ISSN 0378-4347

VOL. 182 NO. 2 MAY 9, 1980
(Biomedical Applications, Vol. 8, No. 2)

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

PUBLICATION SCHEDULE FOR 1980

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	D 1979	J	F	M	A	M	J	J	A	S	O	N	D
Journal of Chromatography	185 186	187/1 187/2 188/1	188/2 189/1 189/2	189/3 190/1	190/2 191 192/1	192/2 193/1 193/2 193/3	194/1 194/2 194/3	195/1 195/2	The publication schedule for further issues will be published later.				
Chromatographic Reviews			184/1	184/2									
Biomedical Applications		181/1	181/2	181/ 3-4	182/1	182/2	182/ 3-4	183/1					

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, if possible 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.

Reviews 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.

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, Biomedical Applications* can be subscribed to separately.

Publication. The *Journal of Chromatography* (including *Biomedical Applications* and *Chromatographic Reviews*) has 22 volumes in 1980. The subscription price for 1980 (Vols. 181–202) is Dfl. 2838.00 plus Dfl. 352.00 (postage) (total *ca.* US$ 1636.00). The subscription price for the *Biomedical Applications* section only (Vols. 181–183) is Dfl. 399.00 plus Dfl. 48.00 (postage) (total *ca.* US$ 229.25). Journals are automatically sent by air mail 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 180) are available at Dfl. 140.00 (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/North-Holland Inc., Journal Information Centre, 52 Vanderbilt Avenue, New York, N.Y. 10017. Tel: (212) 867-9040.

For further information, see page 3 of cover.

Printed in The Netherlands

VOL. 182, NO. 2 JOURNAL OF CHROMATOGRAPHY MAY 9, 1980

(Biomedical Applications, Vol. 8, No. 2)

CONTENTS

(Continued overleaf)

Contents (continued)

Journal of Chromatography, 182 (1980) 137–145
Biomedical Applications

CHROMBIO. 521

VOLATILE METABOLITES IN SERA OF NORMAL AND DIABETIC PATIENTS

ALBERT ZLATKIS* , COLIN F. POOLE and ROSWITHA BRAZELI

Department of Chemistry, University of Houston, Houston, Texas 77004 (U.S.A.)

DONALD A. BAFUS

Northrup Services, NASA, Houston, Texas 77058 (U.S.A.)

and

PETER S. SPENCER

Institute of Neurotoxicology, Albert Einstein College of Medicine, Bronx, N.Y. 10461 (U.S.A.)

(Received November 12th, 1979)

SUMMARY

The profiles of volatile metabolites in serum samples from normal individuals and from individuals with diabetes mellitus with varying degrees of polyneuropathy have been studied. The transevaporator procedure was used to obtain sample extracts which were chromatographed on a highly efficient glass column coated with Silar 10C (106 m × 0.25 mm I.D.). Differences in profiles between normal subjects and diabetic subjects on no drug therapy were noticed. However, correlations between the severity of the neuropathy and the concentrations of certain ketones could not be established. Compounds present both in diabetic and normal sera have been identified by mass spectrometry.

INTRODUCTION

The pattern of polyneuropathy found in cases of prolonged exposure to 2-hexanone is broadly similar to the symmetrical distal polyneuropathy found in many patients with long-standing diabetes mellitus [1]. The toxicity of 2-hexanone is attributed to its oxidative metabolite, 2,5-hexanedione [2, 3]. Longer-chain γ-diketones also produce experimental neuropathy [4]. To test the hypothesis [5] that the diabetic state produces endogenous neurotoxic diketones which could account for the sensory loss affecting many patients with diabetes mellitus, profiling experiments have been undertaken.

*To whom correspondence should be addressed.

Earlier studies on the volatile metabolites in urines of diabetic patients revealed some abnormally high levels of ketones, as well as alcohols and other compounds [6—10]. We have studied the profiles of volatile metabolites in sera from individuals with diabetes with varying degrees of sensory neuropathy to determine quantitative variations of the ketones, especially those that can undergo ω-1 oxidation to form γ-diketones. The overall profiles have also been compared to those obtained from normal individuals to examine other possible qualitative and quantitative changes.

EXPERIMENTAL

Sample preparation

Samples received from Albert Einstein College of Medicine were shipped frozen in dry ice. Upon arrival, the samples were thawed and transferred to 2-ml glass bottles with PTFE-lined screw caps, placing 0.5-ml quantities per bottle. This transfer process was to eliminate any problems associated with the refreezing and rethawing of the samples as they were used. Samples were then stored at $-10°$ until ready for analysis.

Extracts of 50-μl samples were prepared using the transevaporator procedure as described by Zlatkis et al. [11]. The technique was adapted for this work without change, except for a slight modification on the transevaporator which allowed the connections of the glass bead tubes to be made to the body of the transevaporator with a 10/30 ground glass joint (Fig. 1). Headspace samples were not collected because of the low-level profiles that small sample quantities of serum yield [12]. 2-Chloropropane, purified by distillation from phosphorus pentoxide was used as the solvent and 0.4 ml was the volume used for each extract.

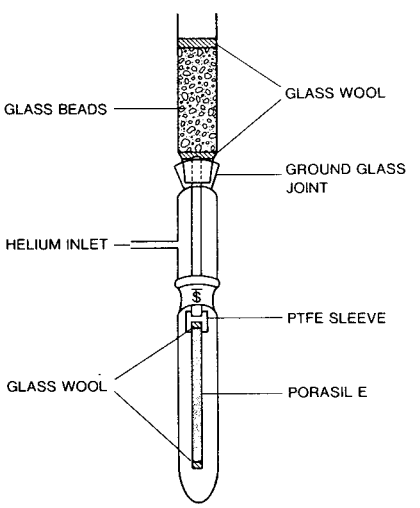


Fig. 1. Schematic diagram of transevaporator sampling apparatus.

Gas chromatography

A Varian 3700 gas chromatograph equipped with flame ionization detector, capillary direct injector insert, and glass-lined detector insert was used in this work. Separations were carried out on a 106 m × 0.25 mm I.D. glass column coated with Silar 10C (Quadrex, New Haven, Conn., U.S.A.). Helium was used as the carrier gas at a flow-rate of 1.5 ml/min and a head pressure of 40 p.s.i. Helium make-up gas was also added at the end of the column to provide a final flow-rate of 30 ml/min through the detector.

A dual trapping system was used in transferring the sample to the analytical column to avoid any losses in efficiency. The volatiles were first stripped from the glass bead tubes which held the concentrates of the extracted samples, and trapped on a precolumn (15 cm × 0.5 mm I.D.) of uncoated stainless steel, as previously described [11]. The precolumn was transferred to the chromatograph where it was connected to the carrier gas inlet via a Swagelok fitting and to a second precolumn (15 cm × 0.25 mm I.D.) of uncoated stainless steel via heat shrinkable PTFE tubing. The second precolumn, which was connected to the analytical column with heat shrinkable tubing, was immersed in liquid nitrogen and the sample transferred to it by heating the first precolumn to 200° with a small current. When the second liquid nitrogen Dewar was removed, the analysis was started. The column was operated isothermally at 40° for 6 min, then programmed at 2°/min to 180° where it was held for an additional 30 min. Peak areas were integrated on a Spectra Physics Autolab System I computing integrator which was tied to the gas chromatograph.

Mass spectrometry

Gas chromatographic—mass spectrometric analyses were made on a Model CH5 mass spectrometer connected to a Spectrosystem 100 data system and coupled to a Varian 1740 gas chromatograph (all from Varian-Mat, Bremen, G.F.R.). Spectra were recorded at 70 eV at an exponential scan mode for a mass range of 21 to 210 every 3 sec. Temperatures were 220° for the analytical ion source and 225° for the separator and transfer lines. Chromatographic conditions and sample transfer conditions were identical to those on the Varian 3700. Sample sizes however were increased to 180 μl of serum.

RESULTS AND DISCUSSION

The transevaporator sampling procedure provides an efficient means of obtaining an extraction profile of small sample quantities and allows for semi-quantitative determinations of a variety of compounds that differ widely in terms of polarity. The reproducibility of the method for a standard mixture of alcohols, ketones, and aldehydes has been previously reported [12].

Fig. 2 shows the chromatogram obtained from a sample of a normal subject. The variations between different normal individuals is small and only slight quantitative changes have been observed. A consistent pattern was obtained even for samples collected in different laboratories or hospitals and presumably therefore under different sample collection procedures. This consistency in the normal profiles facilitates standardization and should help to distinguish any quantitative or qualitative variations found to occur between normal and

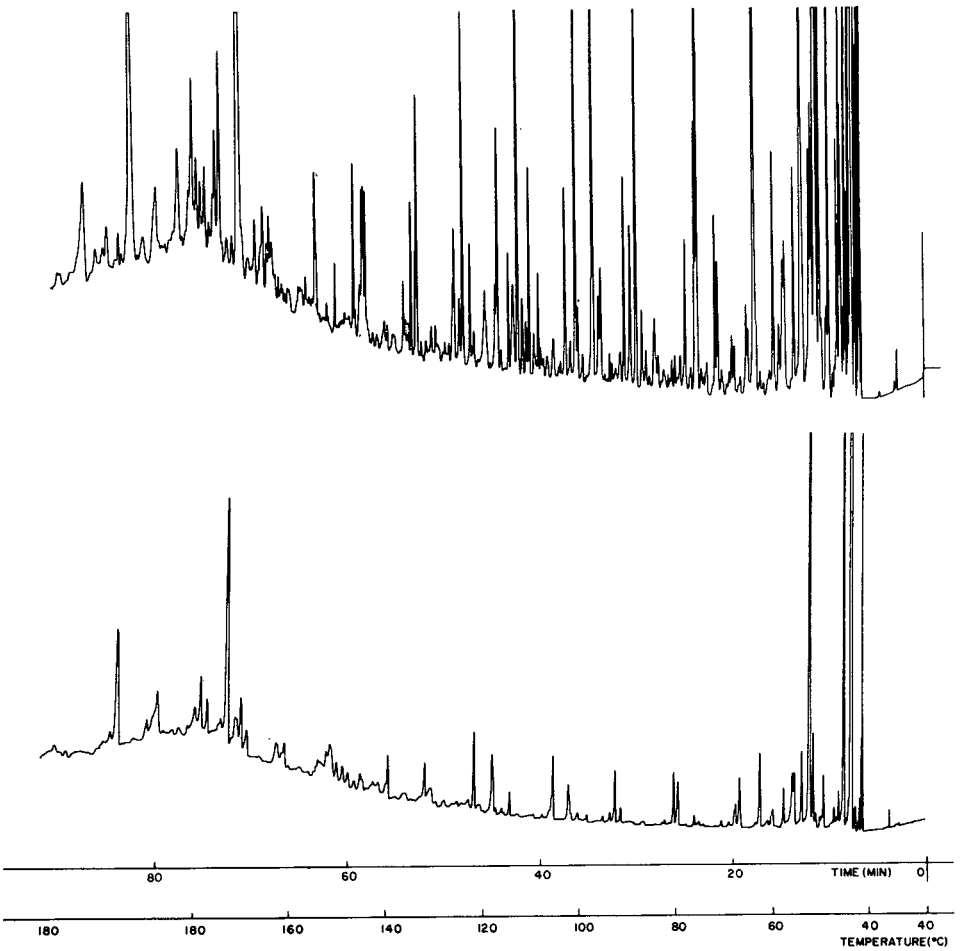


Fig. 2. Chromatograms obtained from normal blood serum (top) and corresponding blank (bottom). Attenuation, 64.

TABLE I

STANDARD DEVIATION OF RETENTION TIME AND PEAK AREA

Five replicate analyses were carried out.

Sample	Retention time			Peak area		
	Mean value (sec)	Standard deviation	Relative standard deviation (%)	Mean value (counts)	Standard deviation	Relative standard deviation (%)
2-Hexanone	1701	11.2	0.66	18046	4118	22.8
4-Heptanone	1860	6.1	0.33	1920	497	25.9
3-Heptanone	1977	9.1	0.46	5258	1064	20.2
2-Heptanone	2089	8.9	0.43	15672	5436	34.7

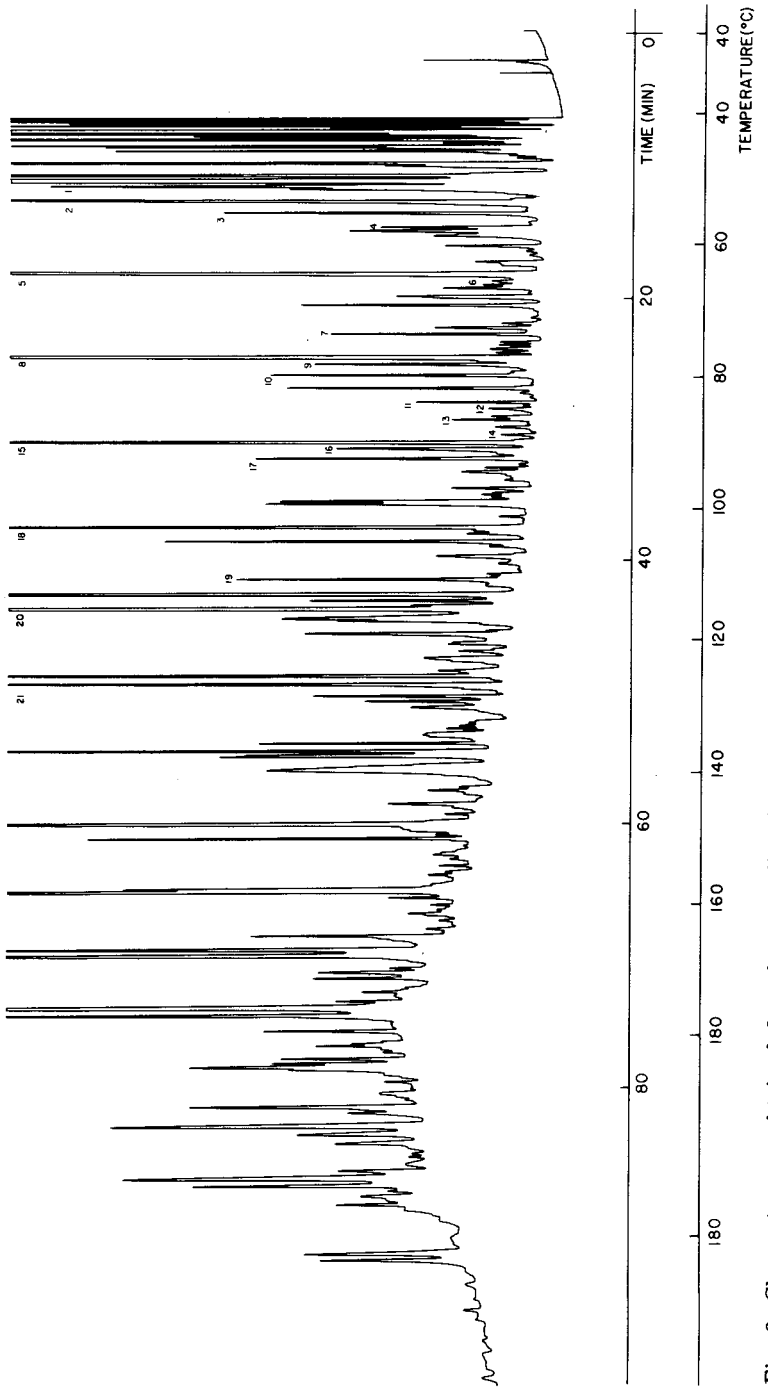


Fig. 3. Chromatogram obtained from long-standing diabetic on no drug therapy. Numbers correspond to compounds listed in Table II. Attenuation, 64.

pathological samples. The standard deviations for some aliphatic ketones identified in a normal control sample, for five replicate runs for both retention time and peak area counts are listed in Table I. The values are close to the average values previously reported for variations for individual peaks in replicate runs of normal serum [11].

Samples from diabetic patients with varying degrees of neuropathy ranging from minimal to severe plus autonomic were investigated. Some of the patients were not on any type of drug therapy while others were receiving insulin or orinase. Significant quantitative variations were found between normal subjects and subjects with long-standing diabetes on no therapy, especially at the high-temperature end of the chromatograms. Qualitative differences were not determined. Fig. 3 shows the chromatogram obtained from a diabetic sample where the patient has a mild case of neuropathy and is not taking medication.

In the analyses of urinary volatile metabolites from insulin-treated diabetics,

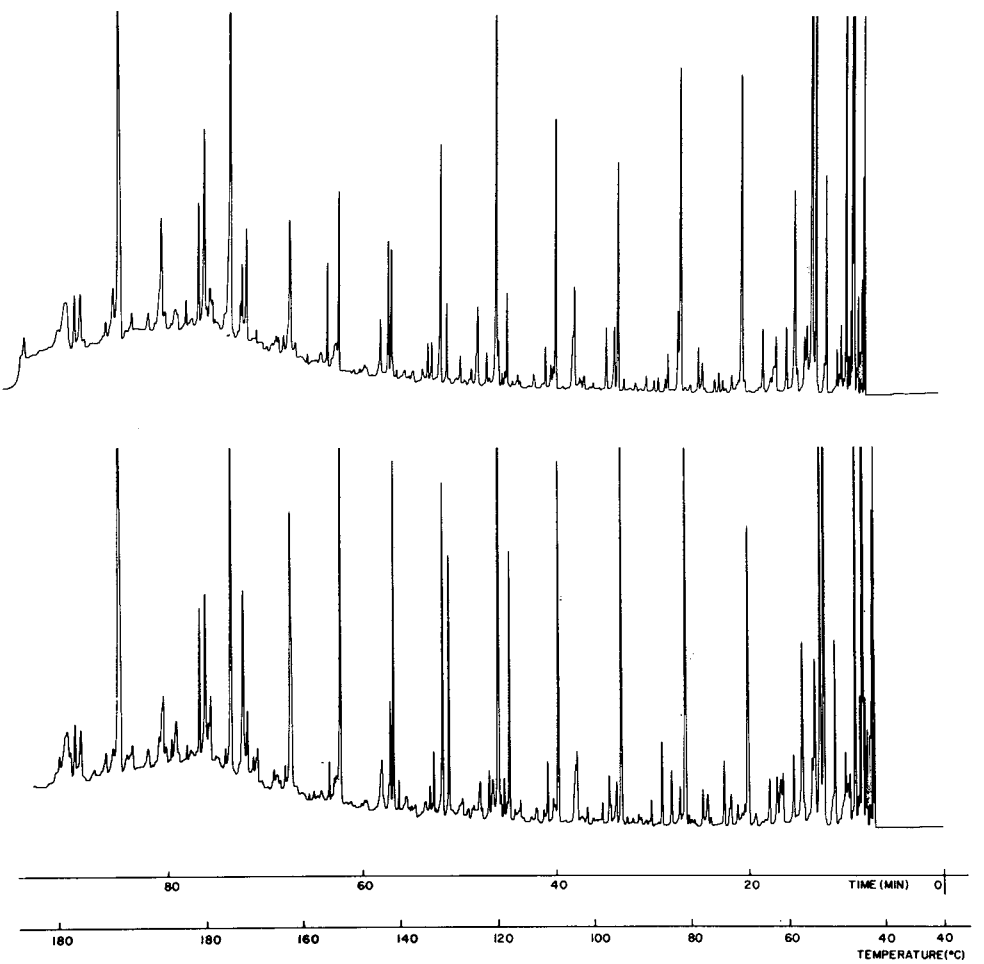


Fig. 4. Chromatograms obtained from diabetic on insulin (top) and from an individual with chemical diabetes (bottom). Attenuation, 160.

characteristic profiles were obtained which differed substantially from normal samples [6]. This distinction was not found in serum samples. Serum profiles from diabetics on insulin closely resembled the profiles obtained from normal individuals. Fig. 4 shows the chromatogram obtained from a diabetic with a moderate case of neuropathy on insulin (top) and from a subject with chemical diabetes (bottom). Individuals with the latter are generally asymptomatic but have abnormal glucose tolerance under standard conditions which is controlled by diet.

Compounds identified by mass spectrometry in diabetic serums were also found in normal serums and are listed in Table II. Sixteen of these compounds had been previously identified [13—15]. Clearly, many of the peaks that are present in large quantities are normal aldehydes. The identity of the aliphatic ketones indicated by mass spectra and listed in Table I were confirmed by comparing their chromatographic retention times with those of the authentic compounds. 2-Hexanone and 3-heptanone were the only ketones found which could undergo ω-1 oxidation to form the γ-diketones. Intermediate metabolites of these ketones which could also lead to the formation of the diketones were not detected nor were the γ-diketones themselves.

TABLE II

VOLATILE COMPOUNDS IDENTIFIED IN HUMAN SERUM

Peak number	Compound	Peak number	Compound
	Formaldehyde	9	*n*-Butanol
	Acetaldehyde	10	2-Hexanone
	Furan	11	*n*-Nona-2,4-dienal
	Propanol	12	4-Heptanone
	Propenal	13	*trans*-2-Methyl-2-butenal
	2-Methylpropenal	14	3-Heptanone
1	2-Propanone	15	*n*-Heptanal
	2-Methyltetrahydrofuran	16	*n*-Pentanol
2	*n*-Butanal	17	2-Heptanone
	Ethanol		Toluene
	2-Propanol	18	*n*-Octanal
3	Benzene		2-Octanone
4	2-Butanone	19	*n*-Pentylbenzene
5	*n*-Pentanal	20	*n*-Nonanal
6	2-Pentanone		Benzaldehyde
7	2-Butenal	21	2-Nonenal
8	*n*-Hexanal		*n*-Decanal
			n-Undecanal

Fig. 5 shows the partial calibration curves for the ketones listed in Table I. They were obtained by injecting known amounts of a standard onto glass bead tubes which were then treated in a manner identical to an actual sample. The curves are linear over the peak area range which the samples contained, and the recovery for the entire sampling procedure was found to average at 70% for each of the ketones.

When individual samples were compared no correlation between the severity of the neuropathy and the concentrations of 2-hexanone or 3-heptanone could be found. Also because of the limited number of control samples we were not

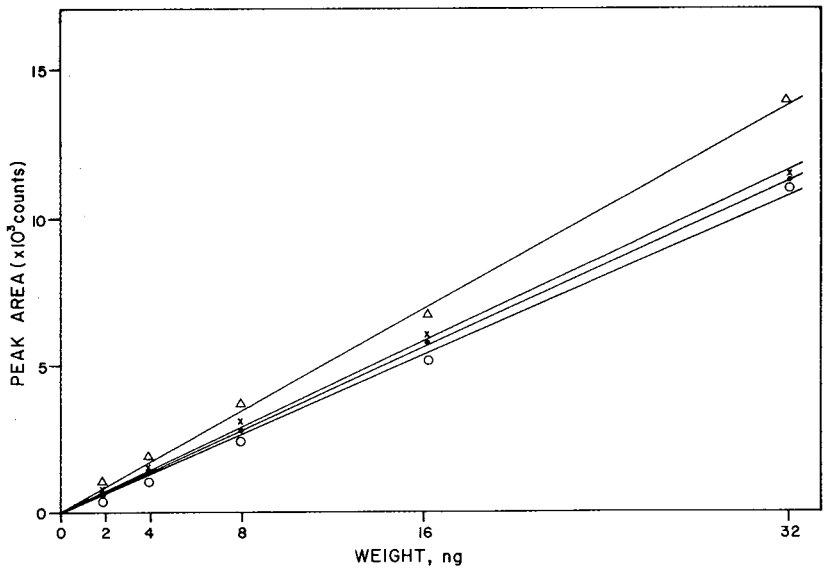


Fig. 5. Calibration curves for ketones identified in serum: 3-heptanone (△); 4-heptanone (×); 2-heptanone (●); 2-hexanone (○).

able to determine if there was any significant concentration differences in 4-heptanone and 2-heptanone in normal versus diabetic samples. It has been established that the concentrations of these two ketones in urine correlate with metabolic disorders related to diabetes [10]. The type of treatment seemed to make no difference. It is possible that the concentrations need to be correlated with blood glucose levels and insulin activity before significant changes in concentration become apparent. In all of the samples analyzed the ketone concentrations were found to be in the low nanogram range and 2-hexanone and 2-heptanone were found to be in the highest concentration while 3-heptanone and 4-heptanone were found to be in the lowest. It should be noted that 2-butanone was not found in greater quantities in the diabetic sera. This is the ketone which is not neurotoxic, but which is able to potentiate the neuropathic property of 2-hexanone [16].

ACKNOWLEDGEMENTS

Supported in part by the National Institute of Occupational Safety and Health Grant No. OH 00535. Thanks are due to Dr. Arnold Sterman for his assistance in this project.

REFERENCES

1 P.S. Spencer and H.H. Schaumburg, Propr. Neuropathol., 3 (1977) 253.
2 P.S. Spencer, M.I. Sabri, H.H. Schaumburg and C.L. Moore, Ann. Neurol., 5 (1979) 501.
3 G.D. DiVincenzo, C.J. Kaplan and J. Dedinas, Toxicol. Appl. Pharmacol., 36 (1976) 511.
4 G.D. Divincenzo, C.J. Hamilton, C.J. Kaplan and J. Dedinas, Toxicol. Appl. Pharmacol., 41 (1977) 547.

5 J.L. O'Donoghue and W.J. Krasavage, Toxicol. Appl. Pharmacol., 48 (1979) A-55.
6 A. Zlatkis, W. Bertsch, H.A. Lichtenstein, A. Tishbee, F. Shunbo, H.M. Liebich, A.M. Coscia and N. Fleischer, Anal. Chem., 45 (1973) 763.
7 H.M. Liebich, O. Al-Babbili, A. Zlatkis and K. Kim, Clin. Chem., 21 (1975) 1294.
8 H.M. Liebich and O. Al-Babbili, J. Chromatogr., 112 (1975) 539.
9 H.M. Liebich, J. Chromatogr., 112 (1975) 551.
10 H.M. Liebich and G. Huesgen, J. Chromatogr., 126 (1976) 465.
11 A. Zlatkis, C.F. Poole, R. Brazell, K.Y. Lee and S. Singhawangcha, J. High Resol. Chromatogr. Chromatogr. Commun., 2 (1979) 423.
12 K.Y. Lee, D. Nurok and A. Zlatkis, J. Chromatogr., 158 (1978) 377.
13 A. Zlatkis, W. Bertsch, D.A. Bafus and H.M. Liebich, J. Chromatogr., 91 (1974) 379.
14 H.M. Liebich and J. Wöll, J. Chromatogr., 142 (1977) 505.
15 A. Zlatkis, K.Y. Lee, C.F. Poole and G.H. Holzer, J. Chromatogr., 163 (1979) 125.
16 K. Saida, J.R. Mendell and H. Weiss, J. Neuropathol. Exp. Neurol., 35 (1976) 207.

Journal of Chromatography, 182 (1980)147—154
Biomedical Applications

CHROMBIO. 509

GAS–LIQUID CHROMATOGRAPHY OF FREE AMINO ACIDS IN THE HYALOPLASM OF RAT CEREBRAL, CEREBELLAR AND OCULAR TISSUES, AND IN SKELETAL AND HEART MUSCLE

JANUSZ GABRYS and JANUSZ KONECKI

Autonomous Environmental Protection Laboratory, Biological and Morphological Institute, Silesian School of Medicine, Katowice (Poland)

(First received August 10th, 1979; revised manuscript received December 7th, 1979)

SUMMARY

The paper deals with the composition of amino acids in the hyaloplasm of cerebral tissue, cerebellum, eyeball, heart muscle and skeletal muscles. The investigations performed showed that: the most numerous groups of peaks were obtained from heart muscle (45), cerebellar tissue (43), skeletal muscle (36), eyeball (29) and cerebral tissue (25); and the highest molar levels corresponded to those of tryptophan in skeletal muscle, heart and cerebellum, proline in the heart, valine in the eyeball, and aspartic acid in the brain. Weight ratios indicated high contents of histidine, tyrosine and phenylalanine in the tissues of the skeletal muscles, the heart and cerebellum.

INTRODUCTION

The present work extends our investigations of the quantitative and qualitative composition of free amino acids contained in the hyaloplasm of different tissues and organs. In our previous work we determined the content of amino acids in the hyaloplasm from the liver and kidneys. This present paper deals with the results obtained from the following organs: frontal lobes of the brain, cerebellum, eyeball without muscles, heart muscle and skeletal muscles.

The analysis of free amino acids by gas—liquid chromatography (GLC) was performed according to the methods of Gehrke et al. [1, 2] and of Kaiser et al. [3]. Preparation of the hyaloplasm was performed as described by Chauveau et al. [4].

MATERIALS AND METHODS

Animals

Ten Wistar strain male rats, aged 3 months, each weighing 180 ± 30 g, were

used. Laparotomy and general perfusion were performed under urethane anesthesia, and perfusion fluid was introduced into the left ventricle. An isotonic solution of 0.75 *M* saccharose and Trition X-100 (0.5% at 4°) was used for perfusion (250 ml per animal). The tissues obtained were homogenized with the perfusion fluid at a ratio of 1:5.

Apparatus

The following equipment was used: Glass homogenizer (25 ml capacity) with a PTFE piston; K-24 and VAC 601 centrifuges (Janetzki Heinz K.-G., G.D.R.); ion-exchange columns were 12 mm × 150 mm; lyophilizing apparatus produced by VEB MLW Labortechnik, (G.D.R.); reaction vessels of our own design equipped with PTFE-lined screw-caps; automatic ultrasonic disintegrator UD-11 produced by Techpan; oil baths with a thermoregulator; evaporator produced by Büchi (Switzerland); Varian 3700 gas chromatograph with data analyzer CDS 111 C and an A 25 recorder; Hamilton 10-μl syringes; automatic pipettes (1000 μl and 100 μl) were produced by Eppendorf, G.F.R.

Reagents

Pure NaCl, 7 *N* ammonia solution and Dowex 50W-X8 100—200 mesh (H^+) were produced by Fluka, Buchs Switzerland. Pure picric acid and gaseous HCl were also products of Fluka. *n*-Butanol Seq. grade and trifluoroacetic anhydride were from Pierce, Rockford, Ill., U.S.A. Standard amino acids were obtained from BDH (Poole, Great Britain), Mann Labs. (New York, N.Y., U.S.A.) and E. Merck (Darmstadt, G.F.R.). A calibration mixture was prepared by adding the successively estimated amino acids. 3% OV-17 on Varaport 30 100—200 mesh was from Varian, Palo Alto, Calif., U.S.A.

Preparation of amino acids

Homogenization. The tissues were homogenized at the ratio of 1:5.

Centrifugation and ultracentrifugation. The investigations were performed at ±4° according to the method of Chauveau et al. [4]. The homogenate was centrifuged at 600 *g* for 15 min, then the supernatant was decanted and centrifuged at 105,000 *g* for 60 min. Cell hyaloplasm was obtained in the supernatant, and 1% picric acid was added at a ratio of 1:5. The solution was centrifuged at 3500 *g* for 15 min, and the proteins were separated in the sediment. The deproteinized supernatant containing an excess of picric acid was introduced into the ion-exchange column.

Ion-exchange. In the following ion-exchange procedure a twenty-fold or greater excess of resin capacity to exchangeable ions placed on the column was maintained; i.e. 6 g of Dowex 50W-X8 100—200 mesh to 25 ml of supernatant. Six grams of ion exchanger placed in a 500-ml vial and covered with 7 *N* NH_4OH were mixed for 60 min. After sedimentation the fluid was decanted. The procedure was repeated two times. The column was then washed with bidistilled water to a pH of 7.5. The ion exchanger was regenerated with 3 *N* HCl (three times), and washed with water until a pH of 6.2 was obtained. Columns (15 mm × 150 mm) were filled half full with the wet resin. The supernatant with the picric acid was passed through the column at a rate of 2 ml/min. The surface was washed with water until the eluate was decolorised.

TABLE I

PERCENTAGE CONTENT OF PEAKS FROM THE SEPARATION OF AMINO ACIDS IN TISSUE HYALOPLASM

$$\text{Percentage content of peaks} = \frac{\text{Area of peak}}{\Sigma \text{ areas of all peaks}} \cdot 100.$$

No.	Percentage content of peaks				
	Brain	Cerebellum	Eyes	Muscle	Heart
1	—	0.10	—	—	0.43
2	2.74	1.51	5.34	3.39	1.42
3	2.65	2.22	5.40	1.63	1.16
4	—	0.34	—	—	0.69
5	11.42	7.27	9.02	4.68	2.21
6	—	1.25	—	—	0.70
7	1.86	1.14	4.51	1.40	0.85
8	—	0.86	0.39	0.17	1.27
9	0.65	1.42	1.64	0.55	1.77
10	—	4.57	0.53	20.49	5.33
11	3.44	1.87	5.94	1.81	1.40
12	—	0.62	0.59	1.87	1.34
13	—	0.24	—	1.38	0.35
14	0.08	0.19	0.14	0.34	0.16
15	—	0.10	—	—	0.19
16	0.14	1.43	—	0.30	2.53
17	—	0.12	—	—	0.25
18	—	—	—	—	0.49
19	20.82	13.73	2.42	0.38	0.32
20	—	0.24	—	—	0.35
21	1.86	1.25	3.37	0.94	0.56
22	—	—	—	—	0.22
23	—	0.20	—	0.25	0.52
24	0.41	0.72	1.25	1.03	0.58
25	—	0.23	—	—	0.35
26	1.57	1.51	—	1.10	1.36
27	16.32	1.70	2.19	0.99	1.65
28	1.80	1.26	2.35	0.78	0.90
29	—	0.32	—	0.25	0.42
30	1.22	1.02	2.38	0.72	0.89
31	0.17	0.76	0.75	0.64	0.75
32	28.93	14.13	30.60	6.07	13.13
33	0.05	0.62	0.36	0.53	0.97
34	0.07	1.31	1.06	1.13	1.84
35	0.06	0.81	0.62	1.17	1.30
36	—	1.39	—	0.81	1.07
37	—	2.09	2.15	1.75	1.38
38	0.24	1.19	1.59	1.16	1.91
39	0.13	2.45	1.06	1.37	2.79
40	1.57	7.89	3.87	18.72	13.69
41	0.46	1.16	0.69	1.26	2.33
42	1.28	13.92	4.94	23.51	21.26
43	—	2.34	1.80	3.24	2.25
44	—	1.90	0.94	1.84	2.64
45	—	2.97	—	1.93	5.04

Then 25 ml of 7 *N* NH_4OH and 30 ml of bidistilled and deionized water were passed through the column. The eluate and the washings were collected and mixed, and 55 ml of the mixture were lyophilized. The procedure was repeated according to the description of Zumwalt et al. [5].

Lyophilization. A 50-ml portion of the eluate and washings were collected in a vessel and quickly frozen in liquid nitrogen. The sample was placed in a 1000-ml condensor. After lyophilization 2 mg of dry sediment were attained and transferred to an esterification vessel.

Derivatization. The reaction was carried out according to the method of Gehrke et al. [2].

Packings and conditions of separation. Columns of Pyrex glass (200 cm × 6.35 mm O.D. × 2 mm I.D.) were packed with 3% OV-17 on Varaport 30 100—200 mesh, and 0.65% EGA on Chromosorb W AW 80—100 mesh. The temperature program was isothermal at 90° for 5 min, then increased at 7°/min up to 230° and then kept isothermal for 10 min. Injection temperature was 170°, flame ionization detector 250°; range 10^{-10}. Nitrogen flow-rate was 10 ml/min, hydrogen 30 ml/min, air 300 ml/min. Chart speed was 1 cm/min, with CDS 111 C computer read-out. Each sample of the amino acids obtained from particular organs and tissues was subjected to chromatographic analysis in triplicate; the total number of chromatograms was 150.

RESULTS AND DISCUSSION

The investigations were carried out for 70 days. During this time 50 final products of derivatized amino acids from the tissues examined were prepared. The products of the derivatization reaction were *n*-butyl N-trifluoroacetyl esters, yellow in colour, which were injected into the OV-17 phase column. Table I shows the number and percentage of peak areas obtained from the various tissues. The analysis, performed according to the method of Amico et al. [6], corresponded to a number of standard amino acids in our laboratory. Thus, it was possible to determine the following amino acids: alanine (2); threonine (3); glycine + serine (5); valine (7); leucine + isoleucine (11); proline (21); methionine (24); histidine (26); asparagine (27); phenylalanine (28); tyrosine (30); glutamic acid + lysine (32); and tryptophan (40). Table II lists identified amino acids according to Zumwalt et al. [7]. Pellizzari et al. [8] and Gehrke et al. [9]. Table I is arranged according to increasing retention time and relative molar response (RMR); it also provides information about the composition of the hyaloplasm of the investigated tissues. This list does not include identification of peak 19 which provided support for the presence of γ-aminobutyric acid, which was identified on the basis of the literature data [10], as well as numerous articles published in the Journal of Neurochemistry. In Table II special attention is paid to glycine, serine, glutamic acid, lysine, leucine, isoleucine and aspartic acid in brain tissue. From among the amino acids that we have in our laboratory, norvaline was chosen as the internal standard.

Table III shows a high weight of the following amino acids: histidine, tyrosine, phenylalanine, proline, aspartic acid and valine in heart muscle, and threonine, valine and methionine in the cerebellar tissue. Tables I—III and Figs. 1—5, showing the peaks, were the basis of our analysis and also helped us draw the final conclusions:

TABLE II

PERCENTAGE CONTENT OF AMINO ACIDS IN TISSUES ACCORDING TO RELATIVE MOLAR RESPONSE

$$\text{Percentage molar} = \frac{\text{area of amino acid}}{100 \cdot \text{RMR of glutamic amino acid}}$$

where area of amino acid = area of the amino acid peak from chromatogram, RMR = relative molar response, RMR of glutamic amino acid = $\frac{\text{Molar response of amino acid}}{\text{Molar response of glutamic acid}}$

Amino acid	Percentage molar				
	Brain	Cerebellum	Eyes	Heart	Muscle
Ala	3.0 ± 0.00	2.4 ± 0.6	5.4 ± 0.6	3.0 ± 2.0	7.2 ± 2.8
Thr	3.0 ± 0.00	3.6 ± 1.4	5.8 ± 1.2	2.1 ± 0.9	3.0 ± 1.0
Gly + Ser	15.00 ± 0.00	13.6 ± 4.4	11.4 ± 1.6	5.0 ± 3.0	10.2 ± 1.8
Val	1.8 ± 0.2	2.2 ± 0.8	5.8 ± 0.2	1.3 ± 1.7	2.5 ± 1.5
Leu + Ile	4.2 ± 0.8	3.4 ± 1.6	6.4 ± 1.6	3.0 ± 1.0	3.3 ± 5.7
Pro	1.8 ± 0.2	1.8 ± 1.2	3.8 ± 0.2	0.7 ± 0.3	1.4 ± 0.6
Met	0.3 ± 0.00	1.1 ± 0.9	1.0 ± 0.00	1.2 ± 1.8	1.6 ± 1.4
His	1.0 ± 0.00	3.4 ± 1.6	2.0 ± 0.00	2.8 ± 1.2	1.7 ± 1.3
Asp	21.6 ± 1.4	3.0 ± 1.0	5.8 ± 10.2	3.4 ± 1.6	1.7 ± 0.3
Phe	1.8 ± 0.2	2.0 ± 0.00	2.6 ± 0.4	1.6 ± 0.4	1.2 ± 0.8
Tyr	1.0 ± 0.00	1.4 ± 0.6	4.6 ± 8.4	1.4 ± 1.6	1.0 ± 1.0
Glu + Lys	37.2 ± 1.8	35.6 ± 7.4	35.2 ± 3.8	32.3 ± 5.7	12.9 ± 1.1
Try	1.2 ± 0.8	17.1 ± 1.9	5.0 ± 1.0	31.1 ± 8.9	41.2 ± 4.8

TABLE III

ANALYSIS OF TISSUE AMINO ACIDS IN 2 mg OF LYOPHYLIZATE OF CELL CYTOSOL

Values were calculated from the formula $G_X = G_S \frac{A_X \cdot RMR_S \cdot M_X}{A_S \cdot RMR_X \cdot M_S}$

where G_X = grams of amino acids in a given number of specimens, A_X = peak area of the investigated amino acid, RMR_S = relative molar response of the internal standard, M_X = molecular weight of the estimated amino acid, A_S = peak area of the internal standard, RMR_X = relative molar response of the estimated compound, M_S = molecular weight of the standard, G_S = grams of internal standard.

