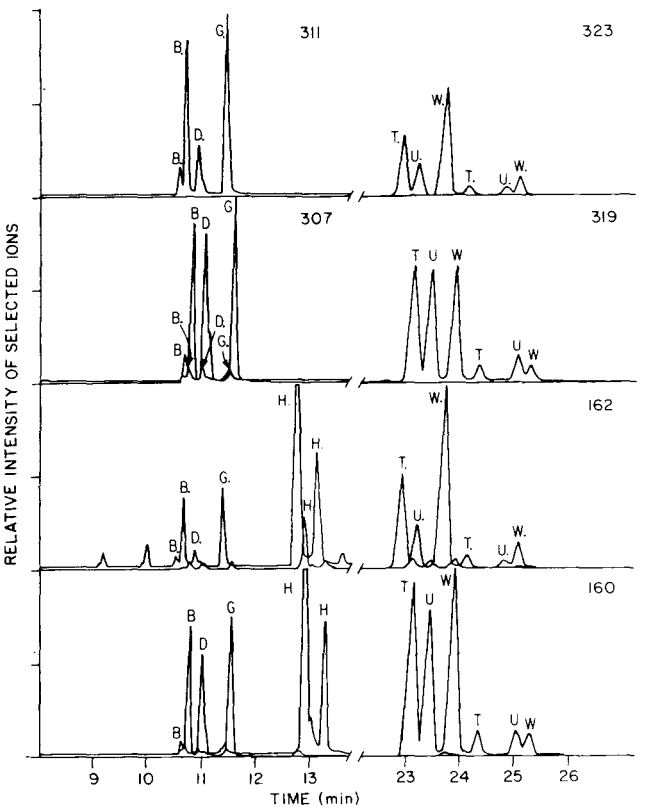


Fig. 3. Fused silica (SP-2100) gas chromatography of trimethylsilyl-O-methyloxime derivatives of a deuterated algal sugar mixture (letters followed by full points) and of a mixture of non-deuterated sugars corresponding to identified deuterated sugars. See Table I for peak identification. The tracings for each mixture are superimposed.

TABLE III

RECOVERIES OF GLUCOSE, MANNOSE, GALACTOSE, FRUCTOSE AND XYLOSE ADDED TO SERUM DEPLETED OF GLUCOSE* AS DETERMINED BY ISOTOPE RATIOS** FOLLOWING CHROMATOGRAPHY WITH FUSED SILICA COLUMN

Added (mg/dl)	Recovery (%)				
	Glucose	Galactose	Mannose	Fructose	Xylose
200	100.2				
100	101.4	102.7	100.7	99.6	
50	100.4				
10		100.9	109.4	101.0	100.7
2		100.2	102.1	99.7	98.0

*Yeast-treated serum [14] containing 1.2 mg/dl of glucose and 0.3 mg/dl of mannose.
**Peak-area ratios of major peaks of glucose, galactose, mannose at *m/z* 319 and of fructose at *m/z* 307 over area at *m/z* 323 for deuterated glucose and the ratio of the xylose peaks at *m/z* 275 over those for deuterated xylose at *m/z* 311.

On the basis of peak-area measurements compared with known amounts of the corresponding unlabelled sugars, these gave the following concentrations in g/dl of the original ampoule: xylose 0.7, arabinose 0.2, ribose 0.7, rhamnose 1.4, mannose 3.4, galactose 2.0 and glucose 6.0. As some sera may contain enzymes capable of isomerizing some of these sugars at room temperature, precautions must be taken to avoid this possible source of error by maintaining the vials to which serum has been added at 5°C during the addition of the labelled sugar mixture and freezing immediately after.

A limited study was made concerning the applicability of the above technique to the determination of glucose, galactose, mannose, fructose and xylose in serum by isotope dilution analysis following chromatography of MO-TMS derivatives with a fused silica column. Analysis of a yeast-treated serum [14] showed only traces of glucose (1.2 mg/dl) and mannose (0.3 mg/dl). Table III shows complete recoveries of various concentrations of glucose, galactose, mannose, fructose and xylose added to that serum depleted of sugar. While much more work and data are still required concerning accuracy and precision in order to be able to recommend specific techniques for sample preparation and SIM measurements, the above results are indicative of the potential usefulness of the combination of a MO-TMS derivatization technique [4], SP-2100 fused silica column chromatography and SIM for the determination of numerous monosaccharides and related sugars.

REFERENCES

1 N.W. Tietz, Clin. Chem., 25 (1979) 833.
2 A. Cohen, H. Hertz, R. Schaffer, L. Sniegoski, M. Welch and E. White, 27th Annual Conference on Mass Spectrometry and Allied Topics, Seattle, WA, June 3—8, 1979, p. 387.
3 K. Jankowski and D. Gaudin, Biomed. Mass Spectrom., 5 (1978) 371.
4 R.A. Laine and C.C. Sweeley, Anal. Biochem., 43 (1971) 533.

5 I. Bjorkhem, R. Blomstrad, O. Falk and G. Ohman, Clin. Chim. Acta, 72 (1976) 353.
6 L. Eldjarn, P. Helland and E. Jellum, in A.P. De Leenheer, R.R. Roncucci and C. Van Peteghem (Editors), Quantitative Mass Spectrometry in Life Sciences II, Proc. Second Int. Symp., Ghent, June 13—16, 1978, Elsevier, Amsterdam, 1978, p. 151.
7 H. Zegota, J. Chromatogr., 192 (1980) 446.
8 P. Størset, O. Stokke and E. Jellum, J. Chromatogr., 145 (1978) 351.
9 G. Petersson, Tetrahedron, 25 (1969) 4437.
10 R.A. Laine and C.C. Sweeley, Carbohyd. Res., 27 (1973) 199.
11 T.W. Orme, C.W. Boone and P.P. Roller, Carbohyd. Res., 37 (1974) 261.
12 W.R. Sherman, N.C. Eilers and S.L. Goodwin, Org. Mass Spectrom., 3 (1970) 829.
13 A.G.W. Bradbury, D.J. Halliday and D.G. Medcalf, J. Chromatogr., 213 (1981) 146.
14 O. Pelletier, F.H. Pryce and J.E. Logan, Clin. Biochem., 13 (1980) 41.

Journal of Chromatography, 231 (1982) 237—245
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1338

ESTIMATION OF BRANCHED-CHAIN α-KETO ACIDS IN BLOOD BY GAS CHROMATOGRAPHY

L.I. WOOLF*, C. HASINOFF and A. PERRY

Kinsmen Laboratory of Neurological Research, Department of Psychiatry, University of British Columbia, Vancouver, B.C. V6T 1W5 (Canada)

(First received December 29th, 1981; revised manuscript received May 5th, 1982)

SUMMARY

Plasma or whole blood is treated with *o*-phenylenediamine dihydrochloride in phosphoric acid under conditions found spectrophotometrically to give maximum yields of the quinoxalinols. The quinoxalinols are extracted and, after removing phosphoric acid, etc., silylated with bis-trimethylsilyltrifluoracetamide in acetonitrile. Other solvents caused instability of the trimethylsilyl(TMS)-quinoxalinols. Gas chromatography on a packed column of trifluoropropyl silicone gave good separation of the TMS-quinoxalinols from one another and from other substances derived from blood. Some representative values for normal arterial and venous human and canine plasma are reported.

INTRODUCTION

Questions posed by an investigation of amino-acid metabolism in septic shock [1] made it necessary to measure the concentrations of α-keto acids in arterial and venous blood from normal subjects, patients and experimental animals. This required a quantitative method of high sensitivity and considerable accuracy if the small arterial—venous differences in concentration were to be calculated with acceptable precision using appropriately small volumes of blood.

There are very few reports of the concentrations of α-keto acids in the blood. The technical difficulties in measuring these concentrations probably account for the paucity of data. There are reports in the older literature of paper or thin-layer chromatographic separation of 2,4-dinitrophenylhydrazones (or other derivatives) of the α-keto acids of blood [2—4], but paper and thin-layer chromatography do not lend themselves to accurate quantitation, *cis—trans*

0378-4347/82/0000—0000/$02.75

isomerism leads to multiple spots, and the identification of some spots has been questioned [5, 6]. Recently, the α-keto acids have been determined by high-performance liquid chromatography of their quinoxalinols [7]. Gas chromatography has been used relatively rarely.

Kallio and Linko [8] described a method for gas—liquid chromatography (GLC) of the esterified 2,4-dinitrophenylhydrazones of α-keto acids. Sternowksy et al. [9] analysed α-keto acids as their silylated oximes, using GLC. The O-methyloximes of the α-keto acids have been analysed by GLC of their trimethylsilyl esters [10, 11]. Liebich et al. [12], in the course of a thorough investigation of the organic acids of blood, separated the oxocarboxylic acids by GLC of their O-methyloxime methyl esters. In some cases *cis—trans* isomerism produces multiple peaks which may overlap.

The α-keto acids react with *o*-phenylenediamine to form stable quinoxalinols [13, 14] and these can be silylated to give derivatives suitable for GLC [15—17]. This technique has been used by Harper and associates [18, 19] to assay α-keto acids in animal tissues and fluids. A modification of the method, which in our hands gave more reproducible results, is described in the present report.

MATERIALS

Gas chromatograph

The instrument used was a Hewlett-Packard HP5840A reporting gas chromatograph equipped with flame ionization detectors. It was used in the single-column mode with SP-2401 stationary phase and in the dual-column mode with all other packings. The columns were nickel, 2.1 mm internal diameter and 1.8 m long, except as noted. Injection ports were glass-lined. The carrier gas was nitrogen flowing at 20 ml/min.

Column packings

The column packing finally adopted was 3% SP-2401 (trifluoropropyl silicone) on 100—120 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). Other packings investigated included 3% SE-30 on 80—100 mesh Supelcoport, 5% SE-30 on 80—100 mesh Anakrom ABS (Analabs, North Haven, CN, U.S.A.), 3% OV-17 on 80—100 mesh Supelcoport, 10% DEGS on 80—100 mesh Anakrom ABS, 10% SP-216 PS (polyester) on 100—120 mesh Supelcoport, 10% SP-1000 (Carbowax + substituted terephthalic acid) on 100—120 mesh Chromosorb W AW (3.05-m column).

Standards and reagents

Except as noted below, all standards were purchased from Sigma (St. Louis, MO, U.S.A.). The purity of the different α-keto acids was determined spectrophotometrically (see below) and, where necessary, correction factors applied for calculating recoveries. The pure quinoxalinols corresponding to several α-keto acids were generous gifts from Dr. B.L. Goodwin, others were prepared by the method of Nielsen [6] and recrystallized from 70% ethanol in water.

Silylation grade dimethylsulphoxide, pyridine, acetonitrile, tetrahydro-

furan and dimethylformamide were purchased from Pierce, Rockford, IL, U.S.A., as were Tri-sil concentrate, bis-trimethylsilyltrifluoracetamide/trimethylchlorosilane (BSTFA/TMCS) and bis-trimethylsilylacetamide (BSA). BSTFA was purchased from Sigma. *o*-Phenylenediamine dihydrochloride (Sigma) was treated with alkali and the precipitated free base was recrystallized four times from chloroform. Final purification was by sublimation in vacuo.

Orthophosphoric acid, "Transistor" grade, 85% H_3PO_4 (Mallinckrodt, Paris, KY, U.S.A.) was diluted to 8 *M* before use. Triethylamine and 2,4-dinitrophenylhydrazine were from Eastman, Rochester, NY, U.S.A., and hydroxylamine hydrochloride from Allied Chemical, Vancouver, Canada. Silicic acid ("Sil-A-200", 60—200 mesh) was from Sigma, and ethyl acetate and methylene chloride ("distilled in glass") were from Burdick & Jackson Labs., Muskegon, MI, U.S.A.

o-Phenylenediamine reagent

Method A: 0.362 g of *o*-phenylenediamine dihydrochloride, as purchased, was dissolved in 15 ml of 8 *M* H_3PO_4.

Method B: 0.215 g of purified *o*-phenylenediamine was dissolved in 15 ml of 8 *M* H_3PO_4 plus 0.67 ml of 6 *N* HCl.

The two methods were found to be equally satisfactory and method A was routinely used in later work. The reagent was prepared immediately before use.

METHOD

To whole blood (2.5 ml plus 2.5 ml of water), plasma (5 ml) or aqueous solution of α-keto acids (5 ml), was added 0.25 μmole α-ketocaproic acid (sodium salt) as internal standard. The *o*-phenylenediamine reagent, 5 ml, was added and the mixture incubated for 90 min at 40°C. The solution was cooled and extracted three times with 20 ml of ethyl acetate each time. To the combined ethyl acetate extracts was added 1 g of silicic acid; the mixture was well shaken and centrifuged. To the clear supernatant were added 2 ml of triethylamine and the mixture was centrifuged. The clear supernatant was evaporated to dryness in vacuo at 40°C in a Buchler Rotary Evapo-Mix. Methylene chloride, 3 ml, was added and the solution evaporated to dryness as before. The addition and evaporation of methylene chloride was repeated twice more.

Acetonitrile, 0.1 ml, and BSTFA, 0.3 ml, were added to the tube which was rotated to dissolve and mix all the dried deposits on the walls. The stoppered tube stood overnight at room temperature.

Between 1 and 2 μl of the mixture, depending on expected peak heights, were injected onto the GLC column, using a glass-lined injection port at 250°C. The column was programmed to rise at 4°C/min from 60°C to 240°C. An attenuation of 2^6 was appropriate for the concentrations of the branched-chain α-keto acids found in normal or pathological plasma, but pyruvic acid usually required a higher attenuation. Known amounts of pure quinoxalinols were silylated with acetonitrile and BSTFA as above and examined by GLC. α-Keto acids were added to water and to plasma to give known concentra-

tions, then the solutions were taken through the whole derivatisation, extraction and GLC as described above to check recoveries.

RESULTS AND DISCUSSION

Organic acids, as well as the quinoxalinols of α-keto acids, were extracted from plasma, blood or aqueous solution and formed trimethylsilyl (TMS) derivatives which gave a multitude of peaks on the gas chromatogram. No attempt was made to identify or quantitate most of these substances.

A 1.8 m column of 3% SP-2401 on 100—200 mesh Supelcoport gave clear separation of TMS-3-isobutylquinoxalinol and TMS-3-(1-methylpropyl)-quinoxalinol (from α-ketoisocaproic acid and α-keto-β-methyl-*n*-valeric acid, respectively), a separation difficult to achieve on other packed columns. Fig. 1 shows the results when a mixture of quinoxalinols and α-hydroxy acids were silylated and chromatographed. When known amounts of branched-chain α-hydroxy and α-keto acids were added to plasma which was then taken through the procedure, recoveries were usually quantitative for the α-keto acids (Table I). The recoveries were calculated, using an internal or external standard, from the GLC peak areas as percentages of the peak areas obtained

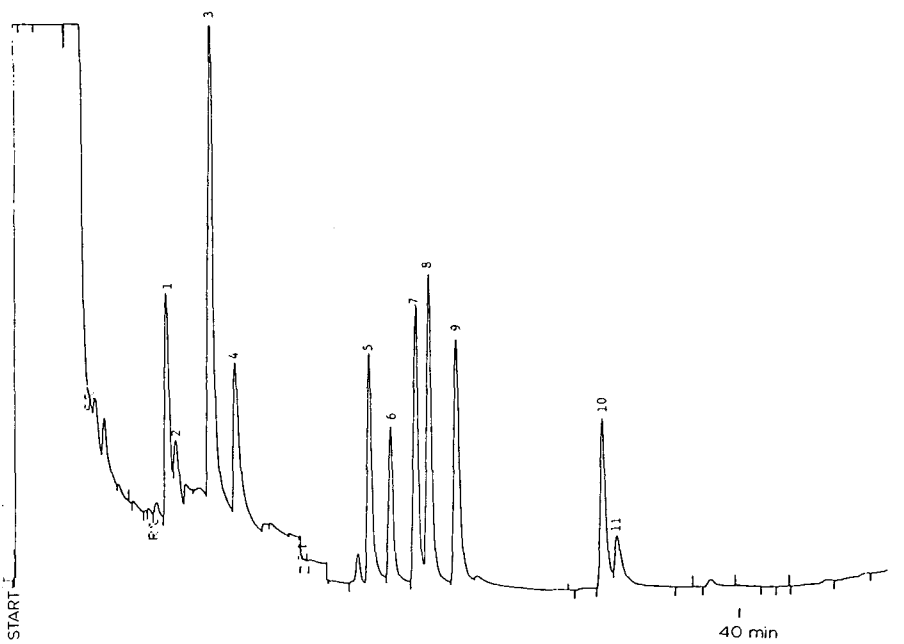


Fig. 1. Chromatogram of trimethylsilyl derivatives of hydroxy acids and of quinoxalinols. 1 = α-hydroxyisovaleric acid; 2 = β-hydroxybutyric acid; 3 = α-hydroxyisocaproic acid + α-hydroxy-β-methyl-*n*-valeric acid; 4 = α-hydroxycaproic acid; 5 = pyruvic acid; 6 = α-keto-isovaleric acid; 7 = α-keto-β-methyl-*n*-valeric acid; 8 = α-ketoisocaproic acid; 9 = α-keto-caproic acid; 10 = *p*-hydroxyphenylpyruvic acid; 11 = phenylpyruvic acid. Column: 1.8 m × 2.1 mm of 3% SP-2401 on 100—120 mesh Supelcoport. Carrier gas: nitrogen at 20 ml/min. Initial temperature 60°C rising at 4°C/min to 240°C. Flame ionization detector.

TABLE I

RECOVERY OF KETO AND HYDROXY ACIDS ADDED TO PLASMA OR WATER

Volume of plasma or water = 5 ml. The results have been corrected for the small amounts of these acids present in the plasma used. The purity of each α-keto acid was determined spectrophotometrically.

Acid	Amount added (μmole)	Amount found (μmole)	Recovery (%)
Pyruvic	0.50	0.55	110
α-Ketoisovaleric	0.22	0.22	100
α-Keto-β-methyl-*n*-valeric	0.20	0.204	102
α-Ketoisocaproic	0.32	0.33	103
α-Ketocaproic	0.306	0.306	100
α-Ketoglutaric	0.50	0.505	101
α-Hydroxyisovaleric	0.25	0.232	93
α-Hydroxyisocaproic	0.225	0.200	89

when equivalent amounts of the appropriate α-hydroxy acids and crystalline quinoxalinols were directly silylated with BSTFA and acetonitrile for GLC. Reproducibility was high (Table II). The concentrations of the branched-chain α-keto acids in arterial, femoral-venous and hepatic venous blood plasma from fasting normal dogs was determined by this method; its sensitivity and accuracy permitted calculation of the arterial—venous differences in concentration in each dog and the biological variation (Table III). Results obtained in fasting normal men are shown in Table IV. Fig. 2 shows a chromatogram of a specimen of normal human plasma: the peaks corresponding to the three branched-chain α-keto acids are clearly separated from each other and from the background, giving area counts adequate for the required precision. The peaks in the positions corresponding to the branched-chain α-hydroxy acids (expected retention times: α-hydroxyisovaleric 7.60, α-hy-

TABLE II

REPRODUCIBILITY OF RESULTS

Five replicate determinations were made using a mixture of α-keto acids in aqueous solution.

Acid	Amount per tube (μmoles)	Relative area*	
		Mean ± S.D.	Coefficient of variation (%)
Pyruvic	0.636	0.893 ± 0.045	5.04
α-Ketoisovaleric	0.724	0.700 ± 0.033	4.71
α-Keto-β-methylvaleric	0.533	0.909 ± 0.018	1.98
α-Ketoisocaproic	0.569	1.176 ± 0.026	2.21
α-Ketocaproic	0.572	—	—

*Area under the GLC peak expressed as a ratio to the area under the peak corresponding to α-ketocaproic acid.

TABLE III

ARTERIAL PLASMA CONCENTRATIONS OF THE BRANCHED-CHAIN α-KETO ACIDS AND THEIR ARTERIAL–FEMORAL VENOUS AND ARTERIAL–HEPATIC VENOUS DIFFERENCES IN CONCENTRATION IN FASTING DOGS (n = 10)

Acid	Concentration (μmoles/l; mean ± S.D.)		
	Arterial	Arterial–femoral venous difference	Arterial–hepatic venous difference
α-Ketoisovaleric	8.4 ± 3.8	−4.1 ± 3.2	0.52 ± 5.1
α-Keto-β-methylvaleric	20.1 ± 8.2	−3.8 ± 5.6	1.14 ± 8.3
α-Ketoisocaproic	25.6 ± 8.5	−6.9 ± 6.8	3.54 ± 6.8

TABLE IV

ARTERIAL PLASMA CONCENTRATIONS OF THE BRANCHED-CHAIN α-KETO ACIDS AND THEIR ARTERIAL–FEMORAL VENOUS DIFFERENCES IN CONCENTRATION IN FASTING NORMAL MEN (n = 10)

Acid	Concentration (μmoles/l; mean ± S.D.)	
	Arterial	Arterial–femoral venous difference
α-Ketoisovaleric	9.36 ± 6.0	1.53 ± 3.8
α-Keto-β-methylvaleric	16.2 ± 9.5	−1.16 ± 7.1
α-Ketoisocaproic	24.29 ± 9.3	−1.10 ± 4.8

droxy-β-methylvaleric 9.42, α-hydroxyisocaproic 9.42) are plainly too small to be distinguished from the background in this specimen. In some specimens it was possible to distinguish small peaks in the positions expected for α-hydroxyisovaleric, α-hydroxyisocaproic and/or α-hydroxy-β-methylvaleric acid, but the areas under these peaks were always much smaller than under the peaks corresponding to the α-keto acids, suggesting that equilibrium in each oxido-reduction was far over towards the branched-chain α-keto acids.

The method of Kallio and Linko [8] was used with mixtures of pure α-keto acids. Using a 1.8-m column of SE-30 it was found that esterified 2,4-dinitrophenylhydrazones of the α-keto acids were well separated except for those of α-ketoisocaproic acid (corresponding to leucine) and α-keto-β-methyl-n-valeric acid (corresponding to isoleucine) which ran as a single peak. Altering the conditions of running did not produce any separation, and since the branched-chain α-keto acids are of particular importance in septic shock the method was abandoned.

Separation of α-keto acids of blood as silylated oximes has been reported [9]. The method involves precipitation of the blood proteins with three volumes of ethanol and evaporation of the ethanolic supernatant before conversion to the oximes. Recovery experiments in which known amounts of various α-keto acids were added to whole blood revealed unacceptably large and variable losses, probably at the evaporation stage. This method was therefore abandoned.

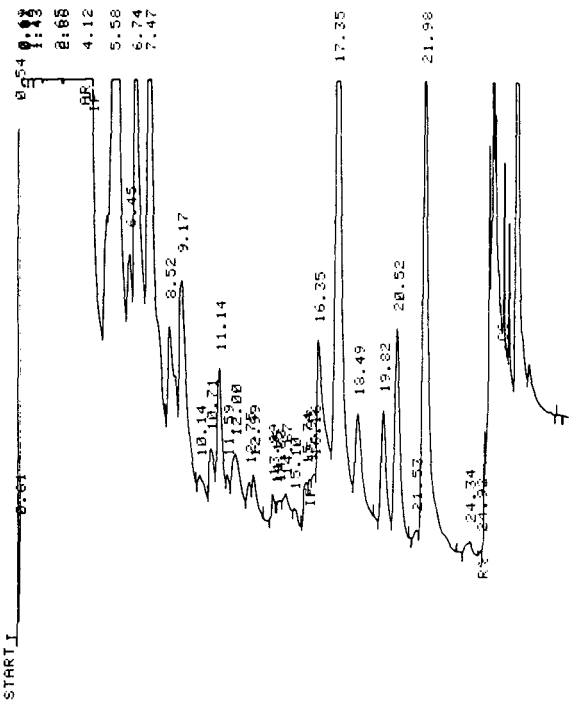


Fig. 2. Chromatogram from normal human plasma. Retention times of the TMS-quinoxalinols from the α-keto acids are: 17.35 min, pyruvic acid (217,400 area count); 18.49 min, α-ketoisovaleric acid (32,050 area count); 19.82 min, α-keto-β-methylvaleric acid (29,640 area count); 20.52 min, α-ketoisocaproic acid (56,140 area count); 21.98 min, α-ketocaproic acid (internal standard, 162,900 area count). Column: 1.8 m × 2.1 mm of 3% SP-2401 on 100—120 mesh Supelcoport. Carrier gas: nitrogen at 20 ml/min. Initial temperature 60° C rising at 4° C/min for 25 min, then at 30° C/min to 240° C. Flame ionization detector.

Quinoxalinols are stable compounds formed by reacting the unstable α-keto acids with *o*-phenylenediamine in acidic aqueous or non-aqueous solution. The absorbance of 3-isobutylquinoxalinol (corresponding to α-ketoisocaproic acid) dissolved in ethyl acetate was found to have a maximum at 330 nm. Quinoxalinols prepared from other aliphatic α-keto acids gave an absorption peak at the same wavelength in ethyl acetate or aqueous solution.

By measuring the absorbance of aqueous solutions at 330 nm, it was found that quinoxalinol formation was maximum at about pH 1.4 with a very large excess of *o*-phenylenediamine dihydrochloride (molar ratio from 100:1 at high concentrations of α-keto acid to 5000:1 at very low concentrations). High concentrations of phosphoric acid were found to increase the yield of quinoxalinols. The method described was found to give optimum, nearly quantitative yields of the quinoxalinols. The quinoxalinols formed were quantitatively extracted by ethyl acetate.

The silicic acid removed pigments extracted by ethyl acetate when whole blood was used. It also served to remove small droplets of aqueous phase carried over with the ethyl acetate. It did not affect recovery of either quinoxalinols or hydroxy acids.

Triethylamine converted the somewhat volatile α-hydroxy (and other) acids to their much less volatile triethylammonium salts which, however, remained soluble in ethyl acetate. Triethylamine also precipitated a small amount of phosphoric acid which was extracted from aqueous solution by ethyl acetate and which would otherwise have interfered at the evaporation stage. Triethylamine did not adversely affect the recovery of any quinoxalinol.

3-Alkylquinoxalinols were rapidly silylated at room temperature in BSTFA alone or in mixtures of pyridine and BSTFA. However, with purely aliphatic substituents at the 3-position, the silylated products were unstable — decomposition was appreciable within 30 min at room temperature and complete within 48 h — making it impossible to repeat GLC on the mixture. Heating the quinoxalinols with pyridine and BSTFA accelerated the decomposition. Each silylated 3-alkylquinoxalinol appeared to be converted (more rapidly on heating) into a compound giving a sharp peak of longer retention time on GLC, but this second compound was itself unstable. It is possible that the first change was a tautomeric transformation from O-trimethylsilyl-3-alkylquinoxalinol to N-trimethylsilyl-3-alkylquinoxalinol [20]. It made no difference to stability whether the *o*-phenylenediamine reagent had been prepared from purified *o*-phenylenediamine or from the dihydrochloride as purchased; pure crystalline 3-alkylquinoxalinols treated with pyridine and BSTFA showed the same instability. (In contrast to the quinoxalinols with straight- or branched-chain purely aliphatic substituents at the 3-position, 3-benzylquinoxalinol gave a stable TMS derivative with both BSTFA—pyridine and BSTFA—acetonitrile.) Of the solvents investigated, only mixtures of acetonitrile and BSTFA avoided this problem. Frigerio et al. [20] also used mixtures of BSTFA and acetonitrile in preparing TMS derivatives of quinoxalinols; these workers reported that use of pyridine caused difficulties in isolation of the derivative. Silylation was much slower in acetonitrile than in pyridine, but the silylated 3-alkylquinoxalinols were stable for at least 48 h; after 96 h some decomposition was apparent. Since as little as 10% of acetonitrile had an appreciable effect in stabilising the TMS-quinoxalinols, it seems likely that the BSTFA contains an impurity that catalyses both the silylation reaction and the subsequent decomposition of the TMS-quinoxalinols and that acetonitrile poisons this catalyst, perhaps by forming a complex with the impurity.

Solid 3-alkylquinoxalinols were found to be stable for over 17 years on the laboratory bench, but solutions in pyridine showed considerable decomposition after standing overnight.

ACKNOWLEDGEMENTS

We wish to thank Miss Rachelle Bouffard for expert assistance. This investigation was supported by a grant from the British Columbia Heart Foundation.

REFERENCES

1 L.I. Woolf, A.C. Groves and J.H. Duff, Surgery, 85 (1979) 212.

2 D. Cavallini, N. Frontali and G. Toschi, Nature (London), 163 (1949) 568.
3 W.J.P. Neish, Methods Biochem. Anal., 5 (1957) 107.
4 P. Lutz, G.M. von Reutern and R.-D. Willigman, in J. Stern and C. Toothill (Editors), Organic Acidurias, Churchill Livingstone, Edinburgh, 1972, p. 137.
5 F.A. Isherwood and R.L. Jones, Nature (London), 175 (1955) 419.
6 K.H. Nielsen, J. Chromatogr., 10 (1963) 463.
7 T. Hayashi, H. Todoriki and H. Naruse, J. Chromatogr., 224 (1981) 197.
8 H. Kallio and R.R. Linko, J. Chromatogr., 76 (1973) 229.
9 H.J. Sternowksy, J. Roboz, F. Hutterer and G. Gaull, Clin. Chim. Acta, 47 (1973) 371.
10 R.A. Chalmers and R.W.E. Watts, Analyst (London), 97 (1972) 951.
11 R.A. Chalmers and R.W.E. Watts, Analyst (London), 97 (1972) 958.
12 H.M. Liebich, A. Pickert and J. Wöll, J. Chromatogr., 217 (1981) 255.
13 O. Hinsberg, Justus Liebigs Ann. Chem., 237 (1887) 340.
14 D.J.D. Hockenhull and G.D. Floodgate, Biochem. J., 52 (1952) 38.
15 N.E. Hoffman and T.A. Killinger, Anal. Chem., 41 (1969) 162.
16 U. Langenbeck, H.-U. Möhring and K.-P. Dieckmann, J. Chromatogr., 115 (1975) 65.
17 U. Langenbeck, A. Hoinowski, K. Mantel and H.-U. Möhring, J. Chromatogr., 143 (1977) 39.
18 F.L. Shinnick and A.E. Harper, J. Nutr., 107 (1977) 887.
19 T.C. Cree, S.M. Hutson and A.E. Harper, Anal. Biochem., 92 (1979) 156.
20 A. Frigerio, P. Martelli, K.M. Baker and P.A. Biondi, J. Chromatogr., 81 (1973) 139.

Journal of Chromatography, 231 (1982) 247—254
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1324

SENSITIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR PROSTAGLANDINS USING A FLUORESCENCE REAGENT, 4-BROMOMETHYL-7-ACETOXYCOUMARIN

HIRONORI TSUCHIYA

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

TOKISHI HAYASHI* and HIROSHI NARUSE

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

and

NOBUHIKO TAKAGI

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

(First received January 19th, 1982; revised manuscript received March 26th, 1982)

SUMMARY

High-performance liquid chromatography of prostaglandins is developed in which a fluorescence reagent, 4-bromomethyl-7-acetoxycoumarin is used to perform the high-sensitivity detection. The reagent reacts with prostaglandins and related compounds to form the ester derivatives, which are separated using a reversed-phase system. Each labeled compound eluted from the column is successively hydrolyzed to the fluorescent coumarin derivative, and this fluorophore is introduced into a flow-through fluorometer. Prostaglandins can be determined in the range of at least 1 nmol to 5 pmol, and the detection limit is about 10 fmol. This system is applied to the analysis of prostaglandins in human seminal fluid.

INTRODUCTION

A number of analytical methods [1] have been developed for the detection and determination of prostaglandins (PGs) and related compounds to study their physiological or pharmacological effects. Among these methods, bioassay [2], radioimmunoassay (RIA) [3] and gas chromatography—mass spectrometry (GC—MS) [4] have been widely used for the analyses of PGs. Bioassay has the possibility of detecting unstable compounds such as thromboxane A_2 (TXA_2) and PGI_2, etc. This method, however, provides approxi-

0378-4347/82/0000—0000/$02.75

mate quantitation and low specific results [5]. RIA has been generally used for the routine analyses of PGs and seems to be better than the other methods in terms of sensitivity. The disadvantage of RIA is the use of radioactive isotopes. Furthermore, time-consuming separative pretreatment such as thin-layer chromatography or column chromatography, etc., is necessary for the measurement of each PG present in a single sample, because it is difficult otherwise to obtain the antibody with little cross-reactivity and high affinity [5]. GC—MS can provide not only specific and sensitive results, but also structural information. However, the cost of the equipment is high and skilled personnel are needed [5], and only relatively few samples can be analyzed using this method.

High-performance liquid chromatography (HPLC) provides specific and reproducible results, and offers the possibility to perform the simultaneous analysis of multiple components with closely similar structures such as PGs. The cost of the equipment is also relatively low compared with GC—MS.

PGs do not show sufficient strong absorption or fluorescence in the UV or visible region; therefore, most HPLC methods [6—11] for their determination are too insensitive to be applied to most biological samples. Several HPLC methods using some reagents which react with PGs to form UV-absorbing [12—15] or fluorescent [16] compounds have been reported, but no method shows sufficient sensitivity.

In a previous paper [17] we reported a highly sensitive HPLC system for the determination of carboxylic acids using a fluorescence labeling reagent, 4-bromomethyl-7-acetoxycoumarin (Br-Mac). This present paper deals with the sensitive detection and determination of PGs by HPLC using this system.

EXPERIMENTAL

Reagents and chemicals

Standard PGs and TXB_2 were kindly supplied by Ono Pharmaceutical (Osaka, Japan). Arachidonic acid (AA) was obtained from Wako (Osaka, Japan). Br-Mac was prepared according to the method reported previously [17]. Dibenzo-18-crown-6 was purchased from Aldrich (Milwaukee, WI, U.S.A.). Redistilled water was used for all investigations. All other reagents and solvents used in this study were of reagent grade.

Apparatus

The apparatus for the HPLC system was constructed as reported previously [17]. All parts were obtained from Japan Spectroscopic Co., (Tokyo, Japan). For the separation of labeled PGs, a Model Tri Rotor I high-performance liquid chromatograph equipped with a Model GP-A 30 solvent programmer was used. HPLC separation was carried out with a stainless-steel column (250 × 4 mm) packed with LiChrosorb RP-18 (particle size 5 μm; Merck, Darmstadt, G.F.R.) by a balanced density slurry packing method. The eluate from a column was mixed with 0.1 *N* sodium hydroxide solution using a Model LCP 150 liquid chromatographic pump and hydrolysis was performed through a coil made of stainless-steel tube (10 m × 0.5 mm). Fluorescent hydrolysate was introduced into a Model FP-110 fluorescence spectrofluorometer (ex-

citation 365 nm, emission 460 nm) connected to a Model RC strip-chart recorder.

The operating conditions for HPLC are given in Fig. 2.

Procedure for the determination of PGs

Human seminal fluid (1—5 μl) was deproteinized with 100 μl of methanol containing 16-methyl-$PGF_{1\alpha}$ (500 pmol) as an internal standard. To the supernatant was added 3 ml of water and PGs were extracted with 7 ml of ethyl acetate after acidifying with 0.1 *N* hydrochloric acid (pH 3—4). The ethyl acetate layer was evaporated to dryness and the residue was dissolved in a small amount of methanol. This solution was transferred to a glass ampoule and methanol was evaporated to dryness. About 10 mg of the finely powdered mixture of $KHCO_3$ and Na_2SO_4 (1:1) were added to this ampoule. Then, after adding 50 μl each of an acetone solution of Br-Mac (20—50 nmol) and dibenzo-18-crown-6 (10 nmol), the derivatization was performed by heating at 80°C for 1 h in the dark. After cooling, an aliquot (20—40 μl) of the reaction solution was injected into the HPLC column.

Calibration curves of PGs were prepared using methanol solutions of authentic PGs which were derivatized under the same conditions as those of the human seminal fluid sample.

RESULTS AND DISCUSSION

A scheme for the reaction of Br-Mac with PGs (represented by PGE_2) and the principle of the detection system are shown in Fig. 1. PGs are derivatized

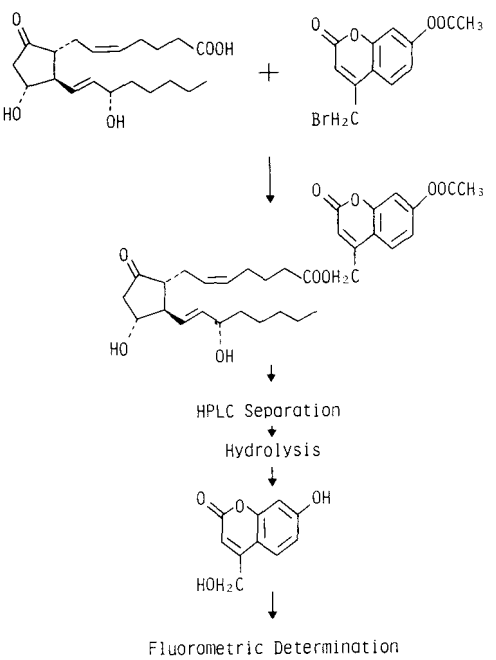


Fig. 1. Scheme of the determination system for prostaglandin E_2 using Br-Mac.

to PG-Mac esters and separated by HPLC. As soon as a mixture of PG-Mac esters is separated on a column, each ester is hydrolyzed to the fluorescent coumarin derivative which is introduced into the spectrofluorometer. Turk et al. [16] used 4-bromomethyl-7-methoxycoumarin (Br-Mmc) as a fluorescence labeling reagent for HPLC of PGs. It was suggested, however, that Br-Mmc had some disadvantages [18]; that is, the fluorescence quantum yields of the derivatives depended on the kind of carboxylic acids and were subject to the solvent effect. On the other hand, whatever the kind of PG, the fluorophore to be detected is common to all PGs in this system. Therefore, the fluorescence quantum yields are hardly effected by the PG moieties. Furthermore, compared with Br-Mmc, a gradient elution technique can be used more effectively, because the fluorescence quantum yield of the hydrolysate is not influenced by the mobile phase constitution (the variation of acetonitrile concentration in water), as reported previously [17].

Compounds prepared as described in the procedure for derivatization were subjected to HPLC and it was found that Br-Mac had good reactivity with all PGs and related compounds examined in this work such as $PGF_{1\alpha}$, $PGF_{2\alpha}$, PGE_1, PGE_2, PGD_1, PGD_2, PGB_1, PGB_2, PGA_1, PGA_2, 6-keto-$PGF_{1\alpha}$, TXB_2 and AA.

A mixture of PGs was subjected to derivatization and then HPLC separation. Fig. 2 shows the separation of derivatives of PGH_2 metabolites and

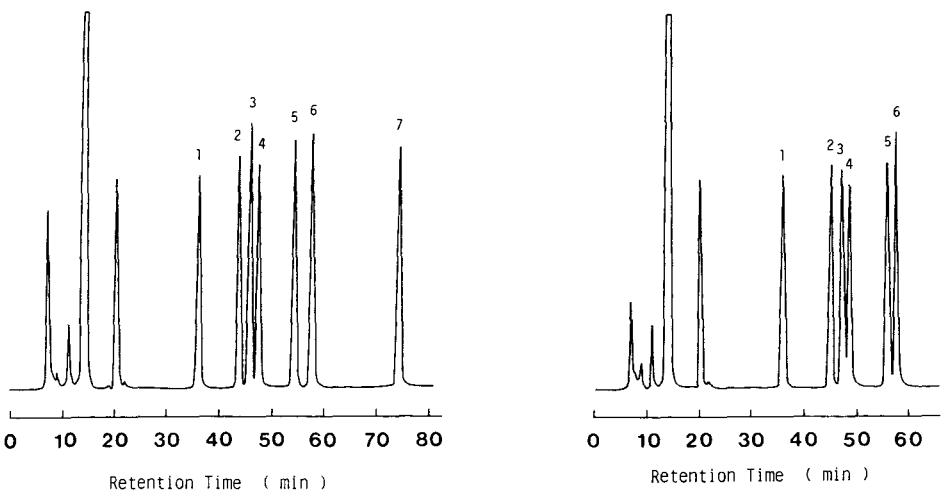


Fig. 2. High-performance liquid chromatographic separation of a mixture of Br-Mac derivatives of prostaglandin H_2 metabolites and arachidonic acid. Peaks: 1 = 6-keto-$PGF_{1\alpha}$; 2 = $PGF_{2\alpha}$; 3 = PGE_2; 4 = PGD_2; 5 = PGB_2; 6 = 2-chlorothioxanthone (internal standard); 7 = arachidonic acid. Chromatographic conditions: column, 250 × 4 mm LiChrosorb RP-18 (5 μm); column and mixing coil temperature, 50°C; mobile phase, aqueous acetonitrile solution 30% (0)—90% (99). The gradient was prepared using a Model GP-A30 solvent programmer (Concave 1, 64 min); flow-rate, 1.0 ml/min; 0.1 *N* sodium hydroxide solution flow-rate, 0.4 ml/min; detector, spectrofluorometer (excitation 365 nm, emission 460 nm).

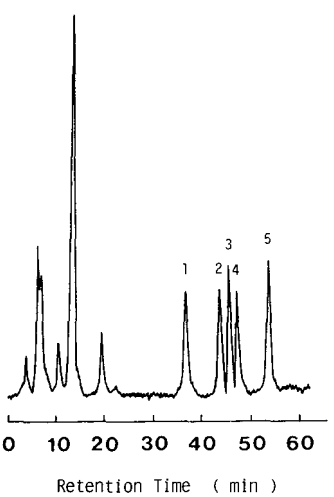
Fig. 3. High-performance liquid chromatographic separation of a mixture of Br-Mac derivatives of prostaglandin 1 groups. Peaks: 1 = 6-keto-$PGF_{1\alpha}$; 2 = $PGF_{1\alpha}$; 3 = PGE_1; 4 = PGD_1; 5 = PGB_1; 6 = 2-chlorothioxanthone (internal standard). Chromatographic conditions as in Fig. 2.

AA using a gradient elution technique. Also, a typical chromatogram of PG_1 derivatives is shown in Fig. 3. Excess reagent and its decomposition products eluted faster than PG- and AA-Mac esters. Five PGs could be chromatographed within about 60 min and a good separation was obtained, as shown in Figs. 2 and 3.

The alkali hydrolysis conditions used in this system are suitable for producing a very strong fluorescence of the hydrolysate as described in the previous paper. Due to this property of Br-Mac, a highly sensitive detection of PGs is expected using this system. Each peak shown in Fig. 4 corresponds to 200 fmol of PG. The detection limit might be at about the 10-fmol level, considering the signal-to-noise ratio. Compared with the HPLC method using Br-Mmc in which the detection limit was reported to be about 70 pmol [16], a much higher sensitivity could be obtained by this system.

A typical result of this method is shown in Fig. 5, which is a plot of the peak height of PG-Mac ester relative to 2-chlorothioxanthone (internal standard), versus the amount of the ester converted into the molarity of unlabeled PG. The plot gave a straight line from at least 1 nmol to 5 pmol (more than 200 pmol is not shown in Fig. 5). These results indicate that the derivatization procedure is suitable for quantitative purposes. PGs other than those shown in Fig. 5 also showed approximately the same results. It seems that the smaller the number of hydroxy groups in the five-membered ring, the higher the reactivity of PGs with Br-Mac.

It has been reported that several PGs were contained in human seminal fluid [19—24] and that PG levels in semen might be related to male infertility [25]. Attempts were made to apply this system to the analysis of PGs in human seminal fluid. After deproteinization with methanol, the supernatant was acidified and then extracted with ethyl acetate. The extract was



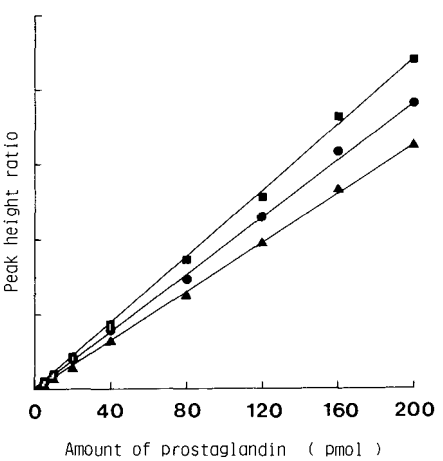


Fig. 4. High-performance liquid chromatogram obtained from a mixture of Br-Mac derivatives of prostaglandin H_2 metabolites. Chromatographic conditions as in Fig. 2. Peak number as in Fig. 2.

Fig. 5. Calibration curves of prostaglandins. ■, PGB_2 ; ●, PGE_2 ; ▲, $PGF_{2\alpha}$.

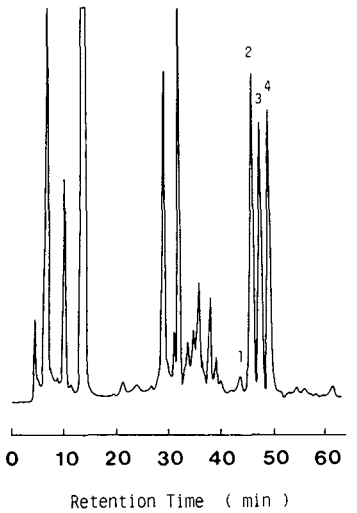


Fig. 6. High-performance liquid chromatogram obtained from human seminal fluid sample. Peaks: 1 = $PGF_{2\alpha}$; 2 = PGE_2; 3 = PGE_1; 4 = 16-methyl-$PGF_{1\alpha}$ (internal standard). Chromatographic conditions as in Fig. 2.

allowed to react with Br-Mac and an aliquot of the reaction solution was injected on to the HPLC column. The results are shown in Fig. 6. Each peak on the chromatogram was identified by comparing the retention time with that of each authentic PG-Mac ester as shown in Fig. 7.

The extract from seminal fluid was also treated with sodium hydroxide solution or acetic acid according to the method of Pike et al. [26]. With alkali treatment, the peaks related to PGE_2 and PGE_1 disappeared and two peaks at the retention times corresponding to PGB_2 and PGB_1 appeared. Conversion of the PGE series to the PGA series was also observed with acid treatment.

Analytical recovery of this method was estimated by adding 100 pmol of $PGF_{2\alpha}$ and 300 pmol each of PGE_2, PGE_1, PGB_2 and PGB_1 to 5 μl of seminal fluid (n = 8). The percentage recoveries and coefficients of variation (C.V., %), using 16-methyl-$PGF_{1\alpha}$ as internal standard, were as follows: $PGF_{2\alpha}$ = 100.0 (8.9), PGE_2 = 104.5 (4.2), PGE_1 = 69.8 (5.2), PGB_2 = 64.8 (9.6), PGB_1 = 66.2 (5.9).

In this study, peaks of $PGF_{2\alpha}$, PGE_2 and PGE_1 were found on the chromatogram obtained from human seminal fluid. It was also found in every experiment that two major peaks eluted faster than the PGE series. These peaks may correspond to those of 19-hydroxy-PGE series, the occurrence of which has been suggested by some workers [23, 24]. It was reported [21, 22] that human seminal fluid contains PGB and PGA series, and their 19-hydroxy analogues in high concentration. In this investigation, seminal fluid was frozen immediately after ejaculation and the chromatogram was obtained after relatively simple preparation. However, no prominent peaks corresponding to the above compounds could be observed on the chromatogram. These results seem to agree with the suggestion by Jonsson et al. [24] that some,

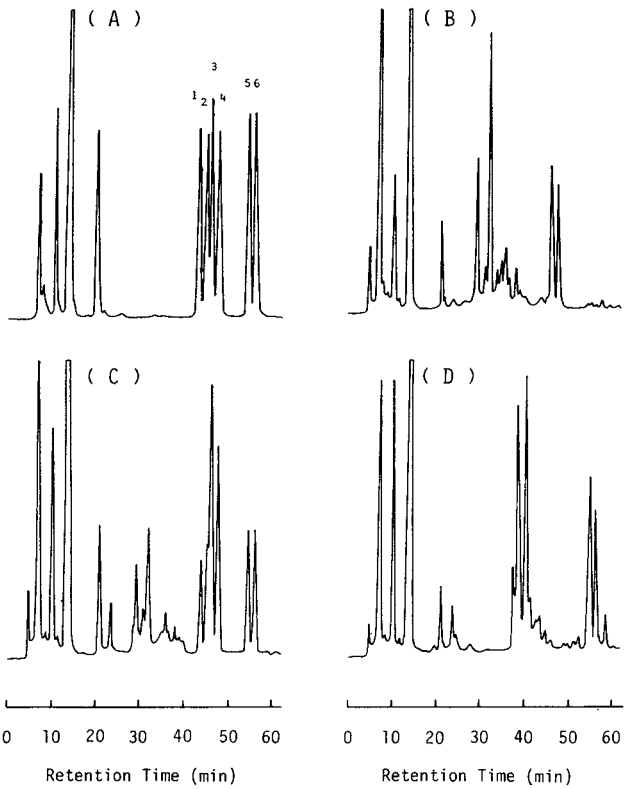


Fig. 7. Identification of the peaks on high-performance liquid chromatograms obtained from human seminal fluid sample. (A) Chromatogram of a mixture of standard PG-Mac esters; Peaks: 1 = $PGF_{2\alpha}$; 2 = $PGF_{1\alpha}$; 3 = PGE_2; 4 = PGE_1; 5 = PGB_2; 6 = PGB_1. (B) Chromatogram obtained from human seminal fluid sample. (C) B spiked with standard PG-Mac esters. (D) B treated with alkali. Chromatographic conditions as in Fig. 2.

if not all, of the PGB and PGA series and their 19-hydroxy analogues are artifacts.

It was found that HPLC using Br-Mac was useful for the detection and the simultaneous determination of several PGs. Compared with other HPLC methods reported previously, this system offered higher sensitivity in the detection and determination of PGs and related compounds. This system was not, however, sufficiently sensitive to be effectively used for the analysis of other biological samples such as plasma and urine, in which the concentrations of PGs have been described to be very low. As described above, the detection limit of the fluorescent hydrolysate of PG-Mac ester was at the low fmol level. But, the reactivity of Br-Mac with PGs was not too good in concentrations lower than pmol. Therefore, it may be necessary to introduce a more reactive group into the reagent for the determination of subpicomoles of PGs using an HPLC system like this.

ACKNOWLEDGEMENTS

The authors wish to thank Ono Pharmaceutical Co., Ltd. for kindly supplying various prostaglandins used in this investigation. This study was partially supported by Grant No. 81-01 from the National Center for Nervous, Mental and Muscular Disorders of the Ministry of Health and Welfare, Japan.

REFERENCES

1 J.M Boeynaems and A.G. Herman (Editors), Prostaglandins, Prostacyclin and Thromboxanes Measurement, Martinus Nijhoff Pub., The Hague, 1980.
2 S. Moncada, S.H. Ferreira and J.R. Vane, Advan. Prostaglandin Thromboxane Res., 5 (1978) 211.
3 E. Grandström and H. Kindahl, Advan. Prostaglandin Thromboxane Res., 5 (1978) 119.
4 K. Gréen, M. Hamberg, B. Samuelsson and J.C. Fröhlich, Advan. Prostaglandin Thromboxane Res., 5 (1978) 39.
5 F. Cattabeni, S. Nicosia, G.C. Folco, D. Longiave and R. Paoletti, in J.M. Boeynaems and A.G. Herman (Editors), Prostaglandins, Prostacyclin and Thromboxanes Measurement, Martinus Nijhoff Pub., The Hague, 1980, p. 56.
6 K. Gréen, M. Hamberg, B. Samuelsson and J.C. Fröhlich, Advan. Prostaglandin Thromboxane Res., 5 (1978) 33.
7 A.R. Whorton, K. Carr, M. Smigel, L. Walker, K. Ellis and J.A. Oates, J. Chromatogr., 163 (1979) 64.
8 G.T. Hill, J. Chromatogr., 176 (1979) 407.
9 M.A. Wynalda, F.H. Lincoln and F.A. Fitzpatrick, J. Chromatogr., 176 (1979) 413.
10 K. Nagayo and N. Mizuno, J. Chromatogr., 178 (1979) 347.
11 S. Inayama, H. Hori, T. Shibata, Y. Ozawa, K. Yamagami, M. Imazu and H. Hayashida, J. Chromatogr., 194 (1980) 85.
12 D.R. Knapp, Handbook of Analytical Derivatization Reactions, Wiley-Interscience, New York, 1979, p. 536.
13 W. Morozowich and S.L. Douglas, Prostaglandins, 10 (1975) 19.
14 M.V. Merritt and G.E. Bronson, Anal. Biochem., 80 (1977) 392.
15 F.A. Fitzpatrick, Anal. Chem., 48 (1976) 499.
16 J. Turk, S.J. Weiss, J.E. Davis and P. Needleman, Prostaglandins, 16 (1978) 291.
17 H. Tsuchiya, T. Hayashi, H. Naruse and N. Takagi, J. Chromatogr., 234 (1982) 121.
18 J.B.F. Lloyd, J. Chromatogr., 178 (1979) 249.
19 S. Bergström and B. Samuelsson, J. Biol. Chem., 237 (1962) 3005.
20 B. Samuelsson, J. Biol. Chem., 238 (1963) 3229.
21 M. Hamberg and B. Samuelsson, Biochim. Biophys. Acta, 106 (1965) 215.
22 M. Hamberg and B. Samuelsson, J. Biol. Chem., 241 (1966) 257.
23 P.L. Taylor and R.W. Kelly, Nature (London), 250 (1974) 665.
24 H.T. Johnsson, Jr., B.S. Middleditch and D.M. Desiderio, Science, 187 (1975) 1093.
25 M. Bygdeman, B. Fredricsson, K. Svanborg and B. Samuelsson, Fertil. Steril., 21 (1970) 622.
26 J.E. Pike, F.H. Lincoln and W.P. Schneider, J. Org. Chem., 34 (1969) 3552.

Journal of Chromatography, 231 (1982) 255—264
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1333

SIMULTANEOUS DETERMINATION OF MORPHINE AND MONOAMINE TRANSMITTERS IN A SINGLE MOUSE BRAIN

KOICHI ISHIKAWA*,*, JOE L. MARTINEZ, Jr. and JAMES L. McCAUGH

Department of Psychobiology, School of Biological Science, University of California, Irvine, CA 92717 (U.S.A.)

(First received January 29th, 1982; revised manuscript received April 17th, 1982)

SUMMARY

A simple procedure for the simultaneous determination of morphine and monoamine transmitters was developed. The procedure consisted of (1) *n*-butanol extraction and (2) separation and quantitative determination by means of high-performance liquid chromatography combined with electrochemical detection. The maximum intracerebral concentration (210 ± 35 ng/g wet tissue) of morphine was detected 30 min after intramuscular injection (10 mg/kg), which agreed with previous research. Noradrenaline was significantly decreased by morphine injection, while dopamine and 5-hydroxytryptamine were unchanged. However, 3-methoxytyramine, a metabolite of dopamine, was increased, suggesting that the drug increased the turnover rate of dopamine. The procedure used revealed a direct correlation between pharmacokinetics (e.g., distribution of morphine) and pharmacodynamics (e.g. changes of monoamine concentrations) of the drug in vivo.

INTRODUCTION

Research in modern experimental pharmacology involves two major subdivisions — pharmacokinetics and pharmacodynamics [1]. Pharmacodynamics is concerned with biochemical and physiological effects of drugs and their mechanisms of actions. The effect of drug is related to the concentration of drug at its site of action. On the other hand, pharmacokinetics, which deals with the absorption, distribution, biotransformation and excretion of drugs, determines the concentration of a drug at its site of action after it is administered.

Some drugs, as one of their actions, change the concentrations of monoamine transmitters in the brain. It is important to measure simultaneously concentrations of the administered drug and the transmitter substances which

*Present address: Department of Pharmacology, Nihon University School of Medicine, Itabashi, Tokyo 173, Japan.

0378-4347/82/0000—0000/$02.75

are affected by the drug in the same sample, because a simultaneous determination will determine whether there is a direct correlation between pharmacokinetics and pharmacodynamics in vivo. Previously, the concentrations of catecholamines and potent inhibitors of aromatic-L-amino acid decarboxylase, α-difluoromethyldopa and α-monofluoromethyldopa were measured in the same sample of mouse brain [2].

While morphine is known to affect the monoaminergic nervous systems, there is no general agreement as to its site of action or underlying mechanism [3, 4]. If, however, a brain assay procedure for the simultaneous determination of morphine and amine transmitters were developed, then it is possible that the precise effect of the drug on monoaminergic systems will be better understood.

Numerous investigators have reported the usefulness of high-performance liquid chromatography combined with electrochemical detection (HPLC—ElCD) for the determination of monoamine transmitters, precursor amino acids and metabolites in biological samples [5—11]. This detector has been reported to be sensitive for substances having phenolic hydroxy group(s) in its molecule [12]. Morphine is a drug which has a phenolic hydroxy group and some reports have demonstrated procedures for the determination of morphine concentrations using HPLC—ElCD [13, 14]. These findings suggest that the detection system should be able to determine morphine and monoamine transmitters simultaneously in biological samples if the appropriate extraction and chromatographic separation procedures are developed. The present study demonstrates a simple procedure for the determination of morphine together with noradrenaline (NA), dopamine (DA), 5-hydroxytryptamine (5-HT), tyrosine, tryptophan and 3-methoxytyramine (3-MT) in a single mouse brain.

MATERIALS AND METHODS

Apparatus

The liquid chromatography system was assembled using commercially available components including a pump (high-pressure precision pump, A-30-S, Eldex Labs., Menlo Park, CA, U.S.A.), six-port injector (Model 7125, Rheodyne, Berkeley, CA, U.S.A.) and electrochemical detector (VMD-101, Yanagimoto, Kyoto, Japan). The working electrode was of glassy carbon. The chromatographic column was an Ultrasphere-ODS (average particle size, 5 μm) prepacked in a stainless-steel column, 25 cm × 4.6 mm I.D. (Altex Scientific, Berkeley, CA, U.S.A.). The chromatography was performed at a constant flow-rate of 0.85 ml/min using a 0.05 *M* sodium citrate citric acid buffer (pH 4.25) containing 1% tetrahydrofuran. The electrode-applied potential was set at 725 mV vs. the Ag/AgCl reference electrode.

Reagents

Reagent-grade chemicals for extraction and chromatography included hydrochloric acid, disodium ethylenediaminetetraacetate, sodium citrate, citric acid, *n*-butanol, *n*-heptane and tetrahydrofuran, and were used without further purification. Morphine sulfate was purchased from Merck (Rahway,

NJ, U.S.A.), and authentic standards for monoamine-related substances from Sigma (St. Louis, MO, U.S.A.). These included noradrenaline·HCl, dopamine·HCl, 5-hydroxytryptamine·HCl, 3-methoxytyramine·HCl, tyrosine and tryptophan. Each substance was separately dissolved in 0.1 *N* hydrochloric acid at a concentration of 1.0 mg/ml as free base. The internal standard was 3,4-dihydroxybenzylamine (Sigma), which was also dissolved in 0.1 *N* hydrochloric acid at a concentration of 1 μg/ml. These standard substances were freshly prepared each month and were stable for over one month when stocked in a refrigerator at 4°C. The working standard solution, including authentic substances, was made from the stock solutions each day of the assay.

Animals

Male Swiss-Webster mice were used. Animals were housed in an air-conditioned room with a 12-h light—dark cycle for a minimum of one week prior to the experiment. Morphine was intramuscularly administered at a dose of 10 mg/kg. The animals were sacrificed 10, 30, 60 or 120 min after the injection by decapitation, which was performed between 10:00 a.m. and 12:00 noon to minimize circadian fluctuation in monoamine levels. After decapitation, the brain was removed as quickly as possible and stored on dry ice until the assay was carried out. The assay was, in general, completed within one week after sacrifice. The control animals received saline injection.

Extraction procedure

Each brain was weighed and transferred to a glass tube containing 500 μl of 0.1 *N* hydrochloric acid, 20 μl of 0.1 *M* EDTA and 500 ng of the internal standard. The brain was homogenized in an homogenizer (Tissuemizer, Janke and Kunkel, G.F.R.) and then transferred to a screw-capped vial (15 ml) which contained 10 ml of *n*-butanol and 4 g of solid sodium chloride. The vial was then shaken on a reciprocal shaker for 60 min. After centrifugation at 5000 rpm for 10 min, 9 ml of the butanol layer were transferred to another vial (50 ml) containing 200 μl of 0.1 *N* hydrochloric acid and 18 ml of heptane. The vial was shaken again on the shaker for 10 min and centrifuged at 5000 rpm for 5 min. A 20-μl volume of the acid aqueous layer was taken directly from the bottom of vial using a microsyringe (710 SNR, Hamilton, Reno, NV, U.S.A.) and injected onto the column.

Calibration

For calibration, authentic standard substances were taken through the entire procedure of extraction. A 400-μl volume of the working standard solution (which contained 400 ng each of NA and 5-HT; 800 ng of DA; 3 μg of tryptophan; 6 μg of tyrosine; and 20 ng of 3-MT) was pipetted into a screw-capped vial containing the same chemicals as described under *Extraction procedure*. The various concentrations of standard substances were derived from those in mouse whole brain. Different amounts of morphine (1—200 ng/tube) were added to the vials and the extraction was then carried out exactly as described in the procedure section. Quantitative determinations of monoamine transmitters and precursor amino acids were based on comparison of relative peak heights to the internal standard.

RESULTS AND DISCUSSION

Determination of chromatographic conditions

Previous determinations of morphine concentration by HPLC—ElCD [13, 14] used different extraction methods. In neither case were the extraction procedures effective for simultaneous determination of morphine and monoamine transmitters in the same sample. Although a preliminary experiment showed that a *n*-butanol—chloroform mixture [15] was useful for the extraction of morphine and indoleamine, it was not effective for the assay of catecholamines. We recently demonstrated a simple procedure for the determination of monoamine transmitters and their precursor amino acids and metabolites in the same sample of mouse whole brain [10]. In that experiment, an organic solvent extraction procedure [16] was used with some modification according to sample size. The procedure consisted of (1) primary extraction of monoamines into *n*-butanol under acidic condition and (2) re-extraction of the substances from the butanol layer into hydrochloric acid by decreasing the solubility of the substances by adding heptane. This procedure may not be specific for the extraction of substances related to monoamine metabolism. The electrochemical detector has been reported to be relatively specific for substances having a phenolic hydroxy group. Since morphine as well as monoamine transmitters have such hydroxy group(s), they can be expected to be specifically measured by the electrochemical detection. On the other hand, the improvement in packing materials for liquid chromatography produces a column which has a high theoretical plate. This fact makes it easier to separate individual substances from a complex matrix of biological origin. If both a specific detector and high efficiency column are available for chromatography, a non-specific extraction procedure can be favorable for a simultaneous determination. Hence, we decided to use butanol extraction procedure in this study.

The selectivity of the electrochemical detector is inversely related to the applied oxidation potential [12]. Fig. 1 shows the change in response current against applied potential (current–potential curve). Morphine, which has one phenolic hydroxy group on position 3, initiated a response current at 425 mV vs. the Ag/AgCl reference electrode. This value was slightly different from that of a previous report [13]. The difference may be due to the character of individual electrodes. The current—potential curve observed for morphine is probably due to one electron oxidation of the phenolic hydroxy group. Codeine, which has no hydroxy group on position 3, did not respond with the same applied voltage. Monoamine transmitters generated their own response current at lower applied potentials than the drug, which were 200 mV for catecholamines and 300 mV for indoleamine. Similar current—potential curves have been reported for catecholamines [11]. On the other hand, the precursor amino acids, tyrosine and tryptophan, needed higher potentials to generate a current. 3-Methoxytyramine, an intermediary metabolite of DA, responded at almost the same applied potential as morphine (not shown in Fig. 1, see ref. 10). For the assay of monoamine transmitters alone, a value under 600 mV is recommended because of low background noise [10]. Although increasing the applied potential produces a greater response current

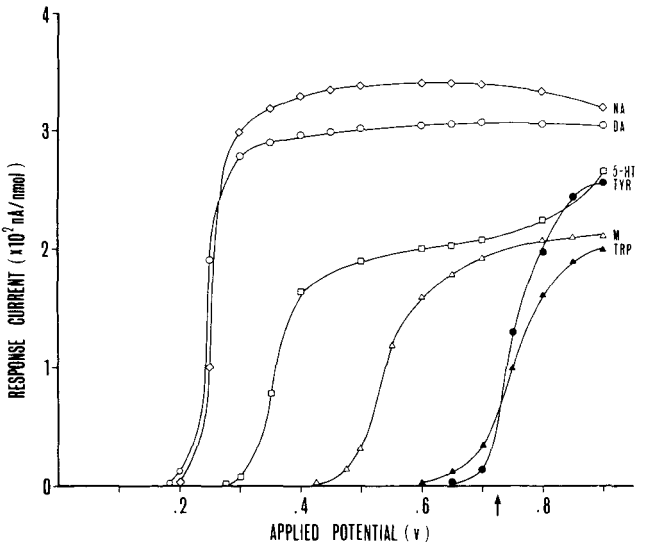


Fig. 1. Current—potential curves for noradrenaline (NA), dopamine (DA), 5-hydroxytryptamine (5-HT), tyrosine (TYR), tryptophan (TRP) and morphine (M). The arrow shows the applied potential used in the present study (725 mV).

for all substances (Fig. 1), higher potentials also make greater background current and noise levels, which decrease the specificity of the detector [12]. The samples obtained by butanol extraction showed no disadvantage in detector specificity for morphine even when the applied potential was increased from 600 mV to 725 mV. This also made it possible to detect precursor amino acids in brain samples (Figs. 1 and 2). Applied potentials above 750 mV produced an unknown peak which interfered with the determination of morphine. The applied potential was therefore selected at 725 mV vs. the reference electrode to detect simultaneously the drug and monoamine transmitters and precursors, since morphine is known to affect both of them [3, 4, 17, 18].

The most important contribution of this study was the selection of a mobile phase that could effectively separate morphine from biogenic substances. Morphine is presumed to have a long retention time on a reversed-phase column because of its physiochemical properties. It is generally thought that decreasing the polarity of the mobile phase results in shortened retention time of polar substances in this type of column. A previous determination of morphine by means of HPLC—ElCD used a high concentration of methanol (85%) in buffer for the separation [14]. However, a high organic concentration in the mobile phase is not suited for electrochemical reactions [12]. In a preliminary experiment, only 20% methanol in buffer was found to decrease electrode efficiency by about 30% of non-methanol buffer for morphine, as well as for monoamine transmitters. This means that the mobile phase containing a high concentration of methanol cannot be used, at least in the present detection system. Tetrahydrofuran is a strong non-polar solvent and was used for adjusting the polarity of the mobile phase. This solvent shortens the retention time of amines about ten-fold as compared to methanol. In a

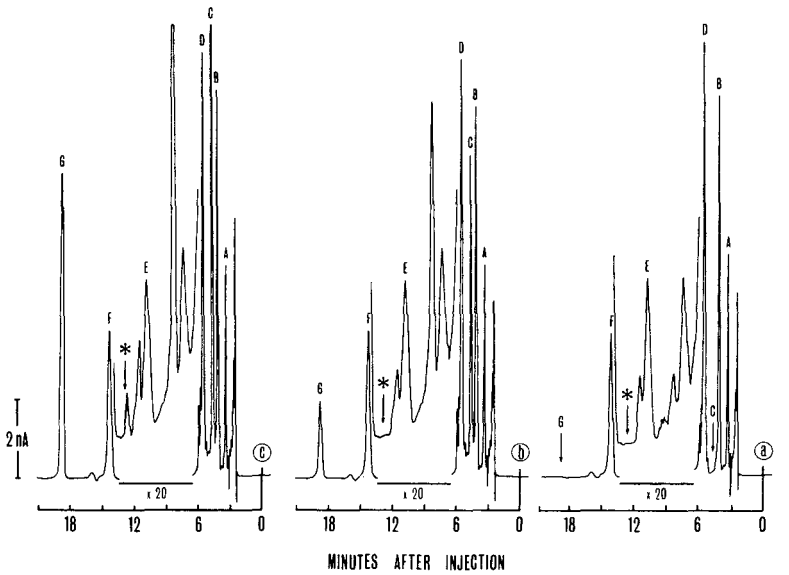


Fig. 2. Chromatograms obtained with different applied potentials: a, 600; b, 725; and c, 750 mV. Asterisks show the retention times for morphine. Peaks: A = noradrenaline; B = 3,4-dihydroxybenzylamine (internal standard); C = tyrosine; D = dopamine; E = 3-methoxytyramine; F = 5-hydroxytryptamine; G = tryptophan.

previous report [10], we successfully used 1% tetrahydrofuran for the separation of substances related to monoamine metabolism. This substance was also effective for the separation of morphine from other biogenic substances and was therefore used as an organic solvent for adjusting the polarity of the mobile phase.

Also, we found that the pH value of the mobile phase critically influenced the retention time of morphine, while ionic strength did not. Decreasing the pH value resulted in a shorter retention time for morphine (Fig. 3). The retention time converged with that of 3-MT when the pH was decreased to 3.0. On the other hand, a pH value of 5.5 yielded a different retention time for morphine from those of 5-HT and tryptophan. Although pH 5.5 was effective for the separation of these substances, it was not appropriate for morphine determination because the peak of morphine showed a significant tailing that was probably due to non-linear absorption of the drug on the column. Furthermore, DA and tyrosine had almost the same retention time in this pH condition, which made it impossible to separate and detect both substances (Fig. 3). Thus, we chose to use a pH of 4.25 in the present experiment. The theoretical plate number was estimated to be about 10,000 per meter for morphine and was greater for monoamine transmitters.

The present electrochemical detector provided linear responses within a wide range of doses from 500 pg to 50 ng (Fig. 4). This made it possible to calculate the concentration by a simple measurement of peak height for morphine as well as monoamine-related substances.

The recovery rates of substances using the *n*-butanol extraction procedure were determined in the presence of tissue samples (Table I). The recovery of

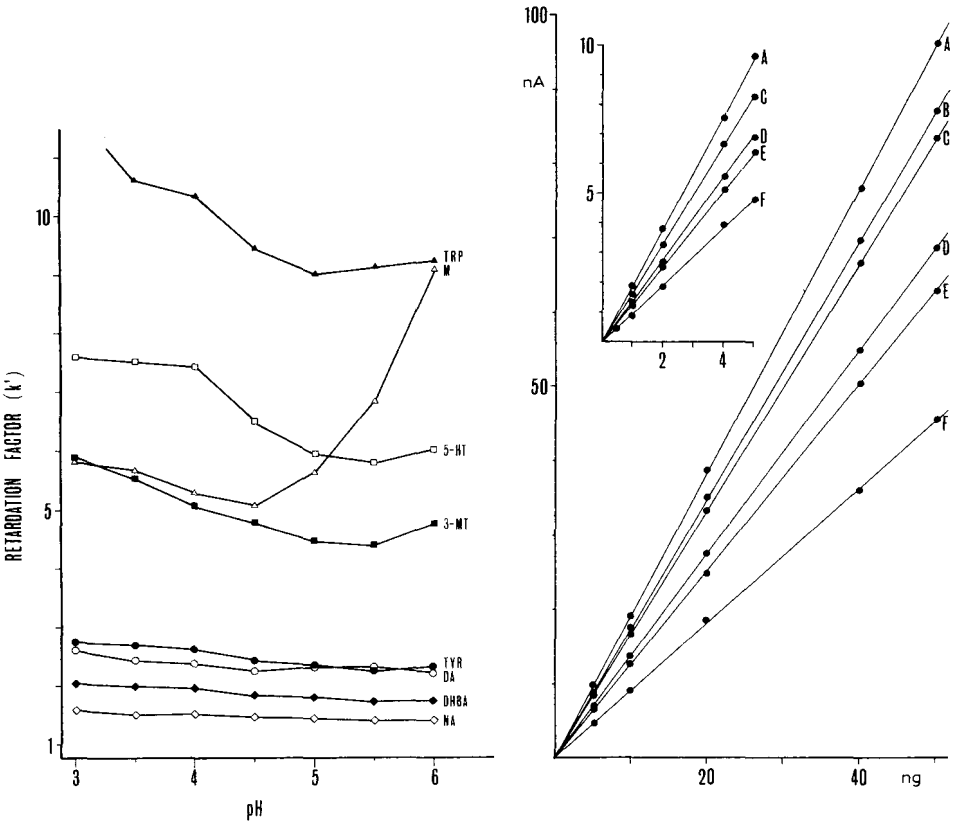


Fig. 3. Effect of pH on the retention of monoamine-related substances and morphine. 3-MT = 3-Methoxytyramine; DHBA = 3,4-dihydroxybenzylamine; other abbreviations as in Fig. 1.

Fig. 4. Standard curves of monoamine transmitters and morphine. A = Noradrenaline; B = 3,4-dihydroxybenzylamine; C = dopamine; D = 5-hydroxytryptamine; E = 3-methoxytyramine; F = morphine.

TABLE I

RECOVERY RATES, DETECTION LIMITS AND RETENTION TIMES FOR THE SUBSTANCES EXAMINED

Substance	Recovery rate* (%)	Detection limit (ng)	Retention time (min)
NA	60.0 ± 3.7	0.2	3.3
DA	64.7 ± 3.4	0.2	5.8
5-HT	54.6 ± 2.9	0.5	14.0
Tyrosine	56.7 ± 2.8	2	4.4
Tryptophan	39.2 ± 4.6	2	19.1
3-MT	51.8 ± 3.6	0.5	10.5
Morphine	72.1 ± 4.8	1	12.2

*The values represent means ± S.D. from 6 determinations.

morphine was over 70%. A preliminary experiment using a *n*-butanol—chloroform mixture showed a greater recovery of the drug than that of the present extraction procedure, while catecholamines were not extracted. These recoveries for morphine and monoamine transmitters were included in the estimation of tissue concentrations. The detection limits and retention times for the substances are also summarized in Table I.

Application to tissue sample

Fig. 5 shows a typical chromatogram for the sample obtained from an animal injected intramuscularly with 10 mg/kg morphine 30 min before sacrifice. The present combination of *n*-butanol extraction and HPLC—ElCD was effective for the simultaneous determination of morphine and substances related to monoamine metabolism (three monoamine transmitters, two precursor amine acids and one intermediary metabolite) in mouse whole brain. The identification of each peak was carried out by a previously reported procedure, in which both chromatographic and electrochemical behavior were compared with the standard substance [7]. Some unknown peaks were recorded on the chromatogram of the present sample. The peaks did not, however, interfere with determination of either morphine or monoamines. A previous report [19] demonstrated that HPLC—ElCD is effective for the determination of catecholamines and indoleamine in dissected regions of the hippocampus (which weighed about 20 mg). This means that the present procedure can be used for the simultaneous determination of regional concentrations of morphine and monoamine transmitters.

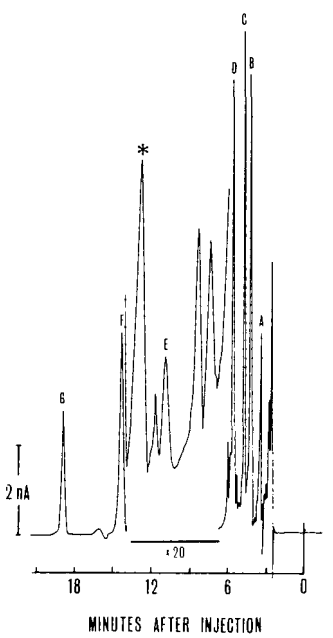


Fig. 5. Representative chromatogram obtained from the brain of animal injected intramuscularly with 10 mg/kg morphine 30 min before sacrifice. See text for the procedures of extraction and chromatography. Peaks as in Fig. 2. The asterisk shows the peak of morphine. The sensitivity under the bar was 20 times greater.

The changes in intracerebral concentrations of morphine and the substances related to monoamine metabolism were measured at different intervals after intramuscular injection of the drug (10 mg/kg). The time course of morphine concentration in the brain (Table II) agreed with previous findings [20]. The maximum value was observed 30 min after an injection. The drug was detectable after 2 h at about 50% of the maximum concentration.

TABLE II

CONCENTRATIONS OF MONOAMINE-RELATED SUBSTANCES AND MORPHINE IN MOUSE WHOLE BRAIN SAMPLE AFTER A 10 mg/kg INTRAMUSCULAR INJECTION

Values are expressed in ng/g wet tissue and means ± S.D. from 6 animals.

Substance	Control	Minutes after injection			
		10	30	60	120
Morphine	—	180 ± 19	210 ± 35	205 ± 15	136 ± 18
NA	468 ± 25	420 ± 54	382 ± 31*	409 ± 16*	417 ± 10*
DA	916 ± 51	999 ± 39	976 ± 44	967 ± 46	990 ± 71
5-HT	509 ± 31	527 ± 49	502 ± 85	518 ± 52	534 ± 36
Tyrosine	7335 ± 610	7404 ± 703	7245 ± 879	7277 ± 482	5544 ± 357*
Tryptophan	3732 ± 523	3733 ± 515	3700 ± 505	3387 ± 351	3148 ± 276
3-MT	25 ± 2	29 ± 5	38 ± 6*	39 ± 3*	35 ± 3*

*Significantly different from control group ($P < 0.01$).

Morphine has been reported to decrease intracerebral levels of catecholamines and indoleamine in both mouse and rat brain [3, 4]. The decreases are followed by increases in the levels of monoamine metabolites [3, 4]. These results suggest that morphine produces an increase in turnover rates of the monoamines. The present results agree, in general, with those of previous findings. Morphine significantly decreased ($P < 0.01$) the level of NA 30 min after an intramuscular injection, a time at which the maximum concentration of morphine was observed (Table II). On the other hand, the levels of DA and 5-HT were not affected by the injection.

Morphine has been also reported to affect the levles of precursor amino acids, tyrosine and tryptophan [17, 18]. The present results revealed a decrease of tyrosine when detected 120 min after injection, while tryptophan was not affected. A metabolite of DA, 3-MT, was significantly increased ($P < 0.01$) by morphine injection. This suggests that morphine increased the turnover rate of DA, confirming a previous report [21]. The procedure reported here does not directly measure the concentrations of metabolites of NA and 5-HT as well as other metabolites of DA. However, in previous studies [10, 19], we reported a procedure for the determination of those metabolites in mouse brain which used the organic layer remaining after the present hydrochloric acid re-extraction. This means that the proposed procedure can measure the concentrations of monoamine metabolites such as 3-methoxy-4-

hydroxyphenylethylene glycol, 3,4-dihydroxyphenylacetic acid, homovanillic acid and 5-hydroxyindoleacetic acid in the sample.

The purpose of this study was to develop a procedure for the simultaneous determination of morphine and monoamine transmitters in a single brain. HPLC—ElCD combined with butanol extraction is a useful technique because the direct correlation between pharmacokinetics and pharmacodynamics of morphine can be uncovered in an in vivo preparation. A more detailed report of the biochemical effects of morphine on monoaminergic neuron systems will appear elsewhere [22]. The procedure may be also applied, with minor modification, to the determination of other drugs having phenolic hydroxy groups.

REFERENCES

1 S.E. Mayer, K.L. Melmon and A.G. Gilman, in A.G. Gilman, L.S. Goodman and A. Gilman (Editors), The Pharmacological Basis of Therapeutics, Macmillan, New York, 1980, p. 1.
2 J. Wagner, M. Palfreyman and M. Zraika, J. Chromatogr., 164 (1979) 41.
3 E.L. Way and F.-H. Shen, in D.H. Clouet (Editor), Narcotic drugs: Biochemical Pharmacology, Plenum Press, New York, 1971, p. 229.
4 E.T. Iwamoto and E.L. Way, in H.H. Loh and D.H. Ross (Editors), Neurochemical Mechanisms of Opiates and Endorphins, Raven Press, New York, 1979, p. 357.
5 S. Sasa and C.L. Blank, Anal. Chem., 49 (1977) 354.
6 L.J. Felice, J.D. Felice and P.T. Kissinger, J. Neurochem., 31 (1978) 1461.
7 D.D. Koch and P.T. Kissinger, J. Chromatogr., 164 (1979) 441.
8 C. Hansson, G. Agrup, H. Rorsman, A.-M. Rosengren and E. Rosengren, J. Chromatogr., 162 (1979) 7.
9 E. Kempf and P. Mandel, Anal. Biochem., 112 (1981) 223.
10 K. Ishikawa and J.L. McGaugh, J. Chromatogr., 229 (1982) 35.
11 R.C. Causon, M.E. Carruthers and R. Rodnight, Anal. Biochem., 116 (1981) 223.
12 P.T. Kissinger, Anal. Chem., 48 (1977) 442A.
13 M.W. White, J. Chromatogr., 178 (1979) 229.
14 J.E. Wallace, S.C. Harris and M.W. Peek, Anal. Chem., 52 (1980) 1328.
15 H. Kupferberg, A. Burkhalter and L.E. Way, J. Pharmacol. Exp. Ther., 145 (1964) 247.
16 A.S. Welch and B.L. Welch, Anal. Biochem., 30 (1969) 161.
17 R.B. Messing, C. Flinchbauch and J.C. Waymire, Neuropharmacology, 17 (1978) 391.
18 A.A. Larson and A.E. Takemori, J. Pharmacol. Exp. Ther., 200 (1977) 216.
19 K. Ishikawa, T. Ott and J.L. McGaugh, Brain Res., 232 (1982) 222.
20 C. Cerletti, S.H. Keinath, R.J. Tallarida, M.M. Reidenberg and M.W. Adler, Substance Alcohol Actions/Misuse, 1 (1980) 65.
21 R. Papeschi, P. Theiss and A. Herz, Eur. J. Pharmacol., 34 (1975) 253.
22 K. Ishikawa, S. Shibanoki and J.L. McGaugh, in preparation.

Journal of Chromatography, 231 (1982) 265—272
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1329

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF CYTOSOLIC GLUTATHIONE S-TRANSFERASE ACTIVITY WITH STYRENE OXIDE

DAVID L. BROWN, Jr.*, WANDA BODA, M.P. STONE and ALAN R. BUCKPITT*

Department of Community and Environmental Medicine and Southern Occupational Health Center, California College of Medicine, University of California, Irvine, CA 92717 (U.S.A.)

(First received January 27th, 1982; revised manuscript received April 17th, 1982)

SUMMARY

A high-performance liquid chromatographic (HPLC) assay for measuring cytosolic glutathione S-transferase activity with styrene oxide is described. After incubating lung or liver cytosol with reduced glutathione and styrene oxide, unreacted styrene oxide is extracted into ethyl acetate. An aliquot of the aqueous phase is evaporated to dryness and reconstituted in the mobile phase for HPLC analysis. The two glutathione conjugates of styrene oxide [S-(1-phenyl-2-hydroxyethyl)glutathione and S-(2-phenyl-2-hydroxyethyl)glutathione] are separated in less than 10 min; quantitation of transferase activity is based on the comparison of the UV absorbance of the two conjugates at 254 nm with synthetic conjugate standards. As little as 1 nmole of either conjugate can be quantitated with good precision. This assay has advantages over previously published methods for measuring styrene oxide glutathione S-transferase activity as it does not depend on the use of relatively unstable and expensive radiolabelled substrates.

INTRODUCTION

Cytochrome P450-dependent metabolism of a number of chemicals results in the formation of highly reactive, electrophilic intermediates that can interact with cellular macromolecules. These interactions have been implicated in toxicities ranging from cellular necrosis to cancer [1, 2]. Alternatively, these highly reactive intermediates can form adducts with reduced glutathione (GSH), a process thought to represent a major detoxification pathway for many electrophiles [3]. In an effort to understand the mechanisms by which certain chemicals are toxic to specific target organs, several studies have examined the role of glutathione conjugation versus other competing detoxification pathways. These studies have often been hampered by the lack of reliable methods for quantitating the glutathione adducts.

*Present address: Department of Biochemistry, University of Vermont, Burlington, VT 05405, U.S.A.

0378-4347/82/0000—0000/$02.75

Glutathione adduct formation with styrene oxide has been used extensively as an in vitro measure of glutathione S-transferase activity [4—6]. Styrene oxide also has been used in isolated perfused organs where attention has focused on the relative importance of epoxide hydration and glutathione conjugation in the overall metabolic fate of this epoxide [7, 8].

Previous methods for measuring glutathione transferase activity with styrene oxide have relied on the use of either carbon-14- or tritium-labelled epoxide, which are currently available only by custom preparation. Quantitation of transferase activity was based on the amount of radioactivity remaining in the aqueous phase after extraction of the unreacted substrate. The sensitivity of this radiochemical assay is limited by high background levels of radioactivity in incubations with boiled enzyme. This problem can be corrected, however, by purifying the substrate just prior to use [9].

Cytosolic glutathione transferase activity also has been measured by utilizing [^{35}S]glutathione followed by thin-layer chromatographic separation of the styrene oxide—glutathione adducts from ^{35}S-labelled reduced and oxidized glutathione [10].

This report presents a rapid, reliable high-performance liquid chromatographic (HPLC) procedure for measuring glutathione S-transferase activity with styrene oxide, which has advantages over previous assays because it does not depend upon the use of relatively unstable and expensive radiolabelled substrate. Quantitation of transferase activity is based on comparison of the UV absorbance of the two styrene oxide—glutathione conjugates eluting from the column with synthetically prepared styrene oxide conjugate standards.

EXPERIMENTAL

Chemicals

Styrene oxide was purchased from Aldrich (Milwaukee, WI, U.S.A.). Reduced glutathione was obtained from Calbiochem (La Jolla, CA, U.S.A.). All other reagents were of analytical-reagent grade or better.

Animals

Male Sprague-Dawley rats (150—225 g) were obtained from Hilltop Breeding Labs. (Chatsworth, CA, U.S.A.) and were kept for at least 5 days before use. Animals were allowed food and water ad libitum.

Preparation of lung and liver cytosol fraction

Animals were killed by decapitation and the lungs were perfused via the pulmonary artery with ice-cold heparinized saline. All further procedures were carried out at 0—4°C. Lungs and livers were removed, rinsed with ice-cold buffer (0.02 *M* Tris—1.15% potassium chloride, pH 7.4), minced and homogenized in 3 volumes of Tris—potassium chloride buffer. After centrifugation at 9000 *g* for 20 min, the supernatant was centrifuged at 105,000 *g* for 1 h. Endogenous glutathione was removed by chromatographing the supernatant on a Sephadex G-25 column previously equilibrated with 0.05 *M* sodium phosphate buffer, pH 7.4 [11]. Protein concentrations were determined by the method of Lowry et al. [12] using bovine serum albumin as standard.

Incubations

A 2-ml incubation contained lung or liver cytosolic protein (at specified protein concentrations), 1 m*M* reduced glutathione and 0.05 *M* sodium phosphate buffer, pH 7.8. Styrene oxide (1.0 m*M* in 20 μl of acetonitrile) was added prior to transferring the incubation vessels from ice to a shaking incubator at 37°C. In time-course studies, enzyme and glutathione were preincubated for 2 min prior to adding styrene oxide. Reactions were terminated by adding 3 ml of ice-cold ethyl acetate and the contents of each vessel were transferred to centrifuge tubes. Unreacted styrene oxide was extracted into ethyl acetate and discarded and an aliquot of the aqueous phase (100—200 μl) was evaporated to dryness under nitrogen for HPLC analysis. The rate of non-enzymatic glutathione conjugate formation was monitored in identical incubations containing boiled cytosolic enzyme.

HPLC analysis

HPLC was performed with a Waters Assoc. (Milford, MA, U.S.A.) M6000A pump, U6K injector and M440 UV detector (fixed wavelength at 254 nm). Samples were reconstituted in a small volume of mobile phase (20% methanol—1% glacial acetic acid—79% water) and an aliquot was injected on to a 30 × 0.39 cm μBondapak C_{18} column (10-μm packing material, Waters Assoc.) at a flow-rate of 1.5 ml/min.

Preparation of standards

Styrene oxide—glutathione conjugate standards were synthesized and partially purified as described by Ryan and Bend [7]. The methanol eluate from the XAD-2 column, which contained both glutathione conjugates, was rotary evaporated and the conjugates were separated by semi-preparative HPLC (Waters Assoc. μBondapak C_{18} column, 30 × 0.78 cm) and collected. After removing the solvent by rotary evaporation, the remaining oily product was dissolved in a small volume of water. The addition of methanol resulted in the precipitation of colorless crystals. Reference standards of each conjugate were prepared in distilled water and were stable for at least 6 months when frozen.

Identification of the conjugates

The two conjugates were separated and purified from incubations of styrene oxide, glutathione and liver cytosolic enzyme by HPLC on a semi-preparative C_{18} column as described above. Solvent was removed by rotary evaporation and the product was dissolved in ^2H_2O for NMR analysis. Proton NMR spectra of each conjugate were recorded on a Brucker WM 250-mHz spectrometer. Structural assignments were based on previously reported spectra [13].

RESULTS AND DISCUSSION

Aliquots of the aqueous phase from incubations containing native cytosolic protein, glutathione and styrene oxide analyzed by HPLC revealed two UV-absorbing peaks that eluted at 5.7 and 6.8 min (Fig. 1A). In identical incubations with boiled cytosolic protein, only a small amount of each conjugate

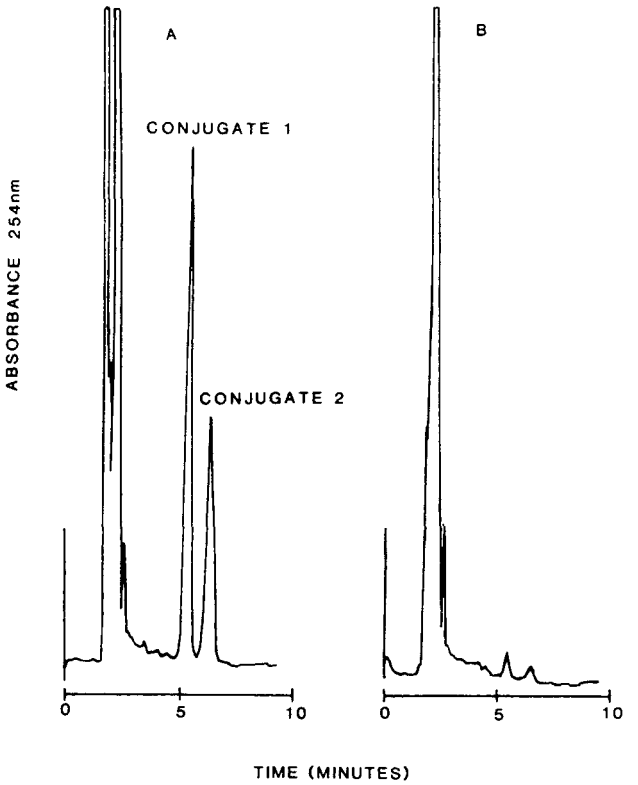


Fig. 1. HPLC profile of 50 μl of the aqueous phase prepared from an incubation containing styrene oxide (1.0 m*M*), reduced glutathione (1.0 m*M*) and (A) 1.0 mg of native liver cytosol or (B) 1.0 mg of boiled liver cytosol. The UV absorbance was monitored at 254 nm. Detector sensitivities were 0.01 and 0.005 a.u.f.s. for A and B, respectively.

was detectable (Fig. 1B). The two peaks were completely absent from incubations lacking styrene oxide or glutathione (data not shown). Extracts prepared from incubations of lung cytosolic enzyme yielded chromatograms which were similar to those obtained with liver cytosolic enzyme (Fig. 1). Synthetically prepared styrene oxide—glutathione conjugates also eluted at 5.7 and 6.8 min.

Separation and quantitation of the two styrene oxide—glutathione conjugates can also be achieved on C_{18} Radial-Pak columns (Waters Assoc., 10 × 0.8 cm) using 35% methanol—1% glacial acetic acid—64% water containing 5 m*M* heptanesulfonic acid at a flow-rate of 3 ml/min. Under these conditions, the elution times of the two conjugates formed from styrene oxide and glutathione were 5.8 and 6.6 min.

Preparative-scale isolation of the two styrene oxide—glutathione conjugates followed by NMR analysis showed conjugate 1 to be S-(1-phenyl-2-hydroxyethyl)glutathione and conjugate 2 to be S-(2-phenyl-2-hydroxyethyl)glutathione, thus confirming the earlier studies of Seutter-Berlage et al. [14], Pachecka et al. [15] and Watabe et al. [13], showing that conjugation of glutathione with styrene oxide occurs at both electrophilic carbon atoms.

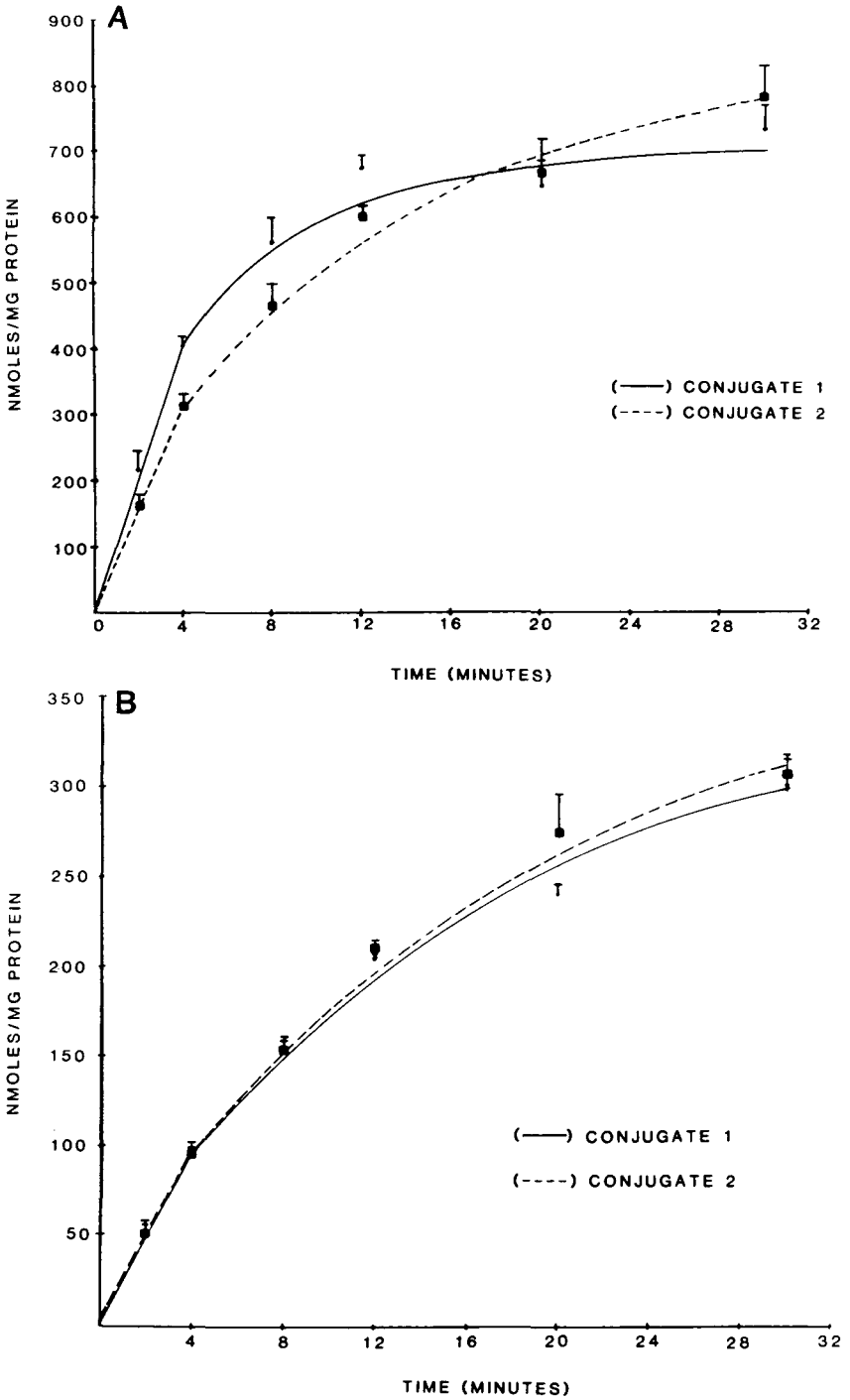


Fig. 2. Time course of formation of conjugates 1 and 2 in incubations containing (A) liver or (B) lung cytosolic enzymes (1 mg), styrene oxide (1.0 m*M*) and glutathione (1.0 m*M*). The rate of non-enzymatic conjugate formation has been subtracted from each value. Points are the means ± standard errors of the means for triplicate incubations.

Calibration graphs, prepared by plotting the product of peak height × absorbance units full-scale versus the amount of synthetically prepared styrene oxide—glutathione conjugate injected, were linear from 0.4 to 4 μg for each conjugate, with correlation coefficients > 0.9996.

The rates of formation of both S-(1-phenyl-2-hydroxyethyl)glutathione and S-(2-phenyl-2-hydroxyethyl)glutathione were linear for 4 min when either liver (Fig. 3A) or lung (Fig. 3B) was used as an enzyme source. Liver cytosolic

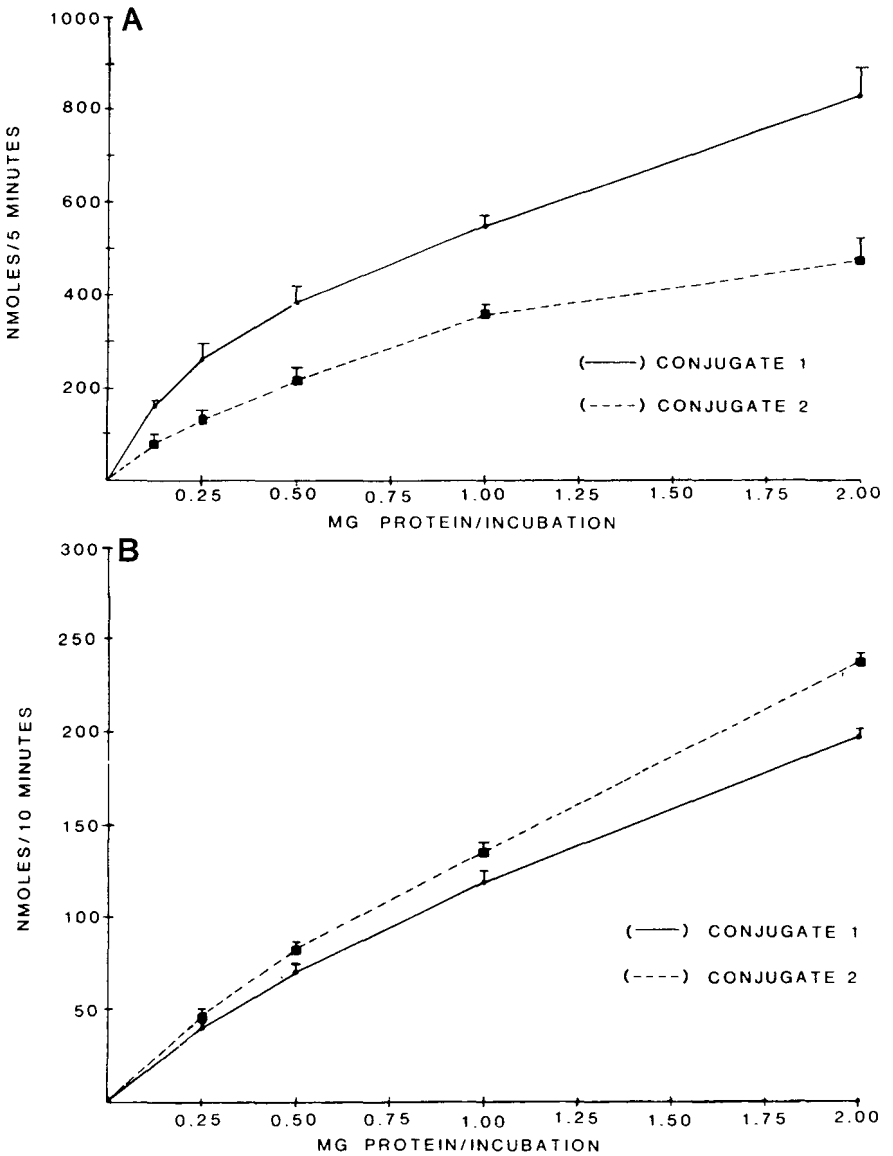


Fig. 3. Effect of protein concentration on the rate of conjugate formation in incubations containing (A) liver or (B) lung cytosolic protein, styrene oxide (1.0 m*M*) and glutathione (1.0 m*M*). The rate of non-enzymatic conjugate formation has been subtracted from each value. Points are the means ± standard errors of the means for triplicate incubations.

enzymes consistently produced conjugate 1 at faster rates than conjugate 2, while pulmonary cytosolic enzymes produced the isomers at approximately equal rates. The ratio of the rates of formation of the two conjugates was not affected by styrene oxide concentration or by the pH of the incubation (6.0—9.0). The rates also were not affected by the type of buffer used (phosphate or Tris). At pH 7.5, non-enzymatic conjugate formation occurred at less than 5% of the enzymatic rate.

Liver cytosolic transferase activities measured with this HPLC method are in good agreement with those of previous studies [4, 5, 16], while activities from lung cytosolic transferase were slightly higher than those reported previously [16]. This may be due to the increased specificity of this method, thereby resulting in lower background levels, or because the lungs were perfused in the current study to remove extraneous blood.

Fig. 3A shows that the formation of both conjugates was linear with protein to 0.25 mg in incubations of liver cytosolic enzymes and to 0.50 mg with lung cytosolic enzymes. These results are consistent with those of studies using radiolabelled styrene oxide [4, 5].

Hence the method presented here provides a rapid means of quantitating cytosolic glutathione S-transferase activity with styrene oxide. Unlike previous methods, the proposed assay does not require the use of radiolabelled substrates, and it is sufficiently sensitive to detect conjugate formation in tissues which generally have low activity, such as lung.

ACKNOWLEDGEMENTS

This work was supported by Grant ES-02475 to Alan R. Buckpitt from the National Institute of Environmental Health Sciences. Financial support for the NMR spectrometer was provided by grant CHE 79-10821 from N.S.F.

REFERENCES

1 J.A. Miller, Cancer Res., 30 (1970) 559—576.
2 J.R. Gillette, in M.J. Coon, A.H. Conney, R.W. Estabrook, H.V. Gelboin, J.R. Gillette and P.J. O'Brien (Editors), Microsomes, Drug Oxidations, and Chemical Carcinogenesis, Vol. 2, Academic Press, New York, 1980, pp. 777—790.
3 L.F. Chasseaud, Advan. Cancer Res., 29 (1979) 175—274.
4 J. Marniemi and M.G. Parkki, Biochem. Pharmacol., 24 (1975) 1569—1572.
5 M.O. James, J.R. Fouts and J.R. Bend, Biochem. Pharmacol., 25 (1976) 187—193.
6 J.S. Felton, J.N. Ketley, W.B. Jakoby, A. Aitio, J.R. Bend and D.W. Nebert, Mol. Pharmacol., 18 (1980) 559—564.
7 A.J. Ryan and J.R. Bend, Drug Metab. Dispos., 5 (1977) 363—371.
8 J. Van Anda, B.R. Smith, J.R. Fouts and J.R. Bend, J. Pharmacol. Exp. Ther., 211 (1979) 207—212.
9 J.W. DePierre and M.S. Moron, Pharmacol. Res. Commun., 11 (1979) 421—431.
10 T. Hayakawa, R.A. LeMahieu and S. Udenfriend, Arch. Biochem. Biophys., 162 (1974) 223—230.
11 D.E. Rollins and A.R. Buckpitt, Toxicol. Appl. Pharmacol., 47 (1979) 331—339.
12 O. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265—275.

13 T. Watabe, A. Hiratsuka, N. Ozawa and M. Isobe, Biochem. Pharmacol., 30 (1981) 390—392.
14 F. Seutter-Berlage, L. Delbressin, F. Smeets and H.C. Ketelaars, Xenobiotica, 8 (1978) 413—418.
15 J. Pachecka, P. Gariboldi, L. Cantoni, G. Belvedere, E. Mussini and M. Salmona, Chem.-Biol. Interact., 27 (1979) 313—321.
16 J.R. Bend, Z. Ben-Zvi, J. VanAnda, D.M. Dansette and D.M. Jerina, in R. Freudenthal and P.W. Jones (Editors), Carcinogenesis — A Comprehensive Survey, Vol. 1, Polynuclear Aromatic Hydrocarbons; Chemistry, Metabolism and Carcinogenesis, Raven Press, New York, 1976, pp. 63—75.

Journal of Chromatography, 231 (1982) 273—281
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1319

SEPARATION AND ANALYSIS OF HAEMATOPORPHYRIN DERIVATIVE COMPONENTS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

PETER A. CADBY, EUGENE DIMITRIADIS, HAMISH G. GRANT and A. DAVID WARD*

Department of Organic Chemistry, University of Adelaide, Adelaide, South Australia 5001 (Australia)

and

IAN J. FORBES

Department of Medicine, University of Adelaide, Queen Elizabeth Hospital, Woodville, South Australia 5011 (Australia)

(First received December 31st, 1981; revised manuscript received March 26th, 1982)

SUMMARY

High-performance liquid chromatography has been used to separate and analyse the components of haematoporphyrin derivative, a material used in cancer phototherapy. Both haematoporphyrin derivative in the solid form and the solution derived from it have been quantitatively analysed on reversed-phase columns. The factors (low pH, presence of ion-pairing reagent and solvent) that are of importance in optimising these separations are discussed.

INTRODUCTION

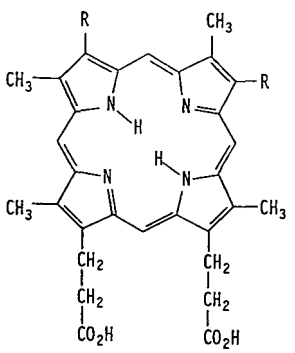
The ability of porphyrins to accumulate in human and animal tumours has been known for some time [1—3] and has been used as a means of identifying tumour tissue [3]. Initially haematoporphyrin was used as the tumour localizing agent but in 1960 Lipson et al. [4] introduced haematoporphyrin derivative (HPD) which they claimed was a superior localizing agent. This material was subsequently used for the identification of a variety of tumour tissues by observing the fluorescence of the porphyrin material within the tumour cells [5—12].

In the early 1970's the use of these porphyrins for cancer therapy was suggested by the work of Dougherty and co-workers [13, 14] and others [15—18]. Dougherty et al. [14] reported in 1975 that HPD, in conjunction

0378-4347/82/0000—0000/$02.75

with locally applied red light delivered 1 day after administration of HPD, prevented recurrences for at least 90 days in about half of the mice bearing a subcutaneous mammary tumour. Kelly et al. [18] showed, at the same time, that HPD activated by white light caused necrosis of human bladder tumour transplanted into mice. Since that time, work on the identification of tumour tissue and the treatment of experimental animals and human patients using HPD has been considerably extended [19, 20].

HPD is prepared [4, 19] in two steps from haematoporphyrin. The first step, involving treatment of haematoporphyrin with acetic acid—concentrated sulphuric acid (19:1), produces a solid which is referred to in the sequel as HPD solid. This solid material is then dissolved in dilute sodium hydroxide solution for up to 60 min, the pH is adjusted to 7.4 using dilute hydrochloric acid then salt and saline solution are added to make the material suitable for injection. This solution, which is that used for treatment by irradiation or for detection by fluorescence of tumour material is referred to in the sequel as HPD clinical.



(1) R = $CH_3CH(OH)$— (haematoporphyrin)
(2), (3) R = $CH_3CH(OH)$—, CH_2=CH—
(4), (5) R = $CH_3CH(OAc)$—, $CH_3CH(OH)$—
(6) R = $CH_3CH(OAc)$— (haematoporphyrin diacetate)
(7), (8) R = $CH_3CH(OAc)$—, CH_2=CH—
(9) R = CH_2=CH— (protoporphyrin)

Both HPD solid and HPD clinical are mixtures of porphyrins as shown by thin-layer chromatography (TLC) [19]. Separation of the methyl esters of the porphyrin components of HPD solid was achieved by silica chromatography and nine components (1—9) were identified in this manner [21]. These compounds differ only in the nature of the side chains (R_1 and R_2) and it is convenient to refer to them by indicating the nature of these side chains. Thus compounds 2 and 3 containing a 2-hydroxyethyl and a vinyl side chain are designated hydroxyvinyl derivatives; 4 and 5 are the hydroxyacetates and 7 and 8 are the vinylacetates. On this designation haematoporphyrin (1) is the dihydroxy compound, protoporphyrin (9) is the divinyl compound and the diacetate derivative (6) of haematoporphyrin becomes the diacetate. The advantage of this designation is that it is possible to discuss the isomeric pairs (e.g. 2 and 3) without necessarily having to indicate the exact structure of both.

While providing information on the composition of the porphyrin mixture, analysis of the methyl esters does not necessarily reflect the composition of the mixture of carboxylic acid used clinically. Bonnett et al. [22] have analysed the porphyrin acids by high-performance liquid chromatography (HPLC) and were able to identify haematoporphyrin (1) the diacetate (6) (partially

resolved only) and protoporphyrin (9) in the mixture. A more recent publication from Bonnett and co-workers [23] describes the further separation of HPD solid and the identification of components 1—8 in the mixture.

In this paper we describe the first complete analysis of both HPD solid and HPD clinical and discuss the factors that are of importance in achieving a complete resolution by HPLC of a very similar group of porphyrins as well as some of the factors that affect the composition of HPD clinical.

EXPERIMENTAL

All solvents were distilled, degassed and filtered through a 0.45-μm Millipore filter prior to use.

HPLC was performed using a Waters Model 6000A solvent delivery system and U6K injector. The detector normally used was a Waters Model 440 UV absorbance type operating at 405 nm. Columns used were Waters μBondapak C_{18} (10 μm) and Waters Radial-Pak C_{18} 5 mm and 8 mm I.D. (5 and 10 μm).

Haematoporphyrin was obtained in the form of its dihydrochloride from Roussel. Protoporphyrin was prepared from haematoporphyrin by brief heating in dimethylformamide. Haematoporphyrin diacetate was prepared by treating haematoporphyrin with acetic anhydride and pyridine and was approximately 80% pure. Haematoporphyrin dimethyl ester, prepared using diazomethane, was separated by HPLC into two peaks, resolved to the baseline, using the conditions described in Fig. 1. Each peak was collected, the solvent was removed and the residue was analysed by mass spectrometry (AEI MS-30).

The ion-pairing reagent (IPR) was tetra-*n*-butylammonium phosphate (Unichrom). The reagent is prepared by diluting the Unichrom concentrate in

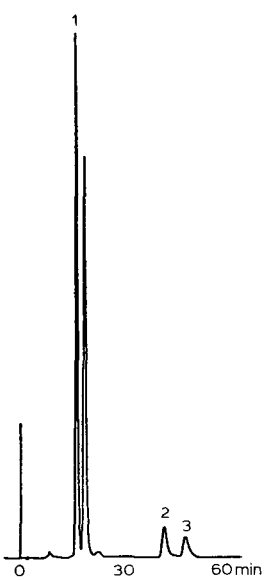


Fig. 1. Chromatogram of haematoporphyrin dihydrochloride in dimethyl sulfoxide solution. Peak numbers correspond to the structure numbers in the text. Column: Waters Radial-Pak C_{18} (5 μm). Solvent: methanol—aqueous IPR, pH 2.0 (80:20); flow-rate: 1.5 ml/min.

distilled water to 1 l, providing a 5 m*M* solution. This solution is then diluted with distilled water (1:1) before use, providing a 2.5 m*M* solution whose pH was adjusted using phosphoric acid and measured with a digital pH meter.

RESULTS AND DISCUSSION

In order to achieve a satisfactory analysis of either form of HPD, any analytical technique must be quantitative, reproducible and preferably relatively rapid. We considered that HPLC would be satisfactory on all these counts. Our initial attempts to reproduce the literature separation [22] using the reported conditions showed that there were considerable differences between the results reported in the literature and the results we could achieve. Accordingly we undertook an investigation into the effect of changing individual parameters in order to ascertain their importance in the separation technique.

Our best separations have been achieved on either a Waters Radial-Pak C_{18} column containing 5-μm adsorbent or a Waters μBondapak C_{18} (10 μm) column. However, the conditions required for best separation and resolution on these columns vary considerably. We attribute this difference to the extent to which the silica in each column has been silanised. With the μBondapak column, any silica remaining unreacted after the treatment with the C_{18} silylating reagent is end-capped with trimethylsilyl groups. The Radial-Pak columns do not receive this treatment. Hence the type of silica, the mesh size and the procedure used for silylation all combine to produce column materials whose performance can vary considerably yet which can all provide good resolution and peak shape of the porphyrin acids.

Initially, two solvent systems were found to be satisfactory, either methanol—water (approx. 8:2) or acetonitrile—water (approx. 6:4). Both solvent mixtures give best results when used at low pH (either added phosphoric acid or acetic acid) although lower pH values resulted in longer retention times. For example, protoporphyrin has a retention time of 60 min at pH 2 but a retention time of 15 min at pH 5 when analysed on the same column using identical conditions except for the pH of the solvent. A lowering of retention time generally resulted in less satisfactory resolution. At higher pH values (4—7) the porphyrin peaks often appeared as quite sharp but tailing peaks superimposed on a broad hump. This behaviour, which has been observed on a number of different columns using different solvents, is tentatively attributed to the aggregation which occurs readily with porphyrin acids. It is known [24] that the monomer is substantially favoured at lower pH values and we suggest that the broad unresolved peaks observed at higher pH values are due to dimers (or higher aggregates).

A feature of the use of the methanol—water solvent system was that it consistently gave two peaks of approximately equal response for haematoporphyrin on a variety of columns. This complete resolution of the two very close peaks (see Fig. 1) could not be achieved using the acetonitrile—water solvent system which generally only partially separated the two peaks. These two peaks were observed in samples of HPD, solid or clinical, as well as in commercial samples of haematoporphyrin from two different sources. The doublet of peaks was also observed when haematoporphyrin dimethyl ester was analysed

using the methanol—water solvent system. The two components of haematoporphyrin and of the dimethyl ester have been separated and collected using semi-preparative conditions and gave substantially single peaks after reinjection and analysis. This result is not compatible with the expected behaviour of two isomers that are equilibrating. A fraction containing only two peaks associated with the dimethyl ester of haematoporphyrin was collected from the HPLC column and subjected to hydrolysis (pH 12, 10 h, room temperature), neutralised and the material was analysed by HPLC. Two peaks of identical elution volume to those of haematoporphyrin were obtained. Mass spectral analysis of each separated component from the ester sample gave a molecular ion at *m/e* 626 corresponding to that of haematoporphyrin dimethyl ester and showed the expected fragmentation pattern for a compound with two hydroxyethyl side chains. We therefore suggest that the two peaks correspond to the two diastereoisomers of haematoporphyrin due to the presence of the two chiral carbon atoms in the hydroxyethyl side chains.

Other peaks in the chromatograms of HPD solid and of HPD clinical can be readily identified. The two monohydroxyethyl monovinyl isomers, 2 and 3, are always present to a small extent in commercially available haematoporphyrin which enables their retention volume to be determined. Acetylation of haematoporphyrin using acetyl chloride—pyridine gives the diacetate (6) as the major product. The diacetate can be separated from other products by chromatography on silica. Minor products from the acetylation reaction are the hydroxy acetates, 4 and 5, and the vinyl acetates, 7 and 8. These pairs can be separated from other components by HPLC (see Fig. 2) esterified and

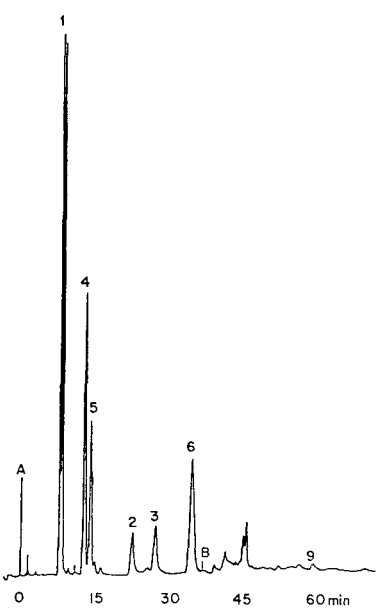


Fig. 2. Chromatogram of HPD solid. Peak numbers correspond to the structure numbers in the text. Column: Waters Radial-Pak C_{18} (5 μm). Solvents: (A) acetonitrile—aqueous IPR, pH 2.5—methanol (3:2:1); (B) methanol—aqueous IPR, pH 2.5 (90:10); flow-rate: 2 ml/min.

identified by spectral methods [21, 25]. We have not, thus far, attempted to ascertain which peak of each particular pair of isomers belongs to a particular structure for that pair.

Notwithstanding the generally highly acceptable performance of the binary solvent systems (methanol or acetonitrile plus aqueous IPR) for analysing HPD clinical, it was found that applying these to the more complex mixtures encountered such as HPD solid, provided less than ideal results. For example, with the methanol-based system, the peak attributed to the diacetate (6) was found to have identical retention time to one of the hydroxy vinyl isomers and no resolution could be obtained of the hydroxy acetate isomers. The acetonitrile based solvent (60% + 40% aqueous IPR, pH 2.5) on the other hand, provided greatly improved separation of the relatively polar components though at the tolerable expense of complete loss of resolution of the haematoporphyrin diastereoisomers.

The aqueous acetonitrile with the IPR solvent also gave no resolution of the haematoporphyrin peaks on the analytical μBondapak column, However, resolution of this peak can be obtained using the aqueous acetonitrile—acetic acid solvent mixture on this column, thus clearly indicating that strong hydrogen bonding agents are required for the selective HPLC separation of the haematoporphyrin diastereoisomers. Consequently, addition of 10—20% methanol to the aqueous acetonitrile solvent gave not only partial resolution of these two components but also provided improved separation of the other components in this region, notably the hydroxy acetates. In this system, the peak attributed to the diacetate (6) was then clearly separated with a longer retention time from the hydroxy vinyl isomers (Fig. 2).

Despite the considerably improved resolution and peak shape afforded by this ternary solvent system in the polar region of the chromatogram, the protoporphyrin peak showed considerable tailing and was virtually unobservable when present in less than 5—8% concentration. This deficiency was overcome by utilising a dual solvent system for each analysis whereby, in the absence of a solvent programmer, the aqueous acetonitrile—methanol—

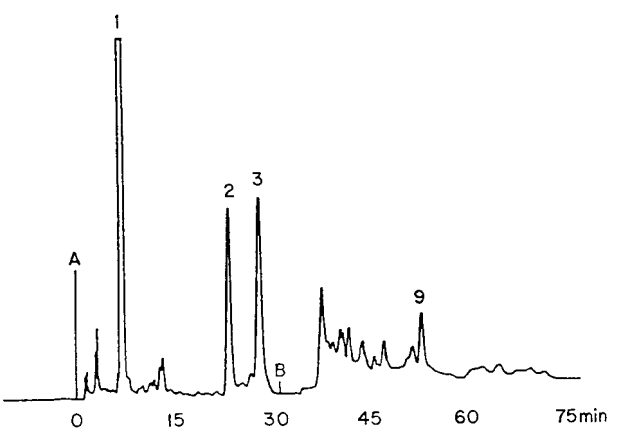


Fig. 3. Chromatogram of HPD clinical. Conditions as for Fig. 2.

IPR mixture was changed to 90% aqueous methanol—IPR (pH 2.5) after elution of the second hydroxy vinyl peak (or diacetate peak if present). This approach provided a chromatogram with greatly improved peak shape and convenient retention volume for protoporphyrin in addition to revealing, by virtue of resultant decreased peak width, a number of previously obscured minor components. The majority of these were observed in the region between the diacetate compound and protoporphyrin but several minor peaks eluted after the latter, usually considered to be the least polar porphyrin of interest in this investigation.

Integration of the chromatogram for HPD clinical (Fig. 3) shows that haematoporphyrin is present to the extent of 45—50%, the hydroxy vinyls comprise 20—25% and protoporphyrin constitutes approximately 3—5% of the total mixture. Minor peaks eluting near haematoporphyrin make up 5—10% of the material and minor peaks eluted by the more polar solvent (B) but excluding protoporphyrin account for 10—15%. However, this last value is less accurate than the others because of the difficulty in allowing for the baseline shift that occurs on changing solvent.

HPD solid is much more variable in composition due to changes that may occur on standing particularly if the material is not adequately washed and dried. However, the diacetate (6) is the major component usually accounting for more than 50% of the freshly prepared material. Depending on how the HPD solid is stored this value may drop considerably on standing. However, in spite of these possible changes the analysis of the HPD clinical made from the HPD solid does not vary to a large extent suggesting that the changes on

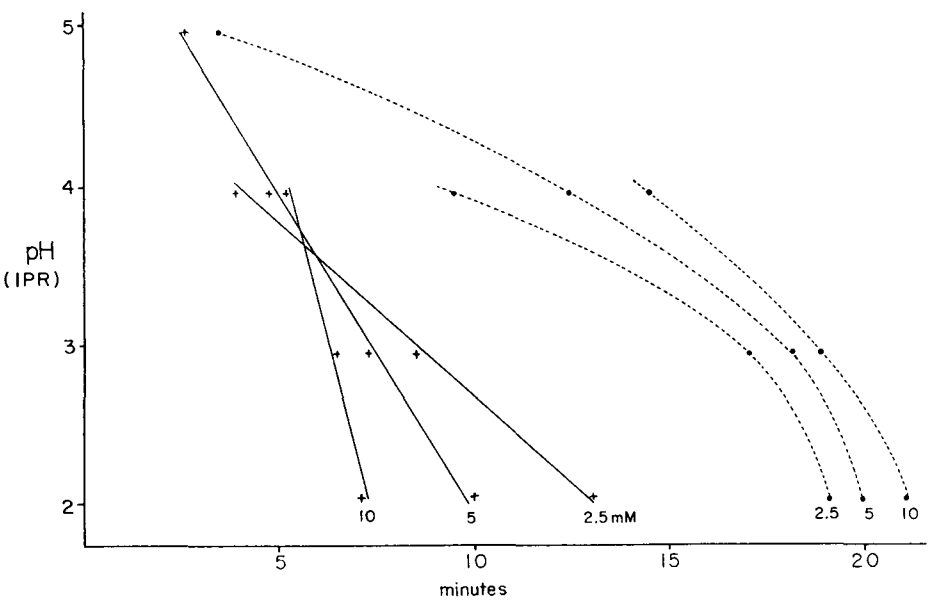


Fig. 4. Plot showing the effect of aqueous IPR solution pH and concentration on the retention time of haematoporphyrin (——) and protoporphyrin (----, multiply time scale × 3). Column: Waters Radial-Pak C_{18} (10 μm); solvent: methanol—aqueous IPR (80:20); flow-rate: 2 ml/min.

storage to HPD solid are similar to those caused by the alkali treatment. The major difference is that the relative amount of the hydroxy vinyls is increased in HPD clinical when it is prepared from fresh HPD solid.

It may be noted that acetonitrile, possessing relatively low hydrogen bonding potential, enhances the resolution of the more polar components as a consequence of minimised solvent—solute interactions. Methanol, on the other hand, appears to provide better resolution and peak shape for the less polar constituents eluting after the diacetate compound. Thus, this dual solvent (or gradient elution) approach would appear to offer the most acceptable compromise for the convenient analysis of HPD solutions.

IPRs have been used quite extensively in the separation of polar, water soluble materials on reversed-phase columns. The role of the IPR is complex and is currently poorly understood [26]. However, at least in part, one reason why IPRs are so useful was shown by the necessity to use them with the Radial-Pak reversed-phase columns which strongly suggests that in this case they function to some extent as an alternative to end-capping by being adsorbed onto the unprotected OH groups on the silica.

Fig. 4 indicates the different effect a change in the ion-pairing concentration has on haematoporphyrin (a polar component) compared to protoporphyrin (a non-polar component). Fig. 4 also shows the substantial change in retention time of these components as the pH of the solution changes.

ACKNOWLEDGEMENTS

We wish to thank Mr. M. Rechsteiner (Waters Associates) for his advice and Mr. L. Paltridge for technical assistance. The work was supported by grants from the University of Adelaide, The Anti-Cancer Foundation of the Universities of South Australia and the National Health and Medical Research Council. We particularly wish to thank Miss E. Laubmann for a very generous donation to the University of Adelaide Cancer Phototherapy Fund.

REFERENCES

1 H. Auter and G.Z. Banzer, Z. Krebsforsch., 53 (1942) 65.
2 F.H.J. Figge, G.S. Weiland and L.O.J. Manganiello, Proc. Soc. Exp. Biol. Med., 68 (1948) 640.
3 D.S. Rasmussen-Taxdal, G.E. Ward and F.H.J. Figge, Cancer, 8 (1955) 78.
4 R.L. Lipson, E.J. Baldes and A.M. Olsen, J. Nat. Cancer Inst., 26 (1961) 1.
5 R.W. Henderson, G.S. Christie, P.S. Clezy and J. Lineham, Brit. J. Exp. Pathol., 61 (1980) 101.
6 R.L. Lipson, E.J. Baldes and A.M. Olsen, Dis. Chest, 46 (1964) 676.
7 R.L. Lipson, J.H. Pratt, E.J. Baldes and M.B. Dockerty, Obstet. Gynecol., 24 (1964) 78.
8 H.B. Gregorie, E.D. Horger, J.L. Ward, J.F. Green, T. Richards, H.C. Robertson and T.B. Stevenson, Ann. Surg., 167 (1968) 820.
9 J.H. Kinsey, D.A. Cortese and D.R. Sanderson, Mayo Clin. Proc., 53 (1978) 594.
10 D.A. Cortese, J.H. Kinsey, L.B. Woolnen, W.S. Payne, D.R. Sanderson and R.S. Fontana, Mayo Clin. Proc., 54 (1979) 635.
11 D.R. Doiron, A.E. Profio, R.G. Vincent and T.J. Dougherty, Chest, 76 (1979) 27.
12 A.E. Profio, D.R. Doiron and E.G. King, Med. Phys., 6 (1979) 523.
13 T.J. Dougherty, J. Nat. Cancer Inst., 51 (1974) 1333.
14 T.J. Dougherty, G.B. Grindey, R. Fiel, K.R. Weishaupt and D.G. Boyle, J. Nat. Cancer Inst., 55 (1975) 115.

15 I. Diamond, S.G. Granelli, A.F. McDonagh, S. Neilsen, C.B. Wilson and R. Jaenicke, Lancet, ii (1972) 1175.
16 S.G. Granelli, I. Diamond, A.F. McDonagh, C.B. Wilson and S.L. Nielsen, Cancer Res., 35 (1975) 2567.
17 J.F. Kelly and M.E. Snell, J. Urol., 115 (1976) 150.
18 J.F. Kelly, M.E. Snell and M.C. Berenbaum, Brit. J. Cancer, 31 (1975) 237.
19 T.J. Dougherty, J.E. Kaufman, A. Goldfarb, K.R. Weishaupt, D.G. Boyle and A. Mittleman, Cancer Res., 38 (1978) 2628.
20 T.J. Dougherty, G. Lawrence, J.E. Kaufman, D.G. Boyle, K.R. Weishaupt and A. Goldfarb, J. Nat. Cancer Inst., 62 (1979) 231.
21 P.S. Clezy, T.T. Hai, R.W. Henderson and L.V. Thuc, Aust. J. Chem., 33 (1980) 585.
22 R. Bonnett, A.A. Charalambides, K. Jones, I.A. Magnus and R.J. Ridge, Biochem. J., 173 (1978) 693.
23 R. Bonnett, R.J. Ridge, P.A. Scourides and M.C. Berenbaum, J. Chem. Soc., Chem. Commun., (1980) 1198; J. Chem. Soc., Perkin I, (1981) 3135.
24 W.I. White, in D. Dolphin (Editor), The Porphyrins, Physical Properties, Part C. Academic Press, New York, 1978, Ch. 7.
25 P.S. Clezy, C.J.R. Fookes and T.T. Hai, Aust. J. Chem., 31 (1978) 365.
26 B.A. Bidlingmeyer, S.N. Deming, W.P. Price, Jr., B. Sachok and M. Petrusek, J. Chromatogr., 186 (1979) 419.

Journal of Chromatography, 231 (1982) 283—289
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1322

THIN-LAYER CHROMATOGRAPHIC DETERMINATION OF INDOLIC TRYPTOPHAN METABOLITES IN HUMAN URINE USING SEP-PAK C_{18} EXTRACTION

DOMENICA TONELLI*, ENRICO GATTAVECCHIA and MASSIMO GANDOLFI

Istituto di Scienze Chimiche, Università di Bologna, Via San Donato 15, 40127 Bologna (Italy)

(First received January 12th, 1982; revised manuscript received April 20th, 1982)

SUMMARY

Tryptophan and some of its indole metabolites were separated by thin-layer chromatography, stained with the Van Urk—Salkowski reagent, and quantitated by scanning densitometry. The application of this technique for the detection of the indoles in urine samples, employing Sep-Pak C_{18} cartridges for extraction, was demonstrated. The proposed method is simple and accurate. The detection limits were 2 μg/ml 5-hydroxytryptophan, 1.75 μg/ml 5-hydroxyindolyl-3-acetic acid, 1.5 μg/ml tryptophan, 0.8 μg/ml indolyl-3-acetic acid, 0.9 μg/ml indolyl-3-butyric acid, 1.75 μg/ml serotonin, and 1.25 μg/ml tryptamine.

INTRODUCTION

An increasing number of investigations involving the determination of indole compounds, especially 5-hydroxyindoles, in biological fluids is being undertaken in several laboratories. Abnormally increased amounts of tryptophan metabolites are associated with many diseases such as malignant carcinoid tumours, schizophrenia, Parkinson's disease, and malignant hyperthermia [1, 2].

Methods of analysis for the determination of tryptophan and its metabolites in biological fluids include thin-layer chromatography (TLC) [3—6], gas—liquid chromatography [7], UV and fluorescence spectrometry [8, 9]. Most of these methods are lengthy and lack sufficient resolving power and specificity. More modern techniques such as gas chromatography—mass spectrometry [10] and radioimmunoassay [11] have been used with varying degrees of success. Recently, reversed-phase high-performance liquid chro-

0378-4347/82/0000—0000/$02.75

matography (HPLC) with fluorescence [12] and electrochemical [13, 14] detection has been successfully employed to determine tryptophan and its metabolites in biological fluids and tissues. TLC has not yet found extensive use in the study of metabolic diseases. Perhaps the main reason is the time-consuming preliminary treatment of the sample necessary to reduce the amount of interfering substances. Although many procedures have been used for analyzing indole compounds in biological fluids by TLC, some are little suited to quantitative determination because of the distorted chromatograms obtained if the samples are not pretreated [15].

This paper describes a convenient TLC method for the assay of tryptophan metabolites in human urine avoiding the need for multiple extractions. The method is based on a rapid extraction procedure from deproteinized urine using Sep-Pak C_{18} cartridges. In particular, we report the separation of tryptophan (TRP), 5-hydroxytryptophan (5-HTRP), tryptamine (T), serotonin (5-HT), indolyl-3-acetic acid (IAA), 5-hydroxyindolyl-3-acetic acid (5-HIAA), and their determination by scanning densitometry using indolyl-3-butyric acid (IBA) as internal standard. Graffeo and Karger [16] developed a rapid and sensitive analytical method for the analysis of the same six indoles in urine. Unfortunately, the selectivity, which is diagnostically important, does not seem to be fully satisfactory for TRP and 5-HIAA (for the latter only at low concentrations) which are masked by the large fluorescence peak in the urine background. Our aim was to develop a simple and rapid assay for tryptophan metabolites which would not offer such a drawback. Briefly, the selectivity arises from three processes: (A) the initial step of sample clean-up, (B) the separation effected by TLC, and (C) the specific reagent used for staining chromatograms.

EXPERIMENTAL

Apparatus

A Camag (Muttenz, Switzerland) TLC/HPTLC 76500 scanner was used for all quantitative measurements, the absorbance of the stained spots being read at 620 nm. In all determinations the instrument was zeroed on a blank area of the thin-layer. A constant drift of the baseline was always observed. The peaks obtained were quantitatively integrated by a Spectra-Physics Minigrator (Darmstadt, G.F.R.). TLC was performed on 0.25-mm precoated layers of silica gel G-60 F_{254} (Merck, Darmstadt, G.F.R.). Aliquots (0.5—1.5 μl) of the solutions to be assayed were spotted quantitatively with a Camag micro-applicator. The plates were developed at room temperature in a twin-trough chamber. Extractions were performed using Sep-Pak C_{18} cartridges, containing octadecylsilane bonded-phase packing (Waters Assoc., Milford, MA, U.S.A.).

Reagents

Analytical reagent grade chemicals were used without further purification. All solutions were made up with deionized and distilled water. All reference compounds were purchased from Sigma (St. Louis, MO, U.S.A.). Stock standard solutions in methanol—citrate buffer (0.1 *M*, pH 4) (1:1, v/v) were prepared containing 510 μg/ml TRP, 550 μg/ml 5—HTRP, 400 μg/ml T, 700

μg/ml 5-HT, 175 μg/ml IAA, 475 μg/ml 5-HIAA, and 200 μg/ml IBA. All solutions were kept refrigerated in tinfoil-wrapped amber bottles, and prepared fresh every four weeks. Working standard solutions were made by dilution to appropriate concentrations with methanol, and discarded after a single use.

Chromatographic procedure

Bright light was avoided throughout the procedure. After spotting, the plate was air-dried and placed in the empty trough of a twin-trough chamber, the second trough containing *n*-hexane. After 15 min of preconditioning, the layer was developed with the solvent system acetone—33% aqueous dimethylamine solution—isopropanol (1.8:2.5:5.6), the run being 9 cm. Then the plate was dried until all traces of solvent had evaporated (10 min). Before staining, the chromatogram was examined under UV light for fluorescing and/or quenching spots. After this examination, the plate was dipped into a solution of the Van Urk—Salkowski reagent [17] for 10 sec, removed and blotted with a dry paper towel. The plate was then heated for 5 min in a 100°C oven connected with a vacuum pump, removed and immersed three times in distilled water to be washed. After the last wash, the plate was dried at 45°C and the stained spots were quantitated by scanning densitometry. The plate was also sprayed with the chromogenic reagent, but the calibration graphs so obtained showed lower correlation coefficients in comparison with those obtained by the dipping procedure, though the slopes were the same. As a consequence, the dipping method was preferred for staining indole compounds.

Extraction procedure

All manipulations were carried out in the dark allowing only slight indirect bulb light. To 50-ml tubes containing 10 ml of urine 1 ml of aqueous zinc sulphate solution (100 g/l) and 1 ml of 1 *M* aqueous sodium hydroxide solution were added. The sample was vortexed for 30 sec to ensure good protein precipitation; 12 ml of 0.2 *M* phosphate buffer (pH 6) were then added and the tubes were centrifuged. The clear supernatant was made up to 25 ml with phosphate buffer; 5-ml aliquots were extracted by passing the supernatant through a Sep-Pak C_{18} cartridge fitted to a Luer-Lok glass syringe. The Sep-Pak C_{18} cartridge was prepared by flushing with 5 ml of methanol followed by 5 ml of aqueous phosphate buffer (0.2 *M*, pH 6). After the deproteinized sample was passed through the cartridge, it was washed with 1.8 ml of redistilled water and 2 ml of methanol. The latter solvent eluted the indole compounds from the cartridge. After washing with a further 5-ml of methanol the cartridge was ready for re-use. The 2-ml methanol fraction was evaporated to dryness in a rotary vacuum evaporator at 40°C. The residue was redissolved in 100 μl of methanol and taken for chromatography.

Preparation of standard curves

The standard curves were prepared by spiking urine samples with varying amounts of TRP, 5-HTRP, T, 5-HT, IAA, 5-HIAA, and with 10 μg/ml IBA as internal standard. The supplemented samples were assayed by the extrac-

tion procedure described above. Results for the standard curves were calculated using the internal standard method. A control urine extract containing only the internal standard was used to estimate the background level associated with each compound. These values were used as corrections for the data obtained before any statistical analysis was carried out. The relative weight response values for each of the tested compounds were determined by at least five independent analyses of calibration standards.

RESULTS AND DISCUSSION

Chromatographic separation

Prior to the analysis of urine samples the chromatographic conditions were optimized for the separation of the six indolic compounds and the internal standard. The solvent system used allowed the effective separation of all the indoles examined as can be seen from the R_F values reported in Table I. Under the conditions described the R_F values were quite reproducible, as demonstrated by the coefficient of variation which was less than 2%. In contrast to other researchers [18] we found no loss of indolic compounds during chromatography.

TABLE I

CHROMATOGRAPHIC PROPERTIES OF INDOLE METABOLITES OF TRYPTOPHAN

Compound	$R_F \times 100$	RRF*	Limit of detection (ng)
5-HTRP	33	0.11	40.0
5-HIAA	42	0.16	35.0
TRP	50	0.37	30.0
IAA	60	0.70	25.0
IBA	67	1.00	20.0
5-HT	80	0.71	35.0
T	93	0.41	25.0

*Relative response factor, derived from the mole ratio, compared to the internal standard IBA. Each value is an average of five independent analyses (C.V. = 6%).

Calibration

Calibration curves for the determination of the tested compounds by TLC were prepared by spotting different amounts of the seven indoles and plotting peak areas against concentration. Altogether almost identical linear calibration curves, passing through the origin by extrapolation, were obtained by diluting the stock standard solutions. Linearity was observed up to 1.5 nmol for IAA and IBA, 3.75 nmol for TRP, 5-HTRP, 5-HIAA and T, and 5 nmol for 5-HT. The wide linear range is more than adequate for tryptophan metabolites and for the clinical investigation of urinary concentrations of indole substances. Allowing a signal-to-noise ratio of 2 the detection limits were estimated and the results are reported in Table I. In practice twice these amounts can be easily quantitated. The precision of the proposed method was deter-

mined by spotting the same amount of each of the seven indoles, varying from 100 to 700 ng, and performing five independent runs. The coefficients of variation averaged less than 8%.

Sample clean-up for TLC analysis

A very important prerequisite for TLC analysis of urine is the preliminary treatment of the sample in order to eliminate the bulk of interfering substances. This is of special importance in relation to the assay of tryptophan metabolites which are difficult to analyze because of their overall lability, as well as their exceedingly small quantities in biological fluids. To minimize losses of indole metabolites (amines and acids) throughout the isolation from urine and to simplify sample clean-up, we have investigated the use of the Sep-Pak C_{18} cartridges for urine extractions. Details of the extraction efficiency of this method are recorded in Table II. The use of the Sep-Pak C_{18} cartridges for urine extraction had several advantages over the solvent extraction methods. It provided chromatographically cleaner extracts because of the partial separation which it produced and effected a significant time saving for extraction because it was a single operation. Moreover, the extraction efficiency was at least equal to that of the solvent extraction methods, the recovery of all indoles being approximately 100%. In agreement with Allan et al. [19], we found that a Sep-Pak C_{18} cartridge could be re-used many times without any loss of performance.

TABLE II

RECOVERIES OF THE TESTED COMPOUND FROM SEP-PAK C_{18} EXTRACTION

Each result represents the average of five assays.

Compound	Theoretical content (μg)	Recovery	
		μg (mean ± S.D.)	%
5-HTRP	55	52.7 ± 1.2	95.8
5-HIAA	47.5	48.2 ± 1.1	101.4
TRP	51.0	50.2 ± 0.8	98.4
IAA	17.5	17.1 ± 0.6	97.7
IBA	20.0	20.3 ± 0.8	101.5
5-HT	70.0	68.2 ± 1.7	97.4
T	40.0	39.4 ± 1.2	98.5

Urinary indole analysis

The chromatogram for a mixture of pure standards is shown in Fig. 1a. This separation was achieved with standard substances and thus represents the best performance of the system. To explore the analysis of the indoles in urine, a 10-ml urine sample was supplemented with 0.5—4 μmol of each indole and 100 μg of the internal standard to simulate pathological conditions and processed as described under Experimental. The fact that indoles

are readily lost during manipulation and evaporation steps [8] showed the convenience of reducing them to a minimum and keeping the evaporation temperature below 40°C. The chromatogram of a urine sample to which tryptophan metabolites were added before analysis is shown in Fig. 1b. As can be seen, the indoles are well separated and free from interference from other naturally occurring constituents of urine except for a small peak which appears between the IAA and IBA peaks. The R_F values of the seven indoles after extraction from urine samples show a slight difference in comparison with the standard solutions, but reproducibility is still good.

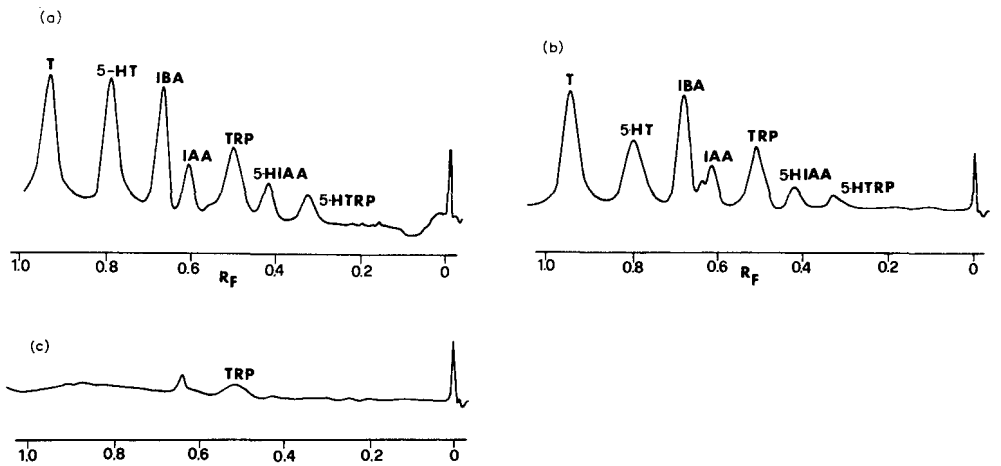


Fig. 1. Chromatograms of (a) a mixture of pure indole standards, (b) urine sample supplemented with pure indole standards, and (c) urine blank.

Analytical recovery of indole compounds added to urine samples

The mean recoveries of the seven indoles added to urine samples are reported in Table III. Since they were not all extracted equally, it was necessary to prepare an extracted standard curve as described under Experimental. The low recoveries obtained for 5-HTRP, 5-HIAA, and 5—HT after extraction from supplemented urine samples indicate that the loss of these indoles occurs during deproteinization. In fact, such a loss cannot be ascribed to the dipping process or to extraction on the Sep-Pak C_{18} cartridge since a standard solution chromatographed directly gives the same response as a standard solution extracted on Sep-Pak C_{18}. Although the recoveries achieved with the extraction procedure described above are sufficient to allow the determination of endogenous levels of tryptophan metabolites, we are presently working to improve the overall extraction procedure further by the use of a Pellicon membrane (Millipore) for deproteinizing urine samples. In conclusion, the proposed technique for the assay of indole tryptophan metabolites in urine, though less sensitive, offers several advantages over other currently used methods. It is a simple, selective and reliable method which can easily be put to routine use for diagnostic application.

TABLE III

RECOVERIES OF THE TESTED COMPOUNDS AFTER THE COMPLETE EXTRACTION PROCEDURE

Each result represents the average of five assays.

Compound	Amount added to 1 ml or urine (μg)	Recovery	
		μg (mean ± S.D.)	%
5-HTRP	27.5	14.3 ± 1.0	52
5-HIAA	23.5	16.2 ± 0.9	69
TRP	25.5	21.2 ± 1.3	83
IAA	8.5	8.2 ± 0.6	97
IBA	10.0	9.8 ± 0.6	98
5-HT	35.0	19.3 ± 1.1	55
T	20.0	17.6 ± 1.2	88

ACKNOWLEDGEMENTS

The authors acknowledge Professor Alberto Breccia for his criticism and support throughout this study. This study was supported in part by the Italian National Research Council (C.N.R.), Finalized Project "Controllo della Crescita Neoplastica", Grant No. 80.01486.96.

REFERENCES

1 T.P. Davis, C.W. Gehrke, Jr., T.D. Cunningham, K.C. Kuo, K.O. Gerhardt, H.D. Johnson and C.H. Williams, Clin. Chem., 24 (1978) 1317.
2 A.M. Krstulovic, P.R. Brown, D.M. Rosie and P.B. Champlin, Clin. Chem., 23 (1977) 1984.
3 L. Edvinsson, R. Hakanson and F. Sundler, Anal. Biochem., 46 (1972) 473.
4 D.J. Byrd, W. Kochen, D. Idzko and E. Knorr, J. Chromatogr., 94 (1974) 85.
5 G.M. Anderson, J. Chromatogr., 105 (1975) 323.
6 H.K.L. Hundt, E.C. Clark and H.C. van der Linde, J. Chromatogr., 182 (1980) 110.
7 P.H. Degen, J.R. DoAmaral and J.D. Barchas, Anal. Biochem., 45 (1972) 634.
8 S.S.M. Hassan, Anal. Chem., 47 (1975) 1429.
9 J.D. Barchas, E. Erdelyi and P. Angwin, Anal. Biochem., 50 (1972) 1.
10 J. Segura, F. Artigas, E. Martinez and E. Gelpi, Biomed. Mass Spectrom., 3 (1976) 91.
11 D.J. Kennaway, R.G. Frith, G. Phillopou, C.D. Matthews and R.F. Seamark, Endocrinology, 101 (1977) 119.
12 T. Flatmark, S.W. Jacobsen and J. Haavik, Anal. Biochem., 107 (1980) 71.
13 D.D. Koch and P.T. Kissinger, J. Chromatogr., 164 (1979) 441.
14 I.N. Mefford and J.D. Barchas, J. Chromatogr., 181 (1980) 187.
15 J.G. Heathcote, D.M. Davies, C. Haworth and R.W.A. Oliver, J. Chromatogr., 55 (1971) 377.
16 A.P. Graffeo and B.L. Karger, Clin. Chem., 22 (1976) 184.
17 A. Ehmann, J. Chromatogr., 132 (1977) 267.
18 T.C. Moore and C.A. Shaner, Plant Physiol., 42 (1967) 1787.
19 R.J. Allan, H.T. Goodman and T.R. Watson, J. Chromatogr., 183 (1980) 311.

Journal of Chromatography, 231 (1982) 291—299
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1310

SIMULTANEOUS ANALYSES OF PHENETHYLAMINE, PHENYLETHANOLAMINE, TYRAMINE AND OCTOPAMINE IN RAT BRAIN USING FLUORESCAMINE

SHINKICHI KAMATA, KENJI IMURA, AKIRA OKADA and YASUNARU KAWASHIMA

First Department of Surgery, Osaka University Medical School, Kita-ku, Osaka (Japan)

and

ATSUSHI YAMATODANI, TAKEHIKO WATANABE* and HIROSHI WADA

Second Department of Pharmacology, Osaka University Medical School, Kita-ku, Osaka (Japan)

(First received June 22nd, 1981; revised manuscript received April 17th, 1982)

SUMMARY

A fluorometric method for the simultaneous analyses of phenethylamine, phenylethanolamine, tyramine and octopamine has been developed. The method involves ion-exchange chromatography, derivatization with fluorescamine, solvent extraction and then separation by thin-layer chromatography. The fluorescent spots are then quantitated by scanning. The detection limits of this method are about 10 pmoles for phenethylamine, phenylethanolamine and tyramine, and 20 pmoles for octopamine. The method was used for simultaneous analyses of putative neurotransmitter amines in whole rat brain.

INTRODUCTION

Recently, it has been suggested that aromatic trace amines, including phenylalkylamines, indoleamines, and imidazoleamines, may play roles in neural transmission in the brain [1—3]. Furthermore, abnormal metabolism of these amines has been shown to be implicated in various pathologic conditions, such as migraine [4], Parkinson's disease [5], schizophrenia [6], phenylketonuria [7], depressive illness [8] and hepatic encephalopathy [9—12].

Many reports have shown that severe liver disorder results in disturbances in metabolism of putative neurotransmitter amines in the brain [9—13], which may explain the pathogenesis of hepatic encephalopathy. To clarify the relation between disturbances in metabolism of brain amines and hepatic encephalo-

0378-4347/82/0000—0000/$02.75

pathy, we needed to analyze brain neurotransmitter amines simultaneously in experimental animals. In particular, since phenethylamine, phenylethanolamine, tyramine and octopamine, which are mainly derived from phenylalanine and tyrosine in the brain [14], undergo similar metabolism and may be interconvertible [15], it was important to determine the changes in contents of these amines in the brain simultaneously.

Recently developed analytical methods, including radioenzymatic, thin-layer chromatographic–mass spectrometric and gas chromatographic–mass spectrometric methods, have revealed the presence of phenethylamine [16–18], phenylethanolamine [18–20], tyramine [21, 22] and octopamine [20, 23–25] in rat brain. These methods, however, require expensive instruments, purified enzymes, or radioactive compounds, and thus a simpler method that can be routinely used in a laboratory is desirable.

We developed a systematic analytical method for biogenic amines [26]. In this paper, we have attempted to measure phenethylamine, phenylethanolamine, tyramine and octopamine simultaneously by converting them to fluorescent fluorescamine conjugates and separating the latter by thin-layer chromatography.

EXPERIMENTAL

All chemicals and solvents were of the purest grade commercially available. Precoated silica gel plates (without fluorescent indicator, thickness 0.25 mm) were from Merck (Darmstadt, G.F.R.). 2-Phenyl[1-^{14}C]ethylamine·HCl (specific radioactivity, 60.6 mCi/mmole) and [1-^{14}C]tyramine (50.0 mCi/mmole) were purchased from Amersham International (Arlington Heights, IL, U.S.A.) and New England Nuclear (Boston, MA, U.S.A.), respectively.

Extraction of fluorescamine derivatives with ethyl acetate

A solution of 1–10 nmoles of each amine in 3 ml of 0.5 *M* sodium borate, pH 8.5, was rapidly mixed with 1.0 ml of fluorescamine solution in acetone (20 mg per 100 ml) at room temperature and stirred on a vortex mixer for 1 min. The mixture was then evaporated to 0.3 ml under nitrogen at 25°C, and the residue was dissolved in 3.0 ml of 4 *M* acetate buffer, pH 3.0–6.5. The fluorescent conjugate was extracted twice with 3.0 ml of ethyl acetate and the fluorescence of the acetate buffer phase was measured in an Hitachi fluorospectrophotometer, Model MPF-4, at 480 nm with excitation at 390 nm. The extraction rate was calculated by comparing the fluorescence before and after extraction and a correction was made with appropriate blanks.

Preparation of the trace amine fraction

Male Wistar rats weighing 350–400 g, some subjected to surgical construction of a portacaval shunt (PCS) 4 weeks earlier [13], were decapitated between 10:00 a.m. and noon. The brain was quickly removed, weighed, and stored at −70°C until use.

The amine fraction containing polyamines, histamine and trace amines, such as phenethylamine, phenylethanolamine, tyramine, and octopamine, was prepared with Amberlite CG-50 and Sephadex G-10 chromatography as reported

previously [13, 26]. Briefly, whole brain (about 2 g) was homogenized in 6.0 ml of ice-cold 3% perchloric acid containing 0.2% disodium EDTA and 1.0 m*M* dithiothreitol in a Polytron homogenizer (Kinematica, Luzern, Switzerland) operated at the maximum setting for 1 min in an ice-bath. The homogenate was centrifuged at 10,000 *g* for 40 min at 4°C. The pH of the supernatant was adjusted to 6.0 by dropwise addition of 5 *M* potassium hydroxide solution and the precipitate was removed by brief centrifugation. The supernatant was applied to a column of Amberlite CG-50 (0.4 cm I.D. × 9.5 cm) equilibrated with 0.1 *M* sodium phosphate buffer, pH 6.0, and the column was eluted with 2.0 ml of distilled water. The resultant eluate was collected (fraction A, containing neutral and acidic amino acids and amine metabolites). The column was washed with 8.0 ml of distilled water and with 4.0 ml of 0.01 *M* sodium phosphate buffer, pH 6.0, containing 1.5% boric acid; then noradrenaline was eluted with 3.0 ml of the same solution (noradrenaline fraction) and dopamine was eluted with 3.0 ml of 4% boric acid (dopamine fraction). After elution of the dopamine fraction, basic amino acids (Lys, Orn, Arg) were eluted with 5.0 ml of 0.1 *M* sodium phosphate buffer, pH 6.9, and with 5.0 ml of 0.1 *N* hydrochloric acid (fraction B). Finally, amines were eluted with 3.0 ml of 0.5 *N* hydrochloric acid, and the eluate was applied to a column of Sephadex G-10 (0.4 cm I.D. × 9.5 cm) equilibrated with 0.1 *N* hydrochloric acid, and the column was eluted with 1.0 ml of 0.5 *N* hydrochloric acid. The resultant eluate was collected (amine fraction — containing polyamines, histamine, and trace amines such as phenethylamine, phenylethanolamine, tyramine, and octopamine) and stored at −80°C for further analysis. Indoleamines, including serotonin and tryptamine, were eluted with 3.0 ml of distilled water (serotonin fraction).

Determination of trace amines

The amine fraction was evaporated to dryness under vacuum and the residue was dissolved in 1.0 ml of 0.5 *M* sodium borate buffer, pH 8.5. The solution was promptly mixed with 1.0 ml of fluorescamine solution in acetone (75 mg per 100 ml) at room temperature with stirring on a vortex mixer for 1 min. The reaction mixture was evaporated to 0.3 ml under nitrogen at 25°C, and then acidified with 1.0 ml of 4 *M* acetate buffer, pH 4.5, and mixed with 4 ml of ethyl acetate on a vortex mixer for 1 min. The ethyl acetate layer was separated and evaporated to 0.2 ml under nitrogen at 25°C. Then, 0.1 ml of distilled water was added and the mixture was evaporated to 0.2 ml under nitrogen at 25°C. The fluorescent compounds were extracted with 20 μl of ethyl acetate and a 5-μl aliquot of extract was used for thin-layer chromatography. Two-dimensional chromatography was performed on a 10 × 10 cm silica gel plate with ethyl acetate—hexane—methanol—water (60:20:25:10) as the first solvent and benzene—dioxane—acetic acid (90:25:5) as the second. The fluorescent spots of phenethylamine, phenylethanolamine, tyramine and octopamine were located under ultraviolet light and measured with a Shimadzu chromatoscanner, Model CS900.

For recovery experiments, 5 nCi of each of [^{14}C]phenethylamine or [^{14}C]-tyramine were mixed with the supernatant fraction of brain homogenate and treated similarly as described above. The spots on the thin-layer chromatogram

corresponding to phenylethylamine and tyramine were scraped off and counted for radioactivity in 10 ml of a scintillation cocktail (ACS II, Amersham) with an Aloka liquid scintillation counter (LCS-700).

RESULTS

Extraction of fluorescamine derivatives of trace amines into organic solvent

To subject fluorescamine derivatives of trace amines to thin-layer chromatography, it is necessary to extract them into an organic solvent. Therefore, the optimal pH for extraction of the derivatives was examined. As shown in Fig. 1, the lower the pH, the better the extraction. However, as fluorescamine derivatives were labile in acidic conditions, pH 4.5 was used in the routine experiments.

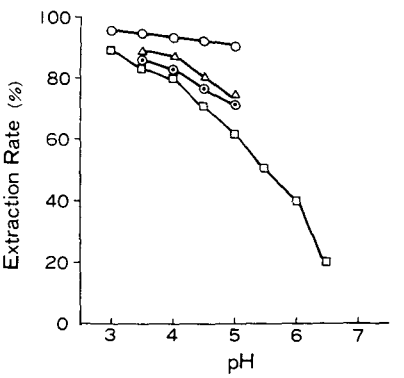


Fig. 1. Effects of pH on extraction rate of fluorescamine derivatives with ethyl acetate. Fluorescamine derivatives of various amines were dissolved in 3 ml of 4 *M* acetate buffer at different pH values and were extracted with 3 ml of ethyl acetate twice. The fluorescence of the acetate buffer phase was measured at 480 nm with excitation at 390 nm as described under Experimental. ○, Phenethylamine; △, phenylethanolamine; ◎, tyramine; □, octopamine.

Determination of trace amines by thin-layer chromatography

Fig. 2 shows the thin-layer chromatogram of fluorescamine derivatives of trace amines on a silica gel plate when developed by ethyl acetate—hexane—methanol—water (60:20:25:10) as the first solvent and benzene—dioxane—acetic acid (90:25:5) as the second. The four major trace amines — phenethylamine, phenylethanolamine, octopamine and tyramine — were separated completely under these conditions. Fluorescent spots were scanned with a Shimadzu chromatoscanner for quantitation. Fig. 3 shows scans of spots corresponding to the four major trace amines from the brains of normal (A) and PCS (B) rats. The minimum amounts detectable by this procedure were 10 pmoles of phenylethylamine, phenylethanolamine and tyramine, and 20 pmoles of octopamine. Fig. 4 shows a standard curve of these trace amines, indicating a linear relationship between fluorescence intensities and the amounts of trace amines over the range from 10—20 to 300 pmoles.

Recovery and reproducibility of the assay

To estimate the recovery and the reproducibility of the present assay meth-

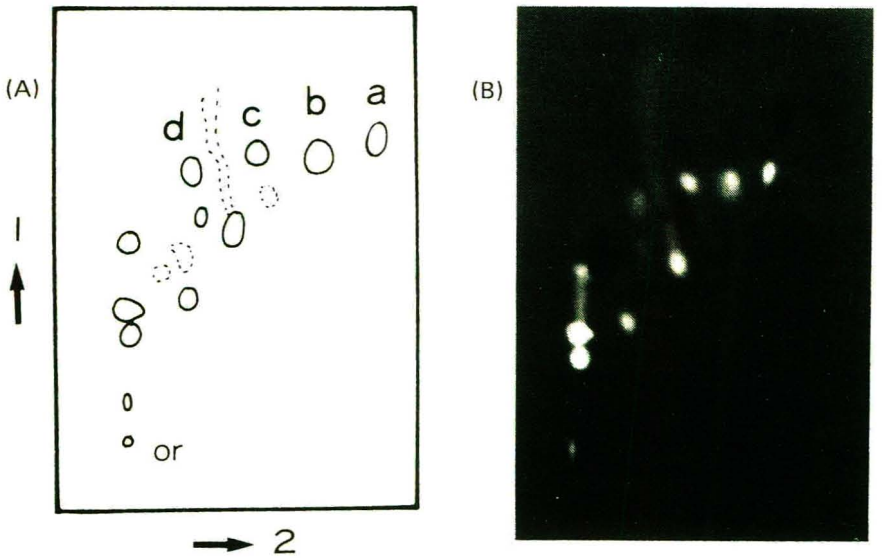


Fig. 2. Two-dimensional thin-layer chromatography of the fluorescamine derivatives of trace amines on silica gel plate (B) and its tracing (A). The sample was applied at the point "or" and developed in two dimensions. Solvent system 1 = ethyl acetate—hexane—methanol—water (60:20:25:10), and solvent system 2 = benzene—dioxane—acetic acid (90:25:5). Spots were located under an ultraviolet lamp. The spots a, b, c, and d show the locations of the fluorescamine derivatives of phenethylamine, phenylethanolamine, tyramine and octopamine, respectively.

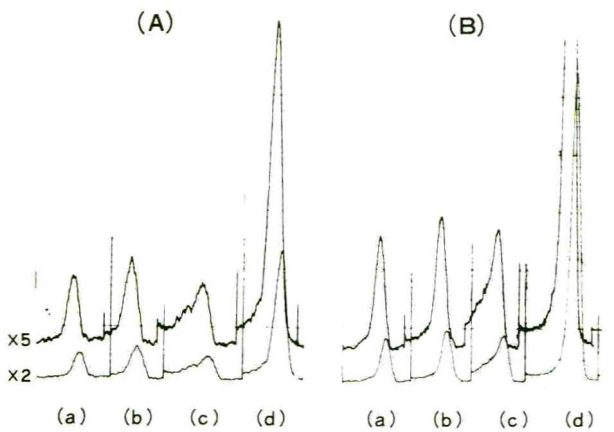


Fig. 3. Typical scanning curves of spots corresponding to phenethylamine (a), phenylethanolamine (b), tyramine (c), and octopamine (d) with a Shimadzu chromatoscanner, Model CS-900 (excitation 365 nm, filter 500 nm). (A) normal rat brain; (B) PCS rat brain.

od, ^{14}C-labelled phenethylamine and tyramine were processed through the same procedure and the corresponding spots on the thin-layer plate were cut out and counted in a liquid scintillation counter as described under Experimental. As shown in Table I, the recoveries of phenylethylamine and tyramine through the whole procedure were 30 ± 5 and 48 ± 18%, respectively, and the

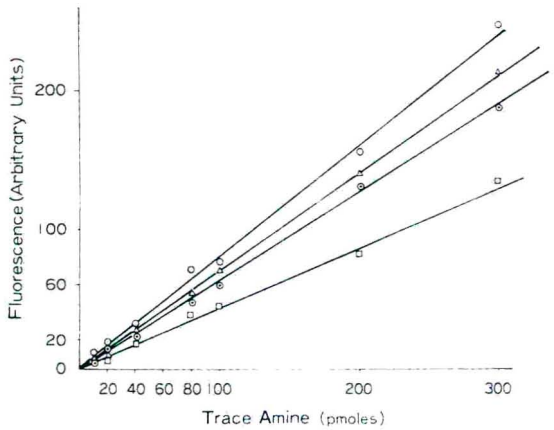


Fig. 4. Standard curves of fluorescamine derivatives of phenetylamine (○), phenylethanolamine (△), tyramine (⊙) and octopamine (□).

TABLE I

RECOVERY AND REPRODUCIBILITY OF THE ASSAY OF ^{14}C-LABELLED PHENYLETHYLAMINE AND TYRAMINE

	Phenylethylamine	Tyramine
Initial radioactivity (dpm)	9508	16,593
Radioactivity after thin-layer separation (dpm)	722 ± 124	1998 ± 783
Recovery* (%)	30 ± 5	48 ± 18
Coefficient of variation (%)	17	39

*The recovery was calculated by multiplying by 4 the counts of spots on the thin-layer plate because the fluorophores were extracted with 20 μl of the ethyl acetate and 5 μl were spotted.

coefficients of variation were 17 and 39% for phenylethylamine and tyramine, respectively.

Trace amine levels of whole brains of normal and PCS rats

The levels of trace amines in whole brains of normal and PCS rats are summarized in Table II. The levels were higher in PCS rats than in normal rats; the levels of phenethylamine, phenylethanolamine, tyramine and octopamine increased by 1.5, 3.0, 2.5 and 4.0 times, respectively, after construction of a portacaval shunt.

DISCUSSION

5-Dimethylaminonaphthalene-1-sulphonyl (Dns) derivatives have been used for determination of trace amines [16, 20—22], but they are not suitable for simultaneous fluorometric analyses of these amines because all the trace amines except phenethylamine form several fluorogenic Dns derivatives [27]. Fluorescamine has been introduced as a highly sensitive fluorogenic reagent for primary amino groups [28—30], but it has been used less extensively than Dns

TABLE II

THE LEVELS OF TRACE AMINES IN WHOLE BRAINS OF SHAM-OPERATED AND PCS RATS

Values are means ± S.D. in pmoles per g wet weight. Numbers of animals are shown in parentheses.

	Normal (5)	PCS (7)
Phenethylamine	7.5 ± 0.4	11.5 ± 2.1*
Phenylethanolamine	7.2 ± 0.4	21.9 ± 1.9**
Tyramine	7.3 ± 1.5	18.5 ± 2.8**
Octopamine	20.6 ± 4.8	80.0 ± 20.3*

*$P<0.05$, **$P<0.01$: significant difference from the value for normal rats.

reagent for determination of primary amines in biological specimens, partly because it is difficult to obtain derivatives of primary amines in biological specimens on a microscale [31]. We resolved this problem by extracting the fluorescamine derivatives with ethyl acetate. Another problem is that fluorescamine derivatives are unstable in acidic solution [32]. Moreover, use of a high temperature in the evaporation of ethyl acetate and complete drying of the fluorescamine derivatives decreased the fluorescence. To avoid destruction of the fluorescamine derivatives, we used 4 *M* acetate buffer, pH 4.5, for extraction. These conditions may be responsible for the low sensitivity of the method for octopamine.

Since the procedure is so long and complex, the recovery and reproducibility was estimated using ^{14}C-labelled phenylethylamine and tyramine: their recoveries were 30 and 48%, respectively. These values are fairly good when the long procedure is considered. The coefficients of variation were 17 and 39% for phenylethylamine and tyramine, respectively, indicating that the reproducibility is not so good but that rough estimation is possible.

There have been many reports on the content of trace amines in biological specimens determined by fluorometric methods [33—35]. However, most of them seem to be unreliable, because the reported levels of amines were considerably higher than those measured by more specific methods. The endogenous levels of trace amines in whole rat brains reported here (Table II) are in good accordance with those [16—25] determined by more sophisticated means, such as radioenzymatic, thin-layer chromatographic—mass spectrometric, and gas chromatographic—mass spectrometric methods. Although an attempt to measure simultaneously trace amines by gas chromatography—mass spectrometry was made by Karoum et al. [36], only phenethylamine, and *m*- and *p*-tyramine were detected in measurable quantities in rat brain. The method reported here is suitable for use in most laboratories as a routine method.

A considerable increase in brain octopamine content has been reported in PCS rats, which have been extensively used as a model of chronic hepatic encephalopathy [37]. Our data (Table II) show that the brain levels of phenethylamine, phenylethanolamine and tyramine as well as octopamine are significantly increased in PCS rats. Although our method cannot distinguish between

m- and *p*-tyramine [21, 22] and *m*- and *p*-octopamine [20], it enabled us to perform simultaneous analyses of phenethylamine, phenylethanolamine, tyramine and octopamine as well as of amino acids, dopamine, noradrenaline and serotonin in whole rat brain. The changes in these parameters after construction of a portacaval shunt have been reported [13, 38].

REFERENCES

1 A.A. Boulton, in E. Costa, E. Giacobini and R. Paoletti (Editors), Advance in Biochemical Pharmacology, Vol. 15, Raven Press, New York, 1976, p. 57.
2 J.M. Saavedra and J. Axelrod, in E. Costa, E. Giacobini and R. Paoletti (Editors), Advances in Biochemical Pharmacology, Vol. 15, Raven Press, New York, 1976, p. 95.
3 R.J. Baldessarini and J.E. Fisher, Biochem. Pharmacol., 27 (1978) 621.
4 M. Sandler, M.B.H. Youdim and E. Hanington, Nature (London), 250 (1974) 335.
5 A.A. Boulton and G.L. Marjerrison, Nature (London), 236 (1972) 76.
6 M. Sandler and G.P. Reynolds, Lancet, i (1976) 70.
7 J.A. Oates, P.Z. Nirenberg, J.B. Jepson, A. Sjoerdsma and S. Udenfriend, Proc. Soc. Exp. Biol. Med., 112 (1963) 1078.
8 M. Sandler, S.B. Carter, B.L. Goodwin and C.R.J. Ruthven, in E. Usdin and M. Sandler (Editors), Trace Amines and the Brain, Marcel Dekker, New York, Basel, 1976, p. 233.
9 J.E. Fischer and R.J. Baldessarini, Lancet, ii (1971) 75.
10 J.E. Fischer and J.H. James, Amer. J. Surg., 123 (1972) 222.
11 R.J. Baldessarini and J.E. Fischer, Nature (London), 245 (1973) 25.
12 J.M. Dodsworth, J.H. James, M.G. Cummings and J.E. Fischer, Surgery, 75 (1974) 811.
13 S. Kamata, A. Okada, T. Watanabe, Y. Kawashima and H. Wada, J. Neurochem., 35 (1980) 1190.
14 J. Axelrod and J.M. Saavedra, in G.E.W. Wolstenholme and D.W. Fitzsimons (Editors), Aromatic Amino Acids in the Brain, Elsevier, Amsterdam, London, New York, 1974, p. 51
15 A.A. Boulton, in E. Usdin and M. Sandler (Editors), Trace Amines and the Brain, Marcel Dekker, New York, Basel, 1976, p. 21.
16 A.A. Boulton, S.R. Philips and D.A. Durden, J. Chromatogr., 82 (1973) 137.
17 J.M. Saavedra, J. Neurochem, 22 (1974) 211.
18 J. Willner, H.F. LeFevre and E. Costa, J. Neurochem., 23 (1974) 857.
19 J.M. Saavedra and J. Axelrod, Proc. Nat. Acad. Sci. U.S., 70 (1973) 769.
20 T.J. Danielson, A.A. Boulton and H.A. Robertson, J. Neurochem., 29 (1977) 1131.
21 S.R. Philips, D.A. Durden and A.A. Boulton, Can. J. Biochem., 52 (1974) 366.
22 S.R. Philips, B.A. Davis, D.A. Durden and A.A. Boulton, Can. J. Biochem., 53 (1975) 65.
23 J.M. Saavedra, Anal. Biochem., 59 (1974) 628.
24 A.J. Harmar and A.S. Horn, J. Neurochem., 26 (1976) 987.
25 P. Molinoff and J. Axelrod, Science, 164 (1969) 428.
26 S. Ogasahara, T. Mandai, A. Yamatodani, T. Watanabe, H. Wada and T. Seki, J. Chromatogr., 180 (1979) 119.
27 N. Seiler, in D. Glick (Editor), Methods of Biochemical Analysis, Vol. 18, Interscience, New York, London, Sydney, Toronto, 1970, p. 259.
28 M. Weigela, S.L. DeBernardo, J.P. Tengi and W. Leimgruber, J. Amer. Chem. Soc., 94 (1972) 5927.
29 S. Udenfriend, S. Stein, P. Böhlen, W. Dairman, W. Leimgruber and M. Weigele, Science, 178 (1972) 871.
30 K. Imai, P. Böhlen, S. Stein and S. Udenfriend, Arch. Biochem. Biophys., 161 (1974) 161.
31 H. Nakamura and J.J. Pisano, J. Chromatogr., 121 (1976) 33.
32 S.D. Bernardo, M. Weigele, V. Toome, K. Manhard and W. Leimgruber, Arch. Biochem. Biophys., 163 (1974) 390.
33 E. Fuscher, B. Heller, H. Spatz and H. Reggiani, Arzneim.-Forsch., 22 (1972) 1560.