Amino acid	Muscular tissue (μg)	Heart muscle (μg)	Cerebellar tissue (μg)
Ala	76.53 ± 0.31	80.01 ± 1.23	75.43 ± 4.11
Thr	101.69 ± 1.65	106.95 ± 3.72	108.34 ± 1.36
Val	99.07 ± 1.58	109.16 ± 9.54	104.13 ± 10.14
Pro	96.93 ± 3.91	120.81 ± 8.83	98.19 ± 15.99
Met	123.19 ± 7.53	155.67 ± 12.38	143.13 ± 9.40
His	188.06 ± 10.14	188.34 ± 8.39	172.59 ± 0.69
Asp	114.70 ± 4.48	120.04 ± 2.61	114.96 ± 3.88
Phe	142.02 ± 5.45	156.19 ± 15.40	148.82 ± 5.54
Tyr	154.43 ± 12.28	174.36 ± 9.32	161.83 ± 17.81

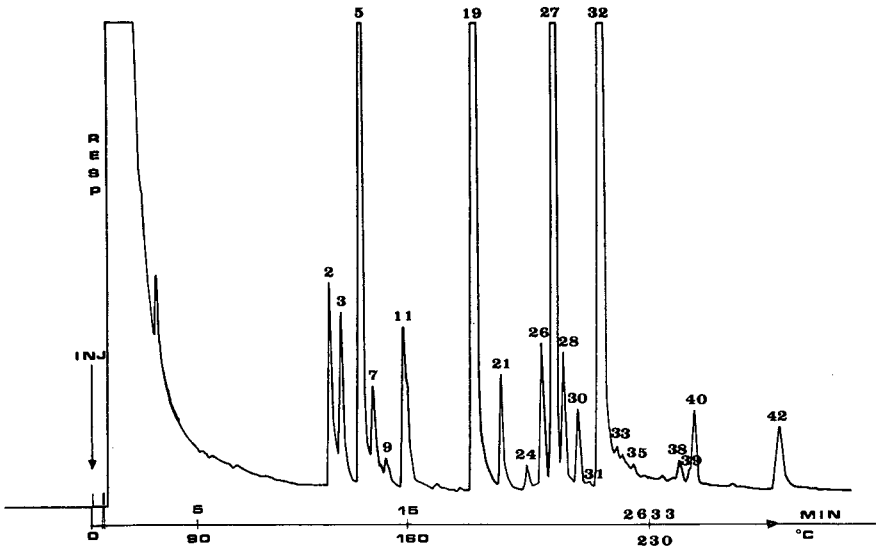


Fig. 1. Chromatogram of amino acids, cleaned by cation exchange, in the hyaloplasm of rat cerebral tissue. Column: 3% OV-17 on Varaport 30. Conditions: initial temperature 90°, isothermal at 90° for 5 min, and then 7°/min up to 230°, and isothermal for 10 min; range $10^{-10} \times 64$.

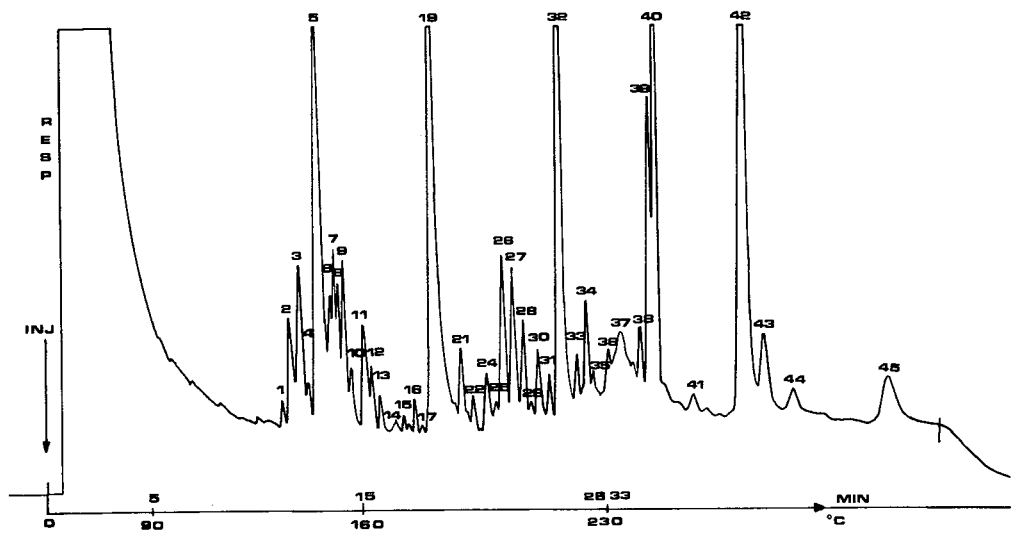


Fig. 2. Chromatogram of amino acids, cleaned by cation exchange, in the hyaloplasm of rat cerebellar tissue. Column and conditions as in Fig. 1.

(1) The method can be used in the investigation of free amino acids in cell hyaloplasm.

(2) A separation column with EGA phase and ornithine as the internal standard should be introduced.

(3) On the basis of the literature data and the available amino acids, a calibration mixture containing more standards should be prepared.

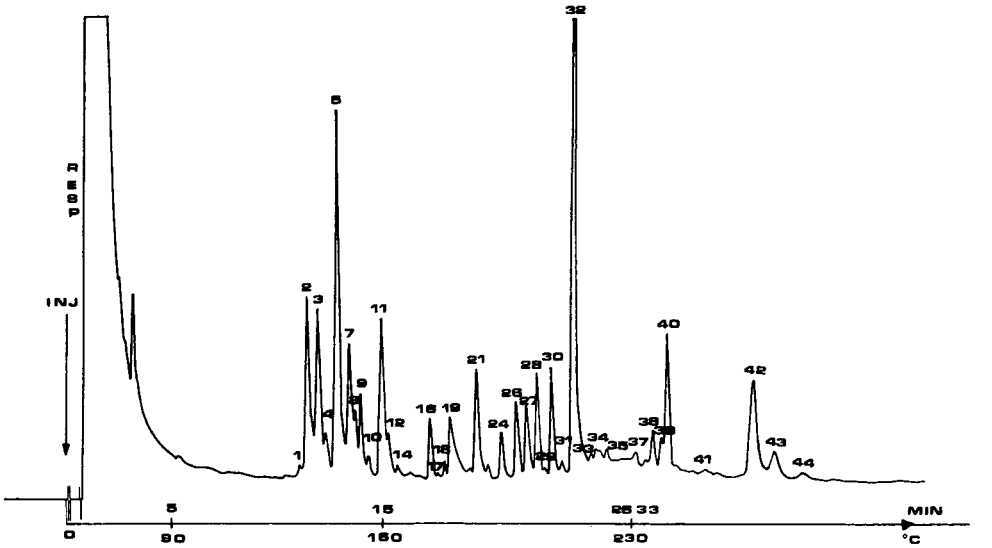


Fig. 3. Chromatogram of amino acids, cleaned by cation exchange, in the hyaloplasm of rat eye-ball tissue. Column and conditions as in Fig. 1.

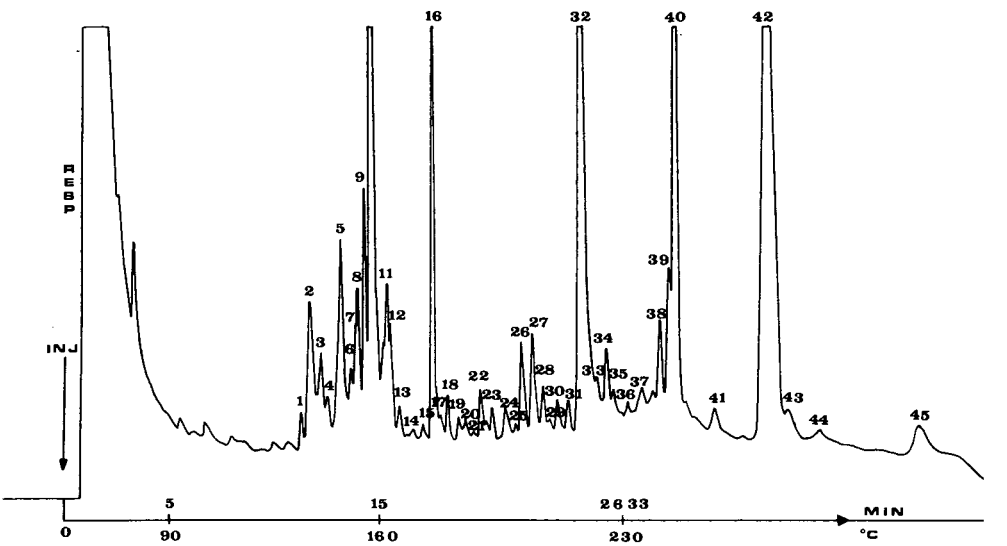


Fig. 4. Chromatogram of amino acids, cleaned by cation exchange, in the hyaloplasm of rat heart muscle tissue. Column and conditions as in Fig. 1.

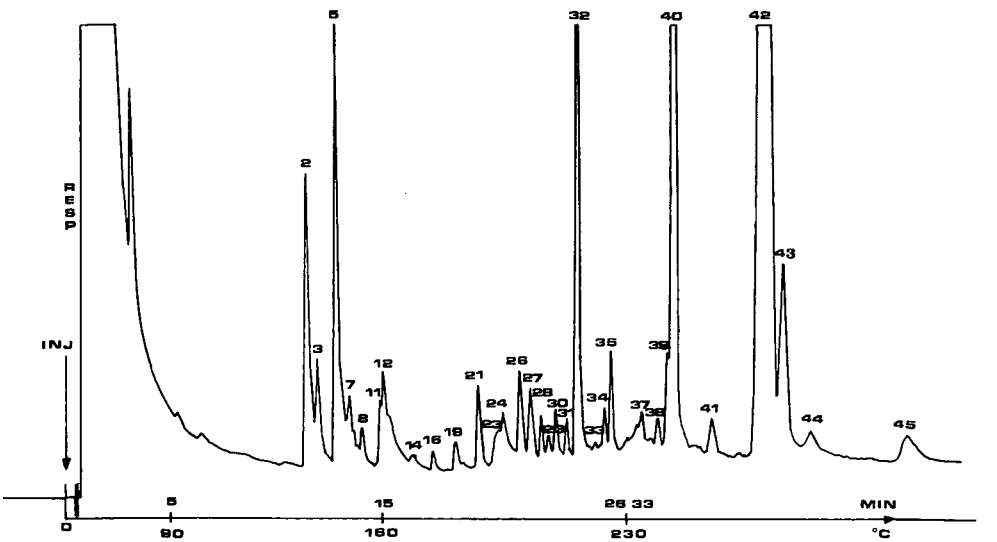


Fig. 5. Chromatogram of amino acids, cleaned by cation exchange, in the hyaloplasm of rat femoral tissue. Column and conditions as in Fig. 1.

REFERENCES

1 C.W. Gehrke, D. Roach, R.W. Zumwalt, D. Stalling and L.L. Wall, Quantitative Gas—Liquid Chromatography of Amino Acids in Proteins and Biological Substances, 1968, Columbia, Mo., p. 1.
2 C.W., Gehrke, R.W. Zumwalt and K. Kuo, J. Agr. Food Chem., 19 (1971) 605—618.
3 F.E. Kaiser, C.W. Gehrke, R.W. Zumwalt and K.G. Aue, J. Chromatogr., 94 (1974) 113—133.
4 I. Chauveau, I. Moule, C. Rouiller and I. Scheebeli, J. Cell Biol., 12 (1962) 17—29.
5 R.W. Zumwalt, D. Roach and C.W. Gehrke, J. Chromatogr., 53 (1970) 171—193.
6 V. Amico, G. Oriente and C. Tringali, J. Chromatogr., 131 (1977) 233—238.
7 R.W. Zumwalt, K. Kuo and C.W. Gehrke, J. Chromatogr., 55 (1971) 267—280.
8 E.D. Pellizzari, J.H. Brown, P. Talbot, R.W. Farmer and L.F. Fabre, Jr., J. Chromatogr., 55 (1971) 281—289.
9 C.W. Gehrke, R.W. Zumwalt, D.L. Stalling, D. Roach, W.A. Aue, C. Ponnamperuma and K.V. Kvenvolden, J. Chromatogr., 59 (1971) 305—319.
10 J. Davis, J. Chromatogr., 69 (1972) 333—339.

Journal of Chromatography, 182 (1980) 155—162
Biomedical Applications

CHROMBIO. 513

EMPLOYMENT OF GAS—LIQUID CHROMATOGRAPHY FOR THE ANALYSIS OF COLLAGEN AMINO ACIDS IN BIOPSY TISSUE

C. PERIER, M.C. RONZIERE, A. RATTNER and J. FREY

Laboratoire de Biochimie, U.E.R. de Médicine, 30 rue Ferdinand Gambon, 42023 Saint-Étienne Cedex (France)

(First received September 18th, 1979; revised manuscript received November 13th, 1979)

SUMMARY

In this paper, gas—liquid chromatography, adapted for the determination of collagen amino acids, is described. This technique was attractive for its sensitivity in that only a small amount of protein such as in 0.5 mg of tissue, especially as obtained from biopsy tissue, was needed for the separation and determination of proline (Pro), 4-hydroxyproline (4-Hyp), 3-hydroxyproline (3-Hyp), lysine (Lys), hydroxylysine (Hyl) and ε-hydroxy-norleucine (ε-OH-Norleu), the characteristic amino acids of collagen. Thus, without purification of collagen, by determining the ratio Hyl/4-Hyp and 4-Hyp/Pro it was possible to determine some anomalies in the collagen content of biopsy tissue (skin or liver). The ratio Hyl/4-Hyp allows an estimation of the lack of hydroxylation of polypeptidic lysine as in the Ehlers-Danlos syndrome type VI; and the ratio 4-Hyp/Pro allows measurement of variations in collagen content in relation to protein, especially in the liver, as in alcoholic cirrhosis.

INTRODUCTION

During the last decade, the gas—liquid chromatography (GLC) of amino acids has been developed as an analytical technique allowing the determination of free amino acids in biological fluids, and of protein amino acids [1]. Thus, it was interesting to examine the possibilities of this technique for the analysis of collagen, especially because of its particular amino acid composition characterized by two amino acids derived from polypeptidic proline (Pro), 4-hydroxypyroline (4-Hyp) and 3-hydroxyproline (3-Hyp) [2], and by two amino acids derived from polypeptidic lysine, 5-hydroxylysine (5-Hyl) and α-amino-δ-semialdehyde adipic acid or lysine aldehyde upon which depends the crosslinking of collagen [3, 4]. The latter amino acids are products of the metabolism of polypeptidic proline and polypeptidic lysine, respectively, during the post-translational step and they thus allow the different steps of collagen metabolism, to be identified.

By utilizing the advantages of GLC (high sensitivity, speed, accuracy), we were able to separate and determine the collagen amino acids, particularly 4-Hyp, 3-Hyp and Hyl. The accuracy of the method as applied to collagen was verified using pure collagen. Its usefulness was emphasized by its very small requirement for biological material, especially as obtained from skin and liver biopsies.

EXPERIMENTAL

Synthesis of ε-hydroxynorleucine

For the identification of ε-hydroxynorleucine, we synthesized this standard according to the method of Gaudry [5]. The structure of the synthesized product was verified by infrared spectrometry, mass spectrometry and the characteristic peak of its N,O-trifluoroacetyl (TFA) butyl ester was identified by GLC [6].

Preparation for biopsy tissue

The size of skin biopsies from adult patients was 0.25 cm^2. After removing fatty tissue from the biopsy material the remaining lipids were extracted with chloroform—methanol (2:1) for 24 h. The samples were then weighed and hydrolysed for 24 h at 100° in 6 *N* HCl in screw-stoppered tubes (2 mg of protein per 1 ml of 6 *N* HCl).

The samples from liver biopsies were hydrolysed directly using the same conditions. The collagen samples were hydrolysed for 6 h under the same conditions since Pro, 4-Hyp and Hyl were liberated after hydrolysis for 6 h.

After hydrolysis the solution was evaporated to dryness in vacuo and the free amino acids were derivatized to their N-TFA butyl esters and analysed by GLC.

N-Trifluoroacetylated butyl ester synthesis

For their separation by GLC, the amino acids were derivatized to the corresponding volatile N-TFA butyl esters, which were synthesized by the method described by Zumwalt et al. [7] and Kaiser et al. [8].

The first step was esterification of the carboxylic moieties in 2 ml of anhydrous *n*-butanol—HCl (1 *N*) at 100° for 2 h (for samples in the range 0.1—10 mg of amino acids). After cooling, the *n*-butanol—HCl was evaporated to dryness under a stream of dry nitrogen at 40°; the second step was acylation with trifluoroacetic anhydride (TFAA) (50 μl in 200 μl of methylene chloride) at 100° for 1 h.

Gas—liquid chromatography

The separation of the derivatized samples was performed in a gas chromatograph (GV Erba Sciences) equipped with a glass column (2 m × 3 mm I.D.). The column was silanized and filled with Gas-Chrom P 100—120 mesh coated with 3% QF-1 mixed with Gas-Chrom P 100—120 mesh coated with 1% SE-30, in the ratio 3:2 (w/w). Nitrogen was used as carrier gas at a flow-rate of 40 ml/min. The column oven temperature was programmed from 90 to 250° at a rate of 4°/min. The injection port was kept at 230° and the detection port at 270°.

Calibration curves were established for each amino acid by using the ratio of amino acid peak height over internal standard (*n*-hexadecane; Merck, Darmstadt, G.F.R.) peak height used as reference [9]. The amount of *n*-hexadecane used as internal standard was 3.45 μmoles per 250 μl of methylene chloride.

RESULTS

Specificity of the GLC separation

We have previously reported [6, 10] the separation of Pro, 4-Hyp and ε-hydroxynorleucine (ε-OH-Norleu). Fig. 1 depicts the chromatogram obtained after separation of standard amino acids. Each derivative gave only one peak. A double peak was obtained for Hyl (Koch Light Labs., Colnbrook, Great Britain), one peak for the isomers D,L-hydroxylysine and another peak for the isomers D,L-*allo*-hydroxylysine. We have verified that in pure collagen and in biopsy tissue there was only one peak for Hyl, since this amino acid was a product of the enzymatic hydroxylation of polypeptidic lysine [11].

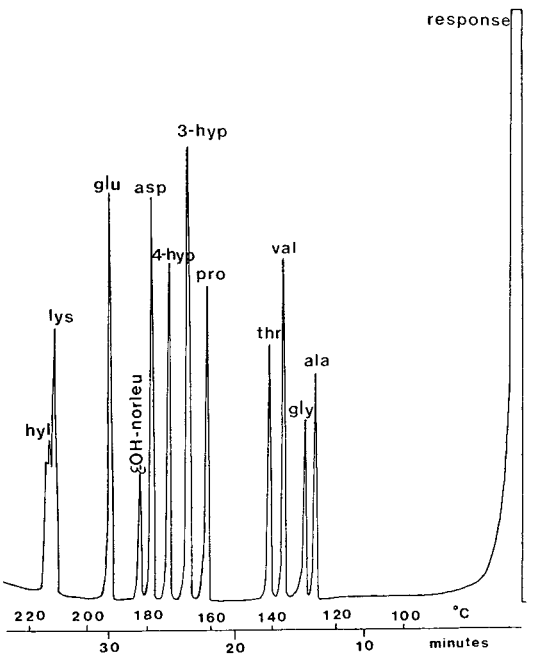


Fig. 1. GLC separation of N-TFA butyl esters of standard amino acids. Column packing: mixture of Gas-Chrom P, 100—120 mesh, coated with 3% QF-1 and coated with 1% SE-30 in the ratio 3:2 (w/w). Carrier gas: nitrogen 40 ml/min. Temperature injection port: 230°. Temperature detection port: 270°. Temperature programme: 4°/min from 90 to 250°.

Use of GLC for the separation of 3-hydroxyproline

The products of hydroxylation of polypeptidic Pro are 4-Hyp, which represents about 12% of the collagen, and 3-Hyp, which exists only in very small quantities compared to the other amino acids except in collagen from

basement membranes. 3-Hyp was purified by Dr. Szymanovicz (Reims, France) and he and his colleagues have described some properties of this amino acid [12]. 3-Hyp particularly was destroyed by oxidation with chloramine T and it was impossible to characterize this amino acid by the colorimetric methods used for the determination of 4-Hyp.

The separation obtained for 3-Hyp and 4-Hyp is shown in Fig. 1; the volatile derivatives of these amino acids gave only one peak of which the homogeneity was determined by mass spectrometry. The mass spectra were obtained on a VG 305 apparatus (Centre de Spectrométrie de Masse, Domaine Universitaire Rockfeller, Lyon, France). The fragmentation observed was different for 4-Hyp and 3-Hyp.

The ions for these two isomeric amino acids are identical, but the relative intensities of the peaks were different for the N,O-TFA butyl esters of 3-Hyp (Fig. 2A) and 4-Hyp (Fig. 2B).

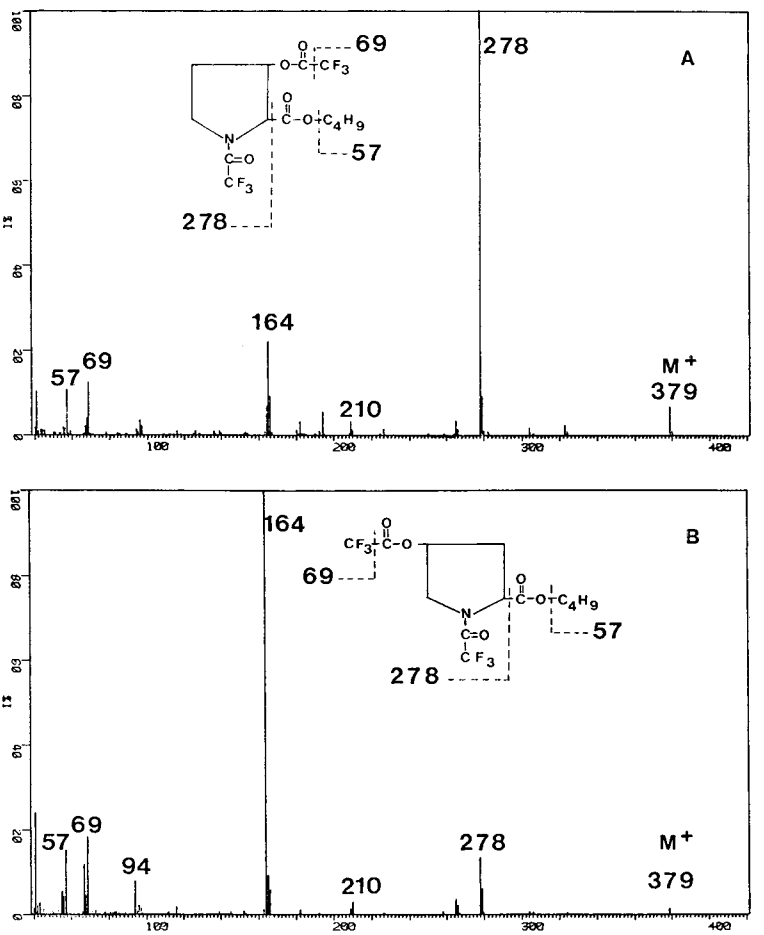


Fig. 2. Mass spectra of the N,O-TFA butyl esters of 3-hydroxyproline (A) and 4-hydroxyproline (B). Ion source energy 70 eV, source temperature 180°.

Use of GLC for the determination of collagen amino acids

The chromatograms obtained from a hydrolysate of collagen type I and type III from human cirrhotic liver (provided by O. Chevalier, C.T.C. Lyon, France) are presented in Fig. 3. Thus it was possible to determine these amino acids by this technique, and we verified that the results obtained with this technique were in good agreement with the results obtained by the usual ion-exchange chromatography. A comparison between the methods is shown in Table I, in which the ratio of each amino acid to 4-Hyp was established for collagen type I and type III.

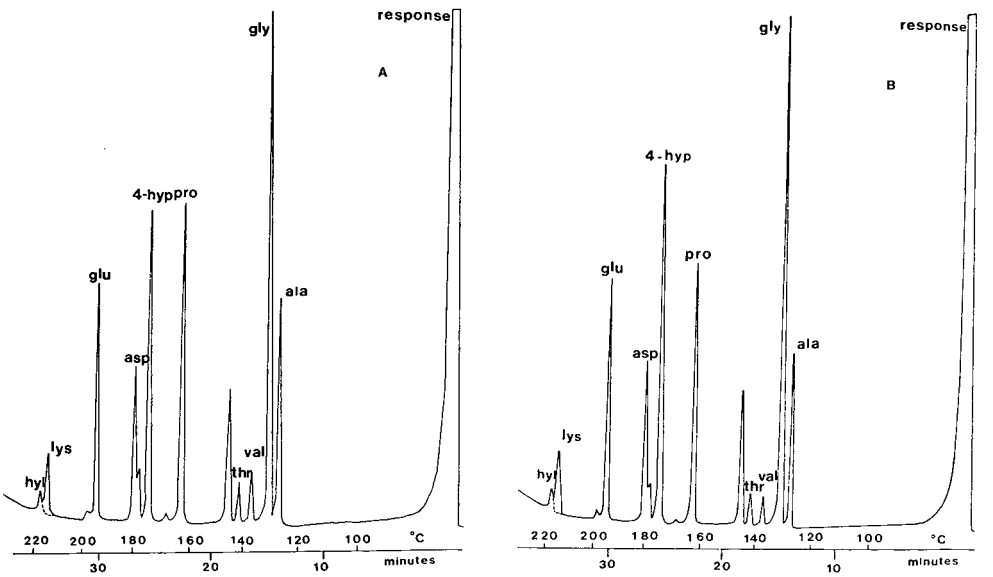


Fig. 3. Separation by GLC of N-TFA butyl esters of amino acids from a hydrolysate of cirrhotic liver collagen type I (A) and type III (B) under the same experimental procedure as in Fig. 1.

TABLE I

COMPARISON OF AMINO ACID COMPOSITION OF HUMAN CIRRHOTIC LIVER COLLAGEN BY GLC AND ION-EXCHANGE CHROMATOGRAPHY WITH REFERENCE TO 4-HYDROXYPROLINE

Amino acid	Collagen type I		Collagen type III	
	GLC	IEC*	GLC	IEC*
Glycine	3.38	3.45	2.81	2.78
Threonine	0.18	0.17	0.13	0.13
Proline	1.13	1.15	0.80	0.86
4-Hydroxyproline	1	1	1	1
Aspartic acid	0.44	0.46	0.39	0.40
Glutamic acid	0.64	0.79	0.58	0.63
Lysine	0.22	0.25	0.20	0.22
Hydroxylysine	0.07	0.09	0.065	0.08

*IEC = ion-exchange chromatography.

Use of GLC for the determination of collagen amino acids in biopsy tissue

The amount of biopsy tissue was small and it was impossible to extract collagen quantitatively from such biological samples. The method of determining the amount of collagen in the tissue studied was to determine the collagen amino acids. The analytical techniques that have been used for determining collagen amino acids in skin biopsy material involved direct colorimetric estimation without separation of the amino acids [13—15]. However, these techniques could not be used for determinations in liver biopsy material since the amount of collagen was very small. We proposed GLC, a useful tool for estimating Hyl/4-Hyp and 4-Hyp/Pro ratios, by determining these amino acids after their separation from skin or liver biopsy material. Thus the chromatograms presented in Fig. 4 were obtained from a hydrolysate of 3 mg of skin biopsy tissue (Fig. 4A) and from a hydrolysate of 0.375 mg of cirrhotic liver biopsy tissue (Fig. 4B). We verified the homogeneity of each peak by GLC—mass spectrometry (MS). In Table II are presented the results obtained for variations of the ratios 4-Hyp/Pro and Hyl/4-Hyp in skin biopsy tissue and liver biopsy tissue without microscopic anomalies. Analysis of these results

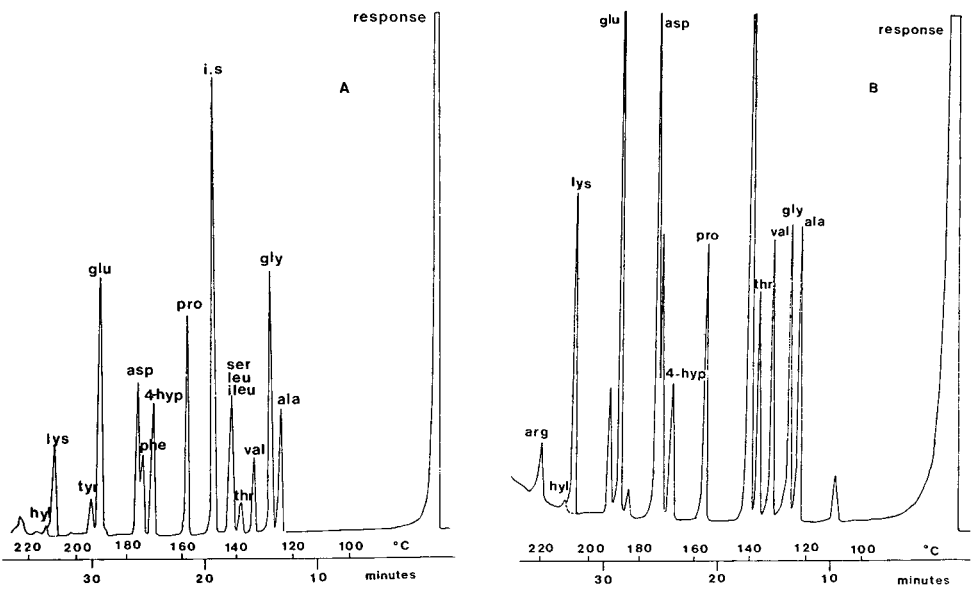


Fig. 4. Separation by GLC of N-TFA butyl esters of amino acids from a hydrolysate of 25 μg of human skin (A) and of 3 μg of human cirrhotic liver biopsy (B) in 2 μl of methylene chloride; experimental procedure as in Fig. 1.

showed that the variations observed for tissue biopsies from different patients were small for both skin and liver for 4-Hyp/Pro and Hyl/4-Hyp. These results were in good agreement with those reported elsewhere [16] for skin biopsy material.

In addition, Hyl/4-Hyp was greater for liver collagen than for skin collagen; and this ratio in liver biopsy tissue was higher than that for purified collagen

TABLE II

DISTRIBUTION OF 4-Hyp/Pro AND Hyl/4-Hyp RATIOS IN SKIN BIOPSIES AND IN LIVER BIOPSIES WITHOUT MICROSCOPIC ANOMALIES

Tissue	4-Hyp/Pro	Hyl/4-Hyp
Human adult skin	$\overline{m}$ = 0.56	$\overline{m}$ = 0.041
(8 biopsies)	σ = 0.028	σ = 0.0087
Human adult liver	$\overline{m}$ = 0.19	$\overline{m}$ = 0.080
(14 biopsies)	σ = 0.027	σ = 0.013

type I and type III (see Table I). This difference could be due to the fact that the Hyl content was determined without separating each type of collagen from liver as collagen type I and type III and basement membrane collagen type A, type B and type E. Indeed, the Hyl content of collagen from basement membranes was higher than that of interstitial collagen type I and type III [17].

DISCUSSION

The results presented in this paper show that GLC is an analytical tool for the separation and determination of collagen amino acids. The advantages of this technique — sensitivity, accuracy, speed — enable its use for determining Pro, 4-Hyp, 3-Hyp and Hyl in a single operation; elsewhere [13, 14], these amino acids have been determined individually in the total hydrolysates by direct colorimetric estimation. In addition, GLC is more sensitive and thus it was possible to repeat these determinations many times from 0.375 mg of liver biopsy tissue since for a chromatographic run only 2 μl from 250 μl of the initial preparation were injected into the column. This possibility was particularly convenient for the determination of Hyl.

Our results, presented in Table II, demonstrate that for skin or liver, these determinations carried out on different samples from different patients yielded a relative constancy for the ratios 4-Hyp/Pro and Hyl/4-Hyp. Indeed, we have verified the constancy of these ratios in duplicate experiments from identical biological samples (skin biopsy). Thus, the values obtained for 4-Hyp/Pro were identical (0.58) and for Hyl/4-Hyp the values lay between 0.037 and 0.039. Thus, under these conditions it was possible to value, in vivo, the lack of hydroxylation of polypeptidic lysine from a skin biopsy as was described by Elsas et al. [18]. In the case reported by these authors the ratio Hyl/4-Hyp was 0.011, and a normal value 0.05 ± 0.009. In addition, it will be possible to determine the variations in collagen content in relation to proteins in a tissue, by measuring 4-Hyp/Pro; this estimation will be particularly suitable for liver biopsies. Indeed, in alcoholic cirrhosis, during which the amount of collagen increases, we have found values for this ratio of between 0.30 and 0.70; thus we could value the extent of hepatic fibrosis. In addition, the ratio 3-Hyp/4-Hyp will allow the measurement of variations in collagen from basement membrane compared to interstitial collagen, since 3-Hyp is a label of collagen from basement membranes.

ACKNOWLEDGEMENTS

This work was supported by grants from D.G.R.S.T. (A.C.C. Chimie Analytique No. 74.7.0763) and C.N.R.S. (RCP 533).

REFERENCES

1 P. Hušek and K. Macek, J. Chromatogr., 113 (1975) 139—230.
2 P.M. Gallop and M.A. Paz, Physiol. Rev., 55 (1975) 418—487.
3 R.C. Siegel, Proc. Nat. Acad. Sci. U.S., 71 (1974) 4826—4830.
4 A. Schneider, E. Henson, O.O. Blumenfeld and P.M. Gallop, Biochem. Biophys. Res. Commun., 27 (1967) 546—551.
5 R. Gaudry, Can. J. Res., 26 (1948) 387—392.
6 C. Perier, M.C. Ronzière, A. Rattner and J. Frey, J. Chromatogr., 125 (1976) 526—529.
7 R.W. Zumwalt, D. Roach and C.W. Gehrke, J. Chromatogr., 53 (1970) 171—193.
8 F.E. Kaiser, C.W. Gehrke, R.W. Zumwalt and K.C. Kuo, J. Chromatogr., 94 (1974) 113—133.
9 J. Desgres and P. Padieu, personal communication.
10 C. Perier, J. Farjanel, M. Therme, M. Brunel and J. Frey, Biochimie, 56 (1974) 1003—1006.
11 U. Poistola, T.M. Turpeenniemi, R. Myllyla and K.I. Kivirikko, in L. Robert and A.M. Robert (Editors), Biochemistry of Normal and Pathological Connective Tissues, C.N.R.S., Paris, 1978, pp. 123—124.
12 G. Szymanovicz, O. Mercier, A. Randoux and J.P. Borel, Biochimie, 60 (1978) 499—503.
13 N. Blumenkrantz and G. Asboe-Hansen, Clin. Chem., 7 (1974) 251—257.
14 N. Blumenkrantz and G. Asboe-Hansen, Clin. Chem., 8 (1975) 177—183.
15 N. Blumenkrantz, S. Ullman and G. Asboe-Hansen, Acta Derm. Venereol., 56 (1976) 93—98.
16 N. Blumenkrantz and G. Asboe-Hansen, Acta Derm. Venereol., 58 (1978) 359—361.
17 M. Rojkind, M.A. Giambrone and L. Biempica, Gastroenterology, 76 (1979) 710—719.
18 L.J. Elsas, R.L. Miller and S.R. Pinnell, J. Pediatr., 92 (1978) 378—384.

Journal of Chromatography, 182 (1980) 163—169
Biomedical Applications

CHROMBIO. 511

QUANTITATION OF THE ANTITUMOR AGENT N-(PHOSPHONACETYL)-L-ASPARTIC ACID IN HUMAN PLASMA AND URINE BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY—SELECTED ION MONITORING

ALAN R. BRANFMAN*, ROBERT J. BRUNI, YOON N. MERRILL and MARJORY CHADWICK

BioMolecular Sciences Section, Arthur D. Little, Inc., Acorn Park, Cambridge, Mass. 02140 (U.S.A.)

JOHN M. STRONG

Laboratory of Chemical Pharmacology, National Cancer Institute, National Institutes of Health, Bethesda, Md. 20205 (U.S.A.)

and

THOMAS J. ERVIN

Department of Medical Oncology, Sidney Farber Cancer Institute, Boston, Mass. 02115 (U.S.A.)

(Received September 17th, 1979)

SUMMARY

N-(Phosphonacetyl)-L-aspartic acid (PALA) is an antitumor agent which is currently under clinical study. A gas chromatography—mass spectrometry—selected ion monitoring assay procedure using [^{13}C]PALA as the internal standard has been developed for the quantitation of PALA in biological samples. Standard curves which related ion intensity peak height ratios (*m/e* 220/221) to PALA concentrations in plasma and urine were described by a non-linear least square analysis with correlation coefficients of $R^2 > 0.995$ and > 0.996, respectively. Over concentration ranges for PALA of 1—60 μg/ml of plasma and 1—160 μg/ml of urine the coefficient of variation from the fitted curve was 4—18%. This methodology has been used to quantitate PALA in human plasma samples in a study on the clinical pharmacology of the drug.

*To whom correspondence should be addressed.

INTRODUCTION

The potent antitumor agent [1] N-(phosphonacetyl)-L-aspartic acid (PALA, NSC 224131) is currently under clinical study by the National Cancer Institute. The drug is reported to act as a transition-state inhibitor of the enzyme aspartate transcarbamylase [2].

A sensitive and specific method of analysis for PALA in plasma was needed to provide clinical pharmacokinetic data. Recently two methods utilizing gas chromatography—mass spectrometry—selected ion monitoring (GC—MS—SIM) have been reported. The method of Strong and co-workers [3, 4] utilizes N-(phosphonacetyl)-L-glutamic acid as the internal standard, and that of Roboz et al. [5] involves quantitation of phosphonacetic acid after hydrolysis of PALA and utilizes phosphonpropionic acid as the internal standard [6]. Our method utilizes [^{13}C]PALA as internal standard. A preliminary account of this work was presented at a recent mass spectrometry conference [7].

EXPERIMENTAL

Materials

PALA disodium salt (77% purity, Lot No. HE 21-84-1) and [^{14}C]PALA (95% purity, sp. act. 32.9 μCi/mg, Lot No. 2333-32) were supplied by Drug Research and Development, Divison of Cancer Treatment, National Cancer Institute, U.S.A. Diazomethane in chloroform was prepared from N-methyl-N'-nitro-N-nitrosoguanidine (Aldrich, Milwaukee, Wisc., U.S.A.) in a diazomethane reaction vessel (Kontes, Vineland, N.J., U.S.A.). Ethereal diazomethane was generated from Diazald® (Aldrich). Saturated HCl in methanol was prepared by bubbling anhydrous HCl gas through methanol for about 30 min.

Synthesis of [^{13}C]PALA

[^{13}C]PALA (Fig. 1, IV) was prepared as follows from [^{13}C]phosphonacetyl chloride (Fig. 1, II) and L-aspartic acid dibenzyl ester (Fig. 1, III) according to the method of Starks Associates [8]. Diazomethane in diethyl ether (ca. 40 mmol) was added dropwise to a solution of 1 g (10.6 mmol) of [^{13}C]chloroacetic acid (1-^{13}C, 90% enriched) KOR Isotopes (Cambridge, Mass., U.S.A.) (Fig. 1, I) in 20 ml of diethyl ether. After 1 h stirring at room temperature, excess reagent and solvent were removed under a stream of nitrogen. The residual methyl chloro-[^{13}C]acetate was added under nitrogen to 7 ml of freshly distilled triethyl phosphite (b.p. 156°, Aldrich) and the solution was heated at reflux for 3 h [9]. The excess reagent was removed by short-path vacuum distillation and the remaining oil was vacuum distilled in a Kugelrohr bulb apparatus (Aldrich) to give 1.7 g (75%) of methyl diethoxyphosphinyl-[^{13}C]acetate, 80% purity by GC. The molecular ion observed at *m/e* 211 by GC—MS confirmed the structure as the desired intermediate. The methyl diethoxyphosphinyl[^{13}C]acetate (1.7 g, 7.9 mmol) was hydrolyzed in 13 ml of concentrated HCl by heating at reflux for 4 h to give 0.74 g (66%) of[^{13}C]-phosphonacetic acid after recrystallization from glacial acetic acid.