34 S. Spector, K. Melmon, W. Lovenberg and A. Sjoerdsma, J. Pharmacol. Exp. Ther., 140 (1963) 229.
35 O. Suzuki and K. Yagi, Anal. Biochem., 75 (1976) 192.
36 F. Karoum, H. Nasrallah, S. Potkin, L. Chuang, J. Moyer-Schwing, I. Phillips and R.J. Wyatt, J. Neurochem., 33 (1979) 201.
37 J.H. James, J.M. Hodgman, J.M. Funovics and J.E. Fischer, J. Neurochem., 27 (1976) 223.
38 S. Kamata, A. Okada, K. Imura, Y. Kawashima, T. Watanabe and H. Wada, in M. Walser and J.R. Williamson (Editors), Metabolism and Clinical Implications of Branched Chain Amino and Keto Acids, Elsevier/North Holland, Amsterdam, 1981, p. 493.

Journal of Chromatography, 231 (1982) 301—310
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1340

DETERMINATION OF THE ANTI-ULCER AGENT GERANYLGERANYLACETONE IN SERUM BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

MICHIRU TANAKA*, JIRO HASEGAWA and JUNZO TSUTSUMI

Kawashima Branch Laboratory, Section of Drug Metabolism and Biopharmaceutics, Eisai Co., Ltd., Gifu (Japan)

and

TAKESHI FUJITA

Section of Drug Metabolism and Biopharmaceutics, Eisai Co., Ltd., Tokyo (Japan)

(First received January 29th, 1982; revised manuscript received April 6th, 1982)

SUMMARY

A highly specific and sensitive method for the determination of the anti-ulcer drug geranylgeranylacetone (GGA) in human serum is described. The extract from serum with hexane was saponified with potassium hydroxide and subjected to silica gel column chromatography to remove interfering substances. GGA in the partially purified extract was then reacted with O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine and measured by selected ion monitoring using gas chromatography—mass spectrometry. A low detection limit (1 ng/ml) and high precision were obtained.

INTRODUCTION

Geranylgeranylacetone (6,10,14,18-tetramethyl-5,9,13,17-nonadecatetraene-2-one) (GGA, Fig. 1), is a newly synthesized polyisoprenoid compound that has been shown to be effective on several experimentally induced ulcers in rats [1]. The evaluation of the clinical usefulness of GGA on gastric ulcers is now in progress.

In order to carry out pharmacokinetic studies, a specific and sensitive method for the determination of GGA in biological samples was required. In this work, gas chromatography—mass spectrometry (GC—MS) has been studied for the separation and determination of GGA in biological fluids.

0378-4347/82/0000—0000/$02.75

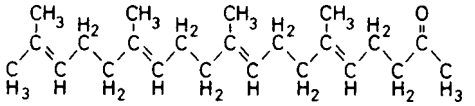


Fig. 1. Structure of all-*trans*-GGA.

The mass spectrum of GGA did not give fragment ions suitable for determination by GC—MS, while an oxime derivative of GGA [GGA-O-(2,3,4,5,6-pentafluorobenzyl)oxime] was found to give a fragment ion with the intensity and *m/z* suitable for the assay.

O-(2,3,4,5,6-Pentafluorobenzyl)hydroxylamine hydrochloride (PFBOA) has been developed as a novel derivatizing reagent for carbonyl compounds for electron-capture GC. Kobayashi et al. [2] reported the usefulness of PFBOA for the GC of low-molecular-weight carbonyl compounds. However, it was found that the resulting oxime of GGA (GGA-O-PFBO) shows four peaks on the gas chromatogram as GGA formed *syn*- and *anti*-oximes of *cis*- and *trans*-GGA.

This paper describes a highly specific and sensitive method using GC—MS which can overcome this disadvantage and is sensitive enough for the measurement of GGA in human serum.

EXPERIMENTAL

Reagents and chemicals

The purity of GGA used in this study was 98.7%, as checked by GC, and was a mixture of *cis*-5-*trans*-9,13,17- (35.3%) and *trans*-5,9,13,17- (64.7%) isomers. Pure *cis*- and *trans*-forms were also used, the purities of which were 95.0% and 98.3%, respectively.

PFBOA was synthesized from pentafluorobenzyl bromide (Aldrich, Milwaukee, WI, U.S.A.) and N-hydroxyphthalimide (Tokyo Kasei, Tokyo, Japan) according to the procedure of Nambara et al. [3] and Youngdale [4]. White leaflets (m.p. 115°C) were obtained. Elemental analysis: calculated for $C_7H_4NOF_5 \cdot HCl$, C 33.69, H 2.02, N 5.61; found, C 33.69, H 1.97, N 5.52%.

A stock PFBOA solution was prepared by dissolving PFBOA in distilled pyridine (25 mg/ml), and was stable for several weeks when stored in the dark in a refrigerator.

Cholestane used as internal standard, was obtained from Applied Science Labs. State College, PA, U.S.A.). Silica gel for column chromatography (70—230 mesh) was purchased from Merck (Darmstadt, G.F.R.). Hexane, ethyl acetate and pyridine were of analytical-reagent grade and distilled prior to use. All other reagents were of analytical-reagent grade and were obtained from commercial sources.

Standards and calibration samples

A stock standard GGA solution was prepared by dissolving 1 mg in 1 ml of ethanol containing 0.2% of α-tocopherol as an antioxidant. Dilute working standard solutions were prepared as follows: the GGA stock solution was diluted with ethanol to give ten batches covering the concentration range

5 ng/ml—5 μg/ml. Calibration samples were prepared as follows: to 1 ml of serum, 0.2 ml of ethanol solution from each batch was added and the mixture was allowed to stand for 30 min.

Glassware

The glassware was cleaned using normal laboratory washers and rinsed with acetone before use.

Extraction and partial purification of GGA from serum

To each 1 ml of serum, 1 ml of ethanol was added and mixed on a vortex mixer. After addition of 0.1 ml of 50% potassium hydroxide solution, the mixture was saponified for 10 min in a boiling water-bath. After cooling, the mixture was shaken with 5 ml of hexane for 10 min and centrifuged for 5 min at 1900 *g*. This extraction procedure was repeated twice and the combined organic layer was submitted to silica gel column chromatography. The silica gel, which had been allowed to swell for at least 2 h in hexane containing 5% of ethyl acetate, was packed in a glass column (7 cm × 1 cm I.D.) with continuous tapping, and washed with 6 ml of hexane. The hexane extract from serum was applied on the top of the column and adsorbed at a flow-rate of about 1 ml/min. After the column had been washed with 20 ml of hexane containing 1% of ethyl acetate, the eluent was changed to hexane containing 10% of ethyl acetate. The first 3 ml of the effluent was discarded and the next 6 ml of the fraction containing GGA was collected and evaporated to dryness under a gentle stream of nitrogen at 50°C and subjected to derivatization.

Derivatization and removal of excess of PFBOA

The residue was dissolved in 0.2 ml of PFBOA solution (25 mg/ml), heated at 60°C for 2 h and 2.5 ml of 1 *N* hydrochloric acid were added. The mixture was shaken mechanically with 5 ml of hexane for 10 min and centrifuged for 5 min at 1900 *g*. The organic layer was separated and 1 ml of an internal standard solution of cholestane (2 μg/ml) was added. After evaporation to dryness under a gentle stream of nitrogen at 50°C, the residue was dissolved in 0.1 ml of ethyl acetate and a 2—4-μl portion was injected into the GC—MS system.

Apparatus

Analyses were carried out on a Shimadzu-LKB 9000 GC—MS instrument with an electron impact source and a total ion current detector. A coiled Pyrex column (1 m × 3 mm I.D.) filled with 3% OV-17 on Gas-Chrom Q (100—120 mesh) was used. The column was operated isothermally at 270°C, the temperatures of the injection port, separator and ion source being kept at 300, 300 and 330°C, respectively. Helium was used as carrier gas at a flow-rate of 30 ml/min. The ionization energy was 25 eV and the trap current was 60 μA.

Calibration graph

The calibration graph was prepared by subjecting human serum calibration samples to the above procedures. The ratio of the peak height of GGA-O-

PFBO at *m/z* 320 to that of cholestane at *m/z* 357 was plotted against GGA concentration.

Serum samples

Serum samples were taken in syringes by venipuncture over a 48-h period after a single oral dose of 150 mg of GGA (as a capsule) to three male healthy volunteers 30 min after breakfast. The serum was obtained by letting the blood clot, followed by centrifugation, and was stored frozen until taken for analysis.

RESULTS AND DISCUSSION

Extraction and partial purification of GGA

As the hexane extract from serum was found to contain endogenous lipids that interfered in the GC—MS analysis, silica gel column chromatography was applied to remove these substances. Each fraction eluted from the column was examined by a thin-layer chromatographic system with flame ionization detection (TLC-FID) (Iatroscan TFG-10, Iatron Labs.), where the silica gel-coated glass rods (Chromarods) were developed in hexane—diethyl ether—formic acid (95:5:4) (Fig. 2). As shown in the TLC-FID trace (Fig. 2), lipids such as cholesterol (Ch), cholesterol esters (ChE) such as cholesterol palmitate and stearate, fatty acids (FA) such as oleic, stearic and palmitic acid and monoglycerides (MG) such as monopalmitate and monoolein in serum were removed by silica gel column chromatography, while triglycerides (TG) such as tristearin and triolein moved together with GGA on the column, and were removed by saponification of serum prior to hexane extraction. Both *cis*- and *trans*-isomers of GGA in serum were demonstrated to be stable during the saponification process.

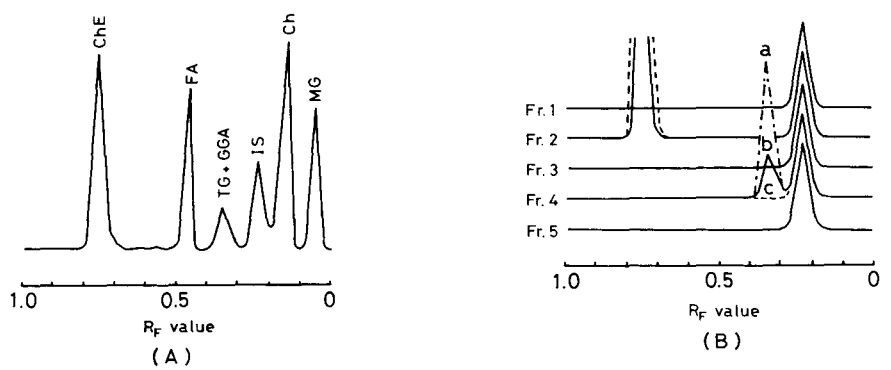


Fig. 2. TLC-FID traces of (A) authentic samples and (B) fractions from silica gel chromatography of hexane extracts from (a) serum spiked with GGA after saponification, (b) serum without saponification and (c) serum after saponification. ChE = cholesterol palmitate or stearate; FA = oleic, stearic or palmitic acid; TG = tristearin or triolein; IS = stearyl alcohol as internal standard; Ch = cholesterol; MG = monopalmitate or monoolein.

Derivatization of GGA to GGA-O-PFBO

GGA gave a complex spectrum under EI ionization (Fig. 3), in which frag-

ment ions with a mass higher than *m/z* 150 were not abundant and the molecular ion was not observed. The CI mass spectrum obtained on a Shimadzu-LKB 9000B GC—MS system using methane, isobutane and ammonia as reagent gas also showed that there was no prominent ion with sufficient intensity for GC—MS analysis.

Investigation of several kinds of derivatives of GGA revealed that GGA-O-PFBO was the most suitable, based on stability, volatility and the fragmentation pattern in the EI mass spectrum.

The influence of the concentration of PFBOA on the reaction in pyridine at 60°C is illustrated in Fig. 4, where it can be seen that the quantitative

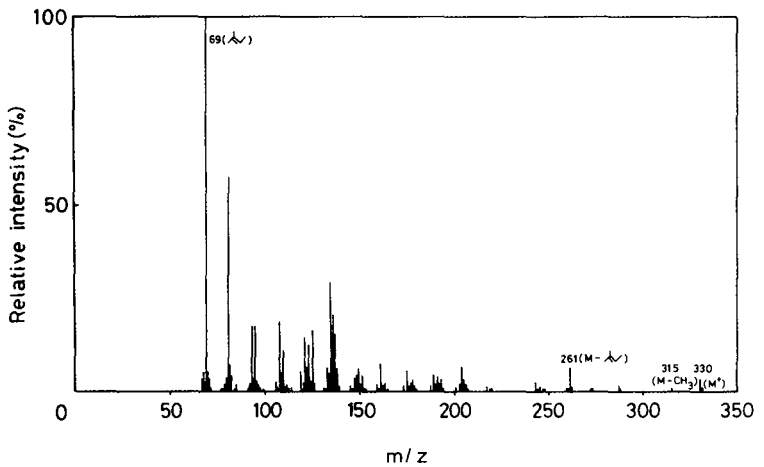


Fig. 3. Electron impact mass spectrum of GGA.

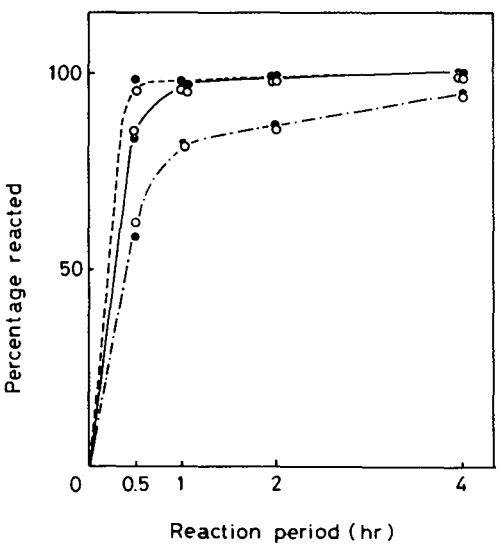


Fig. 4. Effect of the concentration of PFBOA on the formation of *cis*- (○) and *trans*-isomers (●) of GGA-O-PFBO at 60°C. Concentrations: (—○—) 2, (——) 5 and (— — —) 10 mg per 0.2 ml.

formation of GGA-O-PFBO was achieved in 2 h when 5 mg of PFBOA were used.

GC—MS measurement

When GGA-O-PFBO was subjected to GC—MS, four peaks were observed on total ion monitoring, as shown in Fig. 5. Derivatization of many compounds with carbonyl groups to the corresponding oximes has been reported to form *syn-* and *anti-*isomers and to give two peaks on the gas chromatogram [5]. As GGA itself is a mixture of *cis-* and *trans-*isomers, the four peaks of GGA-O-PFBO might be due to the *syn-* and *anti-*isomers of each *cis-* and *trans-*isomer.

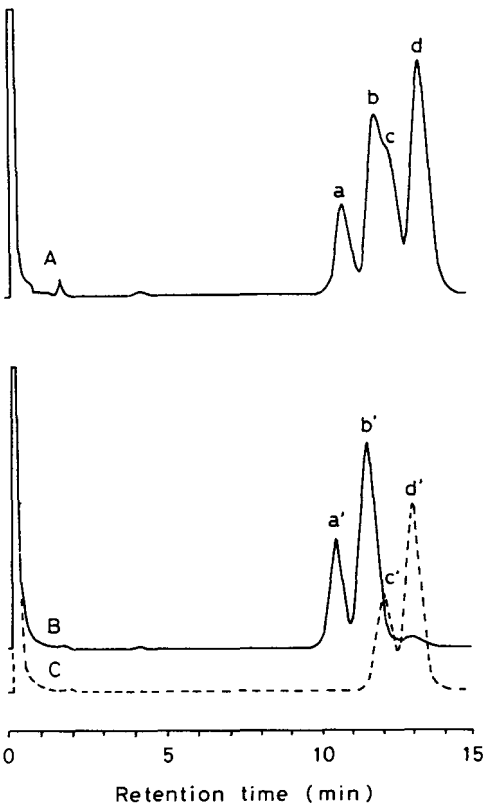


Fig. 5. Total ion current chromatograms of (A) GGA, (B) pure *cis-* and (C) pure *trans-*isomer of GGA after derivatization.

When pure *cis-* and *trans-*isomers of GGA were separately reacted with PFBOA and subjected to GC—MS, the pure *cis-*isomer gave two peaks (a′ and b′) and the pure *trans-*isomer gave two peaks (c′ and d′) (Fig. 5). The retention times and mass spectra of a′, b′, c′ and d′ were idential with those of a, b, c and d, respectively. Peaks a and b were tentatively identified as the *syn-* and *anti-*isomers of *cis-*GGA-O-PFBO, respectively, and peaks c and d as the *syn-* and *anti-*isomers of *trans-*GGA-O-PFBO, respectively [6].

The appearance of multiple peaks on the gas chromatogram is troublesome for GC analysis. However, as shown in Fig. 6, the peak intensity of the fragment ion of *m/z* 320, used as the monitoring ion for measurement of GGA, was very different between *syn*- and *anti*-forms of the *cis*-isomer.

Both spectra showed the molecular ion at *m/z* 525 and the base peak at *m/z* 69, which was the characteristic fragment ion of terpenoids [7]. However, in the *anti*-isomer of *cis*-GGA, an ion of *m/z* 320 was observed as an intense fragment ion, the intensity of which was about eight times that of the *syn*-isomer. Similar mass spectra were also obtained with *trans*-GGA-O-PFBO. This fragment ion was assigned to the structure shown in Fig. 6 with the aid of exact measurement; elemental compsition, $C_{15}H_{15}NOF_5$; accurate mass, 320.107; found, 320.105.

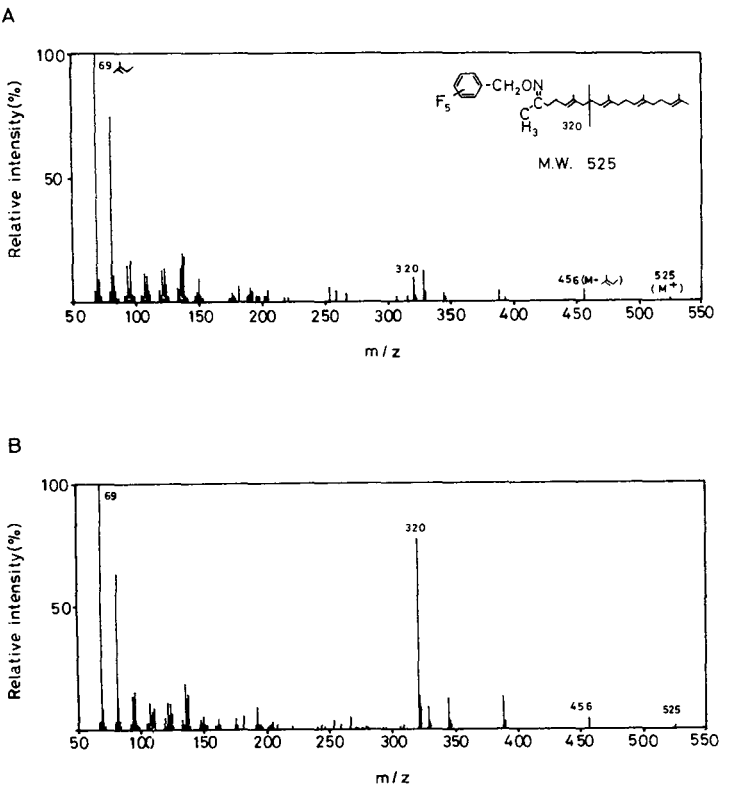


Fig. 6. Electron-impact mass spectra of (A) *syn*- and (B) *anti*-isomer of *cis*-GGA-O-PFBO.

Fig. 7 shows the selected ion monitoring of pure *cis*- and *trans*-GGA after derivatization. The peak height of the *syn*-isomer is 5% of that of *anti*-isomer, as expected from the intensity of the ion at *m/z* 320 in the mass spectrum of each isomer.

The ratio of the amount of *syn*-isomer to that of the *anti*-isomer formed by derivatization was almost constant (Table I).

Consequently, the difficulty with the determination of the concentrations of *cis*- and *trans*-GGA-O-PFBO caused by multiple peaks on the gas chromato-

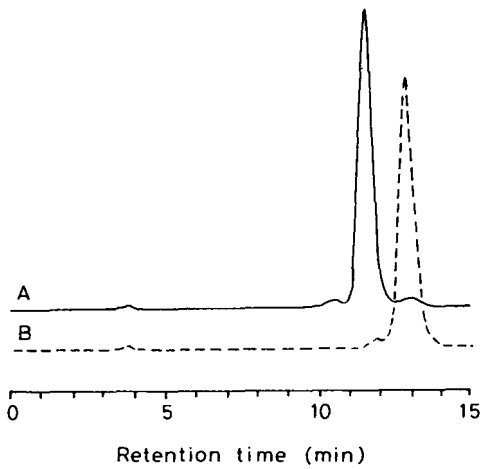


Fig. 7. Mass fragmentograms (*m/z* 320) of (A) *cis*- and (B) *trans*-isomers of GGA-O-PFBO.

TABLE I

RATIO OF AMOUNTS OF *SYN*- AND *ANTI*-ISOMERS FORMED BY DERIVATIZATION OF GGA

Figures represent mean ± S.D. from three measurements by GC with a flame ionization detector.

Isomer of GGA	Amount of GGA reacted (μg)	*Syn*-isomer	*Anti*-isomer
Pure *cis*-	1	32.1±2.3	67.9±2.2
	5	33.1±3.0	66.9±3.0
	10	32.2±2.5	67.8±2.4
Pure *trans*-	1	33.3±2.4	66.7±2.3
	5	32.5±2.8	67.5±2.8
	10	32.6±2.9	67.4±2.8

TABLE II

ACCURACY AND PRECISION OF THE DETERMINATION OF GGA IN SERUM

Isomer	Parameter	Amount of GGA added to serum (ng/ml)				
		1	5	25	100	1000
Cis-	Content (ng/ml)	0.353	1.77	8.83	35.3	353
	Amount found (n=3)	0.341	1.73	8.52	34.8	345
	Precision (C.V., %)	4.7	3.6	2.8	2.4	2.8
Trans-	Content (ng/ml)	0.647	3.24	16.2	64.7	647
	Amount found (n=3)	0.640	3.20	15.9	63.9	638
	Precision (C.V., %)	4.9	3.8	2.6	3.1	2.9

grams could be overcome by focusing on the fragment ion at *m/z* 320 of the *anti*-isomer.

Accuracy and specificity

The selected ion monitoring chromatograms of a control serum sample and the same sample spiked with 100 ng of GGA are shown in Fig. 8. Each serum sample contained 2 μg of cholestane as an internal standard.

The calibration graph was linear in the range 1—1000 ng/ml. The coefficient of variation was less than 5% in the concentration range tested (Table II).

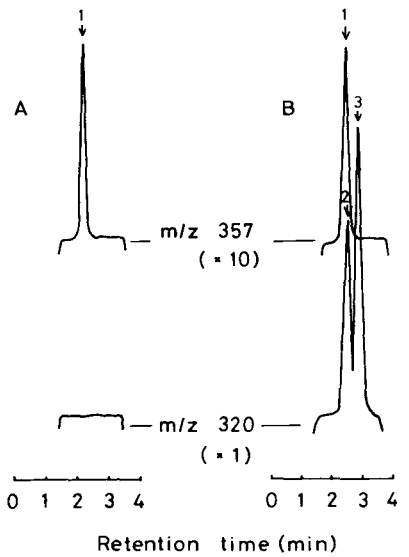


Fig. 8. Examples of chromatograms: (A) extract of blank serum to which 2 μg of cholestane alone had been added; (B) extract of serum to which 100 ng of GGA and 2 μg of cholestane had been added. Peaks 1, 2 and 3 are cholestane, *cis*-GGA and *trans*-GGA, respectively.

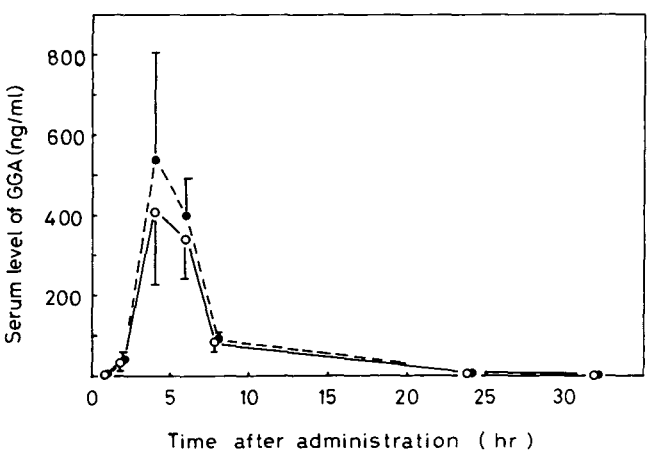


Fig. 9. Average serum levels of *cis*- (○) and *trans*-isomers (●) of GGA following an oral dose of 150 mg to three volunteers. Each bar represents the standard error of means.

Application of the method

The time course of serum GGA levels was examined after an oral dose of 150 mg to three volunteers (Fig. 9). The peak average serum level was observed after about 4 h and could be detected until 32 h after administration, indicating that this method would be appropriate for pharmacokinetic studies in humans.

REFERENCES

1 M. Murakami, K. Oketani, H. Fujizaki, T. Wakabayashi and T. Ohgo, Arzneim.-Forsch., 31 (1981) 799—804.
2 K. Kobayashi, M. Tanaka and S. Kawai, J. Chromatogr., 187 (1980) 413—417.
3 T. Nambara, K. Kigasawa, T. Iwata and M. Ibuki, J. Chromatogr., 114 (1975) 81—86.
4 G.A. Youngdale, J. Pharm. Sci., 65 (1976) 625—626.
5 K. Green, Chem. Phys. Lipids, 3 (1969) 254—259.
6 K.T. Koshy, D.G. Kaiser and A.L. VanDerSlik, J. Chromatogr. Sci., 13 (1975) 97—104.
7 G.R. Waller, Biochemical Applications of Mass Spectrometry, Wiley, New York, 1972, pp. 351—385.

Journal of Chromatography, 231 (1982) 311–319
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1321

ANALYSIS OF METHADONE AND METABOLITES IN BIOLOGICAL FLUIDS WITH GAS CHROMATOGRAPHY—MASS SPECTROMETRY

GUN IL KANG and FRANK S. ABBOTT*

Faculty of Pharmaceutical Sciences, The University of British Columbia, Vancouver, B.C. V6T 1W5 (Canada)

(First received July 14th, 1981; revised manuscript received April 30th, 1982)

SUMMARY

The analysis of methadone and its metabolites in biological fluids by gas chromatography—mass spectrometry is described with deuterated methadone and metabolites as internal standards. The method allowed the determination of 20 ng methadone in 0.5 ml of plasma or saliva. Mean saliva to plasma ratio of methadone for two patients was determined to be 0.51 ± 0.13. Methadone and 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) in urine were measured by selected ion monitoring. Gas chromatography—mass spectrometry was found to have advantages over conventional gas chromatographic methods in terms of ratio analysis. 1,5-Dimethyl-3,3-diphenyl-2-pyrrolidone previously reported as a metabolite was shown to result primarily from the decomposition of EDDP free base.

INTRODUCTION

In order to study methadone metabolism, many analytical methods have been developed to quantify methadone and its metabolites. Gas chromatography (GC), which is the method of choice for the analysis of human samples, has achieved the analysis of 5—15 ng/ml of methadone and the major metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) [1—4]. 2-Ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EMDP), a minor metabolite, could not be quantified in human urine because of a lack of sensitivity and selectivity of GC [5]. A selected ion monitoring (SIM) assay by gas chromatography—mass spectrometry (GC—MS) under electron impact (EI) conditions was described for the quantitation of methadone by monitoring *m/e* 294 (methadone), *m/e* 297 ([$^2H_3$]methadone), and *m/e* 308 for the internal standard, 2-dimethylamino-4,4-diphenyl-5-octanone [6]. By this method, methadone in plasma was assayed with a sensitivity limit of 5 ng/ml. [$^2H_5$]Methadone has been synthesized and utilized for the SIM assay of methadone in human

0378-4347/82/0000—0000/$02.75

plasma using chemical ionization (CI) [7]. The sensitivity limit of this method was similar to that reported using the EI—SIM method. The CI—GC—MS method has also been applied to the enantiomeric assay of [$^2H_3$]methadone and [$^2H_5$]methadone using the $^2H_8$-analogue as the internal standard [8]. Although SIM analysis is adequate to accurately quantitate 5 ng/ml levels of methadone in human plasma, the low abundance of the ions monitored under EI conditions has hindered the development of a more sensitive method. The relatively low sensitivity achieved by using [$^2H_5$]methadone even under CI conditions may be a factor of instrument conditions.

We have previously described the synthesis of deuterated methadone and metabolites [9]. These compounds have been used for the detection of metabolites and for studies of methadone interaction with other drugs. In this report we describe the applicability of the synthesized compounds to the SIM analysis of methadone and its metabolites in biological samples. Our emphasis was placed on the use of a high abundance ion for the analysis of methadone and to the use of ratio analysis.

EXPERIMENTAL

Materials

2-Dimethylamino-4,4-diphenyl-5-nonanone was prepared by the method of Lynn et al. [3]. The product was not distilled but was crystallized from diethyl ether solution as the perchlorate salt. Recrystallization from diethyl ether—ethanol gave crystals, m.p. 137—139°C. Methadone·HCl, [$^2H_3$]methadone·HCl, [$^2H_{10}$]methadone·HCl, EDDP perchlorate, [$^2H_3$]EDDP perchlorate, [$^2H_{10}$]EDDP perchlorate, EMDP·HCl, [$^2H_{10}$]EMDP·HCl, 1,5-dimethyl-3,3-diphenyl-2-pyrrolidone (DDP) and [$^2H_{10}$]DDP were obtained as described [9]. The purity and melting point of EDDP perchlorate were determined by differential scanning calorimetry; m.p. 177°C, recrystallized from diethyl ether—ethanol (literature value [10] m.p. 167—168°C).

Samples

Plasma, saliva, and urine samples were obtained from a pharmacokinetic study of four female methadone maintenance patients which was conducted by the Alcohol and Drug Commission, Vancouver, Canada. Maintenance dosage levels were: patient A, 30 mg/day; and patient B, 90 mg/day. Plasma and saliva samples were taken at 0, 2, 4, 6, 8, 11, 12 and 24 h after the usual daily dose. Urine samples were obtained at 1, 3, 5, 7, 9, 13 and 24 h. A special protocol was used to obtain saliva samples. After the oral dose of methadone was taken, the patients were instructed to rinse the mouth with 250 ml of water. Patients were not allowed to eat or drink just prior to providing a saliva sample. The mouth was rinsed thoroughly with water before the sample was taken. All samples were stored frozen until analysed.

Extraction procedures and standard curve preparation

Plasma and saliva. Saliva was centrifuged in order to remove solids. To plasma or saliva samples (0.5 ml) was added 0.2 ml of internal standard (I.S.), 2-dimethylamino-4,4-diphenyl-5-nonanone perchlorate (a stock solution of I.S.

was prepared to contain 10 mg/ml in methanol which was diluted with water to make a solution equivalent to 200 ng in 0.2 ml water). The solution was diluted to 3 ml with water and 0.1 ml of 1 *N* sodium hydroxide was added. After adding methylene chloride (15 ml) the solution was vortex mixed for 3 min. The aqueous layer was aspirated off and the methylene chloride layer was dried over anhydrous sodium sulfate. The dried methylene chloride was decanted (approximately 13 ml) and evaporated under nitrogen. The residue was dissolved in 50—100 μl of methanol and a 2—5 μl aliquot was injected onto the GC—MS system. Standard curves were prepared by spiking control samples of plasma and saliva (0.5 ml) with methadone in the amounts of 0, 20, 40, 100, 200 and 500 ng. The peak area ratios of methadone/internal standard obtained by monitoring *m/e* 72 were plotted vs. the concentration of methadone.

Urines. After thawing the sample, to 1 ml of the urine was added 0.2 ml of solution containing the internal standards at concentrations of 20 μg of [$^2H_{10}$]-methadone, 10 μg of [$^2H_{10}$]EDDP, and 10 μg of [$^2H_{10}$]DDP per ml. The mixture was diluted to 5 ml with distilled water and the pH was adjusted to 7—8 with 0.1 *N* sodium hydroxide. The mixture was extracted by vortex mixing for 2 min with methylene chloride (15 ml). The methylene chloride extract (13 ml) was dried over anhydrous sodium sulfate and taken to dryness under nitrogen. The residue was taken up in methanol (0.1—0.4 ml) and a 2—5 μl aliquot was injected onto the GC—MS system. For standard calibrations varying amounts of methadone (0.5—1 μg) were added to control urines (1 ml). Blank samples containing only the deuterated internal standards were also prepared in order to subtract background interferences resulting from isotopic impurity and column bleeding. Calibration curves were prepared by plotting the peak area ratios of unlabelled/labelled compound at each of the two ions monitored vs. the known concentration ratio of unlabelled compound to its corresponding labelled internal standard. Monitoring ions were *m/e* 223/*m/e* 233 (methadone), *m/e* 277/*m/e* 280 (EDDP), *m/e* 208/*m/e* 218 (EMDP), and *m/e* 265/*m/e* 275 (DDP).

For the GC analysis of methadone and EDDP in urines, 2-dimethylamino-4,4-diphenyl-5-nonanone (10 μg in 0.2 ml water) was used as the internal standard. The extraction procedures were essentially the same as those described for the SIM analysis of urine samples.

Stability of EDDP

EDDP perchlorate stock solution (1.5 ml, 1 mg/ml in methanol) was evaporated under nitrogen. After adjusting to pH 12 with 1 *N* sodium hydroxide, diethyl ether (25 ml) was added to extract the free base. An aliquot of the ether layer (20 ml) was transferred to a 25-ml volumetric flask and made up to 25 ml with the same solvent. Samples (0.2 ml) were taken for analysis at 0, 1, 2, and 3 days after preparation of the sample which was kept on the bench at room temperature. An internal standard stock solution was prepared with methanol to contain 10 μg of [$^2H_{10}$]DDP and 20 μg of [$^2H_3$]EDDP perchlorate per ml. Sample solution (0.2 ml) was mixed with internal standard solution (0.2 ml) and the mixture was analyzed for DDP and EDDP by monitoring *m/e* 265 and *m/e* 275 for DDP and *m/e* 277 and *m/e* 280 for EDDP. The

standard curves were prepared using various concentrations of EDDP perchlorate and DDP dissolved in methanol. The concentrations of EDDP were expressed as the free base.

Gas chromatography—mass spectrometry and gas chromatography

GC—MS. GC—MS was performed using a Varian MAT 111 gas chromatograph—mass spectrometer. A modified accelerating voltage supply was controlled by a Varian 620L computer to allow ion monitoring. The mass spectrometer was operated with an electron ionization voltage of 70 eV and a source temperature of 285°C. Trap current was 30 μA. A glass column (1.6 m × 2 mm I.D.) packed with 3% OV-17 on 80—100 mesh Chromosorb W HP was used with carrier gas (helium) at 20 ml/min. Column temperature was 200°C, programmed to 270°C at 10°C/min; separator and inlet line temperatures were 290°C, and injector temperature 220°C.

Gas chromatography. The GC analysis was carried out using a Hewlett-Packard Model 5830A chromatograph equipped with a hydrogen flame detector. The glass column, 1.8 m × 2 mm I.D. was packed with 3% OV-17 on 80—100 mesh Chromosorb W HP. Injection temperature was 250°C, oven temperature 210°C, and detector temperature 300°C. The carrier gas (helium) flow-rate was 50 ml/min.

RESULTS AND DISCUSSION

Analysis of methadone in plasma and saliva

Methadone levels in plasma and saliva were frequently found to be too low to use the selective ion at *m/e* 223 for monitoring since with EI the relative abundance of this ion is only 1.6%. In order to enhance the sensitivity of the method the less selective but strong base peak at *m/e* 72 was chosen for monitoring. As shown in Fig. 1, SIM at *m/e* 72 showed high selectivity for methadone with 2-dimethylamino-4,4-diphenyl-5-nonanone as the internal standard, the base peak of which is also *m/e* 72. The lower limit of reproducible quantitation of methadone in 0.5 ml of plasma or saliva taken for extraction was 20 ng.

The sensitivity achieved by this method is comparable to the methods which are routinely used to analyze methadone in human plasma (Table I).

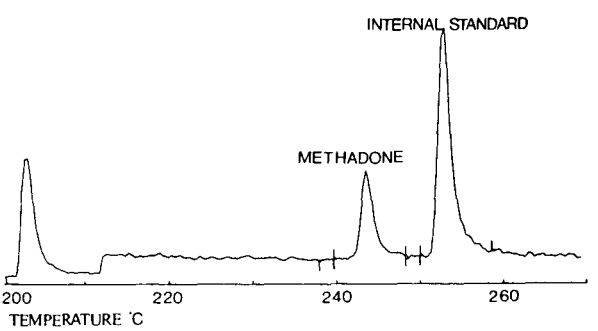


Fig. 1. SIM chromatogram (*m/e* 72) of methadone from saliva; internal standard, 2-dimethylamino-4,4-diphenyl-5-nonanone.

TABLE I

COMPARISON OF QUANTITATIVE METHODS OF METHADONE FOR HUMAN BLOOD SAMPLES

Method*	Sensitivity (sample, final dilution)	I.S.	Reference
GC—FID	15 ng/ml (4 ml plasma, 20 ml chloroform)	SKF-525A	1
GC—FID	15 ng/ml (3 ml plasma, 0.2 ml *n*-butyl chloride)	*n*-Docosane	2
GC—FID	5 ng/ml (15 ml whole blood, 5 μl chloroform)	Methadone analogue	3
GC—ECD	4 ng/ml (1 ml plasma, oxidation to benzophenone) 4-(4-chlorophenyl)-4-phenyl-2-dimethyl-aminobutane (I.S.) forms 4-chlorobenzophenone by oxidation		4
GC—MS (EI)	5 ng/ml (4 ml plasma, 0.1 ml toluene)	Methadone analogue	5
GC—MS (CI)	5 ng/ml (1 ml plasma, 20 μl ethyl acetate)	Methadone-d_5	6

*Abbreviations: FID = flame ionization detector; ECD = electron-capture detector.

The low sensitivity observed is contrary to expectations of using the base peak *m/e* 72 for monitoring and is due to instrumental conditions, especially ion source conditions, variation of which was found to seriously limit sensitivity. Special care was therefore taken to reduce background. The temperatures of separator, inlet line, and ion source were elevated overnight to remove retained impurities before samples were analyzed. This method is more than adequate to analyze the patients' saliva or plasma samples, the concentrations of which were found to vary over a wide range between 0.05—1.0 μg/ml during the 24-h period of the study.

Monitoring drug concentrations in saliva may better reflect the time course of a drug at the receptor site. This was found to be the case for the drug procainamide for which a parallel relationship between time course of the drug in saliva and cardiac action of the drug was observed [11]. This should especially be true for drugs which act on the central nervous system. For drugs such as methadone which are extensively bound to plasma protein, monitoring drug concentration in saliva could better define the activity because the concentrations of a drug in saliva frequently reflect the unbound fraction which crosses the blood brain barrier. It appeared therefore useful to initiate monitoring saliva levels of the drug in steady-state maintenance patients especially in view of the fact that a lack in correlation between methadone concentrations in plasma and symptom complaints of patients have been reported [12].

The mean ratio of saliva to plasma of patient A was 0.55 ± 0.15 (S.D.) with a range of 0.40—0.79. Patient B showed a mean ratio of saliva to plasma of 0.48 ± 0.10 (S.D.) with a range of 0.30—0.58 (Fig. 2). Salivary pH could

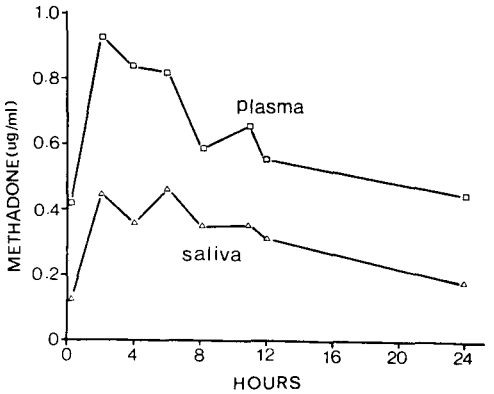


Fig. 2. Methadone concentration in plasma and saliva of a maintenance patient, dose 90 mg/day.

possibly account for the intra-individual variation in the results but pH values were not available. Patient A had difficulty in providing saliva samples and chewing gum (Dentyne, Adams Brands Inc.) was used to stimulate saliva production. Interference from the chewing gum in the analysis was not observed. Adsorption of the methadone to the gum was not proved but was considered to be minimal. If salivary concentrations of methadone in these two patients are a reflection of unbound methadone in plasma then our results agree well with those reported by Horns et al. [12], in which 50% binding of methadone to plasma was reported. This contrasts with the results reported by Lynn et al. [3] where salivary concentrations were found to be much higher than those obtained in whole blood. Our result however was not adequate to draw pharmacokinetic conclusions because of the limited sample size and number of samples.

Analysis of methadone and metabolites in urine

Retention times by SIM of methadone and metabolites were 3.24 (EMDP), 4.0 (EDDP), 4.78 (methadone), and 6.23 min (DDP). When we analyzed methadone and metabolites in urine, GC—MS was a time consuming method compared with GC methods because a separate injection of the sample was required to analyze each metabolite. This was due to the limited range of the ion monitoring mechanism used. GC—MS quantitation with deuterium-labeled internal standards, however, provided ease in work-up procedures. Extractability of labelled and unlabelled compounds from urine using methylene chloride was found to be the same. At pH 7.5 (n=4, 2.5 μg each in 1.0 ml), percent recoveries of methadone, [$^2H_{10}$]methadone, EDDP and [$^2H_3$]EDDP were 84.9 ± 2.6, 83.6 ± 0.2, 94.2 ± 3.8 and 93.9 ± 1.1, respectively. EDDP and [$^2H_3$]EDDP were analyzed in the recovery studies using [$^2H_{10}$]EDDP as the internal standard; methadone and [$^2H_{10}$]methadone using [$^2H_3$]methadone as internal standard.

The calibration equations prepared for methadone and EDDP are shown in Table II. The results indicate that calibration equations can be expressed by using only slope values because intercept values were found to be not significant. Also, after determining the ratio of unlabelled to labelled compound,

TABLE II

CALIBRATION EQUATIONS FOR METHADONE AND EDDP FOR URINE ANALYSIS

Slope and intercept values are calculated by means of computer program (Triangular Regression Package, Computing Centre, The University of British Columbia). Methadone (n=7), EDDP (n=10). Ratio (drug/internal standard) = slope × drug concentration + intercept. Internal standards are [$^2H_{10}$]methadone (4 μg) and [$^2H_{10}$]EDDP (2 μg).

	Methadone	EDDP
Slope	0.326	0.483
Slope standard error	0.0574	0.0033
Intercepts	0.0513	0.0054
Intercept standard error	0.0503	0.0117
Coefficient of determination (r^2)	0.9957	0.9990
Standard ratio (S.D.)* (n=6)	1.305 ± 0.1272	0.968 ± 0.0484
Slope calculated from standard ratio**	0.326	0.484

*Ratios of equal amount of drug to internal standard (standard ratio).
**Standard ratio × 1/internal standard.

quantitation of either methadone or EDDP can be obtained by multiplying the standard ratio, the reciprocal value of the amount of added internal standard, and the observed ratio. Slope values of calibration curves and the slopes calculated from the standard ratio were found to be the same (Table II). The calibration method was used for the analysis of methadone because of instability of the measured ratio which occurred when ions of 10 mass units difference were monitored. The ratio was found changeable depending upon the SIM conditions, particularly due to the instability of the magnetic field. The coefficient of variation of the methadone standard ratio was twice (9.7%) that of EDDP (5.2%).

GC—MS of methadone and EDDP was compared with GC (Table III). The two methods were found to be well correlated for the analysis of urine samples.

Previous experiments with the GC analysis of EMDP in the urine of maintenance patients indicated that the EMDP peak is frequently overlapped either with caffeine or with hydroxycotinine, a metabolite of nicotine [5]. When we attempted to analyze maintenance patient urines for EMDP by monitoring m/e 208 for EMDP and m/e 218 for the internal standard, [$^2H_{10}$]EMDP, we found that the amount of EMDP appeared to be less than 100 ng/ml. We concluded that determination of this metabolite was not necessarily important to study demethylation mechanisms of methadone.

TABLE III

COMPARISON OF GC—FID AND GC—MS (SIM) IN THE ANALYSIS OF METHADONE AND EDDP IN HUMAN URINES

Methadone (0.4—6.8 μg/ml) and EDDP (8—32 μg/ml) were analyzed; n = 14 in both cases.

Methadone	SIM = 0.998 GC + 0.347	r^2 = 0.963
EDDP	SIM = 1.26 GC — 2.13	r^2 = 0.962

STABILITY OF EDDP

In an attempt to obtain information on the minor metabolic pathways of methadone, the level of DDP in urine was determined by monitoring *m/e* 265 for DDP and *m/e* 275 for [$^2H_{10}$]DDP as the internal standard. When the pH of the urine was adjusted to 10.4 for extraction, inconsistency in the reproducibility of the concentration of DDP in a urine sample was found. To investigate this problem, EDDP perchlorate was made alkaline and left to stand at room temperature. A total ion current profile of the methylene chloride extract showed DDP as a significant decomposed product. DDP was identified by thin-layer chromatography (TLC) at R_F 0.8 on Silica Gel F 254 (Brinkman) with ethanol—acetic acid—water (6:3:1). Further study revealed that EDDP free base is easily decomposed to DDP in diethyl ether. Stoichiometric conversion of EDDP to DDP was observed when EDDP and DDP in the solution were measured using labelled compounds as internal standards (Fig. 3). A similar result for the conversion of EDDP to DDP by apparent oxidation reactions was separately reported by Bowen et al. [13]. Minor metabolic transformation of methadone to 2,2-diphenyl-4-dimethylaminopentanoic acid and further metabolism to DDP was suggested [14]. 2,2-Diphenyl-4-dimethylaminopentanoic acid was not detected in urines of maintenance patients by our studies using mass chromatograms in which *m/e* 167 $(C_6H_5)_2$ C^+H for the acid and *m/e* 226 $(C_6H_5)_2$ C^+COOCH_3 for the diazomethane-treated acid were monitored. Our results strongly suggest that DDP is a metabolic artefact and that pH adjustment is important when biological samples are extracted to detect new metabolites if artefact formation is to be avoided.

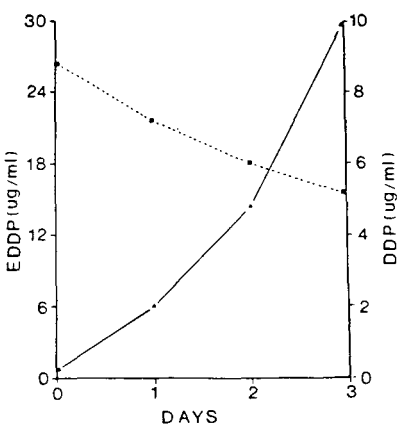


Fig. 3. Air oxidation of EDDP base to DDP (— — — EDDP, —— DDP).

ACKNOWLEDGEMENTS

We thank Dr. Gail D. Bellward for providing us with the patient saliva, plasma, and urine samples, and Mr. Roland Burton for computer analysis of calibration data. The cooperation of the staff and volunteers of the B.C. Alcohol and Drug Commission is gratefully acknowledged. This work in part

was made possible by financial support of the B.C. Health Care Resources Fund.

REFERENCES

1 C.E. Inturrisi and K. Verebely, J. Chromatogr., 65 (1972) 361.
2 H.R. Sullivan and D.A. Blake, Res. Commun. Chem. Pathol. Pharmacol., 3 (1972) 467.
3 R.K. Lynn, R.M. Leger, W.P. Gordon, G.D. Olsen and N. Gerber, J. Chromatogr., 131 (1977) 329.
4 P. Hartvig and B. Näslund, J. Chromatogr., 111 (1975) 347.
5 G.D. Bellward, P.M. Warren, W. Howald, J.E. Axelson and F.S. Abbott, Clin. Pharmacol. Ther., 22 (1977) 92.
6 H.R. Sullivan, F.J. Marshall, R.E. McMahon, E. Anggard, L.-M. Gunne and J.H. Holmstrand, Biomed. Mass Spectrom., 2 (1975) 197.
7 D.L. Hachey, M.J. Kreek and D.H. Mattson, J. Pharm. Sci., 66 (1977) 1579.
8 K. Nakamura, D.L. Hachey, M.J. Kreek, C.S. Irving and P.D. Klein, J. Pharm. Sci., 71 (1982) 40.
9 G.I. Kang, F.S. Abbott and R. Burton, Biomed. Mass Spectrom., 6 (1979) 179.
10 A. Pohland, H.E. Boaz and H.R. Sullivan, J. Med. Chem., 14 (1971) 194.
11 R.L. Guleazzi, L.Z. Benet and L.B. Sheiner, Clin. Pharmacol. Ther., 20 (1976) 278.
12 W.H. Horns, M. Rado and A. Goldstein, Clin. Pharmacol. Ther., 17 (1975) 636.
13 D.V. Bowen, A.L.C. Smit and M.J. Kreek, Advan. Mass Spectrom., 7B (1978) 1634.
14 H.R. Sullivan and S. Due, J. Med. Chem., 16 (1973) 909.

Journal of Chromatography, 231 (1982) 321—331
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1320

GAS—LIQUID CHROMATOGRAPHIC DETERMINATION OF FLURAZEPAM AND ITS MAJOR METABOLITES IN PLASMA WITH ELECTRON-CAPTURE DETECTION*

SAM F. COOPER* and DANIEL DROLET

Centre de Recherche en Sciences de la Santé, Institut National de la Recherche Scientifique, Université du Québec, 7401 rue Hochelaga, Montréal, Québec H1N-3M5 (Canada)

(First received January 15th, 1982; revised manuscript received April 9th, 1982)

SUMMARY

A sensitive and selective gas—liquid chromatographic method, using the electron-capture detector for the quantitative determination of flurazepam and its major blood metabolites is described. After extraction and back-extraction steps, flurazepam (I) is well separated from its main metabolites, N-1-hydroxyethylflurazepam (metabolite II) and N-1-desalkylflurazepam (metabolite III). Metabolite II is quantitated after forming its stable *tert.*-butyldimethylsilyl derivative by reaction with *tert.*-butyldimethylchlorosilane—imidazole reagent. The procedure permits the rapid and selective routine determination of flurazepam and its metabolites (II and III) in plasma with a detection limit of 3 ng/ml for flurazepam (I), 1 ng/ml for metabolite II and 0.6 ng/ml for metabolite III. The procedure is linear over the range of concentrations encountered after administration of a single oral therapeutic dose. No interference from the biological matrix is apparent. The suitability of the method for the analysis of biological samples was tested by studying the variation with time of flurazepam and its metabolites' plasma concentrations in normal human volunteers after a single, therapeutic 30-mg oral dose of flurazepam.

INTRODUCTION

Flurazepam (dihydrochloride) is a 1,4-benzodiazepine derivative (Fig. 1, I) utilized as the most widely prescribed hypnotic agent for the treatment of insomnia in North America [1]. Studies on the biotransformation of flurazepam [2] showed that the drug is extensively metabolized in both man and dog. The major metabolites in human plasma are N-1-hydroxyethylflurazepam (Fig. 1, metabolite II) and N-1-desalkylflurazepam (Fig. 1, metabolite III). Several methods for the quantitative determination of flurazepam and its major plasma and urinary metabolites in biological samples exist in the recent literature,

*Part of M.Sc. thesis of D. Drolet.

0378-4347/82/0000—0000/$02.75

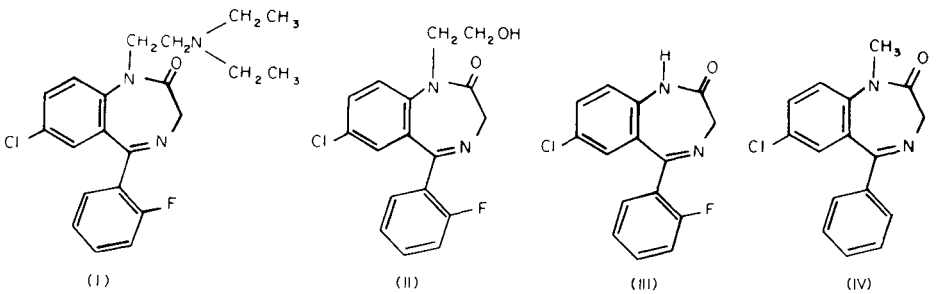


Fig. 1. Structural formulae of flurazepam (I), N-1-hydroxyethylflurazepam (II), N-1-desalkylflurazepam (III) and diazepam (IV).

employing such diverse techniques as spectrofluorometry [3,4], spectrofluorodensitometry [5], radioimmunology [6], differential pulse polarography (DPP) [7], electron-capture—gas—liquid chromatography (EC—GLC) [7—10], high-performance liquid chromatography (HPLC) [11], and gas chromatography—mass spectrometry (GC—MS) [12].

The spectrofluorometric [3,4] and spectrodensitometric methods [5] are however time-consuming and lack the specificity necessary for assaying the drug and its metabolites in a clinical context. The reported immunological method [6] describes the determination of only intact flurazepam (I) in human plasma. The DPP technique [7] was used to quantitate the major urinary metabolites of flurazepam (I), which were present in the submicrogram range in several subjects. De Silva et al. [7] developed an EC—GLC assay to measure flurazepam (I) and its metabolites (II and III) in man. Metabolite II gave an asymmetrical peak due to the polarity of the hydroxyl group. Hasegawa and Matsubara [8] studied the metabolic fate of flurazepam (I) and its several metabolites in human blood and urine by EC—GLC. Their method is time-consuming as the plasma samples were subjected to enzymatic hydrolysis, purification by Amberlite XAD resin and then derivatization of metabolites (II and III) with two different reagents. Aderjan et al. [9] investigated the plasma levels of flurazepam and its metabolites (II and III) in volunteers. Metabolite II was estimated only after enzymatic hydrolysis of the plasma samples. Riva et al. [10] reported a quantitative assay of flurazepam (I) and metabolite III in human plasma by EC—GLC but their method was not applied to human studies. Weinfeld and Miller [11] developed a method to determine only metabolite II by HPLC in human urine. Miwa and Garland [12] reported a specific and sensitive GC—MS technique to determine flurazepam (I) alone in human plasma.

The majority of the above published techniques, however, are either too time-consuming for routine use, too insensitive for the measurement of therapeutic concentrations, too non-specific or too specific and extraordinarily sensitive (e.g. GC—MS) to cause technical problems while developing the assay. The method described in this paper assays the drug and its major plasma metabolites in the absorption phase and over an interval of several biological half-lives after single-dose administration, as is necessary in pharmacokinetic and physiological availability studies in man. Metabolite II is estimated for the first time quantitatively in human plasma as the *tert.*-butyldimethylsilyl

derivative using *tert.*-butyldimethylchlorosilane—imidazole (t-BDMS) as a silylation reagent.

MATERIALS AND METHODS

Flurazepam dihydrochloride (I), its two authentic metabolites (II and III) and diazepam (IV) for chromatographic standards were supplied by Hoffman-la Roche (Vaudreuil, Canada). Diazepam was used as an internal standard for GLC analysis.

Reagents and materials

The following reagents were used: GLC-spectrometric quality benzene and toluene (J.T. Baker, Phillipsburg, NJ, U.S.A.), analytical grade methylene chloride, acetone (J.T. Baker) and methanol (Mallinckrodt, Montreal, Canada). All the above reagents were distilled before use. Analytical grade anhydrous diethyl ether (J.T. Baker) containing less than 1% peroxides, must be used from a can opened not more than five days previously. *tert.*-Butyldimethylchlorosilane—imidazole reagent (t-BDMS) was obtained from Applied Science (State College, PA, U.S.A.). The inorganic reagents were made up in double distilled water. Borate buffer solution (1 *M*) was prepared according to De Silva and Puglisi [13].

Gas—liquid chromatography

A Hewlett-Packard Model 5830A gas chromatograph equipped with a ^{63}Ni (15 mCi) electron-capture detector (ECD) was used in this study; the instrument was linked to a digital integrator (HP 18850A).

The chromatographic conditions for flurazepam (I) and its major metabolites (II and III) in plasma were as follows: a 1.8 m × 2 mm I.D. coiled glass column was packed with 3% OV-17 on 100—120 mesh Gas-Chrom Q (Chromatographic Specialties, Brockville, Canada). In order to avoid adsorption losses of the compounds on the active sites of the column, it was deactivated by injecting 25 μl of REJUV-8 (Supelco, Bellefonte, PA, U.S.A.) daily before use. Column, injector and ECD temperatures were 255°C, 275°C and 300°C, respectively. The argon—methane (95:5) carrier gas flow-rate was 22 ml/min. Under these conditions, the relative retention times of metabolite III, flurazepam (I) and t-BDMS derivative of metabolite II to the internal standard (diazepam) were 1.17, 2.02 and 2.46, respectively (Figs. 2 and 3).

The relative retention time of underivatized metabolite II to the internal standard was 2.21. This indicates that metabolite II was separated in both derivatized and underivatized forms from flurazepam (I) and metabolite III.

Gas chromatography—mass spectrometry

Mass spectra were recorded using a Hewlett-Packard dual electron impact/chemical ionization source gas chromatograph—mass spectrometer (Model 5982A) linked to a laboratory data system (Hewlett-Packard Model 5933A). Operating conditions were as follows: a 1.2 m × 2 mm I.D. coiled glass column was packed with 3% OV-17 on 80—100 mesh Gas-Chrom Q (Chromatographic Specialties). The oven temperature was maintained at 230°C for 2 min then

programmed at 16°C/min to a final temperature of 270°C and held for 6 min. The membrane separator was also programmed at 16°C/min and the transfer line temperature was 240°C. The apparatus was operated in the electron impact mode. Source temperature was 170°C, electron energy 70 eV and trap current 0.35 mA.

Extraction procedure

The extraction procedure in plasma was established using a modification of the extraction procedures described by De Silva and co-workers [7,14] for determination of flurazepam (I) and its major metabolites (II and III) in whole blood by GLC. To 3 ml of plasma in a 15-ml tube with a PTFE-lined cap were added 100 μl of the methanolic solution of diazepam (400 ng/ml) as the internal standard. The plasma sample was made alkaline by the addition of 3 ml of 1 *M* borate buffer solution (pH 9.0). The mixture was agitated with vortex action for 15 sec.

The sample was then extracted with 6.0 ml of benzene—methylene chloride (90:10) for 5 min on an Eberbach mechanical agitator. After centrifuging at 600 *g* for 15 min (Model TJ-6, rotor TH-4, Beckmann, Palo Alto, CA, U.S.A.), the organic layer was transferred to a 15-ml tube with screw cap and the aqueous layer was discarded. The organic layer was back-extracted with 3.0 ml of 4.0 *N* hydrochloric acid for 10 min. The sample was centrifuged for 10 min at 400 *g* and the organic layer was removed and discarded. The aqueous layer was washed with 5 ml of diethyl ether by shaking for 10 min and centrifuging for 10 min. The organic layer was removed by aspiration and discarded. The sample was cooled in an ice-bath and made alkaline by slowly adding 3.5 ml of 4.0 *N* sodium hydroxide solution. The tube was vortexed for 15 sec. The aqueous phase was extracted twice with 2.5 ml of diethyl ether by shaking for 10 min and centrifuging 10 min. The ether extracts were combined in a 7-ml tube, dried with sodium sulfate, then transferred and evaporated off in a 3-ml conical glass-stoppered tube by successive evaporation at 35—40°C with a slow stream of nitrogen. The walls of the tube were rinsed with 200 μl of methanol by vibrating with a Vortex mixer for 1 min. The solution was evaporated to dryness as before and the residue was redissolved in 100 μl of benzene—acetone—methanol (85:10:5). The precise volume of 5.0 μl of the solution was injected into the gas chromatograph for the estimation of flurazepam (I) and metabolite III.

Derivatization of metabolite II

The remainder of the solution was used for the determination of metabolite II. The solution was evaporated to dryness at 60°C with a slow stream of nitrogen. To the residue were added 5 μl of t-BDMS reagent. The mixture was vortexed well for 30 sec in order to rinse the walls of the conical tube and then heated at 60°C for 10 min. To this mixture were added 200 μl of toluene. The solution was vortexed again for 30 sec and the precise volume of 5.0 μl was injected into the gas chromatograph.

Calibration curves

To 3 ml of heparinized blank human plasma in 15-ml tubes were added

(1) 25, 50, 75 or 100 μl of methanolic solution of flurazepam base (400 ng/ml), (2) 10, 20, 30, 40, 50 or 60 μl of methanolic solution of metabolite II (2 μg/ml), (3) 25, 50, 75, 100, 125 or 150 μl of methanolic solution of metabolite III (400 ng/ml) and (4) 100 μl of diazepam solution in methanol (400 ng/ml). The samples were then carried through the complete extraction procedure described above. Quantitation was achieved using the ratio of the peak areas of flurazepam (I), and metabolites II and III to that of the internal standard, diazepam.

Peak area ratios were plotted against weight to obtain the calibration curves. The response of ECD to flurazepam (I) was linear over the range of 3.3—13.3 ng per ml plasma, to metabolite III from 3.3—20 ng per ml of plasma, and to metabolite II from 6.3—38 ng per ml of plasma.

Human studies

Flurazepam dihydrochloride (I, 30-mg capsules) was administered separately to twenty healthy volunteers under controlled conditions. Blood samples (10 ml) were withdrawn from the antecubital vein using heparinized evacuated tubes (Venoject, Kimble-Terumo, Elkton, MD, U.S.A.) at fifteen appropriate time intervals after dosing. The blood samples were centrifuged at 600 *g* for 10 min and the plasma was transferred to another tube and frozen at —20°C until analyzed.

RESULTS AND DISCUSSION

Selectivity

Analytical studies indicate that extracts from blank human plasma do not show peaks that could interfere with the quantitative determination of flurazepam (I) and its major metabolites (II and III) in plasma. Typical chromatograms are shown in Figs. 2 and 3. Although metabolite II could be identified by ECD without derivatization, as indicated by De Silva et al. [7], the detector response of this compound was very low, and in addition, showed tailing due to the polarity of the hydroxyl group. In this procedure, metabolite II was quantitated in plasma as *tert.*-butyldimethylsilyl ether with t-BDMS as a silylation reagent. The main advantages of t-BDMS ethers are: (1) they are 10^4 times more stable to hydrolysis than trimethylsilyl ethers and (2) their mass spectra which usually have their base peak at M^+—57, are less complicated.

De Silva et al. [7] prepared the trimethylsilyl derivative of metabolite II with bistrimethylsilylacetamide (BSA) in pyridine. The resultant silyl ether eluted as a sharp symmetrical peak with a five-fold increase in sensitivity but its retention time was identical to that of metabolite III, the major metabolite of flurazepam in plasma. Owing to this interference, the derivatization of metabolite II with BSA was rendered impractical.

Hasegawa and Matsubara [8] used bistrimethylsilyltrifluoracetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) as a silylation reagent for metabolite II. These authors divided the plasma extract into two parts. One part was methylated to convert metabolite III to N-1-methyl derivative with diazomethane. The other part was silylated to convert metabolite II to its silyl ether. We have observed that the methylation of metabolite III was unnecessary due to its high sensitivity and resolution from other interfering

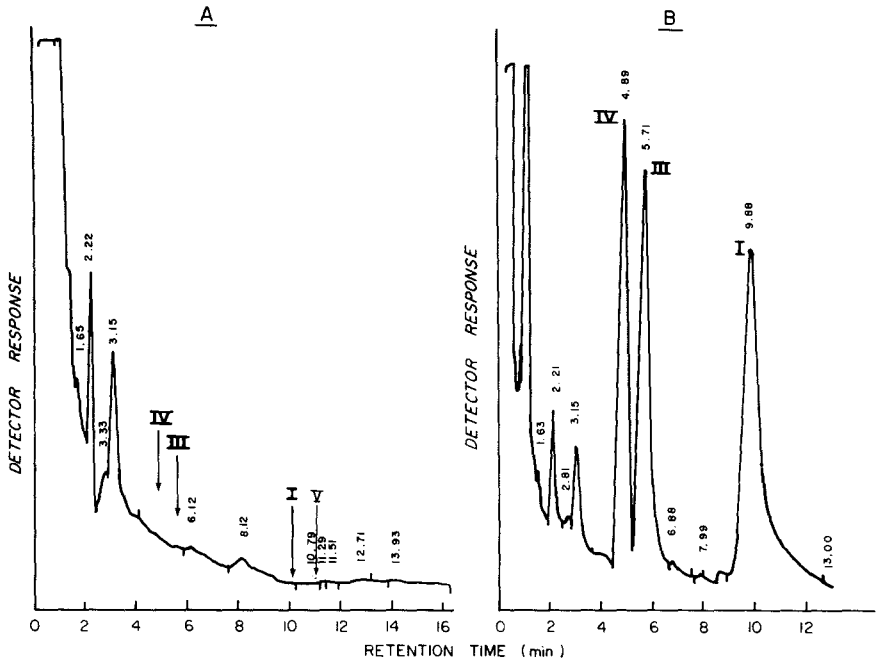


Fig. 2. (A) Chromatogram obtained from an extract of 3 ml of blank human plasma. The arrows show the absence of signals at the retention times of the internal standard (IV), N-1-desalkylflurazepam (III), flurazepam (I) and N-1-hydroxyethylflurazepam (V). (B) Chromatogram obtained from an extract of 3 ml of human plasma containing the compounds IV, III and I.

peaks, whereas, with t-BDMS reagent, the silyl derivative of metabolite II was completely separated from metabolite III (Fig. 3).

Flurazepam (I) and metabolite III were assayed before metabolite II because t-BDMS reagent decreased the sensitivity of compound I. The stationary phase 3% OV-17 gave a good resolution for flurazepam (I), its two major plasma metabolites (II and III) and the internal standard (IV).

Recovery studies

The recoveries of flurazepam (I) and its metabolites (II and III) from 3 ml of spiked plasma were determined using the same internal standardization method as described above. The peak area ratio of each compound and the internal standard (IV) was used as the index of detector performance and overall effiency of the analytical procedure. The reproducibility and recovery results of compounds (I, II and III) are given in Table I. The overall coefficient of variation (C.V.) is below 10%.

Sensitivity

The lower limits for accurate determination of flurazepam (I) and its major plasma metabolites (II and III) were established by spiking 3 ml of blank plasma with dilute methanolic solutions of compounds I, II and III in the range of 10—60 ng, 10—60 ng and 20—120 ng respectively followed by the previously described extraction procedure using diazepam (IV) as an internal standard.

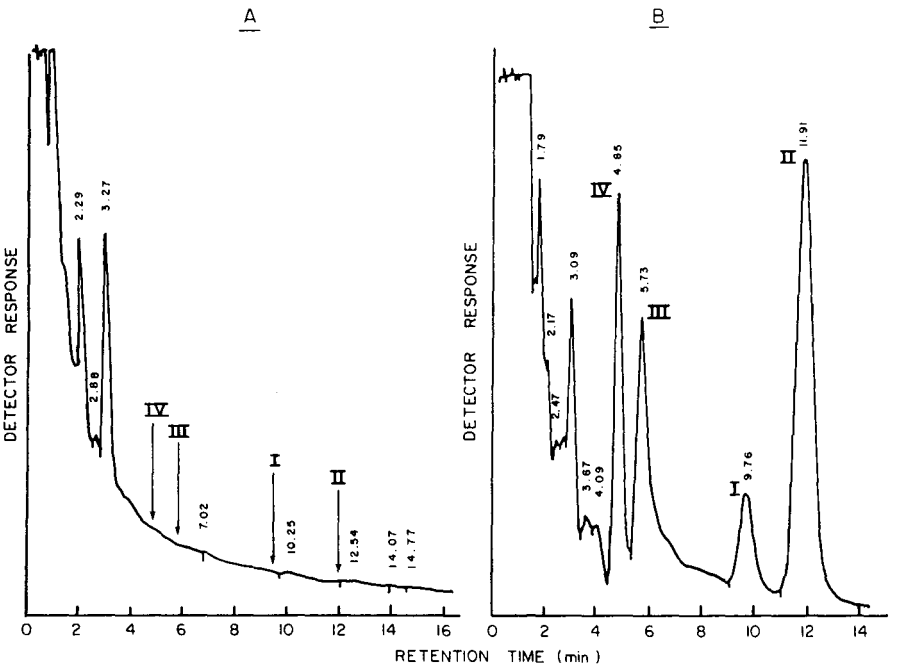


Fig. 3. (A) Chromatogram obtained from an extract of 3 ml of blank human plasma derivatized with t-BDMS. The arrows show the absence of signals at the retention times of the internal standard (IV), N-1-desalkylflurazepam (III), flurazepam (I) and t-BDMS derivative of N-1-hydroxyethylflurazepam (II). (B) Chromatogram obtained from an extract of 3 ml of human plasma containing the compounds IV, III, I and II derivatized with t-BDMS.

The lower detection limit was fixed to the minimum response of ECD to the respective compounds with peak areas up to 40,000 counts. It was found that the lowest detection limits of flurazepam (I), metabolites II and III were 3, 1 and 0.6 ng/ml of plasma respectively with a signal-to-noise ratio of 5.

Precision and accuracy

The ECD response to flurazepam (I) was linear over the range of 3.3—13.3 ng/ml of plasma, to metabolite III from 3.3—20 ng/ml of plasma, and to metabolite II from 6.3—38 ng/ml of plasma. Standard curves constructed by plotting the peak area ratio (y) against the amount (ng added) of I, II and III (x) coincided with the line $y = mx + b$. The linear regression values are: (I), $m = 0.121$, $b = -0.286$, $r = 0.9951$; (II), $m = 0.099$, $b = -0.090$, $r = 0.9980$; (III), $m = 0.097$, $b = 0.027$, $r = 0.9997$. These plots indicate the validity of the peak area ratio method of quantitation using diazepam (IV) as the internal standard.

Gas chromatography—mass spectrometry

The GC—MS data of flurazepam and its major metabolites were first reported by Clatworthy et al. [15]. The mass spectrum of *tert.*-butyldimethylsilyl derivative of metabolite II is shown in Fig. 4 using the electron impact mode. The major ion in the spectrum is at m/z 389 (M^+—57) corresponding to the loss of the *tert.*-butyl group along with the ^{37}Cl isotope peak at 391.

TABLE I

GLC ESTIMATION OF FLURAZEPAM (I) AND METABOLITES II AND III ADDED TO PLASMA

Compound	Compound added (ng per 3 ml plasma)	Compound recovered (ng)	Recovery* (%)	C.V. (%)
Flurazepam (I)	10	8.52	85.2	4.65
	20	16.76	83.8	5.18
	30	25.98	86.6	3.06
	40	32.70	81.8	4.28
Metabolite II**	19	14.40	75.8	5.49
	38	27.88	73.4	4.03
	57	43.80	76.8	4.05
	76	54.16	71.3	3.40
	95	66.72	70.2	2.41
	114	78.68	69.0	2.05
Metabolite III	10	7.86	78.6	4.08
	20	15.32	76.6	6.03
	30	23.04	76.8	5.32
	40	29.04	72.6	4.50
	50	38.40	76.8	4.82
	60	44.28	73.8	4.11

*Each value is the mean of five determinations.
**Determined as *tert.*-butyldimethyl derivatives.

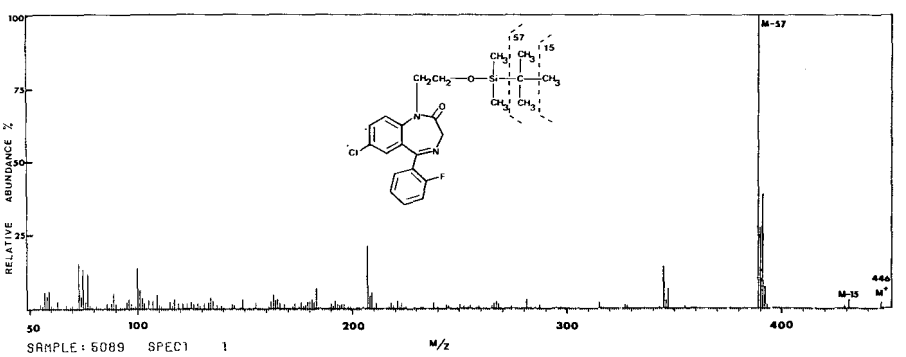


Fig. 4. Mass spectrum of *tert.*-butyldimethylsilyl derivative of metabolite II using the electron impact mode.

The molecular ion is present at m/z 446. The ion at m/z 431 (M^+—15) indicates the loss of a methyl group.

Application of the method to human studies

The lower limit of sensitivity of the procedure as described permits the quantitation of flurazepam (I), whose mean apparent half-life was found to be 2.9 h as reported elsewhere [6,9,12], metabolite II (mean half-lives α-phase 1.9 h and β-phase 14.1 h) as observed by Aderjan et al. [9] and metab-

olite III (mean half-life 93.1 h) as found by Greenblatt et al. [16], after oral administration of a single therapeutic dose. Flurazepam (I) and metabolites II and III could be detected as long as 7, 24 and 264 h post administration, respectively. The average plasma concentrations (as a function of time) of flurazepam (I) and its metabolites (II and III) found in twenty volunteers after a single 30-mg oral dose of flurazepam dihydrochloride are illustrated in Figs. 5, 6 and 7. Plasma levels of flurazepam (I) and its metabolites (II and III) vary considerably between subjects. The pharmacokinetic data of this study are reported elsewhere [17]. This variability in plasma concentrations of in-

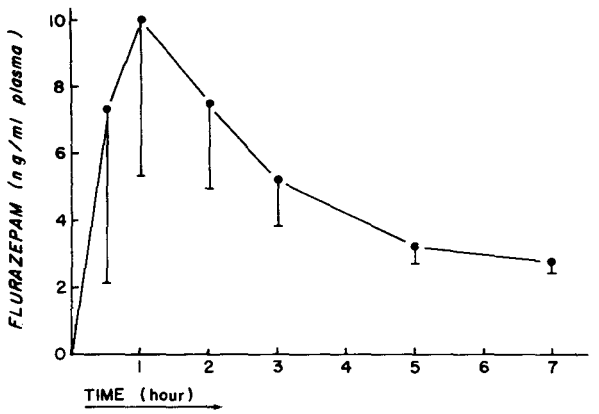


Fig. 5. Average plasma concentration—time profile of flurazepam (I) in 20 volunteers after administration of 30 mg of flurazepam dihydrochloride. Bars indicate 1 S.D.

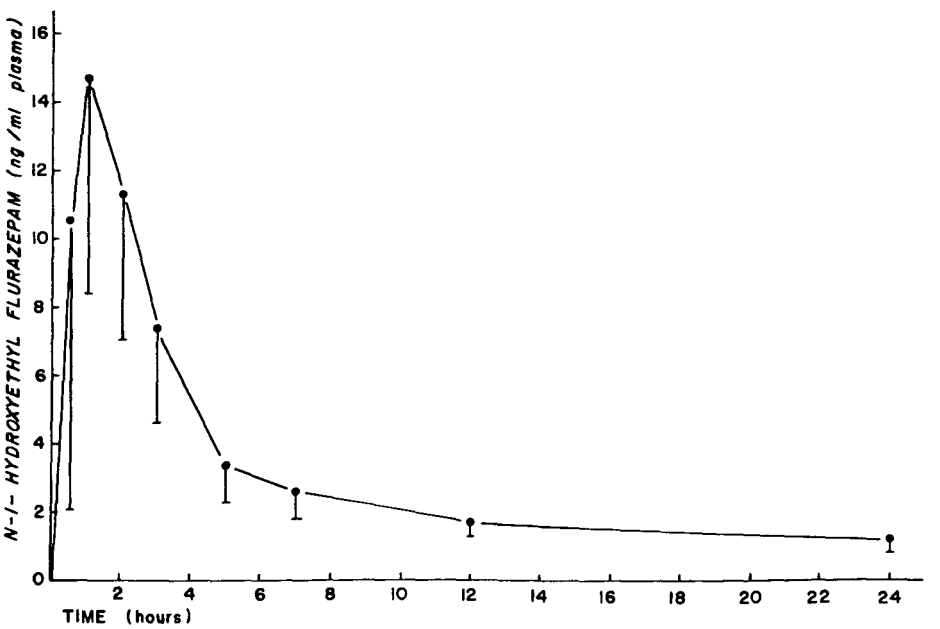


Fig. 6. Average plasma concentration—time profile of metabolite II in 20 volunteers after administration of 30 mg of flurazepam dihydrochloride. Bars indicate 1 S.D.

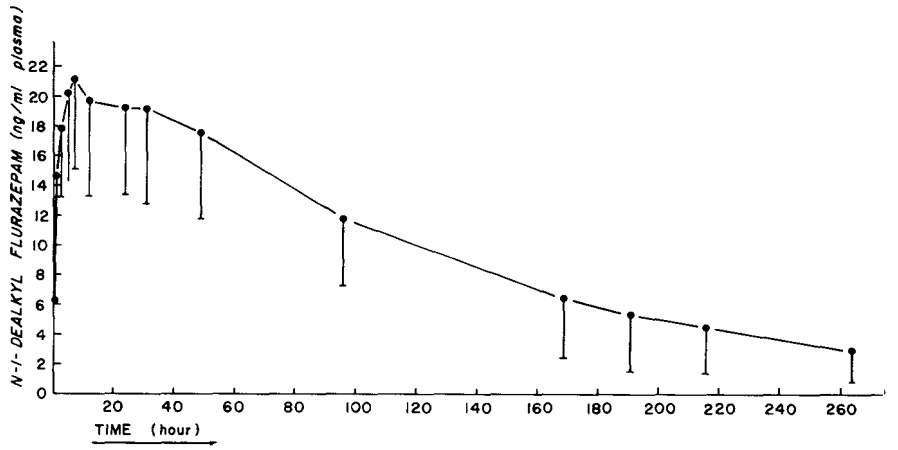


Fig. 7. Average plasma concentration—time profile of metabolite III in 20 volunteers after administration of 30 mg of flurazepam dihydrochloride. Bars indicate 1 S.D.

dividuals under controlled conditions has been found and reported for diazepam [18] and lorazepam [19]. These variations may be ascribed to individual differences in the metabolic rate of elimination.

ACKNOWLEDGEMENTS

The authors express their gratitude to Hoffmann-la Roche Ltd. (Vaudreuil, Quebec, Canada) for providing them with authentic standards of flurazepam, its metabolites and diazepam. They are also grateful to Dr. Robert Massé for mass spectral data and Dr. Kamal Midha for his valuable advice.

REFERENCES

1 Sleeping Pills, Insomnia and Medical Practice, Report of a Study of the Institute of Medicine, National Academy of Sciences, Washington, DC, 1979.
2 M.A. Schwartz and E. Postma, J. Pharm. Sci., 59 (1970) 1800.
3 J.A.F. de Silva and N. Strojny, J. Pharm. Sci., 60 (1971) 1303.
4 S.A. Kaplan, J.A.F. de Silva, M.L. Jack, K. Alexander, N. Strojny, R.E. Weinfeld, C.V. Puglisi and L. Weissman, J. Pharm. Sci., 62 (1973) 1932.
5 J.A.F. de Silva, I. Bekersky and C.V. Puglisi, J. Pharm. Sci., 63 (1974) 1837.
6 W. Glover, J. Early, M. Delaney and R. Dixon, J. Pharm. Sci., 69 (1980) 601.
7 J.A.F. de Silva, C.V. Puglisi, M.A. Brooks and M.R. Hackman, J. Chromatogr., 99 (1974) 461.
8 M. Hasegawa and I. Matsubara, Chem. Pharm. Bull., 23 (1975) 1826.
9 R. Aderjan, P. Fritz and R. Mattern, Arzneim. Forsch., 30 (1980) 1944.
10 R. Riva, M. de Anna, F. Albani and A. Baruzzi, J. Chromatogr., 222 (1981) 491.
11 R.E. Weinfeld and K.F. Miller, J. Chromatogr., 223 (1981) 123.
12 B.J. Miwa and W.A. Garland, Anal. Chem., 53 (1981) 793.
13 J.A.F. de Silva and C.V. Puglisi, Anal. Chem., 42 (1970) 1725.
14 J.A.F. de Silva, I. Bekersky, C.V. Puglisi, M.A. Brooks and R.E. Weinfeld, Anal. Chem., 48 (1976) 10.
15 A.J. Clatworthy, L.V. Jones and M.J. Whitehouse, Biomed. Mass Spectrom., 4 (1977) 248.
16 D.J. Greenblatt, M. Divoll, J. Harmatz, D.S. MacLaughlin and R.I. Shader, Clin. Pharmacol. Ther., 30 (1981) 475.

17 S.F. Cooper, D. Drolet and R. Dugal, Progr. Neuropsychopharmacol., 5 (1981) 293.
18 R. Dugal, G. Caillé, J. Brodeur, S. Cooper and J.G. Besner, Union Med. Can., 102 (1973) 1744.
19 D.J. Greenblatt, R.I. Shader, K. Franke, D.S. MacLaughlin, J.S. Harmatz, M.D. Allen, A. Werner and E. Woo, J. Pharm. Sci., 68 (1979) 57.

Journal of Chromatography, 231 (1982) 333—339
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1313

SIMULTANEOUS DETERMINATION OF GLUCURONIDES OF TRIMETOZINE IN HUMAN URINE BY GAS CHROMATOGRAPHY

K. KAWAHARA* and T. OFUJI

Research Laboratories, Kakenyaku-kako Co., Ltd., 14 Minami-kawaramachi, Shinimiya, Yamashina-ku, Kyoto 607 (Japan)

(First received November 17th, 1981; revised manuscript received April 9th, 1982)

SUMMARY

A gas chromatographic method for the simultaneous determination of four glucuronides (metabolites) of trimetozine excreted in human urine is described. The method involves pretreatment of the urine specimen [i.e. removal of interfering substances by solvent extraction, desalting on an ion-exchange (Amberlite XAD-2) column], and permethylation of glucuronides by reaction with methylsulfinyl carbanion and methyl iodide. The permethylated derivatives were submitted to gas chromatographic separation on an OV-17 column, and their structures were investigated by subsequent gas chromatographic—mass spectrometric analysis. The minimum detectable concentration of each glucuronide is 5 μg/ml when 1 ml of urine is used. The utility of the present method is successfully demonstrated by determining the urinary concentration of four glucuronides following oral administration of trimetozine to human subjects.

INTRODUCTION

Glucuronide formation is one of the detoxication processes of drug metabolism in man. Trimetozine, N-(3,4,5-trimethoxybenzoyl)morpholine, is an antidepressant agent, which is known to be biotransformed in man into four glucuronides [1]. The structures of the metabolites excreted in urine are shown in Fig. 1.

Quantitation of the glucuronides of drugs in general has been performed by determination of the aglycone released by enzymatic or chemical hydrolysis. Such an indirect method, however, suffers the disadvantage that the enzymatic hydrolysis is often influenced by the presence of inhibitors in urine, and that the stability of the aglycone in the reaction medium of chemical hydrolysis is sometimes a prerequisite.