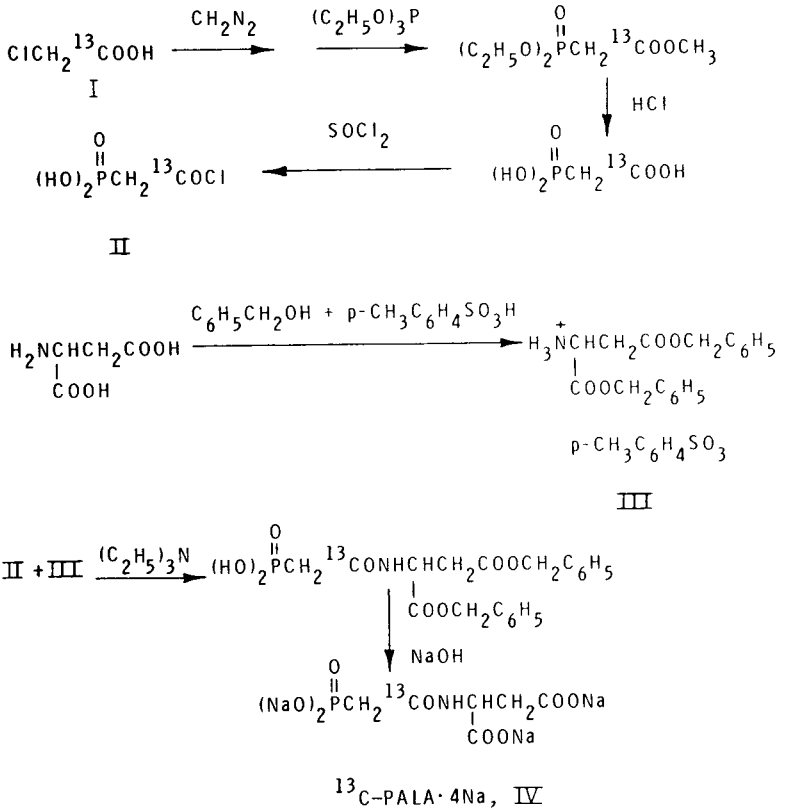


Fig. 1. Synthetic route to the internal standard, N-(phosphon-[^{13}C]acetyl)-L-aspartic acid.

A mixture of 0.74 g (5.2 mmol) of [^{13}C]phosphonacetic acid and 6 ml of thionyl chloride (Aldrich, freshly redistilled before use by maintaining the distillation flask temperature no higher than necessary for slow distillation) was stirred and heated to 50° for 16 h. Excess thionyl chloride was removed under vacuum and with the aid of dry benzene (distilled over sodium metal). The residual oil, [^{13}C]phosphonacetylchloride (Fig. 1, II) was dissolved in 5 ml of dry dioxane (distilled over sodium metal) and used directly for the next step.

L-Aspartic acid dibenzyl ester *p*-toluene sulfonate (3.05 g, 6.28 mmol), prepared according to the method of Zervas et al. [10], was dissolved in 20 ml of dry dioxane containing 1.75 ml (12.6 mmol) of triethylamine. The solution of [^{13}C]phosphonacetyl chloride (Fig. 1, II) in dioxane from the previous step was added dropwise with stirring. The temperature was maintained below 30° during the addition and then at room temperature for two additional hours. The insolubles were filtered off, the filtrate was concentrated in vacuo, and the oily residue was dissolved in methylene chloride. The methylene chloride solution was washed several times with water and then stirred with 45 ml of 0.7 *N* NaOH at 15° for 1 h and at 5° for 17 h. The aqueous layer was washed with methylene chloride and then with diethyl ether, concentrated to ca. 3 ml

and added dropwise to 30 ml of methanol with rapid stirring. The solid product was collected, redissolved in 40 ml of water and treated with 9 g of washed Bio-Rad AG 50W-X8, H^+ form cation-exchange resin (Bio-Rad Labs., Richmond, Calif., U.S.A.). The solution of the free acid was neutralized to pH 8 with aqueous NaOH, concentrated, and the sodium salt precipitated in methanol as before. The vacuum-dried [^{13}C]PALA tetrasodium salt (Fig. 1, IV) weighed 1.0 g (55%). Analysis: calculated for $C_5{}^{13}CH_6NNa_4O_8P \cdot 2.1\ H_2O$: C, 19.14; H, 2.69; N, 3.67; Na, 24.08; P, 8.11; H_2O, 10.08. Found: C, 19.22; H, 2.75; N, 3.59; Na, 23.89; P, 8.18; H_2O, 10.08.

Collection of clinical samples

PALA was administered to patients by a 5-day i.v. infusion. Blood and urine samples were collected at intervals up to 6 days after starting the infusion. Blood samples were transferred to heparinized tubes. Plasma was obtained by centrifugation and was frozen. Plasma and urine samples were stored at −20°.

Extraction of PALA from human plasma and urine

The standard curve samples were prepared in human plasma and urine over ranges of 1—60 μg/ml and 1—160 μg/ml, respectively. [^{13}C]PALA (5 μg) was added to 0.2 ml of each clinical sample and PALA and [^{13}C]PALA were extracted by the procedure reported previously [3]. PALA and [^{13}C]PALA methyl esters were finally extracted into 100 μl of benzene, of which 2 μl were analyzed by GC—MS—SIM. The GC column was preconditioned with 5 injections of 2 μg of PALA tetramethyl ester. Standard curves were constructed with each set of patient samples.

Gas chromatography—mass spectrometry—selected ion monitoring

Quantitation was performed on a DuPont 21-491 mass spectrometer operated at 70 eV and interfaced with a Hewlett-Packard 5700A gas chromatograph. A DuPont 21-095 multiple specific ion detector was used for selected ion current measurements. A DuPont 21-094 data system was used for scanning mode data acquisition and processing. A 1.2 m × 2 mm I.D. glass column packed with 3% OV-17 on 100—120 mesh Gas-Chrom Q (Supelco, Bellefonte, Pa., U.S.A.) was used. The injection port, flame ionization detector, column, glass jet separator and ion source temperatures were set at 250°, 300°, 240°, 275° and 200°, respectively. The helium carrier gas flow-rate was 20—25 ml/min. A GC splitter and a micro needle control valve allowed selected passage of GC effluent into the mass spectrometer via the jet separator.

Standard curves by the NONLIN program

Standard curves were prepared by a least square fitting of the standard curve sample data to the equation:

$$R = (x + A)/(Bx + C)$$

where R is the ion ratio m/e 220 to m/e 221, x is the quantity of PALA added and A, B, and C are constants [11]. The least square analysis was carried out using a computer program called NONLIN [12]. Initial estimates of A, B, and C; limits on the possible values of A, B, and C; and the ion ratio versus concen-

tration data from the analyses of the standard curve samples are given as input to the program. A typical set of R values is presented in Table I.

TABLE I

TYPICAL SET OF R VALUES

x (μg/ml)	R*
0	0.143
10	0.606
20	1.095
30	1.427
40	1.801
50	2.158
60	2.477

*Each R value is a mean of nine replicate measurements.

The initial estimate of B is the ratio of the m/e 221 to m/e 220 ions in the mass spectrum of the PALA tetramethyl derivative and is calculated to be 0.13. C is the amount of internal standard ([^{13}C]PALA) multiplied by the isotopic purity. [^{13}C]PALA (5 μg) was added to 0.2 ml plasma samples and C is calculated to be 5 μg × (1.0/0.2)ml × 0.9 = 22.5 μg/ml. The [^{13}C]PALA used has a 90% isotopic purity. The sum of A and C is approximately the total amount of internal standard used and therefore $A = 5 \times 1.0/0.2 - C = 2.5$. The limits of A, B, and C are set in Table II.

TABLE II

THE LIMITS OF A, B, and C

	A	B	C
Initial estimate	2.5	0.13	22.5
Lower limit	0.5	0.05	10
Upper limit	5	0.3	30

Once the least square best fit values of A, B and C were found, they were used in the rearranged equation:

$$x = (RC - A)/(1 - RB)$$

to calculate the amount of PALA in each unknown plasma sample.

RESULTS AND DISCUSSION

The PALA extraction procedure [3] used here involved addition of the internal standard to samples of plasma or urine and precipitation of the protein and drug with acetone. The drug was extracted into tetrahydrofuran—methanol—hydrochloric acid, dried, and methylated with diazomethane. The recovery

of PALA from plasma and urine averaged 89.2 ± 2.8% (S.D.) and 102.8 ± 2.0% (S.D.), respectively, as determined with ^{14}C-labeled drug.

The methyl derivatives of PALA and [^{13}C]PALA co-chromatograph as a single GC peak (retention time 4 min) which allows simultaneous measurement of the monitored ions in the mass spectrometer. The mass spectral ions of interest were *m/e* 151 and 220 for PALA [13] and *m/e* 152 and 221 for [^{13}C]PALA methyl derivatives. Although measurement of *m/e* 151, the base ion, would result in the highest sensitivity, *m/e* 220 (50% relative intensity) was measured to avoid interference from a substance in plasma which chromatographed close to PALA and possessed an ion at *m/e* 151.

Standard curves which related ion intensity peak height ratios to PALA concentrations in plasma (Fig. 2) and urine (Fig. 3), were described by a non-linear least square analysis with correlation coefficients of R^2 >0.995 and >0.996, respectively. The values ± S.D. for the constants *A*, *B* and *C* were 2.905 ± 0.591, 0.092 ± 0.015 and 19.939 ± 0.948, respectively. Over concentration ranges for PALA of 1—60 μg/ml of plasma and 1—160 μg/ml of urine the coefficient of variation from the fitted curve was 4—18%. The applicability of using internal standards possessing single mass unit differences in selected ion monitoring analysis has been discussed recently by Horie and Baba [14].

Typical ion chromatograms from the analysis of plasma samples from a patient on PALA therapy are shown in Fig. 4. PALA concentrations in the plasma of a cancer patient being treated with PALA at 1800 mg/m^2 /day by continuous i.v. infusion for 5 days are shown in Fig. 5. PALA concentrations varied between 15 and 40 μg/ml during the infusion and decreased to ca. 1 μg/ml, the lower limit of sensitivity of the method by 24 h after the end of the infusion. Further work is in progress on the quantitation of PALA in clinical urine samples and interpretation of the pharmacokinetic data will be reported in a future paper [15].

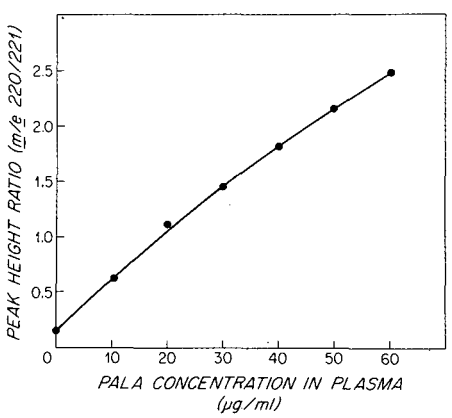


Fig. 2. Standard curve for PALA in plasma.

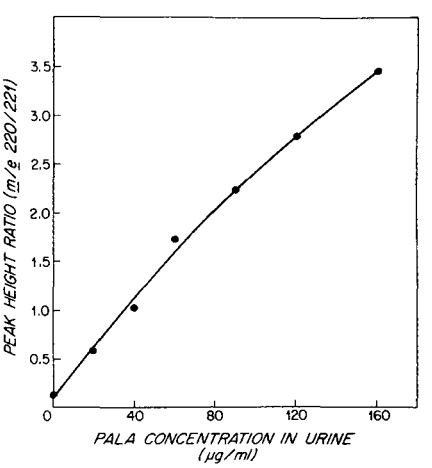
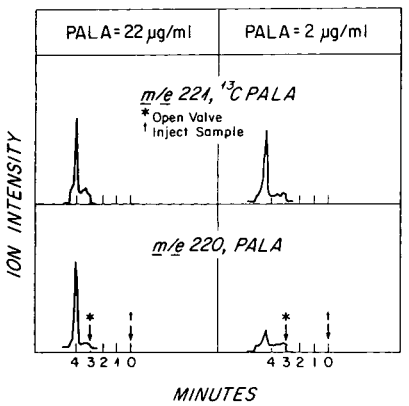


Fig. 3. Standard curve for PALA in urine.



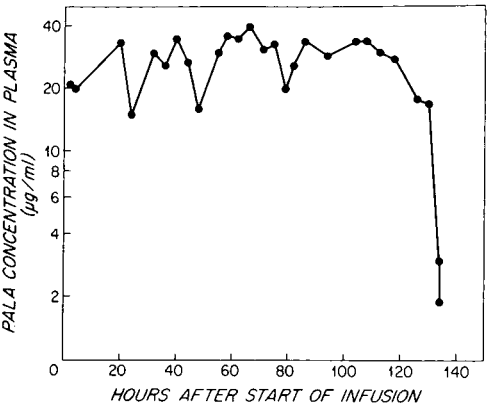


Fig. 4. PALA selected ion chromatograms.

Fig. 5. PALA concentrations in the plasma of a cancer patient.

ACKNOWLEDGEMENT

This investigation was supported by Contract No. N01-CM-87163 from the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health.

REFERENCES

1 R.K. Johnson, T. Inouye, A. Goldin and G.R. Stark, Cancer Res., 36 (1976) 2720.
2 K.D. Collins and G.R. Stark, J. Biol. Chem., 246 (1971) 6599.
3 J.M. Strong, Y.E. Kinney, A.R. Branfman and R.L. Cysyk, Cancer Treat. Rep., 63 (1979) 775.
4 J.M. Strong, Y.E. Kinney, A.R. Branfman and C. Erlichman, 6th International Symposium on Mass Spectrometry in Biochemistry and Medicine, Venice, Italy, June 1979, Abst. 53.
5 J. Roboz, R. Suzuki, E. Rose and T. Ohnuma, 27th Annual Conference on Mass Spectrometry and Allied Topics, June 1979, Abstr. WAMOA3.
6 J. Roboz, R. Suzuki, G. Bekesi and R. Hunt, Biomed. Mass Spectrom., 4 (1977) 291.
7 R.J. Bruni, Y. Merrill, A.R. Branfman, J.M. Strong and T.J. Ervin, 27th Annual Conference on Mass Spectrometry and Allied Topics, June 1979, Abstr. MAMP9.
8 Starks Associates, Buffalo, N.Y., private communication via the National Cancer Institute (U.S.A.).
9 R.M. Davidson and G.L. Kenyon, J. Org. Chem., 42 (1977) 1030.
10 L. Zervas, M. Winitz and J.P. Greenstein, J. Org. Chem., 22 (1957) 1515.
11 B.H. Min, W.A. Garland, K.C. Khoo and G.S. Torres, Biomed. Mass Spectrom., 5 (1978) 692.
12 C.M. Metzler, A Users Manual for NONLIN, Upjohn Company, Kalamazoo, Mich., U.S.A., 1976.
13 A.R. Branfman, K.H. Valia and R.J. Bruni, J. Chromatogr., 151 (1978) 71.
14 M. Horie and S. Baba, Biomed. Mass Spectrom., 6 (1979) 63.
15 M. Chadwick, D.M. Silveira, J.A. MacGregor, A.R. Branfman, R.H. Liss and D.W. Yesair, Cancer Res., in press.

Journal of Chromatography, 182 (1980) 171—177
Biomedical Applications

CHROMBIO. 516

DETERMINATION OF PEPTIDO-AMINOBENZOPHENONE (2-*o*-CHLOROBENZOYL-4-CHLORO-N-METHYL-N′-GLYCYL-GLYCINANILIDE) BY ELECTRON-CAPTURE GAS—LIQUID CHROMATOGRAPHY

TOMOYUKI AGOH, MASAHARU KONISHI and YOSHIO MORI

Department of Analytical Chemistry, Shionogi Research Laboratory, Shionogi & Co. Ltd., Fukushima-ku, Osaka 553 (Japan)

(Received October 26th, 1979)

SUMMARY

A gas—liquid chromatographic method for the determination of peptido-aminobenzophenone (2-*o*-chlorobenzoyl-4-chloro-N-mehtyl-N′-glycyl-glycinanilide, I) in dog plasma was developed. Decomposition of compound I was observed during chromatography. In alkaline medium, compound I in plasma was submitted to ring closure to yield 3-amino-6-chloro-5-(2-chlorophenyl)-1-methylquinolin-2-one (aminoquinolone), and the hexane extract was assayed by gas—liquid chromatography using electron-capture detection. The concentration range of compound I studied was 10—90 ng per 0.5 ml of plasma. Interference from both endogenous and exogenous sources was negligible. The method could be applied to the determination of the plasma level of compound I in dogs following oral administration of a single 5 mg/kg dose.

INTRODUCTION

Peptido-aminobenzophenone (2-*o*-chlorobenzoyl-4-chloro-N-methyl-N′-glycyl-glycinanilide, I) is one of a new series of minor tranquilizers recently developed in our laboratory [1]. Numerous drugs are extensively used as tranquilizers, among them 1,4-benzodiazepines [2].

Peptido-aminobenzophenone is known as a pro-drug of 1,4-benzodiazepine because chlorodiazepam [7-chloro-1,3-dihydro-1-methyl-5-(2-chlorophenyl)-2-H-1,4-benzodiazepin-2-one, II] was identified as a major metabolite in plasma after administration of compound I [3].

In general, as the dosages of minor tranquilizers are low, high sensitivities are required to measure the amounts of these drugs in biological fluids. Pulse polarography [4], fluorimetry [5], radioimmunoassay [6], high-performance liquid chromatography [7] and gas—liquid chromatography (GLC) using

electron-capture detection (EC-GLC) [8] have been used to measure 1,4-benzodiazepines, the most extensively used technique being EC-GLC [9, 10]. The metabolites of compound I in dogs, as shown in Fig. 1, are chlorodiazepam (II), chlorodesmethyldiazepam [7-chloro-1,3-dihydro-5-(2-chlorophenyl)-2H-1,4-benzodiazepin-2-one, III], lorazepam (IV) and an oxalanilic acid derivative [2-(2-chlorobenzoyl)-4-chloro-N-methyloxalanilic acid, V] in urine, and also compounds II and III in plasma [3].

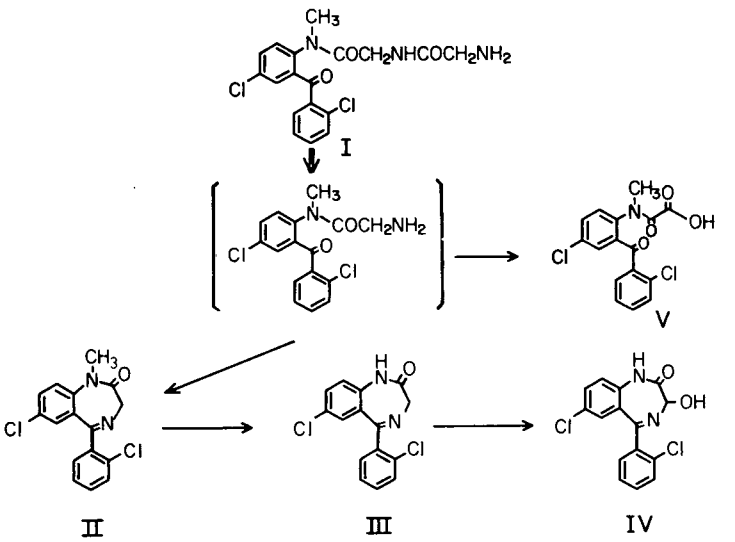


Fig. 1. Postulated metabolic pathway of peptido-aminobenzophenone in dogs [3].

EG-GLC could offer a useful method for measuring the amount of compound I and its metabolites, which show high sensitivities for EC-GLC. But differentiation of compound I from these metabolites is necessary with biological samples.

This paper describes a sensitive and selective EC-GLC method for the determination of compound I in dog plasma.

EXPERIMENTAL

Chemicals and reagents

Compound I and other required compounds (II, III, IV and VI) were synthesized in our laboratory. Solvents used were of a special grade for EC-GLC (Wako, Osaka, Japan), while other chemicals were of reagent grade and were used without further purification.

Gas—liquid chromatography

A Shimadzu Model 4CMPFE gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a 10-mCi ^{63}Ni electron-capture detector was used with a 0.5 m × 3 mm I.D. glass column filled with 3% OV-17 on 100—120 mesh Gas-Chrom Q. The column temperature was kept at 240° after conditioning with a nitrogen

flow-rate of 20 ml/min for 48 h at 330°. The detector and injection port temperatures were 300°. The nitrogen flow-rate was 100 ml/min, and the pulse mode was of an 800-μsec frequency with an 8-μsec width.

The structures and retention times of the compounds in this assay are listed in Table I.

TABLE I

RETENTION TIMES IN EC-GLC OF COMPOUNDS RELATED TO COMPOUND I

	II	III	IV	VI	Diazepam
Retention time (min)	3.9	5.1	1.7	6.0	2.0

Preparation of standard solutions

Aminoquinolone. Solutions containing 6—80 ng/ml of compound VI and 60 ng/ml of diazepam were prepared in toluene.

Internal standard. A solution containing 60 ng/ml of diazepam in toluene was used.

Peptido-aminobenzophenone. Ethanolic solutions in the concentration range 0.2—1.8 μg/ml were used.

Procedure for determination of compound I in plasma

A 12-ml centrifuge tube containing 0.5 ml of plasma, 0.4 ml of distilled water and 0.1 ml of 10 *N* potassium hydroxide solution was gently shaken, then heated on a water bath at 95 ± 1° for 60 min. After cooling, the mixture was extracted twice with 7 ml and 5 ml of *n*-hexane on a mechanical shaker. The *n*-hexane extracts were combined in flasks containing 1 ml of the standard solution of diazepam, then the solvent was evaporated to dryness in vacuo at 40°. The residue was dissolved in 1 ml of toluene and a 2-μl sample was subjected to gas chromatography. Calculations were done using a calibration curve prepared by the peak height ratio method.

RESULTS AND DISCUSSION

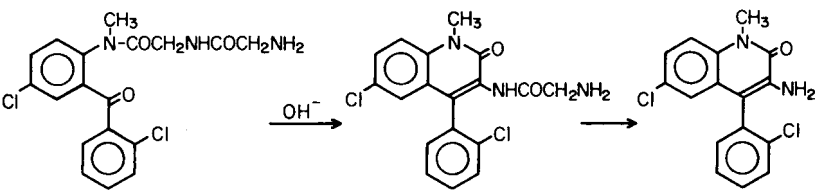
Derivatization of compound I

Compound I was pyrolyzed during GLC to give 2,5′-dichloro-2-methylaminobenzophenone (VII), chlorodiazepam (II) and aminoquinolone (VI) in low yield. Therefore, the following derivatizations of compound I were performed in order to find a suitable compound for analysis by GLC.

Both the carbamate derivative of compound I obtained by reaction with chloromethylformate and the acetyl derivative with acetic anhydride were pyrolyzed during GLC and gave no noticeable peaks.

Reduction of compound I with sodium borohydride produced the benzhydrol derivative in an unsatisfactory yield. Thus, when compound I was heated in ethanol in the presence of potassium carbonate, ring closure leading to the glycylaminoquinolone derivative [6-chloro-5-(2-chlorophenyl)-4-glycylamino-1-methylquinolin-2-one, VIII] occurred, but decomposition of compound VIII was observed during GLC. However, aminoquinolone (VI) obtained after hydrolysis of VIII, as shown below, was thermostable and

possessed good GC properties, which allowed the analysis of compound I. Next, the reaction causing both cyclization and hydrolysis of the glycine moiety to obtain compound VI in one step was investigated. With 5% potassium carbonate in ethanol at 72°, cyclization of compound I to compound VIII was easy, but further conversion into compound VI was incomplete even after 220 min. To complete the reaction, treatment with 1 *N* potassium hydroxide in ethanol was required for 4 h at 75°. Reaction at the higher temperature of 92° with 1 *N* potassium hydroxide in *n*-propanol afforded a considerable reduction in reaction time from 4 to 2 h; the yield was about 85%. With 1 *N* potassium hydroxide in water at 95°, the reaction time was shortened to 30 min, with a yield of 75%.



I VIII VI

To simplify the determination procedure, the assay reaction was performed directly in the plasma solution containing 1 *N* potassium hydroxide without preliminary extraction of compound I. Another merit is that this method prevents sample loss during extraction and enhances the precision of the determination.

Interference

No endogenous plasma components interfered with this assay, as shown in Fig. 2. The inverted peak appearing at the retention time of 6.6 min was assumed to have arisen from endogenous cholesterol.

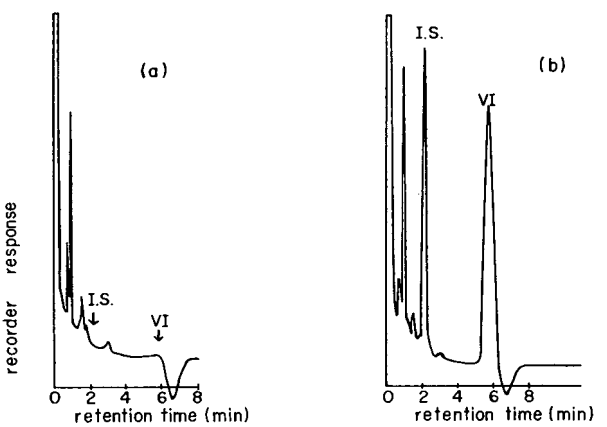


Fig. 2. Gas chromatograms of control dog plasma (a) and of dog plasma after dosing with peptido-aminobenzophenone (b).

Recovery of compounds II and III from plasma containing 2 μg of each compound after the assay reaction was 5% and 7%, respectively, and no interference was observed because they were subjected to alkaline hydrolysis to form aminobenzophenone derivatives having smaller retention times.

Compounds VI and VIII, possible metabolites after administration of compound I, were not found in the plasma samples; their presence would lead to serious errors in the determination.

Choice of an internal standard

Stability of the standard is the foremost requirement in the internal standard method. Because of the strong alkalinity of the reaction medium, a hydrocarbon, 9,10-diphenylanthracene, was tentatively selected as the internal standard, but it caused deterioration of the column within a moderate period of use. Thus, it was replaced by diazepam. Diazepam showed good chromatographic properties and could be separated from all metabolites and endogenous components in plasma. However, as diazepam was hydrolysed by alkali during extraction, it had to be added to the extract fractionated. Accordingly, extraction was performed twice in order to minimize the experimental errors.

Calibration curve

When the ratio of the peak height of compound VI to that of diazepam was plotted against the concentration of compound VI in the range 6—80 ng/ml, the calibration curve obtained gave a satisfactory linearity: $r = 0.999$, C.V. = 3.4%.

Sensitivity

Sensitivities for EC-GLC of compounds related to compound I in this assay are shown in Table II. High electron-capture potency was seen in compound VI as well as in 1,4-benzodiazepine metabolites II and III. In this assay, the limit of detection for compound I was 9 ng per 0.5 ml of plasma.

TABLE II

SENSITIVITIES IN EC-GLC OF COMPOUNDS RELATED TO COMPOUND I

Compound	Molecular weight	Minimum detectable amounts (pg/injection)	Sensitivity (ampere per sec per mole)
II	319	2.0	32
III	305	3.0	15
VI	319	5.0	7
Diazepam	283.5	3.0	31

Extraction solvent

The recovery of compound VI examined with dichloromethane, *n*-hexane, ethyl acetate and benzene, was 99, 97, 93 and 92%, respectively. Considering the interference of endogenous substances and contamination, *n*-hexane, the most non-polar solvent, was selected as the extraction solvent.

Recovery studies

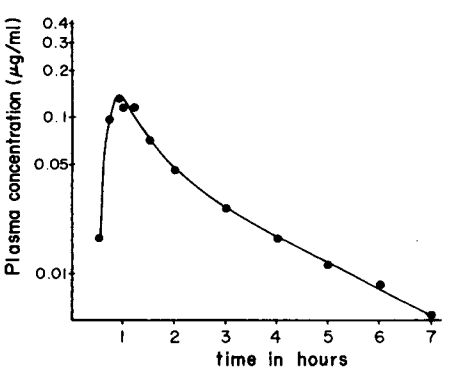
Solutions containing 10, 30, 60, and 90 ng per 0.5 ml of compound I were prepared by adding 50 μl of an ethanolic solution of compound I to 0.5 ml of heparinized dog plasma. Each solution was analyzed according to the procedure described above. The mean percentage recovery of compound I, calculated from a total of 18 analyses, was 75.5% (S.D. = 7.9%).

Metabolites

In the assay procedure described above, the peaks due to the metabolites II and III disappeared due to alkaline hydrolysis of the 1,4-benzodiazepine ring and, consequently, simultaneous determination of the unchanged drug and the metabolites can not be performed. The metabolites should be measured by an alternative procedure, which will be reported in our next paper [11].

Plasma levels of compound I in dogs

The plasma levels of compound I were determined in dogs following a single oral dose of 5 mg/kg, as shown in Fig. 3. The plasma level of compound I reached a peak of 0.15 μg/ml 1 h after administration and decreased rapidly with a half-life of about 1.2 h. The fact that the plasma levels of compound I were quite low suggested that the conversion of compound I into some metabolites, II, III and/or others, was extensive. Further studies on the metabolism and excretion of compound I will be discussed in our next paper [11].



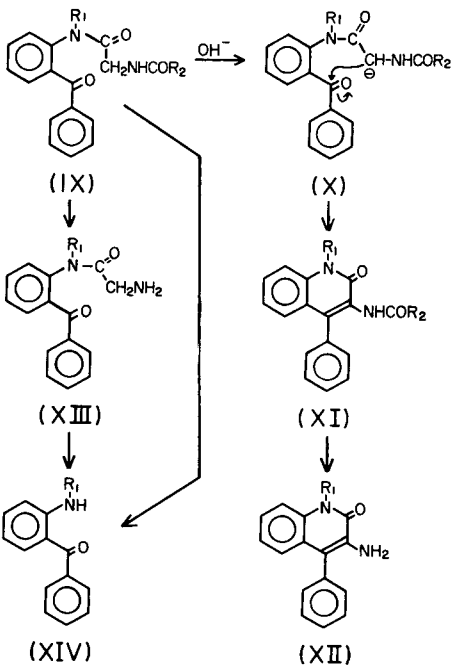


Fig. 3. Plasma concentrations of compound I in a dog after a single 5 mg/kg oral dose of compound I.

Fig. 4. Mechanism of the cyclization reaction of general peptido-aminobenzophenones.

Applications to general peptido-aminobenzophenones

The derivatization of compound I described here can be applied to any peptido-aminobenzophenone series. As shown in Fig. 4, general peptido-aminobenzophenones with the structure represented by compound IX are deprotonized with alkali at the position of the active methylene group to give compound X, which is cyclized to the acylaminoquinolone derivative (XI). This intermediate XI is further hydrolyzed in alkaline medium to form the aminoquinolone derivative (XII). Here, the side-reaction is the hydrolysis of the peptide bond to give an aminobenzophenone derivative (XIV).

ACKNOWLEDGEMENTS

The authors thank Drs. T. Sugasawa, K. Hirai and M. Ogata for preparing the authentic samples.

REFERENCES

1 K. Hirai, T. Ishiba, H. Sugimoto, K. Sasakura, T. Fujishita, Y. Tsukinoki and K. Hirose, Chem. Pharm. Bull., 26 (1978) 1947.
2 D.J. Greenblatt and R.I. Shader, Benzodiazepines in Clinical Practice, Raven Press, New York, 1974, pp. 43—140.
3 T. Sato, M. Konishi, T. Agoh, Y. Nakagawa, K. Iwatani and Y. Mori, Xenobiotica, in press.
4 M.R. Hackman, M.A. Brooks, J.A.F. de Silva and T.S. Ma, Anal. Chem., 46 (1974) 1075.
5 J.A.F. de Silva and N. Strojny, J. Pharm. Sci., 60 (1971) 1303.
6 B. Peskar and S. Spector, J. Pharmacol. Exp. Ther., 186 (1973) 167.
7 T.B. Vree, A.M. Baars, Y.A. Hekster and E. van der Kleijn, J. Chromatogr., 162 (1979) 605.
8 J.A.F. de Silva, I. Berkersky, C.V. Puglisi, M.A. Brooks and R.E. Weinfeld, Anal. Chem., 48 (1976) 10.
9 D.M. Hailey, J. Chromatogr., 98 (1974) 527.
10 J.M. Clifford and W.F. Smyth, Analyst (London), 99 (1974) 241.
11 M. Konishi, T. Agoh, T. Sato, R. Konaka and Y. Mori, Drug Metab. Disp., submitted for publication.

Journal of Chromatography, 182 (1980)179—190
Biomedical Applications

CHROMBIO. 519

QUANTITATIVE GAS CHROMATOGRAPHIC DETERMINATION OF TWO OXIDIZED METABOLITES OF THE DIURETIC MEFRUSIDE IN HUMAN URINE, PLASMA AND RED BLOOD CELLS

H.L.J. FLEUREN*, C.P.W. VERWEY-VAN WISSEN and J.M. VAN ROSSUM

Department of Pharmacology, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen (The Netherlands)

First received October 8th, 1979; revised manuscript received December 10th, 1979)

SUMMARY

A gas chromatographic method is reported for the quantitative analysis of two metabolites of mefruside, viz., 5-oxo-mefruside (mefruside lactone) and its hydroxy-carboxylic acid analogue in human body fluids. Use was made of extractive methylation as the derivatization technique, and quantitation was achieved, with a suitable internal standard, by means of a nitrogen-sensitive detector.

Because the two metabolites are linked chemically through a lactone—open acid equilibrium, interconversion prior to their separation had to be avoided. A pH partitioning study was performed to find optimal separation conditions. The lactone could be extracted quantitatively at pH 7.4, without any trace of co-extracted hydroxy acid. The latter was extracted either at pH 2 directly (in the case of plasma and urine), or after conversion to the lactone at pH 7.4 (in the case of red cells or whole blood). Concentrations down to 25 ng per sample of both compounds could be analysed with a standard deviation of 5%.

The two metabolites of mefruside equilibrated instantaneously between red cells and plasma in vitro. At 37°, the red cell/plasma concentration ratio was 20 for the lactone, but only 0.1 for the open acid compound. 5-Oxo-mefruside was able to displace mefruside from its red blood cell binding sites in vitro.

INTRODUCTION

Recently a few methods for the selective determination of the diuretic mefruside in body fluids have been published [1, 2]. After administration of mefruside to humans less than 1% of the dose was recovered in the urine as the unchanged compound [3]. It seemed logical, therefore, to search for metabolites of this drug in man.

In the rat, two urinary metabolites, which were formed by oxidation of the

*Present address: Department of Clinical Chemistry, Juliana Ziekenhuis, Koning Lodewijk-laan 401, 7314 BD Apeldoorn, The Netherlands.

C-5 atom of the tetrahydrofuran ring of mefruside, constituted together ca. 35% of the dose [4]. These metabolites appeared to be related through a lactone—hydroxy acid equilibrium (Fig. 1). The reaction was shifted completely to the lactone side under acidic conditions (e.g. pH 2) and to the open acid side at alkaline pH (e.g. pH 11), the rates of ring closure and hydrolysis being pH-dependent [5, 6]. In aqueous buffers of pH 7—8, interconversion of both compounds was unmeasurably slow, but it became highly accelerated by the addition of rat plasma or rat liver homogenates. Also, rapid turnover of the two metabolites has been observed in vivo, in dogs [6].

Our attention was drawn to the above metabolites in particular because both substances had been reported to bring about the same diuretic effect as the parent drug after intravenous administration to rats [7]. This raised the possibility that the two compounds would be determinants of drug action also in the human situation. The aim of the present investigations was therefore to develop an assay for these mefruside metabolites and to demonstrate their formation after administration of mefruside to man. Due to their particular chemical constitution, care had to be taken to avoid unwanted interconversion of the compounds in vitro prior to their separation.

MATERIALS AND METHODS

Drugs

5-Oxo-mefruside, 4-chloro-N^1-methyl-N^1-(tetrahydro-2-methyl-5-oxo-2-furanyl)methyl-1,3-benzenedisulfonamide (Fig. 1), and the internal standard, 4-chloro-N^1-methyl-N^1-(3-methoxypropyl)-1,3-benzenedisulfonamide,

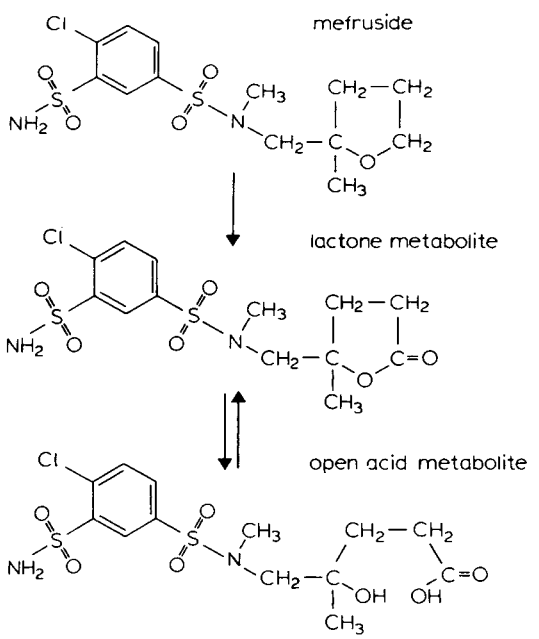


Fig. 1. Structural formulae of mefruside, 5-oxo-mefruside and its hydroxycarboxylic acid analogue.

were supplied by Bayer (Wuppertal, G.F.R.; courtesy of Dr. H. Horstmann). The conformation of the supplied 5-oxo-mefruside was checked by nuclear magnetic resonance measurements and elemental analysis (C, H, N)*, which yielded data confirming the lactone structure, and in agreement with the literature [8]. Stock solutions of 5-oxo-mefruside were prepared in 0.1 *M* aqueous HCl and in diethyl ether, in concentrations of 50 μg/ml and dilutions thereof.

The hydroxy acid analogue of 5-oxo-mefruside, 4-chloro-N^1-methyl-N^1-(2-methyl-2-hydroxy-4-carboxybutyl)-1,3-benzenedisulfonamide, was obtained by dissolving 5-oxo-mefruside (50 μg/ml) in 0.1 *M* NaOH, in which very rapid hydrolysis, with a first-order half-life of 20—25 sec, has been found [6]. Completeness of reaction was checked after 15 min by extracting aliquots (0.1 ml; 5 μg) added to 2 ml of Sörensen phosphate buffer (pH 7.4) twice with 10 ml of diethyl ether. At this pH, only the lactone form can be extracted (as will be shown in the Results section). No trace (<10 ng) was found by the gas chromatographic (GC) method described in this report, indicating complete formation of the hydroxy acid.

Derivatization and gas chromatography

With some modification, the method reported for the assay of mefruside in body fluids [1] could be used. In short, this method consists of extracting drug and internal standard at pH 7.4 into diethyl ether, re-extraction into 0.1 *M* aqueous NaOH, and ion-pair extraction of the anion with tetrahexylammonium as counterion into dichloromethane, under simultaneous methylation with iodomethane, for 15 min at room temperature ("extractive methylation"). After evaporation of the organic phase, the methylated derivatives are re-dissolved in *n*-hexane in order to remove the co-extracted tetrahexylammonium iodide, which is very sparingly soluble in this solvent, so that strong tailing of the corresponding tertiary amine on the GC column is avoided. The methyl derivatives are then concentrated in ethyl alcohol, prior to injection into the gas chromatograph.

5-Oxo-mefruside and its hydroxy acid analogue were extracted as described in the following sections. Furthermore, the assay was modified with respect to the original procedure for mefruside in the dissolution step with *n*-hexane. Intensive sonification, twice for 15 min, with 5 ml of *n*-hexane appeared to be necessary for reproducible and complete dissolution of the more polar metabolites. The wash of the hexane layer with aqueous silver sulphate solution, by which the background signal of the gas chromatogram became reduced [1], could not be employed for 5-oxo-mefruside, as this step removed its methylated derivative almost completely.

All reagents used, GC equipment, and other apparatus were the same as described previously [1]. The methylated derivatives of the internal standard, mefruside and 5-oxo-mefruside eluted from the GC colum (3% SE-30, 265°) at retention times of 2.8, 4.1 and 6.3 min, respectively. The derivative, formed by methylation of the hydroxy acid analogue of 5-oxo-mefruside, had an

*Performed at the Department of Organic Chemistry, University of Nijmegen, Nijmegen, The Netherlands.

identical retention time to the derivative of the latter. This was not unexpected because both compounds pass through an alkaline layer (0.1 *M* NaOH) prior to methylation, so that the lactone is transformed completely to the free acid form, and a common species is generated. A discussion on the structure of this derivative during GC will be given in the Results section below.

pH-dependent distribution between aqueous and organic phases

Lactone partitioning. Into glass tubes containing 2 ml of aqueous buffers varying from pH 2 to pH 10 [citrate–phosphate–borate (0.1 *M*), according to Teorell and Stenhagen], were pipetted 10-ml portions of a 0.5 μg/ml solution of 5-oxo-mefruside in freshly distilled diethyl ether. The tubes were well-closed and vigorously shaken for 5 min. After brief centrifugation, as much as possible of the diethyl ether layer was transferred to another tube. The samples containing the buffers of highest pH were handled first in order to minimize lactone hydrolysis. At pH 11, the half-life of lactone hydrolysis is 27 min [6], which implies that after 5 min in such solution 88% of the drug is still in the lactone form, i.e. a loss of 12%. Because the highest pH we employed was a full pH unit lower (pH 10), we estimated a loss of less than 5% at that pH, which should have an insignificant influence on the shape of the distribution curve.