Marcucci et al. [2] and Nakagawa et al. [3] reported alternative methods, in which glucuronides were converted to their methyltrimethylsilyl de-

0378-4347/82/0000—0000/$02.75

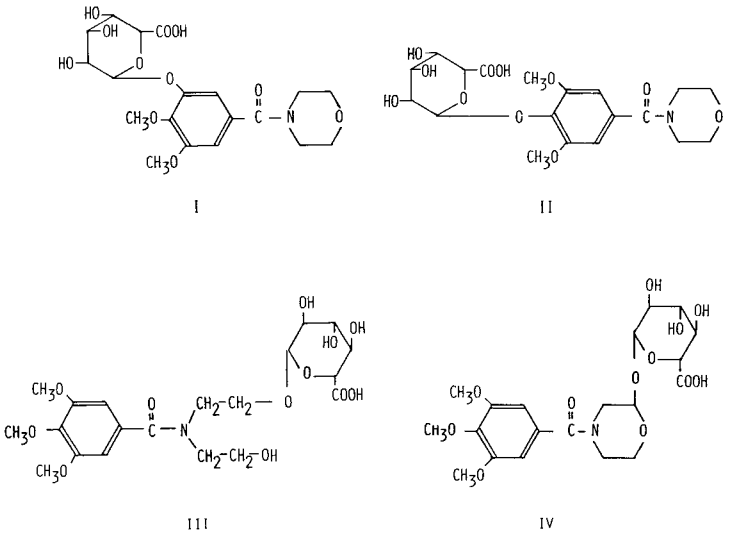


Fig. 1. Chemical structures of I, II, III and IV.

rivatives and determined by gas chromatography (GC). The present paper describes a GC method for the simultaneous determination of four glucuronides of trimetozine after permethylation of hydroxyl and carboxyl groups in the glucuronic acid moiety.

EXPERIMENTAL

Reagents and materials

The reagents used were a 50% dispersion of sodium hydride in oil (Metal Hydrides Inc., U.S.A.), methanol, ethyl acetate and diethyl ether of special grade for pesticide analysis (Wako, Osaka, Japan), chloroform of spectral grade (Nakarai, Kyoto, Japan), methyl iodide, acetic acid, sodium chloride and petroleum ether of reagent grade (Nakarai), and dimethyl sulfoxide of spectral grade (Merck, Darmstadt, G.F.R.).

Tricaprin used as an external standard, and 6-bromo-2-naphthyl-β-D-glucuronide used as an internal standard, were obtained from Sigma (St. Louis, MO, U.S.A.). The internal and external standards were used as an aqueous solution (50 μg/ml) and a chloroform solution (25 μg/ml), respectively.

Amberlite XAD-2 (Rohm and Haas, Philadelphia, PA, U.S.A.) was thoroughly washed successively with two volumes of distilled water and methanol. The resin particles floating on the surface were discarded by decantation. The resin was then transferred to a glass column and washed successively with 5 volumes of distilled water, methanol, distilled water, methanol containing 2% acetic acid, and distilled water, in this order.

The glucuronides of trimetozine (I, II, III and IV in Fig. 1) were isolated from the urine of a dog dosed with trimetozine, using the Amberlite XAD-2 column and thin-layer chromatography followed by recrystallization. The detailed procedure is found in a previous paper [1].

Methylsulfinyl carbanion solution was prepared as follows: 300 mg of sodium hydride were carefully rinsed three times each with 5 ml of anhydrous light petroleum (b.p. 30—60°C). A 5-ml portion of dimethyl sulfoxide was added to the sodium hydride and the mixture was heated at 75°C until evolution of hydrogen had ceased. The mixture was diluted six times with dimethyl sulfoxide.

Drug administration

Two healthy male adults, 26 and 32 years of age, weighing 59 and 53 kg, respectively, each received 200 mg of trimetozine as a capsule after a 16-h fast. The urine specimens were collected just before and at 3, 6, 12 and 24 h after administration.

Sample preparation

In a glass-stoppered test-tube were placed 1 ml of urine, 1 ml of 1 *N* hydrochloric acid and 0.5 g of sodium chloride. After shaking for 5 min, the mixture was washed with 10 ml of ethyl acetate. The trace of ethyl acetate remaining in the aqueous layer was completely removed by shaking with 10 ml of diethyl ether from the aqueous layer under reduced pressure at room temperature. A 1-ml aliquot of internal standard was added to the aqueous layer, and the mixture was placed on an Amberlite XAD-2 column (5 cm × 10 mm I.D.). A 20-ml portion of distilled water was first passed through the column for desalting and the glucuronides were eluted with 20 ml of methanol containing 1.5% acetic acid. The latter eluate was collected and evaporated to dryness in a flask under reduced pressure at 40°C. The residue was dissolved in 0.5 ml of the methylsulfinyl carbanion solution, and kept at room temperature for 1 min. Then 1.5 ml of methyl iodide were added to the reaction mixture which was kept at room temperature for 30 min. The mixture was shaken with 8 ml of distilled water and 12 ml of chloroform for 5 sec then transferred into a glass-stoppered test-tube. After centrifugation at 3000 rpm (1660 *g*) for 2.5 min, the chloroform layer was separated, washed three times with 8 ml of distilled water, and dehydrated with anhydrous sodium sulfate. The chloroform was evaporated to dryness in a flask under reduced pressure at 40°C. The residue was dissolved in 150 μl of chloroform, a 1-μl portion of which was injected into the gas chromatograph.

GC conditions

An Hitachi 073 gas chromatograph equipped with a flame-ionization detector was used. A U-shaped glass column (2 m × 3 mm I.D.) was packed with 1.5% OV-17 on Gas-Chrom Q AW DMCS (80—100 mesh). The column temperature was 285°C, the injection port and detector temperature 295°C; the carrier gas (nitrogen) flow-rate was 60 ml/min. The peak area was measured by a digital integrator (Takeda Riken TR-2213-A).

GC—mass spectrometry conditions

The GC—mass spectrometric (MS) analysis was carried out on an Hitachi 6MG gas chromatograph—mass spectrometer with following conditions: a

column of glass tubing (1 m × 3 mm I.D.) packed with 1.5% OV-17 on Chromosorb W AW DMCS (80—100 mesh); column temperature, 245°C; injection port temperature, 260°C; separator temperature, 260°C; ionization source temperature, 220°C; the carrier gas (helium) flow-rate, 60 ml/min; accelerating voltage, 3.5 kV; ionization energy, 20 eV; trap current, 60 μA.

RESULTS AND DISCUSSION

Recovery of glucuronides from the ion-exchange column

The effect of the elution solvent on the recovery of the four glucuronides (I, II, III, IV) and the internal standard from the Amberlite XAD-2 column was examined by changing the concentration of acetic acid (0 to 1.5% v/v) in methanol. The recoveries of glucuronides were determined by using internal standard added to the eluate; and the recovery of the internal standard itself was determined by using glucuronide I as the standard. The results indicated that the recoveries for all materials were less than 10% when eluted with methanol, but increased rapidly with initial increases in acetic acid concentration up to 0.2%, followed by gradual approaches to the plateau levels (92—100%) between 1.2 and 1.5%. From these results, methanol containing 1.5% acetic acid was chosen for the elution of glucuronides from the Amberlite XAD-2 column.

Permethylation reaction

Permethylation of the glucuronides of trimetozine was achieved by a two-step reaction with methylsulfinyl carbanion and with methyl iodide. The time and temperature dependencies of the reaction were investigated using each of the four glucuronides isolated from the dog urine. The degree of permethylation of the glucuronides and internal standard were evaluated by using tricaprin as an external standard. In examining the time dependence, the reaction temperature was fixed at 25°C for both reaction steps, and in examining temperature dependence the reaction time was fixed at 1 min for the reaction with methylsulfinyl carbanion and at 30 min for the reaction with methyl iodide. The results of the time dependence for the first reaction indicated that glucuronides I, III and IV and the internal standard underwent rapid raction with methylsulfinyl carbanion to give constant yields of permethylated derivatives, whereas glucuronide II suffered a gradual decrease in yields, possibly due to partial decomposition. The results for time dependence of the reaction with methyl iodide indicated that the formation of all the permethylated glucuronides and internal standard was complete 10—30 min after initiation of the second reaction. The results for the effect of temperature on both reaction steps showed that there were almost no changes in the yields of permethylated derivatives between 15°C and 35°C.

Thus, the four glucuronides and the internal standard were permethylated by reaction with methylsulfinyl carbanion for 1 min at room temperature and with methyl iodide for 30 min at room temperature.

GC separation and calibration graph

The permethylated derivatives of the isolated glucuronides and internal

standard each gave a single peak on the gas chromatogram. Fig. 2 shows chromatograms of human urine treated as mentioned above. No interfering peak was observed on the chromatogram of control urine. The retention times of the glucuronides and internal standard are 9.9, 10.6, 12.0, 14.1 and 3.2 min, respectively.

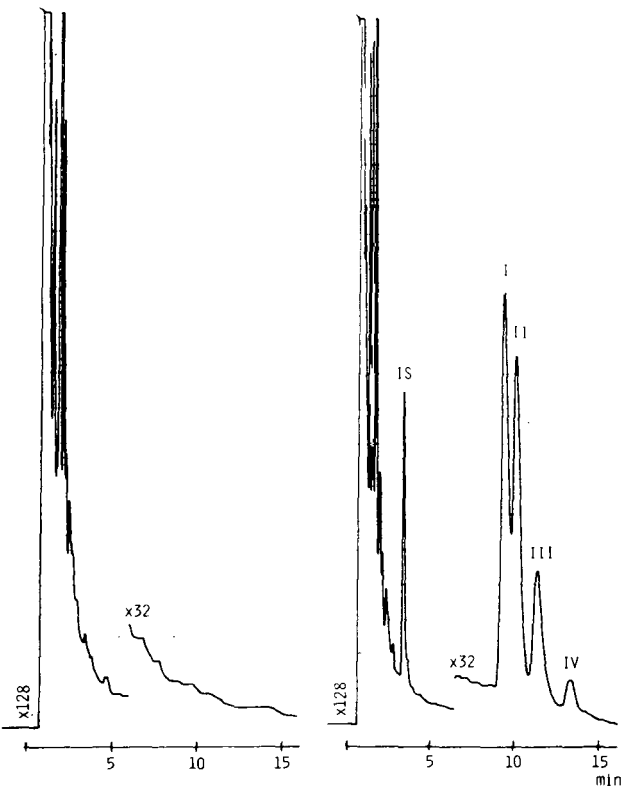


Fig. 2. Chromatograms of urine extracts. Left: blank urine. Right: urine from a volunteer who received 200 mg of trimetozine. IS = internal standard.

The calibration graphs were constructed using a 1-ml aliquot of urine containing 5—200 μg each of I, II, III and IV. The linear relationships were obtained between peak area ratio and sample weight (I, r=0.9995; II, r=0.9978; III, r=0.9986; IV, r=0.9993). The minimum detectable concentration of I, II, III and IV was 5 μg/ml when 1 ml of urine was used.

Mass spectrometry

The structures of the four permethylated glucuronides of trimetozine were confirmed from GC—MS spectra. Typical spectra are shown in Figs. 3 and 4; Table I summarizes the *m/e* values of characteristic peaks of other products. It is known that the fragment ions at *m/e* 75, 101, 141, 169 and 201 are characteristic of the permethylated glucuronic acid moiety [4, 5], and that the ions at *m/e* 116 and 232 are characteristic of the fragments

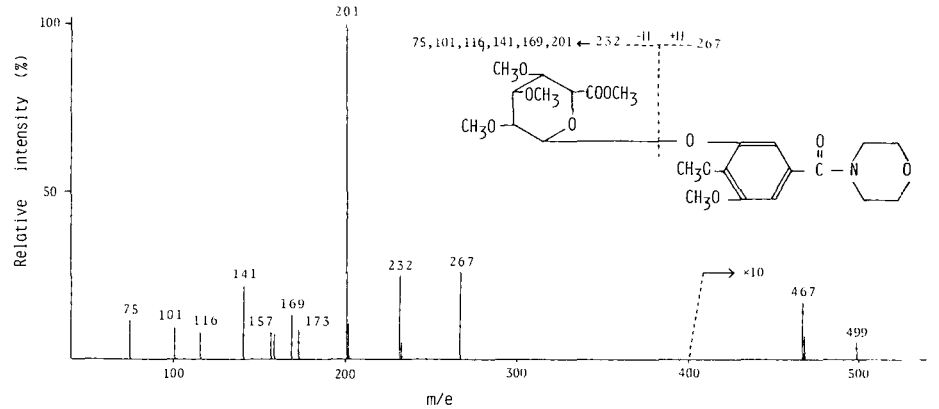


Fig. 3. Mass spectrum of I derivative.

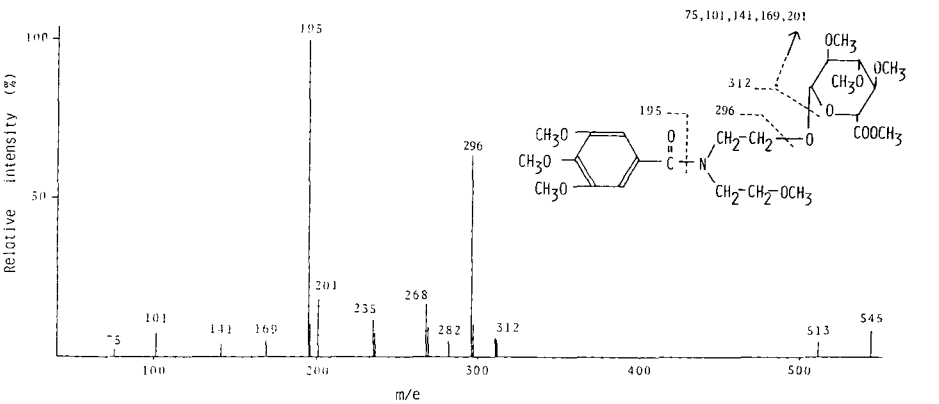


Fig. 4. Mass spectrum of III derivative.

TABLE I

MASS SPECTRA OF PERMETHYLATED DERIVATIVES OF GLUCURONIDES

Glucuronide	Diagnostic ions, *m/e* (relative intensity, %)						
II	75(14.0)	101(9.2)	116(9.1)	141(21.2)	157(9.6)	169(13.8)	173(13.2)
	201(100)	232(37.2)	267(21.5)	499(0.1)			
IV	75(5.8)	101(7.2)	141(4.1)	169(5.4)	195(100)	201(29.6)	280(18.3)
	297(10.1)	529(6.1)					
Internal	75(21.2)	101(19.8)	116(18.1)	141(19.7)	157(7.1)	169(18.0)	201(100)
standard	222(2.1)	224(2.0)	422(0.2)	424(0.2)	454(0.4)	456(0.4)	

of permethylated phenolic glucuronide; also that the ion *m/e* M − 249 is due to the fragment of permethylated aliphatic glucuronide [4, 5]. Compared with these findings, the present results readily show that the derivatives of I, II and internal standard are permethylated phenolic glucuronides, and the derivatives of II and IV are permethylated aliphatic glucuronides.

Urinary excretion in humans

The present method was applied to the determination of four glucuronides of trimetozine excreted in urine after oral doses of 200 mg of trimetozine as a capsule to human subjects. The results (Table II) show that an average of 23% of the administered amount was excreted in the urine as total glucuronides within 24 h.

TABLE II

URINARY EXCRETION OF GLUCURONIDES I, II, III and IV

Values are given in mg.

	h	I	II	III	IV
Volunteer A	0—3	3.1	2.0	ND*	ND
	3—6	7.5	5.6	0.8	0.7
	6—12	14.6	13.0	5.8	3.1
	12—24	8.9	7.0	5.0	1.2
Total		34.1	27.6	11.6	5.0
Trimetozine equivalent		21.6	17.5	6.9	3.0
Volunteer B	0—3	5.0	2.3	ND	ND
	3—6	11.3	6.4	1.2	0.9
	6—12	13.3	8.7	3.5	1.5
	12—24	7.0	4.0	3.0	0.7
Total		36.6	21.4	7.7	3.1
Trimetozine equivalent		23.2	13.6	4.6	1.8

*ND = not determined.

ACKNOWLEDGEMENTS

The authors wish to thank Professor T. Uno and Dr. Nakagawa of the Faculty of Pharmaceutical Sciences, Kyoto University, for many suggestions during this work, and Mr. T. Yamamoto and Mr. N. Kazikawa for isolation of the glucuronides of trimetozine from urine.

REFERENCES

1 M. Tohno, Y. Doi, N. Kajikawa, T. Ofuji, A. Tatematsu, M. Suzuki, H. Yoshizumi and T. Nadai, Pharmacometrics, 14 (1977) 289.
2 F. Marcucci, R. Bianchi, L. Airoldi, M. Salmona, R. Fanelli, C. Chiabrando, A. Frigerio, E. Mussini and S. Garattini, J. Chromatogr., 107 (1975) 285.
3 T. Nakagawa, M. Masada and T. Uno, J. Chromatogr., 111 (1975) 355.
4 R.M. Thompson, N. Gerber, R.A. Seibert and D.M. Desiderio, Res. Commun. Chem. Pathol. Pharmacol., 4 (1972) 543.
5 R.M. Thompson, N. Gerber, R.A. Seibert and D.M. Desiderio, Drug Metab. Dispos., 1 (1973) 489.

Journal of Chromatography, 231 (1982) 341–348
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1314

DETERMINATION OF DIAZEPAM AND ITS PHARMACOLOGICALLY ACTIVE METABOLITES IN BLOOD BY BOND ELUT™ COLUMN EXTRACTION AND REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

SRINIVAS N. RAO, AMIYA K. DHAR, HENN KUTT and MICHIKO OKAMOTO*

Departments of Pharmacology and Neurology, Cornell University Medical College, 1300 York Avenue, New York, NY 10021 (U.S.A.)

(First received December 8th, 1981; revised manuscript received March 30th, 1982)

SUMMARY

A rapid and quantitative analytical micro method for the determination of diazepam and its major pharmacologically active metabolites utilizing high-performance liquid chromatography (HPLC) is reported. The drug and its metabolites were extracted from 50—100 μl samples of whole blood, serum or plasma using Bond Elut™ C_{18} column and quantitated by high-performance liquid chromatography, using Technicon Fast-LC-C-8 (RP 5 μm) bonded column and a mobile phase consisting of 53% methanol, 1% acetonitrile in KH_2PO_4 buffer and 10 μl/l triethylamine. Methyl nitrazepam and medazepam were used as internal and external standards, respectively. The extraction and recovery of diazepam and its major pharmacologically active metabolites, i.e., 3-hydroxydiazepam, desmethyldiazepam and oxazepam from blood were higher than 88% for all compounds. The minimum detection range of each compound was approximately 2.5 ng per 100-μl sample. This micro method of simultaneous quantitation of diazepam and its major pharmacologically active metabolites provides a valuable technique for the study of diazepam pharmacokinetics in a small animal model without disturbance of normal hemodynamics from excess blood loss, as well as in clinical evaluation of pediatric patients.

INTRODUCTION

Since the introduction of diazepam as an anxiolytic sedative hypnotic in 1959, many benzodiazepines have been used clinically, in a wide spectrum of disorders, for controlling insomnia and anxiety and producing central skeletal muscle relaxation. Likewise, several quantitative analytical procedures for determining benzodiazepines and their metabolites in biofluids have been developed utilizing colorimetry [1], spectrofluorometry [2, 3], gas chromato-

0378-4347/82/0000—0000/$02.75

graphy (GC) [4—6], polarography [7], radioimmunoassay [8, 9], and thin-layer chromatography with densitometry [10, 11].

Among all the methods available in the past, GC has provided adequate sensitivity and resolution for clinical investigation when combined with the electron-capture detector [5]. However, time-consuming extraction followed by derivatization procedures for determination of metabolites are required before application to the GC column. Furthermore, some of the benzodiazepines and their metabolites (for example, oxazepam and chlordiazepoxide are highly thermolabile and cannot be subjected to the high temperature needed for GC analysis [6—21].

Since the development of high-performance liquid chromatography (HPLC), efficient high-performance column packing techniques, and also a choice of a wide variety of bonded phase packing materials were made available, HPLC has become a method of choice for determination of drugs and their metabolites in biofluids, which are thermolabile, and have the distinct hydrophobic physicochemical properties suited for HPLC detection.

Several papers reporting analysis of benzodiazepines and their metabolites by the HPLC [11—16] method have been published. Most of these methods [17—19] are suitable for clinical evaluation of benzodiazepines and their representative metabolites concentrations in human plasma samples of 0.5 to 1.0 ml and therefore, the sample volume is not restricted. However, for the study of the pharmacokinetics of benzodiazepines and their metabolites in small animals, the availability of biofluid in amounts required in the previously published procedures, without disturbing hemodynamics, is a major problem.

Therefore, development of a microanalytical procedure which has a high sensitivity and resolution for drug detection from microquantities (50—100 μl) of biofluid is essential. Accordingly, this study reports the further refinement of presently available methods in the literature, utilizing the HPLC system to fit the analysis of diazepam and its metabolites, for example, from microquantity biofluid samples in conjunction with highly efficient Bond Elut^{TM} C_{18} column extraction.

EXPERIMENTAL

Reagents and drugs

Methanol and acetonitrile of analytical spectrophotometric grades, and water of HPLC grade were purchased from Burdick and Jackson Labs. (Muskegon, IL, U.S.A.) and J.T. Baker (Phillipsburg, NJ, U.S.A.), respectively. Inorganic reagents were reagent grade from Fisher Scientific (Pittsburgh, PA, U.S.A.).

The Bond Elut^{TM} C_{18} extraction column was obtained from Analytichem International (Harbor City, CA, U.S.A.).

Preparation of drug reference standards

Pure crystalline forms of diazepam, 3-hydroxydiazepam (temazepam), desmethyldiazepam (nordiazepam), oxazepam, medazepam and methylnitrazepam for reference standards were generously supplied by Hoffmann-La Roche (Nutley, NJ, U.S.A.).

Ten milligrams each of diazepam, 3-hydroxydiazepam, nordiazepam and

oxazepam were weighed and dissolved in 10 ml methanol in a siliconized volumetric flask (1 mg/ml concentration). The solution was further diluted under sonication to reach the final concentration ranging from 35 ng/ml to 25 μg/ml, either in mobile phase solution (for direct injection into HPLC), or in HPLC-grade water, or in blood, plasma or serum (for extraction and recovery studies). Ten milligrams each of medazepam (external standard) and methylnitrazepam (internal standard) were also weighed and dissolved individually in 10 ml methanol. Medazepam was further diluted in the mobile phase solution to achieve a final concentration ranging from 100 ng/ml to 10 μg/ml. Methylnitrazepam was further diluted into HPLC-grade water under sonication to achieve a final concentration of solutions ranging from 25 ng/ml to 25 μg/ml.

Preconditioning of column and extraction procedure

Bond Elut C_{18} columns (1 ml capacity) were positioned in luerlock fittings on the cover of a Vac-Elut™ system. Vacuum pressure was adjusted to 15 in. of mercury. Each column was prewashed repeatedly with a column volume of methanol and then with water through open-and-close processes of the vacuum pressure line.

After the last water wash the column was buffered to pH 9.5 by 100 μl 0.1 *M* sodium borate buffer (pH 9.5). Finally, the column was heparinized by 50 μl 1000 units/ml heparin. The preconditioned columns can be capped and refrigerated for future use.

For extraction, 50 of 100 μg of whole blood, plasma or serum containing the drug and its metabolites were applied to the column. Internal standard methylnitrazepam 100 ng in a 10-μl volume was applied to the column at this step. The sample was drawn through the column by vacuum and the drug and the metabolites were adsorbed on the column matrix. The matrix was then washed twice with water, followed by 50 μl of methanol. This small amount of methanol displaces water from the matrix, but does not elute the drug from the column. Vacuum was then turned off.

A microcentrifuge tube was placed under each extraction column in the Vac-Elut rack for drug elution procedures. With vacuum off, 200 μl of methanol were added to each column in which the drugs were adsorbed. After 2 min of equilibration, vacuum was turned on to elute the materials into the collection tube. Vacuum was turned off and the column was allowed to come to atmospheric pressure. An additional 100 μl of methanol were placed onto the column and the same procedure of elution was repeated in order to complete the elution process. The vacuum was left on for 30 sec in order to collect all the eluent. The eluent totalling 300 μl was dried under a stream of nitrogen. The residue was reconstituted in 25 μl of mobile phase liquid, or with external standard. A 10-μl volume of this reconstituted sample extract solution was injected into the chromatograph.

HPLC analytical procedure

A Hewlett-Packard high-performance liquid chromatograph Model No. 1081, fitted with a Rheodyne syringe loading sample injector with a 10-μl loop was used. The Schoeffel variable-wavelength ultraviolet detector was attached and the wavelength was set at 240 nm (0.002 AUFS).

The column was the Technicon Fast-LC-C-8 (RP 5 μm) bonded prepacked column (150 mm × 4.6 mm; Technicon, Tarrytown, NY, U.S.A.).

The HPLC mobile phase consisted of methanol—2 m*M* KH_2PO_4—acetonitrile (53:46:1). The flow-rate was maintained at 1.3 ml/min at 51 bar. The column temperature was set at 35°C.

Under reversed-phase chromatography conditions, the capacity factor (k') of diazepam was calculated and was 3.90 as defined by $k' = (V_2 - V_0)/V_0$, where V_2 = retention volume. Separation factor (α) values given in Table I were calculated for each compound by dividing the k' value of that compound by the k' value of diazepam.

The chromatogram was recorded on an *X*—*Y* recorder (1 mV gain 0.25 in./min speed) and the data processor was used to calculate the area and/or peak height of each compound analyzed.

RESULTS

Recovery of diazepam and its major metabolites from biofluid by Bond Elut C_{18} column

Fig. 1B shows a chromatogram of diazepam and its metabolite standards from blood extract through a Bond Elut C_{18} column. Blood containing 100 ng each of methylnitrazepam (internal standard), oxazepam, 3-hydroxydiazepam, nordiazepam and diazepam was extracted through the Bond Elut C_{18} column and chromatographed. Fig. 1A shows the chromatogram of the control blood sample which did not contain any of these compounds, but was extracted through the Bond Elut column.

Fig. 2 plots the concentration response relationships of diazepam and its three major metabolites, 3-hydroxydiazepam, nordiazepam, and oxazepam, which were spiked into 100 μl blood, recovered from the Bond Elut C_{18}

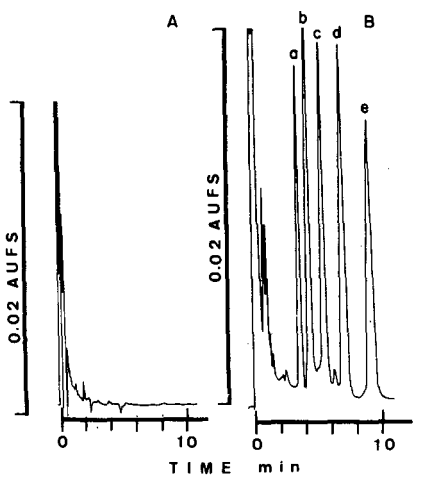


Fig. 1. Chromatograms of (A) blank control blood sample (B) diazepam and its metabolite standards. Blood containing 100 ng each of (a) methylnitrazepam (internal standard), (b) oxazepam, (c) 3-hydroxydiazepam, (d) N-desmethyldiazepam and (e) diazepam was extracted through the Bond Elut C_{18} column.

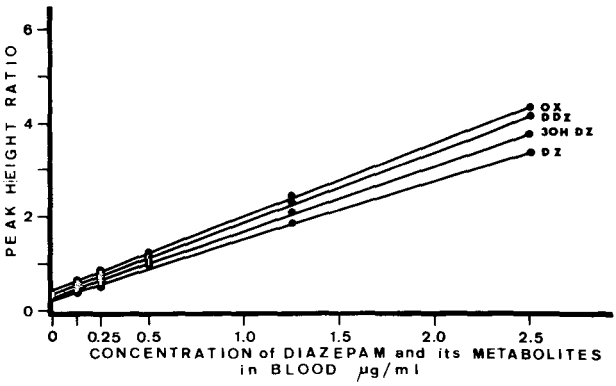


Fig. 2. Recovery concentration response curves of diazepam and its metabolites. Abscissa: initial concentrations of compound in 100 µl whole blood. Ordinate: response expressed as peak height ratio of each compound vs. medazepam as external standard. Curves: OX = oxazepam, DDZ = N-desmethyldiazepam, 3-OHDZ = 3-hydroxydiazepam and DZ = diazepam. Regression slope value ± S.E. (slope) was 1.75 ± 0.07 for oxazepam, 1.59 ± 0.06, for nordiazepam, 1.49 ± 0.06 for 3-hydroxydiazepam and 1.34 ± 0.05 for diazepam. Correlation coefficient for each slope was > 0.994.

TABLE I

HPLC EVALUATION OF SEPARATED DRUG DIAZEPAM AND METABOLITES ON TECHNICON FAST-LC-C-8 BONDED REVERSED-PHASE COLUMN

Compound	Retention time (min) ± S.E.	Capacity factor (k')	Resolution factor (α)
Methylnitrazepam	4.04 ± 0.009	1.05	0.27
Oxazepam	4.84 ± 0.009	1.45	0.37
3-Hydroxydiazepam (Temazepam)	5.99 ± 0.01	2.04	0.52
Desmethyldiazepam (Nordiazepam)	7.57 ± 0.01	2.84	0.73
Diazepam	9.65 ± 0.02	3.90	1.00

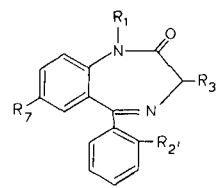
column, and were analyzed by HPLC with medazepam as external standard. As shown in Fig. 2, the relationship was linear for each compound and the quantities tested.

Table I shows HPLC evaluation of separated drugs, methylnitrazepam (internal standard), diazepam, oxazepam, 3-hydroxydiazepam, nordiazepam, and diazepam; retention time, k' and α for each of the above compounds are shown.

Table II shows the efficiency of the extraction and recovery of diazepam and its three major metabolites, i.e., 3-hydroxydiazepam, nordiazepam and oxazepam spiked into 100 µl blood, extracted and recovered through the Bond Elut C_{18} column. The quantities of these compounds tested ranged from 2.5—1000 ng (concentration range 62.5 ng/ml—2.5 µg/ml). As shown in Table II, the percentages of each compound which were recovered were consistently above 88%, on average 93%. Additionally, the recovery efficiency of methylnitrazepam, internal standard was tested and was 96.97 ± 0.54% S.E. at 100-ng quantities.

TABLE II

INTRA-ASSAY RECOVERY OF DIAZEPAM AND ITS METABOLITES FROM BLOOD



Compounds	Chemical structure				Retention time ± S.E. (min)	Spiked blood concentration (ng/100 μl)					
	R_1	R_3	R_7	$R_{2'}$		250	125	50	25	12.5	6.25
						Recovery ± S.E. (n = 12)					
Oxazepam	H	OH	Cl	H	4.84 ± 0.008	93 ± 2.0	93 ± 2.4	96 ± 3.1	88 ± 3.6	92 ± 3.2	98 ± 4.8
3-Hydroxydiazepam	CH_3	OH	Cl	H	5.99 ± 0.01	92 ± 1.63	94 ± 2.2	89 ± 3.3	90 ± 2.7	90 ± 3.29	94 ± 6.0
Desmethyldiazepam	H	H	Cl	H	7.57 ± 0.01	93 ± 1.02	99 ± 2.1	98 ± 2.9	91 ± 2.9	92 ± 3.3	93 ± 5.1
Diazepam	CH_3	H	Cl	H	9.65 ± 0.02	95 ± 2.0	95 ± 2.5	93 ± 2.9	91 ± 2.7	90 ± 3.7	89 ± 4.6

DISCUSSION

The analytical method for simultaneous quantitation of diazepam and its major metabolites from microquantities of blood reported herein, was based on a combination of two processes. One used the Bond Elut C_{18} column to achieve high extraction efficiency and recovery of the parent drug and its metabolites from microquantities of biofluids (50—100 μl); the second used the HPLC system with a Technicon Fast-LC-C-8 (RP 5 μm) column and the mobile phase to identify these compounds with high resolution and accuracy. As shown in the Results section, the quantity response relationship between the amount of diazepam and its metabolites in biofluid and the peak heights (or areas) registered on the HPLC tracings was linear in the quantity range between 2.5 ng and 1 μg. This quantity range will approximate the actual biofluid concentration ranges (50 ng/ml—25 μg/ml) of diazepam and its metabolites which are achieved in clinical situations, and also experimental laboratory conditions, utilizing small animals, from 50—100 μl volume samples, especially since, recently, the smaller volume loop for sample injection has become available for the HPLC system.

The Bond Elut column system was preferable to many extraction systems hitherto reported because of its reproducibility for extraction, and for the simplicity of the procedure. It requires only a one-step extraction and elution and, hence, significantly minimizes loss of drugs. Good and Andrews [20] have recently characterized the Bond Elut column and have extended their studies to determine the type of drugs which can be retained by the Bond Elut column and, hence, be used in combination with the HPLC system for quantitation of the drugs and their major metabolites in biofluid samples. In agreement with these investigators, the efficiency of elution was maximal with a 200-μl methanol volume followed by an additional 100-μl methanol volume in completing elution of benzodiazepines. The column can be preconditioned with an appropriate pH buffer; in our studies pH 9.5 was found to be most appropriate for adsorption of diazepam and its metabolites. When the column was preconditioned with heparin, the collected blood could go directly into the extraction column. Furthermore, on-column hemolysis of red blood cells is accomplished by simple application of HPLC grade water onto the column. Other benzodiazepines and their metabolites such as chlordiazepoxide and flurazepam can be extracted by a Bond Elut column and quantitated by HPLC from a small quantity of biofluid.

Although medazepam has been found suitable as an external standard for the consistency and linearity of detection responses in wide concentration ranges it was found inadequate as an internal standard because of its poor recovery from the Bond-elut column extraction system. In this respect methylnitrazepam was found most suitable as an internal standard in reflecting extractions and recoveries of diazepam and its major metabolites.

In summary, an HPLC method in conjunction with a Bond Elut C_{18} column for efficient extraction of diazepam and its major metabolites from microquantities of biofluid has been reported. The method is relatively simple and also reproducible, therefore, suitable for pharmacokinetic studies in small laboratory animals and/or in pediatric patients where the biofluid sample

volume is the major limiting factor, particularly if normal hemodynamics is to be maintained. Simultaneous detection and measurement of the parent compound and its major metabolites is also essential for other benzodiazepine type drugs because they are biotransformed into several pharmacologically active metabolites and, hence the overall pharmacologic action is considerably complex.

ACKNOWLEDGEMENTS

These studies were supported by Grant No. DA-00591NIDA. The initial findings were reported at the 8th U.P.H.A.R., Tokyo, 1981 (Abstract P560).

REFERENCES

1 P. Jatlow, Clin. Chem., 18 (1972) 516.
2 B.A. Koechliu and L. D'Arconte, Anal. Biochem., (1963) 195.
3 H.A. Schwartz and E. Postma, J. Pharm. Sci., 61 (1972) 123.
4 I.J.A. Zingales, J. Chromatogr., 61 (1971) 237.
5 J.A.F. de Silva, I. Bekersky, C.V. Puglisi, M.A. Brooks and R.E. Weinfield, Anal. Chem., 48 (1976) 10.
6 A.K. Dhar and H. Kutt, Clin. Chem., 35 (1979) 137.
7 M.R. Hackman, M.A. Brooks and J.A.F. de Silva, Anal. Chem., 46 (1974) 1075.
8 W.R. Dixon, J. Earley and E. Postma, J. Pharm. Sci., 64 (1975) 937.
9 B. Peskar and S. Spector, J. Pharmacol. Exp. Ther., 186 (1973) 167.
10 N. Strojny, K. Bratin, M.A. Brooks and J.A.F. de Silva, J. Chromatogr., 143 (1977) 363.
11 C.G. Scott and P. Bommer, J. Chromatogr. Sci., 8 (1970) 446.
12 N. Strojny, C.V. Puglisi and J.A.F. de Silva, Anal. Lett., B11 (1978) 135.
13 T.B. Vree, A.M. Baars, Y.A. Hekster, E. van der Kleijn and W.J. O'Reilly, J. Chromatogr., 162 (1979) 605.
14 T.B. Vree, A.M. Baars, Y.A. Hekster, E. van der Kleijn and W.J. O'Reilly, J. Chromatogr., 162 (1979) 605.
15 T.B. Vree, A.M. Baars, Y.A. Hekster and E. van der Kleijn, J. Chromatogr., 224 (1981) 519.
16 U.R. Tjaden, M.T.H.A. Meeles, C.P. Thys and M. van der Kaay, J. Chromatogr., 181 (1980) 227.
17 B.R. Brodie, L.F. Chasseaud and T. Taylor, J. Chromatogr., 150 (1978) 361.
18 D.J. Greenblatt, R.I. Shader and J. Koch-Wesser, Arch. Gen. Psychiat., 32 (1975) 518.
19 D.J. Greenblatt, R.I. Shader, S.M. MacLeod and E.M. Sellers, Clin. Pharmacokin., 6 (1978) 483.
20 T.J. Good and J.S. Andrews, J. Chromatogr. Sci., 19 (1981) 562.
21 S. Cotler, C.V. Puglisi and J.H. Gustafson, J. Chromatogr., 222 (1980) 95.

Journal of Chromatography, 231 (1982) 349—360
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1326

AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF ANTIPYRINE AND ITS METABOLITES IN URINE

SOME PRELIMINARY RESULTS OBTAINED FROM SMOKERS AND NON-SMOKERS

AKIHIKO NAKAGAWA and KAN-ICHI NAKAMURA*

Analytical and Metabolic Research Laboratories, Sankyo Co., Ltd., Hiro-machi 1-2-58, Shinagawa-ku, Tokyo 140 (Japan)

and

TAKASHI ISHIZAKI and KAN CHIBA

Division of Clinical Pharmacology, Clinical Research Institute, National Medical Center, Tokyo (Japan)

(First received January 14th, 1982; revised manuscript received April 30th, 1982)

SUMMARY

A simple, accurate and fully automated high-performance liquid-chromatographic method was developed for the simultaneous determination of antipyrine (AP), 3-hydroxymethylantipyrine (3HMA), 4-hydroxyantipyrine (4OHA) and norantipyrine (NORA) in urine. This method requires no extraction step and only one chromatographic run with the use of a reversed-phase system. The coefficient of variation (%) (n=8 each) was: 4.14 for AP, 2.31 for 3HMA, 3.48 for 4OHA, and 2.71 for NORA. The method was applied to studies on AP metabolism in three smokers and three non-smokers who received an oral 10 mg/kg dose of AP. These preliminary results suggest that smokers appear to excrete more 4OHA and NORA in the urine than non-smokers.

INTRODUCTION

Antipyrine (AP) plasma or saliva half-life ($t_{1/2}$), or the derived metabolic clearance rates, are widely used to assess activities of the hepatic monooxygenase system in man [1,2]. The elimination rate or clearance of AP alone, however, does not seem to be an absolute indicator of an individual's capacity

0378-4347/82/0000—0000/$02.75

to metabolize drugs. For example, the overall metabolic clearance rate of AP and the clearance of many other drugs that are known to be mainly oxidized are poorly correlated [3]. Several investigators have demonstrated the absence of correlation between the microsomal cytochrome *P*-450 content and the metabolic clearance rate of AP in animals [4,5] and in humans [6—8].

A possible explanation for the observed lack of correlation between the rates of drug metabolism in vivo may be related to the heterogeneity of multiple forms of cytochrome *P*-450 [9], where each metabolic pathway of AP may be involved differently, since the AP metabolism is rather complicated. The primary metabolites of AP are 4-hydroxyantipyrine (4OHA), 3-hydroxymethylantipyrine (3HMA), and norantipyrine (NORA or N-desmethylantipyrine) [10—18]. Previous investigators have documented that a deficiency or absence of one biotransformation in a multimetabolized drug may be overlooked if one measures only the disappearance of the parent drug but not the appearance of its metabolites [19,20]. Hence, to assess AP as a hepatic probe for measuring the oxidative process of drug metabolism in man, investigation of the urinary excretion of the primary AP metabolites should be very important.

Until recently, studies on AP metabolism have been hampered by the lack of convenient methods for determining the different metabolites. The published gas—liquid chromatographic (GLC) methods have precluded the simultaneous determination of AP, 4OHA, 3HMA and NORA [11,17]. The use of high-performance liquid chromatography (HPLC), however, offers a useful tool to overcome difficulties involved in the GLC methods and avoids derivatization of the metabolites [14,15,18]. Among the HPLC methods recently reported, the method of Danhof and Breimer [16] requires two extraction steps and two separate chromatographic runs for the determination of AP, 4OHA, 3HMA and NORA, while that of Eichelbaum et al. [18] involves only one extraction step and one chromatographic run.

We report here on a fully automated HPLC system for the determination of AP and its three major metabolites in human urine which involves no extraction step and only one chromatographic run. This system permits the simple, rapid, sensitive, and quantitative determination of AP, 4OHA, 3HMA, and NORA in the urine. This method was also applied to test the urinary excretion of these metabolites in each of three healthy smoking and non-smoking subjects. Our method can be used as a clinical routine to examine hepatic drug metabolism in man.

EXPERIMENTAL

Chemicals

AP and NORA were obtained from Aldrich (Milwaukee, WI, U.S.A.). 3HMA and 4OHA were synthesized according to the methods described by Yoshimura et al. [21], and Knorr and Pshorr [22], respectively. The structure of the compounds synthesized was confirmed by mass spectrometry (JMS D-300, JEOL, Tokyo, Japan). Glusulase was obtained from Endo Labs. (Garden City, NY, U.S.A.). β-Glucuronidase from bovine liver type B-1, sulfatase from limpet type V, and saccharo-1,4-lactone were purchased from Sigma (St. Louis, MO, U.S.A.). All solvents and chemicals used were of analytical grade and were purchased from Wako (Osaka, Japan).

Apparatus

The automated high-performance liquid chromatograph was constructed with commercially available and laboratory-made components. A solvent-delivery system (M-45 pump) and an automatic sampler (WISP-710B) from Waters Assoc. (Milford, MA, U.S.A.) were used. A variable-wavelength UV detector, UVIDEC-100-III from JASCO (Tokyo, Japan), was operated at 252 nm. The chromatographic data were processed by an HP-3388 reporting integrator from Hewlett-Packard (Avondale, PA, U.S.A.). The interface between the WISP-710B sampler and HP-3388 integrator was newly contrived. To maintain the sample temperature at 37°C during enzymatic hydrolysis or at 5°C for the actual run, a thermostatic bath unit was built in the WISP-710B sampler. The available temperature ranged from −5°C to +50°C within ±1°C. The flow diagram of the total system developed is shown in Fig. 1.

Chromatographic conditions

For rapid and simple HPLC determination of a drug in biological specimens without the tedious clean-up procedure, it is considered advantageous to use a reversed-phase system. We intended to separate AP, 4OHA, 3HMA, and NORA in a single reversed-phase HPLC run. Although we tested and compared several reversed-phase columns (μBondapak C_{18}, Waters; Hibar RP-2 and RP-8, Merck, Darmstadt, G.F.R.; and Radial-Pak C_8 and C_{18}, Waters), only Radial-Pak C_8 (10 μm, 5 mm I.D.) was found to achieve this goal. The mobile phase was methanol—150 m*M* ammonium acetate solution (33.5:66.5) and the flow-rate was set at 1.5 ml/min (ca. 70 kg/cm^2). The concentration of the acetate buffer of the eluent was found to be critical for the separation of AP and 4OHA. The quantitation of each compound was based on the external area method using the HP-3388 integrator (Fig. 1).

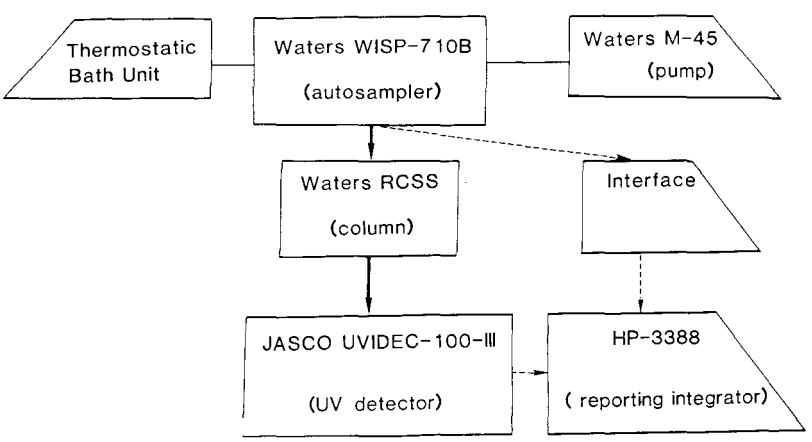


Fig. 1. Flow diagram of automated HPLC system. Thick arrows indicate the sample flow system; broken arrows indicate the operational sequence undergoing the signal.

Sample preparation and calibration curves

To 1.0 ml of urine sample, 1.0 ml of 1.0 *M* acetate buffer (pH 5.2) and 2.0 ml of *n*-hexane were added successively. The solution was mixed for 30 sec with a vortex-type mixer. After centrifuging at 1400 *g* for 5 min, the organic phase was discarded. A few hundred (200—400) microlitres of the aqueous phase were transferred to a micro-sample tube for the analysis of AP and 3 HMA excreted in urine as the free form. To 1.0 ml of the aqueous phase in a sample bottle, 0.5 ml of 10% sodium metabisulfite ($Na_2S_2O_5$) and 1.0 ml of Glusulase solution containing 10,000 Fishman Units (FU) per millilitre of β-glucuronidase were added. The solution was mixed for a few seconds and incubated at 37°C for 2 h in the sampler. Then the temperature of the bath was cooled down to 5°C for the analysis of total 3HMA, and 4OHA- and NORA-glucuronide. The injection volume of each sample was set at 10 μl.

To determine the amount of AP and its three major metabolites excreted in the urine, absolute calibration curves were prepared as follows. The urine was adjusted to pH 5.2 by the same volume of 1.0 *M* acetate buffer and was washed with *n*-hexane. Known amounts of AP and 3HMA, 4OHA and NORA were added and these standard urine solutions were then analyzed according to the procedures described above. The linear calibration curves were obtained by the least-squares method and tested for coefficient correlations.

RESULTS AND DISCUSSION

Sample clean-up procedure

No interfering peaks were eluted under the chromatographic conditions after a 10-μl injection of urine without *n*-hexane treatment. The enzymatic hydrolysis did not affect the separation. However, the life span of the column was found to be prolonged and the analysis time was shortened to 12 min by the pre-treatment. The non-polar endogenous substances were found to be removed effectively by the *n*-hexane washing. The loss by employing this step was negligibly small for AP. The recovery was 100.7 ± 1.2% (14.1 μg/ml of urine, $n=5$). Even for 3HMA excreted as the free form, the recovery was 96.8 ± 0.3% (18.5 μg/ml of urine, $n=5$).

Fig. 2 shows typical chromatograms of urine treated with and without enzymatic hydrolysis by Glusulase after an oral administration of 10 mg/kg AP to a volunteer. The Radial-Pak C_8 column with a radial compression separation system (RCSS) seemed to be suitable for human experiments; the theoretical plate was rather high (Fig. 2), the life span was about 500 runs of hexane-treated samples, and the operating pressure was constantly low.

Enzymatic hydrolysis

The time courses of the enzymatic hydrolysis are shown in Fig. 3A, B and C. The stability of AP metabolites under the enzymatic hydrolysis used was examined. First, in comparison of the two sources of β-glucuronidase, Glusulase and type B-1 β-glucuronidase, the former (Fig. 3B) showed a higher yield and faster equilibrium than the latter (Fig. 3A). Second, in the case of 3HMA and 4OHA conjugates, 1 m*M* saccharo-1,4-lactone perfectly inhibited the enzyme, so that these two metabolites were identified as the corresponding

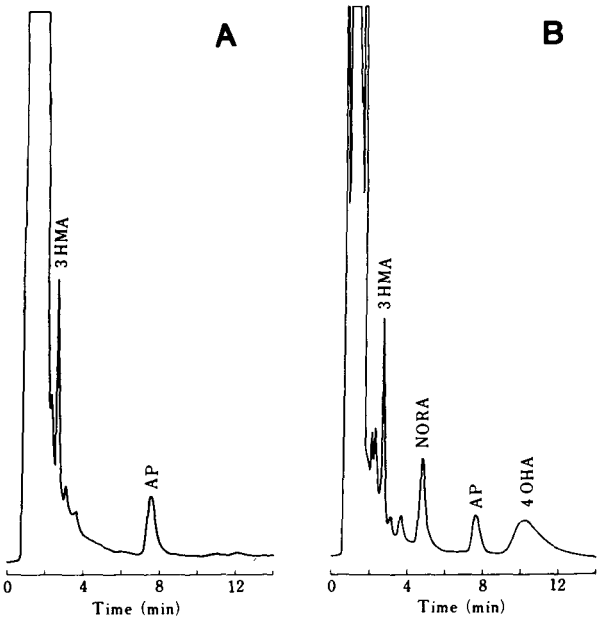


Fig. 2. Chromatograms of urine sample treated (A) without and (B) with enzymatic hydrolysis by Glusulase. The chromatograms indicate peaks corresponding to antipyrine and its metabolites. The drug concentrations are (μg/ml of urine): AP, 15.84; 3HMA free, 20.54; 3HMA total, 40.18; 4OHA, 94.4; NORA, 53.13.

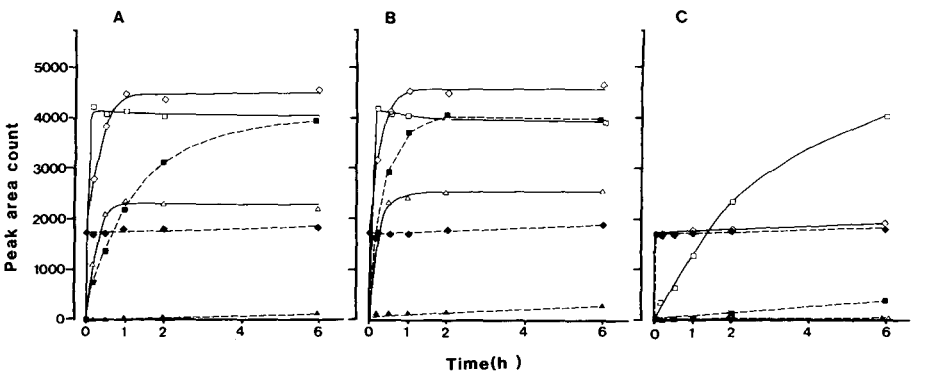


Fig. 3. Time courses of enzymatic hydrolysis under treatment conditions of (A) β-glucuronidase (20,000 FU/ml of urine), (B) Glusulase (20,000 FU β-glucuronidase and 2500 U sulfatase per ml of urine), and (C) sulfatase (100 FU β-glucuronidase and 1000 U sulfatase per ml of urine). ◇, 3HMA, ◆, 3HMA + saccharo-1,4-lactone 1 m*M*, △, 4OHA; ▲, 4OHA + saccharo-1,4-lactone 1 m*M*; □, NORA; ■, NORA + saccharo-1,4-lactone 1 m*M*. $Na_2S_2O_5$, 100 mg/ml of urine, was added to each treatment as an antioxidant.

β-glucuronide form. The behaviour of the NORA conjugate against saccharo-1,4-lactone inhibition was quite different from that of 3HMA- and 4OHA-glucuronide; the rate of hydrolysis was very fast, and this reaction was not inhibited by the generally used amount of saccharo-1,4-lactone (1 m*M*). However, the hydrolysis by sulfatase which contained 100 FU of

β-glucuronidase produced only a trace amount of free NORA, if any, as shown in Fig. 3C. These results indicate that the compounds to be quantitated in human urine include AP, 3HMA-free, 3HMA-glucuronide, 4OHA-glucuronide, and NORA-glucuronide. These findings are in good agreement with those of Inaba and Fischer [17], and Eichelbaum et al. [18], even though the former workers did not measure 3HMA.

We took into account other parameters to be optimized in the quantitative enzymatic hydrolysis, i.e. pH, incubation time, and amount of antioxidant as follows. (1) The favorable pH for hydrolysis with Glusulase ranged from 4.0 to 5.2 for a 3-h incubation with antioxidant ($Na_2S_2O_5$), and under this condition the yield of 4OHA, 3HMA, and NORA was found to be constant. However, since white precipitates were produced below pH 4.6, pH 5.2 was considered optimum. (2) The amounts of Glusulase and antioxidant, and the incubation time correlated to each other during the hydrolysis, so that each variable had to be approximately adjusted. To optimize these variables, the amounts of Glusulase were changed from 2500 to 20,000 FU of β-glucuronidase per ml of urine, and those of $Na_2S_2O_5$ from 0 to 100 mg/ml of urine for 0- to 4-h incubation. Fig. 4A shows the effect of the amount of antioxidant on the stability of NORA, the most unstable metabolite of AP [18], with 20,000 FU of β-glucuronidase per ml of urine. The optimum amount of $Na_2S_2O_5$ was found to be 100 mg/ml of urine, whereas 1 mg $Na_2S_2O_5$ was sufficient to prevent decomposition for 4OHA. (3) The effect of Glusulase potency on the hydrolysis of 3HMA- and 4OHA-glucuronide at 100 mg of $Na_2S_2O_5$ per ml of urine is illustrated in Fig. 4B. The hydrolysis of NORA-glucuronide did not depend upon the potency of Glusulase, and it reached a maximum immediately

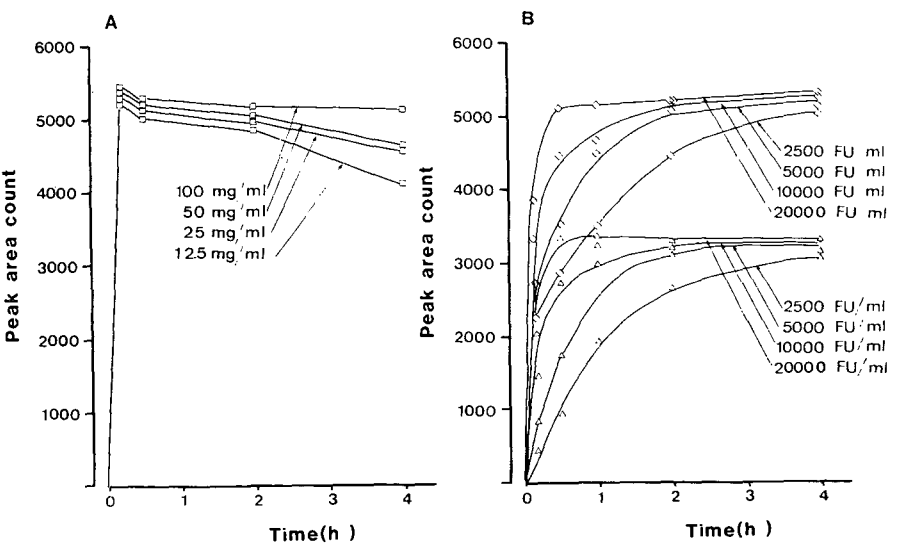


Fig. 4. Effect of (A) $Na_2S_2O_5$ amount on the stability of NORA during enzymatic hydrolysis with Glusulase, 20,000 FU/ml of urine, and (B) Glusulase potency on the hydrolysis of 3HMA-glucuronide (◇) and 4OHA-glucuronide (△) with 100 mg/ml $Na_2S_2O_5$. The amounts of $Na_2S_2O_5$ (A) and Glusulase (B) per millilitre of urine are indicated in the figure.

after the incubation, which is consistent with the finding of Inaba and Fischer [17]. The hydrolysis rates of 3HMA- and 4OHA-glucuronide were time-dependent at each amount of enzyme. At 20,000 FU/ml of urine, these two conjugates gave maximum yields after a 2-h incubation. Thus, the optimum conditions required for the stability of NORA were: 20,000 FU β-glucuronidase plus 100 mg of $Na_2S_2O_5$ per ml of urine plus 2-h incubation at 37°C.

Reproducibility, linearity, and efficiency

This assay differed from other studies, the use of an internal standard was unnecessary since there was no complicated solvent extraction, nor were there evaporation and reconstitution processes. We applied an external standard method by means of a simple clean-up procedure with high recovery and an automatic sampler with good precision and reproducibility on the injection procedures.

The data for reproducibility and linearity obtained from this method are set out in Table I. The results for AP and its three metabolites all gave satisfactory values for coefficients of variation and correlation coefficients. Only 1.0 ml of urine from human subjects, who were administered orally 10 mg/kg AP, was required for the determination of all the compounds, including free and conjugated forms.

TABLE I

REPRODUCIBILITY AND LINEARITY OF THE ANALYSIS OF ANTIPYRINE AND METABOLITES IN URINE

Substance*	Concentration (μg/ml)	Coefficient of variation** (%)	Concentration ranges examined (μg/ml)	Correlation coefficient (r)
AP	10.19	3.95	2.55—40.76	0.9987
	20.38	4.14		
3HMA	25.84	2.02	6.46—103.36	0.9994
	51.68	2.31		
4OHA	50.43	4.33	12.61—201.72	0.9972
	100.86	3.48		
NORA	50.55	3.06	12.64—202.20	0.9993
	101.10	2.71		

*AP = Antipyrine; 3HMA = 3-hydroxymethylantipyrine; 4OHA = 4-hydroxyantipyrine; NORA = norantipyrine.

**Calculated from eight replicates for each substance.

By using our HPLC method, the analysis time required for AP and its metabolites was 12 min, which is fairly compatible with other HPLC methods reported [14,15,18]. Furthermore, in order to evaluate the efficiency of our automated HPLC method, the number of samples that could be analyzed per day was also tested. It was found that a total of 43 measurements for urine specimens (≈ maximum efficiency), which included the parent drug and its

TABLE II

SUBJECT CHARACTERISTICS AND PHARMACOKINETIC PARAMETERS OF ANTIPYRINE IN PLASMA

Subjects	Age (years)	Sex	Body weight (kg)	Average cigarette consumption (No. per day)	Kinetic parameters of antipyrine*		
					$t_{1/2}$ (h)	V_d (l/kg)	MCR (ml per h per kg)
1. T.I.	39	M	65	30—40	11.5	0.622	37.4
2. T.S.	27	M	62	20—30	12.0	0.632	36.6
3. K.S.	23	M	60	20—30	11.7	0.681	40.4
Mean ± S.E.M.	29.7 ± 4.8		62.3 ± 1.5	—	11.7 ± 0.2	0.645 ± 0.022	38.1 ± 1.4
4. T.F.	26	F	54	0	14.7	0.608	28.7
5. T.E.	25	M	56	0	15.2	0.602	27.5
6. S.K.	34	M	78	0	14.9	0.539	25.2
Mean ± S.E.M.	28.3 ± 2.8		62.7 ± 7.7	—	14.9 ± 0.2	0.583 ± 0.027	27.1 ± 1.1

*Antipyrine half-life ($t_{1/2}$) was determined from the linear portion of the plot of plasma concentration versus time on semilog paper. The apparent volume of distribution (V_d) was calculated from the dose of the drug administered divided by the extrapolated y-intercept for drug concentration in plasma at time zero. The metabolic clearance rate (MCR) was calculated as $MCR = 0.693 \cdot V_d / t_{1/2}$.

metabolites in free form and as conjugates, were successfully accomplished with the present method.

Human study

The characteristics of six subjects (three smokers and three non-smokers), who received an oral dose of 10 mg/kg AP with 200 ml of orange juice after an overnight fast, and AP disposition data calculated from a one-compartment model analysis are summarized in Table II. A smoker was defined as a person taking more than 20 cigarettes per day for a year or more. Blood samples (3—5 ml) were collected at time 0, 2, 4, 6, 8, 12 and 24 h. AP in plasma had been measured with the GLC method previously reported [23]. A shorter $t_{1/2}$ or higher metabolic clearance rate in smokers than in non-smokers is compatible with the findings observed previously [24,25].

The automated HPLC method was applied to obtain a preliminary result for ascertaining if our method could be used for human study, and if the enhanced metabolic rate of AP in plasma in relation to the smoking habit may be reflected in the primary metabolites excreted in their urine. Urine samples were collected at the following time intervals: 0—4, 4—8, 8—12, 12—24 and 24—48 h. Urine was frozen at −20°C until analysis.

Shown in Fig. 5 are the mean data of the plasma concentration—time curves of AP and of the urinary excretion rates of AP and metabolites obtained from the three smokers and three non-smokers. Individual data of the amounts (percentage dose of AP) of AP, 3HMA-free, 3HMA-glucuronide, 3HMA-total, 4OHA-glucuronide, and NORA-glucuronide excreted in 0—48 h urine are given in Table II. Smokers tended to excrete higher amounts of 4OHA- and NORA-

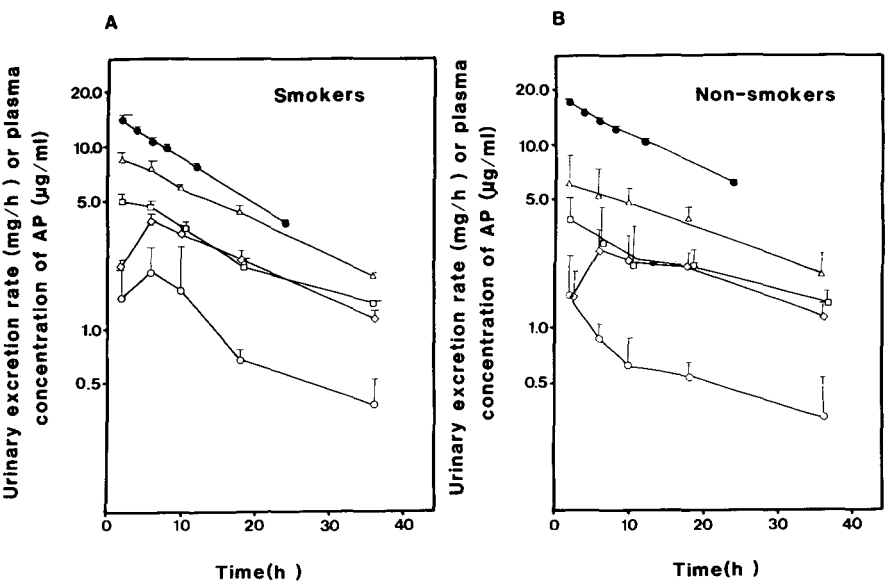


Fig. 5. Plasma concentration—time curves of antipyrine (●) and urinary excretion rates of antipyrine (○), 3HMA (◇), 4OHA (△) and NORA (□) in (A) smokers and (B) non-smokers. The mean ± S.E.M. values obtained from three smokers and three non-smokers are plotted.

TABLE III

CUMULATIVE URINARY EXCRETION OF ANTIPYRINE AND METABOLITES IN 48 h AS PERCENTAGE OF THE DOSE

Subjects*	AP, Free	3HMA			4OHA, Glucuronide	NORA, Glucuronide	Total
		Free	Glucuronide	Total			
Smokers							
1. T.I.	8.55	7.26	7.41	14.67	30.51	17.78	71.51
2. T.S.	6.16	5.90	8.94	14.84	31.04	19.20	71.24
3. K.S.	3.34	6.07	9.25	15.32	28.53	17.20	64.39
Mean ±	6.02	6.41	8.53	14.94	30.03	18.06	69.05
± S.E.M.	±1.84	±0.52	±0.70	±0.24	±0.94	±0.73	±2.85
Non-smokers							
4. T.F.	5.00	5.06	5.62	10.68	18.73	15.97	50.38
5. T.E.	2.75	4.60	8.57	13.17	26.61	11.21	53.74
6. S.K.	4.38	4.19	8.75	12.94	27.63	17.37	62.32
Mean	4.04	4.62	7.65	12.26	24.32	14.85	55.48
± S.E.M.	±0.82	±0.31	±1.24	±0.97	±3.44	±2.28	±4.35

*Subject number is the same as in Table II.

glucuronide. For AP and 3HMA, the differences between smokers and non-smokers appeared to be small. The total amount excreted in urine as a percentage of the AP dose administered tended to be greater in smokers (mean ca. 69%) than in non-smokers (ca. 55%), and the remaining amount (ca. 31—45%) of the administered dose was not detected with the assay method we employed (Table III). Although no exact explanation be offered for these phenomena, we are tempted to assume that AP may likely be metabolized via other pathway(s), as yet uncharacterized or unidentified. Eichelbaum et al. [18] found that on average 67—73% of the dose can be accounted for as AP and its three metabolites plus another minor metabolite, 3-carboxyantipyrine (4—5%). Excluding the latter, our figure of percentage dose seems to be in reasonable agreement with the finding of Eichelbaum et al. [18]. However, the trend towards the observation that AP also tends to be less in non-smokers than in smokers (Table III) remains totally unexplainable with the limited data of the present study.

The slopes of the excretion rates of 4OHA and NORA were fairly coincident with plasma AP disappearance in both smokers and non-smokers (Fig. 5). These preliminary results from human study suggest that the fundamental differences in the metabolic disposition of a drug such as AP, which is known to be oxidized by the hepatic microsomal system [3—6,8,9], exist in relation to the smoking habit, as has been indicated for other oxidized drugs [26—29]. Undoubtedly, whether our automated HPLC method will have a wide applicability to human study awaits further investigation using a large number of subjects with diverse clinical conditions where AP is used as a model marker to test the oxidative process of drug metabolism.

REFERENCES

1 I.H. Stevenson, Brit. J. Clin. Pharmacol., 4 (1977) 261—265.
2 E.S. Vesell, Clin. Pharmacol. Ther., 26 (1979) 275—286.
3 F. Sjöqvist and C. von Bahr, Drug Metab. Dispos., 1 (1973) 469—482.
4 B.E. Statland, P. Astrup, C.H. Black and E. Oxholm, Pharmacology, 10 (1973) 329—337.
5 E.S. Vesell, C.J. Lee, G.T. Passananti and C.A. Shively, Pharmacology, 10 (1973) 317—328.
6 D.D. Davies, S.S. Thorgeirsson, A. Breckenridge and M. Orme, Drug Metab. Dispos., 1 (1973) 411—417.
7 G.C. Farrel, W.G.E. Cooksley and L.W. Powell, Clin. Pharmacol. Ther., 26 (1979) 483—492.
8 D. Vuitton, J.P. Miguet, G. Camelot, C. Delafin, C. Joanne, B. Bechtel, M. Gillet and P. Carayon, Gastroenterology, 80 (1981) 112—118.
9 E.S. Vesell, Clin. Pharmacol. Ther., 22 (1978) 659—679.
10 H. Yoshimura, H. Shimeno and H. Tsukamoto, Biochem. Pharmacol., 17 (1968) 1511—1516.
11 C.E. Hignite, C. Tschanz, D.H. Huffman and D.L. Azarnoff, Drug Metab. Dispos., 6 (1978) 288—295.
12 J.D. Baty and D.A. Price Evans, J. Pharm. Pharmacol., 25 (1973) 83—84.
13 B.B. Brodie and J. Axelrod, J. Pharmacol. Exp. Ther., 98 (1950) 97—104.
14 G.H. Kellerman and M. Luyten-Kellerman, Life Sci., 23 (1978) 2485—2490.
15 M. Danhof, E. de Groot-van der Vis and D.D. Breimer, Pharmacology, 18 (1979) 210—223.

16 M. Danhof and D.D. Breimer, Brit. J. Clin. Pharmacol., 8 (1979) 529—537.
17 T. Inaba and N.E. Fischer, Can. J. Physiol. Pharmacol., 53 (1980) 17—21.
18 M. Eichelbaum, B. Sonntag and H.J. Dengler, Pharmacology, 23 (1981) 192—202.
19 T. Inaba, B.K. Tang, L. Endrenyi and W. Kalow, Clin. Pharmacol. Ther., 20 (1976) 439—444.
20 W. Kalow, B.K. Tang, D. Kadar and T. Inaba, Clin. Pharmacol. Ther., 24 (1978) 576—582.
21 H. Yoshimura, H. Shimeno and H. Tsukamoto, Chem. Pharm. Bull. (Tokyo), 19 (1971) 41—51.
22 L. Knorr and P. Pshorr, Justus Liebigs Ann. Chem., 293 (1896) 49—55.
23 L.F. Prescott, K.K. Adjepon-Yamoah and E. Roberts, J. Pharm. Pharmacol., 25 (1973) 205—207.
24 R.E. Vestal, A.H. Norris, J.D. Tobin, B.H. Cohen, N.W. Shock and R. Andres, Clin. Pharmacol. Ther., 18 (1975) 425—432.
25 P. Hart, G.C. Farrell, W.G.E. Cooksley and L.W. Powell, Brit. Med. J., ii (1976) 147—149.
26 E.J. Pantuck, K.C. Hsiao, A. Maggio, K. Nakamura, R. Kuntzman and A.H. Conney, Clin. Pharmacol. Ther., 15 (1973) 9—17.
27 J.J. Grygiel and D.J. Birkett, Clin. Pharmacol. Ther., 30 (1981) 491—496.
28 D.P. Vaughan, A.H. Beckett and D.S. Robbie, Brit. J. Clin. Pharmacol., 3 (1976) 279—283.
29 W.J. Jusko, J. Pharmacokinet. Biopharm., 6 (1978) 7—39.

Journal of Chromatography, 231 (1982) 361—376
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1317

LIQUID CHROMATOGRAPHIC ASSAY OF PHENOTHIAZINE, THIOXANTHENE AND BUTYROPHENONE NEUROLEPTICS AND ANTIHISTAMINES IN BLOOD AND PLASMA WITH CONVENTIONAL AND RADIAL COMPRESSION COLUMNS AND UV AND ELECTROCHEMICAL DETECTION

STEPHEN H. CURRY*, EDGAR A. BROWN, OLIVER Y.-P. HU and JOHN H. PERRIN

Department of Pharmaceutics, College of Pharmacy, J. Hillis Miller Health Center, University of Florida, Gainesville, FL 32610 (U.S.A.)

(First received December 23rd, 1981; revised manuscript received April 14th, 1982)

SUMMARY

An assay strategy for determining a wide range of phenothiazine, thioxanthene and butyrophenone neuroleptics and antihistamines both alone and in combination in blood and plasma is described. The general method employs liquid chromatography with both conventional and radial compression nitrile bonded columns. Detection is by ultraviolet absorption spectrophotometry or by amperometry depending on the concentrations to be measured. Ultraviolet absorption is suitable down to 10 ng/ml. Below this level amperometry is preferable. The various compounds are used as internal standards for each other. The lower limit of detection is approximately 0.1 ng ml^{-1} with a 10-ml sample. The within-run coefficient of variation is a maximum of 7.3%.

INTRODUCTION

The phenothiazines, thioxanthenes, dibenzazepines and butyrophenones which are used as neuroleptics, antidepressants and antihistamines are weak bases with highly lipophilic non-ionized species. The standard approach [1] to their assay in human plasma involves alkalinization of the sample, extraction of the drug into an organic solvent, reduction of the extract to small volume, and assessment of the drug content by gas chromatography (GC). In the case of the neuroleptics, GC using electron-capture, flame ionization, or thermionic sensitive detection has been successful with chlorpromazine, fluphenazine, perphenazine, thioridazine, butaperazine and haloperidol, while dibenzazepine antidepressants have been successfully assayed by both GC and liquid chromatography (LC) [2—8]. LC was recently successfully applied to biological samples of promethazine, one of the phenothiazine antihistamines

0378-4347/82/0000—0000/$02.75

[9] using electrochemical detection [10, 11]. Between 20 and 30 other drugs within this classification are available world-wide, either as marketed drugs or as drugs under investigation in humans [12]. There is a need for specific methods of assay for all of these compounds [13, 14]. The development of an LC approach with applicability to all of them at concentrations found or anticipated in clinical samples seems desirable. The research described in this paper is a step towards this. A preliminary report of this work has already been presented [15].

EXPERIMENTAL

Apparatus

A Varian 5000 liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) with a Valco injection valve, or, in a few experiments, a Waters Model 440 liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) were used, with detection at a fixed wavelength of 254 nm. A Varian Micropak CN (10 μm) column was routinely used. However, a Waters radial compression separation system with a nitrile-bonded column was also used. Electrochemical detection was achieved using a glassy carbon electrochemical (amperometric) detector (Bioanalytical Systems, Lafayette, IN, U.S.A.) employing an LC-4A controller, and a silver—silver chloride reference electrode. The electrochemical detector potential was +0.9 V. Detector response was recorded using (i) a 1-mV or 10-mV strip chart recorder (Varian), or (ii) a CDS-111 integrator (Varian).

Mobile phase

The mobile phase consisted of 90% acetonitrile or methanol and 10% aqueous ammonium acetate. The ammonium acetate concentration was varied from 0.005 *M* to 0.2 *M*. A flow-rate of 2.0 or 2.5 ml/min was used.

Chemicals and reagents

Reference samples of marketed drugs were obtained from the manufacturers and used as supplied. Small quantities of model metabolites, principally of chlorpromazine, were obtained from the U.S. National Institute of Mental Health and used as supplied. Pesticide-grade mixed hexanes and LC-grade acetonitrile and methanol were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). All other chemicals were analytical grade from Fisher Scientific. The diethyl ether was used from freshly opened small volume containers to ensure that it was peroxide free.

Stock solutions of marketed drugs and their metabolites

These were prepared by dissolving weighed quantities (generally approximately 10 mg) in sufficient methanol to give 1 mg/ml solutions. Dilutions were prepared in methanol to 10 μg/ml. Plasma standards were prepared by diluting appropriate volumes of these solutions with plasma such that the methanol content was never more than 1%. Stock solutions and plasma standards were stable for at least one month stored in the dark at 4°C (methanol solutions) or frozen (plasma solutions).

Extraction procedure using hexane

Plasma samples (1—5 ml) were made alkaline with 1 ml of 1 *N* sodium hydroxide solution, and extracted with mixed hexanes for 30 min. After centrifugation, 9-ml aliquots of the hexane layers were removed and evaporated to dryness at 30°C under a stream of nitrogen. The residues were redissolved in the mobile phase (100 μl) and chromatographed (50 μl samples).

Extraction procedure using diethyl ether

Plasma samples (1—5 ml) were added to tubes containing phosphate buffer (pH 7.4, 0.1 *M*) and extracted with diethyl ether (10 ml) for 30 min. After centrifugation, 9-ml aliquots of the ether layer were treated as described above for hexane extracts.

Extraction procedures for subnanogram levels and for whole blood

Up to 10 ml of plasma or whole blood were added to tubes containing 1 ml of 1 *N* sodium hydroxide solution and extracted twice with 10-ml quantities of mixed hexanes for 30 min, or once with 15 ml of mixed hexanes for 1 h. Measured aliquots of the hexane extracts were combined and evaporated to dryness. The residue was redissolved in 1 ml of 0.1 *N* hydrochloric acid and the compound of interest was extracted into 5 ml of chloroform by shaking gently for 10 min or by vortexing for 1 min, followed by centrifugation. A 4.5-ml aliquot of the chloroform layer was evaporated to dryness and redissolved in 10 μl as described above.

Calibration graphs

These were prepared from spiked plasma containing the appropriate drug at concentrations ranging from 0 to a level above that expected in clinical samples. Quantitation was by measurement of (i) peak height, (ii) the peak height ratio of drug of interest/internal standard, or (iii) integrator response, against drug concentration.

Internal standards

Where appropriate, internal standards were included in the sample of mobile phase used at the re-solution stage, or were added to the plasma to be extracted alongside the drug of interest.

RESULTS

Retention volumes

Table I shows the retention times recorded for 21 marketed drugs, as a function of ammonium acetate concentration in the aqueous solution used in preparation of the mobile phase. Each compound had shorter retention times at higher concentrations of ammonium acetate but no regular pattern was observed. For example the retention time of mesoridazine varied from 81.6 to 8.3 min while that of fluphenazine varied from 8.7 to 2.4 min. There was no case of compounds with identical retention volumes at all ammonium acetate concentrations. The retention volumes of the various compounds make possible the choice of an ammonium acetate concentration and mobile

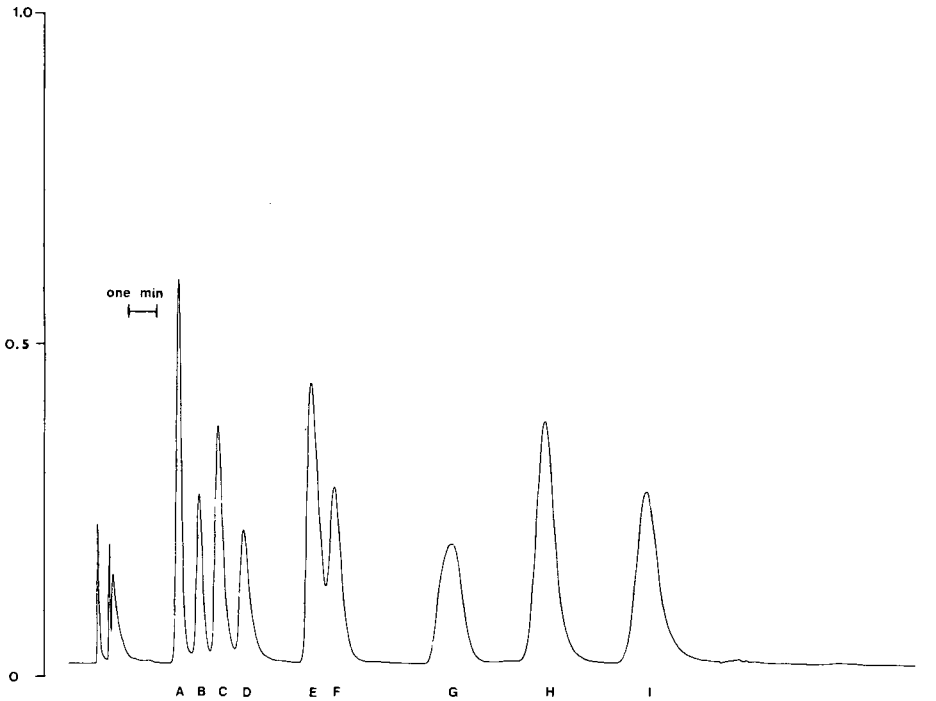


Fig. 1. LC separation of eight phenothiazine drugs plus haloperidol. Peaks and quantities: A = fluphenazine, 50 ng; B = carphenazine, 100 ng; C = trifluperazine, 50 ng; D = butaperazine, 100 ng; E = haloperidol, 250 ng; F = promethezine, 50 ng; G = chlorpromazine, 50 ng; H = promazine, 50 ng; and I = thioridazine, 100 ng. Detection by UV (254 nm); *y* axis in absorbance units.

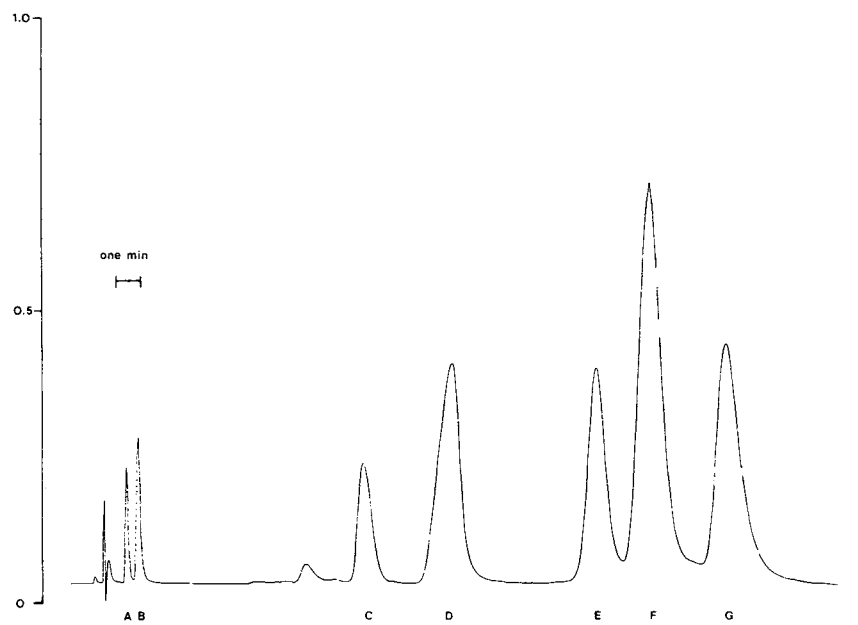


TABLE I

RETENTION TIMES (min) OF 21 MARKETED DRUGS AS A FUNCTION OF AMMONIUM ACETATE CONCENTRATION IN THE AQUEOUS PORTION OF THE MOBILE PHASE

The flow-rate was 2.5 ml/min.

Compound	Concentration of ammonium acetate (*M*)					
	0.005	0.01	0.02	0.05	0.1	0.2
	pH					
	6.24	6.72	6.89	6.91	6.94	6.99
Acetophenazine	18.4	10.1	7.4	5.8	5.1	3.5
Amitriptyline	27.2	13.2	8.8	6.0	5.0	3.3
Benztropine	46.3	20.4	13.2	8.8	7.5	4.8
Butaperazine	28.1	15.6	12.0	8.9	7.4	4.3
Carphenazine	15.5	8.6	6.4	4.8	4.3	3.1
Chlorpromazine	21.7	11.5	8.0	5.4	4.5	3.0
Eluphenazine	8.7	7.0	5.4	3.6	2.9	2.4
Haloperidol	14.0	7.4	5.1	3.4	2.7	2.0
Imipramine	31.6	15.3	10.1	6.8	5.7	3.6
Mesoridazine	81.6	43.3	23.9	16.5	14.0	8.3
Nortriptyline	40.7	21.4	10.7	7.0	6.1	3.9
Ophenadrine	24.6	14.3	8.2	5.4	4.5	3.0
Piperacetazine	34.5	20.3	11.1	7.4	6.2	4.0
Promazine	27.5	16.8	10.0	6.9	5.7	3.7
Promethazine	13.4	8.7	5.7	4.2	3.6	2.5
Thioridazine	29.5	16.7	9.2	6.1	5.3	3.2
Thiothixene	22.1	15.1	10.0	7.5	6.4	3.9
Trifluoperazine	20.1	13.7	9.3	6.9	5.8	3.6
Triflupromazine	17.9	14.0	6.6	4.5	3.7	2.5
Trihexylphendyl	21.1	12.3	7.3	4.8	3.9	2.6
Trimeprazine	17.0	10.2	6.6	4.4	3.7	2.5

phase flow-rate suitable for successful assay of any one of the compounds in the presence of another. Supportive evidence for the identification of a drug in this group is also obtainable. Fig. 1 shows a chromatogram when a mixture of nine of the compounds was examined. The use of methanol in place of acetonitrile gave essentially similar chromatographic results.

Fig. 2 shows the chromatographic separation of eight model metabolites of chlorpromazine. These compounds are produced by ring sulfoxidation, ring hydroxylation, amine oxidation, and N-demethylation of chlorpromazine. Only one pair of compounds was not resolved but they are two relatively minor polar metabolites. Four other model metabolites had retention volumes when 0.005 *M* ammonium acetate solution was used as follows: fluphenazine

Fig. 2. LC separation of eight model metabolites of chlorpromazine. Peaks: A = chlorpromazine N-oxide 5-oxide; B = chlorpromazine N-oxide; C = 7-hydroxychlorpromazine; D = chlorpromazine 5-oxide; E = dedimethylchlorpromazine; F = dedimethylchlorpromazine 5-oxide and demonomethylchlorpromazine unresolved; G = demonomethylchlorpromazine 5-oxide. Detection by UV (254 nm); *y* axis in absorbance units.

5-oxide, 5.2 ml; trifluperazine 5-oxide, 9.5 ml; demonomethylpromazine, 12.0 ml; demonomethylpromazine 5-oxide, 17.3 ml. This evidence shows adequate resolution of the marketed drugs and their metabolites.

Radial compression columns

Fig. 3 shows a sample chromatogram using the radial compression column. Separation was again satisfactory, and chromatogram quality was superior to that obtained with the conventional columns in that the peaks were more symmetrical and had a higher height/width ratio. A listing of retention times using this system is available in a preliminary communication [15].

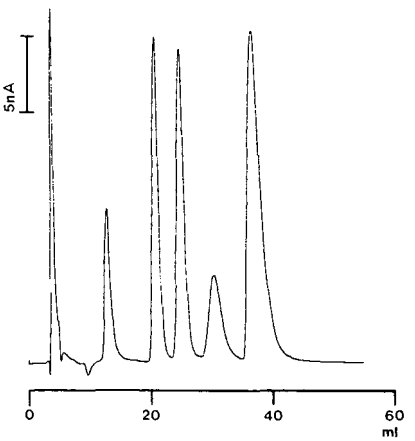


Fig. 3. LC separation of five compounds on a radial compression column. Detection was electrochemical (50 nA full scale deflection with 10-mV recorder). The mobile phase flow-rate was 5 ml/min. The compounds and their retention volumes were: carphenazine (13.0 ml), triflupromazine (20.5 ml), chlorpromazine (25.0 ml), butaperazine (30.5 ml), benztropine (37.0 ml). The first four compounds were at 10 μg/ml; benztropine was at 1 mg/ml. Injection volume 10 μl.