Hydroxy acid partitioning. Aliquots (0.1 ml) of a 50 μg/ml solution of the hydroxy acid analogue of 5-oxo-mefruside in 0.1 *M* NaOH were added to 1.9-ml portions of buffer at pH 2–10 (same buffer as described above). After mixing, 10-ml portions of freshly distilled diethyl ether were added and the tubes were vigorously shaken for 5 min. After short centrifugation (1 min), as much as possible of the organic layers was transferred into new tubes. For samples containing the buffers of lowest pH this separation was performed at once in order to minimize lactone formation. The half-life of ring closure at pH 2 is 3.3 h [6], which means that 93.3% of drug is still in the free acid form after 5 min at this pH, so that the observed distribution at the lowest pH values, too, should be a true reflection of the lipophilicity of the pure open acid species.

Measurement of extraction recovery. To calibrate the above partitioning studies, known amounts of lactone (0.1–0.5 μg) and free acid (0.05–5 μg) were dissolved in diethyl ether (10 ml) and 0.1 *M* NaOH (2 ml), respectively. The sodium hydroxide layers were used directly; the diethyl ether layers were extracted first with 2 ml of 0.1 *M* NaOH for 5 min, and then used in the extractive methylation, together with internal standard (5 μg in 0.1 ml of 0.1 *M* NaOH). The percentage extraction at each pH was calculated by comparing the ratios of the peak areas of drugs and internal standard.

Procedure for the extraction of 5-oxo-mefruside and hydroxy acid analogue from biological samples

In order to determine 5-oxo-mefruside, 2 ml of plasma, 0.5–1 ml of packed red cells (1–2 ml of whole blood), or 1 ml of urine, were extracted at pH 7.4 twice with 10 ml of diethyl ether under the same conditions, including addition of internal standard, as described for mefruside [1].

After this extraction of 5-oxo-mefruside, the same biological samples served to determine the hydroxy acid species. Plasma (2 ml) or urine (0.25—1 ml) was adjusted to pH 2 with a few drops of 2 *M* aqueous HCl and extracted, after renewed addition of internal standard, twice with 10 ml of diethyl ether for 5 min. Red blood cell or whole blood samples, however, extracted at pH 2, yielded a very high background signal in their gas chromatograms and in addition unknown interfering peaks. Therefore, the hydroxy acid in these samples was converted into the lactone form by leaving the tubes at pH 2 for 24 h or longer. Subsequently, the samples were adjusted again to pH 7.4 with solid sodium bicarbonate (100—200 mg/sample); internal standard and buffer at pH 7.4 were added to give a final volume of 2.5 ml and the lactone was extracted in the same way as described at the beginning of this section. Previously, we had verified that ring closure was complete under these conditions, by comparison with known amounts of 5-oxo-mefruside. (Moreover, in assaying urine concentrations of the hydroxy acid, the same quantitative results were found from the lactone conversion procedure as from direct extraction at pH 2.)

Parallel to each series of biological samples, two or three standards of 5-oxo-mefruside and its hydroxy acid counterpart, added to a corresponding volume of buffer at pH 7.4, were extracted at pH 7.4 or pH 2, derivatized and submitted to GC, in order to check the overall procedure. For this calibration, known amounts covering the concentration range of the unknown samples were taken, for example, 0.5, 1 and 2.5 μg of 5-oxo-mefruside in the urine assay.

Sampling of blood and urine, in vitro distribution between plasma and red cells

The method of collection of venous blood from human subjects was the same as described earlier [1]. Briefly, 7-ml heparinized blood samples were centrifuged at 1500 *g* for 3 min at room temperature, immediately after vein puncture, and the plasma was rapidly separated. Because the equilibration of mefruside between plasma and red cells was unmeasurably rapid, and the position of the final equilibrium temperature-dependent, the decrease in blood temperature inherent in routine treatment of blood samples (vein puncture, centrifugation) caused an altered red blood cell/plasma concentration ratio of this drug [1]. A similar phenomenon might be possible for 5-oxo-mefruside and its hydroxy acid counterpart. Therefore, partitioning of these two compounds between plasma and erythrocytes was studied in fresh human blood, according to the methods published previously [1]. Thus, the rate of distribution at 37°, and the difference between plasma concentrations present at in vivo temperature (37°) and those found after routine treatment of the blood (27.5°) were determined. A complicating factor arose from interconversion of the lactone and the open acid in incubations of fresh human plasma. Although different in extent for each plasma sample, hydrolysis of the lactone could account for as much as 25% of the added amount, but ring closure of the hydroxy acid was always less than 5% (at plasma concentrations of 40 μg/ml). This pattern was qualitatively in agreement with observations on the interconversion of the two species in rat plasma [6]. However, because interconversion in human plasma appeared to be finished after 15—30 min, we pre-

incubated the two substances for 30 min in plasma at 37° prior to the red blood cell partitioning experiments, and measured the concentrations of the drug under study in both plasma and red cells. The concentrations used in the present in vitro studies are indicated in the Results section.

Urine from human experiments was adjusted to pH 8 with a few drops of 5 *M* NaOH, prior to extraction. Care was taken to mix the contents of the tube vigorously during pH adjustment, in order to prevent hydrolysis of 5-oxo-mefruside.

Comparison of presumed metabolite with synthetic 5-oxo-mefruside by gas chromatography—mass spectrometry

Electron-impact mass spectra of the methylated derivatives of synthetic 5-oxo-mefruside and mefruside were compared with that of the suspected metabolite by means of a LKB 9000 gas chromatograph—mass spectrometer combination (LKB, Bromma, Sweden), at an accelerating potential of 20 eV, trap current of 60 μA and ion-source temperature at 260°. For GC separation a glass column (1.5 m × 3 mm I.D.) was used, packed with 3% OV-101 on Gas-Chrom Q, 100—120 mesh (Applied Science Labs., State College, Pa., U.S.A.), at an oven temperature of 255°. The retention times of the methylated derivatives of internal standard, mefruside, and 5-oxo-mefruside were 8.5 min, 13 min and 18 min, respectively.

RESULTS AND DISCUSSION

Drug partitioning

The distribution of 5-oxo-mefruside and its open-chain analogue between organic phase and aqueous buffer of varying pH is shown in Fig. 2. The plots indicate that the lactone can be recovered in the ether layer by a single extraction already with a 87% yield at pH 2—8.5, similarly to extraction of mefruside, whereas the carboxylic acid can only be obtained in high yield (76%) below pH 3, and is not extracted at all above pH 7. This latter detail was checked by separate extractions at pH 7.4 (not shown here), using as aqueous phases buffer alone, and buffered plasma, red cells and urine. These experiments confirmed that the quantity of the open acid compound, recovered in the organic phase by two subsequent extractions with diethyl ether at this pH, was not detectable in our GC assay, viz. <10 ng of a total amount of 5 μg. This means that the percentage of hydroxy acid extracted at pH 7.4 is even lower than 0.2%. In this way, the two structural analogues were separated prior to the subsequent steps of the analysis.

Gas chromatographic determination of 5-oxo-mefruside and its open acid counterpart in plasma, red blood cells and urine

Calibration graphs, prepared by adding known amounts of the lactone and the open acid to blank human plasma, urine and red cells, and plotting, after GC analysis, peak area ratios of drugs and internal standard against concentration, were linear and passed through the origin. The standard deviation of the whole procedure, determined from repeated assay of the same samples was ca. 5% (n = 10) for both compounds at concentrations between 0.05 and 10 μg/

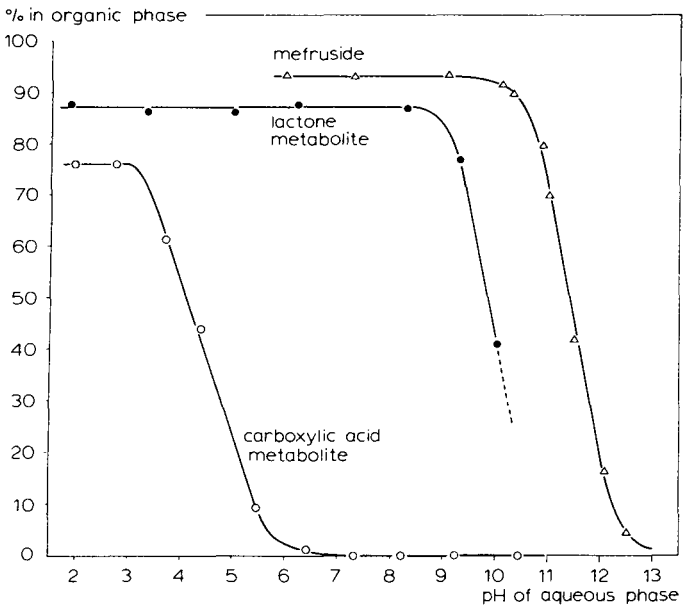


Fig. 2. Plots showing the percentage of extraction of mefruside, 5-oxo-mefruside and the hydroxy acid metabolite against the pH of the aqueous phase (diethyl ether, water, phase volume ratio: $V_{org}/V_{aq} = 5$).

sample. The lowest concentration which could accurately be analysed (i.e. with the above standard deviation) was approximately 25 ng/sample, whereas amounts down to 5—10 ng in biological extracts could still be detected. The recovery of 5-oxo-mefruside by two extractions with diethyl ether at pH 7.4 was 98.5 ± 1% (mean ± S.D., $n = 4$) in the concentration range investigated, independent of the choice of buffer alone, plasma, red cells or urine as the aqueous phase. The corresponding figure for extraction of the hydroxy acid analogue at pH 2 was 94 ± 2% (mean ± S.D., $n = 4$).

Identification of mefruside metabolites in biological samples

The gas chromatograms obtained by analysis of urine from human subjects who had ingested an oral dose of mefruside (25 or 50 mg) as described elsewhere [3], showed a large peak eluting after that of mefruside itself, with a retention time of 6.3 min (Fig. 3), which was the same as that of the methylated derivative of synthetically prepared 5-oxo-mefruside. The peak was present already after extraction of urine at pH 7.4, but became larger when urine had been extracted at pH 2. This extra amount could also be recovered by extraction at pH 7.4 if the urine had previously been allowed to stand at pH 2 for several hours.

The electron-impact mass spectrum of the presumed metabolite, extracted from human urine and separated by gas chromatography, was identical with that of the pure reference compound. The mass spectra of the methyl derivatives of mefruside and 5-oxo-mefruside are shown in Fig. 4. The fragmentation of both compounds is consistent with the scheme of Fig. 5. After being

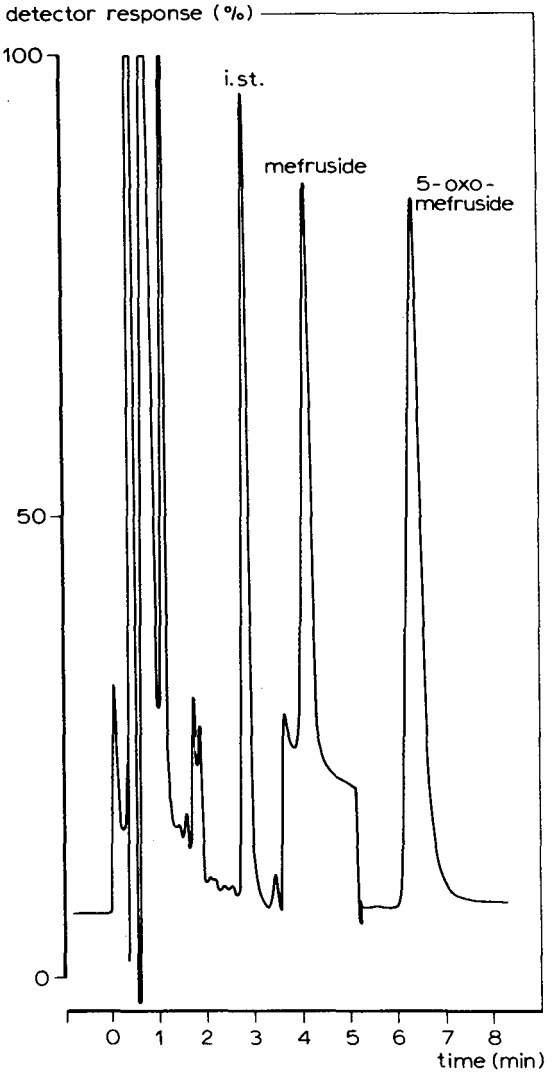


Fig. 3. Typical gas chromatogram showing the presence of mefruside (0.15 μg/ml) and 5-oxo-mefruside (2.7 μg/ml) in human urine, analysed 5 h after intake of a 50-mg dose of mefruside by a normal human subject (i.st. = internal standard).

split off, the tetrahydrofuran ring of both mefruside and its oxidized metabolite appears to be relatively stable, as the *m/e* 85 and 99 fragments are very abundant. It can be seen that a number of peaks in the upper panel are shifted to the right with respect to the mefruside spectrum with 14 *m/e* units (namely 99 vs. 85, 142 vs. 128, and 381 vs. 367), which differences are clearly attributable to the replacement of two hydrogen atoms by oxygen at the C-5 atom of the tetrahydrofuran moiety. Furthermore, the fragmentation pattern of the two compounds is closely comparable with that of a structural analogue with a straight chain (III in Fig. 5), which is used as the internal standard in the GC assay.

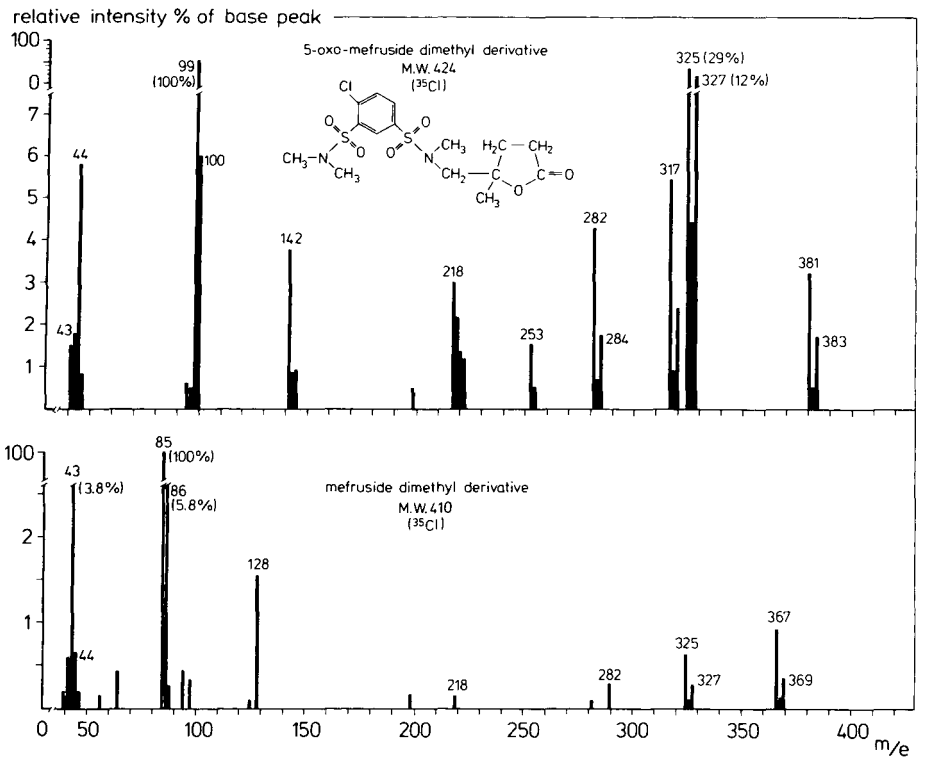


Fig. 4. Electron-impact mass spectra (20 eV) of the methylated derivatives of 5-oxo-mefruside and mefruside, after GC separation.

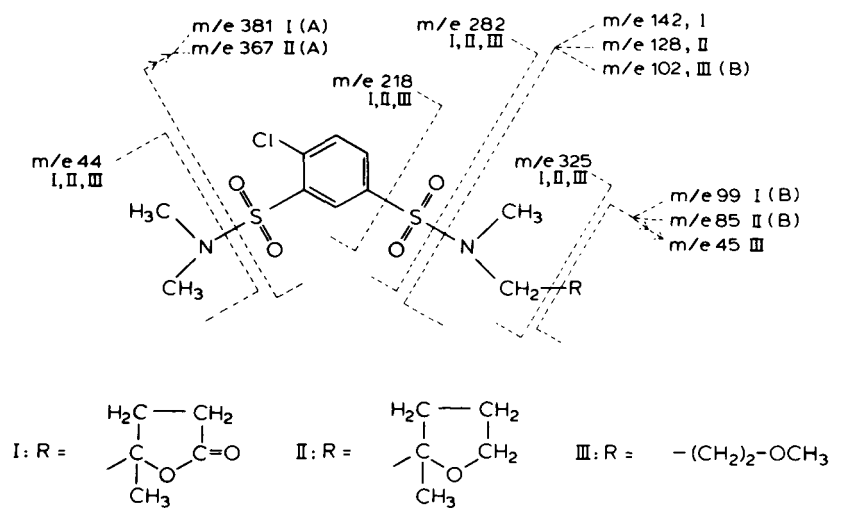


Fig. 5. Common mass fragmentations of the dimethylated derivatives of 5-oxo-mefruside (I, M.W. = 424, ^{35}Cl), mefruside (II, M.W. = 410, ^{35}Cl), and a straight-chain analogue (III, M.W. = 384, ^{35}Cl). (A) Presumably formed after H shift from *m/e* 44 fragment. (B) Base peak.

Only the lactone form of both occurring oxidized metabolites of mefruside has been drawn in Fig. 4. The thermodynamic equilibrium between these two species lies completely at the open-acid side at alkaline pH [6]. There can be no doubt, therefore, that any derivative formed by extractive methylation of either the lactone or the open acid as starting substance, existed in the open-chain form exclusively prior to injection into the gas chromatograph. Whether two, three or even four methyl groups have been introduced at that stage is not known. There is, however, besides the excellent agreement of the mass fragmentation pattern and molecular formula depicted in Fig. 4, another argument to favour the view that *during gas chromatography* the methylated derivative possesses the structure shown. It is well-known that, on heating, γ-hydroxy-carboxylic acids and their ester (and even ether) derivatives are readily converted to the corresponding lactones [9]. Operated at a temperature of 280°, the injection port of the gas chromatograph must amply have provided such a condition. It must be concluded, therefore, that both metabolites of mefruside (i.e. the lactone and the open acid) are chromatographed as the same methylated derivative, with the formula depicted in Fig. 4.

Duhm et al. [4] have proposed N-demethylation of mefruside or its metabolites in the rat to account for a small fraction of dose, leading to carbon dioxide in expired air after administration of the methyl-^{14}C-labelled drug. The methylation which is employed in the present assay could have masked possible biological formation of N^1-demethylated 5-oxo-mefruside or its hydroxy acid congener. The methylated product of this metabolite would be indistinguishable from that originating from 5-oxo-mefruside (or open acid analogue) itself. We were already able to exclude the presence of detectable amounts of demethylated mefruside in human body fluids by the use of propyl iodide as the derivatizing agent [1]. In an analogous way, we now carried out propylation of both urine, plasma and red cell extracts from several human subjects, and did not find a trace of a substance with a GC retention time other than that belonging to the propylated derivative of 5-oxo-mefruside itself. In this way, the selectivity of the assay for 5-oxo-mefruside and its open acid counterpart was considered to be affirmed.

In vitro distribution of mefruside metabolites between plasma and erythrocytes

5-Oxo-mefruside and its hydroxy acid analogue equilibrated instantaneously between plasma and red cells of human blood. Thus, no difference was observed in the red cell/plasma concentration ratios at 37°, whether the blood had been centrifuged immediately after mixing red cells with plasma, or at 1.5, 5, 15 or 45 min after the start of the incubation.

The lactone and the open acid differed greatly in their extent of red cell partitioning. While the lactone reached concentrations in erythrocytes which were ca. twenty times higher than those in plasma, the hydroxycarboxylic acid concentrations in red cells were only one-tenth of those in plasma, at a whole blood concentration of 8 μg/ml for both experiments. For this reason, only the red cell uptake of the lactone was subjected to further investigation. The red cell/plasma concentration ratio appeared to be constant in the concentration range used, 2—15 μg/ml whole blood, and mean ratios of 18 and 21 were found from incubations at 37° in blood from two human subjects.

When blood incubated with the lactone at 37° was centrifuged at room temperature for 3 min (resulting in a blood temperature of 27.5° [1]), the plasma concentrations of the drug were 96.5 ± 1.5% (mean ± S.D., n = 6) of the values obtained when the whole procedure was carried out at 37°. It was concluded therefore that plasma concentrations of 5-oxo-mefruside, found after immediate centrifugation of blood following vein puncture, differ by only ca. 3.5% from the concentrations actually present at in vivo temperature.

Because both 5-oxo-mefruside and mefruside [1] are rapidly taken up by red blood cells, we were interested to see if the binding of the metabolite would influence that of the parent drug. Fig. 6 shows that the erythrocyte/plasma concentration ratio of mefruside, incubated in whole human blood, decreases with increasing concentration of 5-oxo-mefruside. Although the interaction seems of little importance at whole blood concentrations of the lactone below 1 μg/ml, the effect visible at higher concentrations might also play a role in vivo. Some evidence for displacement of mefruside from its red cell binding sites in vivo can be taken from a recent kinetic study with mefruside in human subjects [3].

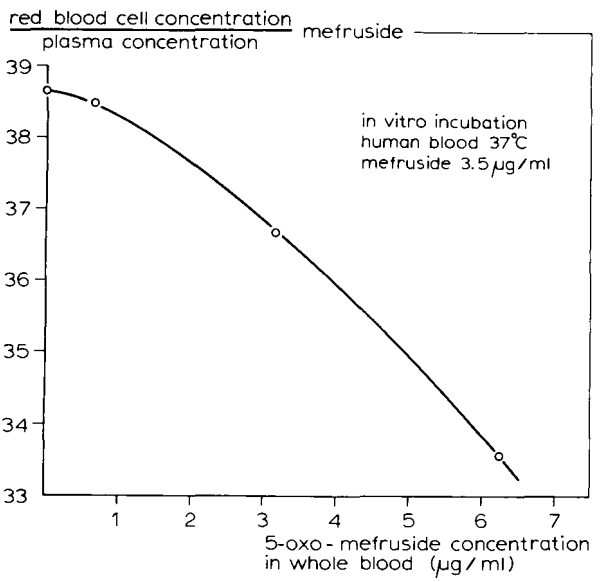


Fig. 6. Graph indicating displacement of mefruside from its red blood cell binding sites by 5-oxo-mefruside in vitro (not included in this figure is a red cell/plasma concentration ratio of 9.4, measured at a lactone concentration of 30 μg/ml in the same blood).

ACKNOWLEDGEMENTS

This work was supported by a grant from the Netherlands Foundation for Medical Research (FUNGO/ZWO). Thanks are due to Mrs. M.P.M. Klumpkens-Janssen for typing the manuscript.

REFERENCES

1 H.L.J. Fleuren, C.P.W. Verwey-van Wissen and J.M. van Rossum, Arzneim.-Forsch., 29 (1979) 1041.
2 G. Oesterhelt and E. Eschenhof, Arzneim.-Forsch., 29 (1979) 607.
3 H.L.J. Fleuren, C.P.W. Verwey-van Wissen and J.M. van Rossum, Eur. J. Clin. Pharmacol., 17 (1980) 59.
4 B. Duhm, W. Maul, H. Medenwald, K. Patzsche and L.A. Wegner, Arzneim.-Forsch., 17 (1967) 672.
5 J. Pütter and K. Schlossmann, Biochim. Biophys. Acta, 186 (1972) 186.
6 K. Schlossmann and J. Pütter, Arzneim.-Forsch., 23 (1973) 255.
7 K. Meng and G. Kroneberg, Arzneim.-Forsch., 17 (1967) 659.
8 H. Horstmann, H. Wollweber and K. Meng, Arzneim.-Forsch., 17 (1967) 653.
9 H. Köper, in E. Müller (Editor), Methoden der Organischen Chemie (Houben-Weyl), Vol. 6/2, Georg Thieme, Stuttgart, 4th ed., 1963, p. 561.

Journal of Chromatography, 182 (1980) 191—200
Biomedical Applications

CHROMBIO. 523

QUANTITATION OF DOXAPRAM IN BLOOD, PLASMA AND URINE

HELEN NICHOL, JOHN VINE and JACK THOMAS

Department of Pharmacy, University of Sydney, N.S.W. (Australia)

and

R. GEORGE MOORE*

Foetal Pharmacology Laboratory, Foundation 41, Women's Hospital, Crown Street, Sydney, N.S.W. (Australia)

(First received September 13th, 1979; revised manuscript received December 27th, 1979)

SUMMARY

Methods for the quantitation of doxapram in blood, plasma and urine have been developed. Following extraction, gas—liquid chromatography was used to separate doxapram from basic metabolites. Doxapram was detected by mass spectrometry for blood and plasma assays, and by flame ionisation for urine assays. The limit of reliable quantitation in blood and plasma was 10 ng and in urine 500 ng, the coefficients of variation being 6.37%, 1.72% and 2.31% respectively. To illustrate the clinical applicability of the assay methods, plasma, blood and urine levels were monitored in a premature newborn following an intravenous infusion of doxapram.

INTRODUCTION

Doxapram [1-ethyl-4-(2-morpholino-ethyl)-3,3-diphenyl-2-pyrrolidinone] is a central nervous system stimulant, acting primarily on the respiratory centres of the brain stem [1, 2]. Doxapram is currently being used in a clinical trial at the Women's Hospital, Sydney, Australia, to evaluate its efficacy in the treatment of recurrent neonatal apnoea in the premature newborn. One aspect of this trial was the determination of the pharmacokinetics of doxapram.

Previous analytical methods for the analysis of doxapram included oxidation of doxapram and doxapram-like materials to the benzophenone and quantitation by UV spectrophotometry [3]. A high-performance ion-exchange chromatographic method using A-5 resin has been used but was unable to separate doxapram from three basic metabolites [4].

*To whom correspondence should be addressed.

A rapid method for the analysis of doxapram in plasma has been reported using gas chromatography (GC) and a nitrogen-sensitive detector [5]. Data were not presented for the concentration range 0.01—0.75 μg per 100-μl sample of blood or plasma, which represents the levels expected in premature newborn.

In order to quantitate doxapram at the low levels present in blood and plasma, an assay based on gas chromatography—chemical ionisation mass spectrometry (GC—MS) with selective ion monitoring was developed. For the quantitation of doxapram in urine, where high sensitivity was not necessary, a GC method using flame ionisation detection (GC—FID) is described.

EXPERIMENTAL

Standards and reagents

Doxapram hydrochloride (Dopram®, A.H. Robins, Sydney, Australia) in multiple-dose vials (20 mg/ml) was used throughout the clinical trial. Doxapram hydrochloride was used as a primary standard (A.H. Robins). Dextromoramide [(+)-1-(3-methyl-4-morpholino-2,2-diphenylbutyryl)pyrrolidine] as the bitartrate salt, in single-dose ampoules, 5 mg/ml (F.H. Faulding, Adelaide, Australia) was used to prepare the internal standard solutions. All reagents and solvents were analytical reagent grade. Anaesthetic diethyl ether B.P. was distilled prior to use.

Qualitative analysis of urine

A cross section of urine samples from patients were extracted and analysed by GC—MS.

Extraction. Urine (3 ml), sodium hydroxide (0.5 ml, 5 *M*) and diethyl ether (10 ml) were placed in a 15-ml glass stoppered centrifuge tube, mixed for 2 min using a vortex mixer and centrifuged for 10 min at 1500 *g*. The diethyl ether layer was transferred to a second 15-ml glass stoppered centrifuge tube and hydrochloric acid (2 ml, 0.1 *M*) added. The mixing and centrifugation steps were repeated, the diethyl ether layer was discarded and sodium hydroxide (0.5 ml, 5 *M*) and diethyl ether (10 ml) were added to the aqueous phase. The mixing and centrifugation steps were repeated and the diethyl ether layer transferred to an evaporation tube (a 15-ml glass tube with a 100-μl capillary tube at the base). The diethyl ether was evaporated to dryness by immersion of the evaporation tube in a water bath at 40°. The tube was then stoppered and placed in ice to condense the diethyl ether vapour remaining in the tube. This evaporation—condensation procedure was then repeated until approximately 10 μl of diethyl ether remained. The sample was then analysed by GC—MS.

GC—MS. GC—MS results were obtained on a Finnigan 3200 quadrupole mass spectrometer fitted with a chemical ionisation source and interfaced to a Finnigan 9500 gas chromatograph. Data acquisition and processing were carried out on-line using a Finnigan 6110 data system. A pan coating method [6] was used to coat Gas-Chrom Q (100—120 mesh) with OV-225 (both from Applied Science Labs., State College, Pa., U.S.A.) using chloroform as the solvent.

A glass column (75 cm × 2 mm I.D.) was packed with 1% OV-225 and conditioned at a temperature of 250° and a methane flow-rate of 20 ml/min over-

night. GC—MS operating conditions: injection port, column, separator oven and transfer line temperatures were 250°, 235°, 260° and 260° respectively, methane (used as a carrier gas and chemical ionisation gas) flow-rate 20 ml/min; source pressure 1 torr; mass range scanned 100—450; scan time 3 sec.

Quantitative analysis of urine

Doxapram in urine was quantitated using a method based on GC—FID.

Extraction. Urine (0.5—2.0 ml), internal standard (dextromoramide 10 μg/100 μl in 0.1 *M* hydrochloric acid), sodium hydroxide (0.5 ml, 5 *M*) and diethyl ether (10 ml) were placed in a 15-ml glass stoppered centrifuge tube and the extraction procedure in the previous section was followed. Approximately half the ethereal concentrate was injected into the chromatography system.

GC—FID. A Hewlett-Packard gas chromatograph Model 5710A equipped with a flame ionisation detector was used. Resolution was achieved on a 75 cm × 2 mm I.D. glass column packed with 1% OV-225 on Gas-Chrom Q (100—120 mesh). The column was conditoned at 250° and a nitrogen flow-rate of 60 ml/min overnight. GC—FID operating conditions were: injection port, column and detector temperatures 250°, 230° and 250° respectively; nitrogen, air and hydrogen flow-rates 60, 240 and 60 ml/min respectively.

Calibration and reproducibility. Known quantities of doxapram hydrochloride (0.5—10 μg) were added to blank urine (0.5—2 ml) and assayed as above. Calibration curves were constructed by plotting the peak height of doxapram to dextromoramide against the amount of added doxapram. Reproducibility was determined by six replicate analyses at the upper (10 μg) and lower (0.5 μg) limits of the calibration range.

Quantitative analysis of blood and plasma

Doxapram in blood and plasma was quantitated using a method based on GC—chemical ionisation mass fragmentography.

Extraction. Blood or plasma (50—100 μl), internal standard (dextromoramide 500 ng/500 μl in 0.1 *M* hydrochloric acid), water (1.4 ml), sodium hydroxide (0.5 ml, 5 *M*) and diethyl ether (10 ml) were placed in a 15-ml glass stoppered centrifuge tube, mixed for 2 min (vortex mixer) and centrifuged for 10 min at 1500 *g*. The diethyl ether layer was transferred to an evaporation tube and evaporated as described above to approximately 10—15 μl. Half this concentrate was analysed.

GC—MS. The Finnigan system described in the section on the qualitative analysis of urine was used except that it was operated in the mass fragmentographic mode and column temperature was increased to 250°. The protonated molecular ions of doxapram (*m/e* 379) and dextromoramide (*m/e* 393) were monitored during the GC—MS run. Peak areas were determined using the Finnigan 6110 data system.

Calibration and reproducibility. Known amounts of doxapram hydrochloride (10—1000 ng for plasma, 10—500 ng for blood) were added to blood or plasma (50—100 μl) and assayed as described above. Calibration curves were constructed by plotting the peak area ratio of doxapram to dextromoramide against the amount of added doxapram. Reproducibility was determined by six

replicate analyses at the upper and lower limits of the calibration curve. Calibration plots were constructed on each day when patient samples were analysed.

Clinical protocol

Doxapram hydrochloride was administered to a premature newborn estimated gestational age 27 weeks, postnatal age 0.5 days, who had developed a recurrent neonatal apnoea. The doxapram was administered as a constant rate intravenous infusion at a rate of 2.4 mg/kg/h for 25 h, followed by an increase in dose rate to 3.8 mg/kg/h for 13.5 h. Blood samples (approximately 0.2 ml) were obtained from heel pricks and collected in heparinized tubes prior to and during the infusion, and for 48 h after cessation of infusion. Blood (50—100 μl) was measured directly into a 15-ml glass tube and the balance of the sample centrifuged and the plasma (50—100 μl) measured into a 15-ml glass tube. Urine samples were collected prior to and during the infusion, and for 48 h after cessation of infusion. The blood, plasma and urine samples were stored at −20° until analysed.

RESULTS AND DISCUSSION

Several chromatographic systems and extraction procedures were investigated in preliminary work attempting to quantitate doxapram in biological fluids by GC—FID. It soon became apparent that a clean-up procedure was required to remove neutral endogenous compounds extracted by diethyl ether. The main problem, thereafter, was the selection of a suitable chromatography

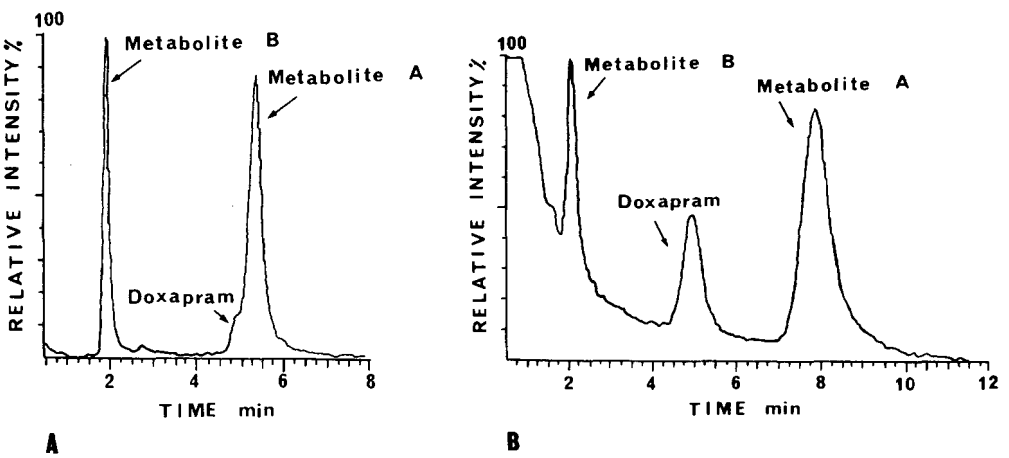


Fig. 1. Chemical ionisation, total ion current chromatograms of urine extracts from a premature newborn after the administration of doxapram. (A) Separation on 3% OV-17 on Gas-Chrom Q, 100—120 mesh; column, 150 cm × 2 mm I.D. Conditions: injection port, column, separator oven and transfer line temperatures 270°, 260°, 270° and 270° respectively; methane flow-rate 20 ml/min. (B) Separation on 1% OV-225 on Gas-Chrom Q, 100—120 mesh; column, 75 cm × 2 mm I.D. Conditions: injection port, column, separator oven and transfer line temperatures, 250°, 235°, 260° and 260° respectively; methane flow-rate 20 ml/min.

system to resolve doxapram from possible basic metabolites of doxapram which had been identified previously in urine in dogs [4]. Unfortunately, authentic samples of these compounds were not available to us and so to verify the authenticity and homogeneity of peaks quantitated as doxapram, GC—MS was used to analyse the eluent from several chromatography systems. A 3% OV-17 column was investigated initially since its use to quantitate doxapram in plasma had been reported [5]. As Fig. 1A illustrates, however, the OV-17 system was not suitable for the analysis of urine since it did not resolve doxapram from a metabolite whose chemical ionisation mass spectrum (Fig. 2B) indicates the structure assigned to metabolite A. The chemical ionisation mass spectrum of the only other urinary metabolite observed in this system is presented in Fig. 2C. There was no significant difference between the chemical ionisation mass spectrum of authentic doxapram and that of doxapram extracted from patient's urine and chromatographed on a 1% OV-225 system. Fig. 1B illustrates the chromatogram of a urine extract on this OV-225 system. Therefore, an OV-225 column was used for subsequent analyses of blood, plasma and urine.

It was decided to develop a method based on GC with chemical ionisation mass fragmentography because: (a) the volumes of blood and plasma available for analysis were small (50—100 μl), (b) the concentrations in some of these samples were expected to be low and (c) the base peak in the chemical ionisation mass spectrum of doxapram was the protonated molecular ion. Dextromoramide was used as internal standard because of its molecular similarity to doxapram and consequently for its mass spectral and chromatographic characteristics (Figs. 2A, 2D and 4B).

A representative mass fragmentogram of ions monitored in a patient's blood sample is illustrated in Fig. 3B. Similar mass fragmentograms were obtained in plasma and no interfering peaks from blank samples were observed (Fig. 3A). The calibration plots obtained from blood and plasma were linear and passed through the origin. The coefficients of variation for six replicate extractions of doxapram from blank blood and plasma are listed in Table I.

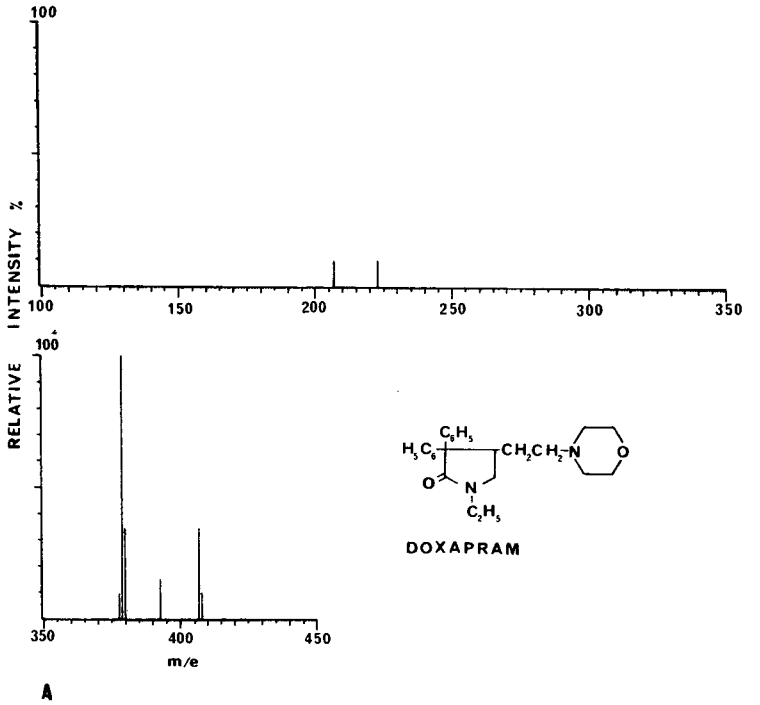
In the analysis of the urine samples where high sensitivity was not necessary, GC—FID was used. A typical chromatogram is illustrated in Fig. 4. A chromatogram resulting from the analysis of a blank urine sample is also shown. The calibration plots obtained were linear and passed through the origin. The coeffi-

TABLE I

REPRODUCIBILITY RESULTS RECORDED AT THE EXTREMITIES OF THE CALIBRATION RANGE FOR THE METHODS

Six replicate extractions were carried out.

Biologic fluid	Method of analysis	Doxapram HCl (μg)	Coefficient of variation (%)
Blood	GC—MS	0.010	6.37
		0.500	4.29
Plasma	GC—MS	0.010	1.72
		1.000	1.70
Urine	GC—FID	0.500	2.31
		10.000	2.42


100
INTENSITY %
100
150
200
250
300
350
100
RELATIVE
350
400
450
m/e
A
DOXAPRAM

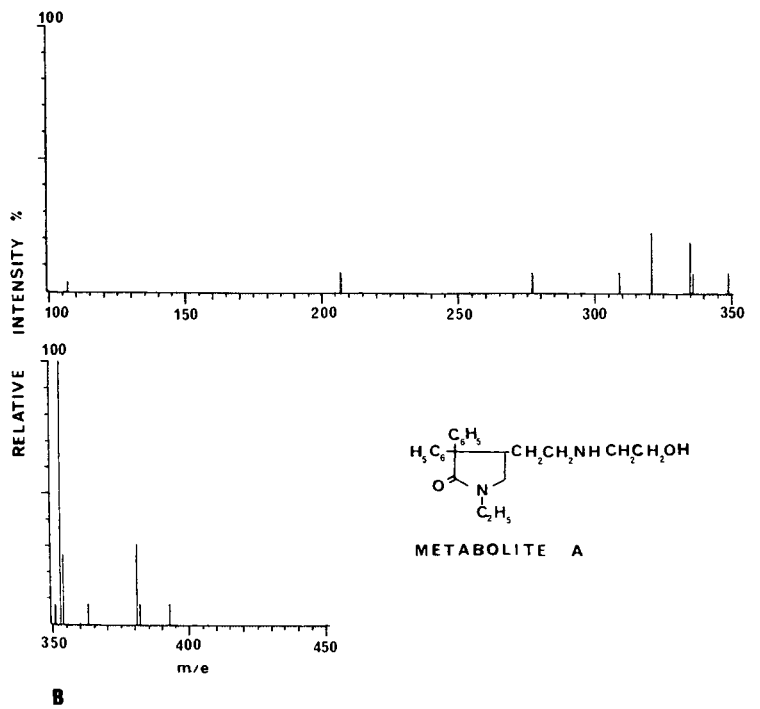

100
INTENSITY %
100
150
200
250
300
350
100
RELATIVE
350
400
450
m/e
B
METABOLITE A

Fig. 2.

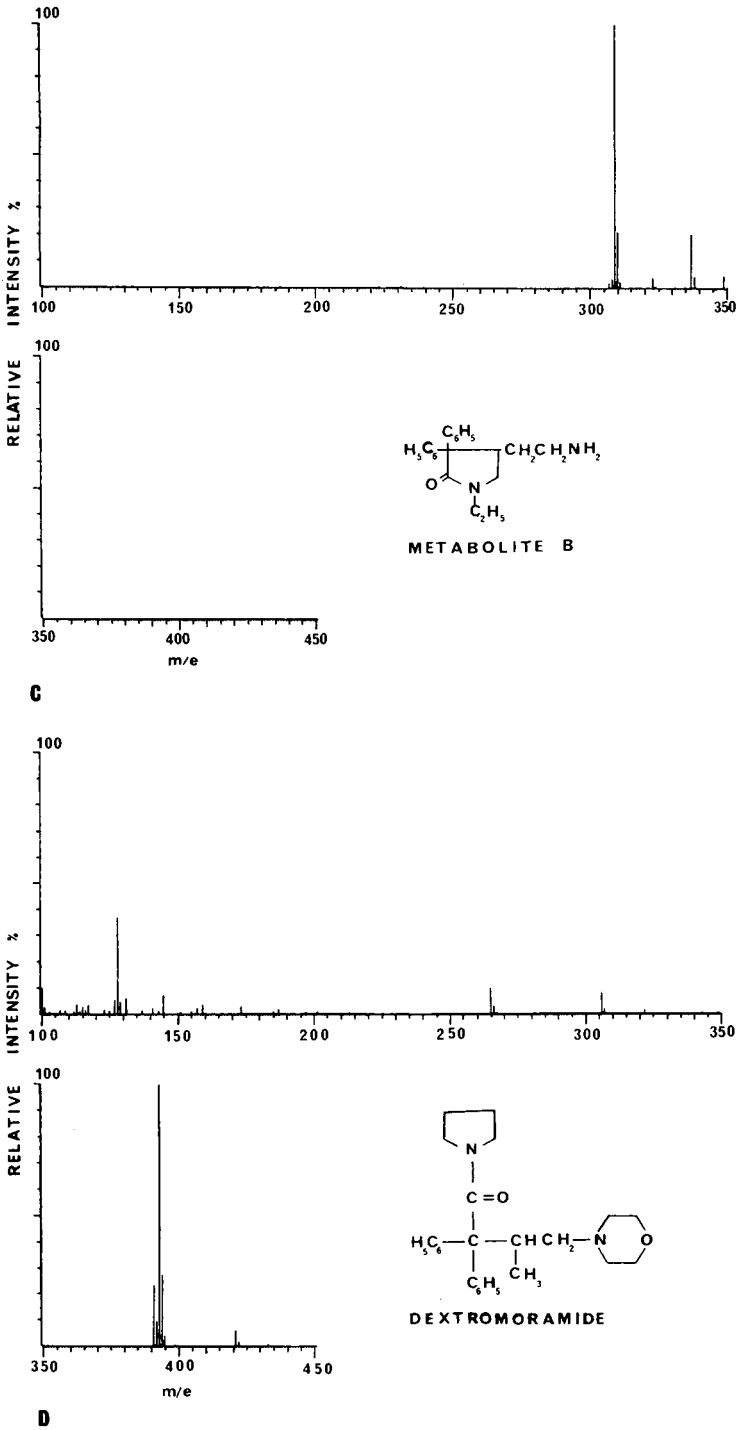


Fig. 2. Chemical ionisation mass spectra of (A) doxapram; (B) metabolite A, (C) metabolite B and (D) dextromoramide, obtained from a urine extract from a premature newborn receiving doxapram (the sample was spiked with internal standard dextromoramide). Separation was achieved on 1% OV-225 (GC—MS conditions, see text).

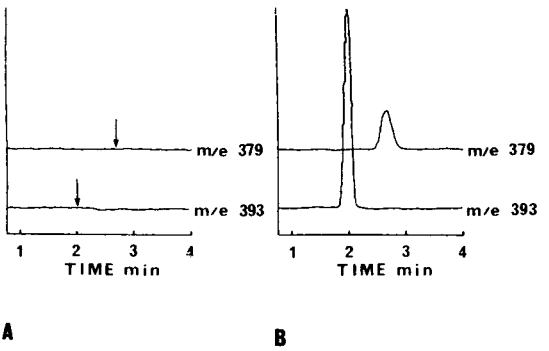


Fig. 3. Mass fragmentograms of blood samples from a premature newborn. The protonated molecular ions of doxapram (*m/e* 379) and dextromoramide (*m/e* 393) were monitored. (A) Blank blood sample; (B) blood sample containing 87 ng doxapram hydrochloride (GC—MS conditions, see text).

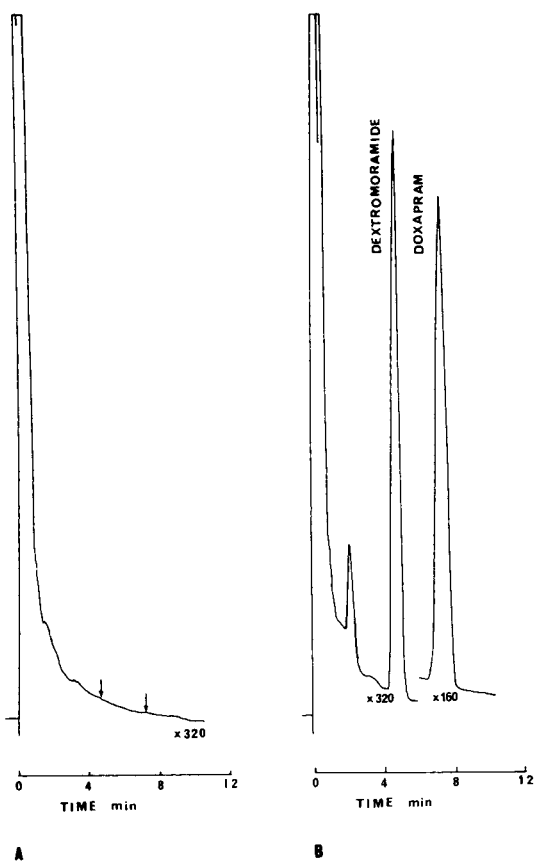


Fig. 4. Gas chromatogram of A, blank urine extract and B, urine extract from premature newborn receiving doxapram. Separation was achieved on 1% OV-225 (GC—FID conditions, see text).

cient of variation for six replicate analyses of doxapram from blank urine containing known amounts of doxapram is listed in Table I.

Fig. 5 presents the plasma and blood levels of doxapram during and after cessation of an infusion of doxapram in a premature newborn of estimated gestational age 27 weeks, postnatal age 0.5 days. The half-life as determined from the post infusion data was 7.7 h in blood and 8.6 h in plasma. Urinary excretion data are presented in Fig. 5, 5.4% of the dose being excreted unchanged.

These quantitative methods have been applied successfully in a study of the pharmacokinetics of doxapram in premature newborns and these results will be presented elsewhere.

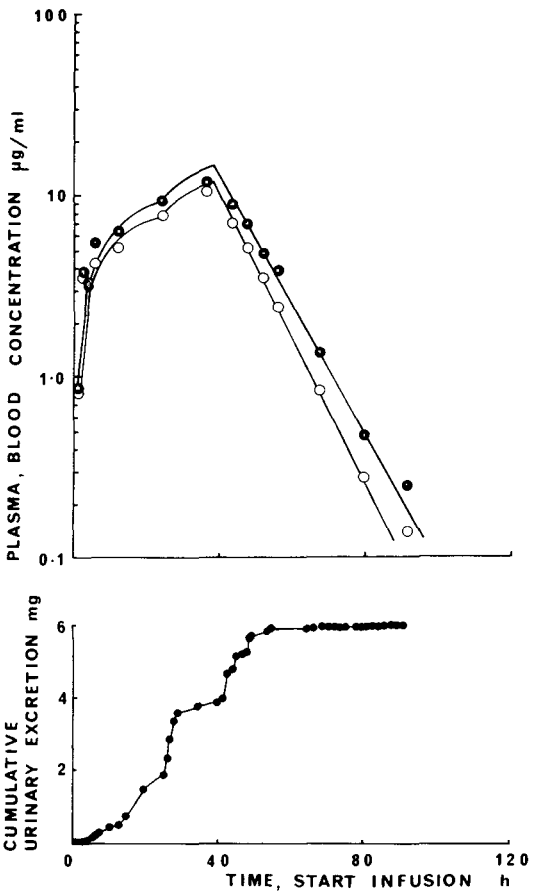


Fig. 5. (Top) blood (○) and plasma (●), and (bottom) urine data during and after cessation of an intravenous infusion of doxapram administered to a premature newborn.

ACKNOWLEDGEMENT

H.N. was supported by a Foundation 41 Postgraduate Scholarship.

REFERENCES

1 J.W. Ward and B.V. Franko, Fed. Proc., Fed. Amer. Soc. Exp. Biol., 21 (1962) 325.
2 H. Kato and J.P. Buckley, J. Pharmacol. Exp. Ther., 144 (1964) 260.
3 R.B. Bruce, J.E. Pitts, F. Pinchbeck and J. Newman, J. Med. Chem., 8 (1965) 157.
4 J.E. Pitts, R.B. Bruce and J.B. Forehand, Xenobiotica, 3 (1973) 73.
5 R.H. Robson and L.F. Prescott, J. Chromatogr., 143 (1977) 527.
6 H.M. McNair and E.J. Bonelli, Basic Gas Chromatography, Varian-Aerograph, Walnut Creek, Calif., 1978, p. 64.

Journal of Chromatography, 182 (1980) 201—210
Biomedical Applications

CHROMBIO. 517

MICRO DETERMINATION OF GENTAMICIN IN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET DETECTION

DIRK M. BARENDS, JOHANNES S.F. VAN DER SANDT and ABRAM HULSHOFF

Farmaceutisch Laboratorium, University of Utrecht, Catharijnesingel 60, 3511 GH Utrecht (The Netherlands)

(First received September 25th, 1979; revised manuscript received December 4th, 1979)

SUMMARY

A procedure for the high-performance liquid chromatographic determination of gentamicin in serum is described using pre-column derivatisation and UV detection. The serum proteins are precipitated with acetonitrile and the gentamicin components in the supernatant are derivatized with 1-fluoro-2,4-dinitrobenzene. The reaction products are chromatographed on a microparticulate C_{18} reversed-phase column and detected at 365 nm. Sample volumes of 50 μl are sufficient for the determination of gentamicin concentrations in, and well below, the therapeutic range.

INTRODUCTION

Gentamicin is a valuable antibiotic in the treatment of patients with gram-negative infections. Monitoring the serum concentration of gentamicin is considered to be helpful in achieving optimal therapeutic levels and in avoiding toxic reactions [1, 2]. Gentamicin serum levels are determined most often by microbiological methods or by radioimmunoassay, but modern chromatographic methods can offer an alternative. A gas—liquid chromatographic method for the assay of aminoglycoside antibiotics in serum has been reported [3, 4]. The method requires a precipitation step and an evaporation step, followed by a two-stage derivatisation of first the hydroxyl groups and then the amino groups.

High-performance liquid chromatographic (HPLC) methods for measuring aminoglycoside antibiotic levels in serum have also been described recently [5—10]. In all these methods fluorescence detection of the derivatized aminoglycosides was used. High-performance liquid chromatographs with UV detectors are rapidly becoming standard equipment in hospital laboratories. An

HPLC method based on UV absorption detection rather than on fluorescence detection should therefore be useful for monitoring the serum levels of these compounds.

In the present investigation a simple HPLC method, based on a method reported by Bangert and Grossman [11], was developed for gentamicin, requiring only 50 μl of serum. The method uses pre-column derivatisation, separation of the components of the reaction mixture on a reversed-phase column and UV detection of the chromatographed products at 365 nm.

MATERIALS AND METHODS

Apparatus and chromatographic conditions

The chromatographic system consisted of a Solvent Delivery System 6000A, a Universal Injector U6K with a 2-ml injection loop (or an Autosampler WISP 710), a UV Absorbance Detector 440 equipped with a 365-nm filter and a μBondapak C_{18} column (30 cm × 3.9 mm I.D., particle size 10 μm) all from Waters Assoc. (Milford, Mass., U.S.A.). Retention times and peak areas were measured with the chromatographic Data System SP4000 of Spectra-Physics (Santa Clara, Calif., U.S.A.)

The mobile phase consisted of 1 g/l tris(hydroxymethyl)aminomethane in water (300 ml/l), adjusted with hydrochloric acid to pH 7, and acetonitrile (700 ml/l). This mixture was passed through a 0.2-μm filter and de-aerated ultrasonically. The flow-rate was maintained at 1.5 ml/min. Chromatography was performed at room temperature.

Reagents and glassware

Demineralised water was used throughout. Hydrochloric acid, 2 mol/l in water, was prepared by diluting 12 mol/l hydrochloric acid (p.a., Merck, Darmstadt, G.F.R.). 1-Fluoro-2,4-dinitrobenzene (FDNB) and tris(hydroxymethyl)aminomethane (Tris) were both p.a. grade from Merck; acetonitrile was "zur Synthese" from Merck.

Garamycin®, an injection of gentamicin sulphate in water containing the equivalent of 40 g of gentamicin per litre, was from Essex (Heist-op-den-Berg, Belgium). The gentamicin components C_1, C_{1a}, and C_2 as their sulphate salts were by courtesy of Schering (Bloomsfield, N.J., U.S.A.); the exact purity and water content of these components were not known. All antibiotic concentrations were calculated relative to potency unless otherwise indicated.

Human serum obtained from ambulant patients was frozen and stored at −18° within three days of collection. Once defrozen it was used the same day.

Centrifuge tubes were of 7 ml capacity with glass stoppers and the conical end drawn to a fine point. Ampoules had a capacity of 1 ml.

Solutions

All solutions and the acetonitrile were passed through a 0.2-μm filter before use.

Solution 1: gentamicin sulphate in water, containing the equivalent of 4 mg of gentamicin per litre.

Solution 2: Tris 20 g/l in water.
Solution 3: FDNB 250 g/l in acetonitrile.
Solution 4: FDNB 170 g/l in acetonitrile.

Procedures

Procedure A: derivatisation of aqueous gentamicin solutions. Dispense into an ampoule 50 µl of solution 1, 50 µl of solution 2, 200 µl of acetonitrile and 20 µl of solution 3. Close the ampoule and place in a water-bath at 80° for 45 min. Inject 175 µl into the chromatograph.

Procedure B: derivatisation of serum samples. Dispense 50 µl of serum into a centrifuge tube, add 50 µl of solution 2 and vortex for 15 sec. Add 200 µl of acetonitrile and vortex for 15 sec, then centrifuge at 2500 *g* for 5 min. Transfer 200 µl of the supernatant into an ampoule, add 20 µl of solution 4. Close the ampoule and place in a water-bath at 80° for 45 min. Inject 175 µl into the chromatograph.

Procedure C: in vivo experiment. A healthy 80-kg volunteer received 1 mg of gentamicin per kg by intramuscular injection. Blood samples were collected at regular time intervals by venipuncture. Serum was separated from the collected blood and stored at −18°. Gentamicin standards were prepared in pooled serum with a gentamicin injection from the same lot that was administered to the subject. Serum samples (in duplicate) and standards were analyzed in one run according to procedure B.

Aqueous dilutions of this injection were also analysed following procedure A. Peak heights of the C_{1a} and of $C_1 + C_2$ derivatives were measured, and the ratio (peak height $C_1 + C_2$ derivatives)/(peak height C_{1a} derivative) was calculated for all serum samples and for the gentamicin injection.

RESULTS

Gentamicin consists of three major components, C_1, C_{1a}, and C_2, which are present in roughly equal amounts. By running the purified gentamicin components through procedure A, the elution order of the gentamicin derivatives was determined. The C_1 derivative eluted a few seconds before the C_2 derivative; however, with the gentamicin mixture, the C_1 and C_2 derivatives appeared as one single peak in the chromatogram. The C_{1a} derivative was well separated from the others, with a retention time of 1.5 min less. The purified gentamicin components yielded the same peak area per mol (by weight) within the experimental error. Fig. 1 shows chromatograms obtained from blank serum, and from serum spiked with gentamicin.

The optimal derivatisation conditions were determined by investigating the effect of pH, FDNB concentration and of reaction time and temperature on the derivatisation of aqueous gentamicin solutions. The results are depicted in Fig. 2—4.

Precision and linearity

Serum samples with five different concentrations of gentamicin, ranging from 1 to 16 mg/l were analyzed in one run using procedure B. The results are summarized in Table I.

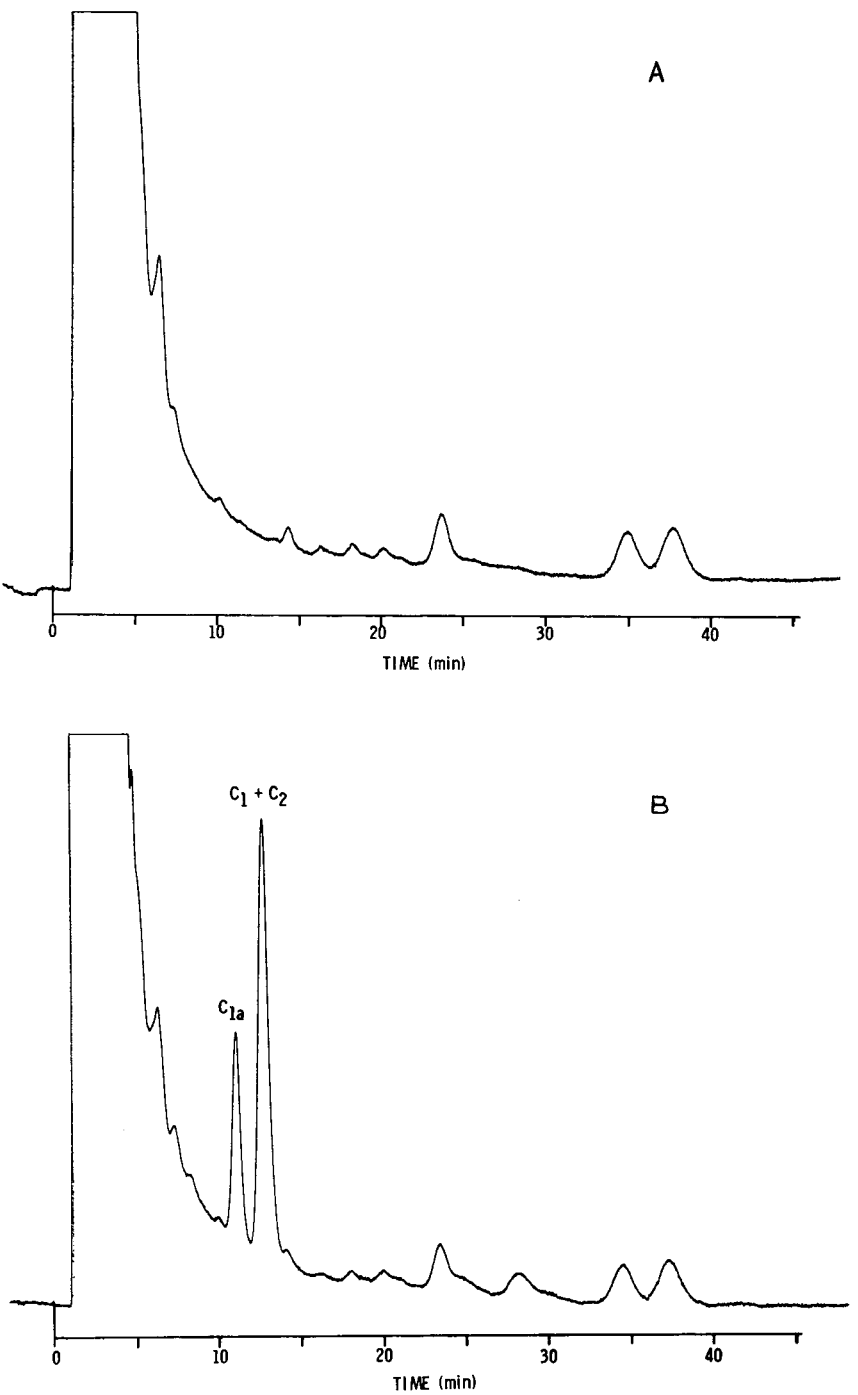


Fig. 1. HPLC of serum samples. Chromatogram A was obtained from 50 μl of blank serum, chromatogram B from 50 μl of blank serum, spiked with gentamicin to a concentration of 4 mg/l. Detector setting was 0.01 a.u.f.s.

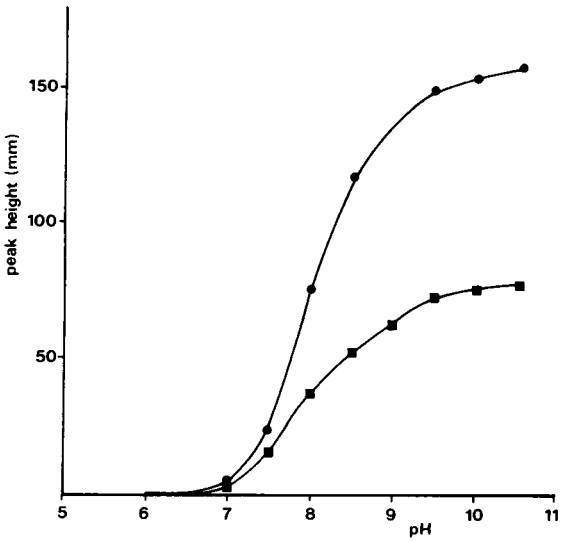


Fig. 2. Effect of pH of the Tris solution on the peak heights of the derivatized gentamicin components C_1 and C_2 (•); and C_{1a} (▪). Experimental details: procedure A, modified by adding different quantities of hydrochloric acid to solution 2. Each point represents the mean value of three or four determinations. Peak heights are based on a detector setting of 0.01 a.u.f.s.

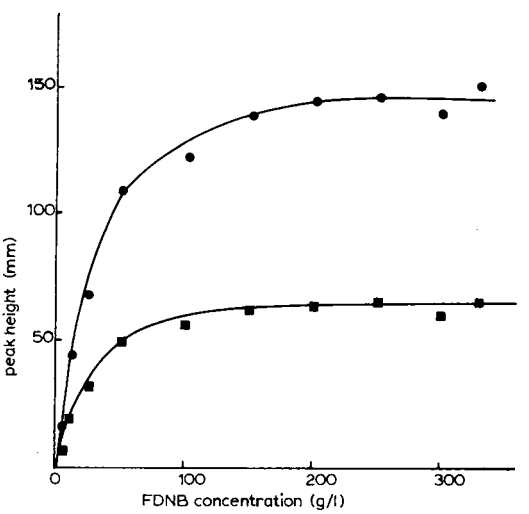


Fig. 3. Effect on FDNB concentration of solution 3 on the peak heights of the derivatized gentamicin components. Symbols as in Fig. 2. Experimental details: procedure A, modified by varying the FDNB concentration of solution 3. Each point represents the mean value of three or four determinations.

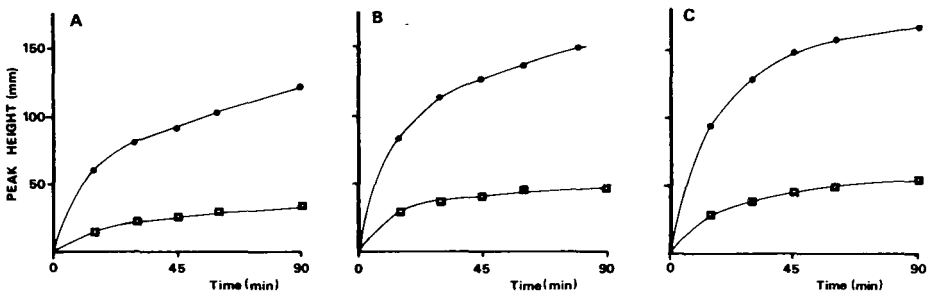


Fig. 4. Effect of the reaction time on the peak heights of the derivatised gentamicin components, at reaction temperatures of 65° (A), 80° (B) and 100° (C). Symbols as in Fig. 2. Experimental details procedure A, modified by varying reaction time and temperature. Each point represents the mean value of three determinations.

TABLE I

PEAK HEIGHTS AND COEFFICIENTS OF VARIATION OF THE DERIVATIZED GENTAMICIN COMPONENTS

Serum samples of 50 μl were used.

Gentamicin concentration (mg/l)	*n*	Component C_{1a}			Components C_1 and C_2		
		PH* (mm)	PH/concentration	C.V. (%)	PH (mm)	PH/concentration	C.V. (%)
1	6	19	19	4.8	40	40	3.7
2	6	32	16	0.9	74	37	3.0
4	5	68	17	2.7	147	37	1.7
8	6	152	19	5.9	312	39	5.4
16	6	311	19	5.5	627	39	4.9

*Peak heights (PH) are based on a detector setting of 0.01 a.u.f.s.

Sensitivity

Samples of pooled serum containing gentamicin at 0.33 mg/l were analyzed according to procedure B. The value of the ratio of the mean peak height of the derivatisation products and the gentamicin concentration was found to be 17 (C.V. = 12%; $n = 6$) for the C_{1a} component, and 35 (C.V. = 4.3%; $n = 6$) for the $C_1 + C_2$ components.

Specificity

Serum samples from a patient subjected to a combined therapy of gentamicin and ampicillin, and serum samples from a patient treated with gentamicin, trimethoprim and sulphamethoxazole, were analyzed by procedure B. No interfering peaks could be discerned in the chromatograms.

Recovery

Recovery was estimated by spiking serum samples with gentamicin at a concentration of 4 mg/l, before and after the acetonitrile treatment, respectively. Both sera were analyzed six times in one run, according to procedure B. Recoveries were found to be 83% (S.D. = 4%; $n = 6$) for the C_{1a} component, and 84% (S.D. = 2%; $n = 6$) for the $C_1 + C_2$ components.

Comparison of sera from different persons

Twenty-seven sera, obtained from different persons, and pooled serum, were spiked with gentamicin, each to the same concentration (6 mg/l). Each of the individual sera, in duplicate, and the pooled serum (six determinations), were analyzed in one run according to procedure B.

The average peak heights of the C_{1a} derivative peak, obtained from the individual sera and from the pooled serum were 101.4 mm (S.D. = 3.2 mm; n = 27) and 100.0 mm (S.D. = 2.2 mm; n = 6), respectively. For the peak of the C_1 + C_2 derivatives an average peak height of 223.6 mm (S.D. = 5.4 mm; n = 27) was obtained with the individual sera, while pooled serum determinations yielded a value of 220.4 mm (S.D. = 5.0 mm; n = 6). (Peak heights were based on a detector setting of 0.01 a.u.f.s.)

In vivo experiment (Procedure C)

The ratio of the combined peak heights of the C_1 and C_2 components to the peak height of the C_{1a} component was found to be the same in the serum samples and in the administered injection, and this ratio remained constant in the serum samples collected at different times after the injection. This ratio was found to be 2.27 (S.D. = 0.09; n = 21) for the serum samples, and 2.29 (S.D. = 0.13; n = 6) for the injection.

Gentamicin serum concentrations, calculated by measuring the C_{1a} derivative peak, did not differ significantly from the serum concentration found by measuring the peak of the C_1 + C_2 derivatives. The average of four values obtained for the gentamicin concentration (one C_{1a}-based and one C_1 + C_2-based, in duplicate) was taken as the final value.

The serum concentration—time curve of gentamicin in the volunteer is shown in Fig. 5. A half-life of 2.4 h was calculated from the data obtained.

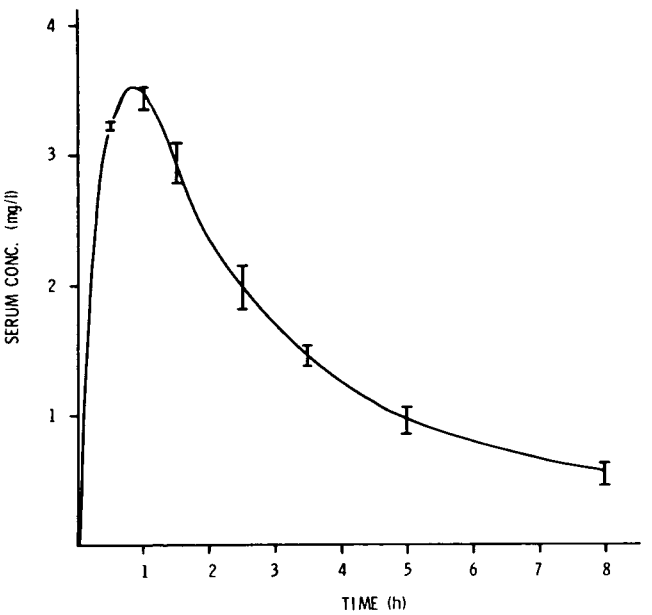


Fig. 5. Serum concentration of gentamicin as a function of time obtained in a volunteer following an intramuscular injection of 1 mg per kg body weight. Each value is the mean (± S.D.) of four values from two determinations (see Results).

DISCUSSION

Deproteinisation

Deproteinisation of gentamicin-containing serum samples, by precipitation with acetonitrile after alkalinisation, has been reported previously [6, 10]. An organic buffer system was chosen for making the samples alkaline, because the usual inorganic buffer compounds tend to precipitate in the acetonitrile—water mixture.

Derivatisation

The peak heights of the derivatized products increased with higher pH values of the added buffer solution. Above pH 9.5 no significant increase in the peak heights was observed (Fig. 2). A mixture of equal volumes of human serum and of Tris solution 2 resulted in a solution of pH 9.6. The peaks of the derivatized gentamicin components increased with higher reagent concentrations, up to an FDNB concentration of about 200 g/l (Fig. 3). FDNB at 250 g/l in acetonitrile was chosen as the derivatisation reagent for procedure A, and at 170 g/l in acetonitrile for procedure B, resulting in the same FDNB concentration in the derivatisation mixture for both procedures. Time—temperature curves were constructed with reaction temperatures of 65°, 80°, and 100°, covering reaction times of up to 90 min (Fig. 4). In none of the resulting plots was a constant peak height achieved, indicating that under the experimental conditions derivatisation was not complete.

As the experimental error was somewhat larger when derivatisation took place at 100° compared with at 80°, the latter temperature was chosen as the derivatisation temperature for the assay. A reaction time of 45 min was selected, in order to keep the time needed for the total assay within acceptable limits. Gentamicin solutions, which were derivatized for 45 min at 80°, showed an increase in peak heights of 14 ± 2% for both product peaks after storage of the reaction mixtures for 24 h at room temperature. When the solutions were injected within 4—5 h of derivatisation, the increase in peak height remained within experimental error.

Stoichiometry of the derivatisation reaction

After derivatisation of equal (molar) amounts of the three purified gentamicin components, the resulting peak areas did not differ significantly. These results are in agreement with a reaction in which every amino group, primary as well as secondary, reacts with one FDNB molecule. Evidence that all of the available amino groups of aminoglycosides can be derivatized with FDNB was also presented by Tsuji et al. [12].

Chromatography

Methanol—water mixtures and acetonitrile—water mixtures were investigated as possible mobile phases. The best results were obtained with the acetonitrile—water mixture described above. The mobile phase was buffered at pH 7 to prevent column damage due to the repeated injection of alkaline samples. Acetonitrile "zur Synthese" was found to be fully satisfactory since detection was performed at 365 nm.

The time required for a complete chromatogram was 45 min, due to the appearance of four small peaks after the gentamicin derivative peaks (Fig. 1). Three of these peaks were observed in the chromatogram from blank serum. One peak, with a retention time of about 28 min, also appeared in chromatograms resulting from aqueous gentamicin solutions, but not in the reagent blank chromatogram; this peak can therefore probably be ascribed to a minor component of the gentamicin complex.

To reduce the analysis time samples were injected at 20-min intervals; this gave a partial overlap of the chromatograms but did not affect the gentamicin peaks. Use of an autosampler made this procedure feasible. Alternatively one might use a flow-rate of 3.0 ml/min; this does not seriously affect the resolution of the peaks, but twice the amount of mobile phase is needed.

Calibration curve and analysis of serum samples

As is apparent from the data in Table I, the calibration curve proved to be a straight line through the origin, over the studied concentration range, for both the C_{1a} and the $C_1 + C_2$ components. No significant differences were observed between the results obtained with pooled serum and with 27 sera from different persons. The method can therefore be applied to the analysis of different serum samples, despite the fact that the derivatisation of the gentamicin components is not complete.

Gentamicin injections from different batches contain different amounts of the C_1, C_{1a}, and C_2 components. With the method described above, gentamicin C_1 and gentamicin C_2 are not resolved. The potencies of the three components may not be the same [13]. It is therefore advisable to construct calibration curves using the same batch of gentamicin that was administered to the patient(s).

From the in vivo experiment it can be concluded that the pharmacokinetic parameters of the three components do not differ significantly. Similar results have been reported previously [14]. This allows computation of the gentamicin concentration in the serum of patients from either one of the two gentamicin peaks, by peak height or by peak area measurements. If the corresponding gentamicin injection solution is not available for calibration, the relatively small differences in potency between the three components might be ignored, and the sum of the peak areas of the two gentamicin peaks can be used for the construction of the calibration curve and for the calculation of the gentamicin level in serum samples from the patient(s), as has been proposed by Anhalt et al. [5].

The serum concentration—time curve, and the gentamicin half-life, obtained by the in vivo experiment, are in good agreement with the data obtained by other investigators using a microbiological assay [15].

Advantages of the proposed method

The method as described above is simple and suitable for automation. Fluorescence detection after derivatisation with *o*-phthalaldehyde [5, 6, 8, 9] or with dansyl chloride [7, 10] offers, in principle, greater sensitivity than UV detection; however, the sensitivity obtained with the proposed method is quite sufficient for monitoring gentamicin levels in the therapeutic range, in serum

samples no larger than 50 μl. The lowest concentration of gentamicin that can be determined in 50-μl serum samples with acceptable precision was found to be about 0.3 mg/l of serum.

ACKNOWLEDGEMENTS

The purified gentamicin components (C_1, C_{1a} and C_2) were a gift of Schering (Bloomsfield, N.J., U.S.A.). We are indebted to Dr. J.B.J. Soons, St. Antoniusziekenhuis Utrecht, for providing many of the serum samples, and to A.H. Teeuw, M.D., for his assistance with the in vivo experiment.

REFERENCES

1 M. Barza and R.T. Scheife, Amer. J. Hosp. Pharm., 34 (1977) 723.
2 M. Barza and M. Lauermann, Clin. Pharmacokin., 3 (1978) 202.
3 J.W. Mayhew and S.L. Gorbach, J. Chromatogr., 151 (1978) 133.
4 J.W. Mayhew and S.L. Gorbach, Antimicrob. Ag. Chemother., 14 (1978) 851.
5 J.P. Anhalt and S.D. Brown, Clin. Chem., 24 (1978) 1940.
6 S. Bäck, I. Nilsson-Ehle and P. Nillson-Ehle, Clin. Chem., 25 (1979) 1222.
7 W.L. Chiou, R.L. Nation, G.W. Peng and S.M. Huang, Clin. Chem., 24 (1978) 1846.
8 S.K. Maitra, T.T. Yoshikawa, J.L. Hansen, I. Nilsson-Ehle, W.J. Palin, M.C. Schotz and L.B. Guze, Clin. Chem., 23 (1977) 2275.
9 S.K. Maitra, T.T. Yoshikawa, C.M. Steyn, L.B. Guze and M.C. Schotz, Antimicrob. Ag. Chemother., 14 (1978) 880.
10 G.W. Peng, M.A.F. Gadalla, A. Peng, V. Smith and W.L. Chiou, Clin. Chem., 23 (1977) 1838.
11 F.K. Bangert and D.M. Grossman, 4th Annual FACSS Meeting, Detroit, Mich, 1977, Abstract No. 164.
12 K. Tsuji, J.F. Goetz, W. VanMeter and K.A. Gusciora, J. Chromatogr., 175 (1979) 141.
13 K.E. Price, J.C. Godfrey and H. Kawaguchi, in D. Perlman (Editor), Structure—Activity Relationships among the Semisynthetic Antibiotics, Academic Press, New York, 1977, p. 326.
14 R.L. Nation, G.W. Peng, W.L. Chiou and J. Malow, Eur. J. Clin. Pharmacol., 13 (1978) 459.
15 R.E. Winters, K.D. Litwack and W.L. Hewitt, J. Infec. Dis., 124 (Suppl.) (1971) 90 (through W.L. Hewitt, in G.G. Jackson (Editor), Gentamicin: Review and Commentary on Selected World Literature, Schering Corporation, Kenilworth, N.J., 1974, p. 57).

Journal of Chromatography, 182 (1980) 211—220
Biomedical Applications

CHROMBIO. 510

ANALYSIS OF THE ANTICANCER DRUGS VP 16-213 AND VM 26 AND THEIR METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

R.J. STRIFE* and I. JARDINE*,**

Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, Purdue University, W. Lafayette, Ind. 47907 (U.S.A.)

and

M. COLVIN

Pharmacology Laboratory, The Johns Hopkins Oncology Center, 601 North Broadway, Baltimore, Md. 21205 (U.S.A.)

(First received August 29th, 1979; revised manuscript received November 30th, 1979)

SUMMARY

A rapid and convenient high-performance liquid chromatographic procedure for the analysis of the clinically useful anticancer agents VP 16-213 and VM 26 is described. The drugs, which are semi-synthetic derivatives of the natural product podophyllotoxin, are extracted from plasma with chloroform. The extracts are evaporated to dryness, reconstituted in methanol, and chromatographed on a reversed-phase microparticle C_{18} column using isocratic elution with a mixture of methanol—water (60:40). Each drug is used as the internal standard for the other. Quantitation to 500 ng/ml (0.85 nmole/ml) plasma is based on peak height ratios using UV detection at 254 nm. Patient plasma concentration versus time data agree well with previously published data obtained using radiolabelled drug.

Investigations into the nature of the hydroxy acid metabolite of VP 16-213, carried out using paired-ion chromatography with tetrabutylammonium bromide and fluorescence detection, are described. Also, a unique separation of VP 16-213 and a possible metabolite, the isomer, picro VP 16-213, is described.

*Present address: Department of Pharmacology, Mayo Clinic, Rochester, Minn. 55901, U.S.A.
**To whom correspondence should be addressed.

INTRODUCTION

VP 16-213 and VM 26 (Fig. 1), two semisynthetic derivatives [1] of the natural product podophyllotoxin, are currently undergoing promising clinical evaluation as anticancer agents [2]. The pharmacokinetic behavior of these drugs in patients has been extensively investigated by Creaven and Allen [3—5] and Pelsor et al. [6] using tritiated VP 16-213 and VM 26. In these studies, the tritiated drugs were extracted with chloroform from biological fluids and subjected to scintillation counting. A simple, rapid, routine and non-radioactive assay for these drugs in plasma was required for further clinical investigation of the pharmacokinetics and metabolism of these drugs. This has been accomplished using reversed-phase high-performance liquid chromatography (HPLC) on a microparticle C_{18} column.

Pelsor et al. [6] and Allen et al. [7] have reported that VP 16-213 is extensively metabolized to a chloroform insoluble hydroxy acid (Fig. 2). They isolated this acid and characterized it by the mass spectrum of the permethylated derivative. This metabolite was further investigated by again utilizing reversed-phase HPLC. Since the *trans*-hydroxy acid of VP 16-213 has not been synthetized, studies proceeded with the easily synthetized *cis*-hydroxy acid. Since the hydroxy acid was poorly retained on the reversed-

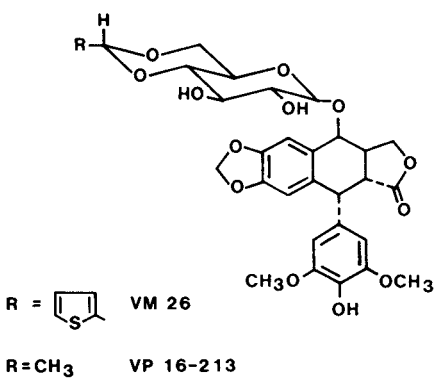


Fig. 1. Structures of VP 16-213 and VM 26.