Blank plasma and recovery experiments

Figs. 4 and 5 show chromatograms for blank extracts. Figs. 6 and 7 show chromatograms for two recovery experiments with spiked plasma. The results for blank samples demonstrate a lack of interfering peaks in the area of the chromatogram in which experimental peaks are expected. The spiked standards demonstrate recovery and clarity of the chromatographic result.

Quantitative data

Table II shows the essential features of a series of calibration graphs for eight phenothiazine drugs extracted from plasma. Table III shows that only with carphenazine was there a significant decrease in recovery with increased plasma volume. The apparent increase in recovery of trifluoperazine is on the borderline of statistical significance. Similar or analogous equations and other observations were obtained with the different extraction modes, the electrochemical detection system, and whole blood up to 10 ml in volume.

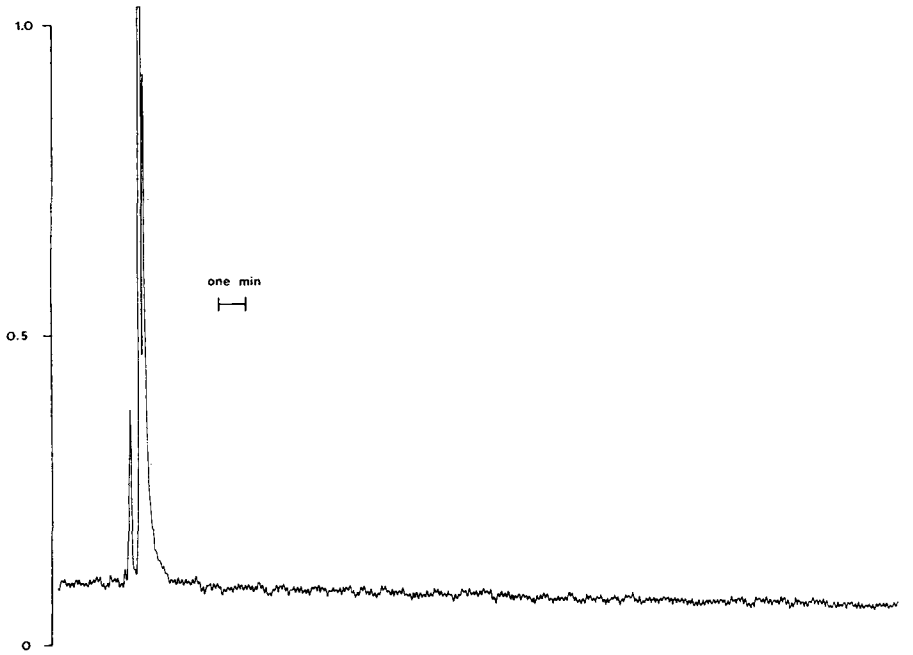


Fig. 4. LC trace of a concentrated hexane extract from blank plasma (5 ml). Detection by UV (254 nm); y axis in absorbance units.

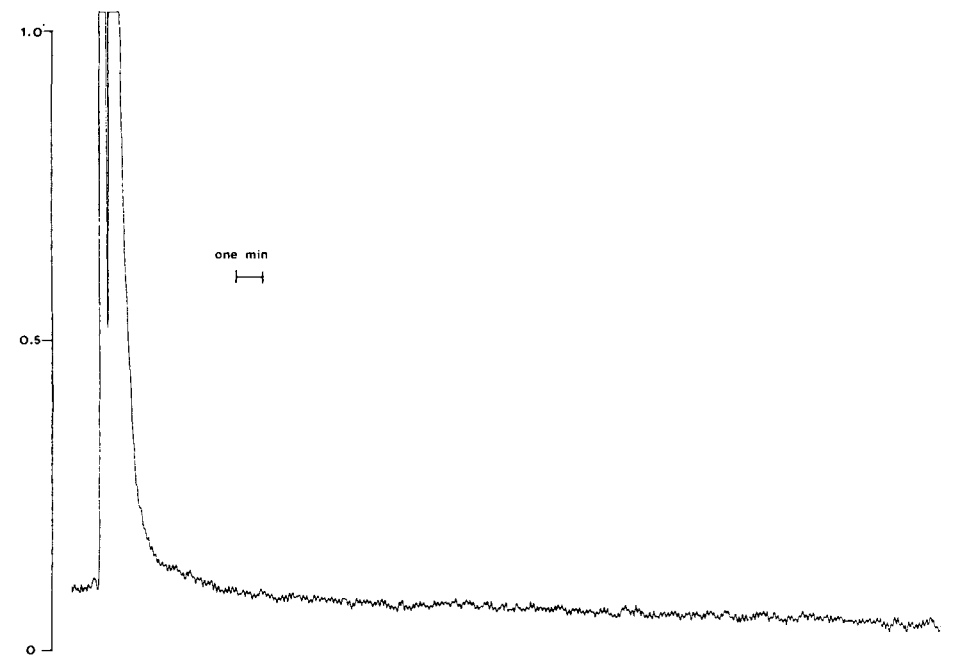


Fig. 5. LC trace of a concentrated diethyl ether extract from blank plasma (5 ml). Detection by UV (254 nm); y axis in absorbance units.

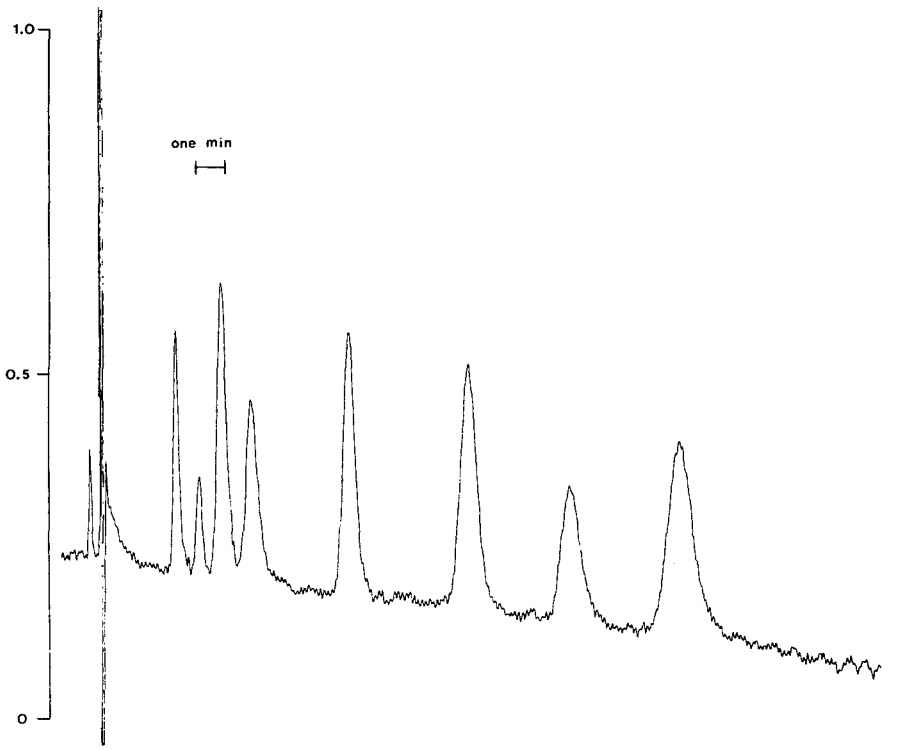


Fig. 6. LC trace of a concentrated hexane extract from plasma spiked with the eight phenothiazine drugs shown in Fig. 1 (haloperidol excluded). Sample was 5 ml containing the drugs at 10 ng/ml. Detection by UV (254 nm); y axis in absorbance units.

TABLE II

CALIBRATION EQUATIONS FOR ASSAY METHODS (HEXANE EXTRACTION FROM 1-ml SAMPLES) FOR EIGHT PHENOTHIAZINE DRUGS IN PLASMA

Model $y = a + bx$ where y is absorbance of the LC response based on peak height, x is the amount (ng) of the drug in the plasma, a is the intercept on the y axis and b is the slope of the straight line graph. The last column indicates the square of the correlation coefficient for each line. Data collected over the range x = 10—500 ng.

Compound	a	x	r^2
Fluphenazine	—0.000735	0.0000365	0.968
Chlorpromazine	—0.000005	0.0000116	0.986
Thioridazine	—0.000722	0.0000084	0.987
Trifluoperazine	—0.001071	0.0000246	0.976
Carphenazine	—0.000574	0.0000053	0.952
Promazine	—0.000151	0.0000159	0.961
Promethazine	—0.000114	0.0000123	0.976
Butaperazine	—0.000988	0.0000078	0.962

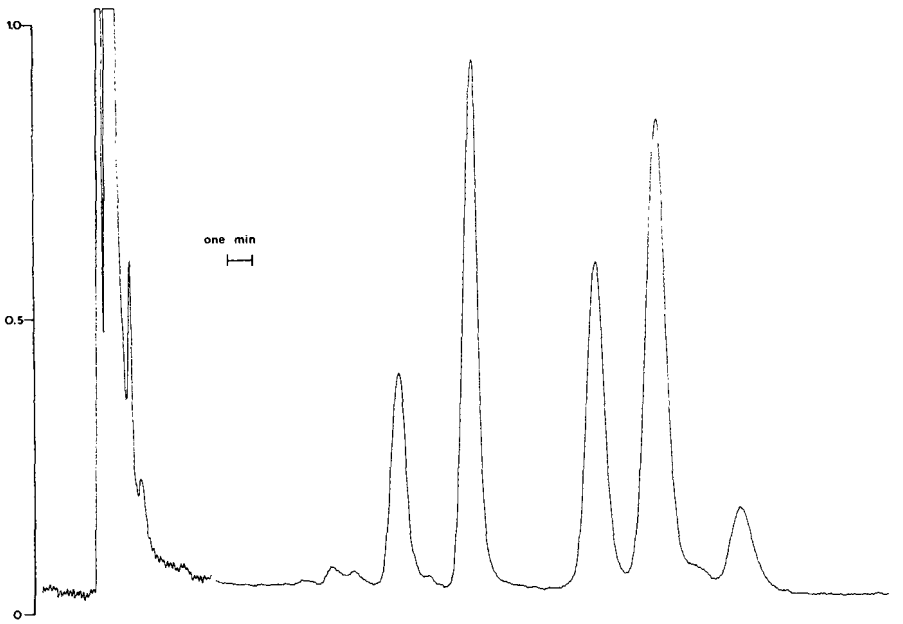


Fig. 7. LC trace of a concentrated diethyl ether extract from plasma containing five of the model metabolites of chlorpromazine shown in Fig. 2. Sample was 5 ml containing the compounds at approximately 10 ng/ml. Detection by UV (254 nm); y axis in absorbance units.

TABLE III

INFLUENCE OF VOLUME OF PLASMA ON EXTRACTION AND INSTRUMENT RESPONSE

Measurements are of peak height at 0.02 a.u.f.s. Plasma at various volumes containing 100 ng of the eight compounds was extracted with hexane as described under Experimental. Carphenazine showed reduced recovery with increased plasma volume (Spearman rank correlation coefficient, $r_s = 1$). The other relationships were clearly non-significant, except for trifluoperazine ($r_s = 0.7, p < 0.1$).

Compound	Volume of plasma (ml)				
	1	2	3	4	5
Fluphenazine	90	79	94	93	86
Carphenazine	54	32	29	22	21
Trifluoperazine	120	120	136	145	146
Butaperazine	72	69	76	80	75
Promethazine	109	130	139	147	141
Chlorpromazine	92	111	107	115	111
Promazine	61	66	66	71	71
Thioridazine	82	90	92	97	96

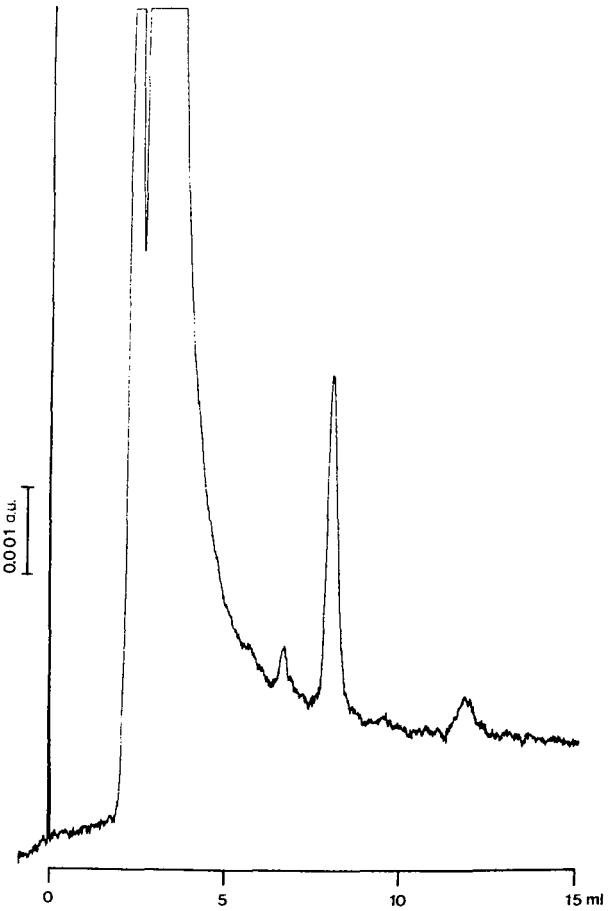


Fig. 8. LC trace of a concentrated hexane extract from a patient receiving chlorpromazine. The patient was receiving 100 mg three times a day and the sample was obtained approximately 2 h after a dose. Sample volume, 2 ml; detection by UV (254 nm); y axis in absorbance units. The trace shows chlorpromazine (8 ml) and small amounts of chlorpromazine sulfoxide (12 ml) and demonomethylchlorpromazine (6 ml to low recovery). The chlorpromazine concentration was 56.0 ng/ml.

At best, the coefficient of variation on repeated assay of plasma or blood containing known quantities of the various drugs was 1.3% (within-day). At worst this value was 7.3% (n = 5 in each case). Between-day coefficient of variation values varied over a wide range (5.0—60.1%) so that daily standardization with both pure drug solutions and spiked plasma or blood was essential.

Detection limits

From Table II, a simple calibration shows that a 1 ng/ml solution of fluphenazine in plasma, when 1 ml was sampled, gave a peak with a height equivalent to 0.0000365 absorbance units. Similarly, a 10 ng/ml solution, when 5 ml were sampled, gave a peak with a height equivalent to 0.0018 absorbance units. This is approximately 40% of full scale deflection when the instrument

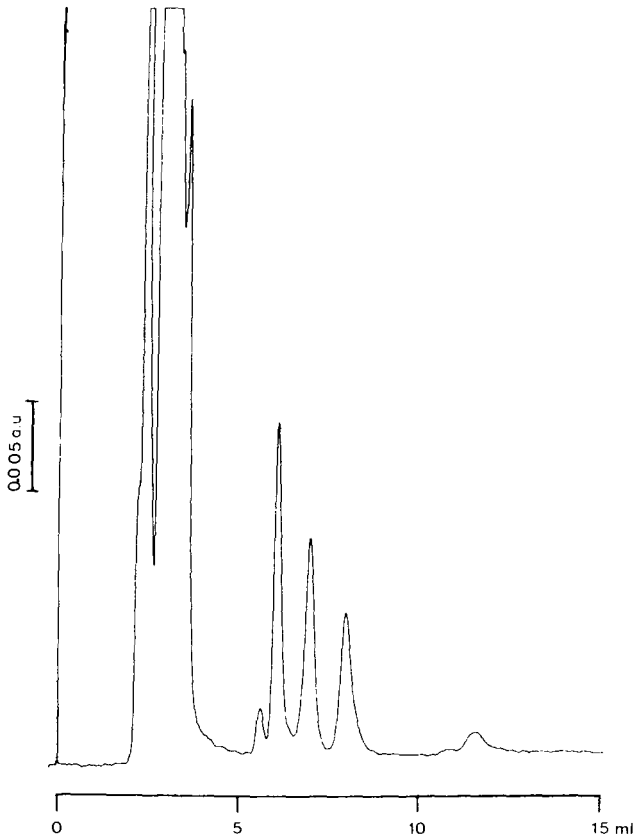


Fig. 9. LC trace of a concentrated diethyl ether extract from the patient of Fig. 8. Sample volume and other details as in Fig. 8. The trace shows chlorpromazine (8 ml), 7-hydroxychlorpromazine (7 ml), demonomethylchlorpromazine (6 ml) and chlorpromazine N-oxide (5.5 ml). The chlorpromazine concentration was 58.3 ng/ml. The metabolites are shown for identification only.

is set at 0.005 a.u.f.s. However, it is necessary to consider the noise level of the instrument response, and also to note that fluphenazine had the highest value of x in Table II. While we consider that in certain circumstances, 1 ng/ml can be assayed by UV detection, generally speaking 10 ng/ml should be considered as the lower limit for this detector. The exact lower limit will vary from laboratory to laboratory. A similar argument leads to the conclusion that electrochemical detection adds approximately two orders of magnitude. The exact detection limit may also be affected by the strength of ammonium acetate used in any particular application.

Clinical samples

Figs. 8—13 show a selection of chromatograms from patients treated with six of the drugs examined in this system. Details are given in the legends. Figs. 11 and 13 were redrawn from LC traces. All others in this paper are direct photographs of the traces.

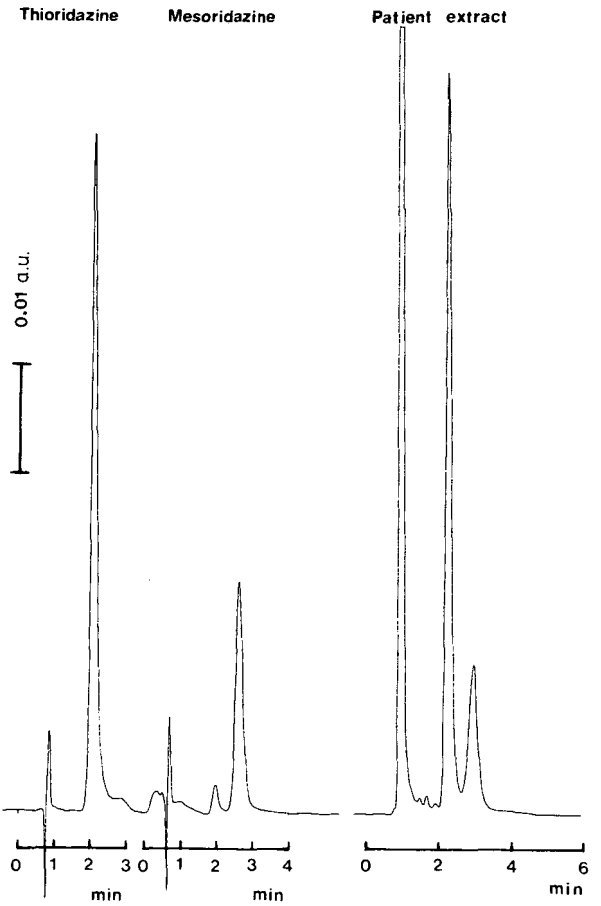


Fig. 10. LC trace of an investigation of a patient receiving thioridazine. The trace shows the drug and its metabolite as standards and in a hexane extract from plasma. The patient was receiving 200 mg thioridazine daily and the sample was collected 20 h post dosage. Detection by UV (254 nm); *y* axis in absorbance units. The thioridazine concentration was 26.1 ng/ml.

DISCUSSION

The compounds considered in this paper are unusual in that such a large number of closely related chemicals is in use in medicine. The drugs are sometimes used alone, and sometimes used in combinations of two or three, and occasionally four. Drug interactions are common and patient monitoring using drug measurements in biological fluids is often sought. Basic principles involved in the assay of these drugs are well established.

The research described in this paper could provide a system for measurement of all of these compounds individually and for most of the likely combinations of drugs. It could also provide a system for obtaining evidence supportive of a drug identification in cases when the drug ingested is unknown. It is based on published principles, and employs, to some extent, established

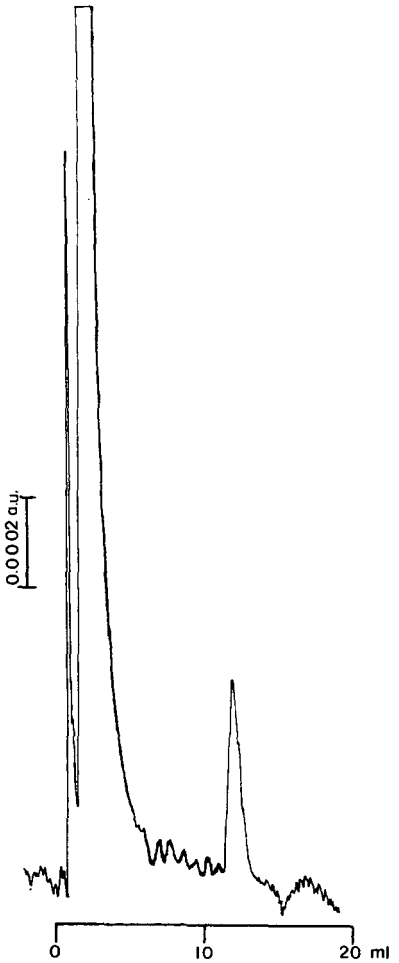


Fig. 11. LC trace of a concentrated hexane extract from a patient receiving trifluoperazine. The last dose was 60 min before the blood sample. Detection by UV (254 nm); *y* axis in absorbance units. The trifluoperazine concentration was 14.4 ng/ml.

LC conditions. We have validated the extractions for a selection of the possible compounds to which they can be applied. It is recommended that the system be used with the following guidelines: (1) for drugs known to occur at concentrations of 10 ng/ml and above, such as chlorpromazine and thioridazine, use UV detection, reserving amperometric detection for low-dose drugs such as trimeprazine; (2) choose an ammonium acetate concentration giving convenient retention times in any particular case, but note that amperometric detection requires relatively high ionic strengths; (3) when an internal standard is needed, choose any compound, from the list, known to be absent from the plasma and to have a retention volume different from that of the compound(s) to be analysed, but note that internal standards must be used with great care [16]; (4) use hexane extracts unless metabolite assays are sought; for the metabolites diethyl ether extracts are essential as many of the metabolites do not extract into hexanes; (5) reserve the hexane—chloroform approach for difficult problems, low concentrations or whole-blood

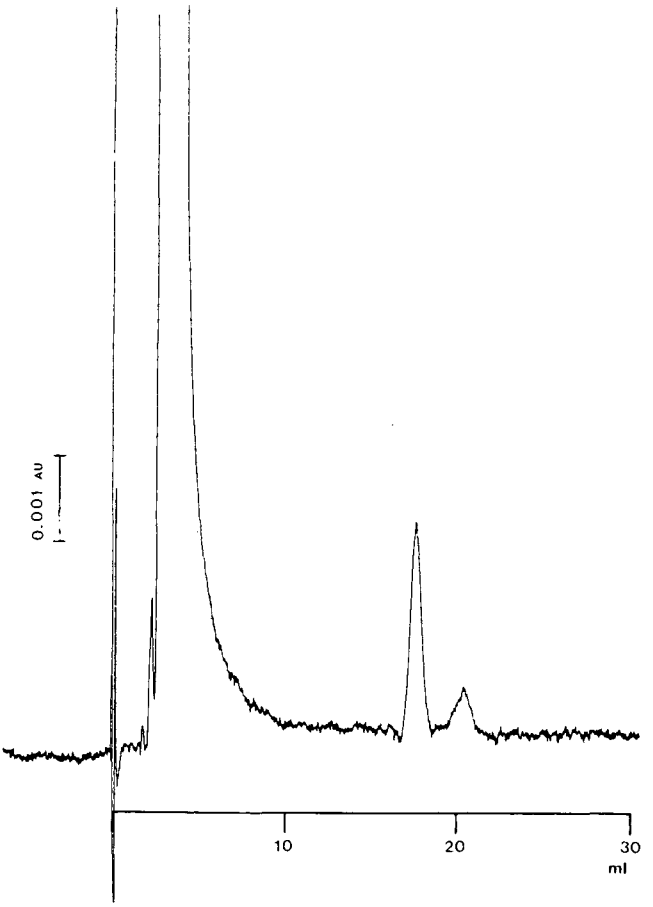


Fig. 12. LC trace of a concentrated hexane extract from a patient overdosed with amitriptyline. Dosage and timing unknown. The trace shows both amitriptyline and nortriptyline. Detection by UV (254 nm); *y* axis in absorbance units. The amitriptyline concentration was 37 ng/ml; the nortriptyline concentration was below 10 ng/ml.

samples; (6) use radial compression columns for speed and high-quality LC traces, but note that the flow-rates and therefore the solvent costs are high with this system. Quite obviously, an assay for a particular compound will have to be validated in the laboratory of application.

The expanded rationale behind some of these recommendations is as follows. UV detection has been found to be more stable than amperometry, which requires time to "warm up", gives drifting baselines, wider solvent bands, and a "noisier" trace, and shows a decay of sensitivity during the working day leading to heavy reliance on the use of the internal standard. However, amperometry is notably more sensitive. As regards extraction, very small quantities were found (< 1 ng/ml) to require prolonged extraction times and the use of large volumes of biological material. This led to "dirty" extracts. The chloroform extraction, which presumably employs an ion-pair principle was found to overcome these problems.

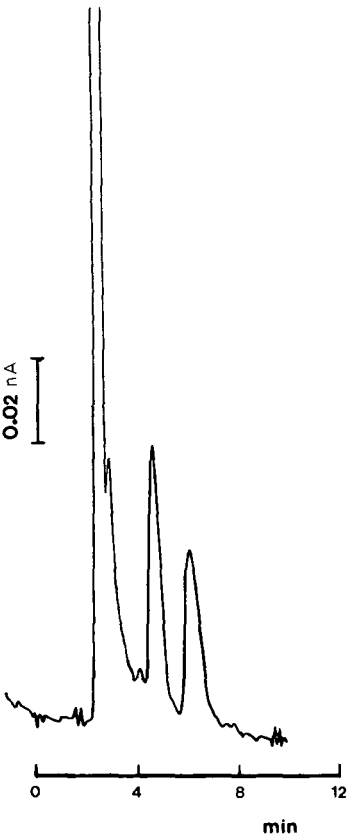


Fig. 13. LC trace of a concentrated hexane—chloroform extract from an experimental subject receiving trimeprazine. The trace shows both trimeprazine (4.5 min) and imipramine (used as an internal standard; 6.5 min). The dose of trimeprazine was 5 mg and the sample was collected 5 h post dosage. Detection by amperometry. The trimeprazine concentration was 0.8 ng/ml.

The use of peak heights, peak areas, as computed by the integrator, and ratios involving internal standards for quantitation purposes were investigated. The peak height ratio method seemed to be more successful for general application in the current study. These matters have been investigated in earlier contributions to this literature [1—9].

The LC approach has a number of advantages over other methods, as well as speed and convenience. For example with chlorpromazine, a serious problem with the GC approach involves the N-oxide. This compound reduces in GC conditions, to both parent drug and demethylated analogues; presumably this is also true for other N-oxides. Unless considerable care in choosing the extraction conditions is exercised, the GC assay of an unchanged drug can be adversely affected by its metabolites. This problem also makes the metabolites themselves difficult to assay, and is compounded by the availability of only a limited supply of model metabolites. In the LC system, all of the compounds are stable, and assayable from the same extraction.

The LC approach is superior to biological assays because of better specificity. For example, while radioimmunoassay methods published for drugs in this group are, in general, of reasonably high specificity, there remains the serious problem of cross-reactivity between unchanged drugs, their demethylated metabolites, and their 7-hydroxylated analogues [17, 18]. Radioreceptor assays are designed to detect the "total neuroleptic" content of biological samples, mixed drugs and their metabolites, and so are by definition non-specific [19, 20]. This makes them unsuitable for studies in pharmacokinetics where data are only of value if they are for a specific known compound.

ACKNOWLEDGEMENTS

We are grateful to the various drug manufacturers for samples of their products: to Dr. A.A. Manian of the U.S. National Institute of Mental Health for samples of model metabolites; and to various members of the staff at the J. Hillis Miller Health Center for assistance with patient and experimental subject samples. All investigations involving human subjects were approved by the relevant human experimentation committees.

REFERENCES

1 S.H. Curry, Anal. Chem., 40 (1968) 1251—1255.
2 A.G. Butterfield and R.W. Sears, J. Pharm. Sci., 66 (1977) 1117—1119.
3 W.F. Heyes and J.R. Salmon, J. Chromatogr., 156 (1978) 309—316.
4 W.F. Heyes, J.R. Salmon and W. Marlow, J. Chromatogr., 194 (1980) 416—420.
5 B.B. Wheals, J. Chromatogr., 177 (1979) 263—270.
6 S.H. Curry and G.P. Mould, J. Pharm. Pharmacol., 21 (1969) 674—677.
7 J.I. Javaid, J. Chromatogr. Sci., 17 (1979) 666—670.
8 C. Eggert-Hansen, Brit. J. Clin. Pharmacol., 3 (1976) 915—923.
9 J.E. Wallace, E.L. Shimek, J. Starchansky and S.C. Harris, Anal. Chem., 53 (1981) 960—962.
10 P. Kissinger, Anal. Chem., 46 (1974) 15R—20R.
11 D. Koch and P.T. Kissinger, Anal. Chem., 52 (1980) 27—29.
12 H.M. van Praag, Psychotropic Drugs: A Guide for the Practitioner, Macmillan, London, 1978.
13 T.B. Cooper, Clin. Pharmacokin., 3 (1978) 14—38.
14 S.H. Curry, in G.D. Burrows and T. Norman (Editors), Psychotropic Drugs: Plasma Concentrations and Clinical Response. Marcel Dekker, New York, 1980.
15 S.H. Curry, E.A. Brown and J.H. Perrin, IRCS Med. Sci., 9 (1981) 166—169.
16 S.H. Curry and R. Whelpton, in E. Reid (Editor), Blood Drugs and Other Analytical Challenges, Ellis Horwood, Chichester, 1978.
17 D.H. Wiles, Psychol. Med., 6 (1976) 407—415.
18 A. Jorgensen, Life Sci., 23 (1978) 1533—1542.
19 I. Creese and S.H. Snyder, Nature (London), 270 (1977) 180—182.
20 A.S. Papadopoulos, Brit. J. Psychiat., 136 (1980) 591—596.

Journal of Chromatography, 231 (1982) 377—391
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1316

SIMULTANEOUS DETERMINATION OF THIORIDAZINE AND ITS S-OXIDIZED AND N-DEMETHYLATED METABOLITES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON RADIALLY COMPRESSED SILICA

CLINTON D. KILTS*

Clinical Psychopharmacology Laboratory, Department of Psychiatry, Box 3870, Duke University Medical Center, Durham, NC 27710 (U.S.A.)

KENNERLY S. PATRICK

Biological Sciences Research Center, University of North Carolina, Chapel Hill, NC 27514 (U.S.A.)

and

GEORGE R. BREESE and RICHARD B. MAILMAN

Departments of Psychiatry and Pharmacology and the Biological Sciences Research Center, University of North Carolina, Chapel Hill, NC 27514 (U.S.A.)

(First received December 22nd, 1981; revised manuscript received March 30th, 1982)

SUMMARY

A method for simultaneously quantifying thioridazine, northioridazine, thioridazine-2-sulfoxide, thioridazine-2-sulfone and thioridazine-5-oxide in serum and plasma is described. Following solvent extraction these compounds were separated by high-performance liquid chromatography on radially compressed silica gel and detected by UV absorbance at 254 nm. Chromatography time is less than 7 min. The relative retention of these compounds as a function of the methanol and methylamine content of the mobile phase is discussed. Practical limits of detection, based upon an assayed plasma or serum volume of 1 ml, were 20 ng/ml for thioridazine-5-oxide and 10 ng/ml for the other compounds. The coefficient of variation for all compounds was less than 13%. The method is compared with more conventional high-performance liquid chromatographic and gas chromatographic methodology.

INTRODUCTION

The clinical pharmacokinetics of antipsychotic drugs have become an area

0378-4347/82/0000—0000/$02.75

of intense study in recent years in an attempt to individualize the pharmacotherapy of psychiatric disorders. Of paramount importance to such studies is the development of analytical methods suitable for determining the concentrations of the parent drug as well as any pharmacologically active metabolites in biological fluids. Thioridazine (Mellaril®), a widely used phenothiazine antipsychotic, typifies many of these considerations. The extent to which its metabolites accumulate following thioridazine administration, and their neuropharmacological activity, makes their quantification a potentially important component when attempting to empirically optimize the balance between therapeutic effect and the incidence of side effects.

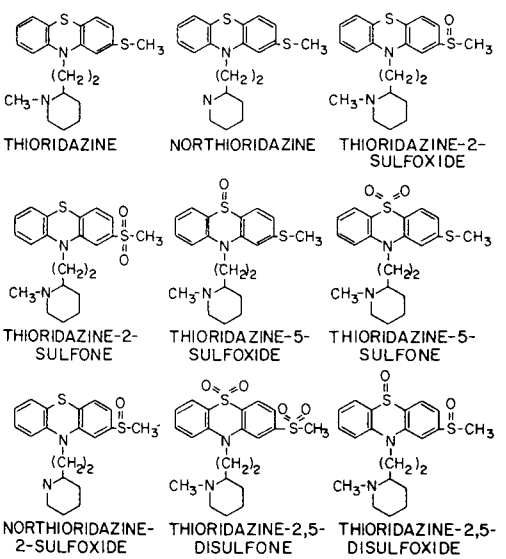


Fig. 1. Structures of thioridazine and its major metabolites.

Thioridazine undergoes extensive transformation in humans with S-oxidation representing the predominant route of metabolism [1, 2]. Unlike other phenothiazines, thioridazine reportedly undergoes little or no metabolic hydroxylation or N-demethylation in humans [3, 4]. The major identified metabolites of thioridazine isolated from human serum and urine are illustrated in Fig. 1. The S-oxidized metabolites differ from thioridazine and one another in a number of important properties: pharmacological activity, both in terms of antipsychotic efficacy [5—8] and side effects [5, 7, 9]; extent of binding to serum [10] or plasma [11] proteins; and time course of accumulation in serum following thioridazine administration [12]. Each of these factors must be considered when attempting to correlate drug concentration with clinical response and necessitates the accurate quantitative determination of thioridazine and its pharmacologically active and major inactive metabolites when performing such studies.

A variety of purification, separation and detection methods have been used to quantify thioridazine and its metabolites. Initial methods involved solvent extraction or ion-exchange chromatography and fluorometric detec-

tion after oxidation of the compounds of interest to fluorophors [13—16]. Such techniques lack selectivity and do not permit the separate quantification of thioridazine and its metabolites. Subsequent efforts also involved a solvent extraction procedure followed by either gas chromatographic (GC) separation with flame ionization detection [2, 3, 17, 18] or high-performance liquid chromatographic (HPLC) separation with fluorometric or ultraviolet (UV) absorbance detection [19—21].

The present study reports a method for the rapid, simultaneous determination of plasma or serum concentrations of thioridazine, northioridazine, thioridazine-2-sulfone, thioridazine-2-sulfoxide and thioridazine-5-oxide. The technique utilizes HPLC separation on radially compressed silica gel with 254-nm UV absorbance detection. Chromatography on radially compressed packed columns is compared with more conventional reversed-phase and adsorption HPLC using packed steel columns, and with GC separation with thermionic (nitrogen—phosphorus) selective detection.

EXPERIMENTAL

Chemicals

The 2,2,4-trimethylpentane, methanol, methylene chloride and hexane were HPLC grade (Fisher Scientific, Fairlawn, NJ, U.S.A.). Methylamine was obtained as a 40% solution in water (Aldrich, Milwaukee, WI, U.S.A.). Diethyl ether (peroxide-free) was reagent grade (Mallinckrodt, St. Louis, MO, U.S.A.). Thioridazine hydrochloride, northioridazine, thioridazine-2-sulfoxide hydrochloride, thioridazine-2-sulfone, thioridazine-5-oxide hydrochloride, thioridazine-2,5-disulfoxide, thioridazine-2,5-disulfone and northioridazine-2-sulfoxide were generously supplied by Sandoz Pharmaceuticals (East Hanover, NJ, U.S.A.). The 2-acetylphenothiazine was from Aldrich.

General

All glassware used in sample preparation, except pipettes, was treated with a 10% solution of dimethyldichlorosilane in toluene, rinsed with methanol, and oven dried at 100°C. Pipettes used to transfer solvents were rinsed with methanol immediately prior to their use. These precautions minimized the loss of thioridazine and its metabolites during sample preparation due to adsorption onto glass surfaces. Screw caps for the conical centrifuge tubes were fitted with PTFE liners. Solutions of thioridazine, its metabolites, 2-acetylphenothiazine or other drugs tested were made in methanol at a concentration of 25 ng/μl (calculated as the free base) and stored in the dark at 4°C.

Sample preparation

A 1-ml aliquot of plasma was added to a 15-ml conical centrifuge tube, and the pH was adjusted to approximately 10.5 by adding 400 μl of 2 *M* sodium hydroxide solution. The mixture was extracted with 2.5 ml of diethyl ether—hexane (3:1, v/v) by mixing for 10 min on a mechanical shaker followed by centrifugation at 500 *g* for 5 min. The organic layer was then transferred to another centrifuge tube containing 1 ml of 0.1 *M* hydrochloric

acid. Extraction and phase separation were performed as in the preceding step and the organic phase was aspirated and discarded. The remaining aqueous phase was washed with 2 ml of the diethyl ether—hexane solvent, the tubes vortexed (20 sec) and centrifuged, and the organic layer was discarded. The aqueous phase was made alkaline (pH > 10) by adding 150 μl of 2 *M* sodium hydroxide solution and the mixture was extracted twice with 1.5 ml volumes of the diethyl ether—hexane solvent. The organic phases were pipetted into another centrifuge tube containing 100 ng of 2-acetylphenothiazine and evaporated to dryness under nitrogen with gentle heating using a heat gun. The residue was dissolved in 60 μl of hexane—methanol—methylene chloride (8:1:1), the tubes vortexed (20 sec), and 20—40 μl aliquots injected into the liquid chromatograph. For GC assay the residue was dissolved in 10 μl of cyclohexane and 2—4-μl aliquots were injected.

Instrumentation

A Laboratory Data Control (LDC, Riviera Beach, FL, U.S.A.) Consta-Metric IIG solvent delivery pump was used in conjunction with a Model 1203 UV absorbance detector (LDC) which was operated at 254 nm with a time constant of 0.5 sec. Detector output was displayed on a 10-mV recorder using a chart speed of 0.25 cm/min. A six-port rotary valve (Model 7125, Rheodyne, Berkeley, CA, U.S.A.) with a 50-μl sample loop was used for sample injection. Chromatographic separations were performed using an RCM-100 radial compression separation system with a cartridge (8 mm I.D.) packed with microparticulate (5 μm diameter) silica gel, both from Waters Assoc. (Milford, MA, U.S.A.). A precolumn filter with a 2-μm frit (Rheodyne) effectively minimized the accumulation of particulate matter on the analytical column. Steel HPLC columns (25 cm × 4.6 mm I.D.) packed with either 8-μm silica gel or C_{18}-bonded 5-μm silica (Spherisorb ODS) were from LDC.

Chromatography

The mobile phase was a mixture of 2,2,4-trimethylpentane—methylene chloride (water saturated)—methanol (8:1:1) containing 0.036% methylamine. The methylamine was added to the methanol prior to mixing. Dissolved gases in the mobile phase were minimized by ultrasonic agitation immediately before use. All separations were performed isocratically at a flow-rate of 2.25 ml/min.

Calculations

Blank-corrected standard curves for the quantification of thioridazine and its metabolites were prepared by assaying a series of standard solutions containing varying amounts (0—1000 ng) of thioridazine and its metabolites. The standard solutions were 1-ml aliquots of drug-free plasma which were subjected to the identical preparative steps used to extract unknown plasma samples. The detector response for each compound was determined for each sample and standard. The concentrations of thioridazine and each metabolite in an unknown plasma sample were calculated from their peak heights relative to the external standard, using the slope and intercept of the standard curve.

UV absorbance spectra

The molar absorptivities of the compounds of interest were determined on a Beckman UV 5230 spectrophotometer (Irvine, CA, U.S.A.). The instrument was standardized for wavelength and absorption versus a solution of potassium dichromate. Values for the standard solution were within ± 0.5 nm and ± 0.5% A of published values [22]. All relevant values were read between 0.47—1.4 au except for the A_{254} for thioridazine-2,5-disulfone. All solutions of thioridazine and its metabolites were prepared in methanol at a concentration of 10 μg/ml.

Gas chromatography

A Varian (Palo Alto, CA, U.S.A.) Model 2440 gas chromatograph was used in conjunction with a thermionic (nitrogen—phosphorous) selective detector (Varian). All separations were performed using dimethyldichlorosilane-treated glass columns (2 mm I.D.) packed with varying stationary phases on 80—100 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.).

Mass spectrometry

Electron impact mass spectra were obtained using a Model 3300 gas chromatograph—mass spectrometer (Finnigan, Sunnyvale, CA, U.S.A.) interfaced with a Finnigan 6100 Data System. Fragmentation was accomplished by electron impact at 70 eV and 500 μA. Spectra were obtained at a scanning rate of 20 a.m.u./sec.

RESULTS AND DISCUSSION

Choice of separation mechanism

Gas chromatography. GC with thermionic (nitrogen—phosphorus) selective detection was initially examined as a means of separating and detecting thioridazine and its metabolites. Previously published GC assays for thioridazine and its S-oxidized and N-demethylated metabolites have utilized flame ionization detection [2, 3, 17, 18], a relatively non-selective and non-sensitive mode of GC detection. This lack of detector sensitivity necessitated the use of large volumes of serum or plasma samples (i.e., 4—5 ml) for accurate determinations. Of these assays, three [3, 17, 18] utilized a stationary phase (OV-17) which incompletely resolved thioridazine and northioridazine as well as thioridazine-2-sulfoxide and thioridazine-2-sulfone. A fourth [2] employed a stationary phase (OV-225) which is not readily compatible with thermionic (nitrogen—phosphorus) selective detection. A further evaluation of the GC retention behavior of thioridazine, northioridazine, thioridazine-2-sulfoxide, thioridazine-2-sulfone and thioridazine-5-oxide on stationary phases of varying polarity and percent loading on the solid support are summarized in Table I. Thermionic (nitrogen—phosphorus) selective detection was promising in that it proved to be two or more orders of magnitude more sensitive than flame ionization detection. However, no combination of stationary phase, column length and column oven temperature (isothermal or programmed) examined resulted in a usable separation of the compounds of interest. Derivatization techniques are often used to resolve phenothiazines from their N-demethylated

TABLE I

GC RETENTION BEHAVIOR OF THIORIDAZINE (THD), NORTHIORIDAZINE (norTHD), THIORIDAZINE-2-SULFOXIDE (THD-2-SO), THIORIDAZINE-2-SULFONE (THD-2-SO_2) AND THIORIDAZINE-5-OXIDE (THD-5-SO) ON COLUMNS OF VARYING POLARITY, LENGTHS AND TEMPERATURES

Chromatography conditions: injector and detector temperatures: 290°C; flow-rates: helium (carrier gas), 35 ml/min; air, 160 ml/min; hydrogen, 3.2 ml/min.

Stationary phase	Column length (feet)	Oven temperature (°C)	Retention time (min)				
			THD	norTHD	THD-2-SO	THD-2-SO_2	THD-5-SO
1% OV-17	3	250	3.0	3.1	8.1	9.2	13.7
3% SP-2250 (OV-17)	6	275	6.0	6.2	14.0	15.5	20.7
3% SP-2250	1.5	260	1.2	1.2	2.8	3.2	5.6
1% OV-25	3	250	4.5	4.8	12.0	13.1	18.5
1% OV-1	3	250	2.4	2.5	4.7	4.8	10.4

metabolites [3, 23]. However, the commonly used acid anhydrides have been reported to reduce the S-oxidized metabolites of chlorpromazine, a structurally related phenothiazine, to their corresponding sulfides [23, 24]. Similarly, the reaction of trifluoroacetic anhydride (100°C, 15 min) with thioridazine-2-sulfoxide, thioridazine-5-oxide and northioridazine-2-sulfoxide resulted in the formation of the corresponding sulfides, as well as degradation products. Furthermore, when thioridazine-5-oxide was chromatographed at relatively high column temperatures (> 230°C) a compound with the same retention time as thioridazine was detected and subsequently identified as thioridazine by electron impact mass spectrometry. It would thus appear that thioridazine-5-oxide undergoes a thermal reduction to thioridazine. Based upon the inability to form stable volatile derivatives of thioridazine or its tertiary amine-containing metabolites, their apparent reduction by commonly used derivatizing reagents, their poor GC resolution and the apparent thermal lability of thioridazine-5-oxide, the use of GC techniques as a means of separating these compounds was discontinued.

High-performance liquid chromatography. Due to the low volatility of thioridazine and its metabolites, HPLC was evaluated as an alternative method of resolving these compounds prior to detection. The retention behavior of these compounds using steel columns with either reversed-phase (C_{18}) or adsorption (silica gel) chromatography were compared. Of the two, the microparticulate silica gel exhibited a greater separation selectivity than that obtained using reversed-phase chromatography. The superiority of adsorption chromatography on silica gel, over reversed-phase chromatography, has been demonstrated previously for the separation of a number of tricyclic antidepressants of closely related structures [25—27].

The recent introduction of compressible, polyethylene HPLC columns packed with various adsorbents has offered an apparent solution to the problem of column dead volume, particularly between the packing material and column wall, that is characteristic of steel HPLC columns [28]. The advantages of using adsorbents under radial compression are thought to arise from a higher degree of interaction between the solute and the homogenously packed adsorbent [28]. A further comparison of the retention behavior of thioridazine, northioridazine, thioridazine-2-sulfoxide, thioridazine-2-sulfone and

thioridazine-5-oxide on conventional steel columns packed with silica gel and on radially compressed microparticulate silica gel was made. Radial compression offered significant improvements in resolution efficiency, and the ability to utilize high mobile phase flow-rates which decreased chromatogram time and permitted a more rapid rate of equilibration between the mobile phase and the column packing. Further, the radial compression separation system offers considerably more ease of operation in terms of lack of conventional column end fittings, lack of unidirectionality of mobile phase flow and longer column lifetime. The chromatographic separation using this method was excellent as can be seen from representative chromatograms of these compounds from standard solutions or from extracts of spiked plasma samples (Fig. 2). Furthermore, the chromatography time is less than 7 min.

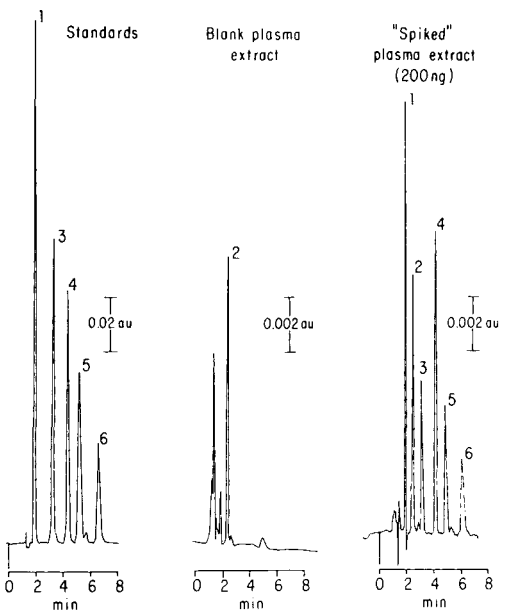


Fig. 2. Chromatograms of 20 μl of a standard mixture containing 25 ng/μl of thioridazine and its metabolites (left) and extracts of blank plasma (center) and plasma to which 200 ng of thioridazine and its metabolites have been added (right). Mobile phase flow-rate was 2.5 ml/min and detector output displayed at a recorder chart speed of 5 mm/min. Peaks: 1 = thioridazine; 2 = 2-acetylphenothiazine; 3 = northioridazine; 4 = thioridazine-2-sulfone; 5 = thioridazine-2-sulfoxide; 6 = thioridazine-5-oxide.

After a preliminary report of our method was described [29], Skinner et al. [21] published a somewhat similar procedure utilizing HPLC separation on silica gel packed in steel columns and 254-nm absorbance detection. However, these authors report that only between 0 to 60% of commercially available prepacked columns yielded usable chromatographic separations. Further, they do not report the ability to be able to separate or detect several important metabolites of thioridazine such as thioridazine-5-oxide as well as mixed 2,5-disulfones or disulfoxides. The use of radially compressed column

packings apparently circumvents these separation problems and increases the already great flexibility of HPLC.

Choice of HPLC detection mechanism

The two most widely used forms of sample detection of HPLC effluents are UV absorption and fluorometric methods. Thioridazine, thioridazine-2-sulfoxide, thioridazine-2-sulfone, thioridazine-5-oxide and northioridazine were found to have little or no native fluorescence. While fluorophors of these compounds have been formed by oxidative mechanisms [14—16, 19], only one study [19] has reported the use of such reactions in conjunction with HPLC and a fluorometer. UV absorption detection would appear to be well suited to the needs for detector sensitivity. The molar absorptivities at 254 nm for thioridazine, northioridazine, thioridazine-2-sulfoxide, thioridazine-2-sulfone and thioridazine-5-oxide exceed 18.2 mM^{-1} cm^{-1} (Table II), and thus permit their detection in small volumes of plasma or serum (0.5—1 ml) following therapeutic doses of thioridazine.

TABLE II

MOLAR ABSORPTIVITIES OF THIORIDAZINE AND ITS METABOLITES

See Experimental section for details.

Compound	ϵ_{254} (mM^{-1} cm^{-1})	λ_{max} (nm)	ϵ_{max} (mM^{-1} cm^{-1})
Thioridazine	31.9	264	40.8
Northioridazine	22.9	264	27.9
Thioridazine-2-sulfone	23.8	266	35.9
Thioridazine-2-sulfoxide	24.0	262	30.5
Thioridazine-5-oxide	18.2	279	44.3
Thioridazine-2,5-disulfoxide	18.1	(broad: 210—275 nm)	
Thioridazine-2,5-disulfone	9.1	234	42.2

Effect of the solvent strength and methylamine content of the mobile phase on solute retention

The relative retention (capacity factor, k') of thioridazine, northioridazine, thioridazine-2-sulfoxide, thioridazine-2-sulfone, thioridazine-5-oxide, northioridazine-2-sulfoxide, thioridazine-2,5-disulfone and thioridazine-2,5-disulfoxide was found to be a function of the methanol and methylamine content of the mobile phase. The order of elution using radially compressed silica gel was inversely related to the polarity of the functional groups of these compounds. Increasing the solvent strength of the mobile phase by increasing the methanol content produced a concentration-dependent decrease in the retention of the S-oxidized metabolites of thioridazine, especially the strongly adsorbed disulfone and disulfoxide (Fig. 3). Conversely, increasing the methanol content of the mobile phase increased the capacity factor for northioridazine. The addition of methylamine to the mobile phase produced a concentration-dependent decrease in the retention of all of the compounds tested

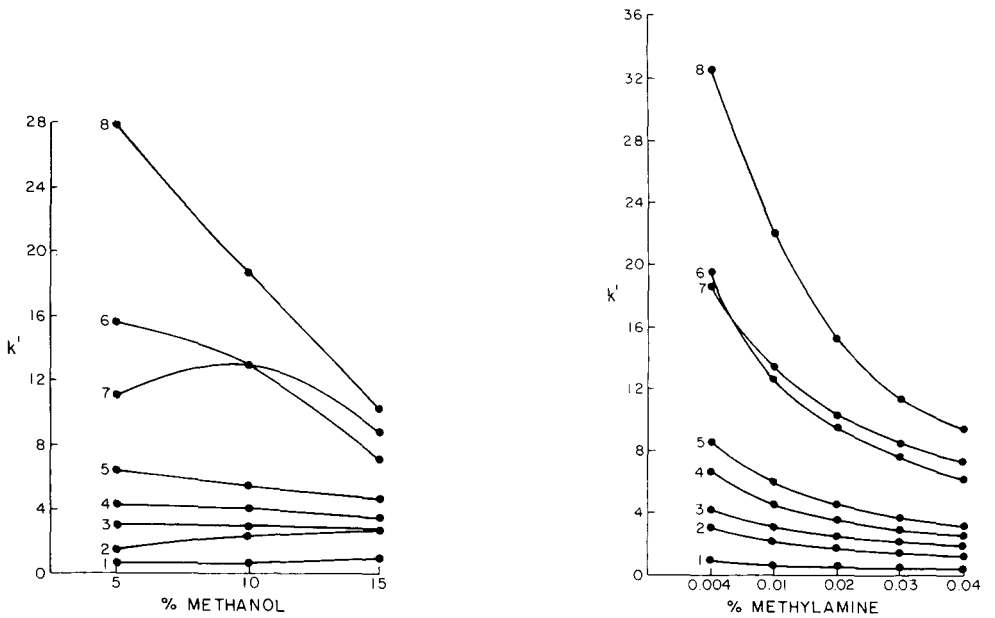


Fig. 3. Effect of methanol content of the mobile phase on the retention of thioridazine and its metabolites. Mobile phase: 2,2,4-trimethylpentane—methylene chloride—methanol (7.5—8.5:1:0.5—1.5) and 0.02% methylamine. The water content of the mobile phase was constant. The capacity factor (k') was calculated by $k' = (t_R - t_{vv})/t_{vv}$ where t_R and t_{vv} are the retention times of the compound of interest and an unretarded compound (chlorobenzene), respectively. Curves: 1 = thioridazine; 2 = northioridazine; 3 = thioridazine-2-sulfone; 4 = thioridazine-2-sulfoxide; 5 = thioridazine-5-oxide; 6 = thioridazine-2,5-disulfone; 7 = northioridazine-2-sulfoxide; 8 = thioridazine-2,5-disulfoxide.

Fig. 4. Effect of methylamine content of the mobile phase on the retention of thioridazine and its metabolites. Mobile phase: 2,2,4-trimethylpentane—methylene chloride—methanol (8:1:1). The water content of the mobile phase was constant. See Fig. 3 for derivation of k' and numbering of curves.

(Fig. 4) and eliminated the peak tailing noted for the later eluting compounds. These effects most probably result from the ability of methylamine to compete with these basic compounds for the acidic silanol groups on the silica surface, and to suppress phenothiazine protonation by increasing the pH of the mobile phase. Caude et al. [30] have similarly noted the need to include a solvent modifier (ethylamine) for the chromatographic separation of a mixture of phenothiazine derivatives on silica gel.

Solvent extraction procedure

Small amounts of isoamyl alcohol in a nonpolar solvent such as heptane are generally used as an extraction solvent for thioridazine and its metabolites [2, 3, 14, 16, 17]. The isoamyl alcohol is thought to prevent the adsorption of these basic compounds to glass surfaces and to minimize the formation of emulsions. However, addition of 1—4% isoamyl alcohol to hexane produced lower extraction recoveries, greater losses during evaporation and increased evaporation time when compared to the combined use of the diethyl ether—hexane solvent, pipettes rinsed with methanol and glassware pretreated

with dimethyldichlorosilane. The rinsing of pipettes with methanol also greatly facilitated the quantitative transfer of the low viscosity extraction solvent. It is essential that the diethyl ether be free of peroxides as their presence results in significant losses of thioridazine, northioridazine and thioridazine-2-sulfone upon evaporation of the extraction solvent to dryness. Therefore the diethyl ether should be periodically tested for peroxides and if present, the diethyl ether should be redistilled over cuprous chloride. The substitution of methyl *tert*-butyl ether (HPLC grade, Fisher) for diethyl ether in the extraction solvent yielded similar extraction recoveries yet greatly decreased evaporation losses, probably due to the much slower rate of peroxide formation of the former solvent. The absolute and relative recoveries from spiked plasma samples are shown in Table III. The relative recoveries of thioridazine and its metabolites were greater than their absolute recoveries at both concentrations examined. Furthermore, the absolute and relative recoveries of the compounds of interest were slightly greater at the higher concentration. The coefficients of variation (C.V.) of the calculated recoveries were 10% or less and did not consistently vary with concentrations.

TABLE III

ANALYTICAL RECOVERIES AND EXTRACTION PRECISION

n = 5 for both concentrations.

Drug	Concentration (ng/ml)	Recovery*			
		Relative	C.V.	Absolute	C.V.
Thioridazine	75	94.7	9.9	75.0	8.9
	500	102.3	2.5	78.0	5.9
Northioridazine	75	83.0	9.0	65.4	5.4
	500	101.7	7.6	73.9	8.1
Thioridazine-2-sulfone	75	92.2	7.7	78.0	2.4
	500	99.1	4.8	91.3	5.5
Thioridazine-2-sulfoxide	75	87.9	4.3	81.6	7.7
	500	103.0	9.7	94.6	6.3
Thioridazine-5-oxide	75	90.2	8.1	72.3	10.0
	500	101.3	8.6	79.2	4.7

*Recoveries were determined from 1-ml aliquots of human serum to which the indicated concentrations of the compounds were added and the samples extracted as in Experimental. The observed detector response was compared to that obtained for either equal volumes of water supplemented to the same concentrations and subjected to an identical extraction procedure (relative recovery) or the same amount of each compound evaporated to dryness, reconstituted and directly injected (absolute recovery). 2-Acetyl phenothiazine (100 ng) was added to all tubes prior to solvent evaporation.

While internal standardization would be expected to improve the accuracy and precision of the method, no single compound has been found which effectively controls for losses of all of these compounds of widely differing polarity during sample preparation. However, 2-dimethylaminoethyl phenothiazine-10-carboxylate hydrochloride (k' = 0.69), which precipitates upon condensing 2-dimethylaminoethanol (Aldrich) with phenothiazine-10-car-

bonyl chloride in refluxing dry toluene, has been initially promising as an internal standard. Routinely, the addition of 100 ng of 2-acetyl phenothiazine (k' = 0.82) to the samples prior to solvent evaporation effectively controls for losses during sample evaporation and variabilities in reconstitution, as well as differences in volume injected and within-run variations in column and detector performance. The use of 2-acetylphenothiazine in this manner as an external standard appreciably increases the precision and accuracy of the method.

A back-extraction step into hydrochloric acid greatly decreased the chromatographic interference from plasma constituents. The addition of 2.5 ml of methylene chloride in the initial and final solvent extraction steps was necessary to extract the most polar metabolites, i.e., thioridazine-2,5-disulfoxide and thioridazine-2,5-disulfone. The diethyl ether—hexane solvent extracted these compounds poorly. The choice of solvents used to dissolve the residue obtained upon evaporation of the extraction solvent was critical. While thioridazine and its metabolites were more soluble in relatively polar solvents (e.g., methanol), and therefore could be highly concentrated prior to injection into the HPLC, the injection of such solvents resulted in a loss of resolution and significant broadening of the peaks of the chromatogram. A mixture of hexane—methylene chloride—methanol (8:1:1) was selected as the sample solvent based upon a combination of sample solubility and compatibility with the mobile phase.

Compound identification

Several approaches were taken to insure the accurate identification of the peaks of the chromatograms. First, the calculated capacity factor for each reference standard was determined and compared to chromatograms of plasma extracts from human patients who had previously received thioridazine. Secondly, the nondestructive nature of UV absorbance detection permitted the collection of the detector effluent at the predetermined retention time of each individual compound. The solvent was evaporated to dryness and the samples reconstituted in 10 μl methanol and directly introduced into the analyzer of the mass spectrometer via the solid probe. Electron impact mass spectra were characterized by parent ions of low relative abundance, yet verified the correct assignment of chemical structure to the chromatographic peaks.

Interfering substances

Using packed steel columns or radially compressed columns containing 10-μm silica, extracts of drug-free plasma were found to contain a compound of unknown identity which coeluted with thioridazine. However, this interfering substance was present in relatively low, consistent amounts and its influence was effectively eliminated by blank-correction of the standard curves. The greater separation efficiency of radially compressed 5-μm silica effectively resolved this unknown plasma component from thioridazine and therefore this packing material is recommended for routine use. The possibility of interconversion of thioridazine and its metabolites during the course of sample preparation [31] and the resulting cross interference was also exam-

ined. The extraction, chromatographic separation and detection of 250 ng of a given metabolite or the parent compound added to separate 1-ml aliquots of drug-free plasma demonstrated the lack of interference by any one compound in the quantification of another.

Seven drugs which are often concurrently administered to pediatric and adult patient populations receiving treatment with thioridazine were also examined for their possible interference with the quantification of thioridazine or its metabolites using this assay procedure (Table IV). None of the drugs tested interfered in the assay of thioridazine or its metabolites. Of the additional antipsychotics tested, chlorpromazine and thiothixene were found to interfere in the determination of thioridazine and thioridazine-2-sulfone, respectively. Furthermore, as the majority of psychoactive drugs are extensively biotransformed to a multitude of appreciably accumulated metabolites of unknown potential for interference, a careful examination of the recent drug history of a given patient prior to sample collection and use of this methodology seems warranted.

TABLE IV

RELATIVE RETENTION (k') OF THIORIDAZINE, ITS MAJOR METABOLITES, OTHER ANTIPSYCHOTICS, AND DRUGS OFTEN ADMINISTERED CONCURRENTLY WITH THIORIDAZINE

Drug	k'	Drug	k'
Imipramine	0.16	Phenobarbital	9.01
Thioridazine	0.41	Thioridazine-2,5-disulfoxide	9.49
Diazepam	0.72	Diphenhydramine	nd*
Northioridazine	1.19	Methylphenidate	nd
Benztropine	1.66		
Thioridazine-2-sulfone	1.86	*Other antipsychotics*	
Thioridazine-2-sulfoxide	2.31	Trifluopromazine	0.24
Thioridazine-5-oxide	3.06	Chlorpromazine	0.43
Phenytoin	4.60	Thiothixene	1.81
Northioridazine-2-sulfoxide	5.71	Haloperidol	2.12
Thioridazine-2,5-disulfone	7.43	Fluphenazine	2.83

*nd = No detector responses.

Accuracy, precision and linearity

The accuracy and within-run precision of the present method were determined by assaying 1-ml aliquots of plasma containing either 75 or 500 ng amounts of thioridazine, northioridazine, thioridazine-2-sulfoxide, thioridazine-2-sulfone and thioridazine-5-oxide (Table V). The deviation of the amount found from the known amount added, and the coefficient of variation, were independent of concentration. Typical blank-corrected standard curves, based upon peak height ratios relative to 2-acetylphenothiazine, were linearly related to concentration with correlation coefficients for each compound being consistently greater than 0.99.

TABLE V

WITHIN-RUN PRECISION AND ACCURACY

n = 9 for both concentrations.

Drug	Concentration (ng/ml)		
	Added	Found	
		X ± S.D.	C.V. (%)
Thioridazine	75	74.0 ± 7.9	10.7
	500	512.6 ± 56.2	11.0
Northioridazine	75	72.7 ± 7.3	10.0
	500	484.5 ± 49.1	10.1
Thioridazine-2-sulfone	75	72.6 ± 6.6	9.0
	500	474.1 ± 39.7	8.4
Thioridazine-2-sulfoxide	75	75.7 ± 9.6	12.7
	500	490.9 ± 38.1	7.8
Thioridazine-5-oxide	75	68.5 ± 7.8	11.4
	500	490.4 ± 44.0	9.0

Sensitivity

The combination of low detector noise following injections of plasma extracts, high extraction efficiencies, and the high molar absorptivities (ϵ_{254}) of thioridazine and its metabolites permit their determination in human plasma at concentrations well below those reported following therapeutic doses of thioridazine. Detection limits were 10 ng/ml for thioridazine, northioridazine, thioridazine-2-sulfone and thioridazine-2-sulfoxide and 20 ng/ml for thioridazine-5-oxide, based upon an assayed plasma volume of 1 ml. Limits were defined by a minimum signal-to-noise ratio of 5 and coefficients of variation for replicate determinations (n = 8) of 15% or less. Concentrations below these values generally yielded coefficients of variation greater than 20% and these values were therefore taken as a lower practical limit of detection. The detection limit for thioridazine-5-oxide may be improved, if desired, by the use of UV absorbance detection at 280 nm, a value closer to the absorption maxima for this compound.

Patient data and summary

The present method permits the accurate quantification of thioridazine and its major metabolites at therapeutic plasma concentrations and in samples obtained for pharmacokinetic analyses. Table VI lists the concentrations of thioridazine and its metabolites in plasma from an adult male schizophrenic patient 1, 2, 4 and 24 h following an initial oral dose (200 mg) of thioridazine and 11 days later (sample obtained prior to morning dose). The present method has been in use in this laboratory for the past eighteen months and is routinely used to assay 20—25 plasma samples, plus a 6 or 7 point standard curve, in a normal working day. Furthermore, the more polar metabolites of thioridazine which are predominantly eliminated in urine [2] (e.g., thioridazine-2,5-disulfoxide, thioridazine-2,5-disulfone and northioridazine-2-sulfoxide) are readily separated and detected by this procedure (Figs. 3 and 4).

TABLE VI

PLASMA CONCENTRATIONS OF THIORIDAZINE AND ITS METABOLITES AT VARIOUS TIMES FOLLOWING THE INITIATION OF A DOSAGE REGIMEN OF 200 mg THIORIDAZINE PER DAY

Times given indicate the interval between drug administration and blood sample collection. The blood sample collected after 11 days was drawn immediately prior to the administration of the morning dose.

Time (h)	Plasma concentration (ng/ml)				
	THD*	norTHD	THD-2-SO_2	THD-2-SO	THD-5-SO
1	7	nd**	nd	nd	nd
2	258	nd	37	415	54
4	358	nd	92	875	173
24	31	nd	78	320	189
11 days	187	nd	98	438	538

*See Table I for definition of abbreviations.
**nd = Non-detectable.

ACKNOWLEDGEMENTS

This work was supported by PHS Grants Nos. HD-10570 and HD-03110. C.D.K. holds a postdoctoral National Research Service Award (MH-07926), and R.B.M. is a Young Investigator Awardee (ES-02087). The authors wish to thank Faygele ben Miriam for assistance in the preparation of this manuscript. We also gratefully acknowledge Dr. J. Garbutt for the collection of clinical samples.

REFERENCES

1 K. Zehnder, F. Kalberer, W. Kreis and J. Rutschmann, Biochem. Pharmacol., 11 (1962) 535.
2 E. Mårtensson, G. Nyberg and R. Axelsson, Curr. Ther. Res., 18 (1975) 687.
3 C.H. Ng and J.L. Crammer, Brit. J. Clin. Pharmacol., 4 (1977) 173.
4 F.A.J. Vanderheeren and R.G. Muusze, Eur. J. Clin. Pharmacol., 11 (1977) 135.
5 A.A. Sugerman, Curr. Ther. Res., 7 (1965) 520.
6 A. Mean, H. Grayson and S. Cohen, J. New Drugs, 6 (1966) 345.
7 R. Axelsson, Curr. Ther. Res., 21 (1977) 587.
8 G. Gardos, J.J. Teece, E. Hartmann, P. Bowers and J.O. Cole, Comp. Psychiat., 19 (1978) 517.
9 G. Kinon, G. Sakalis, L.J., Traficante, M. Aronson, P. Bowers and S. Gershon, Curr. Ther. Res., 25 (1979) 534.
10 G. Nyberg, R. Axelsson and E. Mårtensson, Eur. J. Clin. Pharmacol., 14 (1978) 341.
11 K.A. Freedberg, R.B. Innis, I. Creese and S.H. Snyder, Life Sci., 24 (1979) 2467.
12 R. Axelsson and E. Mårtensson, Curr. Ther. Res., 20 (1977) 561.
13 S. Eiduson, E. Geller and R.D. Wallace, Biochem. Pharmacol., 12 (1963) 1437.
14 J.B. Ragland, V.J. Kinross-Wright and R. Ragland, Anal. Biochem., 12 (1965) 60.
15 T.J. Mellinger and C.E. Keeler, Anal. Chem., 36 (1964) 1840.
16 W.L. Pacha, Experientia, 25 (1969) 103.
17 S.H. Curry and G.P. Mould, J. Pharm. Pharmacol., 21 (1969) 674.

18 E.C. Dinovo, L.A. Gottschalk, B.R. Nandi and P.G. Geddes, J. Pharm. Sci., 65 (1976) 667.
19 R.G. Muusze, J. Chromatogr. Sci., 12 (1974) 779.
20 J.R. McCutcheon, J. Anal. Toxicol., 3 (1979) 105.
21 T. Skinner, R. Gochnauer and M. Linnoila, Acta Pharmacol. Toxicol., 48 (1981) 223.
22 G.W. Haupt, J. Opt. Soc. Amer., 42 (1952) 441.
23 D.N. Bailey and J.J. Guba, Clin. Chem., 25 (1979) 1211.
24 G. Alfredsson, B. Wode-Helgodt and G. Sedvall, Psychopharmacol., 48 (1976) 123.
25 J.H.M. van den Berg, H.J.J.M. de Ruwe, R.S. Deelder and T.H.A. Plomp, J. Chromatogr., 138 (1977) 431.
26 F.L. Vandemark, R.F. Adams and G.J. Schmidt, Clin. Chem., 24 (1978) 87.
27 R.A. de Zeeuw and H.G.M. Westenberg, J. Anal. Toxicol., 2 (1978) 229.
28 G.J. Fallick and C.W. Rausch, Amer. Lab., Nov. (1979) 87.
29 C.D. Kilts, R.B. Mailman, E. Hodgson and G.R. Breese, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 40 (1981) 238.
30 M. Caude, L.X. Phan, B. Tertain and J.-P. Thomas, J. Chromatogr. Sci., 13 (1975) 390.
31 I.A. Zingales, J. Chromatogr., 44 (1969) 547.

Journal of Chromatography, 231 (1982) 393—399
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1327

SIMULTANEOUS DETERMINATION OF AZATHIOPRINE AND 6-MERCAPTOPURINE IN SERUM BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

KIMIKO TSUTSUMI, YOSHIE OTSUKI and TOSHIO KINOSHITA*

School of Pharmaceutical Science, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108 (Japan)

(First received January 21st, 1982; revised manuscript received March 30th, 1982)

SUMMARY

The simultaneous determination of azathioprine and its metabolite 6-mercaptopurine in serum by reversed-phase high-performance liquid chromatography is described. 6-Mercaptopurine was converted to a derivative, 6-mercaptopurine-N-ethylmaleimide, which is stable against autoxidation, on reaction with N-ethylmaleimide. Since the N-ethylmaleimide derivative was more hydrophobic than the parent compound, it could be extracted into ethyl acetate together with azathioprine and the derivative was retained on the reversed-phase column better than 6-mercaptopurine. In addition, 6-mercaptopurine-N-ethylmaleimide absorbed at the same wavelength (280 nm) as azathioprine. Consequently, this derivatization procedure enabled the simultaneous extraction, separation, and detection of these compounds.

INTRODUCTION

Azathioprine [AZA, 6-(1-methyl-4-nitroimidazol-5-ylthio)-purine] has been utilized as an effective immunosuppressive agent in organ transplantation and it is transformed in vivo to the active moiety, 6-mercaptopurine (6MP). 6MP itself is also used as an antiplastic agent. Their use should be carefully controlled in order to avoid severe adverse effects. In addition, there are great interindividual differences in the sensitivity and tolerance to these drugs. These facts prompted us to investigate the simultaneous monitoring of AZA and 6MP.

Numerous methods for the determination of AZA and/or 6MP have been proposed such as colorimetry and ultraviolet (UV) spectrometry involving ion-exchange column chromatography [1—3], but these methods gave only poor sensitivity. Although gas chromatography—mass spectrometry [4, 5] was reported as a sensitive tool for the assay of 6 MP, the need to use a mass spectrometer has prevented its wide application in routine laboratories. High-

0378-4347/82/0000—0000/$02.75

performance liquid chromatography (HPLC) on ion-exchange column [6—8] has been limited to the analysis of 6MP and it has not been applied to routine drug monitoring. Reversed-phase HPLC [9—11] has recently been introduced in this field to give high sensitivity and selectivity, but the simultaneous determination of AZA and 6MP has not yet been achieved by this method. This may be due to the fact that their absorption maxima are a long way apart, that their retention times differ greatly from each other, and that the mercapto group of 6MP is highly sensitive to autoxidation and it should be treated in the presence of a stabilizing agent which also causes the decomposition of AZA. In addition, 6MP is hardly soluble in organic solvents and it cannot be extracted together with AZA. Perchloric acid or trichloroacetic acid are effective for the extraction of 6MP, but the use of strong acid necessitated a tedious neutralization step in the course of sample pretreatment.

In the present study, 6MP was converted to a stable derivative (6MP-NEM) by reaction with N-ethylmaleimide (NEM) prior to chromatography. This technique made the addition of the stabilizing agent unnecessary, and solved the problems caused by the differences in absorption wavelengths, retention times, and solubilities between the two compounds. As a result, the simultaneous separation and determination of AZA and 6MP on reversed-phase HPLC was achieved.

EXPERIMENTAL

Materials

AZA was obtained from Nippon Wellcome (Osaka, Japan) and 6MP was purchased from Sigma (St. Louis, MO, U.S.A.). 2-Ethyl-4-oxoquinazoline (2EOQ) was the gift of Dr. Y. Okamoto in our laboratory. Stock solutions of these compounds were prepared by dissolving 5 mg in 50 ml of ethanol and storing at 4°C. The NEM reagent was prepared fresh daily by dissolving 30 mg of NEM (Wako, Osaka, Japan) in 2 ml of 0.05 *M* phosphate buffer, pH 7.0. HPLC-grade acetonitrile was obtained from Kanto (Tokyo, Japan). Mobile phase A was 0.01 *M* KH_2PO_4 aqueous solution containing 9% acetonitrile; mobile phase B was 0.01 *M* KH_2PO_4 aqueous solution containing 50% acetonitrile. Other reagents and solvents were of analytical grade. Hyland Q-Pak Chemistry Control Serum I was purchased from Travenol Labs. (IL, U.S.A.).

Procedure

To 200 μl of serum were added 5 μl of ethanol containing 250 ng of 2EOQ as the internal standard. The sample was mixed with 100 μl of the NEM reagent solution and allowed to stand at room temperature for 1 h. Then 1.8 ml of ethyl acetate were added, and the solution was mixed by an automatic mixer. The resulting mixture was then centrifuged at 1800 *g* for 5 min. A 1.5-ml aliquot of the supernatant was transferred to another centrifuge tube. After duplicate extractions, the organic phases were combined and evaporated to dryness under reduced pressure below 30°C. The residue was dissolved in 100 μl of mobile phase A and a 90-μl aliquot of the solution was injected into the chromatograph.

Chromatographic procedure

The liquid chromatograph consisted of a Waters Model 6000A pump, a U6K universal injector, a μBondapak C_{18} (particle size, 10 μm) column (all from Waters Assoc., Milford, MA, U.S.A.) equipped with a Soma UV detector S-310 A (Soma Optic Co., Tokyo, Japan) operated at 280 nm, and a Shimadzu Model R-111 recorder (Shimadzu, Kyoto, Japan) with a range of 1 mV under the full-scale setting of 0.005 or 0.002. The column was first eluted with mobile phase A for 26 min to separate AZA and 6MP-NEM and then eluted with mobile phase B for 1 min to wash the column. Both mobile phases were delivered at a constant flow-rate of 1.5 ml/min.

Extraction recoveries

The extraction recoveries of AZA and 6MP were examined using sera spiked with 500 ng/ml and 100 ng/ml of these compounds according to the procedure. The peak heights for spiked AZA were compared with the peak heights obtained from the same amount of standard. Also, the peak heights for 6MP-NEM were compared to the peak heights obtained from the same amount of 6MP that had been derivatized but not extracted.

Identification of the NEM derivative of 6MP

After 6MP was allowed to react with the NEM reagent according to the procedure, 6MP-NEM was purified by injecting the reaction mixture into the chromatograph and eluting with mobile phase A. The fraction of 6MP-NEM was collected and extracted with ethyl acetate. The extract was evaporated to dryness. The product was identified as 6MP-NEM by mass spectrometry. 6MP-NEM: *m/z* (percentage relative abundance) = 110 (76); 125 (44); 152 (100); 277 (M^+, 8).

RESULTS AND DISCUSSION

Absorption spectra of AZA, 6MP and 6MP-NEM in mobile phase A are shown in Fig. 1. Although the absorption maximum of 6MP is located away from that of AZA, that of 6MP-NEM appears at the same wavelength (280 nm) as that of AZA.

The reaction conditions for the pre-column derivatization of 6MP with NEM were examined using 500 ng/ml 6MP at room temperature. AZA remained unchanged during the derivatization. Fig. 2 shows the peak height of the 6MP derivative plotted against the concentration of NEM in the derivatization reagent. The peak height increased with the NEM concentration and continued to increase above 15 mg/ml NEM, but 15 mg/ml NEM was adopted in the assay procedure because the solubility of NEM is limited. The peak height also increased with the reaction time until 120 min, but 60 min was adopted in the procedure to minimize the time for the pretreatment.

AZA can be extracted with ethyl acetate or acetonitrile but 6MP cannot be extracted with such organic solvents. Therefore, perchloric acid and trichloroacetic acid have been used for the pretreatment of biological samples. However, the use of these strong acids was always followed by tedious neutralization steps. On the other hand, 6MP-NEM could be readily extracted with

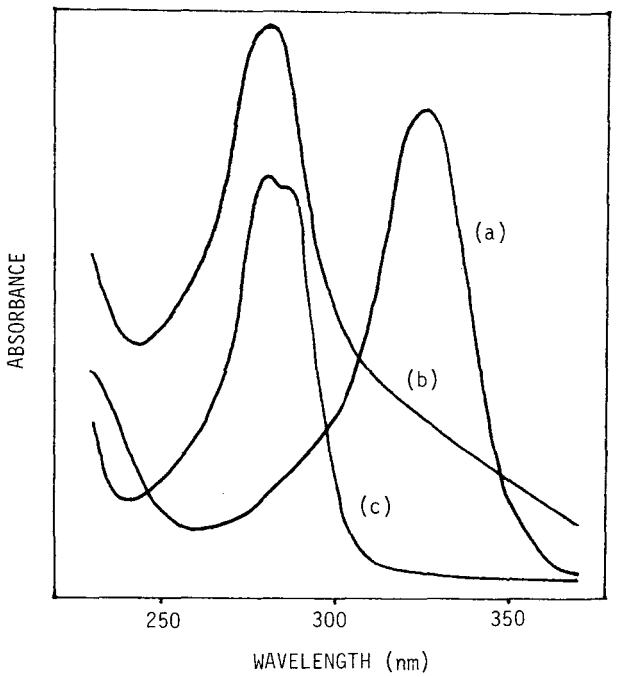


Fig. 1. Absorption spectra of (a) 6MP, (b) AZA and (c) 6MP-NEM dissolved in mobile phase A.

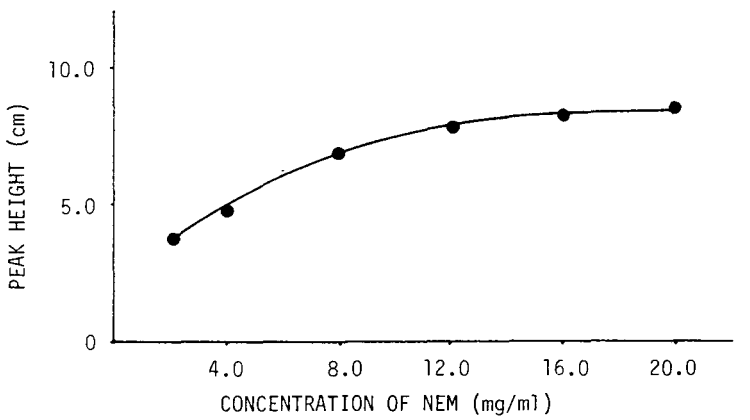


Fig. 2. The peak height of 6MP-NEM plotted against the concentration of NEM in the derivatization reagent.

ethyl acetate. The extraction of AZA and 6MP was examined using volumes of ethyl acetate ranging from 0.6 ml to 2.4 ml. Satisfactory recovery was obtained using 1.8 ml of ethyl acetate as listed in Table I.

Fig. 3a shows the chromatogram obtained from a standard mixture of AZA, 6MP and the internal standard (2EOQ) without extraction. The retention times of AZA, 6MP-NEM and 2EOQ were 13.4, 18.6 and 28 min, respectively. Fig. 3b and c display chromatograms of the ethyl acetate extracts of serum

TABLE I

EXTRACTION RECOVERIES OF AZA AND 6MP FROM SERUM

Values (in per cent) are expressed as mean ± S.D., $n = 5$.

Compound	Concentration	
	500 ng/ml	100 ng/ml
AZA	98.3 ± 3.72	87.2 ± 7.73
6MP	91.8 ± 4.78	82.9 ± 6.91

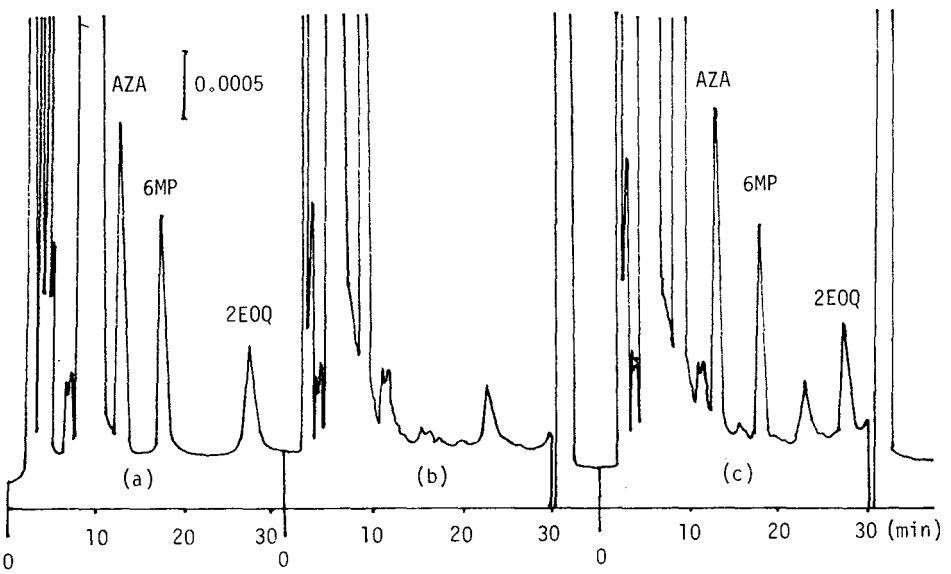


Fig. 3. Chromatograms obtained from: (a) a standard mixture of AZA (50 ng), 6MP (50 ng) and internal standard (250 ng) without extraction; (b) serum blank; and (c) serum spiked with AZA (50 ng) and 6MP (50 ng) with extraction according to the procedure.

blank and serum spiked with AZA, 6MP and 2EOQ, respectively. Since the serum samples show big unknown peaks at a retention time of about 45 min when eluted with mobile phase A, the mobile phase was changed to B at 26 min to reduce the separation time. This procedure enabled the injection of the next sample 40 min after the previous injection. AZA, 6MP-NEM and the internal standard were separated well from serum components, whereas the peak of underivatized 6MP appeared at the retention time of 3 min and overlapped with those of serum components. This indicates that the derivatization of 6MP is also useful for the removal of the peak of 6MP from the region where interfering peaks of serum components appear.

Standard curves for AZA and 6MP were linear in the range 10—500 ng/ml and passed through the origin. The signal-to-noise ratios of AZA and 6MP at a concentration of 10 ng/ml were 5.8 and 2.5 with 0.002 a.u.f.s., respectively. In addition, 2 ng/sample each of AZA and 6MP in serum was found to be detectable as shown in Fig. 4.

The within-run and day-to-day variability of the assay for AZA and 6MP

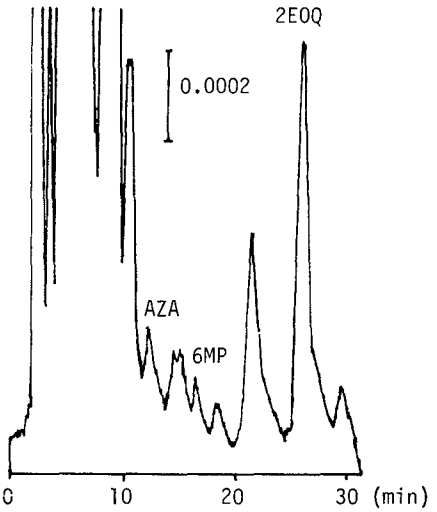


Fig. 4. Chromatogram obtained from serum spiked with AZA (2 ng) and 6MP (2 ng) with extraction according to the procedure.

TABLE II

COEFFICIENTS OF VARIATION (%) FOR ANALYSES

$n = 5$.

Compound	Concentration (ng/ml)	C.V. (%)	
		Within-run	Day-to-day
AZA	250	1.96	2.65
	50	2.64	4.92
6MP	250	3.39	4.98
	50	4.25	5.78

were studied as summarized in Table II. The variabilities resulted in coefficients of variation of less than 6%.