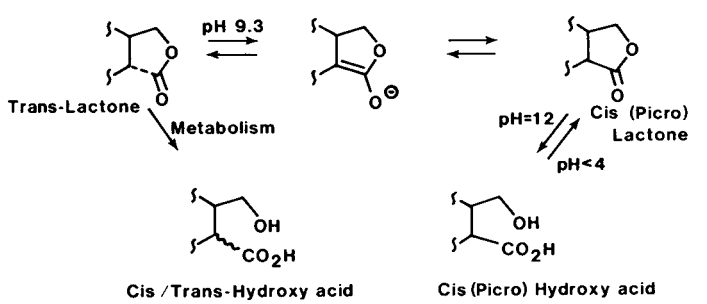


Fig. 2. Chemical interconversions of the lactone ring of the natural product podophyllotoxin and of derivatives such as VP 16-213 and VM 26 (all *trans*-lactones).

phase C_{18} column, paired-ion chromatography was applied to the analysis. Fluorescence detection was employed to achieve specificity and sensitivity of analysis. Initial isolation of the presumed hydroxy-acid metabolite of VP 16-213 from plasma was by neutral adsorption on XAD-4 resin.

Reports have appeared in the literature [8] suggesting that podophyllotoxin undergoes epimerization in vivo to the much less biologically active picropodophyllotoxin (Fig. 2). This possibility for VP 16-213 was investigated. This necessitated the development of a separation of VP 16-213 from picro VP 16-213, Separation of VP 16-213 and its picro isomer could not be achieved on a reversed-phase C_{18} column but was effected on a reversed-phase microparticle phenyl column.

EXPERIMENTAL

Materials

VP 16-213 and VM 26 were a generous gift from Drs. H. Friedli and H. Stähelin (Sandoz, Basle, Switzerland). Picro VP 16-213 and picro VP 16-213 hydroxy acid were synthesized as described below. Stock solutions (1 mg/ml) were made up in methanol and stored in a refrigerator at 5° for no longer than one week.

Methanol distilled in glass was obtained from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.). Water was used as obtained from the building purified water system. Ethyl acetate and chloroform, CHROM-AR grade were purchased from Mallinckrodt (St. Louis, Mo., U.S.A.). Tetra-*n*-butylammonium bromide X grade was obtained through Scientific Products (McGaw Park, Ill., U.S.A.) from Eastman Chemicals (Rochester N.Y., U.S.A.). Ceric ammonium sulfate and 85% phosphoric acid were obtained from Fischer Scientific (Chicago, Ill., U.S.A.). XAD-4 resin was supplied by Supelco (Bellefonte, Pa., U.S.A.). Precoated thin-layer chromatography (TLC) plates of silica gel (0.25 mm) on glass with fluorescent indicator were obtained from EM Laboratories (Elmsford, N.Y., U.S.A.). Extractions were carried out in 16 × 125 mm culture tubes with PTFE-lined caps (Scientific Products). All glassware was routinely washed in chromic acid.

Apparatus

A Waters Assoc. (Milford, Mass., U.S.A.) Model ALC 202 high-pressure liquid chromatograph equipped with a UV detector at 254 nm and a Model U6K injector was used. Fluorescence detection was accomplished by running the column effluent through a 35-μl flow cell (Aminco, Silver Springs, Md., U.S.A.) inserted in the cell holder of an Aminco-Bowman SPF spectrofluorometer. Analyses of VP 16-213 and its acid metabolite and of VM 26 were performed using a 30 cm × 3.9 mm I.D. column prepacked with μBondapak C_{18} (10 μm) from Waters Assoc. An identical column prepacked with μBondapak Phenyl (10 μm, with a phenyl group chemically bonded to μPorasil at 16% by weight) from Waters Assoc. was used to analyze picro VP 16-213.

HPLC conditions

Analyses were performed with a flow-rate of 1.0—1.2 ml/min at an inlet pressure of 1600—2200 p.s.i. For the analysis of VP 16-213 and VM 26, the solvent was methanol—water (60:40) although this ratio was altered slightly (e.g., to 58:42) depending on C_{18} column performance. The separation of picro VP 16-213 and VP 16-213 on the phenyl column was achieved using methanol—water (50:50). Paired-ion chromatography of the VP 16-213 hydroxy acids was accomplished by dissolving tetra-*n*-butylammonium bromide to the appropriate molarity directly in the mobile phase. All solvents were clarified with a 0.5-μm MF-Millipore filter (Millipore Corporation, Bedford, Mass., U.S.A.). Injection volume was generally 20 μl.

Separation of VP 16-213, picro VP 16-213 and picro VP 16-213 hydroxy acid by thin-layer chromatography

The compounds were spotted in methanol and the TLC plates were developed in ethyl acetate. The spots were dark under short-wave UV light and were also visualized with a 1% solution of ceric ammonium sulfate in 85% phosphoric acid (w/w). The reagent was diluted 50/50 volume for volume with water to facilitate spraying. The R_F values were: VP 16-213, 0.57 (light red); picro VP 16-213, 0.49 (light orange—red); picro VP 16-213 hydroxy acid, origin to 0.03 (light red).

Synthesis of picro VP 16-213

Picro VP 16-213 was synthesized by using a modification of a known procedure for the conversion of podophyllotoxin to picropodophyllotoxin [9]. 16 mg of VP 16-213, 2.5 ml of 1.2 *M* sodium acetate and 3.75 ml of ethanol were heated together with stirring for 2.5 h at 75°. The reaction medium was about pH 9. The ethanol was removed with a nitrogen stream and the pH was adjusted to 6 with 1 *N* HCl. The product was extracted with 10 ml of chloroform and the extract volume was reduced to about 0.5 ml. The product was then precipitated with diethyl ether. The ether—chloroform layer was decanted and reduced in volume by slow evaporation to produce more product as fine needles (m.p. 210—212°). The product was pure by TLC and HPLC and its mass spectrum exhibited all of the major ions found in the mass spectrum of VP 16-213. Particularly diagnostic were *m/z* 588 (M^+) and *m/z* 382 ($C_{21}H_{18}O_7$; the aglycone fragment and base peak).

Synthesis of picro VP 16-213 hydroxy acid

To 10 mg of VP 16-213 and 1 ml of water were added exactly two equivalents of accurately standardized 0.1 *N* NaOH. The mixture, pH about 12, was heated with stirring at 45° for 50 min. After cooling to room temperature, two equivalents of accurately standardized 0.1 *N* HCl were added, causing a precipitate to form. The mixture was immediately lyophilized and the residue was repeatedly swirled with 2-ml portions of ethyl acetate until most of the product had been taken up. As the combined extracts were reduced in volume with a nitrogen stream, the product crystallized out (m.p. 182—186°, decomposition).

The product was pure by TLC and HPLC and was very polar as suggested by

its low R_F and low retention on the reversed-phase column. Its retention time could be increased by using paired-ion chromatography at pH 7 with tetra-*n*-butylammonium bromide, indicating that it was acidic. The carbonyl stretching frequency had shifted from 1775 cm^{-1} (lactone) to 1710 cm^{-1}. The mass spectrum showed no melcular ion but the base peak for the aglycone fragment appeared at *m/z* 400, indicating successful hydrolysis of the lactone ring. Permethylation [10] shifted the aglycone fragment base peak to *m/z* 442, which would be expected for the hydroxy acid. The permethylated product also revealed *m/z* 676 ($M^{\ddagger}$), *m/z* 645 ($M-CH_3O$) and *m/z* 644 ($M-CH_3OH$) Under acidic conditions the underivatized product recyclized to picro VP 16-213. These observations are consistent with the product being picro VP 16-213 hydroxy acid (*cis*-acid).

Calibration and analysis of VP 16-213 from plasma

To 1 ml of plasma in a culture tube were added 10 µl of VM 26 solution (1 mg/ml) as internal standard. The sample was extracted with 5 ml of chloroform by gentle rocking for 15 min. Following brief centrifugation approximately 4.5 ml of the chloroform layer were transferred to a conical centrifuge tube and evaporated to dryness at 40° with a nitrogen stream. Methanol (50 µl) was added and the tube was sealed and vortexed. The samples were centrifuged 5—10 min to precipitate any particulate matter and 20 µl of supernatant were injected onto the HPLC system with the µBondapak C_{18} column. A calibration curve from 0.5 to 30 µg/ml was established by analyzing spiked samples in triplicate and plotting peak height ratios of VP 16-213:VM 26, after subtraction of background values, versus plasma concentration of VP 16-213. Patient samples were analyzed similarly by splitting the collected plasma sample (usually about 2–3 ml) and carrying out duplicate analyses using the zero time sample as background.

Analysis for picro VP 16-213 hydroxy acid

Before use, XAD-4 resin (40—80 mesh) was extensively washed with 1 *N* NaOH, water, acetone, chloroform, methanol, 1 *N* HCl, water and methanol, respectively. XAD-4 columns were poured in methanol into disposable pipettes with methanol-washed glass-wool plugs. The resin was then rinsed with 10 ml of methanol and then 20 ml water. The columns were stored in water and were not allowed to run dry.

To 1 ml of plasma in a centrifuge tube were added 1.5 ml of methanol to precipitate proteins. The sample was vortexed and then centrifuged for 10 min at high speed in a clinical centrifuge. The supernatant was decanted into a test tube and the volume was reduced to less than 0.5 ml with a nitrogen stream at 40°. The sample was then diluted by addition of 1 ml of water and was loaded onto the XAD-4 column. After rinsing with 20 ml of water, the compound was eluted with 3 ml of methanol into a conical centrifuge tube. The sample was blown dry at 40° with nitrogen, reconstituted in 50 µl of methanol, sealed, vortexed, centrifuged and injected (20 µl) on the HLPC system.

Analysis for picro VP 16-213

Picro VP 16-213 was extracted from spiked plasma with chloroform, as with

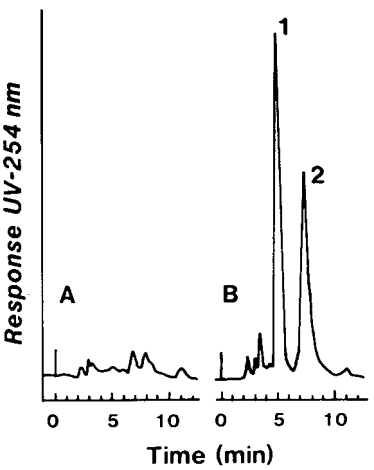
VP 16-213. Clinical samples were analyzed on a μBondapak Phenyl column to determine whether any discernable amount of picro VP 16-213 was present.

RESULTS AND DISCUSSION

The separation and analysis of VP 16-213 and VM 26 are illustrated in Fig. 3B, a chromatogram of a patient plasma extract 2 h after infusion of VP 16-213. VM 26 was added as the internal standard. The two compounds are well resolved, appearing with retention times of 5 min and 7.5 min for VP 16-213 and VM 26, respectively. The plasma background at this sensitivity is low (Fig. 3A). Both compounds are essentially quantitatively extracted by this method.

The calibration curve from 30 μg/ml of plasma to the practical limit of detection of VP 16-213, 500 ng/ml (0.85 nmole/ml) by least squares analysis gave a linear correlation coefficient of $r^2 = 0.9998$. The calculated sample estimate of the coefficient of variation was on the order of 2.5% for each set of data values used to establish the standard curve.

The analysis of VP 16-213 in the plasma of a 30-year old man with testicular carcinoma is shown in Fig. 4. The patient received a constant i.v. infusion of 170 mg VP 16-213 over one-half hour. The patient was also receiving cyclophosphamide and cis platinum. The profile over 24 h agrees well with typical curves obtained by Allen and Creaven [4] and Pelsor et al. [6] using radiolabelled drug.



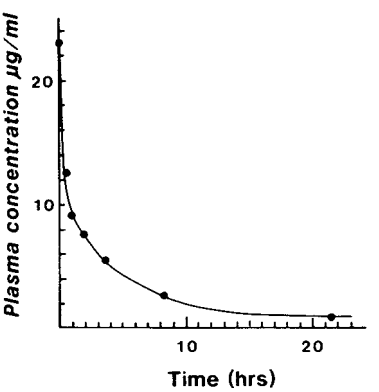


Fig. 3. HPLC UV traces of patient plasma samples treated by the chloroform extraction procedure. (A) Pre-infusion chromatogram; (B) 2-h post-infusion chromatogram with 10 μg/ml VM 26 as internal standard. Chromatographic conditions: 30 cm × 3.9 mm I.D. column with μBondapak C_{18} (10 μm); solvent, methanol—water (60:40); flow-rate, 1.0 ml/min at an inlet pressure of ca. 1600 p.s.i. Peaks : 1 = VP 16-213 (9.5 μg/ml); 2 = VM 26 (10 μg/ml).

Fig. 4. Post-infusion plasma decay curve of VP 16-213 for a patient receiving the drug. Each point represents the average of two separate determinations from 1-ml plasma samples.

The described assay for VP 16-213 is directly applicable to VM 26. For the analysis of VM 26, VP 16-213 is used as the internal standard. The use of these drugs as complementary internal standards is possible since the drugs are not at present used in combination.

Some relevant transformations of VP 16-213, which are analogous to known reactions of podophyllotoxin [11], are summarized in Fig. 2. Reaction conditions to produce these analogs of VP 16-213 were modifications of similar reactions of podophyllotoxin. The *trans*-hydroxy acid cannot be produced by direct alkaline hydrolysis, since the compound rapidly epimerizes via the enolate to the *cis*-lactone and is then opened to the *cis*-hydroxy acid, i.e., the hydrolyzed picro compound. Picro VP 16-213 may be selectively produced at pH 9. However, although the *trans*-hydroxy acid of podophyllotoxin has been synthetized [12], no synthesis of the *trans*-hydroxy acid of VP 16-213 has been reported to date. Work therefore proceeded with the *cis*-hydroxy acid. At acidic pH values this compound rapidly recyclizes to picro VP 16-213. Therefore, pH-controlled extraction was not feasible. Thus, XAD-4 resin was used to adsorb the acid from plasma since neutral or mildly basic conditions were required. Recovery was greater than 90%. The resin required extensive cleaning, and the C_{18} column was used to monitor the cleaning process. Even so, many UV absorbing interferences were adsorbed from plasma by the resin and subsequently eluted with methanol. Ion-pairing did not selectively move the acid away from the UV interferences. Therefore, fluorescence detection was examined.

Podophyllotoxins possess native fluorescence [13]. Picro VP 16-213 hydroxy acid can be excited at approximately 292 nm to fluoresce at 329 nm. By running the effluent from the HPLC system through a flow-cell inserted in the block of a typical spectrophotofluorometer, specificity for this acid was obtained. Because of the arrangement of the fluorescence detection system, the ultimate sensitivities available from fluorescence detection of this compound were not obtained, but low-microgram levels were easily measured. Paired-ion chromatography was also used at pH of about 6—7 to increase the retention time of the acid by adding tetrabutylammonium counter cation to the mobile phase. This helped to remove the acid from fluorescent interferences. A chromatogram of about 2 μg/ml of standard picro VP 16-213 *cis*-hydroxy acid is illustrated in Fig. 5B (broken trace), along with an extracted patient plasma sample obtained 2 h after infusion of VP 16-213. Background interferences (Fig. 5A) are low. Of interest is the observation that the picro VP 16-213 hydroxy acid standard has an ion-paired retention time of 5 min, whereas the metabolite has a retention time of only 4 min. Because the metabolite exhibited ion-pairing properties and its retention time does not match that of the *cis*-hydroxy acid (picro hydroxy acid), this constitutes indirect evidence that it may indeed be the *trans*-hydroxy acid. This implies that the hydrolysis may be enzymatically directed by, for example, an esterase. Assuming that the fluorescence response of the *cis*-hydroxy acid is comparable to that of the *trans*-hydroxy acid, and taking into account the 90% recovery from the XAD-4 extraction, the level of the hydroxy acid represented by the chromatogram of Fig. 5 is about 1—2 μg/ml of plasma. This agrees with the levels predicted by the pharmacokinetic model reported by Allen and Creaven [4] and Pelsor et al. [6]. This metabolism is currently being investigated further.

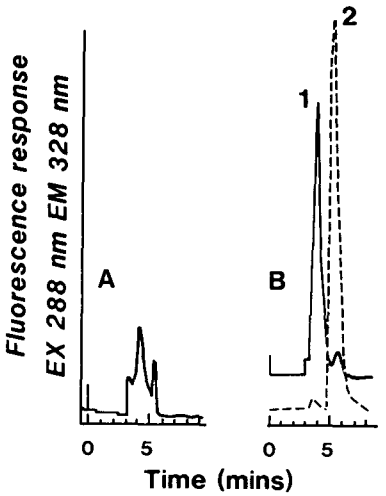


Fig. 5. HPLC fluorescence traces of patient plasma samples treated by XAD-4 extraction procedure. (A) Pre-infusion chromatogram; (B) 2 h post VP 16-213 infusion chromatogram. Chromatographic conditions: 30 cm × 3.9 mm I.D. column with μBondapak C_{18} (10 μm); solvent, methanol—water (50:51) with 0.5 m*M* tetra-*n*-butylammonium bromide at 1.0 ml/min at an inlet pressure of ca. 2000 p.s.i. Peaks: 1 = metabolite of VP 16-213, presumed to be the *trans*-hydroxy acid compound; 2 = standard (2 μg/ml) picro VP 16-213 hydroxy acid (*cis*-hydroxy acid) run separately under the same conditions.

The fluorescence of these compounds can be applied not only to the analysis of the main metabolite but also to the analysis of the parent drugs. The chloroform extracts of plasma are fairly clean with respect to UV detection and extremely clean with respect to fluorescence detection. Therefore, it is not anticipated that background fluorescence will limit the ultimate sensitivity of detection greatly. Since with a UV detector 10 ng of VP 16-213 is easily seen on injection of pure compound, there is reason to expect that quantitation to at least this level with a dedicated flow-cell in a fluorometric detector designed for efficient light collection would be possible. This would then allow the analysis of these drugs in other biological fluids, such as cerebrospinal fluid, and tissue, where lower levels are encountered. It would also allow the analysis of the drugs in smaller collected volumes of plasma.

The separation of VP 16-213 and picro VP 16-213, obtained using a μBondapak Phenyl reversed-phase column, is shown in Fig. 6. The separation of these two components was not possible on a reversed-phase C_{18} column. In view of the scattered reports [8] on the production of picropodophyllotoxin under biological conditions, the possibility of picro VP 16-213 being produced as a metabolite of VP 16-213 was investigated.

VP 16-213 and picro VP 16-213 are both very soluble in chloroform and have very similar chromatographic properties. Creaven and Allen [3] checked the purity of radiolabelled VP 16-213 extracted by chloroform from patient plasma. using several TLC systems. However, they did not use the TLC system which we have found to separate the two components (see Experimental). Further, they did not explicitly address the question of picro VP 16-213 as a potential

metabolite, at least in their publications. A slight possibility existed then that the pharmacokinetic data they derived were based on scintillation counting of VP 16-213 and picro VP 16-213 combined. Likewise, the assay on a C_{18} column would have been unreliable because these two components are not separated in this system. However, to date, no picro VP 16-213 has been detected in clinical plasma samples.

In the event that VP 16-213 and VM 26 are ever used in combination therapy, as recently suggested [14, 15], the separation of picro VP 16-213 from VP 16-213 will be useful since the picro compound can be used as the internal standard and the assay can be conducted on a reversed-phase phenyl column.

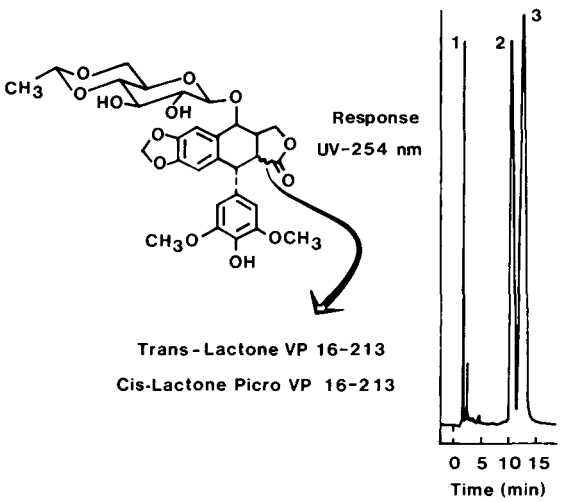


Fig. 6. HPLC separation of VP 16-213 (*trans*-lactone) and picro VP 16-213 (*cis*-lactone). Chromatographic conditions: 30 cm × 3.9 mm I.D. column with μBondapak Phenyl; solvent, methanol—water (50:50); flow-rate, 1.2 ml/min at an inlet pressure of ca. 2000 p.s.i. Peaks: 1 = picro VP 16-213 hydroxy acid; 2 = VP 16-213; 3 = picro VP 16-213.

CONCLUSION

A rapid, practical assay is reported for the analysis of the promising anti-cancer agents VP 16-213 and VM 26. The assay is based on isocratic reversed-phase microparticle C_{18} HPLC of chloroform extracts of plasma. Each drug functions as the internal standard for the other.

The acid metabolite of VP 16-213 appears to be the *trans*-hydroxy acid, although more definitive work is still needed.

Picro VP 16-213, a potential metabolite based on literature precedent, is apparently not produced in vivo.

Finally, the analytical potential of using detection of the native fluorescence of these podophyllotoxin derivatives is demonstrated.

ACKNOWLEDGEMENTS

The authors thank Drs. H. Friedli and H. Stähelin of Sandoz, Basle, Switzerland for their extremely generous gift of VP 16-213 and VM 26. We also thank R. Hemmig for technical assistance.

This work was supported by a grant from the American Cancer Society through the Purdue Cancer Center. Dr. Jardine is the recipient of an Eli Lilly and Co. research fellowship. Dr. Colvin is supported by NIH grant CA 00103.

REFERENCES

1 C. Keller-Juslén, M. Kuhn, A. von Wartburg and H. Stähelin, J. Med. Chem., 14 (1971) 936.
2 M. Rozencweig, D.D. Von Hoff, J.E. Henney and F.M. Muggia, Cancer, 40 (1977) 334.
3 P.J. Creaven and L.M. Allen, Clin. Pharmacol. Ther., 18 (1975) 221, 227.
4 L.M. Allen and P.J. Creaven, Eur. J. Cancer, 11 (1975) 697.
5 L.M. Allen, Drug Metab. Rev., 8 (1978) 119.
6 F.R. Pelsor, L.M. Allen and P.J. Creaven, J. Pharm. Sci., 67 (1978) 1106.
7 L.M. Allen, C. Marcks and P.J. Creaven, Proc. Amer. Ass. Cancer Res., 17 (1976) 6.
8 W.J. Gensler, C.D. Murthy and M.H. Trammell, J. Med. Chem., 20 (1977) 635.
9 W.J. Gensler and C.D. Gatsonis, J. Org. Chem., 31 (1966) 3224.
10 P.A. Leclercq and D.M. Desiderio, Anal. Lett., 4 (1971) 305.
11 J. Renz, M. Kuhn and A. von Wartburg, Justus Liebigs Ann. Chem., 681 (1965) 207.
12 M. Kuhn and A. von Wartburg, Experientia, 19 (1963) 391.
13 S. Udenfriend, D.E. Duggan, B.M. Vasta and B.B. Brodie, J. Pharmacol. Exp. Ther., 120 (1957) 26.
14 L. Allen, Proc. Amer. Ass. Cancer Res., 19 (1978) 125.
15 L.M. Allen, Cancer Res., 38 (1978) 2549.

Journal of Chromatography, 182 (1980) 221—225
Biomedical Applications

CHROMBIO. 522

Note

Origin of the two peaks for 2-keto-3-methylvaleric acid produced by the oximation of the keto acids occurring in maple syrup urine disease

O.A. MAMER*, J.A. MONTGOMERY and V.Y. TAGUCHI

The Mass Spectrometry Unit, McGill University, 1130, Pine Avenue West, Montreal, Quebec H3A 1A3 (Canada)

(Received November 5th, 1979)

(+)-2-Keto-3*S*-methylvaleric acid is the product of the transamination in mammals of L-(+)-isoleucine (2*S*-amino-3*S*-methylvaleric acid). It is also the precursor of 2*S*-methylbutyryl CoA which leads into the conventional *S* pathway of mammalian isoleucine catabolism. Furthermore, the 3*S* chiral center in this keto acid may racemize through non-enzymic keto—enol tautomerism to yield (−)-2-keto-3*R*-methylvaleric acid. This *R* acid can be enzymically reaminated to L-(+)-alloisoleucine (2*S*-amino-3*R*-methylvaleric acid), and is also the precursor of 2*R*-methylbutyryl CoA, the entry point for a recently illuminated *R* pathway of isoleucine catabolism [1]. Thus the *R* and *S* stereomers of 2-keto-3-methylvaleric acid have different catabolic fates and both are physiologically important [2], contrary to the impression conveyed by Langenbeck et al. [3].

A compound having only one center of asymmetry at a carbon atom exists in two forms, *R* and *S* which cannot be distinguished by any physical means except by the observation of the direction of the angle of displacement of transmitted plane polarized light [4]. Several authors have published findings, however, that are interpreted to suggest that the trimethylsilyl (TMS) derivatives of the oximes of (+)-2-keto-3*S*-methylvaleric and (−)-2-keto-3*R*-methylvaleric acids are readily separated by gas chromatography on silicone liquid phases (ref. 5, Figs. 1 and 2; ref. 6, Fig. 1) and on Dexsil 300 as the TMS derivatives of the 3-(*sec.*-butyl)-2-quinoxalinol condensation products of *o*-phenylenediamine and the *R* and *S* keto acids [3]. This is clearly impossible. Enantiomers such as these two keto acids and any possible derivatives which do not introduce a second center of molecular asymmetry (i.e., generation of diastereomers) cannot be chromatographically separated in any racemic or non-chiral system. Gas

*To whom correspondence should be addressed.

chromatography using chiral stationary phases can of course be used to effect separation of enantiomers [7].

The gas chromatographic presentation of a pair of peaks due to the TMS derivatives of 2-ketoxime-3-methylvaleric acid is therefore not related to the relative quantities of the *R* and *S* isomers present for example in the urine and serum of maple syrup urine disease patients as reported by Jakobs et al. [6], but rather is related to the relative proportions of the *E* and *Z* (i.e., *anti* and *syn*) isomers determined by the ketoxime moiety itself. It is interesting to note further that the early eluting member of these two peaks was originally attributed to the L-isomer without substantiating evidence of any kind [5] and that this impossible assignment has been accepted by later authors.

We wish to report here the results of a study that identifies unequivocally the structures of the two oximes of this 2-keto acid.

EXPERIMENTAL

Preparation of oximes

D,L-2-Keto-3-methylvaleric acid (sodium salt, Sigma, St. Louis, Mo., U.S.A.) was converted in 50-mg quantities to its oxime derivatives by two methods: oxime mixture I by the method of Sternowsky et al. [5], and oxime mixture II by the method of Lancaster et al. [8]. Oxime mixture I was obtained in pyridine solution and was converted subsequently to the TMS derivatives as reported [5]. Oxime mixture II was obtained as the crude solid remaining from the evaporated diethyl ether extract. A redissolved aliquot of II was also converted to the TMS derivatives as reported [9]. Analysis of both oxime mixtures was effected on an LKB 9000 mass spectrometer under the following conditions: column, glass (2 m × 6 mm O.D.); 6% OV-101 on Chromosorb W HP (100—120 mesh); 125° isothermal; injector and separator, 280°; ion source, 290°, 70 eV, 60 μA. Under these conditions the derivatives of interest had retention times of approximately 10 min.

Reaction of oxime mixture II with copper(II) sulphate

An aqueous solution (3 ml) of oxime mixture II (10 mg) was made pH 10 with dilute aqueous sodium hydroxide. Saturated aqueous copper(II) sulphate (200 μl) was added and the resulting mixture was allowed to stand at room temperature for 10 min. A fine pale blue precipitate was removed by filtration through a Pasteur pipette packed with glass wool. The blue—green supernatant was acidified with concentrated hydrochloric acid, saturated with sodium chloride and extracted with 3 volumes of diethyl ether. The residue left from the evaporation of the diethyl ether was converted to the TMS derivatives and analyzed as described above.

RESULTS AND DISCUSSION

The bis-TMS derivatives of the *Z* and *E* oximes of 2-keto-3-methylvaleric acid are easily separable by gas chromatography using a 2-m 6% OV-101 column as illustrated in Fig. 1. Peaks D and E are the relative proportions of the *E* and *Z* isomers obtained when the oximation step is carried out in pyri-

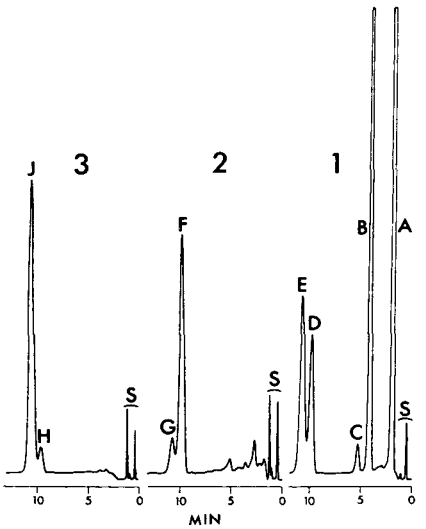


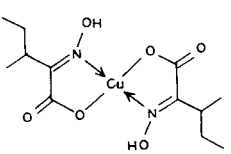
Fig. 1. Gas chromatographic analyses of the TMS derivatives of the isomeric oximes of 2-keto-3-methylvaleric acid. Chromatograms: (1) oxime mixture I prepared in pyridine according to Sternowsky et al. [5], peaks A, B, and C are artifacts produced in this procedure; (2) oxime mixture II prepared in aqueous alkali according to Lancaster et al. [8]; (3) the oxime mixture extracted from the supernatant obtained following the precipitation of the *E* oxime in mixture II with aqueous copper (II) sulphate. Peaks E, G, and J are produced by the *Z* oxime and have the mass spectrum reproduced in Fig. 2. Peaks D, F, and H are due to the *E* oxime and have the mass spectrum reproduced in Fig. 3. The peaks labelled S are solvent and reagent peaks that are gate valve attenuated.

dine according to the method of Sternowsky et al. [5]. Peaks A, B, and C appear to be artifactual, the probable result of the reaction of excess hydroxylamine in pyridine with the silylating reagent.

Peaks F and G are the relative proportions of the oxime isomers formed in aqueous alkaline solution according to the method of Lancaster et al. [8]. Peak G, the oxime that co-elutes with the oxime of 2-ketoisocaproic acid is very much reduced in favour of the isomer that elutes earlier (peak F).

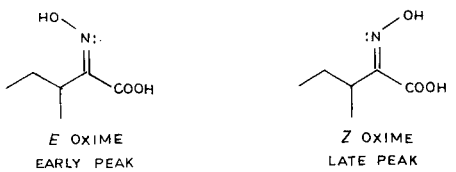
The method of Lancaster et al. [8] therefore bears the advantage over that of Sternowsky et al. [5] in that there is less mutual interference in the determination of 2-keto-3-methylvaleric and 2-ketoisocaproic acids in biological fluids. Secondly, the oximes have been shown to be much more completely extracted from urine and serum when made in situ [8] than the corresponding native keto acids which are oximated by Sternowsky et al. [5] only after extraction.

Treatment of oxime mixture II with an excess of aqueous copper (II) sulphate resulted in the selective precipitation of the *E* oxime as a copper chelate having the probable structure:



Stable chelation requires that the hydroxy group bound to nitrogen be directed away from the carboxyl group (*E*) so that it cannot interfere with coordination between the metal ion and the nitrogen atom. Oximes are known to form chelates involving the nitrogen atom rather than the oxygen atom [10].

The oxime not complexing with copper is therefore the *Z* isomer and corresponds to peak J (Fig. 1), the late eluting member of the pair which co-elutes with the oxime of 2-ketoisocaproic acid.



E OXIME
EARLY PEAK

Z OXIME
LATE PEAK

Peaks E, G, and J have identical mass spectra (Fig. 2) and retention times. The mass spectrum of the *E* isomer (Fig. 3) is very similar to that of the *Z* isomer, the major difference being the relative intensities of the $M^{+\cdot}-CH_3\cdot$ ions (*m/z* 274). The loss of the methyl radical from the ester TMS group in the

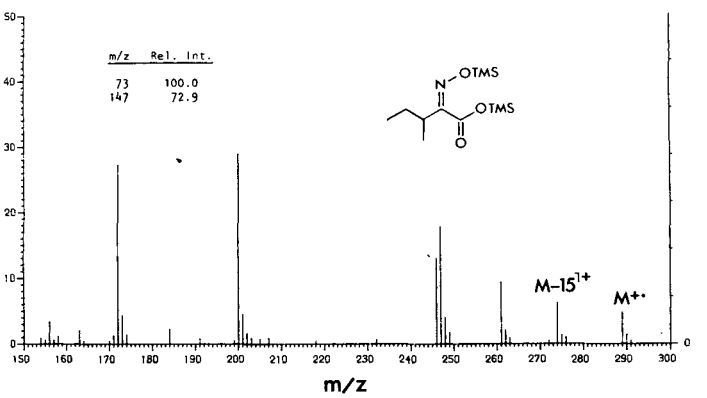


Fig. 2. Mass spectrum of (*Z*)-2-ketoxime-3-methylvaleric acid as the bis-TMS derivative.

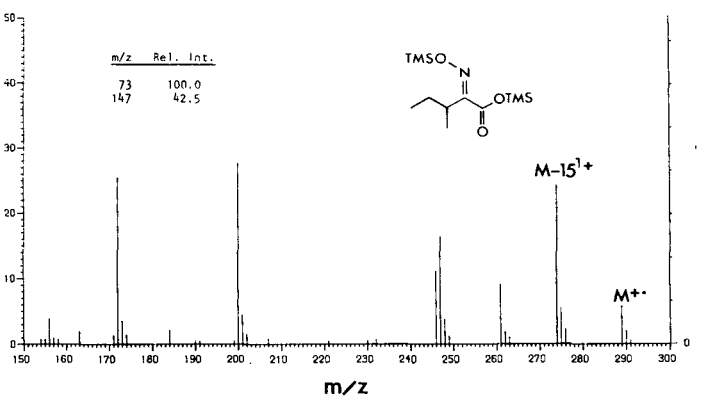
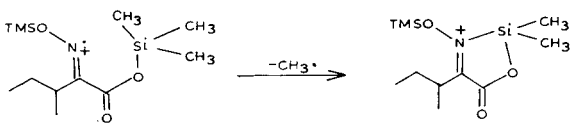


Fig. 3. Mass spectrum of (*E*)-2-ketoxime-3-methylvaleric acid as the bis-TMS derivative.

molecular ion is assisted in the case of the *E* derivative by the formation of a ring-stabilized even-electron fragment ion with the ion center on the nitrogen atom (Scheme 1). The trimethylsiloxy group presents a barrier to such ring formation in the *Z* isomer however, and this stabilizing force is therefore denied to this $M^{+\cdot}-CH_3\cdot$ ion, thus accounting for its reduced intensity relative to the *E* isomer.



SCHEME I

The 2-keto acids which lack branching substitution on the 3-carbon (e.g., pyruvic, 2-ketobutyric, 2-ketovaleric, 2-ketocaproic, and 2-ketoisocaproic acids) appear to form predominantly a single oxime in pyridine while those with substituent branching on the 3-carbon (e.g., 2-keto-3-methylvaleric and 2-ketoisovaleric acids) form a pair of oximes in comparable yields. In oxime formation, the relative size of the two groups bonded to the carbonyl moiety is a major factor influencing the proportions of *E* and *Z* isomers formed. In the first group of acids, the carboxyl group is larger than the alkyl group unbranched at the 3-carbon, and therefore the *E* isomer predominates. With the second group in aprotic solvents (e.g., pyridine), the bulk associated with 3-carbon branching makes the *E* isomer less favoured and so both the *E* and *Z* oximes are formed in comparable yields. In aqueous alkali however, the carboxylate anion is highly solvated and is effectively much larger than in aprotic solvents thus favouring once again the formation of the *E* oxime which elutes free of 2-ketoisocaproic oxime interference.

ACKNOWLEDGEMENTS

The authors wish to thank Drs. W.J. Holland and K.G. Rutherford of the Department of Chemistry of the University of Windsor for helpful discussions.

REFERENCES

1 O.A. Mamer, S.S. Tjoa, C.R. Scriver and G.A. Klassen, Biochem. J., 160 (1976) 417, and references listed therein.
2 B.H. Baretz, C.P. Lollo and K. Tanaka, J. Biol. Chem., 254 (1979) 3468.
3 U. Langenbeck, U. Wendel, A. Mench-Hoinowski, D. Kuschel, K. Becker, H. Przyrembel and H.J. Bremer, Clin. Chim. Acta, 88 (1978) 283.
4 N.L. Allinger, M.P. Cava, D.C. De Jongh, C.R. Johnson, N.A. LeBel and C.L. Stevens, Organic Chemistry, Worth Publishers, New York, 1971, p. 46.
5 H.J. Sternowsky, J. Roboz, F. Hutterer and G. Gaull, Clin. Chim. Acta, 47 (1973) 371.
6 C. Jakobs, E. Solem, J. Ek, K. Halvorsen and E. Jellum, J. Chromatogr., 143 (1977) 31.
7 H. Frank, G.J. Nicholson and E. Bayer, J. Chromatogr., 146 (1978) 197.
8 G. Lancaster, P. Lamm, C.R. Scriver, S.S. Tjoa and O.A. Mamer, Clin. Chim. Acta, 48 (1973) 279.
9 O.A. Mamer, J.C. Crawhall and S.S. Tjoa, Clin. Chim. Acta, 32 (1971) 171.
10 T. Moeller, Inorganic Chemistry, Wiley, New York, 1952, p. 240.

Journal of Chromatography, 182 (1980) 226—231
Biomedical Applications

CHROMBIO. 514

Note

Simultaneous determination of bupivacaine and 2,6-pipecoloxylidide in serum by gas—liquid chromatography

L.J. LESKO* and J. ERICSON

Clinical Pharmacokinetics Laboratory, Massachusetts College of Pharmacy, 179 Longwood Avenue, Boston, Mass. 02115 (U.S.A.)

G. OSTHEIMER

Department of Anesthesia, Boston Hospital for Women, Boston, Mass. 02115 (U.S.A.)

and

A. MARION*

Clinical Pharmacokinetics Laboratory, Massachusetts College of Pharmacy, 179 Longwood Avenue, Boston, Mass. 02115 (U.S.A.)

(First received September 24th, 1979; revised manuscript received December 12th, 1979)

Bupivacaine (Marcaine®) is a local anesthetic of the anilide type used for epidural analgesia during labor and delivery. Bupivacaine is a highly lipid-soluble drug which can cross the placenta quite easily. The major metabolite of bupivacaine is 2,6-pipecoloxylidide (PPX) which is formed in the liver by oxidative dealkylation of the parent drug. It is important to be able to measure bupivacaine and PPX in the maternal and fetal circulation in order to evaluate the toxicity and disposition kinetics of bupivacaine.

Several gas chromatographic (GC) methods have been reported for the measurement of serum bupivacaine. Some of these methods are time-consuming, laborious, or not specific or sensitive enough to measure PPX [1--4]. A modified GC procedure for bupivacaine has been reported to detect PPX in blood but the authors provided no information describing the linearity, reproducibility, sensitivity and recovery of the method. Furthermore, this method required 2 ml of whole blood and a double-extraction procedure [5].

*Present address: Drug Concentration Laboratory, University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, Mass. 01605, U.S.A.

A recent method for the determination of bupivacaine by GC appears to be rapid and simple, but it, too, does not measure PPX [6]. In all of these GC methods of assay a flame-ionization detector was used and no attempts to use the more sensitive nitrogen—phosphorus detector have been reported.

This report describes a GC method designed for rapid, simultaneous determination of bupivacaine and PPX in serum using a nitrogen—phosphorus detector. The method is suitable for monitoring bupivacaine and PPX serum concentrations in maternal and fetal circulation during labor and delivery.

EXPERIMENTAL

Reagents and chemicals

The following reagents and chemicals were used: bupivacaine hydrochloride (Sterling-Winthrop Research Institute; lot No. XB0-4-68), 2,6-pipecoloxylidide (Sterling-Winthrop Research Institute, New York, N.Y., U.S.A.) and lidocaine hydrochloride (Astra Pharmaceutical Products, Framingham, Mass., U.S.A.; lot No. XH 99 A). Diethyl ether (Fisher Scientific, Pittsburgh, Pa., U.S.A.), methanol (Burdick and Jackson Labs., Muskegon, Mich., U.S.A.) and all other solvents and reagents were of analytical grade quality and were used as received.