CONCLUSION

The present method involving the derivatization of 6MP offers the following advantages: (1) derivatized 6MP can be extracted together with AZA into ethyl acetate giving excellent recovery; (2) the absorption maximum of 6MP appears at 280 nm at which wavelength AZA also absorbs; (3) 6MP-NEM is well retained on the reversed-phase column and appears close to AZA on the chromatogram; (4) the stabilizing agent needed for the mercapto group can be eliminated.

The present method thus facilitates the rapid and sensitive simultaneous determination of AZA and 6MP.

ACKNOWLEDGEMENT

The authors wish to thank Mr. Ken Tokunaga (Nihon Waters Ltd., Tokyo, Japan) for his kind advice about the chromatographic technique.

REFERENCES

1 T.L. Loo, J.K. Luce, M.P. Sullivan and E. Frei, III, Clin. Pharmacol. Ther., 9 (1967) 180.
2 J.J. Coffey, C.A. White, A.B. Lesk, W.I. Rogers and A.A. Serpick, Cancer Res., 32 (1972) 1283.
3 A.H. Chalmers, Biochem. Med., 12 (1975) 234.
4 J.M. Rosenfeld, V.Y. Taguchi, B.L. Hillcoat and M. Kawai, Anal. Chem., 49 (1977) 725.
5 S. Floberg, P. Hartvig, B. Lindström, G. Lönnerholm and B. Odlind, J. Chromatogr., 225 (1981) 73.
6 D.J. Nelson, C.J.L. Bugge, H.C. Krasny and T.P. Zimmerman, J. Chromatogr., 77 (1973) 181.
7 H.J. Breter and R.K. Zahn, J. Chromatogr., 137 (1977) 61.
8 H.J. Breter, Anal. Biochem., 80 (1977) 9.
9 J.L. Day, L. Tterlikkis, R. Niemann, A. Mobley and C. Spikes, J. Pharm. Sci., 67 (1978) 1027.
10 T.L. Ding and L.Z. Benet, J. Chromatogr., 163 (1979) 281.
11 S.N. Lin, K. Jessup, M. Floyd, T.P.F. Wang, C.T. van Buren, R.M. Caprioli and B.D. Kahan, Transplantation, 29 (1980) 290.

Journal of Chromatography, 231 (1982) 401—409
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1336

SIMULTANEOUS DETERMINATION OF DANTROLENE AND ITS METABOLITES, 5-HYDROXYDANTROLENE AND NITRO-REDUCED ACETYLATED DANTROLENE (F 490), IN PLASMA AND URINE OF MAN AND DOG BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

E.W. WUIS*, A.C.L.M. GRUTTERS, T.B. VREE and E. VAN DER KLEYN

Department of Clinical Pharmacy, Sint Radboud Hospital, University of Nijmegen, Geert Grooteplein Zuid 10, 6526 GA Nijmegen (The Netherlands)

(First received February 2nd, 1982; revised manuscript received March 18th, 1982)

SUMMARY

A reversed-phase high-performance liquid chromatographic method is described for the simultaneous determination of the skeletal muscle relaxant dantrolene and its metabolites, 5-hydroxydantrolene and nitro-reduced acetylated dantrolene (F 490), in plasma and urine of man and dog. The substances are detected spectrophotometrically at 375 nm. The detection limits are 0.02 mg/l. A preliminary extraction step into a chloroform—butanol mixture is required for the plasma samples. The method is suitable for pharmacokinetic studies of dantrolene.

INTRODUCTION

Dantrolene sodium, 1-{[5-(*p*-nitrophenyl)-furfurylidene]-amino}-imidazolidine-2,4-dione sodium salt hydrate, first reported by Snyder et al. [1], is used as a skeletal muscle relaxant which appears to act by blocking muscle contraction beyond the neuromuscular junction [2—5]. It is used for the symptomatic relief of clinical spasticity resulting from serious disorders such as cerebral palsy, stroke, spinal cord injury, and multiple sclerosis [6—13]. More recently, it has also been recommended for the prevention and treatment of malignant hyperthermia, a syndrome recognized as one of the causes of anaesthesia-related deaths [14—16].

Metabolism of dantrolene (see Fig. 1) has been shown to proceed through both reductive and non-reductive pathways [17]. The nitro group of dantrolene is reduced to the corresponding amine, and in man and some animals subsequently acetylated to yield nitro-reduced acetylated dantrolene (F 490).

0378-4347/82/0000—0000/$02.75

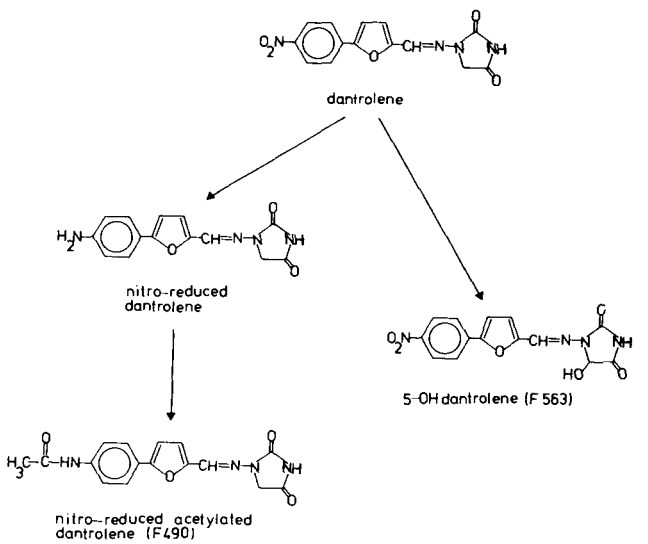


Fig. 1. Proposed metabolic scheme of dantrolene [18].

Of these reduced metabolites, which do not possess muscle relaxant properties [18], only F 490 has been detected in human blood [17,19] and urine [17,20]. Oxidation of dantrolene results in 5-hydroxydantrolene [21], a metabolite with muscle relaxant effects; the activity is less than dantrolene on an equimolar basis [18]. This 5-hydroxy metabolite has been identified in human blood [17,19,20,22,23] and urine [17,20].

Several analytical methods have been developed for the determination of dantrolene in blood and urine, including spectrophotofluorimetry [24,25], differential pulse polarography [17], high-performance liquid chromatography (HPLC) [26,27], and a qualitative colorimetric procedure [28]. The fluorimetric and polarographic methods require complicated differential analytical techniques to measure the drug in the presence of its metabolites. Meyler et al. [22], who used the fluorimetric procedure for plasma concentration measurements in volunteers and patients, encountered a discrepancy in the method which was not further investigated. They found that the total fluorescence of an extract from plasma containing dantrolene and 5-hydroxydantrolene (direct method) was considerably less than the fluorescence measured after the extract had passed through a Sephadex column and the two fractions containing 5-hydroxydantrolene and dantrolene, respectively, had been combined (indirect method).

The first reported HPLC procedure for dantrolene has the disadvantage of using a non-aqueous mobile phase [19,26]. In a short communication Hackett and Dusci [27] reported a reversed-phase HPLC procedure with, however, limited sensitivity and selectivity. Only dantrolene itself was measured, in concentrations down to 0.25 mg/l. To investigate the pharmacokinetics of dantrolene in volunteers and patients a new reversed-phase HPLC procedure was developed which allows the measurement of the drug and its metabolites at levels as low as 0.02 mg/l.

MATERIALS AND METHODS

Reagents and chemicals

All chemicals were of analytical grade. Three separate standard solutions were prepared in N,N-dimethylformamide containing 0.5, 0.5 and 0.3 mg/ml dantrolene sodium, 5-hydroxydantrolene, and nitro-reduced acetylated dantrolene (F 490), respectively, (a gift from Eaton Laboratories, Norwich, NY, U.S.A.). Ultrasonic treatment was used to increase the rate of dissolution of F 490. The extraction fluid was chloroform—1-butanol (95:5, v/v). Phosphate buffer (pH 6.8) was 50% (v/v) of dibasic sodium phosphate·$2H_2O$ solution (11.88 g/l) and 50% (v/v) of monobasic potassium phosphate solution (9.08 g/l). The mobile phase was acetonitrile—phosphate buffer (pH 6.8) (33.3:66.6, v/v).

Apparatus and chromatographic conditions

A liquid chromatograph (Kipp Analytica No. 9208) with a variable-wavelength detector (Schoeffel Instruments; SF 770 Spectroflow, GM 770 Monochromator), and a column packed with CP-Spher C_8 (Chrompack, Middelburg, The Netherlands; Cat. No. 28502, particle size 8 μm, 25 cm × 4.6 mm I.D.) was used. The flow-rate was 1.5 ml/min (pressure approx. 5 MPa). Samples were introduced by means of a 50-μl loop. The substances were detected at 375 nm, and the peak heights were measured.

Plasma

Calibration curve. A mixture of the standard solutions was diluted with blank plasma (concentrations 0.02—4 mg/l). To 1.0 ml of each solution were added 0.5 g of ammonium sulfate and 4.0 ml of the extraction fluid. The solutions were shaken for 10 min in a rotary mixer (Cenco Instruments, Cat. No. 23426). After centrifugation for 10 min at 2000 *g* (Heraeus Christ, type UJ1S), an aliquot of the lower layer was collected and evaporated to dryness in a hot (50°C) water bath under nitrogen. The residue was mixed with 1.0 ml of the mobile phase on a vortex mixer and injected onto the column. A control with N,N-dimethylformamide in blank plasma was treated in the same manner.

Samples. To 1.0 ml of plasma were added 0.5 g of ammonium sulfate and 4.0 ml of the extraction fluid; this mixture was further treated as described for the calibration curve.

Urine

Calibration curve. A mixture of the standard solutions was diluted with blank urine and diluted further 1 in 10 with the mobile phase to final concentrations of 0.02—4 mg/l. These solutions were directly injected onto the column. A control with N,N-dimethylformamide in blank urine was treated in the same manner.

Samples. Urine samples of 100 μl were diluted 1 in 10 with the mobile phase and directly injected.

Dog experiment

A male beagle dog of 12 kg body weight was anaesthetized with pento-

barbitone and subsequently given 12 mg of dantrolene sodium (dantrium) intravenously. Blood samples were collected at scheduled intervals. Urine samples were collected by means of a catheter for the first 7 h, thereafter spontaneously voided urine was used.

Recovery

Solutions in urine, prepared as described under the calibration curve for urine, and extracts from water and plasma, using the procedure as described under *Calibration curve* for plasma, were compared to a direct assay of standards in the mobile phase. The recoveries were determined for three different concentrations.

Stability

The standard solutions, which were kept protected from light at 4°C, were periodically measured spectrophotometrically (Beckman spectrophotometer, Model 3600), and by the described HPLC procedure.

RESULTS

A chromatogram of a plasma sample obtained from a patient treated with a daily oral dose of 5 mg/kg body weight dantrolene sodium is given in Fig. 2.

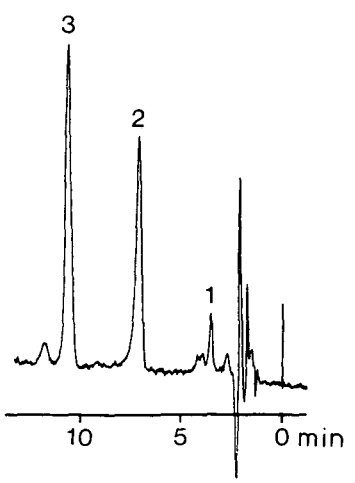


Fig. 2. Liquid chromatogram of patient plasma containing 0.705 mg/l dantrolene (3), 0.624 mg/l 5-hydroxydantrolene (2), and 0.055 mg/l F 490 (1).

Calibration curves in plasma and urine showed good linearity between peak heights and concentrations from 0.02 to 4.0 mg/l (r^2 = 0.999 for all substances). Chromatograms of blanks did not show any interfering substances at the detection wavelength of 375 nm. Retention times and capacity ratios are given in Table I.

The detection limit of dantrolene and its metabolites was 1 ng, defined as three times the noise level. The precision of the determination was measured for two different concentrations; the coefficients of variation are given in

TABLE I

RETENTION TIMES OF DANTROLENE AND ITS METABOLITES

For chromatographic conditions, see text.

Substance	Retention time (min)	Capacity ratio (k')
Dantrolene	10.9	3.5
5-Hydroxydantrolene	7.1	2.0
F 490	4.1	0.7

TABLE II

COEFFICIENTS OF VARIATION

For description of analytical procedure, see text.

Substance	Plasma			Urine		
	Concentration (mg/l)	Coefficient of variation (%)	n*	Concentration (mg/l)	Coefficient of variation (%)	n*
Dantrolene	0.051	3.5	6	0.043	7.0	6
	1.63	3.9	5	1.30	5.7	6
5-Hydroxydantrolene	0.066	3.1	6	0.055	6.0	6
	2.13	4.3	5	1.68	6.3	6
F 490	0.039	6.1	4	0.031	5.8	6
	1.24	3.9	5	1.07	6.3	6

*n = number of determinations.

TABLE III

RECOVERIES OF DANTROLENE AND ITS METABOLITES

For description of analytical procedure, see text.

Substance	Recovery* (%)		
	Water (extracted)	Plasma (extracted)	Urine
Dantrolene	97 ± 4	99 ± 8	103 ± 7
5-Hydroxydantrolene	100 ± 3	86 ± 2	99 ± 1
F 490	101 ± 4	89 ± 4	101 ± 4

*Means and standard deviations of three different concentrations (n = 3): dantrolene 3.25, 0.406, and 0.051 mg/l; 5-hydroxydantrolene 4.20, 0.525, and 0.066 mg/l; F 490 2.68, 0.335, and 0.042 mg/l.

Table II. The results of the recovery experiments are mentioned in Table III.

The standard solutions of all three substances did not show any deterioration for at least three months when kept at 4°C and when protected from light. Upon standing in light, however, F 490 deteriorates extremely quickly. On the chromatogram two peaks develop (Fig. 3). After 4 h at room temperature only about 50—60% of the peak heights relative to a freshly prepared solution could be measured (Fig. 4).

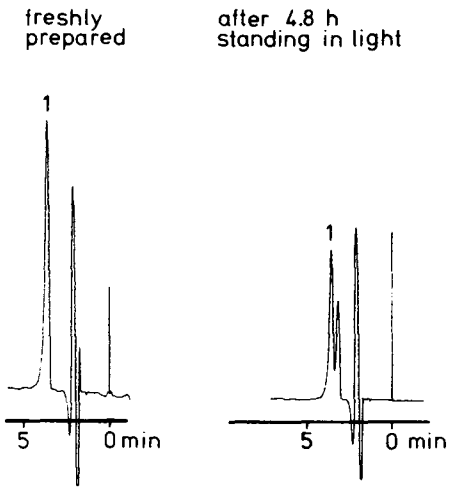


Fig. 3. Liquid chromatograms of a solution containing F 490 (1), showing instability upon standing in light.

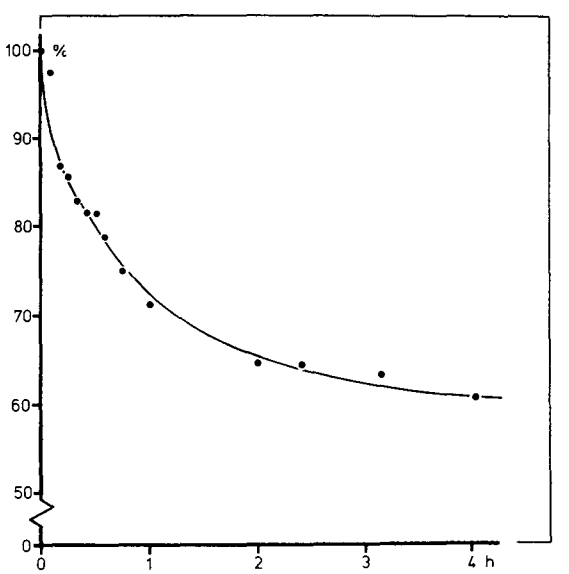


Fig. 4. Degradation of a solution containing F 490 upon standing in light.

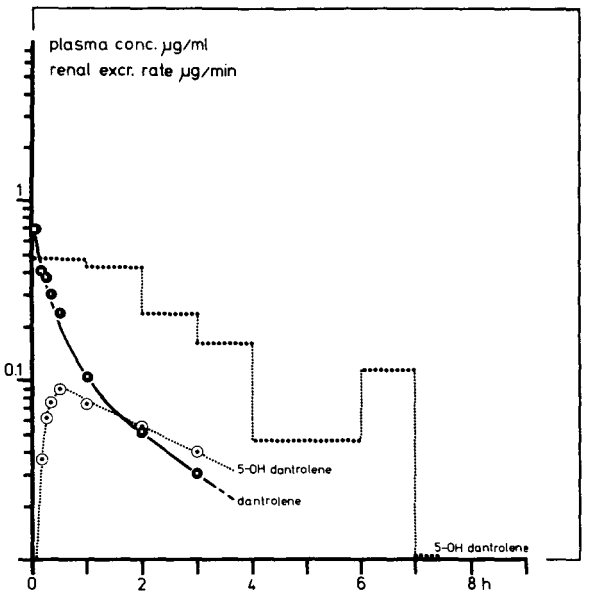


Fig. 5. Plasma concentration and renal excretion rate profiles of dantrolene and 5-hydroxydantrolene in a dog after intravenous administration of 1 mg/kg dantrolene sodium.

Fig. 5 shows the plasma concentration and renal excretion rate profiles of dantrolene and 5-hydroxydantrolene in a dog after intravenous administration of 1 mg/kg body weight dantrolene sodium. No dantrolene is excreted unchanged in dog urine.

DISCUSSION

The very low solubility of dantrolene and its metabolites in many solvents, including water, and the degradation by light, are complicating factors in the analysis. Dantrolene sodium is slightly soluble in water (15 mg/l), but it hydrolyzes quickly. The extremely insoluble (less than 1 mg/l) free acid dantrolene precipitates. The water solubilities of 5-hydroxydantrolene and F 490 are less than 10 mg/l (Data Sheet, Eaton Laboratories). Protection from light during the whole procedure appears to be important, especially for F 490, which is very unstable upon standing in light (see Figs. 3 and 4). N,N-dimethylformamide appeared to be a suitable solvent in preparing standard solutions. The solutions were stable for at least three months when kept protected from light at 4°C. No interference from this solvent was observed in the chromatographic assay.

Extraction of 1 ml of plasma with 4 ml of the chloroform—1 butanol (95:5) mixture yielded good recoveries. For the recovery of dantrolene from plasma (see Table III) a higher value than reported by Saxena et al. [26] was found; they used 80 ml of a chloroform—butanol (70:30) mixture.

On the chromatogram, the peaks of parent drug and metabolites were well resolved (see Fig. 2). With the reversed-phase method of Hackett and Dusci

[27] a relatively poor resolution between dantrolene and 5-hydroxydantrolene was obtained.

Slightly different chromatographic conditions may be needed for measurements in urine samples because of interference by endogenous substances with low retention times.

At present only a few pharmacokinetic studies of dantrolene have been published. Varying values for plasma concentrations and drug metabolite ratios have been reported. Vallner et al. [19] found dantrolene plasma concentrations between 0.03 and 0.2 mg/l, with slightly higher values for the metabolites in patients on chronic oral therapy, with daily dosages ranging from 50 to 200 mg. However, Lietman et al. [20] and Meyler [23] found plasma dantrolene concentrations of between 1 and 2 mg/l in patients on daily dosages of 4—12 mg/kg and 200—400 mg, respectively; the concentration of the 5-hydroxymetabolite appeared to be only 30—50% that of the parent compound. In a preliminary survey of patients on low dosages (0.7—5 mg/kg) we found large inter-individual differences in plasma concentrations and drug metabolite ratios, with plasma concentrations of 0.2—2.0 mg/l, 0.1—1.0 mg/l, and about 0.02 mg/l for dantrolene, 5-hydroxydantrolene, and F 490, respectively. In the dog experiment dantrolene and 5-hydroxydantrolene could be detected in plasma up to 3 h after the intravenous dose. The amount of 5-hydroxydantrolene excreted in the urine accounted for less than 1% of the dose. No dantrolene or F 490 could be detected in the urine.

The reported differences in the concentrations of the drug and of the metabolites have stimulated the investigation of the clinical pharmacokinetics of dantrolene. The described method provides adequate sensitivity and selectivity to make it applicable to pharmacokinetic studies.

REFERENCES

1 H.R. Snyder, C.S. Davis, R.K. Bickerton and R.P. Halliday, J. Med. Chem., 10 (1967) 807.
2 J.E. Desmedt and K. Hainaut, Biochem. Pharmacol., 28 (1979) 957.
3 K.G. Morgan and S.H. Bryant, J. Pharmacol. Exp. Ther., 201 (1977) 138.
4 R.M. Pinder, R.N. Brogden, T.M. Speight and G.S. Avery, Drugs, 13 (1977) 3.
5 C. Sengupta, U.A. Meyer and E. Carafoli, FEBS Lett., 117 (1980) 37.
6 A. Chantraine and C. Van Ouwenaller, Nervenarzt, 51 (1980) 50.
7 R.L. Joynt and J.A. Leonard, Jr., Dev. Med. Child Neurol., 22 (1980) 755.
8 R.E. Keenan, M.E. Kolb and M.L. Horne, Clin. Ther., 1 (1977) 48.
9 M. Van Laere, P. Leus and C. Mastelinck, J. Belge Med. Phys. Rehabil., 1 (1978) 189.
10 J. Mai and E. Pedersen, Acta Neurol. Scand., 59 (1979) 309.
11 O. Miglietta, Amer. J. Phys. Med., 56 (1977) 293.
12 G.E. Molnar, L.A. Evangelista and R. Kathirithamby, N.Y. State J. Med., 78 (1978) 1233.
13 R. Weiser, T. Terenty, P. Hudgson and D. Weightman, Practitioner, 221 (1978) 123.
14 S.K. Pandit, S.P. Kothary and P.J. Cohen, Anesthesiology, 50 (1979) 156.
15 D.K. Faust, S.D. Gergis and M.D. Sokoll, Anesth. Analg., 58 (1979) 33.
16 O.M. Jardon, D.W. Wingard, A.J. Barak and J.F. Conolly, J. Bone Joint Surg., 61A (1979) 1064.
17 P.L. Cox, J.P. Heotis, D. Polin and G.M. Rose, J. Pharm. Sci., 58 (1969) 987.
18 K.O. Ellis and F.L. Wessels, Naunyn-Schmiedeberg's Arch. Pharmacol., 301 (1978) 237.

19 J.J. Vallner, I.L. Honigberg, J.T. Stewart and A.B. Peyton, Curr. Ther. Res., 25 (1979) 79.
20 P.S. Lietman, R.H.A. Haslam and J.R. Walcher, Arch. Phys. Med. Rehabil., 55 (1974) 388.
21 R.L. White and T.J. Schwan, J. Pharm. Sci., 65 (1976) 135.
22 W.J. Meyler, H.W. Mols-Thürkow and H. Wesseling, Eur. J. Clin. Pharmacol., 16 (1979) 203.
23 W.J. Meyler, Thesis, State University of Groningen, Groningen, The Netherlands, 1978.
24 R.D. Hollifield and J.D. Conklin, J. Pharm. Sci., 62 (1973) 271.
25 R.D. Hollifield and J.D. Conklin, Arch. Int. Pharmacodyn. Ther., 174 (1968) 333.
26 S.J. Saxena, I.L. Honigberg, J.T. Stewart and J.J. Vallner, J. Pharm. Sci., 66 (1977) 751.
27 L.P. Hackett and L.J. Dusci, J. Chromatogr., 179 (1979) 222.
28 J.D. Conklin and R.J. Sobers, J. Pharm. Sci., 62 (1973) 1924.

Journal of Chromatography, 231 (1982) 410—417
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1311

Note

Quantitation of Dns-amino acids from body tissues and fluids using high-performance liquid chromatography

V.T. WIEDMEIER*, S.P. PORTERFIELD and C.E. HENDRICH

Department of Physiology, Medical College of Georgia, Augusta, GA 30912 (U.S.A.)

(First received November 9th, 1981; revised manuscript received April 7th, 1982)

The development and refinement of high-performance liquid chromatography (HPLC) in recent years has led to considerable interest in this technique as a means for accomplishing complex separations of biologically important compounds. Standard techniques for the separation of amino acids are complex [1], costly and by comparison with HPLC, time consuming.

(5-Dimethylaminonaphthalene-1-sulfonyl chloride (Dns-Cl) reacts with both primary and secondary amino acids and the highly fluorescent derivatives have been separated using thin-layer [2] and column [3] chromatography. These derivatives provide high sensitivity and are stable over relatively long periods.

The formation of Dns-amino acids involves a series of reactions and the product yield appears to be dependent on the relative amounts of Dns-Cl and amino acid and on conditions using during incubation [4]. Careful control of reaction conditions which favor formation and inhibit decomposition of Dns-amino acids revealed the product yield to be independent of the Dns-Cl:amino acid ratio over a 1000-fold range [5]. Previous studies also emphasized the importance of carefully controlling reaction pH and temperature to optimize product yield [6]. An objection to the use of Dns-Cl for amino acid derivatization is that multiple derivatives of several amino acids may form. We have selected conditions such that formation of multiple derivatives is minimal and does not pose a problem.

Finally, pre-column derivatization requires no special equipment, can be carried out at room temperature and the resulting derivatives are stable over days permitting the use of automatic sampling devices. Considerable work has been published related to the chemistry of the reaction between Dns-Cl and amino acids [4—6]. Less information is available relating the application of the method to separation and quantitation of amino acids in biological samples.

In this paper we present a method for sample preparation, derivatization

0378-4347/82/0000—0000/$02.75

with Dns-Cl and quantitation of amino acids in biological tissues and fluid using HPLC.

EXPERIMENTAL

Apparatus

A high-performance liquid chromatograph (Beckman Instruments) consisting of a Model 421 microprocessor, two Model 110A pumps, a Model 210 sample injector with a 50-μl sample loop and a C-R1A recorder-integrator was used. Fluorescence was detected using a fluorometer (Gilson Spectra/Glo) with a standard flow-cell, 7-51X excitation filter and 3-72M emission filter. Prepacked reversed-phase columns (Ultrasphere-ODS, 5 μm, 25 cm × 4.6 mm) were used for separation. Reagents (water, acetonitrile and tetrahydrofuran) were HPLC grade (Baker). Dns-Cl dissolved in acetone (Pierce) was diluted to a final concentration of 6 mg/ml with acetone.

Standards

Standards were prepared by dissolving the appropriate amount of various amino acids (Sigma) in 0.5 *M* sodium bicarbonate (pH 8.5) to yield a 10 m*M* concentration. Twenty-one amino acids were prepared in this way and included taurine, asparagine, glutamine, serine, aspartic acid, hydroxyproline, glutamic acid, threonine, glycine, alanine, arginine, methionine, proline, valine, phenylalanine, tryptophan, leucine, isoleucine, lysine, tyrosine and histidine. Volumes of 20-μl of each were then mixed together in small tubes and 80 μl of sodium bicarbonate added for a total volume of 0.5 ml (0.4 nmoles of each amino acid per μl).

Extraction procedures

Although much smaller amounts of tissue can be utilized, we generally extracted the amino acids from either 1.0 g of brain or liver or 1.0 ml of serum. All extraction procedures were conducted in an ice bath and a refrigerated centrifuge. The brain and liver were homogenized in 2.0 ml of ice-cold 80% ethanol and the serum was added to this volume of 80% ethanol. The tissues were then centrifuged at 1500 *g* for 15 min. This extraction was repeated twice more and the supernatants pooled and evaporated to dryness in a vacuum oven. The residue was dissolved in distilled water (2.0 ml/g of tissue or ml of serum) and frozen until analyzed for amino acid content. Known quantities of ^{14}C-labeled amino acids were added to serum and tissue samples and put through these extraction procedures. Our recovery rates ranged from 87—93%. These are the methods of Dickerson and Pao with some modification [7].

Preparation of Dns derivatives

Aliquots of brain and plasma extract were added to equal volumes of sodium bicarbonate (0.5 *M*, pH 8.5) and mixed. Standard and samples were then derivatized together. The standard was comprised of 0.1 ml sodium bicarbonate (pH 8.5) 25 μl of the standard mixture (10 nmoles of each amino acid) and 0.1 ml Dns-Cl. Samples mixed at the same time consisted of 0.1 ml of plasma

or tissue extract in sodium bicarbonate, 25 μl of the standard mixture and 0.1 ml Dns-Cl. The pH of the standard and samples after adding Dns-Cl is 9.2—9.5. Twenty samples and 4—5 standards were derivatized for assay the following day, covered tightly and placed in the dark at room temperature overnight. Dns derivatization is complete in 3—4 h and if left overnight we have found no decrease in fluorescence. Water (0.8 ml) adjusted to a pH of 8.5 with sodium hydroxide, was then added, the samples and standard centrifuged to remove any precipitate and 50 μl injected onto the column. This volume represents 0.5 nmoles of each amino acid in both standard and sample. The presence of the internal standard prevents confusion when quantitating peaks which are not completely resolved and is especially useful when one of these peaks is absent. The 50-μl volume placed on the column contains the amino acids from 1.25 mg of tissue or 2.5 μl of plasma.

Chromatography

Resolution of the peaks was accomplished using gradient elution with the mobile phase in pump A consisting of 10 m*M* sodium acetate buffer, pH 4.18— tetrahydrofuran, (95:5) and that in pump B acetonitrile—tetrahydrofuran (90:10). The mobile phase was begun at a flow-rate of 1.0 ml/min at 10%B and increased to 40%B over 30 min at which time an isocratic hold lasting 15 min was instituted. At the end of the isocratic period B was increased to 100% over 3 min and maintained at 100% for an additional 12 min. The column was then re-equilibrated with A—B (90:10) for 8 min.

RESULTS

Separation of a standard mixture of amino acids is shown in Fig. 1. Most of the products of the Dns derivatization procedure including dansic acid and unhydrolyzed Dns-Cl elute with or very close to the solvent front. The other major by-product, Dns-amide (DAM), elutes from the column at 31 min and serves as a convenient midrun marker. Each peak in Fig. 1 represents 0.5 nmoles of the amino acid at 1/5 the maximal sensitivity of the fluorometer. Duplicate analysis of samples derivatized as described yields excellent reproducibility with variations of 0—4%. An advantage of this method is that the secondary amino acids hydroxyproline (HPR) and proline (PRO) are also derivatized and easily identified (Fig. 1).

The formation of multiple derivatives of the amino acids lysine (LYS) and tyrosine (TYR) did not occur when the pH was carefully controlled during derivatization and only the di-Dns derivatives were formed. Dns derivatization at a lower pH (8.5) results in the formation of multiple derivatives of tyrosine with a second peak eluting at 28 min (Fig. 2). Dns derivatization of the epsilon amino group of lysine may also occur if the pH is too low resulting in multiple derivatives of this amino acid. This poses no problem since ϵ-Dns-LYS elutes as a well defined peak after, and separated from, glycine (GLY).

A chromatogram from a liver sample showing the levels of various amino acids is shown in Fig. 3. Although some unidentified peaks are apparent they are minor and elute at times different from those of the standard. It is

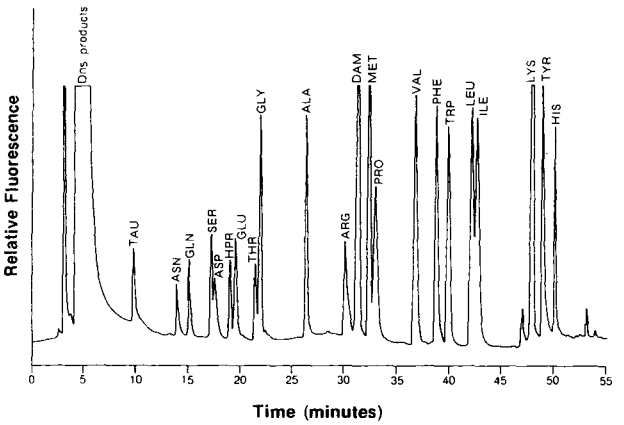


Fig. 1. A chromatogram showing the relative fluorescence and retention times of a mixture od 21 Dns-amino acids. Each amino acid peak represents 0.5 nmoles. Gradient elution, mobile phase: A = 10 m*M* sodium acetate buffer, pH 4.18—tetrahydrofuran (95:5); B = acetonitrile—tetrahydrofuran (90:10), flow-rate 1.0 ml/min. Peaks: TAU = taurine; ASN = asparagine; GLN = glutamine; SER = serine; ASP = aspartate; HPR = hydroxyproline; GLU = glutamate; THR = threonine; GLY = glycine; ALA = alanine; ARG = arginine; DAM = Dns-amide; MET = methionine; PRO = proline; VAL = valine; PHE = phenylalanine; TRP = tryptophan; LEU = leucine; ILE = isoleucine; LYS = lysine; TYR = tyrosine; HIS = histidine.

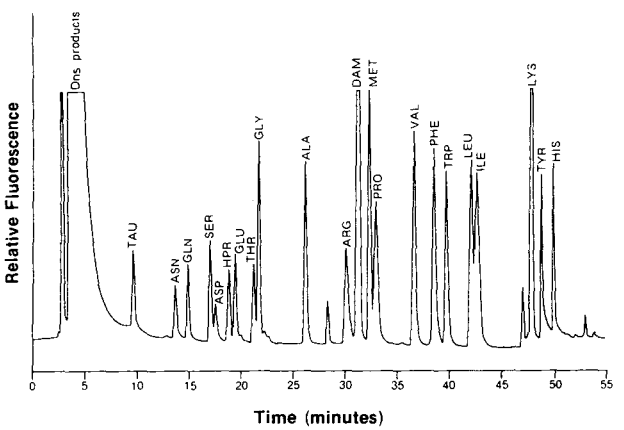


Fig. 2. A chromatogram of a standard mixture of Dns-amino acids, derivatized at pH 8.5. A second derivative of tyrosine (mono-Dns-tyrosine) elutes at 28 min. Separation procedure and abbreviations as in Fig. 1.

apparent that certain amino acids (ASN, HPR, ARG, MET, TRP and HIS) are present in small amounts or absent from this sample.

The ability to reproduce retention times is essential when accomplishing a complex separation such as a mixture of amino acids. Using the system described we have found the retention times to be very consistent from one chromatogram to the next. The retention times and variability of early, middle and late eluting peaks were determined. Taurine (TAU), valine (VAL) and lysine (LYS) were selected and the means ± S.E. for ten determinations were:

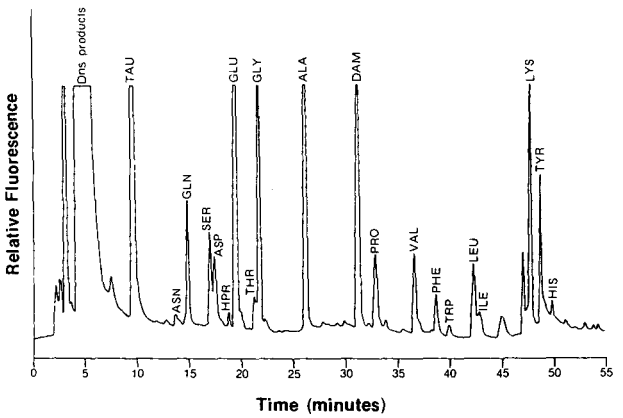


Fig. 3. A sample of rat liver chromatographed after Dns derivatization without internal standard. Separation procedure and abbreviations as in Fig. 1.

TABLE I

PERFORMANCE DATA INDICATING THE WITHIN-RUN AND RUN-TO-RUN PRECISION AS INDICATED BY COEFFICIENTS OF VARIATION (C.V.)

Concentration of each amino acid, 0.5 nmoles. In all cases $n = 6$.

Amino acid	Mean*	C.V. (%)
		Precision within-run
Glutamine	38	1.4
	42	3.9
	41	3.9
	39	1.9
	40	1.6
	40	2.2
Alanine	154	0.9
	155	0.9
	156	1.3
	155	1.2
	154	0.7
	155	1.6
Lysine	516	0.5
	506	2.8
	493	1.7
	500	1.6
	508	2.6
	514	2.1
		Precision run-to-run
Glutamine	40	3.6
Alanine	155	0.5
Lysine	506	1.8

*Mean = relative area ($\times 10^3$) computed by the integrating recorder under conditions described in the Experimental section.

TAU 9.2 ± 0.09 min, VAL 38.64 ± 0.06 min and LYS 47.5 ± 0.02 min.

Data in Table I demonstrate the precision achieved for three amino acids from the beginning, middle and end of the chromatograms. The run-to-run data were obtained over a 3—4 month period.

Even though retention times are reproducible we chose to utilize an internal standard for each peak to insure that (a) if variations in retention time occurred we could still identify the peaks and (b) if variations in the derivatization reaction occurred it would affect standard and sample to the same degree. Fig. 4 is a chromatogram of sample and standard (0.5 nmoles of each amino acid) derivatized together. Knowing the area of the standard alone (integration of peaks as in Fig. 1) and of the area of the sample + standards (as in Fig. 4) it is simple to quantitate the amount of amino acid in each peak.

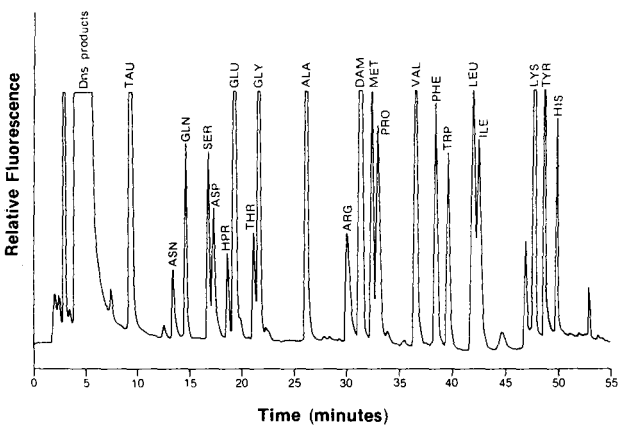


Fig. 4. Chromatogram of a sample of rat liver Dns derivatized with internal standards as in Fig. 1. The same separation procedure and abbreviations as in Fig. 1.

The figures presented here are from chromatograms obtained from liver and standards. We have used the same technique to analyze samples of brain and plasma, varying only the amount of sample derivatized or the amount injected onto the column (to prevent overloading). We observe only minor differences and find that the technique works equally well for all tissues tested thus far.

DISCUSSION

The technique for separation and quantitation of derivatized amino acids described herein provides several advantages over other methods. Pre-column derivatization with Dns-Cl is rapid and economical and in contrast to pre-column derivatization with *o*-phthalaldehyde (OPA) the product formed is very stable. We have run repeat chromatograms on derivatized samples over a 96-h period and find no detectable decrease in fluorescence. This observation is of critical importance when the use of autosamplers and large numbers of samples are involved. Pre-column derivatization with OPA precludes use of an automatic sampler since the OPA-amino acid adducts formed degrade at different rates and thus result in peak height changes with time [8]. A further

disadvantage of OPA is that it does not react with secondary amino acids such as proline and hydroxyproline [9]. More recently a system was presented which permits simultaneous detection of OPA derivatized primary and secondary amino acids [10].

The short analysis time required (55 min) is yet another advantage. Sample preparation is fast and simple which permits a technician to learn the technique and do the complete analysis at a fraction of the time and cost required by an amino acid analyzer. The ability to quantitate primary and secondary amino acids during a single run is yet another advantage of this method since the reaction between Dns-Cl and amino acids is linear and quantitative over a wide range of concentrations (up to 1000 times excess Dns-Cl) [5].

In contrast to the findings of Hill et al. [11] we encountered no problem resolving threonine and glycine in either tissue samples or plasma. In agreement with their findings we noted several peaks which were especially susceptable to changes in the concentration of buffer. A decrease in buffer concentration to 5 m*M* causes methionine and proline to co-elute and arginine to be retained longer, appearing after the Dns-amide peak. Retention of arginine is also especially sensitive to changes in pH; a decrease causes an increase in retention time while an increase moves arginine closer to alanine. The susceptability of arginine and Dns-amide peaks to changes in mobile phase composition have also been observed by others [3,12]. Serine and aspartate may also co-elute if the pH is decreased.

We have found pre-column derivatization with Dns-Cl to be highly reliable and reproducible. Repeat analyses of the same samples derivatized at various times have produced variations of less than 5%, an error which can be attributed to dilution etc. during sample preparation (Table I) and is in the range of variation observed in the separation of OPA-amino acid adducts [13].

The use of HPLC and pre-column derivatization to form fluorescent adducts can provide a useful tool for the rapid, economical separation and quantitation of amino acids in biological fluids and tissues. Extension of this technique may prove useful in peptide analysis and separation and quantitation of other biogenic amines.

ACKNOWLEDGEMENTS

This work was supported by NIH Grant No. R01 HD11411 and the Eagles Max Baer Heart Fund.

REFERENCES

1 D.H. Spackman, W.H. Stein and S. Moore, Anal. Chem., 30 (1958) 1190.
2 W.R. Gray, Methods Enzymol., 25 (1972) 121.
3 Y. Tapuhi, N. Miller and B.L. Karger, J. Chromatogr., 205 (1981) 325.
4 D.J. Neadle and R.J. Pollitt, Biochem. J., 97 (1965) 607.
5 Y. Tapuhi, D.E. Schmidt, W. Lindner and B.L. Karger, Anal. Biochem., 115 (1981) 123.
6 C. Gros and B. Labouesse, Eur. J. Biochem., 7 (1969) 463.
7 J.W.T. Dickerson and S.K. Pao, Biol. Neonate, 25 (1975) 114.
8 B.R. Larsen and F.G. West, J. Chromatogr. Sci., 19 (1981) 259.
9 M. Roth, Anal. Chem., 43 (1971) 880.

10 Y. Ishida, T. Fujita and K. Asai, J. Chromatogr., 204 (1981) 143.
11 D.W. Hill, F.H. Walters, T.D. Wilson and J.D. Stuart, Anal. Chem., 51 (1979) 1338.
12 J.M. Wilkinson, J. Chromatogr. Sci., 16 (1978) 547.
13 K. Lenda and G. Svenneby, J. Chromatogr., 198 (1980) 516.

Journal of Chromatography, 231 (1982) 418—424
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1331

Note

Simple and rapid high-performance liquid chromatographic method for analysis of nucleosides in biological fluids

RAM P. AGARWAL*

Section of Medical Oncology, The Evans Memorial Department of Clinical Research, and the Departments of Medicine and Pharmacology, Boston University Medical Center, Boston, MA 02118 (U.S.A.)

and

PIERRE P. MAJOR and DONALD W. KUFE

Sidney Farber Cancer Institute, 44 Binney Street, Boston, MA 02115 (U.S.A.)

(First received January 21st, 1982; revised manuscript received April 17th, 1982)

Patients with severe combined immunodeficiency (adenosine deaminase deficiency variant) lack both T- and B-cell function and have been reported to have elevated plasma dAdo* levels [1—4]. The adequacy of enzyme replace ment therapy appears to correlate with decreases in plasma dAdo concentrations [4, 5]. Recent in vitro studies have demonstrated a marked enhancement of dAdo toxicity to lymphoid cells, when ADA activity is inhibited by specific ADA inhibitors [6]. The neurotoxic complications following accumulation of Ado and dAdo in the plasma and cerebrospinal fluid of patients treated with a potent ADA inhibitor, deoxycoformycin [7, 8], and the neurotoxicity caused by dAdo and Ado in experimental animals [9—11] suggest that these nucleosides might play a role as neurotransmitters. Another adenosine analogue, Ara-A, a useful antiviral agent [12, 13], is now being evaluated in patients for its antileukemic properties [14—16]. These observations demonstrate the need for rapid, reproducible and sensitive methods for separation and quantification of Ado, dAdo, Ara-A and their metabolites in body fluids.

*Abbreviations used: ADA = adenosine deaminase (adenosine aminohydrolase, E.C. 3.5.4.4); Ado = adenosine; dAdo = 2'-deoxyadenosine; Ara-A = 9-β-D-arabinofuranosyladenine (arabinosyladenine); Ara-Hyp = 9-β-D-arabinofuranosylhypoxanthine (arabinosylhypoxanthine); DCF = deoxycoformycin; HPLC = high-performance liquid chromatography.

0378-4347/82/0000—0000/$02.75

Recently, several methods have been reported for the separation of nucleosides [17–31]; except for two [30, 31], most of these methods require the use of either large volumes of body fluids, tedious protein precipitation procedures and/or radioactive chemicals. Furthermore, the separation of ribosyl-, deoxyribosyl- and arabinosylpurines present in a mixture has not met with success. In order to separate them one has to employ special techniques, e.g., periodation or use of borate. The method we report here is simple, rapid and reproducible, and has been applied successfully to quantify Ado, dAdo, Ara-A and their degradation products in clinical samples of body fluids.

EXPERIMENTAL

Chemicals

Purine bases and nucleosides were purchased from P.L. Biochemicals (Milwaukee, WI, U.S.A.). Ara-A was a gift from Parke Davis (Ann Arbor, MI, U.S.A.). Adenosine deaminase (specific activity 195 units/mg protein) was obtained from Sigma (St. Louis, MO, U.S.A.). Potassium dihydrogen phosphate (analytical-reagent grade) was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.) and HPLC-grade methanol from Fisher Scientific (Fairlawn, NJ, U.S.A.). All other reagents were of the highest grade available through commercial suppliers.

Equipment

Throughout the study we used the following equipment: Altex Model 312A liquid chromatograph (Altex Scientific, Berkeley, CA, U.S.A.), equipped with two pumps (Model 110), a microprocessor solvent programmer (Model 410), a UV—visible variable-wavelength detector (Model 155-100), a syringe injector valve with a 100-μl sample loop, a reporting integrator (Model 3380A; Packard, Avondale, PA, U.S.A.) and a reversed-phase Ultrasphere-ODS 5-μm column (150 × 4.6 mm; Altex).

Methods

The eluents for HPLC were 10 m*M* KH_2PO_4 (solution A) and 30% methanol in 10 m*M* KH_2PO_4 (solution B), pH 4.9. Each solution was prepared fresh daily by diluting from a 500 m*M* KH_2PO_4 stock solution (which was stored at 4°C) and filtering through an HA 0.45-μm Millipore filter (Millipore, Bedford, MA, U.S.A.).

Plasma, cerebrospinal fluid and urine samples were prepared by filtering under centrifugal force through Amicon Centriflo CF25 ultrafiltration membrane cones (Amicon, Lexington, MA, U.S.A.) at 1100 *g* for 20 min. This method is rapid for deproteinization and gives excellent recoveries of nucleosides. Excellent recoveries of inosine and xanthosine from human serum by use of ultrafiltration have also been reported by Hartwick et al. [30]. The filtered samples were either analyzed immediately or stored at −20°C.

Aliquots of 10–20 μl were injected directly for HPLC analysis. The elutions were performed by using a linear gradient of 10–30% of solution B in solution A, achieved in 15 min at a flow-rate of 1 ml/min. Absorbance was monitored at 254 nm.

Evaluation of recovery of Ara-A and Ara-Hyp from human plasma. Plasma samples, with or without 2 μM deoxycoformycin, an inhibitor of ADA, were incubated for 15 min prior to the addition of known amounts of Ara-Hyp and Ara-A. Following a 20-min incubation at room temperature the samples were filtered through Amicon Centriflo ultrafiltration membranes as described above. The ultrafiltrates were then analyzed by HPLC and the amounts of Ara-A and Ara-Hyp quantified.

Confirmation of nucleoside identity of peak-shift method. The peak-shift method was employed for additional and definitive identification of the peaks having the retention times of adenosine, deoxyadenosine and Ara-A. The fractions corresponding to these peaks on HPLC were collected separately and each was treated with one unit of ADA. After incubation for 15—20 min at room temperature the proteins were removed either by filtration through Amicon Centriflo ultrafiltration membranes or by heating the mixture for 1 min in a boiling water-bath, cooling on ice and centrifugation. The samples were then re-analyzed by HPLC. Disappearance of the original peaks and appearance of peaks corresponding to their deaminated products established the identity of these compounds.

RESULTS AND DISCUSSION

Chromatographic separation

Fig. 1 presents an HPLC profile of a number of nucleosides and purine bases. The separation of Ara-A and its metabolite, Ara-Hyp, is shown in Fig. 2. Ado, dAdo and Ara-A are clearly separated. Furthermore, the metabolic

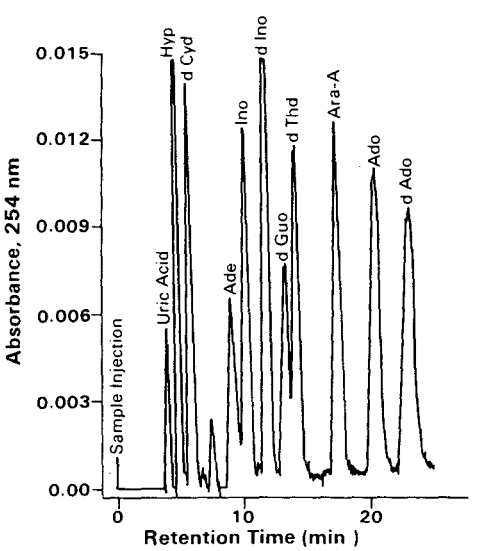


Fig. 1. Separation of a standard mixture of nucleosides and bases by HPLC. Injection volume, 10 μl; column, Ultrasphere ODS; flow-rate, 1 ml/min. Peaks: uric acid, hypoxanthine (Hyp), deoxycytidine (dCyd), adenine (Ade), inosine (Ino), deoxyinosine (dIno), deoxyguanosine (dGuo), thymidine (dThd), arabinosyl adenine (Ara-A), adenosine (Ado) and deoxyadenosine (dAdo).

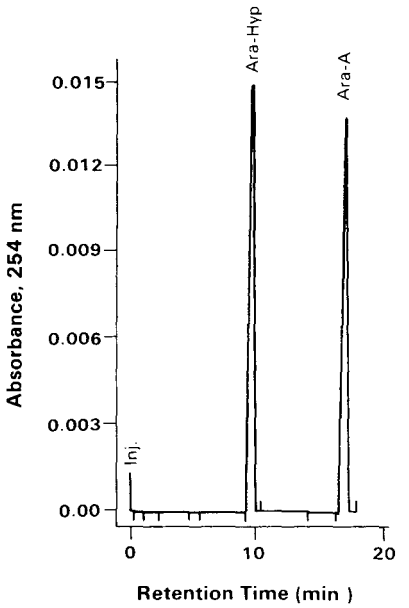


Fig. 2. HPLC separation of arabinosylhypoxanthine (Ara-Hyp) and arabinosyladenine (Ara-A). A 10-μl aliquot of a mixture containing 13.0 μg/ml each of Ara-A and Ara-Hyp was injected.

products of these nucleosides are also resolved in a single run. For example, Ado, Ino, dIno, Ara-Hyp, adenine, hypoxanthine and uric acid are all resolved. Although not illustrated, this procedure can also be used to resolve guanosine from deoxyguanosine and other nucleosides such as deoxycoformycin (Table I).

The retention times show very little day-to-day variation. The small differences did not cause either overlapping or alterations in the pattern illustrated

TABLE I

RETENTION TIMES OF SOME BASES, RIBONUCLEOSIDES, DEOXYRIBONUCLEOSIDES AND ARABINOSIDES

Compound	Retention time (min)*	Compound	Retention time (min)*
Uric acid	3.88 ± 0.61 (85)	Deoxyinosine	11.75 ± 0.76 (85)
Hypoxanthine	4.64 ± 0.59 (85)	Guanosine	11.90 ± 0.45 (5)
Deoxycytidine	5.64 ± 0.64 (85)	Deoxyguanosine	13.13 ± 0.76 (85)
N^1-Methyladenosine	6.67 (2)	Thymidine	14.10 ± 0.83 (85)
Deoxycoformycin	7.91 ± 0.62 (3)	5-Bromodeoxyuridine	17.01 (2)
Adenine	8.40 ± 1.09 (79)	Arabinosyladenine	17.24 ± 0.64 (45)
Arabinosylhypoxanthine	9.61 ± 0.31 (26)	Adenosine	20.00 ± 1.32 (85)
Inosine	10.18 ± 0.64 (85)	Deoxyadenosine	22.38 ± 1.66 (85)

*Mean ± S.D. (n).

in Fig. 1. These small differences were due to the mechanical adjustment rather than to changes in resolution, and therefore remained constant and were reflected in all profiles on that particular day. Therefore, a common practice has been to run a mixture of standards once in the morning and at the end of the day. Table I summarizes the retention times of all standard compounds examined in runs performed over a 2-year period.

Maintenance of the column

After prolonged use, aberrant separation profiles were sometimes obtained. This problem was usually corrected by washing the column for 15—30 min in the following order: first distilled water, then 1 m*M* phosphoric acid, followed by distilled water, 70% (v/v) methanol and distilled water. All solutions were filtered through Millipore filters (HA, 0.45 μm). At the end of the day the column was flushed with 20—30 ml of 30% methanol in 10 m*M* KH_2PO_4. Storage of the column, when it is not in use for more than 3 days, in 70% methanol prolongs its useful life. In our laboratory, the same column has been in use for more than 2 years for analyzing over 2000 samples. The quality and retention times of the peaks have been excellent.

Calibration graphs and accuracy

The relationship between the concentration and the peak area of nucleosides was linear over a wide range of concentrations. Using 100-μl samples, we could detect as low as 10 pmole of Ara-A (or Ado or dAdo). This sensitivity is similar to that recently reported with another HPLC method, which used radioactive nucleosides and protein precipitation [26].

The recoveries of Ara-A and Ara-Hyp added to plasma were 95 ± 4% and 101 ± 2.5%, respectively. However, in the absence of DCF the recovery of exogeneously added Ara-A was slightly lower (83%) and there was correspondingly a slightly larger amount of Ara-Hyp (110%) resulting from deamination of Ara-A. This suggests that blood samples for the determination of Ara-A or other adenosine analogues must be collected in tubes containing DCF. The DCF will inhibit red blood cell ADA, which would also degrade Ara-A following sample collection. In addition, keeping the samples cold and removing the red blood cells from plasma as soon as possible could further minimize degradation of adenosine analogues.

Application

The application of this method to human samples of blood, urine and cerebrospinal fluid from a patient treated with deoxycoformycin and Ara-A is shown in Fig. 3. For example, the concentrations of dAdo (a natural nucleoside) are so low that the peaks of this nucleoside are undetectable in either plasma or urine of normal or leukemic subjects (not shown here). However, this nucleoside accumulates following treatment of patients with an ADA inhibitor, DCF (see left and right panels of Fig. 3 and refs. 7 and 8). An accumulation of dAdo has also been reported in the plasma and urine of patients with genetic deficiency of ADA [5]. Furthermore, treatment of leukemic patients with DCF and Ara-A caused accumulation of Ara-A in the plasma, urine and cerebrospinal fluid of the patients (Fig. 3). On treatment with

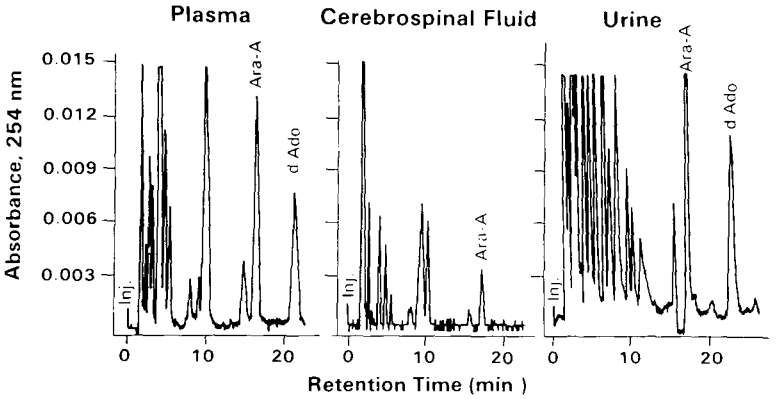


Fig. 3. HPLC profiles of plasma, cerebrospinal fluid and urine from a leukemic patient. The patient was treated with arabinosyladenine i.v. infusion (300 mg/m^2, 180 min) following a single i.v. dose of deoxycoformycin (15 mg/m^2). The samples were prepared as described under *Methods*. A 20-μl volume of the plasma and cerebrospinal fluid and 5 μl of urine were used for analysis.

Ara-A alone the drug was rapidly deaminated to Ara-Hyp and the peaks of Ara-A were undetectable (not shown here). The identification of dAdo and Ara-A was further confirmed by the peak-shift method. Details of pharmacokinetic studies of DCF and Ara-A combination will be published elsewhere.

The method presented here offers the advantage of rapid and simple preparation of samples of either plasma, urine or cerebrospinal fluid and uses volumes as small as 0.5 ml. There is no need to extract the nucleosides or to remove or modify sugar moieties to obtain adequate separation. In pharmacokinetic studies, when amounts of clinical material are small and large numbers of assays are required, the present method is very useful. We are presently using this method for measuring Ara-A, Ara-Hyp, Ado and dAdo in the body fluids of patients receiving treatment with Ara-A/deoxycoformycin. However, the method should be useful to other investigators performing similar studies. Further, with slight modification, this method has been adapted to resolve arabinosyl cytosine, arabinosyl uracil and fluorouracil and is being used in pharmacokinetic studies of these drugs.

This method has been used successfully to assay Ado and dAdo in the body fluids of patients treated with DCF [7, 8]. The method should also find use in monitoring the levels of purines and nucleosides in patients with defects of purine metabolism, e.g., hypoxanthine—guanine phosphoribosyl transferase (E.C. 2.4.2.8) deficiency (Lesch—Nyhan syndrome) [32], purine nucleoside phosphorylase (E.C. 2.4.2.1) deficiency [33] and severe combined immunodeficiency—ADA deficiency, etc.

ACKNOWLEDGEMENTS

The authors thank Michael Pimental and Alvaro Lopez for their skillful assistance. This work was carried out during the tenure of Grant No. CH-161 from the American Cancer Society (RPA).

REFERENCES

1 E.R. Giblett, G. Anderson and F. Cohen, Lancet, ii (1972) 1067.
2 G.C. Mills, F.C. Schmalstieg, K.B. Trimmer, A.S. Goldman and R.M. Goldblum, Proc. Nat. Acad. Sci. U.S., 73 (1976) 2867.
3 H.A. Simmonds, A. Sahota, C.F. Potter and J.S. Cameron, Clin. Sci. Mol. Med., 54 (1978) 579.
4 R. Hirschhorn, V. Roegner, A. Rubinstein and P.S. Papageorgious, J. Clin. Invest., 65 (1980) 768.
5 R. Hirschhorn, P.S. Papageorgiou, H.H. Keserwala and L.T. Taft, N. Engl. J. Med., 303 (1980) 377.
6 D.A. Carson, J. Kay and J.E. Seegmiller, Proc. Nat. Acad. Sci. U.S., 74 (1977) 5677.
7 P.P. Major, R.P. Agarwal and D.W. Kufe, Cancer Chemother. Pharmacol., 5 (1981) 193.
8 P.P. Major, R.P. Agarwal and D.W. Kufe, Blood, 58 (1981) 91.
9 I. Haulica, L. Adabei, D. Brainisteanu and F. Topoliceanu, J. Neurochem., 21 (1973) 1019.
10 J.W. Phillis and J.P. Edstrom, Life Sci., 19 (1976) 1041.
11 J.W. Phillis, J.K. Kostopoulos, J.P. Edstrom and S.W. Ellis, in H.P. Baer and G.I. Drummond (Editors), Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides, Raven Press, New York, 1979, p. 373.
12 F.M. Schabel, Jr., Chemotherapy (Basel), 13 (1968) 321.
13 C.A. Alford and R.J. Whitley, J. Infect. Dis., 133, Suppl. (1976) A101.
14 G.P. Bodey, J. Gottlieb, K.B. McCredie and E.J. Freireich, in D. Pavan-Langston, R.A. Buchanan and C.A. Alford (Editors), Adenine Arabinoside: An Antiviral Agent, Raven Press, New York, 1975. p. 281.
15 D.P. Gray, M.R. Grever, M.F.E. Siaw, M.S. Coleman and S.P. Balcerzak, Proc. Amer. Ass. Cancer Res., 22 (1981) 488.
16 W. Plunkett, R. Benjamin, L. Feun, M. Keating and E.J. Freireich, Proc. Amer. Ass. Cancer Res., 22 (1981) 177.
17 P.R. Brown, S. Bobick and F.L. Heinley, J. Chromatogr., 99 (1974) 587.
18 M. Uziel, L.H. Smith and S.A. Taylor, Clin. Chem., 22 (1976) 1451.
19 R.A. Hartwick and P.R. Brown, J. Chromatogr., 143 (1977) 383.
20 J.F. Kuttesch, F.C. Schmalstieg and J.A. Nelson, J. Liquid Chromatogr., 1 (1978) 97.
21 U.E. Honegger, S.S. Bogdanov and P.R. Bally, Anal. Biochem., 82 (1977) 262.
22 A.M. Krstulovic, P.R. Brown and D.M. Roise, Anal. Chem., 49 (1977) 2237.
23 H.G. Schneider and A.J. Glazko, J. Chromatogr., 139 (1977) 370.
24 B. Bakay, E. Nissinen and L. Sweetman, Anal. Biochem., 86 (1978) 65.
25 A. McBurney and T. Gibson, Clin. Chim. Acta, 102 (1980) 19.
26 C.A. Koller, P.L. Stetson, L.D. Nichamin and B.S. Mitchell, Biochem. Med., 24 (1980) 179.
27 E.C. Reynolds and L.R. Finch, Anal. Biochem., 82 (1977) 591.
28 A.M. Krstulovic, R.A. Hartwick and P.R. Brown, Clin. Chim. Acta, 97 (1979) 159.
29 R. Hartwick, A. Jeffries, A. Krstulovic and P.R. Brown, J. Chromatogr. Sci., 16 (1978) 427.
30 R.A. Hartwick, D. VanHaverbeke, M. McKeag and P.R. Brown, J. Liquid Chromatogr., 2 (1979) 725.
31 S.P. Assenza and P.R. Brown, J. Chromatogr., 181 (1980) 169.
32 M. Lesch and W.L. Nyhan, Amer. J. Med., 36 (1964) 561.
33 E.R. Giblett, A.J. Ammann, D.W. Wara, R. Sandman and L.K. Diamond, Lancet, i (1975) 1010.

Journal of Chromatography, 231 (1982) 425—432
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1337

Note

High-performance liquid chromatographic differentiation of urinary free porphyrins

E. GAETANI, C.F. LAURERI and M. VITTO
Istituto di Chimica Farmaceutica e Tossicologica dell'Università di Parma, Parma (Italy)
and
E. ROCCHI*, P. GIBERTINI, F. FARINA, M. CASSANELLI and E. VENTURA
Istituto di Semeiotica Medica dell'Università di Modena, Modena (Italy)
(First received October 16th, 1981; revised manuscript received April 13th, 1982)

Naturally occurring porphyrins in biological material have rather similar physicochemical properties which make their separation difficult.

Several extraction, chromatographic, and fluorometric methods have been presented; however, only a combination of them allows a precise but time-consuming identification of the different carboxylic compounds and of their isomeric forms [1—7].

So we welcome the opportunity to separate and quantitate the different porphyrins in biological materials by high-performance liquid chromatography (HPLC). This technique has become important in recent years and is becoming as widely used for relatively involatile substances as gas chromatography is for volatile ones [8].

A good separation of porphyrin polycarboxylic acids may be obtained by HPLC with a preliminary conversion of free acids into their methyl esters, but this procedure, although simple, requires several hours, it may be incomplete, and it introduces some problems as further reactions occur at vinyl groups, which finally may lead to a loss of material [9—11]. On the other hand, few papers in the literature present HPLC methods available for the separation of porphyrins as free acids in biological material: some deal with porphyrin detection in concentrations far greater than the normal range of urinary excretion [11, 12], others employ time-consuming gradient elution devices coupled with pre-running of sample [13].

We propose here a new simplified HPLC method of separating the free urinary porphyrins, followed by fluorometric detection to optimize their esti-

0378-4347/82/0000—0000/$02.75

mation within the range of normal human excretion. This procedure is then compared with a traditional solvent extraction method in normal subjects and liver patients.

EXPERIMENTAL

Instrumentation

The experiments for the solvent extraction method were performed on a Zeiss PQM II spectrophotometer. HPLC was performed on a Varian Aerograph 8500 high-pressure liquid chromatograph equipped with a Varian fluorimetric detector (Fluorichrom); the excitation filter was a 400-nm interference filter, and the emission filter was a 490-nm cut-off filter; the lamp was in the HI position, with gain × 1, attenuation × 10; a Varian Model A 25 recorder, a stop-flow injector, and a 10-μl S.G.E. syringe were also used.

Solvent extraction procedure

For many years we have commonly detected urinary coproporphyrin and uroporphyrin by the solvent extraction method of Fernandez et al. [3], described in detail elsewhere. The copro- and uroporphyrin HCl eluates were spectrophotometrically quantitated with zero absorbance set with distilled water.

HPLC procedure

The column was a stainless steel (25 × 0.2 cm) RP-18 MCH (Varian, Palo Alto, CA, U.S.A.). As mobile phase 20% acetonitrile (Carlo Erba RPE, Milan, Italy) in a solution of 0.25 *M* methanesulfonic acid (Merck, Darmstadt, G.F.R.) in bidistilled water, was used. The flow-rate was 60 ml/h; and the column temperature 25° C.

Quantitative evaluation was obtained by correlating peak areas, obtained by the height times width at half-height, to known concentrations of relative reference solutions.

Reference standard

We prepared five portions of standard solutions containing uro- and coproporphyrin in 3 *M* hydrochloric acid at a known scalar concentration ranging from 5 to 300 μg/l (see Fig. 3A, B).

Sample preparation

A 5-ml volume of urine, previously treated with 0.1 ml of a solution containing 1 mg/ml chloranil (Carbo Erba RPE) in glacial acetic acid [14], was passed through a column of anion-exchange resin AG 1-X8 (Cl^-) 100—200 mesh, 4 × 0.7 cm (Prefilled Econo-Column™, Bio-Rad Labs, Richmond, CA, U.S.A.); after three washings with 4 ml of distilled water, porphyrins were eluted with two portions of 2 ml of 3 *M* hydrochloric acid (Carlo Erba RPE). (We used a sample/eluate ratio of 5:4 to make up for losses during purification [4].) Then, 10 μl of the eluate were injected into the HPLC column.

RESULTS AND DISCUSSION

As porphyrins in a strongly acidic solution show their maximum relative

quantum yield by fluorescence, we decided to employ a mobile phase of pH near 1, so as to ionize pyrrolic nitrogens, and to use $CH_3SO_3^-$, as a counterion; thus, a sensitivity greater than 5 μg/l for coproporphyrin and 1 μg/l for uroporphyrin can be obtained.

To evaluate column durability in such extreme conditions, we fluxed solvent continuously through it for 48 h and measured its efficiency before and after treatment. Column efficiency did not diminish appreciably.

The results are summarized in Figs. 1—3 and Table I and II.

The uroporphyrin values in both Table I and Table II are indicated as "traces" when the amount ranged between 0 and 0.5 μg/l. In Table II we have reported only the octa-, hepta- and tetracarboxylic porphyrins recorded in the HPLC chromatograms; however, in all the porphyria cutanea tarda patients we

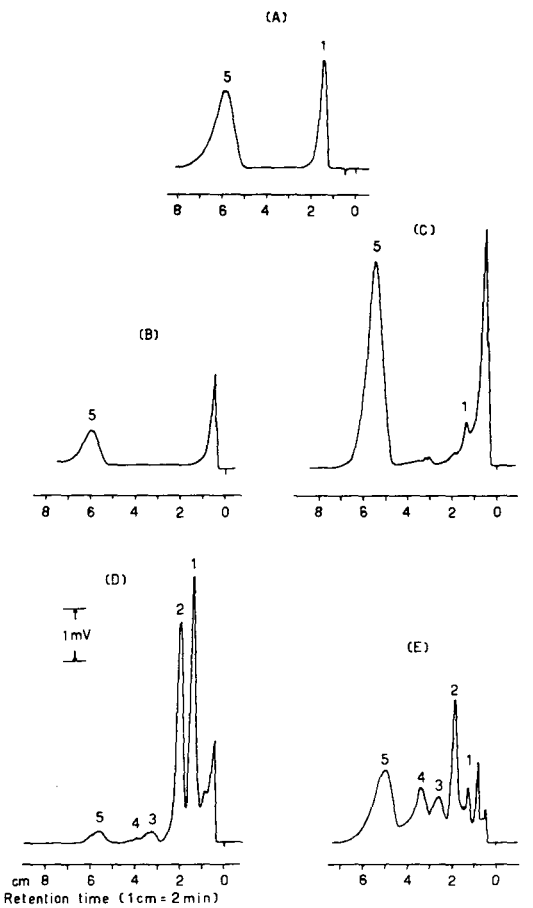


Fig. 1. Examples of porphyrin HPLC chromatograms of (A) standard solution, and the urine of (B) control subjects, (C) liver cirrhosis + cholestasis, (D) porphyria cutanea tarda patient, (E) HCl coproporphyrin extract obtained by solvent extraction method from the same porphyria cutanea tarda patient's urine. 1 = uroporphyrin; 2 = heptacarboxyporphyrin; 3 = hexacarboxyporphyrin; 4 = pentacarboxyporphyrin; 5 = coproporphyrin.

TABLE I

URINARY PORPHYRIN EXCRETION IN CONTROLS, DETECTED BY SOLVENT EXTRACTION AND HPLC

No.	Age	Sex	Coproporphyrin (μg/l)		Uroporphyrin (μg/l)	
			Solvent extraction	HPLC	Solvent extraction	HPLC
1	48	F	68	9	5	Traces
2	42	F	28	20	11	14
3	48	M	52	35	22	5
4	25	M	31	28	3	Traces
5	30	F	38	12	5	Traces
6	55	F	46	25	6	Traces
7	53	M	35	6	13	Traces
8	45	M	71	40	19	Traces
9	50	M	29	25	12	1
10	40	F	79	23	23	Traces
$\bar{x}$	43.6		47.7	22.3	11.9	
S.D.	9.6		18.9	10.9	7.3	
S.E.	3.0		5.9	3.4	2.3	

TABLE II

URINARY PORPHYRIN EXCRETION IN PATIENTS WITH DIFFERENT LIVER DISEASES

CAH = Chronic active hepatitis; LC = liver cirrhosis.

Diagnosis		Age	Sex	Coproporphyrin (μg/l)		Uroporphyrin (μg/l)		
				Solvent extraction	HPLC	Solvent extraction	HPLC	
							8C*	7C**
1	CAH	17	F	105	25	31	Traces	—
2	CAH	44	M	84	20	7	4	—
3	LC	50	F	86	26	6	3	—
4	LC	46	M	46	17	5	1	—
5	LC	40	F	42	30	6	1	—
6	LC	49	M	87	47	22	18	—
7	CAH + cholestasis	35	M	144	70	17	11	—
8	LC + cholestasis	24	M	230	117	29	Traces	—
9	LC + cholestasis	54	M	429	232	72	26	—
10	LC + cholestasis	42	F	181	90	49	14	—
11	LC + cholestasis	59	M	235	47	29	Traces	—
12	LC + cholestasis	54	M	200	93	30	Traces	—
13	Porphyria cutanea tarda	52	M	130	20	762	316	101
14	Porphyria cutanea tarda	48	M	62	23	828	576	149
15	Porphyria cutanea tarda	50	M	73	15	1025	348	287

*8C = uroporphyrin.
**7C = heptacarboxyporphyrin.

easily recorded minor amounts of hexa- and pentacarboxylic porphyrins as Fig. 1D shows.

Fig. 2 summarizes the statistical analysis of urinary coproporphyrin results obtained with both methods.

The mean (± S.E.) of urinary coproporphyrin in controls is 47.7 (± 5.9) μg/l detected by solvent extraction method, and 22.3 (± 3.4) μg/l detected by HPLC. The corresponding values in patients are as follows: 75.0 (± 10.2) μg/l in chronic liver disease without cholestasis, 236.5 (± 40.8) μg/l in chronic liver disease with cholestasis, and 88.3 (± 28.0) in porphyria cutanea tarda patients detected by the solvent extraction method; and 27.5 (± 4.3) μg/l in chronic liver diseases without cholestasis, 108.1 (± 26.5) μg/l in chronic liver disease with cholestasis, and 19.3 μg/l (± 2.3) in porphyria cutanea tarda detected by the HPLC method.

The analysis of variance (ANOVA) of controls vs. each group of patients shows a significant increase of coproporphyrin excretion by the solvent extraction method in all the three groups, mainly in cholestatic liver disease, while we documented a significant increase by HPLC only in the last group.

The coefficient of variation of the proposed method has been calculated from series of ten replicate experiments at four different concentrations of coproporphyrin I ranging from 5 to 300 μg/l, the coefficient of variation was always within 10%, agreeing with the results of Doss and Schmidt [4]. The coefficient of variation at 5, 50 and 300 μg/l was 9.1, 3.5 and 8.0%, respectively. Also the coefficient of variation of ten replicate analyses of uroporphyrin I at 50 μg/l was 8.5%.

The coefficient of variation of the solvent extraction method was 8% (ten replicate analyses) in our laboratory.

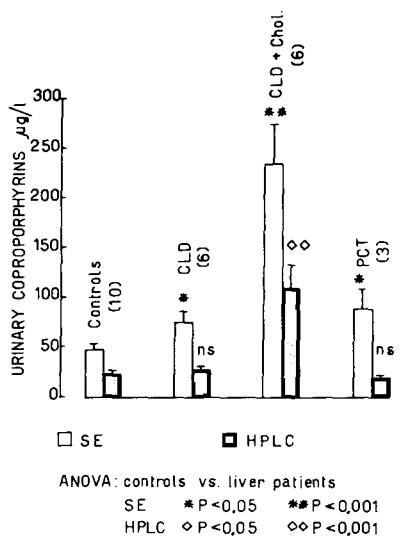


Fig. 2. Statistical analysis of urinary coproporphyrin results ($\bar{x}$ + S.E.; ANOVA = analysis of variance). CLD = chronic liver disease; PCT = porphyria cutanea tarda; SE = solvent extraction.

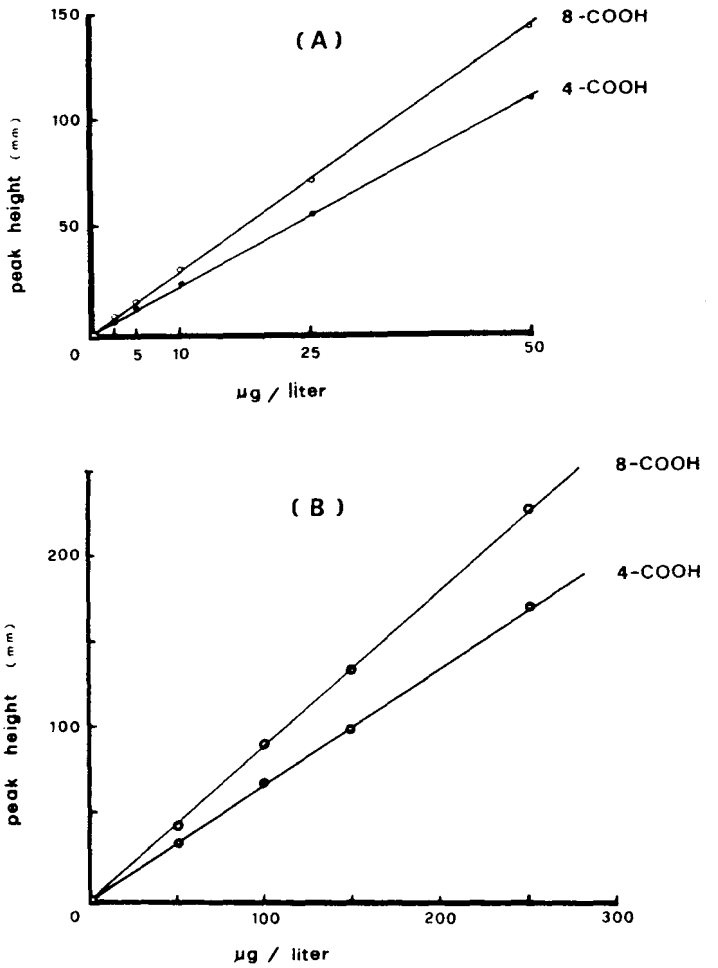


Fig. 3. Plots of peak height vs. concentration of uroporphyrin (8-COOH) and coproporphyrin (4-COOH) in normal range (A) and in pathological range (B).

The amount of urinary coproporphyrin is almost double when detected by the solvent extraction method for both the controls and the liver patients (see Fig. 2).

As to recovery, the two methods overlapped and the fluorometric scanning of HPLC free-porphyrin eluate at an acid pH greatly increases the sensitivity of the proposed method. We believe that 50% of the coproporphyrin detected by the traditional technique may be due to the imprecision of the method itself.

For the same reason the uroporphyrin excretion seems usually within a few µg/l of urine in healthy subjects, and in chronic liver disease without cholestasis (Tables I and II); so far only a small number of early-morning urine samples have been studied.

In pigment-rich urines we observed a "true" increase of coproporphyrins (with both methods) as an effect of impaired biliary secretion of cholephil anions; this agrees with our and other previous observations [15].

As regards the coproporphyrin excretion in porphyria cutanea tarda patients, we recorded increased values with the solvent extraction method according to our and other previous reports [16]. However, we found a "true" coproporphyrin (4-COOH) excretion within the normal urinary range in our three porphyria cutanea tarda patients, and some penta- or hexacarboxylic porphyrins in the HPLC chromatograms (Fig. 1D). Moreover, the HCl coproporphyrin extract from a porphyria cutanea tarda patient's urine obtained by solvent extraction showed a predominance of hepta- and uroporphyrin on subsequent HPLC analysis (Fig. 1E). This confirms the lack of specificity of the solvent extraction method in detecting coproporphyrin when a predominance of uroporphyrin is present in the sample.

The "uroporphyrin class" determined by the solvent extraction method represents the sum of octa- and heptacarboxylic porphyrins, which, on the contrary, are easily differentiated by the HPLC method.

The usefullness of measuring the ratio of uro- to heptacarboxylic compounds in the differentiation of porphyria cutanea tarda from other chronic hepatic porphyrias have been widely emphasized [17].

We therefore agree that the solvent extraction and other similar methods may be regarded as useful, as in the past, for screening or monitoring porphyric patients, but more complex and time-consuming chromatographic techniques are required for the differentiation of porphyrias.

The HPLC of porphyrin free acids and fluorometric scanning which we propose fulfils the need for an easy and quantitative analysis of individual compounds even within the normal range. Further investigations are in progress on a larger number of patients and controls to differentiate individual carboxylic porphyrins and their isomers in other biological materials such as faeces, plasma and liver homogenates.

ACKNOWLEDGEMENT

Part of the present research was supported by Grant 289/79.019996.04/115. 1158 from the Consiglio Nazionale delle Ricerche, Rome, Italy.

REFERENCES

1 S. Schwartz, L. Zieve and C.J. Watson, J. Lab. Clin. Med., 37 (1951) 843.
2 C. Rimington, Ass. Clin. Pathol. Broadsheet, 36 (1961) 1.
3 A.A. Fernandez, R.J. Henry and H. Goldenberg, Clin. Chem., 12 (1966) 463.
4 M. Doss and A. Schmidt, Z. Klin. Chem. Klin. Biochem., 9 (1971) 415.
5 E. Reinking and E.J. Van Kampen, Clin. Chim. Acta, 9 (1964) 592.
6 S. Schwartz, B. Stephenson and D. Sarkar, Clin. Chem., 22 (1976) 1057.
7 M. Doss, Hoppe-Seyler's Z. Physiol. Chem., 350 (1969) 499.
8 A.H. Jackson, Semin. Hematol., 14 (1977) 193.
9 M. Evans, D.E. Games, A.H. Jackson and S.A. Matlin, J. Chromatogr., 115 (1975) 325.
10 C.H. Gray, C.K. Lim and D.C. Nicholson, Clin. Chim. Acta, 77 (1977) 167.
11 R. Bonnett, A.A. Charalambides, K. Jones, I.A. Magnus and R.J. Ridge, Biochem. J., 173 (1978) 693.
12 E. Englert, Jr., E.E. Wales, Jr., A.W. Wayne and R.C. Straight, Gastroenterology, 74 (1978) 1031.

13 E. Englert, Jr., A.W. Wayne, E.E. Wales, Jr. and R.C. Straight, J. High Resolut. Chromatogr. Chromatogr, Commun., 2 (1979) 570.
14 S. Schwartz, P. Edmonson, B. Stephenson, D. Sarkar and H. Freyholtz, Ann. Clin. Res., 8 (1976) 156.
15 C.J. Watson, D. Sutherland and V. Hawkinson, J. Lab. Clin. Med., 37 (1951) 8.
16 M.E. Grossman and M.B. Poh-Fitzpatrik, Med. Clin. North Amer., 64 (1980) 807.
17 M. Doss, W. Meinhof and D. Look, S. Afr. J. Lab. Clin. Med., 17 (1971) 50.

Journal of Chromatography, 231 (1982) 433–438
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1312

Note

Thiamin analysis and separation of thiamin phosphate esters by high-performance liquid chromatography

DORIS M. HILKER* and ANDREW J. CLIFFORD*

Department of Nutrition, University of California, Davis, CA 95616 (U.S.A.)

(First received November 6th, 1981; revised manuscript received April 17th, 1982)

High-performance liquid chromatography (HPLC) has been proposed for the analysis of water-soluble vitamins. Methods for analyzing thiamin in food [1–3], tissues [4–6], urine [7], and commercial vitamin preparations [8, 9] have been published. In general, either prior to [4, 6, 7] or after [1, 5] HPLC, the methods require reacting thiamin and its phosphate esters with alkaline ferricyanide to form a fluorescent thiochrome which is then measured. Although commercial vitamin-rich preparations have been analyzed for thiamin [8, 9] using UV detection, only one report [2] has described thiamin analysis of a food item using UV detection. While UV detection methods are less sensitive than those employing fluorescence detection of thiochrome, they are nevertheless, much simpler in terms of sample preparation and avoid the danger that naturally occurring compounds such as polyphenols will interfere with the oxidation reaction involved in thiochrome formation [10] and lead to spurious results.

Presented here is a rapid, sensitive, and reproducible method of sample preparation and analysis for thiamin in food (breakfast cereal) and in urine specimens. An extension of the method which allows for separating and quantifying the following reagent standards: thiamin (Th), thiamin disulfide (TDS), thiamin monophosphate (TMP), thiamin diphosphate (TDP) and thiamin triphosphate (TTP) in a single run is also presented.

*Present address: Department of Food Science and Human Nutrition, University of Hawaii, Honolulu, HI 97822, U.S.A.

0378-4347/82/0000—0000/$02.75

EXPERIMENTAL

Chemicals

All reagents were of the highest chemical purity available and all reagents were made up in Milli-Q (Millipore, Bedford, MA, U.S.A.) reagent-grade water. Disposable C_{18} reversed-phase columns (3 ml) were obtained from Analytichem International (Harbor City, CA, U.S.A.). Chemical reagents were obtained from the following sources: acetonitrile (LC grade) from Alltech (Los Altos, CA, U.S.A.); ammonium phosphate (Ultrex grade) from J.T. Baker (Phillipsburg, NJ, U.S.A.); Th, TDS, TMP, and TDP from ICN Pharmaceuticals (Plainview, NY, U.S.A.); TTP was a generous gift from Central Research Laboratories of Sankyo (Tokyo, Japan).

Instruments

The liquid chromatograph was a Varian LC 5060 (Palo Alto, CA, U.S.A.) with a 300 × 4.6 mm, weak anion-exchange column (MicroPak AX-5) with a 5-μm diameter particle size and a diamine functional group. Column effluent was monitored at 245 nm using a Varichrom-5 UV detector and recorded on a Varian strip-chart recorder (4.25 mm/min). The liquid chromatograph was connected in series with low-dead-volume stainless-steel tubing to a Hewlett-Packard 8450 spectrophotometer (Palo Alto, CA, U.S.A.) having an 8-μl flow-cell to permit spectrophotometric scanning of peaks as they emerged from the UV detector flow-cell.

Preparation and HPLC of thiamin reagent standards

A freshly prepared aqueous standard solution of thiamin (0.2 m*M*) was prepared and aliquots of this standard were loaded on the HPLC column and eluted at 30°C isocratically with 5 m*M* $NH_4H_2PO_4$ (pH 2.85) pumped at a flow-rate of 0.5 ml/min. Additional aliquots of the thiamin standard solution were carried through the sample clean-up procedure used for urine and crude homogenates of breakfast cereal.

Preparation and clean-up of cereal and urine samples for thiamin analysis

A breakfast cereal was chosen as the representative food and prepared for thiamin analysis in the following way. The cereal was ground to a fine powder in a mortar. A 1-g sample was suspended in 100 ml of 0.1 *N* hydrochloric acid and autoclaved at 121°C for 30 min. The suspension was centrifuged at 14,000 *g* for 15 min at room temperature. Aliquots of the supernatant were applied to disposable C_{18} reversed-phase columns whose particle size was 10 μm. The columns were washed twice with water and twice with methanol. Thiamin was then eluted with a mixture of 5 m*M* $NH_4H_2PO_4$ (pH 2.85)—acetonitrile—phosphoric acid (3.9:1:0.1, v/v/v) and a 10-μl aliquot of the eluent was applied to the HPLC column. A casual sample of human urine was diluted with 9 volumes of water and an aliquot applied to the C_{18} reversed-phase column. The column was then washed and the thiamin eluted and aliquots were loaded on the HPLC column as described for the cereal sample.

HPLC of cereal and urine extracts for thiamin

Thiamin was eluted isocratically from the HPLC column at 30° C with 5 m*M* $NH_4H_2PO_4$ (pH 2.85), flow-rate 0.5 ml/min, and quantitated by monitoring the UV absorption of the eluting peaks at 245 nm. The identity and purity of the eluent peak containing thiamin was confirmed by spectrophotometrically scanning the leading and trailing edges of the thiamin peak. Peak heights of thiamin from the cereal and urine samples were compared with that of the thiamin reagent (0.2 m*M*) taken through the same clean-up and HPLC procedures.

Preparation and HPLC of thiamin ester reagent standards

A freshly prepared aqueous standard solution containing a mixture of the following forms of thiamin: Th (0.2 m*M*), TDS (0.15 m*M*), TMP (0.2 m*M*), TDP (0.3 m*M*), and TTP (0.3 m*M*) was applied to the HPLC column. The various forms of thiamin were separated using the following buffers: buffer A, 870 ml of acetonitrile plus 130 ml of 5 m*M* $NH_4H_2PO_4$ (pH 2.85); buffer B, 5 m*M* $NH_4H_2PO_4$ (pH 2.85); and buffer C, 750 m*M* $NH_4H_2PO_4$ (pH 4.0). The elution program consisted of 100% buffer A for 15 min followed by a gradient of buffer B from 0—100% at 4%/min. This was followed by a gradient of buffer C from 0—100% at 2.5%/min. The flow-rate was 0.4 ml/min for the first 15 min, then was increased to 1.0 ml/min during the next 2 min and remained at this flow-rate throughout the analysis. At these flow-rates the column pressure ranged from 40—70 atm. The column was maintained at 30° C. The chemical identity of the peaks eluted from the HPLC column was confirmed from their characteristic UV spectra.

RESULTS

Thiamin elutes from the HPLC column in 4 min as is shown in Fig. 1. The peak height was reproducible as shown in Table I and was in linear proportion

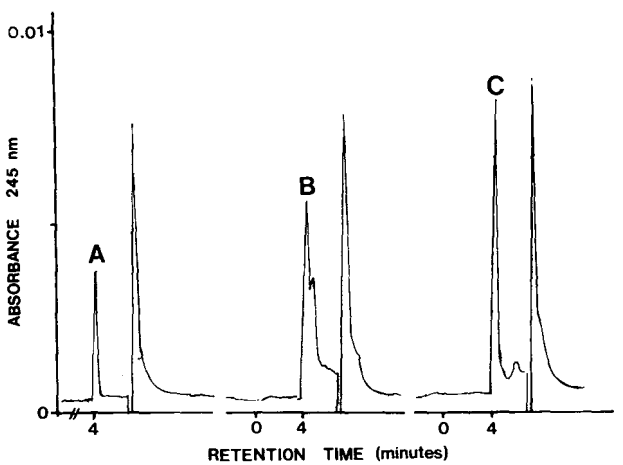


Fig. 1. HPLC of thiamin reagent standard (A), thiamin in breakfast cereal (B) and in urine (C) after clean-up on C_{18} reversed-phase columns. Absorbance range 0.01 with detection at 245 nm. The peak following thiamin was due to the solvent mixture used for eluting the thiamin from C_{18} reversed-phase columns.

TABLE I

REPRODUCIBILITY AND LINEARITY OF PEAK HEIGHT RESPONSE AT THREE CONCENTRATIONS OF REAGENT STANDARD THIAMIN

Run No.	Thiamin concentration (ng per 10 μl)		
	2	3	5
1	5.46*	9.14	12.70
2	4.45	9.40	13.46
3	5.21	9.53	12.19
4	4.45	8.76	12.46
Mean peak height ± S.D.	4.89 ± 0.52	9.21 ± 0.34	12.70 ± 0.55
Coefficient of variation (%)	5.1	3.7	4.3

*Values are peak height in cm.

from 0.5 to 10 ng Th. The HPLC chromatograms for the cereal and urine sample, and also standard thiamin are shown in Fig. 1. Recovery of standard thiamin taken through the cereal and urine sample clean-up procedure was quantitative. The cereal shows a small shoulder peak not completely separated from the thiamin. Other water-soluble vitamins including pyridoxin, riboflavin and niacin coelute with a retention time of 4.8 min. However, these water-soluble vitamins are, for all practical purposes, quantitatively removed from the C_{18} reversed-phase columns with the two water and two methanol washes. Calculation of thiamin in the cereal from the peak height gave a value of 75.5 μg/g cereal compared to the 53.5 μg/g calculated from the information on the cereal carton. The thiamin in urine chromatographed with a sharp peak and was not contaminated with other vitamins. This sample of urine contained 11.4 μg/ml of urine as calculated from the HPLC chromatogram.

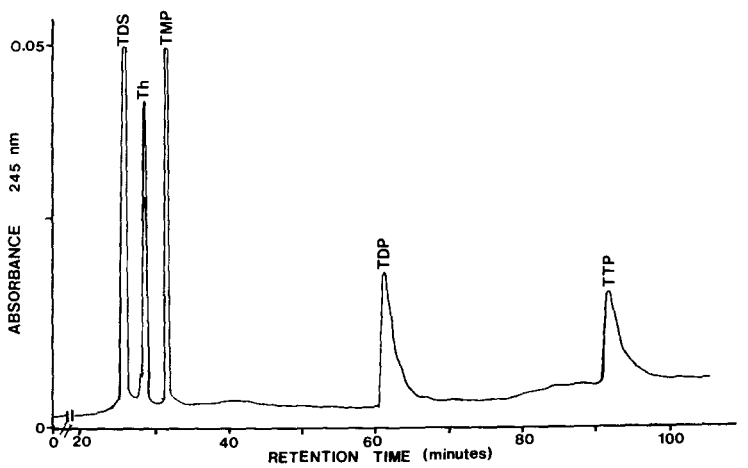


Fig. 2. HPLC chromatogram of TDS, Th, TMP, TDP, and TTP at concentrations of 0.15, 0.2, 0.2, 0.3 and 0.3 m*M*, respectively. The absorbance range was 0.05 with detection at 245 nm.

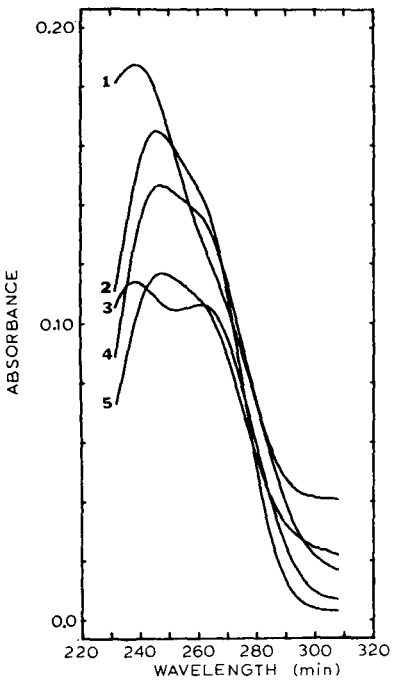


Fig. 3. UV absorption spectra of the various forms of thiamin after HPLC. The absorbance values are relative. Curves: 1 = TDS; 2 = TMP; 3 = Th; 4 = TDP; 5 = TTP.

A HPLC chromatogram showing the separation of Th and TDS from the phosphate esters is shown in Fig. 2. The five compounds were eluted within 100 min. The TDS which does not occur in tissues or blood can be used as an internal standard. The UV spectra for the various forms of thiamin as they came off the HPLC column are shown in Fig. 3. Thiamin has a characteristic UV spectrum with two absorption maxima, one at 245 nm and the other at 265 nm, while the UV spectra of the three phosphate esters are similar with maximum absorption at 248 nm. TDS has one absorption maximum at 245 nm.

DISCUSSION

Previously published methods of thiamin assay by HPLC involve first, the conversion of thiamin to a fluorescent thiochrome compound before or after elution from HPLC and detection with a fluorescent detector. The major advantage of measuring fluorescent thiamin derivatives is the high sensitivity. However, compounds including polyphenols can interfere with the oxidation reaction involved in the thiochrome formation [10]. The advantage of the method described here is that it is fast and simple; it does not require the conversion of thiamin to thiochrome and fluorescence detection; and it is sufficiently sensitive for foods, for urine, and possibly for many common biological tissues. The clean-up procedure is simple and rapid in removing major interfering substances. While no attempt was made in our study to maximise sensitivity, this can be increased by adjusting the absorbance range.

The separation of thiamin and the various phosphate esters along with the practically simultaneous scanning of their absorption spectra in Fig. 3 demonstrate the use of this technique in confirming the identity of the eluted peaks. Since a ternary buffer system was employed for this separation, thiamin elutes at a later time (28 min) than when buffer B was used isocratically (Fig. 1). Another advantage of the described method is that it can be upgraded to separate Th, TDS, TMP, TDP, and TTP in a single run. This capability is extremely useful for kinetic studies on various forms of thiamin. With further modification the method could be adapted for use in the analysis of tissues for these thiamin compounds.

ACKNOWLEDGEMENTS

The authors thank Dr. Mitsuo Yamazaki, Central Research Laboratories, Sankyo Company, Tokyo 140, Japan, for providing thiamin triphosphate reagent standard.

These studies were supported by the U.S.D.A., Western Regional Cooperative Project W-143.

REFERENCES

1 T. van de Weerdhof, M.L. Wiersum and H. Reissenweber, J. Chromatogr., 83 (1973) 455.
2 R.B. Toma and M.M. Tabekhia, J. Food Sci., 44 (1979) 263.
3 C.Y.N. Ang and F.A. Moseley, J. Agr. Food Chem., 38 (1980) 483.
4 K. Ishii, K. Sarai, H. Senemori and T. Kawasaki, J. Nutr. Sci. Vitaminol., 25 (1979) 517.
5 M. Kimura, T. Fujita, S. Nishida and Y. Itokawa, J. Chromatogr., 188 (1980) 417.
6 H. Sanemori, H. Ueki and T. Kawasaki, Anal. Biochem., 107 (1980) 451.
7 R.L. Roser, A.H. Andrist, W.H. Harrington, H.K. Naito and D. Lonsdale, J. Chromatogr., 146 (1978) 43.
8 K. Callmer and L. Davies, Chromatographia, 7 (1974) 644.
9 R.B.H. Wills, C.G. Shaw and W.R. Day, J. Chromatogr. Sci., 15 (1977) 262.
10 B. Panijpan, P. Vilartsakdanon, K.Rungruangsak and S.L. Vimokesant, Int. J. Vit. Nutr. Res., 48 (1978) 262.

Journal of Chromatography, 231 (1982) 439—444
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1315

Note

Measurement of vitamin A and vitamin E in human serum by high-performance liquid chromatography

WILLIAM J. DRISKELL*, JANE W. NEESE, CLYDE C. BRYANT and MARK M. BASHOR

Nutritional Biochemistry Branch, Clinical Chemistry Division, Center for Environmental Health, Centers for Disease Control, Public Health Service, U.S. Department of Health and Human Services, Atlanta, GA 30333 (U.S.A.)

(First received December 15th, 1981; revised manuscript received April 21st, 1982)

In the past few years, high-performance liquid chromatography (HPLC) has begun to be used for measurement of metabolites important in nutrition. Among those most amenable to HPLC quantitation are vitamin A and vitamin E, because they are present in serum in high enough concentration to be detected easily by their ultraviolet absorption and because there appear to be no substances in serum that interfere in the HPLC assay. Several procedures for measuring vitamin A and vitamin E separately by HPLC have been published [1—5]. Recently, Bieri et al. [6] and De Leenheer et al. [7] have described HPLC procedures which allow the simultaneous determination of vitamin A and vitamin E from a common serum extract. This paper describes another HPLC method for the simultaneous measurement of vitamin A and vitamin E. A major difference between the three approaches is in the choice and number of internal standards. The procedure of Bieri et al. [6] employs two internal standards, retinyl acetate and tocopherol acetate. The method described in this paper, developed for routine high-volume service use, utilizes only retinyl acetate, which greatly decreases the run time. De Leenheer et al. [7] also use only one internal standard, but it is tocol, a synthetic analogue of vitamin E. The procedure is applied to the determination of the stability of vitamins A and E in serum samples. The HPLC procedure and the older trifluoroacetic acid colorimetric assay for vitamin A [8], which has been used in this laboratory in connection with the Health and Nutrition Evaluation Survey (HANES), are compared to determine the degree of correlation between the two methods.

EXPERIMENTAL

Materials

All-*trans*-retinol and all-*trans*-retinyl acetate, both in a pure crystalline form, were from Sigma (St. Louis, MO, U.S.A.) and *d*-alpha-tocopherol was from ICN (Cleveland, OH, U.S.A.). Methanol and *n*-hexane were HPLC grade from Fisher Scientific (Atlanta, GA, U.S.A.) and used without further purification. The absolute, undenatured alcohol was from National Distillers and Chemical Corporation (New York, NY, U.S.A.).

A normal human serum pool was established specifically for the vitamins A and E stability studies. Human serum samples used in the HPLC procedure versus colorimetric vitamin A procedure comparison were from HANES survey.

High-performance liquid chromatography

The HPLC pump was an Altex 110A (Berkeley, CA, U.S.A.) equipped with a pulse dampener. The injector was an Altex 210 with a 50-μl loop. Injection was with a 50-μl syringe from Hamilton Company (Reno, NV, U.S.A.). A Waters Assoc. (Milford, MA, U.S.A.) Model 450 detector and a Beckman (Fullerton, CA, U.S.A.) 10-in. recorder were used. The column was a 25 cm × 3.9 mm prepacked Waters μBondapack C_{18} (10 μm particle size). A guard column (Waters), 22 × 3 mm, packed with Waters C_{18} Corasil was attached on-line before the main column. Elution was performed by methanol—water (96:4) at a flow-rate of 2 ml/min (pressure of 45 bar). The column effluent was monitored at 290 nm.

Sample preparation

Serum (100 μl) was pipetted into a 75 × 10 mm test tube. To the serum was added 100 μl ethanol containing a known amount of retinyl acetate in the range of 75 to 100 μg/dl. The tube was vortexed for 5 sec. Hexane (200 μl) was added and the mixture vortexed for 30 sec (the tube was bounced as it was being vortexed to assure thorough mixing of the two layers.) Centrifugation was carried out for 1 min at 1000 *g*. A Pasteur pipet was used to remove about 150 μl of the top layer, which was transferred to another 75 × 10 mm test tube. Tubes were placed in a room temperature water bath, and the hexane was evaporated with a stream of nitrogen. The residue was redissolved in 100 μl ethanol; 50 μl of the solution was injected for chromatography.

Quantitation

Retinol and alpha-tocopherol were quantitated from a standard curve of peak height ratios. To prepare a standard curve, a constant amount of retinyl acetate was combined with five different concentrations of retinol in a range of 20—100 μg/dl and with five different concentrations of alpha-tocopherol in a range of 0.4—2.4 mg/dl. The solution of retinol, retinyl acetate, and alpha-tocopherol was chromatographed and the peak height ratios recorded. A linear relationship between peak height ratios (peak height of retinol or alpha-tocopherol: peak height of retinyl acetate) and concentration ratios (concentration of retinol or alpha-tocopherol: retinyl acetate) was found. The equations for

retinol and alpha-tocopherol were, respectively: $y = 1.61x$ and $y = 0.068x$, where y = peak ratios and x = concentration ratios.

RESULTS

A typical chromatogram of a serum extract containing retinyl acetate as an internal standard is shown in Fig. 1. Each of the peaks of interest was well separated from the others. The total time for the assay with a flow-rate of 2 ml/min was about 6 min. The speed of elution was limited to about 4 ml/min flow-rate because of a deterioration of resolution at higher flow-rates. Beta- and gamma-tocopherol eluted together as one peak, immediately before the alpha-tocopherol peak.

Long-term and within-day precision were excellent. In Table I are shown the results of a precision study in which the same serum sample was assayed fourteen times during one day and sixteen times over two months. The within-day coefficients of variation (C.V.) were 3.6% for retinol and 4.5% for alpha-tocopherol with means of 76.6 μg/dl and 747.5 μg/dl, respectively. The long-term overall coefficients of variation were 3.6% for retinol and 4.2% for alpha-tocopherol with means of 76.8 μg/dl and 735 μg/dl, respectively. Efficiency of recovery of known amounts of retinol and alpha-tocopherol added to serum was 97 ± 2% for retinol and 97 ± 2.5% for alpha-tocopherol (means ± S.E. for eight trials each). In serum, the lower detection limit was estimated to be 10 μg/dl for retinol and 80 μg/dl for alpha-tocopherol.

The standards (retinol, alpha-tocopherol, and retinyl acetate) were stable for

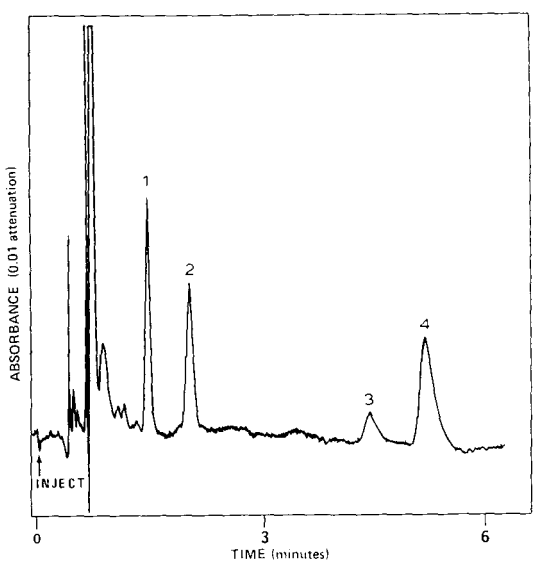


Fig. 1. HPLC chromatogram of a serum extract. Peaks: 1 = retinol; 2 = retinyl acetate; 3 = beta- and gamma-tocopherol; 4 = alpha-tocopherol. Column: 25 cm × 3.9 mm packed with Waters μBondapak C_{18} (10 μm particle size), eluent: methanol—water (96:4) flow-rate: 2.0 ml/min, detector: 290 nm.

TABLE I

SHORT AND LONG-TERM PRECISION OF THE HPLC PROCEDURE FOR RETINOL AND ALPHA-TOCOPHEROL

	Retinol (short-term)* (μg/dl)	Retinol (long-term)** (μg/dl)	Alpha-tocopherol (short-term)* (μg/dl)	Alpha-tocopherol (long-term)** (μg/dl)
	80	79	710	790
	76	80	790	700
	75	71	770	700
	73	77	710	710
	79	76	705	740
	78	80	730	760
	78	74	785	700
	73	74	760	765
	71	76	785	780
	79	73	780	705
	80	81	710	725
	78	77	700	780
	78	75	775	720
	74	81	755	750
		75		720
		80		715
$\bar{X}$	76.6	76.8	747.5	735
S.D.	2.8	2.8	33.5	30.6
C.V. (%)	3.6	3.6	4.5	4.2

*Simple assays, each taken through the entire procedure including extraction, performed over one day. Results listed in chronological order of analysis.

**Daily mean of multiple analyses, each taken through the entire procedure including extraction, performed on sixteen different days over a period of two months. Results are listed in chronological order of assay.

at least two weeks in absolute ethanol at −20°C. After about two weeks of storage a slight shoulder would occasionally appear on the retinyl acetate peak. Retinol and alpha-tocopherol were stable almost indefinitely in absolute ethanol at −20°C. Serum extracts stored in absolute ethanol at −20°C are stable for at least two weeks. This stability permits the convenience of performing the extractions on one day and performing the chromatography on a later day. The lifetime of the HPLC column used for these assays was very long: Over 700 runs were made on the same column with no serious decrease in resolution. The resin in the guard column was changed once during this time.

The HPLC procedure and the trifluoroacetic acid colorimetric procedure were compared for retinol. Three hundred individual serum samples that had been assayed colorimetrically in the HANES study were used. Fig. 2 shows a regression line plot comparing the two methods. The mean for the colorimetric assay was 34.56 μg/dl and the mean for the HPLC method was 32.00 μg/dl. The slope and correlation coefficient were 0.997 and 0.973, respectively. The only obvious outliers were the six samples that show much higher values in the colorimetric assay than the HPLC assay (see Fig. 2). It would be speculation to try to explain the cause of the outliers; but it should be pointed out that the

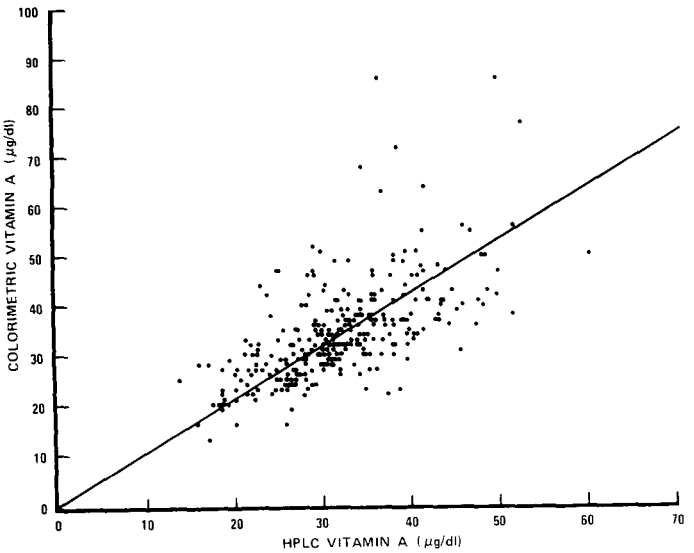


Fig. 2. Regression line plot, utilizing error in both variables model [9], comparing the vitamin A HPLC assay and the TFA colorimetric assay for 300 individual serum samples. The 95% confidence range for the intercept was 0.83—7.6 μg/dl; therefore, regression was forced through zero.

accuracy of the colorimetric assay depends on the presence of only normal carotenoids in serum, i.e., carotenoids that absorb maximally near 450 nm. The contribution of the normal carotenoids to the colorimetric reaction can be cancelled out by subtracting a factor derived from the absorbance of the extract at 450 nm. Although the great majority of carotenoids are normal, there are some naturally occurring short-chain carotenoids which absorb hardly at all at 450 nm [10]. The presence of these would give a falsely high value for retinol.

The question of the stability of vitamin A and vitamin E in serum under various conditions of storage was addressed using the HPLC technique. Samples from a single-donor human serum pool established expressly for the stability study were stored at 25°C, (1 day), 4°C (4 weeks), —20°C (16 months) and —70°C (16 months). Samples were assayed at intervals during these periods of storage. Vitamins A and E in serum were found to be stable. The values were constant within the limits of the precision of the assay, and the chromatographic peaks were clean with no shoulders or other indication of breakdown of the vitamins. In addition, four other single donor human serum pools, which were established in August, 1977; April, 1978; August, 1978; and April, 1979; and used as controls for the original colorimetric assay and later for the HPLC assay, were stable. Vitamin A values were constant during the year or more of use in the colorimetric assay and during the use of approximately one year in the HPLC assay. Vitamins A and E in serum were found to be stable to freezing and thawing (seventeen freezing and thawing cycles over a period of five weeks).

DISCUSSION

The measurement of serum vitamin A and vitamin E is a very appropriate application of HPLC. The procedure is fast and reliable with a minimum of downtime in our experience. As long as a guard column is used, the HPLC columns, which are relatively expensive, last almost indefinitely; thus, the only major continuing material expense is the eluting solvent, which amounts to about 12 ml of methanol per assay. The small serum sample requirement of 100 μl is especially convenient when infants' serum is involved. Interferences, to which colorimetric assays are often subject, are not a problem with HPLC. The precision with a coefficient of variation of between 3 and 4% for vitamin A is superior to the precision of the trifluoroacetic acid colorimetric assay with a coefficient of variation of between 6 and 7%, observed in this laboratory. Our stability studies suggest that it is unlikely that any error is introduced into the measurements because of degradation of the vitamins during storage of the serum. The simultaneous measurement of the two vitamins in the HPLC assay will make vitamin E measurement, which has not been done extensively in health surveys very convenient to do and might help to elucidate in humans the significance of the sparing effect of vitamin E on vitamin A observed in animals [11].

We use a single internal standard, retinyl acetate, while Bieri et al. [6] use both retinyl acetate and tocopheryl acetate as internal standards. Although it would seem to be desirable to have a structurally related internal standard for each measured metabolite, in practice, we have found that results equal in precision and accuracy are obtained with either one or two internal standards. Eliminating tocopheryl acetate as an internal standard shortens the chromatographic run time by 20% and also reduces by one the number of peaks to be measured.

REFERENCES

1 K. Abe and G. Katsui, Vitamins (Japan), 40 (1975) 259.
2 K. Abe, K. Ishibashi, M. Ohmae, K. Kawabe and G. Katsui, Vitamins (Japan), 51 (1977) 275.
3 M.G.M. de Ruyter and A.P. de Leenheer, Clin. Chem., 22 (1976) 1593.
4 A.P. de Leenheer, V.O. de Bevere, A.A. Cruyl and A.E. Claeys, Clin. Chem., 24 (1978) 585.
5 B. Nilsson, B. Johansson, L. Jansson and L. Holmberg, J. Chromatogr., 145 (1978) 169.
6 J.G. Bieri, T.J. Tolliver and G.L. Catignani, Amer. J. Clin. Nutr., 32 (1979) 2143.
7 A.P. de Leenheer, V. de Bevere, M.G.M. de Ruyter and A.E. Claeys, J. Chromatogr., 162 (1979) 408.
8 J.B. Neeld, Jr. and W.N. Pearson, J. Nutr., 79 (1963) 454.
9 P.J.M. Wakkers, H.B.A. Hellendoorn, G.J. op de Weegh and W. Heerspink, Clin. Chem. Acta, 64 (1975) 173.
10 W. Vetter and G. Englert, in O. Isler (Editor), Carotenoids, Birkhauser Verlag, Basel, Stuttgart, 1971, p. 189.
11 T. Moore, Vitamin A, Elsevier, Amsterdam, 1957, p. 231.

Journal of Chromatography, 231 (1982) 445—450
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1309

Note

Determination of tolazoline in plasma by gas chromatography—mass spectrometry

ROBERT M. WARD*

Departments of Pediatrics and Pharmacology, The Milton S. Hershey Medical Center, The Pennsylvania State University, Hershey, PA 17033 (U.S.A.)

MEHROO J. COOPER

Department of Pharmacology, University of Minnesota, Minneapolis, MN 55455 (U.S.A.)

and

BERNARD L. MIRKIN

Division of Clinical Pharmacology, Departments of Pediatrics and Pharmacology, University of Minnesota, Minneapolis, MN 55455 (U.S.A.)

(First received March 9th, 1981; revised manuscript received April 15th, 1982)

Tolazoline [4,5-dihydro-2-(phenylmethyl)-1H-imidazole] has been used extensively since 1939 for its histaminergic, alpha-adrenergic antagonist and vasodilator activities [1]. Tolazoline is administered frequently to newborn infants as a pulmonary vasodilator [2]. Since repeated administration to neonates has been associated with undesirable side effects, careful monitoring of the dosage regimen may be necessary [3]. Few methods have been reported for the determination of tolazoline. Farkas [4], Boon and Sudds [5] and Mollica et al. [6] reported methods for the assay of tolazoline in pharmaceutical preparations, while Brodie et al. [7] described a colorimetric method for its determination in biological fluids and tissues. This colorimetric technique requires 1—5 ml of plasma, detects 5—25 μg of tolazoline per sample and has been used to study tolazoline pharmacokinetics in a limited number of adult humans [7] and animals [7,8]. None of the reported assays for tolazoline is suitable for small sample volumes and low concentrations. Consequently, pharmacokinetics of tolazoline have not been reported in young animals or infants.

We report a new procedure for the determination of tolazoline in plasma which is chemically specific for tolazoline and applicable to small sample

0378-4347/82/0000—0000/$02.75

volumes. Tetrahydrozoline [4,5-dihydro-2-(1,2,3,4-tetrahydro-1-naphthalenyl)-1H-imidazole] is added to the plasma sample as an internal standard. After solvent extraction, tolazoline and the internal standard are converted to trifluoroacetyl (TFA) derivatives and quantitated by gas chromatography—mass spectrometry with selected ion monitoring. The clinical applicability of the method is shown by the determination of tolazoline concentrations in a few neonatal patients.

EXPERIMENTAL

Standards and reagents

Tolazoline and tetrahydrozoline were kindly furnished by CIBA Pharmaceutical Co. and Pfizer Labs., respectively. Standard solutions were prepared in methanol and only freshly prepared solutions were used. Trifluoroacetic anhydride (TFAA) and triethylamine (TEA) were obtained from Sigma. All solvents were spectrophotometric grade. Water was glass distilled.

Instrument

A Hewlett-Packard 5992b gas chromatograph—mass spectrometer with a jet orifice separator was used. The 91.4 cm × 2 mm I.D. glass column was packed with SP-2100 (Supelco). The injector temperature was maintained at 240°C, while the column temperature was programmed to increase from 190°C to 230°C at 15°C/min beginning 1.0 min after injection. The helium carrier gas flow-rate was 20 ml/min. The electron energy was 70 eV with an electron multiplier voltage of 2.2—2.6 kV according to the parameters established each day by the Hewlett-Packard autotune program using perfluorotributylamine as a standard.

The Hewlett-Packard peakfinder program was used to obtain spectra of the derivatives of tolazoline and the internal standard by scanning in 0.1 a.m.u. increments at 330 a.m.u./sec with recording of the chromatogram of the total ion current and of the spectra of ions comprising the peaks in the chromatogram. A background spectrum can be selected manually or automatically from the minimum total ion current during the analysis and later subtracted by computer from the spectra being analyzed. Although the scanner is computer-focused to 0.1 a.m.u., small day-to-day variations in the instrument result in fluctuations of ± 0.1 a.m.u. in the ions recorded in the spectrum of a given compound. Due to this variation, the ions in the spectra of Fig. 1C and D are labeled to the nearest a.m.u. With the Hewlett-Packard single ion monitoring program, the ion currents of as many as six ions, focused to 0.1 a.m.u., are recorded individually and in summation (Fig. 2). Ratios of the peak areas of selected ions can be obtained.

Derivatization

Toluene (200 μl), TEA (5 μl) and TFAA (25 μl) were added to 2—8 μg of tolazoline and 10 μg of tetrahydrozoline in conical centrifuge tubes. The tubes were stoppered, shaken on a Vortex mixer and heated for 40 min at 60° C in a shaking water bath. The tubes were cooled immediately and 200 μl water

added to each. The tubes were shaken on a Vortex mixer, centrifuged and an aliquot of the toluene layer injected onto the column.

Quantitation of plasma samples

For extraction of tolazoline from plasma, toluene was selected over ethyl acetate due to variation in the ethyl acetate volume separating from the aqueous layer both after extraction and after derivatization. Water-saturated ethyl acetate was avoided because of potential inactivation of TFAA by hydrolysis during derivatization. Although the volume of 5 *N* sodium hydroxide solution was varied from 25 to 150 μl/ml plasma, extraction was maximum with 50 μl. Addition of salt sufficient to saturate the plasma layer improved the linearity of standard curves. Vigorous vortex mixing for 1 min frequently produced a gel-like emulsion of the plasma proteins. To maintain alkaline conditions, freezing was selected over perchloric acid precipitation to denature the proteins and improve separation of the plasma proteins from toluene during later centrifugation. If the gel-like layer remained after centrifugation, it was disrupted with a glass rod and the sample recentrifuged. Vortex mixing for 60 sec was found to be equivalent to extraction by mechanical shaking (Dubnoff shaker) for 5—10 min. No difference in extent of extraction was noted when mechanical shaking was varied from 5 to 20 min.

Thus, the following procedure was utilized for quantitation of tolazoline in plasma. To 1.0 ml plasma containing tolazoline were added 2 μg of internal standard, 1.0 g sodium chloride, 5.0 ml toluene and 50 μl 5 *N* sodium hydroxide solution. The samples were mixed for 60 sec on a Vortex mixer, frozen at 0°C for 10 min and centrifuged for 10 min at 5000 *g* to separate the layers. If necessary the organic layer was stirred with a glass rod and the sample was recentrifuged. The organic layer was transferred to another tube and evaporated to dryness at 50°C in vacuo. The sample was then derivatized as described earlier and an aliquot injected onto the column. Quantitation of plasma samples was achieved with selected ion monitoring (SIM) by comparison of the peak area *m/e* 352.1 for tolazoline to the peak area of *m/e* 392.1 for tetrahydrozoline. Secondary ions of *m/e* 283.1 and 295.2 for tolazoline and tetrahydrozoline, respectively, were also monitored to insure that the primary ions monitored were unique to these compounds. For calculation of recoveries of tolazoline and tetrahydrozoline, the peak areas at the appropriate *m/e* of the extracted plasma samples were compared to the peak areas of samples to which an equal amount of standard and internal standard were added prior to derivatization.

RESULTS AND DISCUSSION

To insure completeness of reaction, the conditions for derivatization were varied and optimum conditions were established as follows: 25 μl TFAA, 5 μl TEA, 200 μl toluene, and incubation at 60°C for 40 min.

Fig. 1A shows the structure of tolazoline and the mass spectra of the TFAA derivatives of tolazoline and the internal standard obtained with the Hewlett-Packard peakfinder program when *m/e* 84 to 505 were scanned (Fig. 1B, C and D). Although the scan was initially extended to *m/e* 800, only the di-TFA

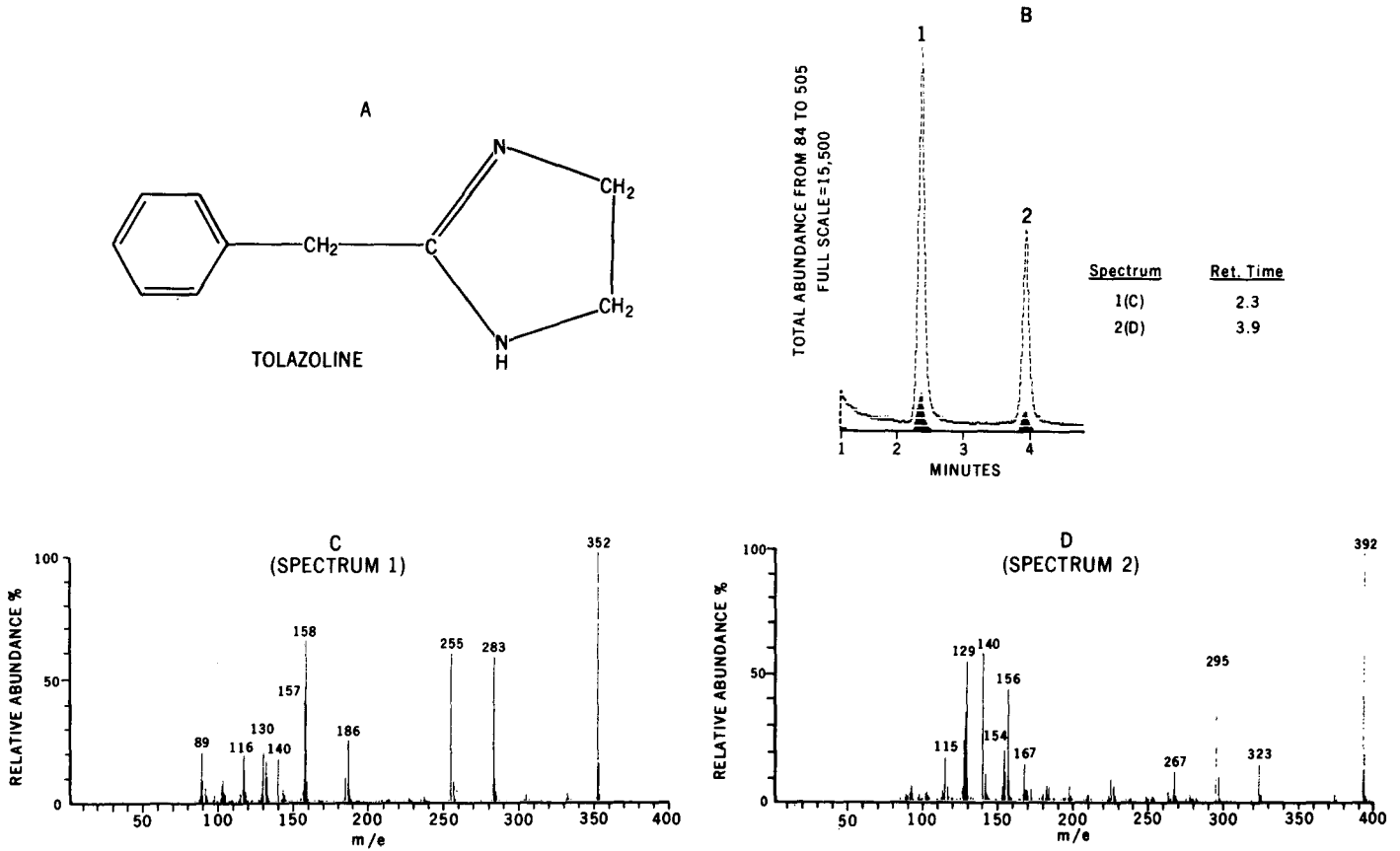


Fig. 1. (A) Tolazoline structure, mol.wt. 160.2; (B) chromatogram of the TFAA derivatives of tolazoline (1) and internal standard, tetrahydrozoline (2) after extraction from plasma; (C) electron impact mass spectrum of peak 1, tolazoline di-TFA; (D) electron impact mass spectrum of peak 2, tetrahydrozoline di-TFA.

derivative of both compounds was obtained. Representative selected ion chromatograms, monitoring the ions *m/e* 352.1 and 283.1 for tolazoline di-TFA and *m/e* 392.1 and 295.2 for tetrahydrozoline di-TFA, are shown for a standard (Fig. 2A) and a patient sample (Fig. 2B). An unidentified compound containing *m/e* 295.2 (a secondary ion of tetrahydrozoline di-TFA) with a retention time of 2 min was observed occasionally in patient samples, but did not interfere with quantitation.

The stability of the derivatives was tested by keeping the samples at 0°C for one month and reinjecting the toluene layer; no deterioration was observed after this period.

A recovery of 59.2 ± 5.5% (mean ± S.D., $n = 26$) was obtained when tolazoline was added to plasma in the range of 2—8 μg/ml. The recovery of the internal standard (concentration = 10 μg/ml) was 67.9 ± 9.6% (mean ± S.D., $n = 33$).

Ten plasma standard curves with 1-ml samples (peak area 352.1/peak area 392.1 × 100% vs. [tolazoline]) including 54 points over the concentration

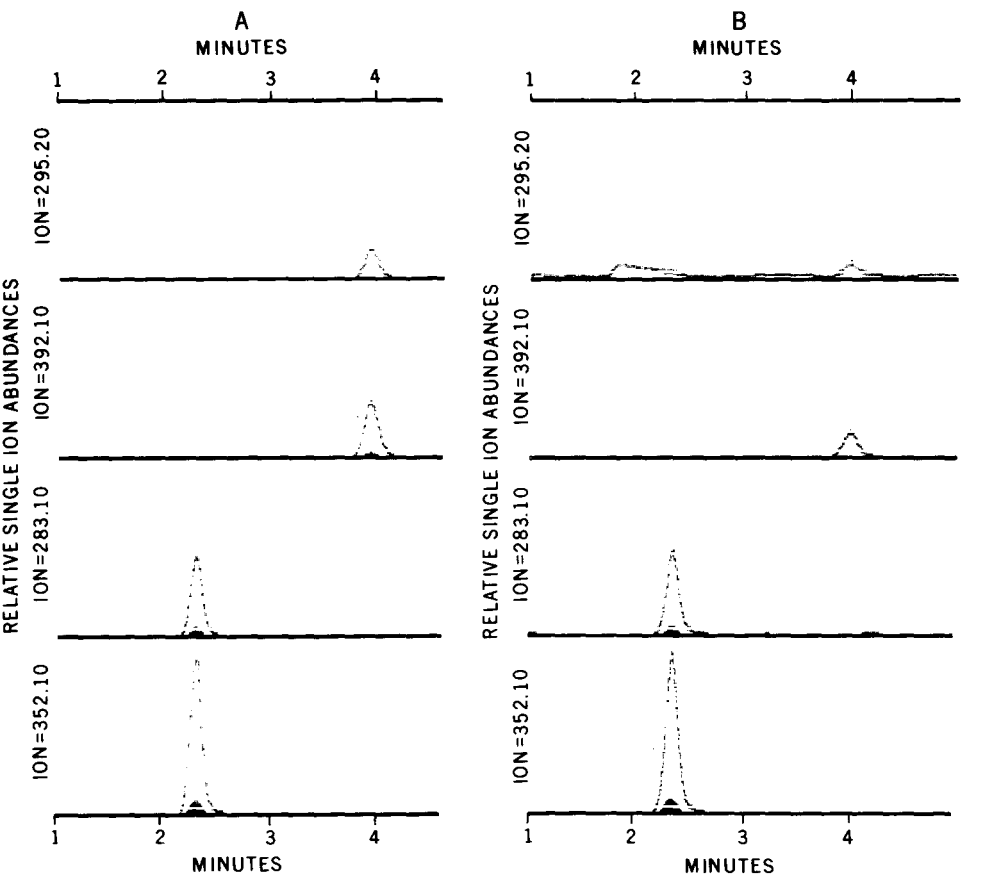


Fig. 2. GC—MS SIM chromatograms of TFAA derivatives of tolazoline, ions *m/e* 352.1 and 283.1 and tetrahydrozoline, ions *m/e* 392.1 and 295.2. (A) Blank plasma and (B) patient plasma sample.

range of 0.01—50.00 μg/ml were linear (correlation coefficient of 0.996 ± 0.006, mean ± S.D.) with a slope of 0.0121 ± 0.0017 and passed close to the origin. When 100-μl plasma samples were analyzed, 400 μl blank ovine plasma were added to facilitate extraction. Using the micro-method with 100-μl plasma samples and 0.5 μg internal standard, five standard curves from 1.0—30.0 μg/ml (40 points) had a slope of 0.0876 ± 0.0016 (mean ± S.D.) and linear correlation coefficients of 0.996 ± 0.003. Precision of this method was examined by five analyses of 100-μl aliquots of a lamb plasma sample and produced a coefficient of variation of 5.6%. Accuracy of analysis was examined by repeated injections (n = 8 per sample) of low (0.5 μg/ml) and high (10.0 μg/ml) concentration samples of tolazoline and produced coefficients of variation of 4.9% and 2.3%, respectively.

Using 200-μl samples, six plasma tolazoline concentrations were determined in four infants receiving the drug at different dosages and were found to range from 2.7—9.8 μg/ml.

The following drugs which are often used by clinicians concomitantly with tolazoline were found not to interfere with the assay: ampicillin, gentamicin, pancuronium bromide, heparin, dopamine, morphine and furosemide.

The present extraction and derivatization procedure has been performed with 1-ml volumes, but when sample size is limited the entire procedure can be successfully performed with plasma volumes as low as 100 μl. This makes the method particularly attractive for application in the neonatal field.

This method is currently being used to study the pharmacokinetics and renal excretion of tolazoline in lambs and newborn infants.

ACKNOWLEDGEMENTS

This work was supported in part by NIGMS Grant No. 5T32 G M07466-02. The authors gratefully acknowledge technical assistance provided by Rebecca R. Thorsen and Catherine H. Daniel with secretarial assistance provided by Jody Hower.

REFERENCES

1 R.P. Ahlquist, R.H. Huggins and R.A. Woodbury, J. Pharm. Exp. Ther., 89 (1947) 271.
2 B.W. Goetzman, P. Sunshine, J.D. Johnson, R.P. Wennberg, A. Hackel, D.F. Merten, A.L. Bartoletti and N.H. Silverman, J. Pediatr., 89 (1976) 617.
3 J.V. Aranda, A. Portuguez-Malavasi, J. Collinge and E. Outerbridge, Pediatr. Res., 12 (1978) 422 (abstract).
4 E. Farkas, Pharmazie, 21 (1966) 600.
5 P.F.G. Boon and W. Sudds, J. Pharm. Pharmacol., 19 (1967) 88S.
6 J.A. Mollica, G.R. Padmanabhan and R. Strusz, Anal. Chem., 45 (1973) 1859.
7 B.B. Brodie, L. Aronow and J. Axelrod, J. Pharm. Exp. Ther., 106 (1952) 200.
8 B.M. Boulos, W.L. Jenkins and L.E. Davis, Amer. J. Vet. Res., 33 (1972) 943.

Journal of Chromatography, 231 (1982) 451—458
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1323

Note

Occurrence and measurement of nifedipine and its nitropyridine derivative in human blood plasma

J. DOKLADALOVA*, J.A. TYKAL, S.J. COCO, P.E. DURKEE, G.T. QUERCIA and J.J. KORST

Quality Control Division, Pfizer Inc., Eastern Point Rd., Groton, CT 06340 (U.S.A.)

(First received January 19th, 1982; revised manuscript received April 6th, 1982)

Nifedipine, [dimethyl 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridinecarboxylate] is a potent coronary vasodilator [1]. For pharmacokinetic studies in which nanogram amounts of nifedipine in blood plasma have been measured, the literature [1—4] invariably describes the use of gas—liquid chromatography (GLC) with either electron-capture detection or selective ion monitoring.

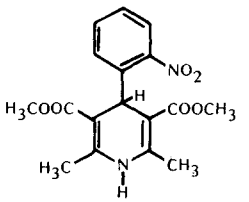
Two basic analytical approaches have been used in these studies. In the procedure reported by Higuchi and Shiobara [1], as well as that by Kondo et al. [2], nifedipine is oxidized to its more stable nitropyridine derivative [dimethyl 2,6-dimethyl-4-(2-nitrophenyl)-3,5-pyridine dicarboxylate] II, prior to GLC analysis. Despite the loss of specificity in this approach, both groups of authors have found it necessary as, under the chromatographic conditions employed, nifedipine was found to partially degrade to the nitropyridine derivative II.

Conversely, Jakobsen et al. [3] and Testa et al. [4], utilizing similar chromatographic conditions, have found nifedipine to be stable and have measured the drug directly.

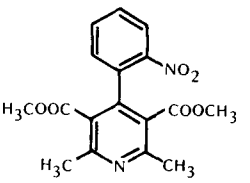
In the present work, the GLC methodology of Jakobsen et al. [3] has essentially been employed with similar results: no degradation to nitropyridine derivative II during chromatography was observed. This was shown by repeated injections of nifedipine extracted from blank plasma samples to which known nifedipine amounts had been added prior to analysis.

The procedure has been used to quantitate nifedipine in the plasma of subjects from a pharmacokinetic study. In addition to nifedipine which was measured directly, the nitropyridine derivative II was also quantitated. Positive

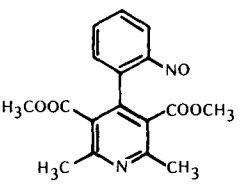
0378-4347/82/0000—0000/$02.75



(I)



(II)



(III)

identification of the last-named substance as well as final proof that the nitropyridine derivative is a metabolite, since it is not formed during the plasma preparation or in contact with blood in vitro, contribute to confirm Jakobsen's observation [3] of the presence of a tentatively identified metabolite in plasma of patients treated with nifedipine. Prior to this time, the nitropyridine substance II was postulated as a precursor of known nifedipine metabolites [2,5], but had not been unequivocally found in plasma. The identity of the nitropyridine derivative II was confirmed by high-performance liquid chromatographic (HPLC) analysis.

EXPERIMENTAL

All sample handling and extraction steps were carried out under gold fluorescent lighting (e.g. General Electric, F40G0) to prevent light degradation of nifedipine.

Materials

Toluene, methanol, hexane, ethyl acetate, acetonitrile, were all glass distilled and supplied by Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Nifedipine, nitropyridine derivative and nitrosopyridine derivative were obtained from Bayer (Leverkusen, G.F.R.). Diazepam was supplied by U.S.P.C. (Rockville, MD, U.S.A.). Water was purified by a water conditioning system purchased from Continental Water Systems Corporation (El Paso, TX, U.S.A.).

In addition, the reagents included methanol treated with *n*-hexane and *n*-hexane treated with methanol. (Reagents were prepared by transferring 500 ml methanol and 400 ml *n*-hexane to a 1-l separatory funnel, shaking the mixture and allowing the phases to separate, the bottom layer being methanol treated with *n*-hexane, the upper layer, *n*-hexane treated with methanol.)

All glassware used during the extraction procedure was cleaned with chromic

acid, washed with distilled water, air-dried and rinsed with methanol.

Instruments

Gas—liquid chromatograph. The Hewlett-Packard 5750 Research gas chromatograph was equipped with a ^{63}Ni electron-capture detector (attenuated at 10 × 16) and a glass 1.83 m × 2.0 mm I.D. column packed with 2% OV-17 on Gas-Chrom Q (100—120 mesh). Argon—methane (95:5) was used as a carrier gas at a flow-rate of 32 ml/min. Injection port temperature was maintained at 245°C, column temperature at 235°C, detector temperature at 280°C. Injection volume corresponded to 3 μl, recorded chart speed to 0.64 cm/min.

A vortex evaporator (Buchler) was used at 40°C to remove the solvents from samples.

High-performance liquid chromatograph. The HPLC system consisted of a solvent pump (Waters Assoc., Model 6000A); injection valve, loop type (Valco, Model 6V6UHPA); a column, 200 × 6 mm O.D. × 4 mm I.D., Nucleosil® 5 C_{18}, particle size 5 ± 1.5 μm, Macherey, Nagel and Co., Düren, G.F.R., No. 715260; UV detector at 235 nm, range 0.04 (Schoeffel, Model 770); recorder 1 mV (Varian, Model 25). Mobile phase consisting of water—methanol—acetonitrile (55:36:9) was degassed and used at a flow-rate of 1.0 ml/min.

Preparation of samples for GLC analysis

Plasma (1 ml) was placed in a 15-ml centrifuge tube to which 50 ng diazepam (internal standard) had been added. Sample was extracted twice with 2 ml of toluene, each time centrifuging the sample (ca. 1000 *g* for 10 min). Each toluene extract was transferred to a clean 15-ml centrifuge tube using Pasteur disposable pipets. The combined toluene extracts were evaporated to dryness on an evaporator. The sample was reconstituted with 1.0 ml methanol treated with hexane. The methanol solution was washed with 3 ml hexane treated with methanol and centrifuged. The hexane layer was discarded using disposable pipets and the methanol evaporated to dryness. The sample was reconstituted with 200 μl of toluene before injection.

Preparation of standards for GLC analysis

Control plasma samples (1 ml) were spiked with 5, 10, 15, 25, and 50 ng nitropyridine derivative, with 10, 25, 50, 100 and 200 ng nifedipine and with 50 ng diazepam as internal standard. The samples were processed as described above. The ratios of nifedipine peak height to diazapam peak height and of nitropyridine derivative peak height to diazepam peak height were calculated and the calibration curves constructed.

Preparation of samples for HPLC analysis

After completing the GLC analysis, the sample was evaporated to dryness and redissolved in 100 μl of methanol before injection on the HPLC column.

RESULTS AND DISCUSSION

Nifedipine (I) in solution undergoes facile degradation. Daylight-induced

decomposition to the nitrosopyridine derivative III and to the nitropyridine derivative II under UV irradiation has been reported [4]. In addition, nifedipine is known to be thermally unstable. It is not surprising, therefore, that controversy exists in the literature concerning the detection and quantification of this substance in complex matrices (e.g., blood plasma). In particular, the GLC methodology used for quantitation is subject to question since higher temperatures (typically 230°C—250°C) are involved and the possibility of thermal degradation of nifedipine to its nitroderivative during chromatography cannot be discounted.

Jakobsen et al. [3] and Testa et al. [4] emphasize that they did not observe degradation of nifedipine during GLC analysis and therefore claim their assay being superior to the indirect method which involves oxidation of nifedipine to the more stable nitropyridine derivative II prior to analysis [1,2]. The last-mentioned approach may be used for nifedipine analysis only when the absence of the nitropyridine derivative in the original sample can be demonstrated.

The sensitivity reported by Jakobsen et al. [3], which is required for clinical studies, could not be achieved using the Hewlett-Packard 5750 research gas chromatograph. Therefore, the samples had to be concentrated prior to analysis. Five-fold concentration of the extract was accompanied by increase in the chromatographic background. Consequently, a more extensive clean-up procedure had to be developed. This procedure is applicable to the simultaneous quantification of nitropyridine derivative and nifedipine. Nitrosopyridine derivative III is separated from these two substances eluting first from the chromatographic column. Linearity of the procedure was established for 10—200 ng nifedipine/ml plasma and 10—50 ng nitropyridine derivative/ml plasma. Concentrations below 10 ng/ml can be reliably estimated for both substances. Amounts down to 1 ng can be detected. Reproducibility and accuracy of the procedure described in Table I will deteriorate when whole blood or fast-prepared lower quality plasma are analyzed.

Our results obtained during the analysis of 1480 clinical blood plasma samples and during additional studies of the effect of fresh whole blood and fresh plasma on nifedipine stability, lead us to conclude that the nitropyridine derivative is indeed present in the blood of subjects to whom nifedipine had been administered. The following experiments justify our conclusion. Daily calibration curves were constructed by analyzing a series of blank plasma samples spiked with known amounts of nifedipine and internal standard (diazepam). Concurrently, a five-month stability study (10 and 200 ng nifedipine/ml in frozen plasma) was conducted. In addition, nifedipine and internal standard were added to the subjects' control plasma (plasma from blood samples taken before administration of nifedipine) and analyzed. Only nifedipine was observed in the chromatograms obtained from these experiments (see Fig. 1A and B). However, in the chromatograms from subjects' plasma samples taken after nifedipine administration, the nitropyridine derivative II was observed in addition to nifedipine (Fig. 1C). The kinetic profile of the nitropyridine compound was similar to that of nifedipine for all subjects studied (for example, see Fig. 2).

In order to confirm the identity of the nitropyridine derivative II in plasma

TABLE I

REPRODUCIBILITY AND ACCURACY OF NIFEDIPINE AND NITROPYRIDINE DETERMINATION IN BLOOD PLASMA

Compound added (ng/ml)	Number of parallel determinations	Recovery (%) (average)	Relative standard deviation (%)
*A. Nifedipine determination**			
10	5	96.0	4.5
25	9	105.3	9.0
50	9	98.9	4.8
100	9	100.9	3.7
200	8	98.6	1.1
*B. Nitropyridine derivative determination***			
5	5	72.0	11.9
10	9	90.0	7.5
15	5	105.8	8.2
25	10	104.0	6.4
50	5	91.6	1.9

*Amounts of nitropyridine derivative added varied from 5 to 50 ng per ml plasma.
**Amounts of nifedipine added varied from 10 to 100 ng per ml plasma.

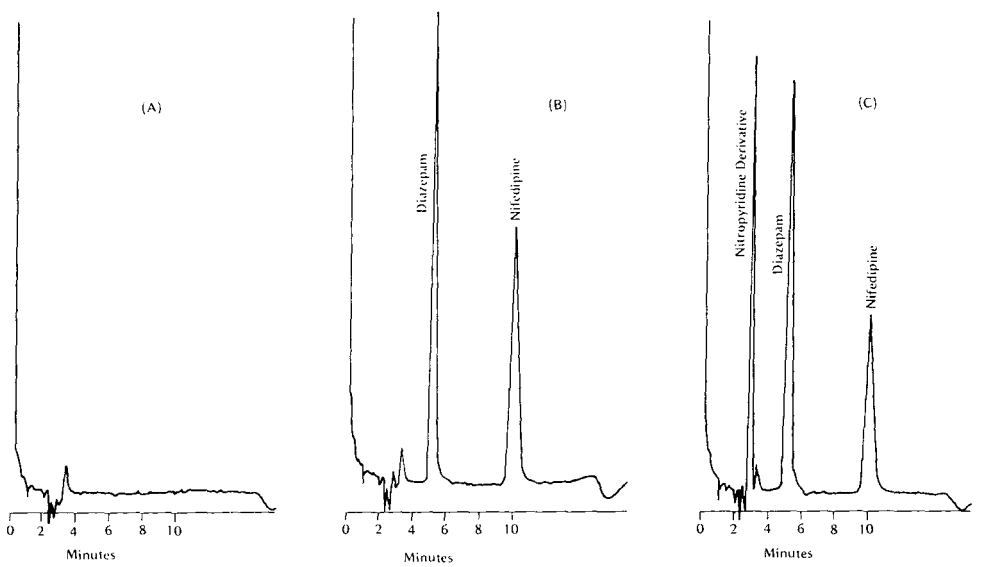


Fig. 1. GLC analysis of (A) control plasma before administration of nifedipine; (B) control plasma before administration of nifedipine spiked with 100 ng/ml nifedipine and 50 ng/ml diazepam; and (C) plasma, 20 min after administration of nifedipine.

from subjects to whom nifedipine had been administered, HPLC analysis of control plasma extract containing II and of a typical plasma extract exhibiting high nitropyridine content in the GLC analysis were carried out. In both instances a peak appeared at the retention time of the nitropyridine derivative (compare Fig. 3A, B and C). The peak identity was further confirmed by col-

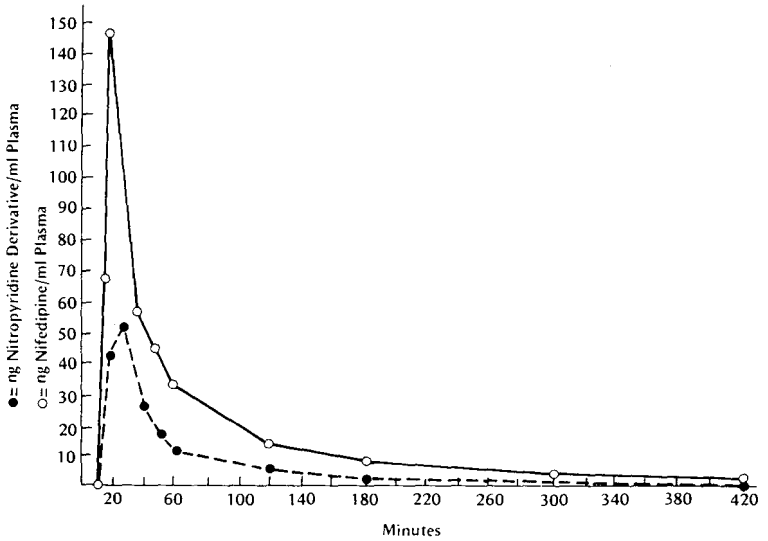


Fig. 2. Typical kinetic profile of nifedipine (○—) and nitropyridine derivative (- - ● - -) for a subject administered the drug.

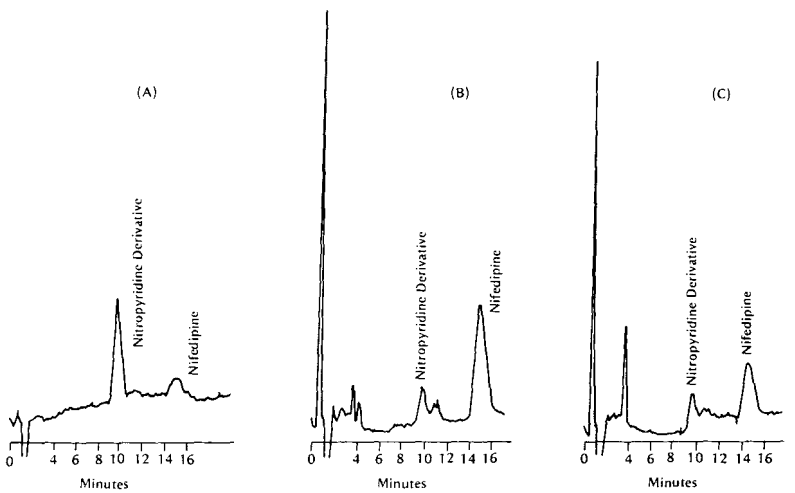


Fig. 3. HPLC chromatograms of (A) reference substances, nitropyridine derivative and nifedipine, demonstrating their retention times; (B) control plasma spiked with 100 ng nitropyridine derivative and 200 ng nifedipine per ml plasma; (C) subject's plasma taken 30 min after nifedipine administration.

lecting the material eluted under the HPLC peak and subsequently reanalyzing it by GLC. A chromatogram typical of the nitropyridine derivative was obtained, confirming the identity of the substance.

The possibility of nitropyridine derivative being formed from nifedipine during the plasma preparation or in contact with blood in vitro was investigated by adding known amounts of nifedipine (equivalent to 25 ng/ml and 200 ng/ml) to fresh whole blood and to fresh fast-prepared plasma. In total, sixteen experiments were carried out. Simultaneously, calibration curves for both the

TABLE II

OCCURRENCE OF NITROPYRIDINE DERIVATIVE IN FRESH PLASMA AND WHOLE BLOOD CONTAINING NIFEDIPINE

Nifedipine added (ng/ml)	Nitropyridine derivative determined (ng/ml)	Nifedipine determined (ng/ml)	Percentage recovery of nifedipine
Analysis of nifedipine added to fresh blood plasma			
25	< 2	25.5	102.0
	< 2	24.9	99.6
	< 2	28.4	113.6
	< 2	26.7	106.8
		Average	105.5
200	ca. 2	186.8	93.4
	ca. 2	188.1	94.1
	ca. 2	181.2	90.6
	ca. 2	176	88.0
		Average	91.5
Analysis of nifedipine added to fresh whole blood			
25	< 2	19.7	78.8
	< 2	23.0	92.0
	< 2	20.5	82.0
	< 2	24.3	97.2
		Average	87.5
200	ca. 2	158.4	79.2
	ca. 2	165.5	82.8
	ca. 2	124.3	62.2
	ca. 2	169.7	84.9
		Average	77.3

nitropyridine derivative and nifedipine were constructed. The results (Table II) clearly indicated that nifedipine in direct contact with either fresh human blood plasma or fresh whole blood is not significantly converted to the nitropyridine derivative during the processing of the samples prior to freezing and analysis. Lower nifedipine recoveries (Table II) obtained, in particular when whole blood was analyzed, were caused by lower extraction efficiency from this medium. However, they do not influence the conclusion on the stability of nifedipine under given experimental conditions.

Since the samples of plasma from subjects administrated nifedipine, prepared under conditions identical to those described above, invariably showed the presence of both nifedipine and its nitropyridine derivative (the amount of nitropyridine derivative expressed as percentage of nifedipine varied from subject to subject from 17 to 90%), it is concluded that the nitropyridine derivative, which has been postulated as a precursor of known nifedipine metabolites [2,5], is present in the blood of subjects who have been administered nifedipine. Consequently, a selective assay for nifedipine without preliminary oxidation to the nitropyridine derivative must be employed in order to accurately assess the content of nifedipine in plasma or serum samples.

REFERENCES

1 S. Higuchi and Y. Shiobara, Biomed. Mass Spectrom., 5 (1978) 220.
2 S. Kondo, A. Kuchiki, K. Yamamoto, K. Akimoto, K. Takanashi, N. Awata and I. Sugimoto, Chem. Pharm. Bull., 28 (1980) 1.
3 P. Jakobsen, O. Lederballe Pedersen and E. Mikkelsen, J. Chromatogr., 162 (1979) 81.
4 R. Testa, E. Dolfini, C. Reschiotto, C. Secchi and P.A. Biondi, Farmaco, Ed. Prat., 34 (1979) 463.
5 S. Ebel, H. Schutz and A. Hornitschek, Arzneim.-Forsch., 28 (1978) 2188.

Journal of Chromatography, 231 (1982) 459—462
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1339

Note

Simultaneous determination of carbidopa, levodopa and 3,4-dihydroxyphenylacetic acid using high-performance liquid chromatography with electrochemical detection

ERKKI NISSINEN* and JYRKI TASKINEN

Orion-Yhtymä OY, Orion Pharmaceutical Co., Research Laboratories, P.O. Box 19, SF-00101 Helsinki 10 (Finland)

(First received January 25th, 1982; revised manuscript received May 6th, 1982)

The combination of levodopa (3,4-dihydroxyphenylalanine) and carbidopa (L-α-hydrazino-3,4-dihydroxy-L-α-methylcinnamic acid) in the treatment of Parkinson's disease has proved to be very successful [1]. The effect of levodopa on Parkinson's disease is considered to be due to its metabolic product dopamine (3-hydroxytyramine), which is produced by decarboxylation of levodopa [2]. Carbidopa instead is a decarboxylase inhibitor and is used to block the extracerebral metabolism of levodopa allowing more levodopa to reach the brain for conversion to dopamine [2].

Carbidopa, levodopa and its metabolites dopamine and 3,4-dihydroxyphenylacetic acid (DOPAC) have so far been determined separately or simultaneously by rather tedious methods [3—5]. Of the available methods high-performance liquid chromatography (HPLC) with electrochemical detection offers the best specificity and sensitivity for determination of catecholamines in any tissue [6]. However, the recent reports for the simultaneous determination of carbidopa and levodopa [7—9] are not sensitive or selective enough for quantification of these compounds with their metabolites in human plasma.

We report here a method for the simultaneous determination of these compounds from plasma using adsorption on alumina and HPLC with electrochemical detection. This method has been applied to the quantification of carbidopa, levodopa and DOPAC sampled continuously in human plasma after an oral dose of levodopa with carbidopa.

EXPERIMENTAL

Reagents

Dihydroxybenzylamine (DHBA), dopamine and DOPAC were purchased from Sigma (St. Louis, MO, U.S.A.). Levodopa and carbidopa were obtained from Orion Pharmaceutical (Helsinki, Finland). Sodium octanesulfonic acid was from Eastman Kodak (Rochester, NY, U.S.A.). Alumina, activity grade I for chromatography, was from Merck (Darmstadt, G.F.R.) and it was prepared by the method of Anton and Sayre [10]. All other chemicals were of reagent grade and purchased from commercial sources.

Sample preparation

Blood samples (5 ml) were drawn by venipuncture and transferred immediately to tubes containing 0.1 ml of 10% metabisulfite and 0.1 ml of 1% EDTA in physiological saline solution. Samples were centrifuged at 600 *g* at 4°C; the plasma was removed and stored at −20°C until analysis.

Plasma samples (1 ml) were spiked with 50 ng of DHBA (the internal standard) in tubes containing 0.1 ml of 2.5 m*M* metabisulfite. Acid-washed alumina (100 mg) was added followed by 1.0 ml of 0.5 *M* Tris–EDTA, pH 8.6. The tubes were stoppered and shaken mechanically 5 min on a multitube vortexer. The samples were centrifuged, the supernatants discarded and the alumina was washed twice with 10 ml of water. The catecholamines were eluted with 0.5 ml of 0.2 *M* perchloric acid, and 20 μl of the eluate were injected into the liquid chromatograph.

Chromatography

The liquid chromatography–electrochemical detection system consisted of a Waters Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.), a Rheodyne Model 7125 injector valve with a 20-μl sample loop (Rheodyne, Cotati, CA, U.S.A.), and a 3.2 mm × 250 mm 5 μm ODS Spherisorb column fitted with a 3.2 mm × 30 mm precolumn (Altex Scientific, Berkeley, CA, U.S.A.).

The electrochemical detector consisted of a TL-5 glassy carbon electrode and LC-4A potentiostat (Bioanalytical Systems, West Lafayette, IN, U.S.A.). The working electrode was set at +0.70 V relative to an Ag/AgCl reference electrode. The sensitivity was set 5 nA full-scale.

The elution of catecholamines from the column was carried out in isocratic mode at ambient temperature. The mobile phase was composed of 100 m*M* NaH_2PO_4, 20 m*M* citric acid, 1.25 m*M* sodium octanesulfonic acid, 0.15 m*M* sodium EDTA in 8% methanol. The pH was adjusted to 3.2 with sodium hydroxide. The flow-rate was 1 ml/min.

RESULTS AND DISCUSSION

As seen in Fig. 1A, all the compounds studied — levodopa, carbidopa, DOPAC, dopamine and DHBA (the internal standard) — are completely separated in 10 min, thus allowing the handling of about 40 samples per day. Fig. 1B shows a chromatogram of blank plasma after adsorption on alumina

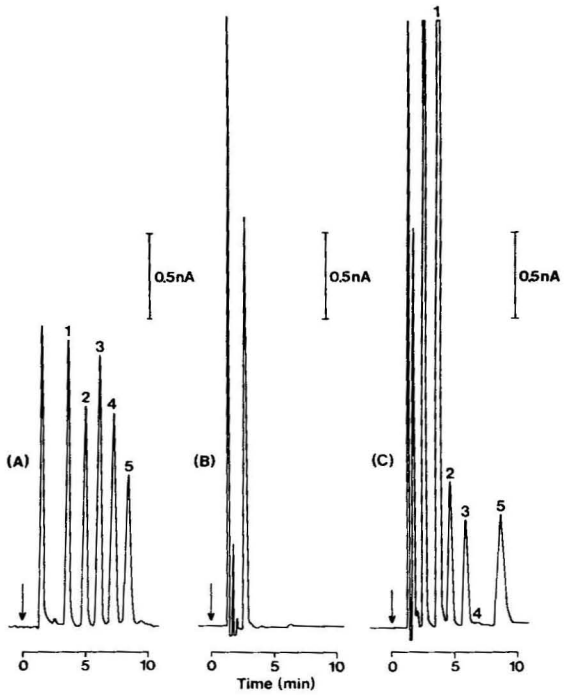


Fig. 1. Chromatograms of (A) catecholamine standards, (B) predose control plasma, and (C) plasma obtained from a volunteer 60 min after oral administration of 100 mg of levodopa with 25 mg of carbidopa and containing the internal standard (50 ng/ml). Peaks: 1 = levodopa; 2 = DHBA; 3 = DOPAC; 4 = dopamine; 5 = carbidopa. Chromatographic conditions as described under Experimental.

and perchloric acid extraction. Using the chromatographic conditions described no interfering peaks were detected. Fig. 1C demonstrates a typical chromatogram of a plasma sample from a volunteer after 1 h of concomitant oral administration of levodopa and carbidopa.

The recoveries of added catecholamines (50 ng/ml, n = 10) from plasma were: levodopa 60.7 ± 7.6%, carbidopa 51.3 ± 7.3%, DOPAC 54.1 ± 8.5% and DHBA 72.2 ± 7.8%. Repeated determinations (n = 10) of four parallel standard preparations at the concentration 50 ng/ml gave the following coefficients of variation: levodopa 1.7%, carbidopa 3.6%. DOPAC 3.8% and DHBA 3.2%. Standard curves were linear over the range 1—500 ng/ml, except for carbidopa, for which the lower limit of detection was 15 ng/ml.

The described HPLC method with electrochemical detection provides a fast, reliable, uncomplicated method to determine serum levels of levodopa—carbidopa combination dosage up to 12 h after administration. It has been successfully applied to the analysis of over 700 plasma samples from human volunteers with various levodopa—carbidopa dosage combinations.

REFERENCES

1 R.L. Falck, Drug Intell. Clin. Pharm., 10 (1976) 84.

2 C.C. Porter, L.S. Watson, C.D. Titus, J.A. Tataro and S.S. Byer, Biochem. Pharmacol., 11 (1962) 1067.
3 K. Sasahara, T. Nitanai, T. Habara, A. Ninomiya, T. Morioka and E. Nakajima, Ann. Rep. Sankyo Res. Lab., 30 (1978) 65.
4 S. Vickers, E.K. Stuart, J.R. Bianchine, H.B. Hucker, M.E. Jaffe, R.G. Rhodes and W.J.A. Vandenheuvel, Drug Metab. Dispos., 2 (1974) 9.
5 J. Seki, Y. Arakawa, K. Imai, Z. Tamura, S. Yoshiue, Y. Mizuno, K. Yamada, M. Matsue and H. Narabayashi, Chem. Pharm. Bull., 29 (1981) 789.
6 P.T. Kissinger, C.S. Bruntlett and R.E. Shoup, Life Sci., 28 (1981) 455.
7 V.D. Gupta and A. Gupta, J. Pharm. Sci., 69 (1980) 1145.
8 S.T. Hamid and J. Walker, Anal. Chim. Acta, 105 (1979) 403.
9 P.A. Asmus and C.T. Freed, J. Chromatogr., 169 (1979) 303.
10 A.H. Anton and D.F. Sayre, J. Pharmacol. Exp. Ther., 138 (1962) 360.

Journal of Chromatography, 231 (1982) 463—466
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1328

Note

Vancomycin quantitation by high-performance liquid chromatography in human serum

JOHN BRUCE LUNDY McCLAIN*

Infectious Disease Service, Walter Reed Army Medical Center, Washington, DC 20012 (U.S.A.)

RODOLPHO BONGIOVANNI

General Support Laboratory, Clinical Investigation Department, Walter Reed Army Medical Center, Washington, DC 20012 (U.S.A.)

and

SCOTT BROWN

Clinical Microbiology Service, Department of Pathology, Walter Reed Army Medical Center, Washington, DC 20012 (U.S.A.)

(First received January 25th, 1982; revised manuscript received April 20th, 1982)

Since its introduction, vancomycin has found broad application in therapy of gram-positive infections where resistance or allergy has precluded the use of penicillin [1, 2]. Administration of vancomycin is complicated by renal insufficiency since drug is only excreted via glomerular filtration and accumulates to high levels if the dose is not reduced [3]. When the glomerular filtration rate is rapidly changing as in a seriously ill patient with hemodynamic instability, prediction of therapeutic levels is difficult and monitoring of drug levels must be done to prevent subtherapeutic or toxic levels. The major adverse effect of vancomycin is ototoxicity which has been reported when blood levels have reached the 80 μl/ml range [4]. Currently, therapeutic monitoring of vancomycin levels is done using a microbiologic assay which takes from 24 to 48 h. We developed a rapid method of vancomycin assay which would be useful in the therapeutic range needed for most infections which does not require vancomycin extraction or internal standards.

METHODS

All reagents were of analytical grade and were commercially available. Vancomycin standard 1150 mg of activity per g was a gift of Eli Lilly Research Corp. (Indianapolis, IN, U.S.A.). Vancomycin measurements were determined relative to potency. Water was deionized and filtered. Serum used in assay was from a common donor. Other antibiotic specimens were obtained from commercial sources of antibiotic standards. Chromatography was performed on a Waters Associates Model 6000 solvent pump, Model 440 ultraviolet detector at a wavelength setting of 280 nm, sensitivity of 0.005 a.u.f.s., and a flow-rate of 2 ml/min. We found that vancomycin gave maximal absorption at 280 nm. A Waters reversed-phase μBondapak C_{18} column (30 cm × 3.9 mm I.D., 10 μm particle size) was used with a mobile phase of 12% acetonitrile and 88% 0.01 *M* 1-heptane sulfonic acid (PIC-B7, Waters). Under isocratic conditions quantitation was based on peak height and standardized against a sample with known amount of vancomycin by activity.

Sample preparation

A 500-μl volume of serum was combined with 1 ml of cold trichloroacetic acid (TCA) and mixed in a vortex mixer. The mixture was then spun at 2500 *g* for 15 min in a refrigerated Sorvall DPR 6000 centrifuge. The supernatant was removed to screw-top test tubes. Then 4 ml of diethyl ether were added, mixed, and the organic supernatant was decanted removing the TCA; 100 μl of the aqueous layer were injected on the column.

Microbiologic assay was done using standard methods employing *Bacillus subtilis* ATCC 6633 obtained commercially [5]. Clinical specimens were bioassayed using the Microbiology Laboratory's vancomycin standards.

Initial studies were performed with aqueous and serum specimens with known amounts of the same vancomycin standard added. The samples were split and run simultaneously by microbiologic and high-performance liquid chromatographic (HPLC) methods. Clinical specimens were frozen after bioassay and chromatographed together. Preliminary work indicated no loss of vancomycin activity over a four-week interval in frozen specimens.

RESULTS

Fig. 1 demonstrates the HPLC peaks after TCA precipitation for serum, aqueous vancomycin and vancomycin in serum. Retention time for vancomycin is 16 min. Total sample analysis time is 20 min.

We found vancomycin to be in the aqueous layer of the ether extraction only. No vancomycin was detected in the ether fraction even when levels of 128 μg/ml were tested, which is far above the levels used clinically.

Fig. 2 shows correlation of peak heights before and after precipitation with TCA in aqueous and serum samples. As one may see, the activity loss in aqueous specimen is the same as activity loss in serum specimens; 87% of activity is recovered in the precipitated aqueous and serum samples compared to unprecipitated samples.

Fig. 3 demonstrates comparison between microbiologic assay and HPLC

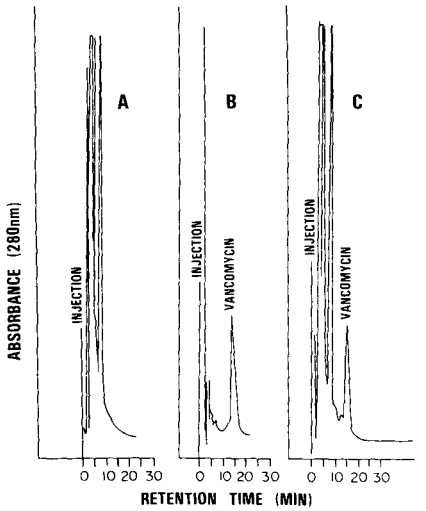


Fig. 1. (A) Chromatogram of blank serum treated with TCA. (B) Chromatogram of an aqueous solution of vancomycin (25 μg/ml) treated with TCA. (C) Chromatogram of serum spiked with vancomycin (25 μg/ml) and treated with TCA. Ultraviolet detection at 280 nm, sensitivity 0.005 a.u.f.s.

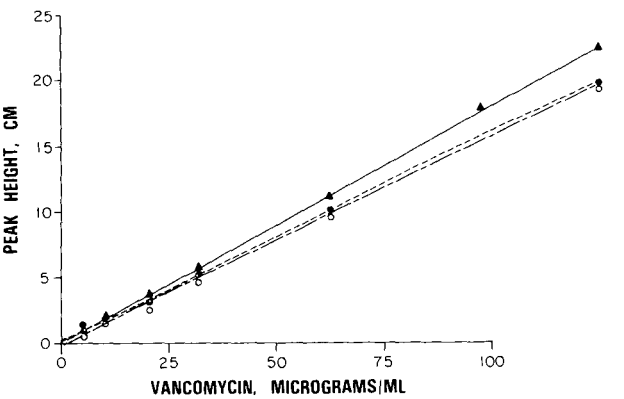


Fig. 2. Correlation of peak heights before and after precipitation with TCA in aqueous and serum samples. ▲, Aqueous vancomycin, $y = 0.18x + 0.03$, correlation coefficient $r = 1.00$; •, TCA-precipitated aqueous vancomycin, $y = 0.16x + 0.20$, $r = 1.00$; ○, TCA-precipitated vancomycin-spiked serum, $y = 0.16x + 0.11$, $r = 1.00$. Conditions: 100 μl injected on column; ultraviolet detection at 280 nm, sensitivity 0.005 a.u.f.s.

methods when the same vancomycin standard was used in each method. Standard deviation of the assay at 5 μg/ml was ± 0.25 μg.

Fifteen clinical specimens from patients who had vancomycin levels determined by bioassay as part of vancomycin therapy were run by HPLC. Our method showed a correlation coefficient $r = 0.87$. Different lots of vancomycin standard were used by the Microbiology Laboratory and the HPLC Laboratory in these clinical comparisons.

The following drugs were found not to interfere with the assay: penicillin G, nafcillin, ampicillin, cephalothin, amphotericin, amikacin, tobramycin, gentamicin, and tetracycline.

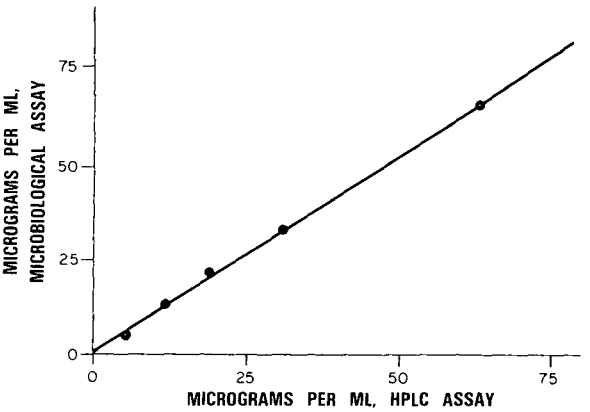


Fig. 3. Comparison between the present method and a microbiological assay for a series of spiked serum samples. $y = 1.05x + 0.64$, $r = 1.00$.

DISCUSSION

Microbiologic assay is the current standard of vancomycin determination. HPLC offers a rapid and accurate alternative vancomycin assay. The advantage of our method is its simplicity, requiring no extractions, internal standards or derivatization and its usefulness in the range of drug concentrations that vancomycin normally achieves in serum. The previously reported method of Uhl and Anhalt [6] differs from our method in several aspects: the different composition of the mobile phase, the use of cold TCA in a refrigerated centrifuge and the absence of an extraction step. The use of cold TCA may be responsible for the increased recovery of vancomycin in our method. Our assay is not useful in as low a range as the method of Uhl and Anhalt, but we decided levels below 5 μg/ml would not be clinically important to measure. Vancomycin is not altered in the body to any extent and, since the body's only method of excretion is via glomerular filtration, renal failure makes the blood levels of the drug very difficult to estimate even with a nomogram. There is no safety factor as with aminoglycosides or penicillin where the drug is altered in the body giving one some cushion in dose determination. Therapeutic monitoring with short turn-around times may greatly improve the quality of patient care in patients treated with vancomycin.

REFERENCES

1 F.W. Cook and W.E. Farrar, Ann. Intern. Med., 88 (1978) 813.
2 E.W. Hook and W.D. Johnson, Amer. J. Med., 65 (1978) 411.
3 H.E. Nielsen, H.E. Hansen, B. Korsager and P.E. Skov, Acta Med. Scand., 197 (1975) 261.
4 J.E. Gervaci, F.R. Heilman, O.R. Nichols and W.E. Wellman, Proc. Staff Meet., Mayo Clin., 33 (1958) 172.
5 E.H. Lennette, A. Batows, W.J. Hausler and J.P. Truant, Manual of Clinical Microbiology, American Society for Microbiology, Washington, DC, 3rd ed., 1980, p. 487.
6 J.R. Uhl and J.P. Anhalt, Ther. Drug. Monit., 1 (1979) 75.

Journal of Chromatography, 231 (1982) 467–472
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1318

Note

High-performance liquid chromatography of Ro 10-9359 (Tigason) and its metabolite Ro 10-1670 in human plasma

JEAN-GUY BESNER*, SYLVAIN MELOCHE, ROBERT LECLAIRE, PIERRE BAND and SYLVIE MAILHOT

Faculty of Pharmacy, University of Montreal, P.O. Box 6128, Montreal, Quebec H3C 3J7 (Canada)

(First received December 29th, 1981; revised manuscript received March 30th, 1982)

Retinoids, analogues of vitamin A, have prophylactic and therapeutic activities in various types of experimental cancers [1—3] and dermatologic disorders [4]. All-*trans*-retinoic acid (tretinoin) is currenctly used in dermatology in topical preparations only due to its systemic toxicity [5]. Its isomer, 13-*cis*-retinoic acid is less toxic and is used orally with success in chemoprevention of lung and bladder carcinogenesis [6, 7] and in various dermatosis [8—10]. The Ro 10-9359 (Tigason), an aromatic retinoid, has a better therapeutic index than all-*trans*-retinoic acid and is experimentally used in lamellar ichtoyosis [11], epidermolytic hyperkeratosis [11], erythrokeratoderma [11] and psoriasis [12—14].

This report describes a sensitive and selective high-performance liquid chromatography (HPLC) analytical procedure for Ro 10-9359, its metabolite Ro 10-1670, and endogenous retinol in plasma, using all-*trans*-retinyl acetate as the internal standard (Fig. 1).

MATERIALS AND METHODS

All manipulations were carried out in the dark allowing only slight indirect light, to prevent photoisomerisation reactions.