Internal standard. A solution of lidocaine hydrochloride (500 mg/l) was prepared by dissolving 50.0 mg of lidocaine hydrochloride (equivalent to 43.3 mg of lidocaine base) in 100 ml of methanol. A second solution containing lidocaine hydrochloride (5 mg/l) was prepared by dilution with methanol.

Drug standards. Stock solutions of bupivacaine hydrochloride (500 mg/l) and PPX (500 mg/l) were prepared by dissolving 50 mg of bupivacaine hydrochloride (equivalent to 45 mg of bupivacaine base) and 50 mg of PPX, respectively, in 100 ml of methanol. Working drug solutions were prepared by diluting the original stock solutions 100-fold with methanol. Drug serum standards were prepared by spiking blank control serum with appropriate microliter volumes of working drug solution to obtain five serum standards with the following concentrations of bupivacaine and PPX: 0.1, 0.2, 0.4, 0.8 and 1.6 μg/ml.

Instrument and chromatographic conditions

A Perkin-Elmer Sigma 3 gas chromatograph fitted with a nitrogen—phosporus detector in the nitrogen mode was used for chromatography. A glass column, 1.8 m × 2 mm I.D., packed with 3% OV-17 on Chromosorb W HP (100—120 mesh) was used for separation (Perkin—Elmer, Norwalk, Conn. U.S.A.) The column was conditioned for 16 h at 280° with a nitrogen flow-rate of 20 ml/min. The detector end of the column was disconnected during the conditioning process. The recorder was a Perkin—Elmer Model 023 with a range of 0—1 mV; the chart speed was 0.5 cm/min.

The zone temperature (injector and detector block) was 260° and the rubidium bead temperature in the detector was set at 450°. The oven temperature was programmed from 180° to 240° at a ramp rate of 20°/min.

The initial hold time was 8 min at 240°. The gas flow-rates were as follows: nitrogen (carrier gas) 16 ml/min, hydrogen 2.25 ml/min and air 120 ml/min. Peak heights were measured to the nearest half millimeter with a metric ruler.

PROCEDURE

A 0.1-ml sample of patient serum and 0.1 ml of lidocaine hydrochloride stock solution (5 mg/l) were added to a small, disposable, acid-washed glass test-tube. The serum was made alkaline (pH adjusted to 11) by adding 0.5 ml of sodium carbonate solution (0.2 mole/l) and extracted with 1.0 ml of diethyl ether by vortex mixing for 15 sec. The organic phase (upper) was separated by centrifugation at 1600 *g* for 10 min and transferred to a clean, disposable, glass test-tube. The ether was evaporated at ambient temperature under a gentle stream of nitrogen. The residue was reconstituted with 20 μl of methanol by vortex mixing, and 10 μl were injected into the gas chromatograph. The concentrations of bupivacaine and PPX in the serum sample were determined from a calibration curve of peak height ratio (drug/internal standard) versus drug concentration in serum standards carried through this procedure.

RESULTS

A chromatogram obtained for a spiked serum sample containing lidocaine, PPX and bupivacaine extracted and assayed as previously described is shown in

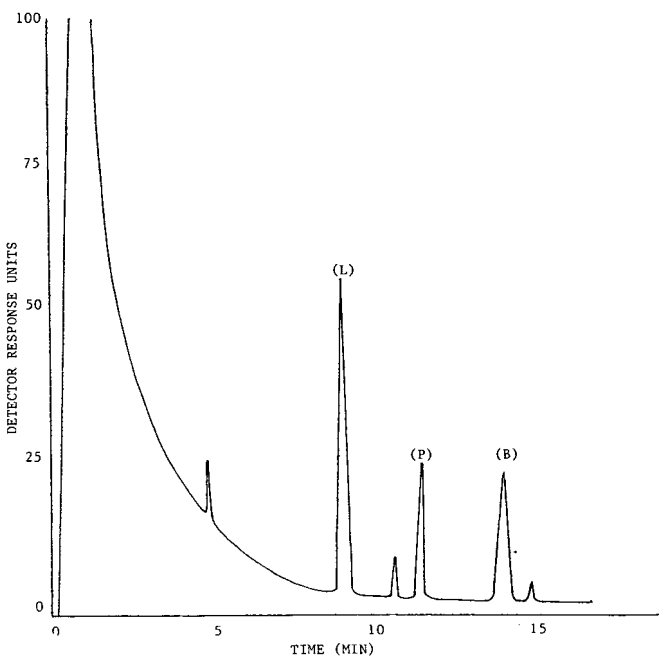


Fig. 1. Gas chromatogram of a mixture of lidocaine (L), PPX (P) and bupivacaine (B) added to drug-free serum; the concentration of each is 0.8 μg/ml.

Fig. 1. Three symmetrical drug peaks appear in the chromatogram with lidocaine emerging first from the column followed in sequence by PPX and bupivacaine. The retention times for lidocaine, PPX and bupivacaine were 9, 11.5 and 14 min, respectively. The drug peaks were well-removed from the solvent front and completely resolved from any extraneous serum peaks.

Calibration curves of peak height ratio versus concentration were obtained by analyzing serum standards containing bupivacaine and PPX in concentrations ranging from 0.1 to 1.6 μg/ml. The calibration curves were linear over the concentration range studied. The least-squares linear regression line which represents the best fit of the bupivacaine data has a slope of 0.89 and a y-intercept (where y = peak height ratio of drug to internal standard) of 0.07 (r = 1.0). The corresponding line for the PPX data had a slope of 0.77 and a y-intercept of 0.02 (r = 1.0).

The precision of the method was determined by repeated analysis of spiked serum samples containing low (0.1 μg/ml) and high (0.8 μg/ml) concentrations of bupivacaine and PPX. The mean concentration, standard deviation and coefficient of variation for intra- and inter-day analysis are shown in Table I. The within-day coefficient of variation for low concentrations of bupivacaine and PPX was 9.6 and 7.9%, respectively, while at high concentrations the coefficient of variation was 6.1 and 3.6%, respectively. The day-to-day precision for low and high concentrations of bupivacaine and PPX was between 1.7 and 11.8%.

TABLE I

PRECISION DATA FOR THE DETERMINATION OF BUPIVACAINE AND PPX IN SERUM

Drug	Intra-day			Inter-day		
	n	Mean (μg/ml ± S.D.)	C.V. (%)	n	Mean (μg/ml ± S.D.)	C.V. (%)
Bupivacaine						
Low conc.	5	0.093 ± 0.0088	9.46	10	0.110 ± 0.0100	9.09
High conc.	8	0.800 ± 0.0489	6.11	10	0.790 ± 0.0212	2.68
PPX						
Low conc.	5	0.089 ± 0.0070	7.86	10	0.110 ± 0.0126	11.4
High conc.	8	0.840 ± 0.0300	3.57	10	0.840 ± 0.0092	1.10

The analytical recovery of bupivacaine, PPX and lidocaine from serum was determined by comparing the peak heights for each drug obtained by analyzing extracted, spiked serum specimens, to the peak heights obtained by analyzing unextracted methanolic solutions of each drug containing an amount of drug equivalent to the amount in the spiked serum specimens. The recovery data for each drug are shown in Table II.

The sensitivity (least amount of bupivacaine and PPX measurable) of this method was 0.005 μg. Using 0.1 ml of patient serum and the analytical procedure described in this paper, the lowest concentration that can be measured accurately is 0.1 μg/ml. Greater sensitivity may be possible by increasing instrument sensitivity, increasing serum volume, using whole blood,

TABLE II

ANALYTICAL RECOVERY OF BUPIVACAINE, PPX, AND LIDOCAINE ADDED TO SERUM

Drug	*n*	Present (μg/ml)	Found (μg/ml)	Mean recovery (%)	C.V. (%)
Bupivacaine	5	0.80	0.76	95.1	3.8
PPX	5	0.80	0.73	91.2	10.7
Lidocaine	5	0.80	0.64	79.9	3.7

or increasing injection volume. Concentrations of bupivacaine and PPX of 0.05 μg/ml in serum were readily measurable by including such modifications in the method of analysis.

DISCUSSION

The ability to simultaneously measure the serum concentration of bupivacaine and PPX may be of particular value to the clinician who may attempt to correlate serum levels of these substances with signs of toxicity in the neonate after delivery or the ability of the neonate to metabolize bupivacaine in the post-partum period. The method described is relatively simple, rapid and well-suited for the clinical or pharmacokinetics laboratory. Only 0.1 ml of serum is required for analysis.

Previously published assay procedures for bupivacaine did not measure the concentration of PPX conveniently, if at all. The analysis of PPX by this procedure is based on the use of a nitrogen–phosphorus detector which has greater selectivity and sensitivity than the flame-ionization detector utilized in previously reported assays. The resolution of the bupivacaine, PPX and lidocaine peaks from each other and from extraneous serum or solvent peaks after only a single extraction step is made possible by temperature programming.

The use of an internal standard maximizes the precision and accuracy of the assay by making quantitation independent of transfer volumes or injection volumes. Lidocaine was selected as an internal standard because of its structural similarity to bupivacaine and PPX, because it is extracted and chromatographs well, and because of the good detector response to lidocaine. It is unlikely that a maternal or neonatal serum sample will contain lidocaine and bupivacaine simultaneously but, if that were to occur, virtually any other anilide-type local anesthetic may be used as an internal standard.

OV-17 was selected for the GC column because it had been used for the separation of local anesthetics in the past. In conjunction with the nitrogen–phosphorus detector drug-free serum extracts were analyzed and were found not to produce any extraneous peaks at retention times that would interfere with the quantitation of bupivacaine and PPX. Using the experimental conditions described herein, the relative retention times for lidocaine, PPX and bupivacaine were 0.64, 0.82 and 1.0. Extraneous serum peaks in the chromatogram may be avoided in one of two ways: first, the serum aliquot may be mixed with an equal volume of methanol to precipitate protein, and after centrifuging, extract the supernatant with diethyl ether; second, back-extract

the ether phase with hydrochloric acid (0.1 mole/l) and re-extract with a fresh aliquot of ether. The recovery of the drugs was not reduced when the first approach was used but when back-extracting in the second approach the recovery of PPX was noticeably decreased. Organic solvents other than ether (for example, chloroform and hexane) were not suitable in the single extraction procedure.

It is worthwhile to use small, disposable, glass test-tubes in this assay to facilitate evaporation of the ether phase and complete reconstitution of the residue with only a small volume of methanol. Disposability also minimizes the problem of contamination arising from the use of phosphorus-containing soaps used to clean reusable glassware. Phosphorous contaminants may be adequately removed by acid washing and methanol rinsing. If such a contaminant is present it will emerge most often under the lidocaine peak.

Whenever bupivacaine is present care must be taken to evaporate the ether phase at ambient temperature under nitrogen rather than at elevated temperatures in order to minimize any loss of bupivacaine through volatilization encountered in this procedure.

This method has been used to measure the concentration of bupivacaine and PPX in serum samples obtained from the maternal vein, umbilical artery and umbilical vein at the time of delivery. Interference from endogenous substances has not been observed. We have not determined the potential interference of other co-administered drugs in the measurement of bupivacaine and PPX. Approximately 20—25 serum samples may be conveniently assayed in a day by one analyst. This method has also been found to be useful for therapeutic monitoring of serum lidocaine concentrations.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the technical assistance of Marion Dorscheimer in the preparation of this manuscript.

REFERENCES

1 F. Reynolds and A.H. Beckett, J. Pharm. Pharmacol., 20 (1968) 704.
2 G.T. Tucker, Anaesthesiology, 32 (1970) 255.
3 A. Berlin, B. Persson and P. Belfrage, J. Pharm. Pharmacol., 25 (1973) 466.
4 L.E. Mather and G.T. Tucker, J. Pharm. Sci., 63 (1973) 306.
5 F. Reynolds and G. Taylor, Anaesthesia, 25 (1970) 14.
6 E. Zylber-Katz, L. Granit and M. Levy, Clin. Chem., 24 (1978) 1573.

Journal of Chromatography, 182 (1980) 232—236
Biomedical Applications

CHROMBIO. 515

Note

Micro estimation of plasma theophylline by gas—liquid chromatography with on-column butylation and nitrogen-specific detection

GARY MARTIN SCHIER and IGNATIUS ENG THO GAN

Department of Biochemistry, The Wollongong Hospital, Crown Street, Wollongong, N.S.W. 2500 (Australia)

(First received September 26th, 1979; revised manuscript received December 11th, 1979)

Theophylline (1,3-dimethylxanthine) is well-established as a bronchodilator in the management of patients with asthma [1—3], and has proved effective in the treatment of apnea and bradycardia in premature and low birth-weight infants [4—7]. Measurement of plasma theophylline levels is useful, because of pronounced intersubject differences in the biological half-life of theophylline in adults [8, 9], children [10], and infants. Because plasma theophylline has a relatively narrow therapeutic range [5, 11] and considerable plasma variability among patients on the same oral dose [5, 12], safe and effective use of theophylline depends on information obtained by plasma monitoring. Although estimation of plasma theophylline has been provided by various analytical techniques [10, 13—19], gas—liquid chromatographic (GLC) methods have proved to be some of the more popular procedures [12, 20—30]. Most of these procedures have relied upon sample volumes of 1 ml to achieve sufficient analytical precision with the commonly used flame ionisation detectors (FID). The need for a micro analysis for paediatric use was apparent and a number of methods using nitrogen—phosphorus-specific detectors [26, 29], FID [27] and electron-capture detectors [24], were developed to fulfil this requirement. For routine use these methods have a number of disadvantages, since they require double solvent extraction [26], temperature programming of the GLC for resolution of the components [26], the use of aqueous tetrabutylammonium hydroxide [27], and have low extraction efficiencies [29], or are unduly complicated [24]. In view of these limitations, we developed a method involving the extraction of theophylline from 20 μl of plasma with 100 μl of extracting solvent. This is then followed by evaporation and on-column butylation [31], with detection by means of a nitrogen-specific detector.

MATERIALS AND METHODS

Apparatus

A Packard 427 gas chromatograph equipped with a Model 905 nitrogen—phosphorus detector was used. The instrument was fitted with a coiled glass column (1.8 m × 2 mm I.D.) packed with Gas-Chrom Q (100—120 mesh) coated with 3% OV-17. Operating conditions were: helium (carrier gas) flow-rate 30 ml/min, hydrogen 4.6 ml/min, air 100 ml/min; injection port and detector temperatures 280°; column temperature was isothermal at 230°.

Reagents

Theophylline and 7-β-hydroxypropyltheophylline were obtained from Sigma (St. Louis, Mo., U.S.A.) Chloroform (Spectrosol®) and propan-2-ol (Spectrosol®) were obtained from Ajax Chemicals (Sydney, Australia). The column packing (3% OV-17 on Gas-Chrom Q 100—120 mesh) was obtained from Applied Science Labs., State College, Pa., U.S.A. Tetrabutylammonium hydroxide (0.02 *M* in methanol) was prepared from 25% tetrabutylammonium hydroxide obtained from Matheson, Coleman and Bell (Norwood, Ohio, U.S.A.). The internal standard (7-β-hydroxypropyltheophylline, 11.91 mg) was dissolved in 100 ml of propan-2-ol. A 10-ml aliquot was diluted to 500 ml with 50% chloroform—propan-2-ol to give a combined extracting solution and working internal standard.

Extraction and on-column derivatisation of plasma theophylline

To 20 μl of plasma were added 100 μl of internal standard—extracting solution, followed by vortex mixing for 30 sec. Following centrifugation at 800 *g* for 2 min, the lower layer was transferred by pasteur pipette to a conical tube and evaporated to dryness with a stream of nitrogen at 40°. The residue was reconstituted with vortex mixing in 20 μl of 0.02 *M* tetrabutylammonium hydroxide solution; 1 μl was then used for injection into the gas chromatograph.

Standard solutions

A series of plasma standards with concentrations of 125, 100, 75, 50 and 25 μmol/l were prepared by adding pooled sera to 1 ml of a stock solution of theophylline in water (45, 36, 27, 18 and 9 mg per 100 ml) in 10-ml volumetric flasks. Aliquots of these solutions were kept frozen at −20°, and then thawed at 37° before use.

Quantitation

Theophylline levels in plasma samples were quantitated by reference to the standards. These were plotted as the ratio of the peak height of theophylline to that of the internal standard, versus the plasma theophylline concentration (μmol/l).

Spectrophotometric method for theophylline

The method of Koysooko et al. [32] was used to determine theophylline in plasma. Two millilitres of plasma were extracted with 10 ml of chloroform—

isopropanol (20:1, v/v). Eight millilitres of the organic phase were removed and extracted with 2 ml of NaOH (0.1 mol/l). Absorbance of the aqueous phase was measured in a quartz cuvette at 277 nm with a Model SP 1800 spectrophotometer (Pye-Unicam, Cambridge, Great Britain).

RESULTS AND DISCUSSION

Chromatography

Fig. 1 shows a typical chromatogram from an extract of plasma spiked with theophylline (50 μmol/l), with retention times calculated from the time of injection as follows: solvent front, 0.4 min; theophylline, 3.3 min; internal standard, 5.0 min.

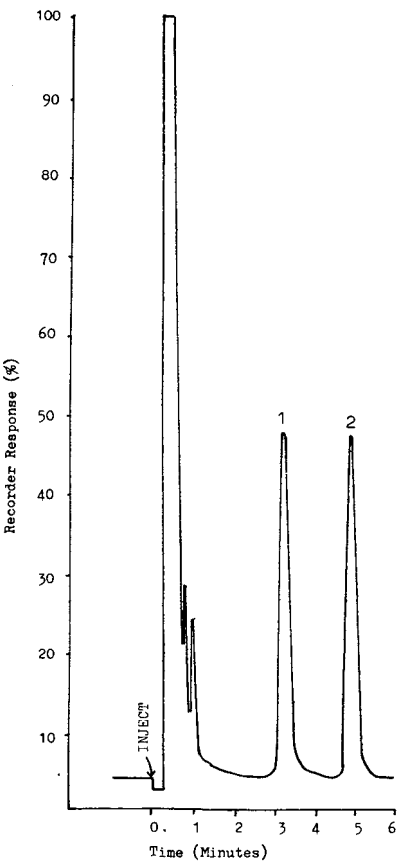


Fig. 1. Gas chromatogram of an extract from 20 μl of plasma containing 50 μmol/l of theophylline. Column, 3% OV-17 on Gas-Chrom Q 100—120 mesh, 1.8 m × 2 mm I.D. glass; attenuation 32×; column temperature 260°. Peaks: 1 = theophylline, 2 = 7-β-hydroxypropyltheophylline.

Linearity

The linearity of the assay method was established by analysing duplicate aliquots of the plasma standards. Analysis of the results by the least-squares method resulted in a straight line with a correlation coefficient of 0.999.

Precision and detection limit

The within-day precision was determined by analysing ten 20-μl aliquots of pooled serum with a theophylline concentration of 100 μmol/l. The result was 101 ± 2.0 (S.D.) μmol/l with a coefficient of variation of 2.0%. In a similar manner, aliquots of the same pooled sera were analysed daily for ten days and the between-run precision was 98 ± 3.8 (S.D.) μmol/l, with a coefficient of variation of 3.9%.

For practical use, the lower limit of theophylline estimation was 5 μmol/l.

Extraction efficiency and recovery

The extraction efficiency and recovery of theophylline were determined by carrying out the above procedure on twenty samples of plasma to which theophylline had been added to the 100 μmol/l level. The peak heights were compared to the peak heights of an equivalent amount of standard which had been dried and subjected to the same derivatisation procedure. The mean recovery following extraction was 76% with an S.D. of 3.5%.

Interference

To check for possible interference, several drugs (phenobarbital, caffeine, theobromine, phenytoin, primidone, carbamazepine, salicylic acid, acetylsalicylic acid and uric acid) were added to plasma at the 100 μmol/l level and analysed in the same manner as the theophylline standards. No interference could be detected, although the following drugs eluted on the same chromatogram: caffeine, 2.3 min; theobromine, 3.7 min; and phenobarbital, 4.1 min. To check for possible non-specific interference, several drug-free sera were subjected to the new procedure. Again, no interference was detected, although a peak with a retention time of 2.3 min (corresponding to caffeine) was observed in some sera.

Accuracy

Ten specimens of plasma obtained from patients taking theophylline medication were analysed for theophylline by the spectrophotometric method [32] and the method described here. The mean value obtained with the spectrophotometric method was 66 μmol/l and with this method 62 μmol/l. When values from this assay were compared to those obtained with the spectrophotometric assay, the correlation coefficient was 0.952.

Using the above method it is possible to determine ten plasma theophylline levels within 90 min. The analytical procedures of extraction, evaporation and chromatography can be completed in less than 10 min per specimen. The excellent precision, accuracy and small sample requirement make this procedure ideally suited to the analysis of theophylline in plasma from neonates and infants.

REFERENCES

1 M.M. Weinberger and E.A. Bronsky, J. Pediatr., 84 (1974) 421.
2 M.M. Weinberger and E.A. Bronsky, Clin. Pharmacol. Ther., 17 (1975) 585.
3 G. Hambleton, M. Weinberger, J. Taylor, M. Cavanaugh, E. Ginchansky, S. Godfrey, M. Tooley, T. Bell and S. Grinberg, Lancet, i (1977) 381.
4 J.A. Kuzemko and J. Paala, Arch. Dis. Child., 48 (1973) 404.
5 D.C. Shannon, F. Gotay, I.M. Stein, M.C. Rogers, I.D. Todres and P.M.B. Moylan, Pediatrics, 55 (1975) 589.
6 R. Uauy, D.L. Shapiro, B. Smith and J.B. Warshaw, Pediatrics, 55 (1975) 595.
7 J.F. Lucey, Pediatrics, 55 (1975) 584.
8 J.W. Jenne, E. Wyze, F.S. Rood and F.M. MacDonald, Clin. Pharmacol. Ther., 13 (1972) 349.
9 P.A. Mitenko and R.I. Ogilvie, Clin. Pharmacol. Ther., 14 (1973) 509.
10 E.B. Truitt, Jr., C.J. Carr, H.M. Bubert and J.C. Krantz, Jr., J. Pharmacol. Exp. Ther., 91 (1947) 185.
11 P.A. Mitenko and R.I. Ogilvie, New Engl. J. Med., 289 (1973) 600.
12 G.F. Johnson, W.A. Dechtiaruk and H.M. Solomon, Clin. Chem., 21 (1975) 144.
13 A.J. Plummer, J. Pharmacol. Exp. Ther., 93 (1948) 142.
14 J.A. Schack and S.H. Waxler, J. Pharmacol. Exp. Ther., 97 (1949) 283.
15 R.C. Gupta and G.D. Lundberg, Anal. Chem., 45 (1973) 2403.
16 R.D. Thompson, H.T. Nugasawa and J.W. Jenne, J. Lab. Clin. Med., 84 (1974) 584.
17 C.V. Manion, D.W. Shoeman and D.L. Azarnoff, J. Chromatogr., 101 (1974) 169.
18 M. Weinberger and C. Chidsey, Clin. Chem., 21 (1975) 834.
19 M. Riechert, J. Chromatogr., 146 (1978) 175.
20 A. Arbin and P.O. Edlund, Acta Pharm. Suecica, 11 (1974) 249.
21 B. Wesley-Hadzija, Brit. J. Pharmacol., 1 (1974) 515.
22 V.P. Shah and S. Riegelman, J. Pharm. Sci., 63 (1974) 1283.
23 W. Dechtiaruk, G.F. Johnson and H.M. Solomon, Clin. Chem., 21 (1975) 1038.
24 A. Arbin and P.O. Edlund, Acta Pharm. Suecica, 12 (1975) 119.
25 J.D. Lowry, L.J. Williamson and V.A. Raisys, J. Chromatogr., 143 (1977) 83.
26 C.J. Least, Jr., G.E. Johson and H.M. Solomon, Clin. Chem., 22 (1976) 765.
27 D. Perrier and E. Lear, Clin. Chem., 22 (1976) 898.
28 M. Sheehan and P. Haythorn, J. Chromatogr., 117 (1976) 393.
29 B. Vinet and L. Zizian, Clin. Chem., 25 (1979) 156.
30 L.J. Dusci, L.P. Hackett and I.A. McDonald, J. Chromatogr., 104 (1975) 147.
31 M. Kowblansky, B.M. Scheinthal, G.D. Cravello and L. Chafetz, J. Chromatogr., 76 (1973) 467.
32 R. Koysooko, E.F. Ellis and G. Levy, Clin. Pharmacol. Ther., 15 (1974) 454.

Journal of Chromatography, 182 (1980) 237–240
Biomedical Applications

CHROMBIO. 524

Note

Simple, rapid procedure for the determination of valproate and ethosuximide in plasma by gas–liquid chromatography

N. GRGURINOVICH and J.O. MINERS

Department of Clinical Pharmacology, Flinders Medical Centre, Bedford Park, South Australia 5042 (Australia)

(First received October 9th, 1979; revised manuscript received December 18th, 1979)

Therapeutic monitoring of the anticonvulsants ethosuximide (Zarontin, Parke Davis, Sydney, Australia) and valproate (Epilim, Reckitts, Sydney, Australia) is a routine aid in the clinical management of epileptic patients treated with these drugs. With the exception of a recently developed enzyme immunoassay for valproate [1], gas–liquid chromatography (GLC) is the only technique available for the measurement of plasma valproate and it also remains an attractive alternative for the determination of ethosuximide. Since requests for plasma ethosuximide estimations are infrequent in this department, and consequently uneconomic to perform by the enzyme multiplied immunoassay technique, we therefore examined a range of conditions which would be suitable for the gas chromatographic (GC) determination of both valproate and ethosuximide.

Although GC conditions suitable for the determination of valproate and ethosuximide have been reported [2], sample preparation for the assay included evaporation of the plasma extract which, due to the volatility of valproic acid, may result in loss of this drug. Furthermore, precision data were not reported. While valproate may be analysed by GLC following the direct injection of diluted plasma [3], use of this method results in accumulation of denatured proteins in the column which may necessitate frequent replacement of the stationary phase. A number of simple procedures which require only a single extraction from plasma have been described [4, 5] for the analysis of valproate by GLC. Similarly, ethosuximide in plasma may be assayed by GLC after a single extraction [6–8], although such methods generally employ an evaporation step to concentrate the sample. This report describes a rapid GC procedure for the simultaneous analysis of valproate and ethosuximide using the stationary phase Carbowax 20M–terephthalic acid. The method employs a

single extraction from plasma but does not require evaporation of the extract or derivatisation of the sample. The assay is reproducible and applicable to the routine therapeutic monitoring of both adult and paediatric patients.

EXPERIMENTAL

Gas chromatography

Analyses were performed on a Hewlett-Packard Model 5720A gas chromatograph fitted with a flame ionisation detector. The following chromatographic conditions were employed; a glass column (1.6 m × 2 mm I.D.) containing 10% Carbowax 20M—terephthalic acid on Chromosorb W HP, 80—100 mesh (Applied Science Labs, State College, Pa., U.S.A.), column temperature 180°, injector port and detector temperatures 250°, and carrier gas (nitrogen) flow-rate 40 ml/min.

Reagents and standards

Pure samples of ethosuximide and methsuximide were donated by Parke Davis. Sodium valproate was donated by Reckitts and octanoic acid was supplied by Sigma (St. Louis, Mo., U.S.A.). Other reagents and solvents were of analytical grade.

Standard solutions of sodium valproate and ethosuximide were prepared containing 50, 250, 500, 750 and 1000 mg/l in distilled water.

A 0.1-ml aliquot of valproate was combined with a 0.1-ml aliquot of the appropriate ethosuximide standard and then diluted with 0.3 ml of drug-free plasma to give final concentrations of 10, 50, 100, 150 and 200 mg/l. The internal standards, octanoic acid for valproate determination and methsuximide for ethosuximide determination, were prepared by dissolving 60 mg of the compounds in 100 ml of methanol. For analytical samples containing a single drug, only the appropriate standard and a single internal standard need be used.

Extraction

To 0.5 ml of analytical sample and standards in a conical tip glass tube were added 0.1 ml of the internal standard solution, 0.4 ml of saturated potassium dihydrogen phosphate and 0.5 ml of chloroform. The mixture was vortexed for 1 min and then centrifuged at 1500 *g* for 3 min. An aliquot (1—2 μl) of the organic phase was then injected into the gas chromatograph.

For paediatric samples, 0.1 ml of the analytical sample and standards were extracted by the above procedure using 1/5th of the reagent and solvent volumes described. The extractions were performed in 1.5-ml Eppendorf microtubes and the organic phase was separated during 1 min on an Eppendorf Model 5412 high-speed centrifuge at 6500 *g*.

Unknown concentrations were determined by comparison of the valproate/octanoic acid and ethosuximide/methsuximide peak height ratios with those of the calibration curve. A factor of 0.87 must be employed in the calculation of valproate concentration since standards were prepared from the sodium salt.

RESULTS AND DISCUSSION

Fig. 1B shows a chromatogram obtained following this procedure for a patient plasma sample containing valproate (45 mg/l) and ethosuximide (55 mg/l). Sharp, symmetrical peaks with retention times of 45, 70, 180 and 360 sec are obtained for valproic acid, octanoic acid, ethosuximide and methsuximide respectively. Drug-free plasma gave no interfering peaks under the chromatography conditions described (Fig. 1A).

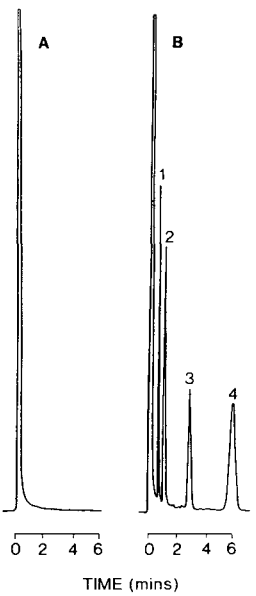


Fig. 1. Chromatograms of plasma extracts (attenuation 2^6). (A) Drug-free plasma; (B) patient sample containing valproate (45 mg/l) and ethosuximide (55 mg/l). Peaks: 1, valproate; 2, octanoic acid; 3, ethosuximide; 4, methsuximide.

Linear calibration curves passing through the origin were obtained for plots of valproate and ethosuximide to the appropriate internal standard peak height ratio versus concentration in the range 10—200 mg/l. The mean slopes of the linear responses were 0.0239 ± 0.0012 and 0.0219 ± 0.0017 (n = 14) for valproate and ethosuximide respectively. Analyses of repetitive samples (n = 20) at concentrations of 25 and 100 mg/l had coefficients of variation, respectively, of 2.8 and 3.7% for valproate, and 8.5 and 5.0% for ethosuximide. The mean recoveries of valproate and ethosuximide from plasma were 86 ± 3 and 73 ± 4% over the range 10—200 mg/l.

The anticonvulsants phenytoin, phenobarbitone, primidone and carbamazepine, as well as salicylate and paracetamol, do not interfere with the determination of valproate or ethosuximide. Moreover, in over a year's operation no other drugs, metabolites or endogenous plasma constituents have been found to interfere with the analysis.

In summary, a simple and rapid GC procedure for the estimation of valproate and ethosuximide in plasma has been developed. The procedure is reproducible and has been applied to the routine analysis of both paediatric and adult patients.

REFERENCES

1 A. Izutsu, D. Leung, C. Araps, P. Singh, A.Jaklitsch and D.S. Kabakoff, Clin. Chem., 25 (1979) 1093.
2 A.J. Kupferberg, in C.E. Pippenger, J.K. Penry and H. Kutt (Editors), Antiepileptic Drugs: Quantitative Analysis and Interpretation, Raven Press, New York, 1978, p. 147.
3 C. Jakobs, M. Bojasch and F. Hanefeld, J. Chromatogr., 146 (1978) 494.
4 M.H. Wood, D.C. Sampson and W.J. Hensley, Clin. Chim. Acta, 77 (1977) 343.
5 A.J. Fellenberg and A.C. Pollard, Clin. Chim. Acta, 81 (1977) 203.
6 J.B. Bonitati, Clin. Chem., 22 (1976) 341.
7 L. Sun and I. Szafir, Clin. Chem., 23 (1977) 1753.
8 W. Godolphin and J. Thoma, Clin. Chem., 24 (1978) 483.

Journal of Chromatography, 182 (1980) 241—245
Biomedical Applications

CHROMBIO. 525

Note

Gas chromatographic determination of valproic acid in human plasma

ANTOINE SIOUFI*, DANIELLE COLUSSI and FRANÇOISE MARFIL

Ciba-Geigy, Centre de Recherche Biopharmaceutique, B.P. 308, 92506 Rueil-Malmaison Cedex (France)

(First received November 12th, 1979; revised manuscript received December 14th, 1979)

Valproic acid is an anti-epileptic drug. A high-performance liquid chromatographic method [1] and many gas chromatographic (GC) methods have been described for its determination in body fluids. In some GC methods, valproic acid is derivatized [2—6].

In most of the published assays, however, no derivatization is used, and the usual procedure entails only one extraction step. Different solvents were used for this extraction step: heptane [7], carbon tetrachloride [8, 9], diethyl ether [10—13], toluene [14], chloroform [13, 15—20], dichloromethane [21, 22] and carbon disulphide [23]. In two procedures [24, 25], no extraction is required. One method [26] involves a large-scale extraction in a separating funnel, followed by back-extraction. Another method [27] also employs back-extraction, and is designed to measure low plasma concentrations of valproic acid down to 1 μg/ml. The other methods cited above have a sensitivity between 5 and 20 μg/ml.

A number of problems are encountered in the estimation of valproic acid. The free acid is volatile and is hence lost on evaporation; it also has a tendency to be adsorbed on the column.

In the method described below there is no evaporation or derivatization; after a one-step extraction a very polar stationary phase is used, and the technique is suitable for the analysis of valproic acid down to a concentration of 1 μg/ml.

*To whom correspondence should be addressed.

EXPERIMENTAL

Chemicals

Sodium valproate was stored in a desiccator under vacuum. The aqueous solutions of sodium valproate and octanoic acid (Aldrich 15 375-3; Aldrich, Milwaukee, Wisc., U.S.A.) are stable at +4° for more than three months.

Equipment

A gas chromatograph (Carlo Erba, Fractovap 2400T) equipped with a flame-ionization detector was used. The glass column (2 m × 2.8 mm I.D.) is operated at 175° and the injector at 200°, with a nitrogen flow-rate of 35 ml/min, an air flow-rate of 300 ml/min and a hydrogen flow-rate of 30 ml/min. The column packing is 10% SP 216 PS on 100—120 mesh Supelcoport (Supelco 1 1879; Supelco, Bellefonte, Pa., U.S.A.).

Extraction

To 1 ml of plasma in a 4-ml stoppered glass tube are added 0.3 ml of the internal standard solution (19.1 μg of octanoic acid), 0.5 ml of water and 0.25 ml of 4 *N* hydrochloric acid. After gentle shaking on a Vortex, 0.5 ml of carbon disulphide is added; the mixture is again gently shaken a further 2 min and centrifuged for 10 min. The upper layer is removed. (It may be necessary to stir the carbon disulphide with a Pasteur pipette to break the emulsion.) The organic phase is then transferred to another tube.

Gas chromatography

A 3-μl portion of the organic layer is injected into the gas chromatograph.

The sodium valproate concentration is calculated from the peak-area ratio by reference to a calibration curve. This curve is obtained by extraction of plasma spiked with increasing amounts of sodium valproate (from 1 to 100 μg/ml) and a constant amount of internal standard (19.1 μg per ml of plasma).

RESULTS

Sensitivity, reproducibility and accuracy

Table I gives the results obtained when the described procedure was applied to spiked plasma samples. The coefficients of variation are calculated on the basis of six replicate analyses at each concentration. Concentrations down to 1 μg of sodium valproate per ml of plasma can be accurately determined. At lower concentrations there is no detectable chromatographic peak.

Plasma interference

Fig. 1 shows the chromatograms of an extract of human plasma and of the same plasma spiked with 10 μg of sodium valproate. There is no interference from the normal constituents of plasma.

DISCUSSION AND CONCLUSION

An inter-laboratory variability test of the results of valproate determinations

TABLE I

PRECISION AND RECOVERY OF THE DETERMINATION OF SODIUM VALPROATE APPLIED TO SPIKED PLASMA SAMPLES

Amount of sodium valproate added (μg/ml)	Amount of sodium valproate found (μg/ml)	Mean (μg/ml)	Precision/ reproducibility (C.V., %)	Recovery/ accuracy (%)
1	0.98			98
	0.96			96
	0.96	0.96	5.8	96
	0.96			96
	0.86			86
	1.03			103
2	2.02			101
	2.00			100
	1.90	1.96	2.4	95
	1.95			97
	1.99			100
	1.92			96
10	9.0			90
	9.1			91
	10.3	9.9	6.8	103
	10.7			107
	9.9			99
	10.2			102
20	19.7			98
	22.1			111
	18.7	20.0	6.8	94
	20.8			104
	18.4			92
	20.1			100
100	98			98
	96			96
	99	103	5.5	99
	108			108
	107			107
	109			109
			Mean ± S.D.:	99 ± 5.9

by different methods [13] showed that the sodium valproate used by all the investigators as a standard may give deceptively high values, as it is hygroscopic. The salt must be dried and stored in a desiccator under vacuum, as recommended here. The use of the same solvent for extraction and chromatography also avoids the problem of loss on evaporation.

There is no mention in the literature of the presence of conjugated valproic acid in plasma. Approximately one quarter of an 800-mg dose is excreted as free and conjugated valproate in the urine [2]. The main metabolite of valproic acid is 3-keto-valproic acid, and 4- and 5-hydroxyvalproic acids are also present [3, 28, 29]. The method described here may be considered specific. Schäfer and Lührs [8] indicated that the three metabolites are unstable and non-volatile, so that they had to convert them into stable and easily volatilized derivatives. In the present method no such a treatment was applied.

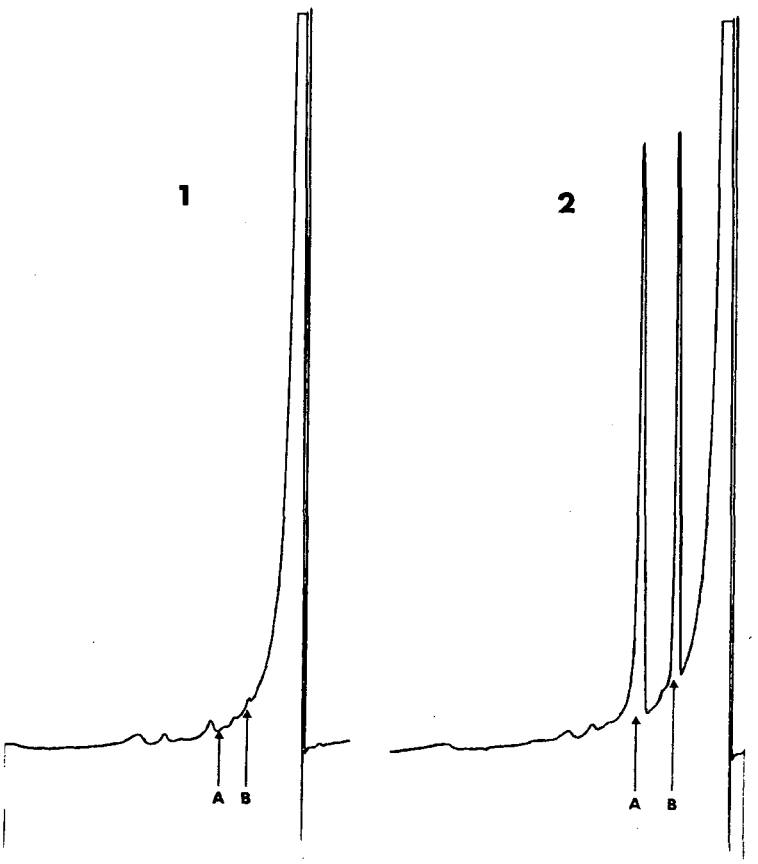


Fig. 1. Examples of chromatograms. (1) Human plasma blank. (2) 19.1 μg/ml of internal standard (A) and 10 μg/ml of sodium valproate (B) in human plasma.

According to what is known about the pharmacokinetics of valproic acid [30—34], the described method is convenient for measuring plasma concentrations until they decline to levels of ten to twenty times less than the peak concentration. Moreover, its sensitivity of around 1 μg of sodium valproate per ml of plasma permits analyses to be made in very small samples, which is useful because the drug is frequently given to children.