Reagents

All solid chemicals used were of reagent grade. Solvents used for extraction and chromatography were of chromatoquality (99%).

0378-4347/82/0000—0000/$02.75

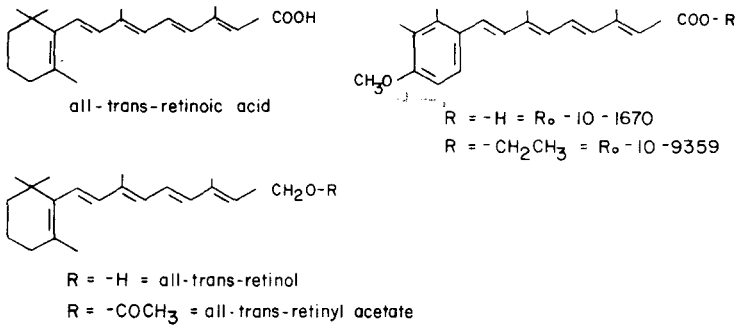


Fig. 1. Chemical structures of assayed retinoids.

Columns

The columns used were 250 mm × 4.6 mm I.D. stainless steel containing a 5-μm Spherisorb ODS reversed-phase packing.

Instruments

A dual-pump Perkin-Elmer, Model 601 high-performance liquid chromatograph was used, equipped with a Rheodyne injector permitting injection of volumes between 1 and 175 μl. The detection was carried out with a variable-wavelength UV—VIS spectrophotometer set at 350 nm, and the recorder was set at 1 mV. Peak areas were calculated with a Varian Model 485 digital integrator equipped with filtering and baseline tracking devices.

Analytical standards

All-*trans*-retinoic acid, all-*trans*-retinol and all-*trans*-retinyl acetate were purchased from Eastman Kodak (Rochester, NY, U.S.A.) and Ro 10-9359 and Ro 10-1670 were obtained from Hoffmann-La Roche (Vaudreuil, Canada). All these substances have a purity greater than 99%. Stock solutions of all retinoids were prepared by diluting 10 mg of pure substance in 100 ml of methanol. These solutions were stable for at least four months if stored at −30°C in the dark.

Mobile phase

The mobile phase was an acetonitrile—water (76:24) mixture containing 1% of ammonium acetate. The flow-rate was 1.5 ml/min, keeping the pressure between 80—95 bar which is well below the critical pressure of 205 bar.

Buffer

A 1 *M* phosphate buffer was prepared by adding 68.5 ml of an 85% phosphoric acid solution to 600 ml of water; the pH was adjusted to 6.00 with 10 *N* sodium hydroxide solution, and the volume completed to 1 l.

Procedure

In 15-ml PTFE-screw-cap tubes containing 1 ml of plasma, 1 μg of retinyl acetate as internal standard was added and the plasma was vortexed for 10 sec; then, 2 ml of buffer were added followed by 3 ml of extracting solvent

containing a mixture of diethyl ether—ethyl acetate (75:25), and the tubes were agitated for 10 min with a reciprocal movement agitator and centrifuged at 800 *g* for 5 min. The organic layer was removed and the extraction procedure repeated on the residual plasma fraction with fresh solvent. The two organic fractions were then combined, and evaporated in the dark under dry nitrogen on a hot plate set at 25°C. The dry residue was dissolved in 200 μl of extracting solvent and a 20-μl aliquot was injected for the assay.

The calibration curves were prepared by adding known amounts (Table I) of retinoids to 1 ml of plasma containing 1 μg of all-*trans*-retinyl acetate. Three spiked samples were assayed for each concentration and peak area ratios were used for the quantification of retinoids.

TABLE I

PEAK AREA RATIOS OF PLASMA Ro 10-1670, Ro 10-9359 AND ALL-*trans*-RETINOL VERSUS INTERNAL STANDARD OBTAINED FOR THE CALIBARATION CURVES

Amount of retinoids added to plasma (ng/ml)	Ratio (mean value ± S.D.)		Amount of retinol added to plasma (ng/ml)	Ratio (mean value ± S.D.) all-*trans*-retinol/ internal standard
	Ro 10-9359/ internal standard	Ro 10-1670/ internal standard		
100	0.22 ± 0.01	0.30 ± 0.02	0	0.55* ± 0.02
250	0.46 ± 0.02	0.55 ± 0.01	125	0.73 ± 0.00
400	0.61 ± 0.03	0.78 ± 0.05	250	0.81 ± 0.05
500	0.95 ± 0.04	1.06 ± 0.06	400	1.03 ± 0.06
700	1.34 ± 0.08	1.77 ± 0.04	500	1.13 ± 0.02
800	1.44 ± 0.10	1.93 ± 0.12	600	1.17 ± 0.03
1000	1.98 ± 0.13	2.52 ± 0.02	1000	1.70 ± 0.08

*This value corresponds to endogenous plasma all-*trans*-retinol.

RESULTS AND DISCUSSION

The HPLC analytical procedure reported allows the separation and quantitative determination of Ro 10-9359, its principal metabolite, Ro 10-1670 [15] and of endogenous retinol, using their UV absorbance at 350 nm and by measuring the peak area ratios relative to retinyl acetate chosen as internal standard. The method is rapid and accurate with a sensitivity limit of 25 ng/ml for each compound. Mean values ± S.D. for the elaboration of standard curves are reported in Table I for the three retinoids. The coefficients of variation are less than 7% of the mean for each concentration of the three substances studied. Routine analysis performed on plasma spiked with different concentrations of standards, were also within the variations mentioned earlier. The regression coefficients (r^2) for the standard curves were higher than 0.98 for Ro 10-9359, Ro 10-1670 and retinol. The overall recovery from plasma determined by comparison of peak areas from plasma extracts with standards injected under the same conditions, is 90 ± 5% for each substance considered and is constant for each concentration analysed.

A typical chromatogram obtained from a plasma sample containing five different retinoids is shown in Fig. 2. The retention times for Ro 10-1670, retinol, all-*trans*-retinoic acid, Ro 10-9359 and retinyl acetate are 6, 8.2, 9.5, 11 and 13.5 min, respectively. Fig. 2 illustrates the net separation between all these retinoids. The peaks are well defined with no interfering substances, these being partly eliminated by the extraction procedure or eluted within 4 min. This method has been proved to be as accurate as the one published for the dosage of 13-*cis*-retinoic acid [16]. The method was applied to determine the plasmatic concentration of Ro 10-9359 and its metabolite Ro 10-1670 in a patient receiving a 25-mg capsule of Ro 10-9359 three times a day. Following 30 days of treatment, a blood sample was withdrawn immediately before the morning dose. Fig. 3 illustrates the chromatogram obtained after analysis of this blood sample. The plasmatic concentrations calculated are 176 ng/ml, 138 ng/ml and 400 ng/ml for Ro 10-9359, Ro 10-1670 and endogenous retinol, respectively.

To ensure the reproducibility of the results, some precautions must be taken. All technical manipulations must be performed in the dark (or at least in very indirect daylight) in order to avoid photoisomerisation reactions. The evaporation of the organic phase must be carried out at a temperature not exceeding 25°C to avoid thermal degradation of the retinoids [17]. Once the extraction procedure is completed, the chromatographic analysis must be

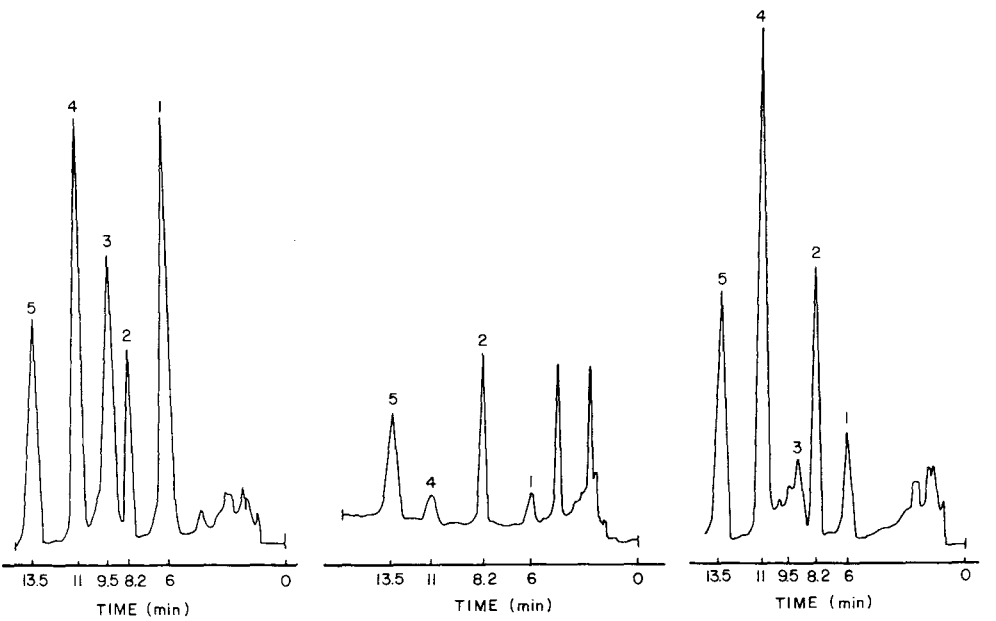


Fig. 2. Typical chromatogram of HPLC analysis of (1) Ro 10-1670, (2) retinol, (3) all-*trans*-retinoic acid, (4) Ro 10-9359 and (5) retinyl acetate (internal standard).

Fig. 3. HPLC chromatogram illustrating plasmatic concentrations from a patient of (1) Ro 10-1670, (2) retinol, (4) Ro 10-9359 and (5) retinol acetate (internal standard).

Fig. 4. Chromatogram illustrating evidence of the degradation of all-*trans*-retinoic acid (3) in plasma extract.

performed as quickly as possible. In the event that immediate HPLC analysis is impossible, the samples can be stored at −30°C in the dark for a maximum period of 24 h without significant degradation. The samples when redissolved are subject to rapid degradation, especially for Ro 10-9359, with a decrease in main peak area and occurrence of secondary peaks. This phenomenon has also been observed with all-*trans*-retinoic acid and is less striking than for Ro 10-1670. The method described in this paper differs from the assay reported by Hänni et al. [18]. Firstly, all-*trans*-retinyl acetate was chosen instead of retinoic acid as internal standard because of better stability and secondly the peak areas were evaluated instead of peak heights. We have discarded the possibility of using all-*trans*-retinoic acid due to its marked instability in our analytical conditions. The chromatogram shown in Fig. 4 illustrates the degradation of all-*trans*-retinoic acid, observed during the manipulations. The peak height is decreased and secondary peaks appear due to degradation of retinoic acid during the extraction procedure. The use of peak areas instead of peak heights insures a more precise and reproducible determination by circumventing column modification and peak broadening over a period of time. Furthermore, compared to an adsorption column, the results obtained with a reversed-phase system show a better reproducibility on a long term basis. All analytical methods should also consider endogenous retinol, even when its analysis is not the object of the exercise. The normal endogenous retinol level is between 300 and 700 ng/ml in man [19]. It has been demonstrated by Keilson et al. [20] that all-*trans*-retinoic acid affects the mobilisation of retinol. The same phenomenon could occur with other retinoids. Our method permits the separation of retinol from the retinoids studied, and thus permits its simultaneous determination. The procedure, rapid and practical, is particularly suitable for pharmacokinetic studies.

ACKNOWLEDGEMENT

The authors are grateful to Hoffmann-La Roche, Canada, for the generous supply of Ro 10-9359 and Ro 10-1670.

REFERENCES

1 E.W. Chu and R.A. Malmgren, Cancer Res., 25 (1965) 884.
2 U. Saffioti, R. Montesano, A.R. Sella Kumar and S.A. Borg, Cancer, 20 (1967) 857.
3 R.C. Moon, C.J. Grubbs and M.B. Sporn, Cancer Res., 36 (1976) 2626.
4 W. Bollag and A. Hanck, Acta Vitamin. Enzymol., 31 (1977) 113.
5 A.M.P. Janssen de Limpens, Brit. J. Dermatol., 103 (1980) 319.
6 C.D. Port, M.B. Sporn and D.G. Kaufman, Proc. Amer. Cancer Res., 16 (1975) 21.
7 P.J. Becci, H.J. Thompson, C.J. Grubbs, R.A. Squire, C.C. Brown, M.B. Sporn and R.C. Moon, Cancer Res., 38 (1978) 4463.
8 G.L. Peck and F.W. Yoder, Lancet, ii (1976) 1172.
9 G.L. Peck, T.G. Olsen, F.W. Yoder, J.S. Strauss, D.T. Downing, M. Pandya, D. Butkus and J. Armand-Battandier, N. Engl. J. Med., 300 (1979) 329.
10 M.L. Reed, J. Stanley, F. Stengel, J.L. Shupack and D.M. Benjamin, Arch. Dermatol., 115 (1979) 605.
11 L. Tamayo and R. Ruiz-Maldonado, Dermatologica, 161 (1980) 305.
12 B. Dahl, K. Mollenbach and F. Reymann, Dermatologica, 154 (1977) 261.
13 T. Frederiksson, Dermatologica, 157 (Suppl. 1) (1978) 13.

14 A. Lassus, Brit. J. Dermatol., 102 (1980) 195.
15 R. Hänni, Dermatologica, 157 (Suppl. 1) (1978) 5.
16 J.-G. Besner, R. Leclaire and P.R. Band, J. Chromatogr., 183 (1980) 346.
17 M.G. de Ruyter, W.E. Lambert and A.P. de Leenheer, Anal. Biochem., 98 (1979) 402.
18 R. Hänni, D. Hervouet and A. Busslinger, J. Chromatogr., 162 (1979) 615.
19 L.S. Goodman and A. Gilman, The Pharmacological Basis of Therapeutics, Macmillan, New York, 6th ed., 1980, p. 1589.
20 B. Keilson, B.A. Underwood and J.D. Loerch, J. Nutr., 109 (1979) 787.

Journal of Chromatography, 231 (1982) 473–477
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1325

Note

Determination of prenalterol in plasma by high-performance liquid chromatography with fluorescence detection

C.J. ODDIE, G.P. JACKMAN* and A. BOBIK

Clinical Research Unit, Alfred Hospital, Commercial Road, Prahran, Victoria 3181 (Australia)

(Received January 19th, 1982)

Prenalterol, S-(−)-(4-hydroxyphenoxy)-3-isopropylamino-2-propanol, is a relatively new beta-adrenoceptor agonist with positive cardiac inotropic effects in man [1] making it of possible use in the treatment of congestive heart failure [2–6] or in overcoming beta-blockade produced by oxprenolol or metoprolol [4–7]. Some pharmacokinetic information has been obtained using gas–liquid chromatographic (GLC) assays using electron-capture detection [8] or mass spectrometric detection [9]. However, these assays while being of satisfactory sensitivity for most purposes involve time-consuming extraction and derivatisation steps and have not been evaluated for use in patients who may be taking other medication. We describe a relatively simple, yet sensitive, assay for prenalterol using high-performance liquid chromatography (HPLC) which is relatively free of interference from many common cardiovascular drugs which may be administered in addition to prenalterol. The detection of prenalterol is by its endogenous fluorescence on low wavelength excitation, a technique which we have successfully employed to detect catecholamines, serotonin and a beta-adrenergic blocking drug, pindolol [10–12].

EXPERIMENTAL

Reagents

Prenalterol hydrochloride was obtained from Ciba-Geigy (Basle, Switzerland). Stock solutions (1 g/l) were prepared in 0.1 *M* hydrochloric acid containing 0.3 m*M* EDTA and stored at 4°C for up to one month. More dilute solutions were prepared fresh daily. Acetonitrile 190 nm HPLC grade was obtained from Waters Assoc. (Milford, MA, U.S.A.). Diethyl ether, analytical grade was

0378-4347/82/0000–0000/$02.75

washed with 1 *M* sodium hydroxide, 1 *M* hydrochloric acid and water before use. Water was redistilled from alkaline potassium permanganate before use. All other reagents were of analytical grade.

Chromatographic system

A 5000 series liquid chromatograph with universal loop injector (Varian, Palo Alto, CA, U.S.A.) was used with a Spherisorb 5 μm ODS reversed-phase column (250 × 4.6 mm I.D.) (Laboratory Data Control, Riviera Beach, FL, U.S.A.). The detector was a Schoeffel FS970 fluorimeter (Schoeffel, Westwood, NJ, U.S.A.) with a deuterium arc source. Maximum prenalterol fluorescence was found on excitation at 220 nm while emission was selected with a glass filter (bandpass 320—400 nm).

Plasma samples

Drug-free venous blood was obtained from healthy human subjects receiving no other medication, and from volunteers receiving prenalterol 2.5 mg intravenously as a 5-min infusion. Plasma used to test for possible interferences was obtained from out-patients receiving medication for cardiac disorders, but not taking prenalterol. Blood was collected into plastic tubes containing lithium heparin and centrifuged for 10 min at 1000 *g* in a refrigerated centrifuge. Plasma was separated and stored at −20°C in plastic tubes until assayed.

Extraction of prenalterol and HPLC estimation

Plasma (2 ml) in stoppered 25-ml glass tubes is extracted with diethyl ether (10 ml) by 2 min shaking after the addition of sodium chloride (200 mg) and 2 *M* sodium carbonate (200 μl). The phases are separated by brief centrifugation and the ethereal phase transferred to clean tubes containing 100 μl of 0.1 *M* phosphoric acid adjusted to pH 3.0 with sodium hydroxide into which prenalterol is extracted by 2 min shaking. The diethyl ether is aspirated after brief centrifugation. Phase separation is simplified at each step by freezing the aqueous phase in a dry ice—ethanol bath. Aliquots (50 μl) of the extract are injected directly onto the chromatographic column. The mobile phase was 0.01 *M* perchloric acid—acetonitrile (4:1) at a flow-rate of 2 ml/min. Calibration is by the assay of known amounts of prenalterol (1 to 40 ng/ml) added to control plasma. The mean calibration curve from nine assays was $y = 8.14$ (S.D. 1.50)$x + 0.77$ (S.D. 3.65) where y is peak fluorescence intensity in nA and x is plasma concentration in ng/ml.

RESULTS AND DISCUSSION

Prenalterol isolated from plasma chromatographed with a retention time of 5.8 min and was well resolved from peaks present in control plasma (Fig. 1). Recovery of prenalterol in nine assays, assessed by the injection of known amounts of prenalterol onto the chromatographic column, averaged 70 ± 5% S.D. The standard curve from these assays is linear and shows only slight variation between assay days. The linearity has been found to extend to at least 200 ng/ml. Precision of the assay was assessed by replicate assays of known standards added to control plasma (Table I). The coefficient of variation (C.V.)

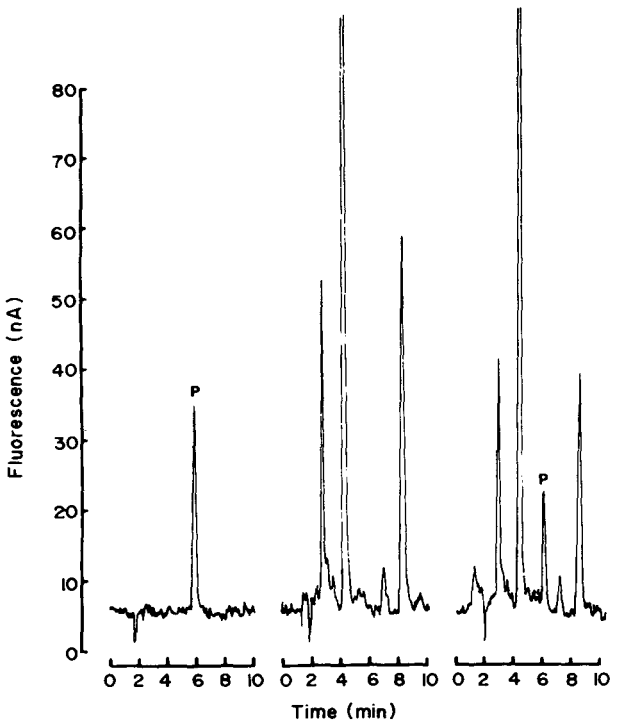


Fig. 1. Chromatographic traces. From left to right; 5 ng prenalterol standard (P) injected; extract of control plasma; extract of plasma containing 2.8 ng/ml prenalterol (P).

TABLE I

PRECISION OF THE ASSAY

Prenalterol (ng/ml)		No. of assays	C.V. (%)
Added	Found ± S.D.		
1	1.1 ± 0.16	4	15
2	2.1 ± 0.23	5	11
5	5.0 ± 0.22	6	4
10	10.1 ± 0.40	6	4
25	26.8 ± 2.1	6	8
100	110.5 ± 6.3	6	6

of 15% at 1 ng/ml is an improvement over the GLC assays, C.V. 20% at 5 ng/ml with electron-capture detection [8] and 14% at 2.5 ng/ml with mass spectrometry [9].

Selectivity was assessed by analysing plasma from patients likely to be taking a variety of cardiovascular drugs (see *Plasma samples*). Drugs known to have been prescribed in this group were methyldopa, amiloride, chlorothiazide, cyclopenthiazide, diazepam, digoxin, frusemide, glycerol trinitrate, metoclopramide, naproxen, pindolol, quinidine, sorbide nitrate and thyroxine. Interfering peaks were observed in only one sample from a patient taking nitra-

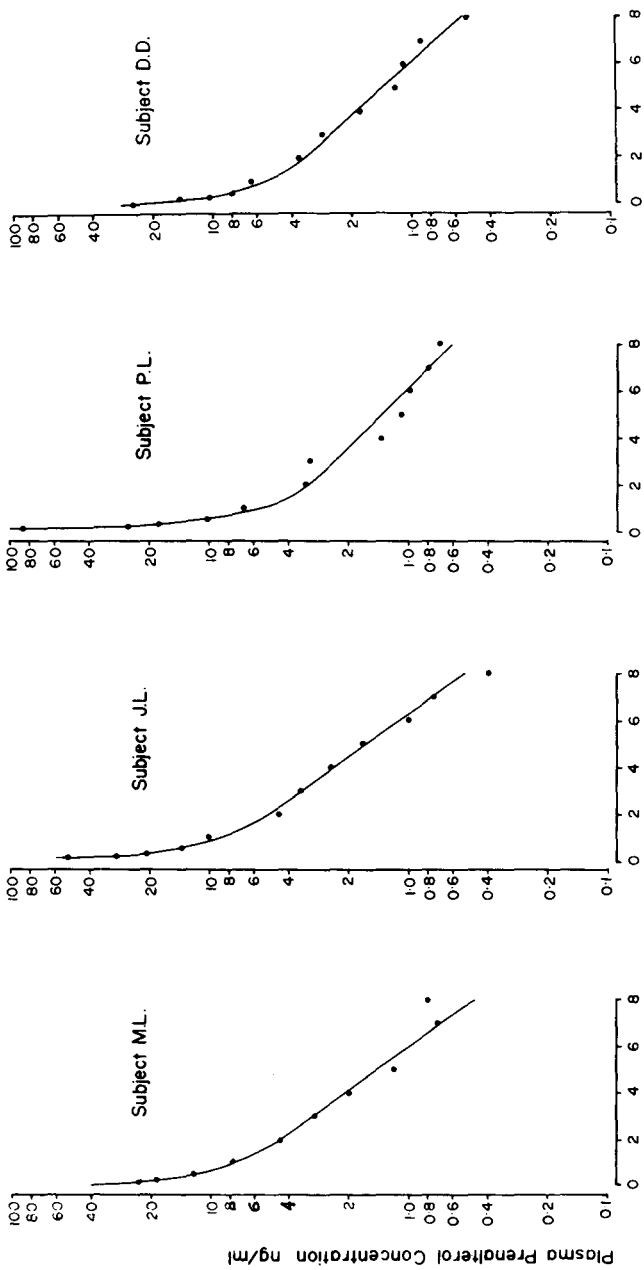


Fig. 2. Plasma levels of prenalterol following 2.5 mg given as a 5-min intravenous infusion, in four normal subjects.

zepam. The assay was used initially to determine the plasma concentrations of prenalterol in four normal subjects administered 2.5 mg intravenously. The plasma concentration—time curves in these subjects (Fig. 2) are similar to those previously reported using GLC assays [3, 6, 7, 9].

In summary, the assay described has the advantage over the GLC assays of employing a simpler extraction procedure and improved sensitivity without derivatization. While internal standardisation may be included in the assay as with the GLC assay [8] it is not necessary and may lead to drug interference. The relative freedom from interference by commonly used cardiovascular drugs makes the assay suitable for patient monitoring.

REFERENCES

1 E. Carlsson, C.G. Dahlöf and A. Hedberg, Naunyn Schmiedebergs Arch. Pharmacol., 300 (1977) 101—105.
2 G. Johnsson, L. Jordö, P. Lundberg, O. Rönn, I. Welin-Fogelberg and J. Wikstrand, Eur. J. Clin. Pharmacol., 13 (1978) 163—170.
3 O. Rönn, E. Fellenius, C. Graffner, G. Johnsson, P. Lundberg and L. Svensson, Eur. J. Clin. Pharmacol., 17 (1980) 81—86.
4 T. Lysbo Svendsen, O.J. Hartling and J. Trap-Jenssen, Eur. J. Clin. Pharmacol., 18 (1980) 219—223.
5 A. Weiss, B. Pfister, P. Imhof, P.H. Degen, D. Burckhardt and U.C. Dubach, Eur. J. Clin. Pharmacol., 18 (1980) 383—390.
6 D.H.T. Scott, G.R. Arthur, R.N. Boyes and D.B. Scott, Brit. J. Clin. Pharmacol., 7 (1979) 365—370.
7 O. Rönn, C. Graffner, G. Johnsson, L. Jordö, P. Lundberg and J. Wikstrand, Eur. J. Clin. Pharmacol., 15 (1979) 9—13.
8 P.H. Degen and M. Ervik, J. Chromatogr., 222 (1981) 437—444.
9 C. Graffner, K.J. Hoffman, G. Johnsson, P. Lundberg and O. Rönn, Eur. J. Clin. Pharmacol., 20 (1981) 91—97.
10 M. Bangah, G. Jackman and A. Bobik, J. Chromatogr., 183 (1980) 255—259.
11 G.P. Jackman, V.J. Carson, A. Bobik and H. Skews, J. Chromatogr., 182 (1980) 277—284.
12 G.P. Jackman, Clin. Chem., 27 (1981) 1202—1204.

Journal of Chromatography, 231 (1982) 478—484
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1332

Note

Rapid and simple method for determination of lorcainide, a new antiarrhythmic drug, and its major metabolite, norlorcainide, by high-performance liquid chromatography

VERNE SIMON and PITAMBAR SOMANI*

Division of Clinical Pharmacology, Departments of Pharmacology and Medicine, Medical College of Ohio, C.S. 10008, Toledo, OH 43699 (U.S.A.)

(First received January 29th, 1982; revised manuscript received April 1st, 1982)

Lorcainide is a new antiarrhythmic drug [1, 2] which is being clinically evaluated for the management of ventricular dysrhythmias in this country [3]. Since antiarrhythmic drugs in general have a relatively narrow therapeutic index, it is quite important to measure their plasma concentrations and this would also be the case for lorcainide in patients receiving this new drug. It is also important to determine whether or not one or more metabolites are present in the blood of such patients. Lorcainide and its major metabolite, norlorcainide, have been identified and quantitated by gas chromatography [4, 5]. Yee and Kates [6] recently described a high-performance liquid chromatographic (HPLC) method to determine these drugs; however, the extraction efficiency from the plasma in their method was only 38% for lorcainide and 41% for norlorcainide. Their procedure also involved detection at 196 nm, which is not possible with any HPLC system equipped with the more common fixed UV detector at 254 nm. We report an improvement in the HPLC method for rapid determination of plasma lorcainide and norlorcainide which would make therapeutic drug level monitoring more practical.

EXPERIMENTAL

Instrument and chromatographic conditions

All HPLC components were manufactured by Waters Associates and included: 6000A solvent delivery system, U6K injector, and Model 440 absorbance detector with wavelength fixed at 254 nm. The wave forms were recorded on a Houston OmniScribe Model B 5217-1 strip chart recorder. The column was Waters μBondapak phenyl reversed-phase, 30 cm × 3.9 mm

0378-4347/82/0000—0000/$02.75

I.D., with particle size of 10 μm. The flow-rate was 1 ml/min which produced a precolumn pressure of 68.95 bars. Detection was at 254 nm with sensitivity at the highest setting, 0.005. The chart speed was 0.5 cm/min.

Chemicals and reagents

Lorcainide hydrochloride {N-(4-chlorophenyl)-N-[1-(1-isopropyl)-4-piperidinyl] benzeneacetamide hydrochloride} and norlorcainide [N-(4-chlorophenyl)-N-(4-piperidinyl)benzeneacetamide], as the free base, were provided by Janssen Pharmaceutical (New Brunswick, NJ, U.S.A.). D-600 or gallopamil hydrochloride, {α-isopropyl-α-[(N-methyl-N-homoveratryl)-α-aminopropyl]-3, 4,5-trimethoxyphenylacetonitrile hydrochloride}, the internal standard, was obtained from Knoll (Ludwigshafen, G.F.R.). Acetonitrile and *n*-pentane (HPLC grade) were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Isopropanol (spectrophotometric grade) was obtained from Mallinckrodt (St. Louis, MO, U.S.A.); all other chemicals were reagent grade.

Mobile phase preparation

For 2 l of mobile phase, 5.44 g of potassium dihydrogen phosphate were dissolved in ca. 1000 ml of distilled water in a 2-l beaker; 750 ml of acetonitrile were added and the pH was adjusted to 2.3 using 42% phosphoric acid. The mobile phase was then transferred to a 2-l volumetric flask and distilled water was added to the 2-l mark. A type FH 0.5-μm Millipore filter was wetted by filtration of a few milliliters of acetonitrile and the mobile phase was then filtered, discarding the first small portion.

Extraction procedure

Method I. Glass tubes with PTFE-lined screw caps were rinsed with *n*-pentane. A small amount (1—3 ml) of either control or patient's plasma was added along with D-600 (1.6 μg). The tubes were gently vortexed (1 sec). A solution containing 2 *M* sodium hydroxide and 4 *M* sodium chloride was added (200 μl) along with *n*-pentane (5 ml) and isopropanol (200 μl). The tubes were placed on a nearly horizontal wheel and rotated at 40 rpm for 20 min followed by bench-top centrifugation for 10 min. The plasma layer was frozen by immersion in dry ice—ethanol and the organic layer decanted into conical centrifuge tubes and evaporated to dryness in warm water (initial temperature 45°C, final temperature 80°C). To the cooled tubes, 0.8 *M* phosphoric acid (150 μl) and *n*-pentane (750 μl) were added. After vigorous vortexing (90 sec) the aqueous layer was frozen in dry ice—ethanol, and the organic layer was removed by aspiration. The pH of the aqueous layer was adjusted to 6.6 by addition of 3 *M* sodium hydroxide (50 μl). The solution was filtered through a 0.2 μm polytetrafluoroethylene microfilter (Anspec, Ann Arbor, MI, U.S.A.), and a 100—180 μl portion of the solution was injected into the HPLC system.

Method II. Extraction method II was quite similar to method I except that the *n*-pentane—isopropanol layer (5 ml) was extracted directly with 0.8 *M* phosphoric acid (150 μl) without evaporation. The aqueous layer was treated as in method I except that filtration was not necessary.

Preparation of calibration standards

Aqueous solutions containing equal amounts of 0.025 or 0.005 μg/μl lorcainide hydrochloride and norlorcainide (free base) were used in establishing standard curves. The solutions contained $2.4 \cdot 10^{-4}$ and $4.9 \cdot 10^{-5}$ *M* phosphoric acid, respectively, as an aid in solubilization of the norlorcainide free base. The D-600 solution used contained 0.04 μg/μl aqueous D-600 hydrochloride. Throughout this paper the concentration of norlorcainide refers to the free base while that of lorcainide and D-600 refers to the hydrochloride salt.

RESULTS AND DISCUSSION

The retention times for norlorcainide, lorcainide and D-600 on the μBondapak phenyl column were 7.0, 9.8 and 13.2 min, respectively. The retention times were strongly dependent on solvent composition and pH (see also Yee and Kates [6]). The chromatogram of plasma of a patient being treated with oral lorcainide is shown in Fig. 1 and it may be seen that the three drugs

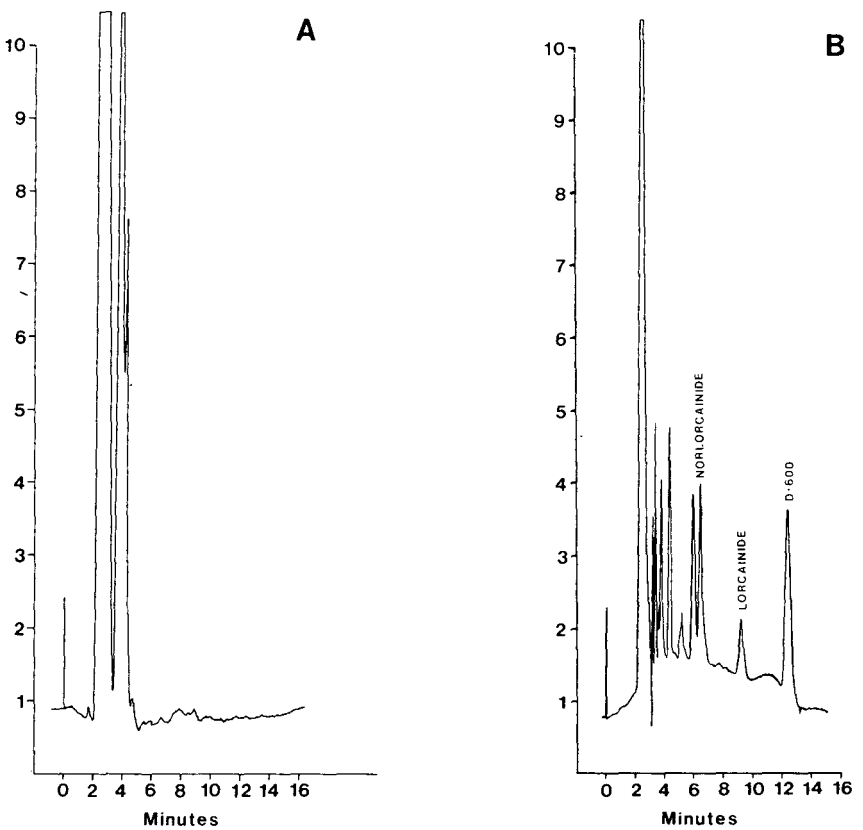


Fig. 1. Chromatogram showing the blank plasma (A) and the peaks for norlorcainide, lorcainide and the internal standard, D-600, in the plasma of a 72-year-old white male patient being treated with 300 mg/day oral lorcainide (B). The extract was injected into the chromatograph at time 0 and the retention time in minutes is indicated along the horizontal axis.

can be easily identified in this patient. The standard curves of lorcainide and norlorcainide were established by adding the two drugs to the plasma from healthy volunteers in concentrations from 0.06 μg/ml to 0.9 μg/ml, followed by the extraction procedure as described above by both methods I and II. Peak height ratios of lorcainide or norlorcainide to D-600 (h_L/h_D or h_N/h_D) were determined. Linear regression, with predetermined zero y-intercept, of the drug concentration (y-axis) on peak height ratio (x-axis) yielded the following equations for method I

$$[N] = (1.117 \pm 0.018 \text{ S.D.})\, h_N/h_D \quad (n = 37) \tag{1}$$

$$[L] = (1.307 \pm 0.012 \text{ S.D.})\, h_L/h_D \quad (n = 37) \tag{2}$$

and for method II

$$[N] = (1.262 \pm 0.016 \text{ S.D.})\, h_N/h_D \quad (n = 33) \tag{3}$$

$$[L] = (1.319 \pm 0.012 \text{ S.D.})\, h_L/h_D \quad (n = 33) \tag{4}$$

where [N] = calculated plasma concentration of norlorcainide free base (μg/ml), and [L] = calculated plasma concentration of lorcainide hydrochloride (μg/ml). All four of the above equations are based on the peak height (h_D) of 1.6 μg D-600 per ml plasma added prior to the extraction procedure. The coefficients of the peak height ratios were proportional to the quantity of internal standard added.

In order to evaluate the assay results of the HPLC, eqns. 1—4 were used to generate calculated versus actual values for lorcainide and norlorcainide concentrations for both extraction methods. Linear regression analysis of calculated concentration on actual concentration was carried out in all four cases, and the results for method I are shown in Figs. 2 and 3. Excellent correlation between actual and measured concentration was found for both lorcainide and norlorcainide and, based upon these results, the four equations for calculated versus actual concentration are as follows:

Extraction method I

$$[N]_{\text{calcd.}} = 0.013 + 0.97\,[N]_{\text{actual}};\ r^2 = 0.954$$

$$[L]_{\text{calcd.}} = 0.003 + 0.99\,[L]_{\text{actual}};\ r^2 = 0.992$$

Extraction method II,

$$[N]_{\text{calcd.}} = 0.0022 + 0.96\,[N]_{\text{actual}};\ r^2 = 0.987$$

$$[L]_{\text{calcd.}} = 0.014 + 1.02\,[L]_{\text{actual}};\ r^2 = 0.994$$

Reproducibility of the procedure was evaluated by replicate analysis of five plasma samples containing equal concentrations of lorcainide and norlorcainide at each of three different concentrations. For extraction method I,

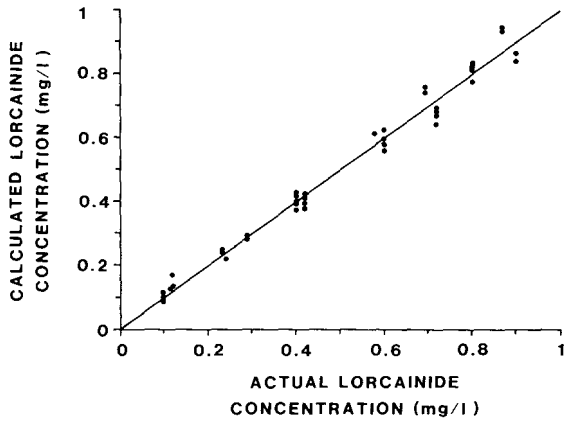


Fig. 2. Linear regression analysis of the actual versus calculated lorcainide concentration. The amount of lorcainide added to control plasma is shown along the horizontal axis and the calculated concentration from the peak height ratio (eqn. 2) is shown along the vertical axis. Note that an excellent correlation (r^2 = 0.992) exists for actual versus calculated concentration of the drug (y = 0.003 + 0.99x; $P < 0.001$).

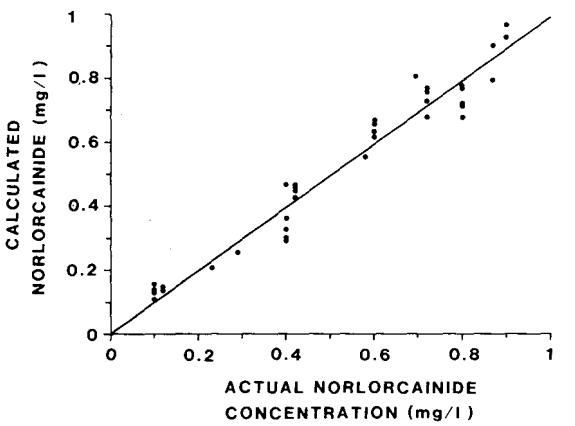


Fig. 3. Linear regression analysis of the actual versus calculated norlorcainide concentration. The amount of norlorcainide added to the control plasma is shown along the horizontal axis and the calculated concentration from the peak height ratio (eqn. 1) is shown along the vertical axis. Note that an excellent correlation (r^2 = 0.954) exists for actual versus calculated concentration of the drug (y = 0.013 + 0.97x; $P < 0.001$).

studies were done at concentrations of 0.1, 0.4 and 0.8 μg/ml and the coefficients of variation for the calculated concentrations were 23.2, 20.8 and 5.8%, respectively, for norlorcainide and 12.1, 4.9 and 2.5%, respectively, for lorcainide. Similar replicate studies were done at concentrations of 0.06, 0.3 and 0.9 μg/ml for extraction method II of the two drugs and coefficients of variation of 9.5, 5.0 and 5.2%, respectively, for norlorcainide and 3.4, 1.6 and 4.9%, respectively, for lorcainide were found. The results of the reproducibility studies are summarized in Table I. The poorer reproducibility observed for method I may be due to the injection of only some of the samples without prior filtration.

TABLE I

REPRODUCIBILITY OF LORCAINIDE/NORLORCAINIDE DETERMINATIONS IN HUMAN PLASMA

Method I, n = 37; method II, n = 33.

Concentration of lorcainide or norlorcainide (μg/ml)	C.V.* (%)			
	Norlorcainide		Lorcainide	
	Method I	Method II	Method I	Method II
0.06	—	9.5	—	3.4
0.1	23.2	—	12.1	—
0.3	—	5.0	—	1.6
0.4	20.8	—	4.9	—
0.8	5.8	—	2.5	—
0.9	—	5.2	—	4.9

*C.V. = coefficient of variation, as determined by the equation $\text{C.V.} = \frac{\text{S.D.}}{\text{mean}} \times 100$.

The extraction efficiency was determined by comparing the peak height after injection of the entire aqueous extract of the drug with the peak height for direct injection of the same quantity of the drug in a standard aqueous solution (Fig. 4). The recovery of lorcainide and norlorcainide was 75 ± 11% (n = 37) and 49 ± 11% (n = 37), respectively, for extraction method I, and

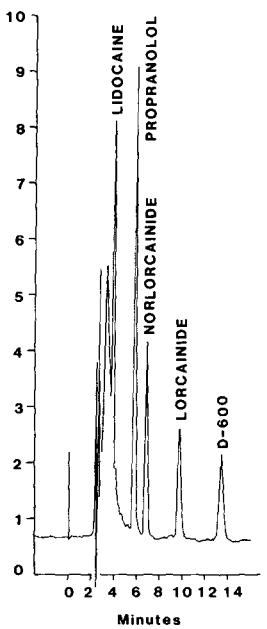


Fig. 4. Chromatogram of an aqueous solution containing a mixture of lidocaine, propranolol, norlorcainide, lorcainide and D-600. It may be noted that the peaks for each of the five drugs can be easily identified.

65 ± 5% (n = 33) and 43 ± 6% (n = 33), respectively, for extraction method II. Yee and Kates [6] reported extraction efficiency of 38 ± 0.6% and 41 ± 1% for lorcainide and norlorcainide, respectively, by their procedure using heptane—isoamyl alcohol.

Since with detection at 254 nm one must work at the highest sensitivity setting (0.005), a 2.0-fold (extraction method I) or 1.7-fold (extraction method II) increase in lorcainide recovery over the previously available method is an important advantage.

Extraction by method I permits daily assay of about 18 samples, while extraction by method II permits daily assay of about 24 samples. Because method II is faster without too great a loss in sensitivity, we now routinely use extraction method II for determination of lorcainide and norlorcainide in our patient population. If sample volume permits, the use of 2 or 3 ml of plasma is helpful. Concentrations as low as 0.008 μg/ml can be measured by our method, although the usual therapeutic concentrations of lorcainide and norlorcainide in patients receiving 200—400 mg per day of the parent drug range between 0.1 and 1 μg/ml for lorcainide and 0.1 and 1.5 μg/ml for norlorcainide, respectively. These results demonstrate that our procedure can be used easily in most clinical laboratories equipped with a standard HPLC system with detection at 254 nm for therapeutic drug monitoring of this new antiarrhythmic agent.

ACKNOWLEDGEMENT

We wish to thank Ms. Debra LeBarr for excellent secretarial help in preparation of this manuscript.

REFERENCES

1 H. Kesteloot and R. Stroobandt, Arch. Int. Pharmacodyn. Ther., 230 (1977) 255.
2 W.K. Amery, J.J.P. Heykants, R. Xhonneux, G. Towse, P. Oettel, D.A. Gough and P.A.J. Janssen, Acta Cardiol., 36 (1981) 207.
3 P. Somani, Amer. J. Cardiol., 48 (1981) 157.
4 U. Klotz, P. Müller-Seylitz and P. Heimburg, Clin. Pharmacokin., 3 (1979) 407.
5 R. Woestenborghs, M. Michiels and J. Heykants, J. Chromatogr., 164 (1979) 169.
6 Y.-G. Yee and R.E. Kates, J. Chromatogr., 223 (1981) 454.

Journal of Chromatography, 231 (1982) 485–491
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1335

Note

High-performance liquid chromatographic analysis of imidazopyrazole (NSC 51143) in serum

GARRY W. BOSWELL* and JORDAN L. COHEN*

Clinical Pharmacokinetic Laboratory, School of Pharmacy, University of Southern California, 1985 Zonal Avenue, Los Angeles, CA 90033 (U.S.A.)

(First received February 2nd, 1982; revised manuscript received April 30th, 1982)

Imidazopyrazole [2,3-dihydro-1H-imidazo(1,2-6)pyrazole, NSC 51143, IMPY] is an investigational antineoplastic agent which selectively inhibits DNA synthesis [1]. Pre-clinical studies have shown IMPY to have significant anti-tumor activity especially against LI210 leukemia cells, including those variants resistant to similar chemotherapeutic agents [2]. The suggested mechanism of action is inhibition of ribonucleotide reductase and in mice the drug showed the capacity to synchronize tumor, bone marrow and duodenal crypt cells in the S phase of the cell cycle [3]. Phase I clinical trials of this novel agent have been initiated in children and adults with initial doses of 150 mg/m^2 body surface area [4], and an obvious need to collect early pharmacologic disposition data exists.

A limited number of methods for determining IMPY in biological media have been preliminarily reported, including liquid scintillation of radiolabelled drug [5], radioimmunoassay [6] and electron-capture gas chromatography (GC) [7]. The former two methods require reagents not readily available and lack evidence of specificity, while the latter suffers from the lack of ruggedness generally associated with electron-capture detection when applied to analysis of biological specimens. We have recently reported a GC method employing nitrogen-specific detection [8] which has been used in support of phase I clinical and pharmacokinetic studies of IMPY in children. While this has

*Present address: Letterman Army Institute of Research, San Francisco, CA 94129, U.S.A.

0378-4347/82/0000—0000/$02.75

proved sensitive enough to support phase I clinical and pharmacokinetic studies of IMPY in children, the overall reliability and ruggedness of high-performance liquid chromatography (HPLC) has prompted development of the present method which has comparable sensitivity and improved reproducibility.

EXPERIMENTAL

Chemicals and reagents

IMPY was obtained from the National Cancer Institute (Bethesda, MD, U.S.A.) and used directly. The internal standard, 2,3-dihydro-1-benzoylimidazo(1,2-6)pyrazole (ϕ-IMPY), was synthesized using a modification of a known reaction [9] in which a two-fold molar excess of benzoyl chloride (Gold Label; Aldrich, Milwaukee, WI, U.S.A.) was added slowly to 10 ml of a solution of 500 mg of IMPY in 2.5 *N* sodium hydroxide solution in an ice bath. After standing for 10 min, this was heated at 50°C for 10 min then cooled, and the resulting precipitate was separated and recrystallized three times from methanol. The structure was confirmed by ultraviolet and infrared spectroscopy, nuclear magnetic resonance and GC—mass spectrometry. The m.p. was 165—166° C. The structures of IMPY, ϕ-IMPY and the pentafluorobenzoyl (PFB) derivative of IMPY are shown in Fig. 1. Acetonitrile was HPLC grade (Omnisolve; MCB, Cincinnati, OH, U.S.A.), all other chemicals and solvents were reagent grade. Distilled water was purified by passing it through a reverse-osmosis four-filter system (Millipore, Bedford, MA, U.S.A.). Stock standard solutions of IMPY were prepared in purified water at concentrations of 10—100 μg/ml. Internal standard solutions were prepared in methanol at a 100 μg/ml concentration. These were refrigerated at 4°C and found to be stable for several weeks.

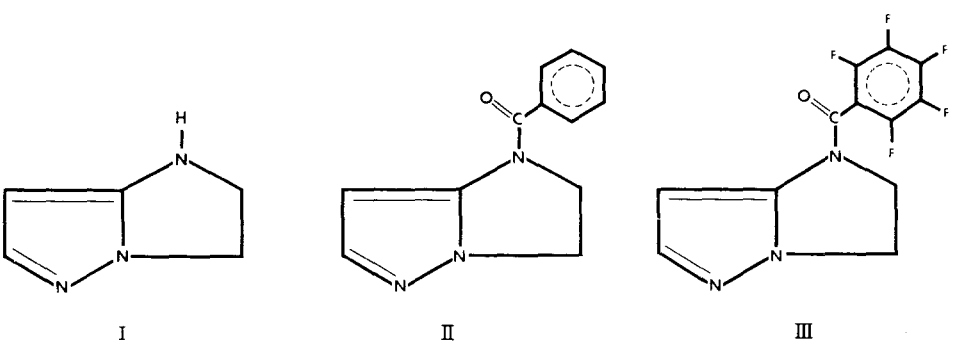


Fig. 1. Chemical structures of imidazole-pyrazole (IMPY, I), 2,3-dihydro-1-benzoylimidazo-(1,2-6)pyrazole (ϕ-IMPY, II), and the derivatized drug (PFB-IMPY, III).

Chromatographic conditions and instrumentation

A Waters Assoc. (Milford, MA, U.S.A.) Model 202 liquid chromatograph equipped with a Model U6K injector and a Model 440 UV detector was used

for the analyses. Chromatography was performed on a 25 cm × 4.6 mm I.D. stainless-steel RP-8, 5 μm, Ultrasphere (Altex, Berkeley, CA, U.S.A.) column with an MPLC 3-cm, 10 μm precolumn (Brownlee, Santa Clara, CA, U.S.A.). The mobile phase consisted of acetonitrile—water (45:55) at a flow-rate of 1.5 ml/min and a pressure of 71.4 bar. The separation was run at ambient temperature at a wavelength of 254 nm.

Extraction procedure

To 1.0 ml of a serum sample or standard in a 15-ml centrifuge tube were added 7 ml of dichloromethane, 50 μl of internal standard solution (5 μg of ϕ-IMPY), 2 g of sodium chloride and 1 ml of 1.0 *M* carbonate buffer, pH 10.5. The tube was mechanically shaken for 30 min, centrifuged at 850 *g* for 10 min and the organic layer was filtered through Whatman No. 1 paper into a clean tube containing 1.7 g of sodium sulphate. This was vortexed for 1 min, allowed to stand for 10 min and the organic phase transferred to a tube containing 5 μl of pentafluorobenzoyl chloride. This was heated at 50°C for 30 min and then 0.5 ml of methanol was added. The tube was again heated at 50°C for 15 min to react the excess acylating reagent and then evaporated to dryness under dry air at room temperature. The residue was redissolved in 2 ml of dichloromethane and shaken with 5 ml of 1.0 *M* carbonate buffer, pH 10.5. The organic layer was transferred to a 5-ml conical centrifuge tube, evaporated to dryness under dry air and reconstituted in 50 μl of methanol; 5—20 μl were injected into the liquid chromatograph.

Quantitation

Standard curves were generated over the range 0.1—20 μg/ml in serum and IMPY concentrations were determined by calculating peak-height ratios of drug to internal standard.

Mass spectrometric analysis

The structure of the derivatized IMPY was assessed using a gas chromatograph—mass spectrometer—computer system (Hewlett-Packard Model 5992B/9825A, Santa Clara, CA, U.S.A.). GC was performed using a coiled glass column (1.2 m × 2 mm I.D.) packed with 3% OV-101 on 100—120 mesh Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.) operated isothermally at 170°C with an injector temperature of 250°C. The instrument was equipped with a jet separator and used a 70-V electron-impact ionization source.

Rabbit pharmacokinetic studies

The procedure was used to analyze the in vivo disposition of IMPY administered intra-arterially at doses of 250—1000 mg/m^2 to New Zealand white rabbits. Serial blood samples were drawn from an indwelling ear artery canula up to 12 h after drug administration. The plasma was immediately separated from the cells and quick-frozen in a methanol—dry-ice bath (—68°C) until analyzed for IMPY.

RESULTS AND DISCUSSION

Typical chromatograms from a human serum blank and spiked human serum are shown in Fig. 2. Under the analytical conditions described, retention times for ϕ-IMPY and derivatized IMPY were 3.5 and 6.9 min, respectively. No significant interferences from extracted blank rabbit and human serum were observed. Peak shape was generally symmetrical and allowed calculation of IMPY concentrations from peak-height ratio measurement. Standard curves prepared from spiked human and/or rabbit serum were linear over the range 0.1—20 μg/ml (r = 0.997). Between-run reproducibility and recovery were examined over this working concentration range with typical coefficients of variation in the 3—7% range (see Table I). The practical limit of sensitivity from a 1.0-ml serum sample which produced a 3:1 signal-to-baseline-noise ratio was 80 ng/ml. This is within the same magnitude as that reported in a gas chromatographic electron-capture procedure [7] and in our own GC—nitrogen-

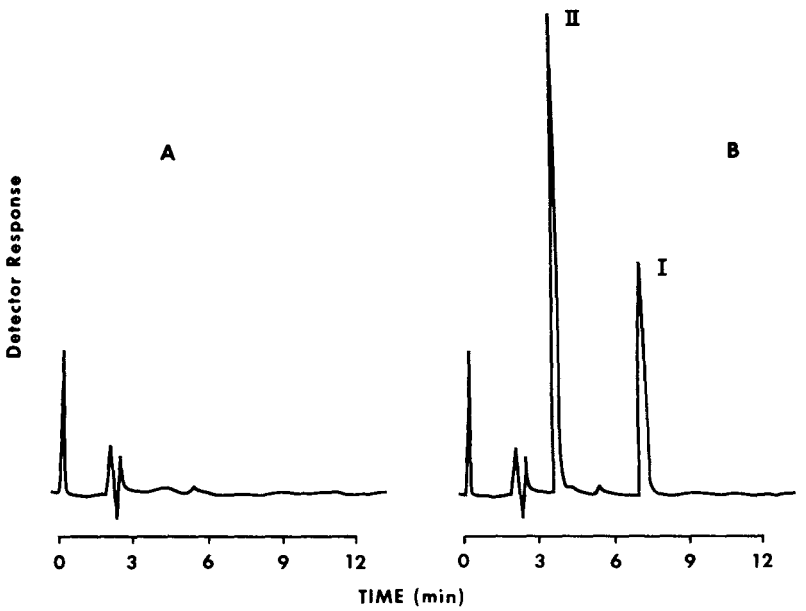


Fig. 2. High-performance liquid chromatograms of (A) extracted serum blank and (B) human serum standard spiked with 2.52 μg/ml IMPY and internal standard at an attenuation of 0.02 a.u.f.s.

TABLE I

RECOVERY AND BETWEEN-RUN VARIABILITY OF IMPY-SPIKED SERUM SAMPLES

IMPY added (μg/ml)	IMPY found (μg/ml)	n	S.D.	C.V.
0.10	0.113	4	0.0044	3.9
2.48	2.42	4	0.065	2.7
7.23	7.13	4	0.374	5.3
12.25	11.96	4	0.1026	0.9

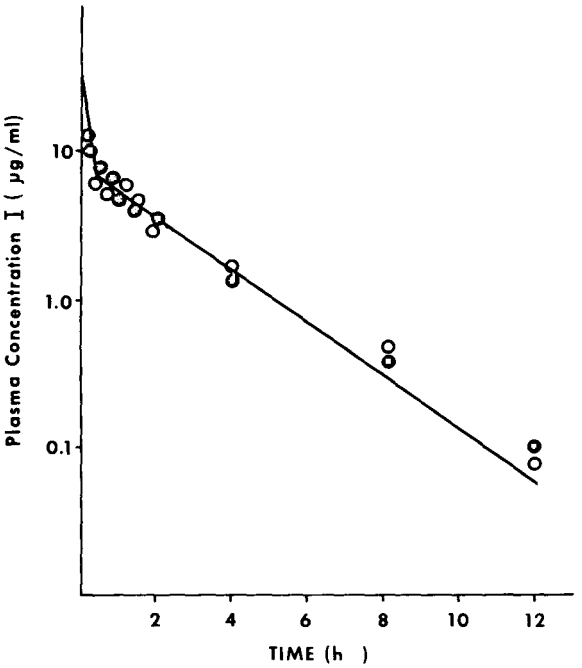


Fig. 3. Serum time course of IMPY in a rabbit receiving a 250 mg/m^2 intra-arterial bolus determined by GC analysis (○) and by HPLC (●) analysis.

detection procedure [8]. The entire procedure reported here required about 4 h and allows analysis of 20—25 specimens per analyst per working day.

The present procedure and the nitrogen-detection GC procedure [8] were compared by drawing and analyzing duplicate serum samples from a rabbit receiving 250 mg/m^2 IMPY as an intra-arterial bolus. The comparative results are seen in Fig. 3. The overall reliability of the HPLC procedure was better in our laboratory, probably due to the relative instability of the derivatized bromo-IMPY, which is used as the internal standard in the GC procedure. Between-run reproducibility was also substantially better in the present procedure.

The identity of the derivatized IMPY chromatography peak was confirmed by collecting the appropriate mobile phase fraction and, following solvent removal, subjecting the material reconstituted in methanol to GC—mass spectrometric analysis for comparison with authentic PFB-IMPY, which had been prepared in our laboratory. These were identical and demonstrated a parent peak at *m/e* 303 and a base peak at *m/e* 195, which corresponds to the pentafluorobenzoyl fragment. Other characteristic peaks occurred at *m/e* 167, 108 and 81, as seen in the proposed fragmentation pattern in Fig. 4.

The pharmacokinetic behavior of IMPY was determined in rabbits receiving intra-arterial doses ranging from 250 to 1000 mg/m^2. The serum time course was followed up to 12 h and was fitted to a two-compartment open model using the non-linear regression program AUTOAN/NONLIN [10]. The parameters derived for three animals each, at the 250 mg/m^2 and 1000 mg/m^2 dose,

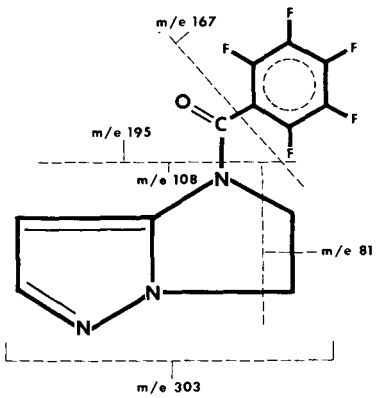


Fig. 4. Proposed mass fragmentation of 2,3-dihydro-1-pentafluorobenzoyl-imidazo(1,2-6)-pyrazole (III). See text for conditions.

TABLE II

PHARMACOKINETIC PARAMETERS FOR IMPY IN RABBITS FOLLOWING INTRA-ARTERIAL BOLUS DOSES

Three animals were used at each dose level.

Parameter*	1000 mg/m² dose (mean ± S.D.)	250 mg/m² dose (mean ± S.D.)
α (min^{-1})	0.105 ± 0.074	0.21 ± 0.14
β (min^{-1})	0.007 ± 0.002	0.008 ± 0.003
$t_{1/2\beta}$ (min)	109.8 ± 24.9	87.9 ± 23.0
k_{10} (min^{-1})	0.040 ± 0.05	0.134 ± 0.18
k_{12} (min^{-1})	0.043 ± 0.03	0.048 ± 0.052
k_{21} (min^{-1})	0.028 ± 0.011	0.026 ± 0.013
V_d (l/kg)	0.92 ± 0.98	0.233 ± 0.33
$V_{d_{ext}}$ (l/kg)	3.31 ± 1.92	2.27 ± 0.37
Dose (mg)	237 ± 26	50.2 ± 2.0

*Symbolism for pharmacokinetic parameters is that of AUTOAN [10].

are illustrated in Table II. There are at present no data from rabbit studies in the literature to allow comparison. There has been a report by Malspeis et al. [7] that IMPY, administered as an intravenous dose of 250 mg/m² to a single dog, demonstrated saturability in its kinetics and that typical log—linear disappearance was not observed until 2 h following the dose. The wide dose range selected here was designed, in part, to examine whether this could be demonstrated in rabbits. The data indicate widely varying parameter estimates between animals but do not suggest any non-linear kinetic behavior. More studies are needed in animals and humans to examine this issue.

In our previous paper describing the GC—nitrogen-detection analysis of IMPY, it was observed that the measured IMPY from a spiked serum sample declined significantly if allowed to stand unfrozen for a few hours [8]. This phenomenon was observed in the present procedure as well and all specimens should be rapidly frozen after collection to avoid the introduction of error caused by some unknown in vitro degradation of IMPY in serum.

REFERENCES

1 H.L. Ennis, L.H. Moller, J.J. Wang and O.S. Selawry, Biochem. Pharmacol., 20 (1971) 2639.
2 R.W. Brockman, S.C. Shaddix, J.W. Carpenter, N.F. BuBois and R.F. Struck, Proc. Amer. Assoc. Cancer Res., Abstract 81 (1978) 19.
3 A. Krishran and R. Ganapathi, Cancer Res., 40 (1980) 1103.
4 B. Yap, W. Murphy, M.A. Burgess, M. Valdivieso and G.P. Bodey, Cancer Treatm. Rep., 63 (1979) 1849.
5 L.M. Allen, Proc. Amer. Assoc. Cancer Res., Abstract 433 (1979) 107.
6 K.L.L. Fong, D.H.W. Ho, G.P. Bodey, B.S. Yap, R.S. Benjamin, N.S. Brown and E.J. Freireich, Proc. Amer. Assoc. Cancer Res., Abstract 826 (1979) 204.
7 L. Malspeis, J.J.V. DeSousa, A.E. Staubus and H.B. Bhat, Proc. Amer. Assoc. Cancer Res., Abstract 617 (1979) 153.
8 G.W. Boswell and J.L. Cohen, J. Pharm. Sci., (1982) in press.
9 S.B. Matin and M. Rowland, J. Pharm. Sci., 61 (1972) 1235.
10 A.J. Sedman and J.G. Wagner, AUTOAN — A Decision Making Pharmacokinetic Computer Program, Publication Distribution Service, Ann Arbor, MI, 1974.

Journal of Chromatography, 231 (1982) 492—496
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1334

Note

High-performance liquid chromatographic procedure for simultaneous determination of SKF 78729A and its N-acetyl metabolite

R.N. GUPTA*

Department of Pathology, St. Joseph's Hospital, Hamilton, Ontario L8N 1Y4 (Canada)

M.R. ACHONG

Department of Medicine, St. Joseph's Hospital, Hamilton, Ontario L8N 1Y4 (Canada)

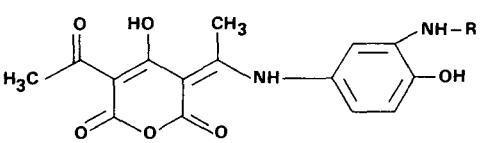
and

F. ENG

Department of Pathology, St. Joseph's Hospital, Hamilton, Ontario L8N 1Y4 (Canada)

(First received February 2nd, 1982; revised manuscript received March 29th, 1982)

SKF 78729A {5-acetyl-4-hydroxy-3-[1-[(3-amino-4-hydroxyphenyl)amino] ethylidene] 2H-pyran-2,6(3H)-dione hydrochloride} is a compound which can inhibit immediate-type allergic reactions in vitro and in animal models [1]. It is currently being tested in humans as an inhibitor of allergen-provoked bronchospasm. This paper describes a high-performance liquid chromatographic (HPLC) procedure for the simultaneous estimation of SKF 78729A and its N-acetyl metabolite (Fig. 1) in human plasma.



1. R=H
2. R=$COCH_3$
3. R=COC_2H_5

Fig. 1. Structural formulae: 1 = SKF 78729A; 2 = N-acetyl derivative of SKF 78729A; 3 = N-propionyl derivative of SKF 78729A.

MATERIALS AND METHODS

Reagents were of analytical grade and the solvents were distilled in glass by the supplier (Caledon Labs., Georgetown, Canada). Solutions of 1 mg/ml of each of SKF 78729A (1) and its N-acetyl metabolite (2) were prepared in 1% acetic acid in methanol. A solution of 0.1 mg/ml of the N-propionyl derivative of SKF 78729A (3) was prepared in 1% acetic acid in methanol. Compounds 1, 2 and 3 were obtained from Smith Kline and French Canada, Mississauga, Canada. These solutions were stored in dark bottles at 4°C. Plasma standards, 4 μg/ml, of 1 and 2 were prepared from drug-free plasma and appropriate volumes of solutions of 1 and 2. Plasma standards of 2, 1, 0.5 and 0.25 μg/ml, of compounds 1 and 2, were prepared by serial dilution. Plasma standards were divided into 1-ml aliquots and stored frozen. Working internal standard solution (2 μg/ml) was prepared when required by diluting stock solution of 3 1:50 with 1% aqueous acetic acid. After an oral dose of 500 mg of SKF 78729A had been given to healthy volunteers, blood was collected in heparinized evacuated blood collecting tubes. Plasma was collected in plastic test tubes and kept frozen until analyzed.

Sample preparation

Bond Elut® C_{18} disposable extraction columns of 1-ml capacity and a Vac Elut® system were purchased from Analytichem International (Harbor City, CA, U.S.A.). The columns were washed under suction once with 2% acetic acid, twice with methanol and finally with 2% acetic acid. Plasma (0.5 ml) and working internal standard solution (0.5 ml) were applied to the washed columns placed in the suction rack with the suction turned off. Pressure was applied to the columns with a stream of nitrogen so that the liquid passed through the column in 40—60 sec. The columns were washed twice with 0.2% acetic acid and the washings discarded. The columns were then eluted with 0.5 ml of methanol containing 2 μl per 100 ml mercaptoethanol. The eluate was collected in 75 × 12 mm disposable glass tubes and evaporated at room temperature with a current of nitrogen. The residue in each tube was dissolved in 50 μl of mobile phase and 20 μl were injected into the liquid chromatograph.

Chromatography

The assay was performed with an HPLC system consisting of Model 100 A pump, and an Hitachi Model 100-40 variable-wavelength absorption detector set at 330 nm (Altex, Berkeley, CA, U.S.A.). Injections were made by means of a Rheodyne Model 7125 syringe loading injector with a 20-μl loop (Rheodyne, Cotati, CA, U.S.A.). The chromatogram was recorded and the peaks were integrated with a Model 3390A integrator (Hewlett-Packard, Avondale, PA, U.S.A.).

A 10-μm PRP-1 (XAD resin), 15 cm × 4.1 mm I.D. (Hamilton, Reno, NV, U.S.A.) column was used. The mobile phase was prepared by mixing acetonitrile (700 ml) and glass-distilled water (1 l) containing 70% perchloric acid (0.5 ml) and 24% methanolic solution of tetramethylammonium hydroxide (0.5 ml). The pH of the mobile phase was 3.0. The flow-rate was 0.8 ml/min with a back pressure of 5.54 MPa (800 p.s.i.).

RESULTS AND DISCUSSION

SKF 78729A and its N-acetyl metabolite cannot be extracted efficiently into water-immiscible solvents such as chloroform or ethyl acetate. These compounds are isolated in yields of 70—90% by the procedure described in this report. The ratios of drug/internal standard and/or metabolite/internal standard remain constant in this recovery range. The recovery of drug is reduced drastically if the mixture of plasma and internal standard passes through the column in less than 30 sec. Extraction with acetonitrile and separation of organic layer by freezing the aqueous layer is less efficient than extraction by Bond-Elut columns.

Fig. 2B shows a chromatogram of the extract of drug-free plasma. There are virtually no peaks due to endogenous components of plasma. For convenience, blood is withdrawn from patients in commercially available evacuated blood-collecting tubes. The undeclared additives used in the manufacturing of those tubes affect trace drug analysis either by causing interference or by affecting the distribution of drug in cells and plasma water. It has been claimed that heparinized Venoject® brand tubes are suitable for drug analysis [2]. However, blood collected in these tubes produces additional peaks when analyzed for SKF 78729A by the described procedure. Blood collected in newly formulated blue-capped heparinized Vacutainer® brand tubes (Becton Dickinson, Orangeburg, NY, U.S.A.) does not produce any peak when analyzed for compound 1 by the present procedure. It has recently been claimed that drug concentrations are not significantly affected when blood is collected in these tubes [3].

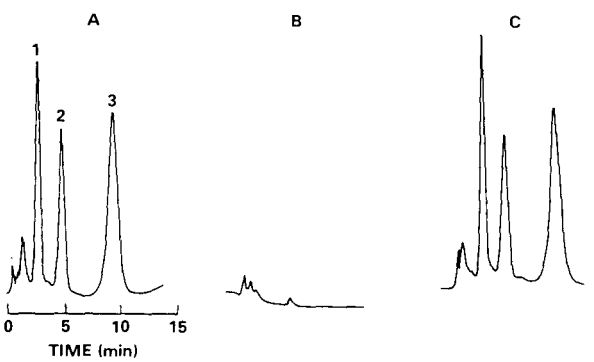


Fig. 2. Chromatograms (detector = 0.05 A full scale; plotter attenuation = 2; 4 mV) of: (A) a mixture of methanolic solutions of compounds 1, 2 and 3, (b) an extract of drug-free plasma, (c) an extract with added 1 and 2 (1 μg/ml). Peaks: 1 = SKF 78729A; 2 = metabolite; 3 = internal standard.

N-Acetylation of primary aromatic amino groups is a major inactivation step for many drugs [4]. However, formation of the N-acetyl derivative of SKF 78729A has not yet been unequivocally established in man. We have analyzed the extracts of plasma obtained from volunteers who had ingested oral doses of compound 1 with different columns (Ultrasphere-ODS, 5 μm, 25 cm × 4.6 mm I.D., and Ultracil-Octyl, 10 μm, 25 cm × 4.6 mm I.D.),

and different composition of mobile phase (acetonitrile—methanol—water and 2-ethylhexylphosphoric acid). In every case the retention time of the additional peak present in the volunteers specimen was identical with that of the N-acetyl derivative of the drug.

Fig. 2C shows a chromatogram of an extract of plasma standard. The peaks of drug, metabolite and internal standard are sharp and are well separated. The standard curve is linear for the range tested (0.25—4 μg/ml) for both the drug and its N-acetyl metabolite either by comparing the ratios of peak heights or by comparing the ratios of peak areas of the drug or of the metabolite and the internal standard. The calibration curve passes through the origin. Analysis of plasma supplemented with the drug and its metabolite showed a within-batch coefficient of variation (C.V.) of 6.5% for the drug (n = 10, mean = 1.8 μg/ml) and 7.6% for the metabolite (n = 10, mean = 1.8 μg/ml). Analysis of plasma over a period of ten weeks showed a between-batch C.V. of 7.5% for the drug (n = 20, mean = 1.9 μg/ml) and 4.0% for the metabolite (n = 20, mean = 1.9 μg/ml).

This procedure has been used to study the bioavailability of SKF 78729A in humans. An example of a plasma concentration—time curve for the drug and its metabolite in a volunteer given 500 mg of SKF 78729A orally is shown in Fig. 3. The procedure allows detection of 200 ng/ml of drug or of metabolite. For the detection of as low as 25 ng/ml of drug or of metabolite 1 ml of the specimen is used and the residue of plasma extract is dissolved in 25 μl of mobile phase.

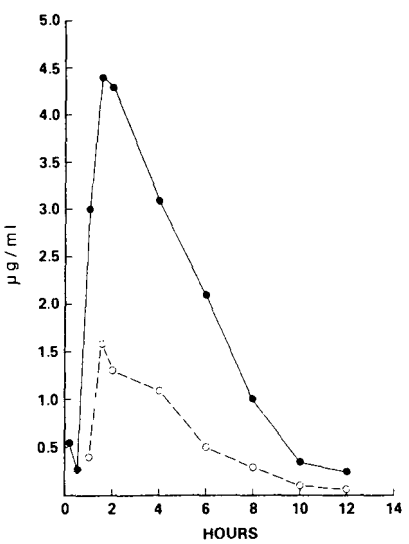


Fig. 3. Plasma drug concentration—time curve in a healthy volunteer after ingestion of 500 mg of SKF 78729A. (•), SKF 78729A; (○), N-acetyl derivative.

ACKNOWLEDGEMENT

This work was supported by a grant from Smith Kline and French, Canada Ltd.

REFERENCES

1 L.W. Chakrin and R.D. Krell, J. Pharmacol. Exp. Ther., 207 (1978) 756.
2 R.C. Veith and C. Perera, N. Engl. J. Med., 300 (1979) 504.
3 B. Vinet, Clin. Biochem., 14 (1981) 105.
4 S.C. Glauser, Med. Clin. North Amer., 58 (1974) 945.

Related articles published in *Journal of Chromatography*, Vols. 242—245

Volume 242

Volume 243

Author Index

Subject Index

Errata

J. Chromatogr., 229 (1982) 111—120
Page 111, the authors and their affiliations should read:
U. TIMM* and M. ZELL
Biological and Pharmaceutical Research Department, F. Hoffmann-La Roche & Co. Ltd., 4002 Basle (Switzerland)
and
L. HERZFELD
Chemical Laboratory Dr. Herzfeld, Basle (Switzerland)

J. Chromatogr., 230 (1982) 373—380
Page 380, ref. 9 should read:
9 T.L. Ding and L.Z. Benet, J. Chromatogr., 163 (1979) 281.

Vol. 231, No. 2

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

MANUFACTURERS' LITERATURE

N-1689

HPLC BOOKLET

LKB Produkter has issued a 68-page booklet, entitled: "High Performance Liquid Chromatography for the Biochemist", that describes the basic principles of gel filtration, ion exchange and reversed-phase chromatography. Each section includes typical application examples, a presentation of separation principles and factors affecting resolution, as well as practical information on the choice of columns and mobile phases, sample handling and column maintenance. Twenty application examples are included which illustrate the speed, resolution and capacity of HPLC.

N-1688

CATALOGUE

New products for electrophoresis, chromatography, HPLC, cell biology, and immunochemistry are described in Bio-Rad Labs. 156-page 1982 catalogue. New products described this year include a complete system for electrophoresis; new affinity gels for purifying calmodulin, lectins and fibronecion; a spring-clamp chromatography column rack; beads that separate B and T cells by affinity chromatography; a premixed gel filtration standard; and a family of carbohydrate analysis HPLC columns.

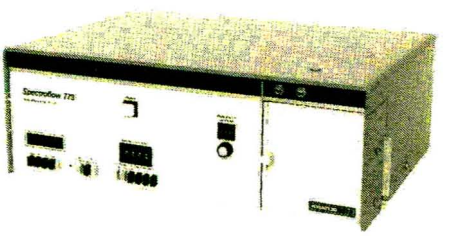
For further information concerning any of the news items, apply to the publisher, using the reply cards provided, quoting the reference number printed at the beginning of the item.

NEW PRODUCTS

N-1694

HPLC COLUMNS

J.T. Baker have introduced a series of HPLC columns for the fast separation of peptides, oligonucleotides, and proteins with molecular weights up to 150,000 for globular compounds, and up to 300,000 for fibrous materials. The columns are said to have good permeation and high resolving power for large biopolymers due to chemically modified wide-pore silica gel. These columns are called BAKERBOND™ Wide Pore Octadecyl, Octyl, Cyano and Diphenyl. Analysis times are said to be less than 1 h and recovery better than 95% with conservation of the activity. The columns can be used in analytical and preparative work.



N-1687

HPLC DETECTOR

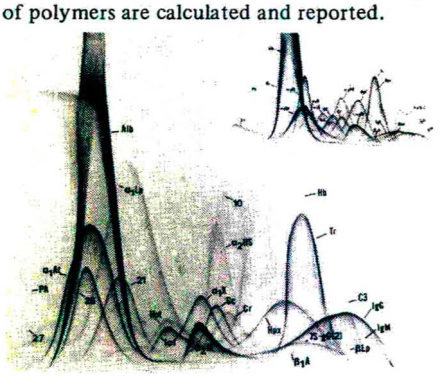
Kratos Analytical Instruments recently introduced the Spectroflow 773, a sensitive variable-wavelength UV–VIS detector for HPLC. The instrument operates at any wavelength from 190–700 nm, at any sensitivity

level from 0.001–2.999 a.u.f.s. The typical noise levels are less than 2×10^{-5} a.u. The Spectroflow 773 is claimed to be the most sensitive detector of this type available today. The detector has an automatic baseline zero, a built-in elution timer, dual range recorder outputs, and front panel self diagnosis. The flow cell reduces the effects of changes in refractive index, while improving the flow characteristics of the cell to minimize band broadening. The Spectroflow 773 is suitable for high sensitivity detection of microbore LC separations and high speed HPLC.

N-1681

STERIC EXCLUSION CHROMATOGRAPHY SOFTWARE

New software for the Apple II Plus–Vista 401 chromatography data system combination for polymer characterizations, formulations and comparisons by means of steric exclusion chromatography has been introduced by Varian. This software and the data system combination with Varian's Series 500 HPLC instruments enables automated on-line calibrations and analyses to be performed with either narrow or broad molecular weight distribution standards. Varian's range of size exclusion chromatography columns enables a wide range of polymer analyses to be carried out quickly and accurately. Molecular weight distributions, mean average molecular weights, polydispersity index, intrinsic viscosity and the peak molecular weight of polymers are calculated and reported.



N-1690

IMMUNOELECTROPHORESIS MAP

A paper obtainable from Bio-Rad describes a method that can be used to develop a reproducible crossed immunoelectrophoresis map of normal mouse serum in order to detect abnormalities in crossed IEP plates of diseased states.

N-1685

AGAROSE ELECTROPHORESIS SYSTEM

Providing all the advantages of agarose gel at the cost of cellulose acetate, Beckman Instruments' Paragon™ Agarose Gel Electrophoresis System combines good technical performance with the convenience of a kit. The system is said to offer excellent resolution, reproducibility and sensitivity. Separations are performed in about 45 min, and the patterns are said to be sharp and clear. The Paragon system consists of five reagent kits, each designed for a specific chemistry. Each ready-to-use kit comes with appropriate reagents, supplies and agarose gels for 100 determinations.

N-1680

SENSITIVE SILVER STAIN

The new Bio-Rad Silver Stain is said to be up to 50 times more sensitive than Coomassie Blue for detecting proteins in polyacrylamide gels. Silver staining takes only 2 h using the three stable reagents provided in the Bio-Rad kit. It allows detection of trace polypeptides with no need to overload or preconcentrate the sample. It is said to be sensitive enough to serve as a fast alternative to autoradiography. The Bio-Rad Silver Stain is suitable for SDS-PAGE gels, electrofocusing gels and non-SDS-PAGE gels. The Silver Stain kit includes oxidizer concentrate, silver reagent concentrate and powdered developer. All three are prepared for use by simply adding water, and are provided in sufficient quantity to stain up to 24 slab gels.

N-1686

GEL FILTRATION STANDARD

Bio-Rad's premixed gel filtration standard is a lyophilized mixture of molecular weight markers for calibrating gel filtration chromatography columns. The standard eliminates the difficulty of mixing ingredients and provides an invariable yardstick for molecular weight calibration or for testing column performance under native conditions. The standard contains five molecular weight markers balanced to generate uniform response at 280 nm, and is packaged in small quantities to eliminate the variation that can occur with aliquoting, freezing and thawing large batches. The gel filtration standard can be used to determine the gel filtration medium which is the best for the specific application, or to test the efficiency of a gel filtration system.

Reader service card

JOURNAL OF

CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

use this card for more information on advertisements and news items

I would like to receive, without any obligation, further information on

News items: _____

Advertisements: _____

I am especially interested in the fields as indicated below:

- ☐ 6040 Analytical Chemistry
- ☐ 6050 Chromatography
- ☐ 6150 Mass Spectrometry
- ☐ 6140 Spectroscopy
- ☐ 6240 Pharmaceutical Chemistry
- ☐ 6241 Medicinal Chemistry
- ☐ 6242 Clinical Chemistry
- ☐ 6260 Environmental Chemistry

Name: _____

Position: _____

Address: _____

_____

please use envelope; mail as printed matter

ELSEVIER SCIENCE PUBLISHERS

Advertising department

P.O. BOX 211,

1000 AE AMSTERDAM – THE NETHERLANDS

please use envelope; mail as printed matter

ELSEVIER SCIENCE PUBLISHERS

Advertising department

P.O. BOX 211,

1000 AE AMSTERDAM – THE NETHERLANDS

Reader service card

JOURNAL OF

CHROMATOGRAPHY

INTERNATIONAL JOURNAL ON CHROMATOGRAPHY, ELECTROPHORESIS AND RELATED METHODS

use this card for more information on advertisements and news items

I would like to receive, without any obligation, further information on

News items: _____

Advertisements: _____

I am especially interested in the fields as indicated below:

- ☐ 6040 Analytical Chemistry
- ☐ 6050 Chromatography
- ☐ 6150 Mass Spectrometry
- ☐ 6140 Spectroscopy
- ☐ 6240 Pharmaceutical Chemistry
- ☐ 6241 Medicinal Chemistry
- ☐ 6242 Clinical Chemistry
- ☐ 6260 Environmental Chemistry

Name: _____

Position: _____

Address: _____

_____

PUBLICATION SCHEDULE FOR 1982

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	234/1 234/2 235/1 235/2	236/1 236/2	237/1 237/2 237/3	238/1 238/2 239	240/1 240/2 241/1	241/2 242/1 242/2	243/1 243/2 244/1 244/2	245/1 245/2 245/3	246/1 246/2 247/1	The publication schedule for further issues will be published later.		
Chromatographic Reviews		251/1		251/2								
Biomedical Applications	227/1	227/2	228	229/1	229/2	230/1	230/2	231/1	231/2			

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 209, No. 3, pp. 501–504. 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.

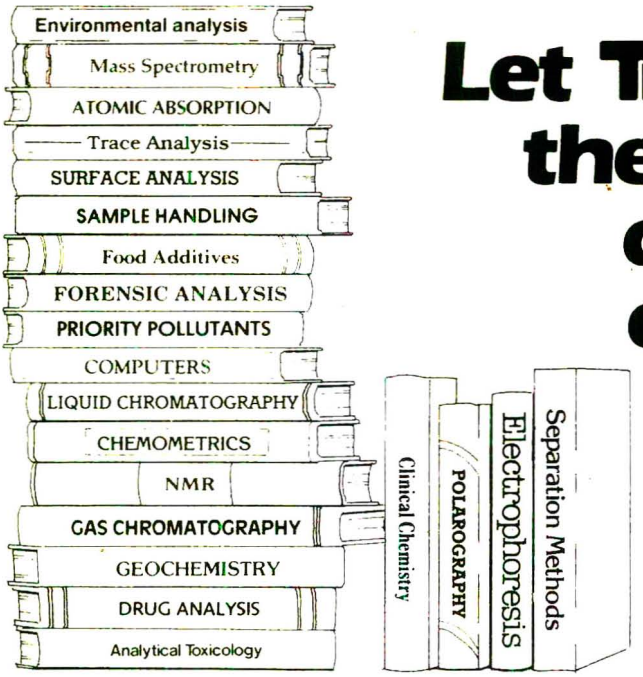
References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the lay-out of the reference list. Abbreviations for the titles of journals should follow the system 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.

News. News releases of new products and developments, and information leaflets of meetings should be addressed to: The Editor of the News Section, Journal of Chromatography/Journal of Chromatography, Biomedical Applications, Elsevier Scientific Publishing Company, P.O. Box 330, 1000 AH Amsterdam, The Netherlands.

Advertisements. Advertisement rates are available from the publisher on request. The Editors of the journal accept no responsibility for the contents of the advertisements.



Let TrAC cover the whole of analytical chemistry for you!

TrAC trends in analytical chemistry

TrAC - Trends in Analytical Chemistry is a topical monthly which provides you with an easy-to-read interdisciplinary digest of current developments and new ideas in the analytical sciences.

TrAC's authors are leading scientists, its articles practically oriented, its coverage international.

TrAC covers the broader issues which affect those who develop and use analytical methods, such as the impact of computers on the analytical laboratory and the implications of new governmental regulations.

TrAC comes from the publishers of the **Journal of Chromatography** and **Analytica Chimica Acta.**

Subscription Information
Personal edition: 12 issues -
U.K. £ 20.00,
USA and Canada US$ 42.50,
Europe 91.50 Dutch guilders,
Japan Yen 12400,
Rest of World 95.50 Dutch guilders

Library edition: Vol 1
(16 issues + Compendium) -
US$ 133.25 or 260 Dutch guilders.

Prices include air delivery worldwide.

Send now for a free sample copy to

Elsevier Scientific Publishing Company

P.O. Box 330
1000 AH Amsterdam
The Netherlands

52 Vanderbilt Avenue
New York, NY 10017
U.S.A.

Elite-Inn Yushima IF
3-28-1 Yushima, Bunkyo-ku,
Tokyo 113, Japan

7242