REFERENCES

1 R. Farinotti, M.C. Pfaff and G. Mahuzier, Ann. Biol. Clin., 36 (1978) 347.
2 S. Willox and S.E. Foote, J. Chromatogr., 151 (1978) 67.
3 O. Gyllenhaal and A. Albinsson, J. Chromatogr., 161 (1978) 343.
4 N.L. Tupper, E.B. Solow and C.P. Kenfield, J. Anal. Toxicol., 2 (1978) 203.
5 R.N. Gupta, F. Eng and M.L. Gupta, Clin. Chem., 25 (1979) 1303.
6 H.M.G. Cremers and P.E. Verheesen, Pharm. Weekbl., 109 (1974) 522.
7 A.J. Fellenberg and A.C. Pollard, Clin. Chim. Acta, 81 (1977) 203.
8 H. Schäfer and R. Lührs, Arzneim.-Forsch., 28 (1978) 657.
9 W. Oelkers, G. Stoffels, H. Schäfer and H. Reith, Arzneim.-Forsch., 27 (1977) 1088.

10 U. Klotz, Arzneim.-Forsch., 27 (1977) 1085.
11 U. Klotz, T. Rapp and W.A. Müller, Eur. J. Clin. Pharmacol., 13 (1978) 55.
12 P. Loiseau, A. Brachet and P. Henry, Epilepsia, 16 (1975) 609.
13 D. Schmidt, B. Ferrandes, D. Grandjean, R. Gugler, C. Jakobs, S. Johannessen, U. Klotz, W. Kochen, H.J. Kupferberg, J.W.A. Meijer, A. Richens, H. Schäfer, H.U. Schulz and A. Windorfer, Arzneim.-Forsch., 27 (1977) 1078.
14 F.J.E. Vajda, O.H. Drummer, P.M. Morris, J.J. McNeil and P.F. Baldin, Clin. Exp. Pharmacol. Physiol., 5 (1978) 67.
15 W. Löscher and H. Esenwein, Arzneim.-Forsch., 28 (1978) 782.
16 M.H. Wood, D.C. Sampson and W.J. Heusley, Clin. Chim. Acta, 77 (1977) 343.
17 S.I. Johannessen, Arzneim.-Forsch., 27 (1977) 1083.
18 J.P. Davison and J. Sinn, Clin. Chem., 24 (1978) 991.
19 D.J. Berry and L.A. Clarke, J. Chromatogr., 156 (1978) 301.
20 M. Punkka, M. Reunanen, U. Lamminsivu and R. Punkka, Acta Neurol. Scand., 57 (Suppl. 67) (1978) 286.
21 W. Godolphin and N. Urquhart, Clin. Biochem., 10 (1977) 9.
22 J. Balkon, J. Anal. Toxicol., 2 (1978) 207.
23 J.C. Libeer, S. Scharpé, P. Schepens and R. Verkerk, J. Chromatogr., 160 (1978) 285.
24 C. Jakobs, M. Bojasch and F. Hanefeld, J. Chromatogr., 146 (1978) 494.
25 B. Pileire, J. Chromatogr., 162 (1979) 446.
26 L.J. Dusci and L.P. Hackett, J. Chromatogr., 132 (1977) 145.
27 C.J. Jensen and R. Gugler, J. Chromatogr., 137 (1977) 188.
28 T. Kuhara, Y. Hirokata, S. Yamada and I. Natsumoto, Eur. J. Drug Metab. Pharmacokinet., 3 (1978) 171.
29 W. Kochen, H. Imbeck and C. Jakobs, Arzneim.-Forsch., 27 (1977) 1090.
30 R. Gugler, Eur. J. Clin. Pharmacol. Ther., 21 (1977) 125.
31 U. Klotz and K.H. Antonin, Clin. Pharmacol. Ther., 21 (1977) 736.
32 E. Perucca, G. Gatti, G.M. Frigo and A. Crema, Brit. J. Clin. Pharmacol., 5 (1978) 313.
33 J. Bruni, B.J. Wilder, L.J. Willmora, R.J. Perchalski and H.J. Villareal, Clin. Pharmacol. Ther., 24 (1978) 324.
34 E. Perucca, G. Gatti, G.M. Frigo, A. Crema, S. Calzetti and D. Visintini, Brit. J. Clin. Pharmacol., 5 (1978) 495.

Journal of Chromatography, 182 (1980) 246—251
Biomedical Applications

CHROMBIO. 518

Note

High-performance liquid chromatographic assay for the metabolites of nitrofurantoin in plasma and urine

BETTY-ANN HOENER and JOY L. WOLFF

Department of Pharmacy, School of Pharmacy, University of California, San Francisco, San Francisco, Calif. 94143 (U.S.A.)

(First received October 8th, 1979; revised manuscript received December 4th, 1979)

We have previously published a high-performance liquid chromatographic assay for the quantitation of the urinary tract anti-infective nitrofurantoin in plasma and urine [1]. Subsequently, we isolated and identified, following anaerobic metabolism, two metabolites of nitrofurantoin [2]. These were the amino, 1-[[(5-amino-2-furanyl)methylene]amino]-2,4-imidazolidinedione, and the open-chain nitrile, 1-[[(3-cyano-1-oxopropyl)methylene]amino]-2,4-imidazolidinedione [2].

The purpose of the present investigation was to develop an assay for both the amino and cyano metabolites of nitrofurantoin. Since the assay was to be used for our bioavailability and pharmacokinetic studies, we wanted to develop a rapid, sensitive method for measuring low levels of both metabolites in plasma and urine. The high-performance liquid chromatographic assay we describe here is rapid, sensitive and accurate. Furthermore no prior extraction and/or derivatization of the plasma or urine samples is required.

EXPERIMENTAL

Apparatus

We used a high-pressure liquid chromatograph, Model ALC/GPC 244, Waters Assoc. (Milford, Mass., U.S.A.) characterized by a constant solvent flow at working pressures up to 420 kg/cm^2. This model includes a U6K universal injector and a dual-channel, fixed-wavelength ultraviolet absorption detector. The instrument was fitted with a 250 mm × 3.2 mm I.D. 5-μm particle size, LiChrosorb C_{18} reversed-phase column (Altex, Berkeley, Calif., U.S.A.). The chromatograph was operated isocratically at a flow-rate of 2 ml/min. The wavelengths of detection were fixed at 280 and 365 nm. A dual-pen recorder was used

(OmniScribe Model A5211-1; Houston Instruments, Austin, Texas, U.S.A.). Chart speed was 2.5 cm/min and full-scale response was 1 mV.

Reagents

Chemicals. The amino and cyano metabolites were prepared by catalytic hydrogenation of nitrofurantoin over 5% palladium on charcoal (ICN Pharmaceuticals, Plainview, N.Y., U.S.A.) [2]. After lyophilization, the amino was stored as the dry powder at −20°. Since the cyano tends to polymerize when the crystals are exposed to air, it was stored in a solution of methanol at −20°. The purity of each was >99.5% as confirmed by UV and chemical ionization mass spectroscopy [2]. The 5-nitro-2-furoic acid, Lot 090447, was purchased from Aldrich (Milwaukee, Wisc., U.S.A.).

The methanol (glass-distilled, Burdick and Jackson Labs., Muskegon, Mich., U.S.A.), and distilled water (glass-redistilled and stored in glass) were filtered through a 0.45-μm filter (FHLPO 4700 and HAWPO 4700; Millipore, Bedford, Mass., U.S.A.) before use. The acetonitrile (glass-distilled, Burdick and Jackson Labs.), and other chemicals were ACS reagent grade or better.

Mobile phase. The mobile phase consisted of methanol—0.02 *M* acetic acid (2.5:97.5). It was prepared by mixing 25 ml of methanol with 975 ml of water and adding 1.2 ml of glacial acetic acid. This mobile phase was degassed under vacuum before use.

Biological fluids. Human plasma that had been stored at −20° was obtained from the blood bank of the University of California, San Francisco. The plasma was thawed to room temperature before use. Human urine from a female donor was collected daily.

Procedure

Plasma. Add 5 μl of a 520 μg/ml solution of 5-nitro-2-furoic acid, the internal standard, to 100 μl of plasma. Shake well, add 500 μl of acetonitrile and shake to mix. Centrifuge for 10 min at 3000 *g* to precipitate the proteins. Pour the supernatant into a 4-ml glass tube. Evaporate to dryness under nitrogen. Dissolve the residue in 200 μl of distilled water and inject onto the chromatograph. Injection volumes of 30—120 μl were satisfactory for the entire range of plasma concentrations. The sensitivity of the 365-nm detector was set at 0.01 a.u.f.s. for detection of the amino metabolite and the 5-nitro-2-furoic acid. The 280-nm detector was set at 0.01 a.u.f.s. for detection of the cyano metabolite. Operate the chromatograph at 2 ml/min at room temperature using the methanol—0.01 *M* acetic acid (2.5:97.5) mobile phase. Retention times for the 5-nitro-2-furoic acid, cyano metabolite and amino metabolite are 2, 6 and 8 min respectively.

Urine. Add 5 μl of the 520 μg/ml solution of 5-nitro-2-furoic acid to 50 μl of urine and 50 μl of distilled water. Shake well and inject onto the chromatograph. Injection volumes of 30—75 μl were satisfactory for the entire range of urine concentrations when the detector sensitivity was set at 0.01 a.u.f.s. at 365 nm and 0.05 a.u.f.s. at 280 nm. All other chromatographic conditions were identical to those described above for plasma.

Standard curves were prepared by adding the cyano metabolite, the amino metabolite and the 5-nitro-2-furoic acid to plasma or urine. The concentration

of each metabolite is determined by comparing the metabolite:internal standard peak height ratio to its standard curve of peak height ratios versus metabolite concentration. With all curves we made a straight-line fit of the data by least squares linear regression analysis using the PROPHET system, a specialized computer resource developed by the Chemical/Biological Information Handling Program of the National Institutes of Health, U.S.A.

Stability study

Plasma. Prepare plasma samples containing 20 μg/ml of the amino metabolite and 20 μg/ml of the cyano metabolite and store at −20°. We performed analyses on six separate days for one month. Thaw the solution on the day of analysis, add the internal standard and proceed as described above for the plasma standard curve. On each day of analysis construct a standard curve and determine the metabolite concentrations of the stability study by comparing the peak height ratios with those of the standard curve.

Urine. Prepare urine samples containing 15 μg/ml of the amino metabolite or 120 μg/ml of the cyano metabolite and store at −20°. We performed analyses on six separate days over a period of three weeks. Thaw the solutions on the day of analysis, add internal standard and proceed as described above for urine standard curves. On each day of analysis construct a standard curve and determine the concentration of each metabolite by comparing the peak height ratios with those of the standard curve.

RESULTS AND DISCUSSION

Pharmacokinetic studies of nitrofurantoin following intravenous and oral administration have shown that only 30—50% of the nitrofurantoin is recovered unchanged in the urine [3—11]. At the time of these studies none of the metabolites had been identified in man. Products of the metabolic reduction of nitrofurantoin, which could account for up to 50—70% of the administered dose, have been implicated in the polyneuropathy, and possibly the pulmonary hypersensitivity that have been reported following long-term administration of nitrofurantoin [12—14]. These metabolites are probably also the end products of the metabolic pathway responsible for the mutagenic activity of nitrofurantoin in bacterial and mammalian tissue culture systems [12, 15, 16]. Since we wanted to study the disposition of nitrofurantoin and its reduced metabolites in man, we have developed a high-performance liquid chromatographic assay for quantitating the amino and cyano metabolites of nitrofurantoin in plasma and urine. The assay is rapid, sensitive and accurate. No extraction and/or derivatization is required. Fig. 1 shows a chromatogram for the quantitation of both metabolites in plasma. The retention times for the internal standard, the cyano metabolite and the amino metabolite are 2, 6 and 8 min respectively. The peak height ratio indicates a cyano concentration of 12 μg/ml and an amino concentration of 12 μg/ml. Standard curves were constructed by adding known amounts of each metabolite and the internal standard to plasma and plotting the peak height ratio versus concentration in μg/ml. Over a period of two months we constructed six plasma standard curves each containing five concentrations of both the amino and cyano metabolites. With 30 points the

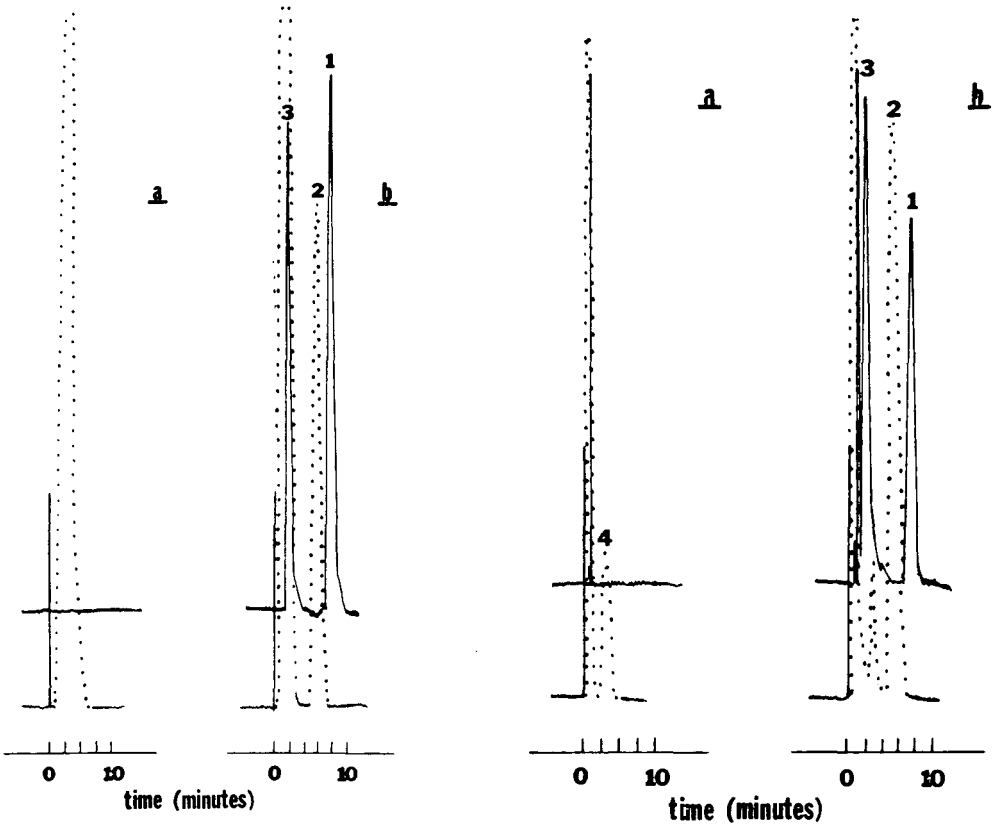


Fig. 1. Chromatograms of (a) blank plasma and (b) plasma with the amino metabolite (1), the cyano metabolite (2), and 5-nitro-2-furoic acid, the internal standard (3). The detector was set at 365 nm (0.01 a.u.f.s.) (——), and 280 nm (0.01 a.u.f.s.) (· · ·). The peak height ratios indicate an amino concentration of 12 μg/ml and a cyano concentration of 12 μg/ml.

Fig. 2. Chromatograms of (a) blank urine and (b) urine with the amino metabolite (1), the cyano metabolite (2), and 5-nitro-2-furoic acid, the internal standard (3). The detector was set a 365 nm (0.01 a.u.f.s.) (——), and at 280 nm (0.05 a.u.f.s.) (· · ·). The peak height ratios indicate an amino concentration of 12 μg/ml and a cyano concentration of 105 μg/ml. The peak labelled (4) is endogenous xanthines (see text for explanation).

regression line for the amino in plasma was $y = (0.138 \pm 0.009)\,x - (0.556 \pm 0.139)$ with a correlation coefficient of 0.93. With 30 points the regression line for the cyano was $y = (0.082 \pm 0.004)\,x - (0.040 \pm 0.073)$ with a correlation coefficient of 0.95. For both the amino and cyano the concentration range was 4—25 μg/ml.

Fig. 2 shows a chromatogram for the quantitation of both metabolites in urine. The retention times for the internal standard, the cyano metabolite and the amino metabolite are 2, 6 and 8 min respectively. The peak height ratios indicate a cyano concentration of 105 μg/ml and an amino concentration of 12 μg/ml. Over a period of two months we constructed six standard curves. With 29 points the regression line for the amino in urine was $y = (0.085 \pm 0.004)$

x − (0.250 ± 0.050) with a correlation coefficient of 0.97 over the concentration range of 4—25 μg/ml. With 29 points the regression line for the cyano was y = (0.0127 ± 0.0004) x − (0.186 ± 0.029) with a correlation coefficient of 0.98 over the concentration range of 20—130 μg/ml.

The preliminary stability studies were performed over a period of one month for plasma and three weeks for urine. The results showed that the amino metabolite is not stable for longer than 2—3 days even when stored frozen at −20°. The cyano metabolite appears to be stable. However, it appears that some, although not all, of the amino breaks down to the cyano. Thus, we would advise that both plasma and urine samples be analyzed for nitrofurantoin metabolites as soon as possible after collection.

Both metabolites were analyzed at wavelengths close to their absorption maxima. The cyano has a λ_{max} = 278 nm and the amino a λ_{max} = 348 nm [2]. As shown in Fig. 2 the only potential interference from endogenous substances appears to be in urine at 3 min on the 280-nm detector. We have tentatively identified this peak as "xanthines" since caffeine, theophylline and several dimethylpurines co-chromatograph with this peak under the assay conditions.

With urine samples, where no precipitation is necessary, analyses can be performed in less than 10 min per sample. In the case of plasma, centrifugation after protein precipitation is necessary because direct injections of plasma onto the chromatograph result in increases in operating pressure caused by the build-up of proteins in the column. The evaporation to dryness also requires additional time, usually about 15 min. When plasma and urine samples are collected from volunteers or patients we divide them into two fractions. We analyze the first fraction for nitrofurantoin using our previously published method [1]. The second fraction is used to analyze for the amino and cyano metabolites. Both methods are simple, rapid and accurate. We are currently using them for bioavailability and pharmacokinetic studies on nitrofurantoin and its metabolites following intravenous and oral administration to normal volunteers.

ACKNOWLEDGEMENTS

We wish to thank Michel B. Aufrère and Dennis R. Doose for the catalytic hydrogenation of the nitrofurantoin. The nitrofurantoin was a gift of Eaton Laboratories, Norwich, N.Y., U.S.A.

1 M.B. Aufrere, B. Hoener and M.E. Vore, Clin. Chem., 23 (1977) 2207.
2 M.B. Aufrere, B. Hoener and M. Vore, Drug Metab. Disp., 6 (1978) 403.
3 A.R. DiSanto, D.J. Chodos, J.P. Phillips, K.A. DeSante and R.G. Stoll, Int. J. Clin. Pharmacol., 13 (1976) 220.
4 J.D. Conklin and F.H. Hailey, Clin. Pharmacol. Ther., 10 (1969) 534.
5 G.L. Mattok, R.D. Hossie and I.J. McGilveray, Can. J. Pharm. Sci., 16 (1974) 63.
6 T.R. Bates, J.A. Sequeira and A.V. Tembo, Clin. Pharmacol. Ther., 16 (1974) 63.
7 D. Cadwallader, J. Amer. Pharm. Ass., NS15 (1975) 409.
8 H.A. Rosenberg and T.R. Bates, Clin. Pharmacol. Ther., 20 (1976) 227.
9 H.K. Reckendorf, R.G. Castringius and H.K. Spingler, Antimicrob. Ag. Chemother., 1961 (1962) 531.

10 J. Schirmeister, F. Stetani, H. Willmann and W. Hallaver, Antimicrob. Ag. Chemother., 1965 (1966) 223.
11 T.B. Vree, Y.A. Hekster, A.M. Baars, J.E. Damsma and E. van der Kleijn, J. Chromatogr., 162 (1979) 110.
12 S.M. Cohen, in G.T. Bryan (Editor), Carcinogenesis — A Comprehensive Survey, Vol. 4, Nitrofurans, Raven Press, New York, 1978, Ch. 6, p. 171.
13 M. Geller, D.K. Flaherty, H.A. Dickie and C.E. Reed, J. Allerg. Clin. Immunol., 59 (1977) 445.
14 A.I. Jacknowitz, J.L. LeFroch and R.A. Prince, Amer. J. Hosp. Pharm., 34 (1977) 759.
15 Y. Tazima, T. Kada and A. Murakami, Mutat. Res., 32 (1975) 55.
16 J.M. Klemencic and C.Y. Wang, in G.T. Bryan (Editor), Carcinogenesis — A Comprehensive Survey, Vol. 4, Nitrofurans, Raven Press, New York, 1978, Ch. 4, p. 99.

Journal of Chromatography, 182 (1980) 252—256
Biomedical Applications

CHROMBIO. 520

Note

Rapid high-performance liquid chromatographic method for the determination of probenecid in biological fluids

P. HEKMAN, P.A.T.W. PORSKAMP, H.C.J. KETELAARS and C.A.M. VAN GINNEKEN

Department of Pharmacology, University of Nijmegen, P.O. Box 9101, 6500 HB Nijmegen (The Netherlands)

(First received October 12th, 1979; revised manuscript received December 14th, 1979)

In the course of a study on the effect of probenecid (Fig. 1, I) on the renal excretion of other drugs we felt the need to develop a rapid and sensitive quantitative method for the detection of I in plasma and urine. The various

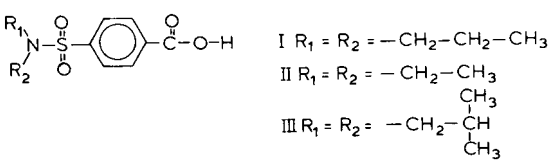


Fig. 1. Structural formulae: I, dipropylsulfamoyl benzoic acid (probenecid); II, diethylsulfamoyl benzoic acid; III, diisobutylsulfamoyl benzoic acid.

older spectrophotometric methods that have been reported [1—3] lack the required sensitivity and specificity, whereas the more recently introduced gas chromatographic procedures [4—7] make derivatization of I necessary, which is a complicating and time-consuming step in the analysis.

In this case high-performance liquid chromatography is the method of choice. The procedure described in this report combines sensitivity and specificity with ease of handling. Chromatography is performed in a soap chromatography mode using a C_8 hydrocarbon stationary phase. As internal standards the diethyl analog (II) and the diisobutyl analog (III) of probenecid are applied.

MATERIALS AND METHODS

Materials

Probenecid was obtained from Sigma (St. Louis, Mo., U.S.A.). Compounds II and III were synthesized from *p*-carboxybenzenesulfonylchloride and the appropriate amines, according to a procedure adopted from Mieler [8]. Stock solutions of I, II and III were prepared in 5% sodium bicarbonate. LiChrosorb RP-8 (5 μm) and sodium dodecyl sulphate were obtained from E. Merck (Darmstadt, G.F.R.). All other reagents were of analytical grade.

Apparatus

The equipment used was a high-pressure liquid chromatograph of Waters Assoc. (Milford, Mass., U.S.A.) consisting of an M6000A pump, a 46K universal injector and an M440 absorbance detector.

Chromatographic conditions

A stainless-steel column (15 cm × 0.46 cm I.D.) was packed with LiChrosorb RP-8 (5 μm). Chromatography was performed in a soap chromatography mode using a mobile phase of methanol—water—acetic acid with 0.005 *M* sodium dodecyl sulphate added as a counter-ion. For plasma samples the mobile phase contained methanol—water—acetic acid in a volume ratio of 49.5:49.5:1; for urine samples the ratio was 64.5:34.5:1. The flow-rate was fixed at 1.5 ml/min. Some typical examples of chromatograms obtained from plasma and urine samples are given in Fig. 2.

Experimental

Extraction procedure. Samples of plasma (1.0 ml) or urine (1 ml) were trans-

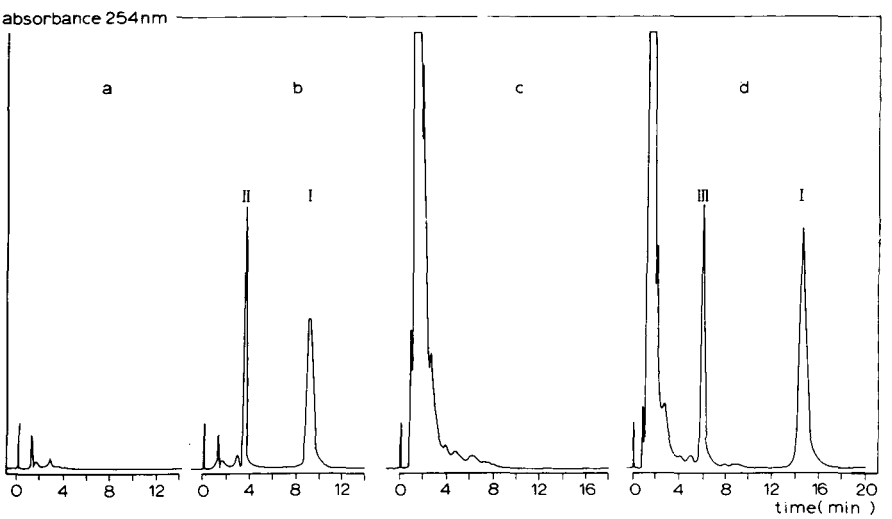


Fig. 2. Chromatograms obtained from plasma and urine samples. (a) Blank plasma; (b) plasma containing compounds I and II; (c) blank urine; (d) urine containing compounds I and III.

ferred to conical test-tubes, spiked with 1 ml of internal standard solution and acidified by the addition of 1 ml of 2 *N* HCl. Extraction was carried out by shaking this mixture mechanically with ethyl acetate (5 ml) for 10 min. After centrifugation the ethyl acetate layer was transferred to another test-tube and evaporated to dryness under a stream of dry, filtered air in a waterbath of 45°. The residue was dissolved in 250 μl of methanol, an aliquot of which (10 μl) was injected into the liquid chromatograph.

Calibration procedure. Samples of blank plasma (1.0 ml) or urine (1 ml) were spiked with various known amounts of probenecid, ranging from 0.5 to 40 μg, and with a fixed amount of internal standard (usually 25 μg). Since interfering peaks necessitated different mobile phases for plasma and urine samples, optimal chromatographic conditions required different internal standards. In the case of plasma, compound II was used as internal standard, in the case of urine compound III. The calibration samples were extracted as outlined above and after injection into the liquid chromatograph peak height ratios of probenecid to internal standard were plotted against the amount of probenecid added.

RESULTS AND DISCUSSION

Both for probenecid and compounds II and III the efficiency of the extraction process was estimated by comparing peak heights obtained after injection of standard solutions to peak heights obtained after injection of extracted standard solutions. The mean recoveries found were 98 ±2% (n = 6) for probenecid and for compound III, and 95 ± 2% (n = 6) for compound II. This recovery appeared to be constant over the concentration range 0.5—40 μg/ml. The detection limit of probenecid with the method described was below 0.5 μg/ml, but reproducible analytical results were obtained only when the concentration was 0.5 μg/ml or higher. Then the following equations are obtained for the calibration graphs from plasma

$$C_{\text{probenecid}} = 3.64 \times \text{amount compound II} \times \frac{\text{peak height probenecid}}{\text{peak height compound II}}$$

and from urine

$$C_{\text{probenecid}} = 0.563 \times \text{amount compound III} \times \frac{\text{pH probenecid}}{\text{pH compound III}}$$

Over the whole range studied the standard deviation of the plasma and urine determination was 2—5%.

This method of probenecid analysis was successfully applied to pharmacokinetic studies, details of which will be reported elsewhere. As an example of these investigations Fig. 3 shows the plasma curve obtained after oral administration of a 500-mg dose of probenecid to a human volunteer. Although this curve suggests a linear pharmacokinetic profile, experiments with higher doses clearly indicate the occurrence of nonlinear, saturable elimination. Whereas after a single 500-mg dose plasma concentrations are near to the limit of detection at 24 h after dosing, this period may be substantially prolonged

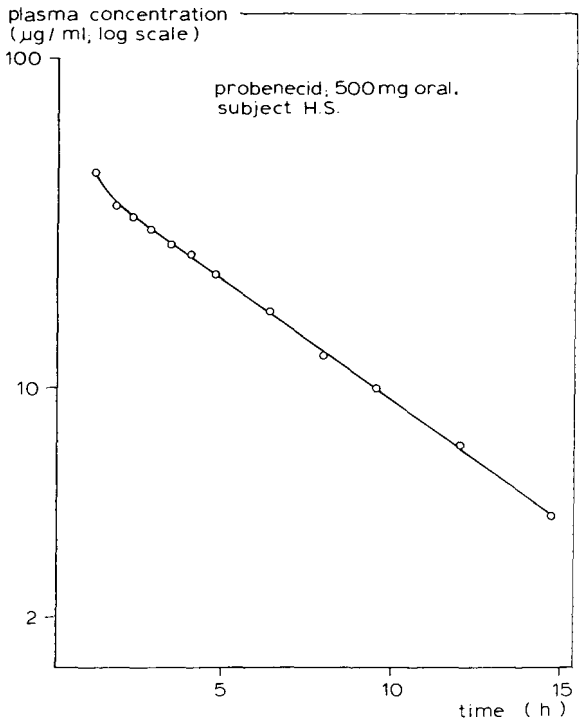


Fig. 3. Plasma concentration curve obtained after oral administration of 500 mg of probenecid to a human volunteer.

after application of higher doses. Less than 5% of a 500-mg dose is recovered unchanged from urine, the rest is metabolised. One of the metabolites is the probenecid acyl glucuronide (IV, Fig. 4), which can be measured in urine after hydrolysis with the aid of β-glucuronidase and which accounts for about 25%

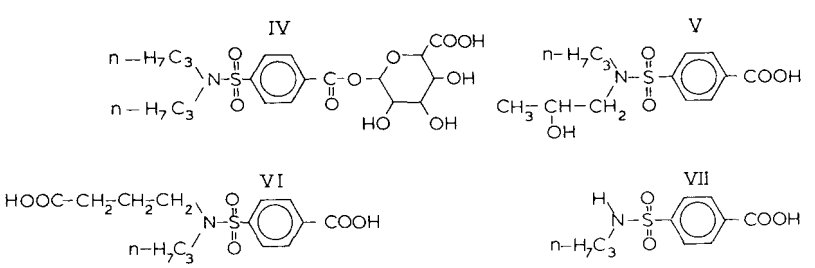


Fig. 4. Structural formulae of the principal metabolites of probenecid: IV, acyl glucuronide of probenecid; V, *sec.*-hydroxylated metabolite; VI, carboxy metabolite; VII, monopropyl metabolite.

of the dose. For the analysis of the other metabolites (Fig. 4) some modifications of the procedure described above are necessary. Extraction of these metabolites from urine is preferably performed with a 5-ml aliquot of a dichloromethane—diethyl ether (1:2) mixture. As internal standard the diethyl

analog (compound II) is used. Liquid chromatographic separation of the metabolites requires a mobile phase with a higher water content than used for probenecid itself. By using a mobile phase of methanol—water—acetic acid (39.5:59.5:1) with 0.005 *M* sodium dodecyl sulphate, the cumulative renal excretion of compounds V and VI was found to represent about 20% and that of compound VII about 5% of the total dose administered. Under these chromatographic conditions compounds V and VI appeared as a single peak in the chromatograms, showing a retention time of 5.9 min. The retention times of compounds VII and II (internal standard) were 4.5 and 7.7 min, respectively.

REFERENCES

1 E.K. Tillson, J. Pharmacol. Exp. Ther., 111 (1954) 385.
2 K.H. Beyer, Arch. Int. Pharmacodyn., 48 (1954) 97.
3 P.G. Dayton, J.F. Yu, W. Chen, L. Berger, L.A. West and A.B. Lutma, J. Pharmacol. Exp. Ther., 159 (1963) 278.
4 K. Sabik and C.D. Klaassen, J. Pharm. Sci., 60 (1971) 745.
5 A.G. Zacchei and L. Weidner, J. Pharm. Sci., 62 (1973) 1972.
6 E. Watson and S. Wilk, J. Neurochem., 21 (1973) 1569.
7 W.D. Conway and S. Melethil, J. Chromatogr., 115 (1975) 222.
8 C.S. Mieler, U.S. Pat. 2,608,507 (1952).

Journal of Chromatography, 182 (1980) 257—261
Biomedical Applications

CHROMBIO. 526

Note

Separation and analysis of azosemide in urine and in serum by high-performance liquid chromatography

RUTH SEIWELL and CRAIG BRATER*

Department of Pharmacology, Division of Clinical Pharmacology, University of Texas Health Science Center at Dallas, 5323 Harry Hines Blvd., Dallas, Texas 75235 (U.S.A.)

(First received October 17th, 1979; revised manuscript received December 18th, 1979)

Azosemide, 5-(4-chloro-5-sulphamyl-2-thenylamino-phenyl)tetrazole, is a new diuretic in the initial stages of testing which resembles the potent diuretic furosemide in chemical structure (Fig. 1), and in its site of action [1—3]. We have developed an assay for azosemide by high-performance liquid chromatography (HPLC) which allows facile measurement in serum and in urine. A previous assay utilized fluorometric detection and involved derivatization and extraction of the samples [3]. This paper describes a new assay of azosemide in serum and in urine using reversed-phase HPLC with ultraviolet detection and requiring no extraction or derivatization of the sample. An internal standardization technique employed phenobarbital as the standard. The method is simple,

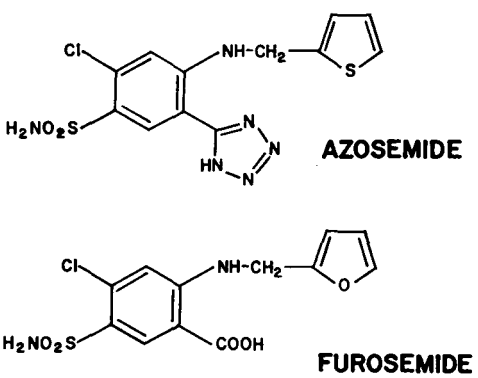


Fig. 1. Chemical structures of azosemide and furosemide.

*To whom correspondence should be addressed.

reliable and has been automated for urine samples to allow ease of drug measurement, a prerequisite for large-scale clinical evaluation and characterization of the drug.

EXPERIMENTAL

Materials

Azosemide was obtained from Merrell-National Labs. (Cincinnati, Ohio, U.S.A.) and the phenobarbital from Sigma (St. Louis, Mo., U.S.A.). Distilled-in-glass acetonitrile was obtained from Burdick and Jackson Labs. (Muskegon, Mich., U.S.A.) and glacial acetic acid (A.C.S. grade) from Mallinckrodt (St. Louis, Mo., U.S.A.). Water used in the assay was triple distilled and filtered (0.45 μm pore size). All solvents were degassed before use.

Chromatography

All analyses were performed using a Perkin-Elmer (Norwalk, Conn., U.S.A.) Series 3 high-performance liquid chromatograph with a Perkin Elmer Model LC 65-T variable-wavelength ultraviolet detector. A Dupont (Wilmington, Del., U.S.A.) Zorbax ODS column (25 cm × 4.6 mm C_{18} reversed-phase, 5 μm particle size) preceded by a 7 cm × 2.1 mm precolumn packed with CO:PELL ODS (Whatman, Clifton, N.J., U.S.A.) was used to perform the separations. Azosemide and phenobarbital were measured at 239 nm and a gradient elution using acetonitrile and acetate buffered water, pH 4.05 (0.6 ml glacial acetic acid added to 1 l of water and buffered to pH 4.05 with 4 *N* sodium hydroxide) was employed. A linear gradient from 10 to 40% acetonitrile over a 10-min period was delivered at a flow-rate of 2 ml/min. The system was then purged with 40% acetonitrile for 2 min and allowed to equilibrate for 5 min before the next sample was injected.

Sample preparations

To each 0.3-ml volume of filtered urine (0.45-μm pore size) were added 0.3 ml distilled water and 50 μl of the internal standard (0.412 mg/ml phenobarbital in ethanol). The samples were prepared in 1-ml serum vials, capped, vortexed and a volume of 10 μl was injected automatically using a Perkin-Elmer Model 420 autosampler. To serum samples were added 20 μl of phenobarbital followed by the addition of 0.4 ml acetonitrile with mixing to precipitate serum proteins. Each sample was then centrifuged and the supernate was decanted and evaporated to dryness. The residue was reconstituted in 50—100 μl of buffer and 5—20 μl was injected manually.

Drug quantitation

Azosemide in a patient sample was quantified by comparing the peak height ratio of azosemide to phenobarbital (A/P) in the sample to the A/P of standard samples of urine or serum containing known amounts of azosemide and phenobarbital. A linear plot of the peak height ratio versus known concentration of standard samples was constructed. For a given peak height ratio of a patient sample a corresponding amount of standard drug was obtained from the curve and then converted to a specific concentration of drug per sample.

RESULTS AND DISCUSSION

Fig. 2 depicts a typical chromatogram of the analysis of a human urine sample. The retention time of azosemide was 10 min and the total analysis time was 17 min. No other substances with a retention time comparable to that of azosemide were seen during the sample analyses. Commonly used drugs which were tested for and showed no interference included acetaminophen, aspirin, chlorothiazide, chlorpromazine, hydrochlorothiazide, procainamide, quinidine, sulfamethazole, theophyllin, and tolbutamide.

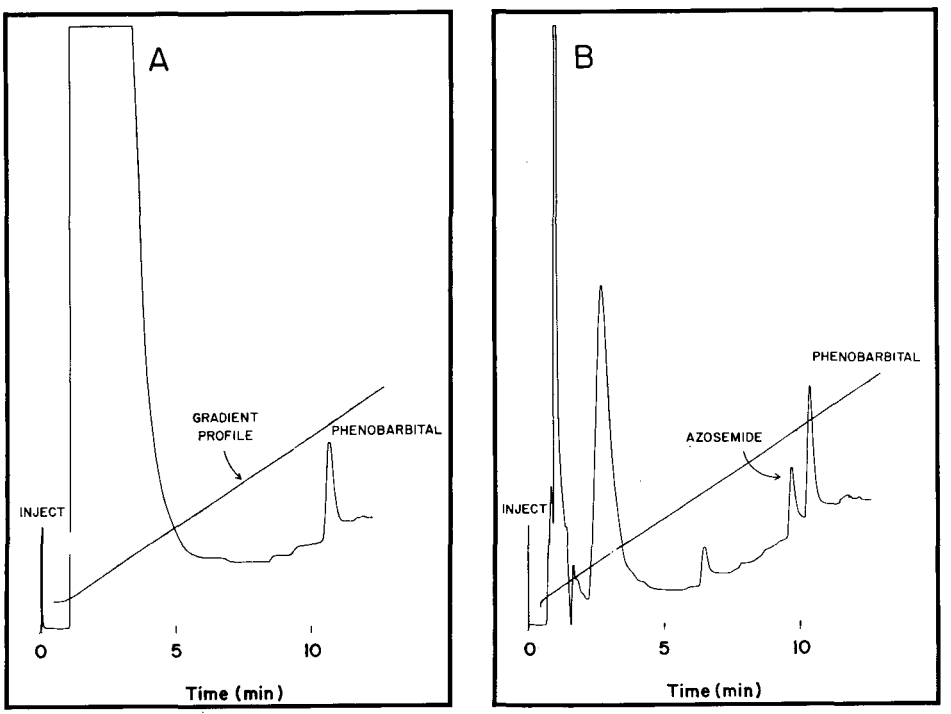


Fig. 2. Typical chromatogram of an aliquot of urine from a subject before (A) and 2 h after (B) receiving 40 mg of azosemide by mouth.

Recovery of drug was assessed by comparison of serum samples processed in the normal manner (i.e., deproteinated, centrifuged, filtered and evaporated) with a standard curve obtained from non-processed samples. Recovery of azosemide was 97.4% at each concentration tested.

The relationship between the peak height ratio (peak height of azosemide/peak height of phenobarbital) and the concentration of standard drug used to calculate the amount of azosemide in urine and serum samples was linear over the concentration range studied, verified by the consistently high correlation coefficient ($r > 0.99$) obtained from a linear regression analysis of the data.

As expected, the plot reliably passed very near the origin. The precision of the method was also tested over the concentration range studied. The coefficient of variation of ten measurements at each of several concentrations varied

from 1.6% at 0.86 μg/ml to 13.3% at 0.055 μg/ml. We considered the lower limit of reliable detection of azosemide to be 0.05 μg/ml. Concentrations as low as 0.007 μg/ml were detected but this was at or below the limits of the standard curve in some cases and thus was not considered reliable.

The chromatographic determination described above resulted in good separation and quantitation of azosemide and consequently allowed a preliminary pharmacokinetic study of azosemide. Fig. 2 shows a chromatogram of human urine prior to administration of azosemide and a trace 2 h after an oral dose of 40 mg of the drug. The concentration of diuretic in urine samples ranged from 0.007 to 1.651 μg/ml and in serum samples from 0.052 to 1.073 μg/ml.

The profiles of drug excretion rate (urine) and drug concentration (serum) with time for one subject are shown in Fig. 3. A lag time of approximately 60 min was observed in the appearance of azosemide in the serum. From the profile of concentration or excretion rate with time, the half-life of drug absorption was estimated to be 49 min in both urine and serum and the half-life of drug elimination was estimated at 89 min.

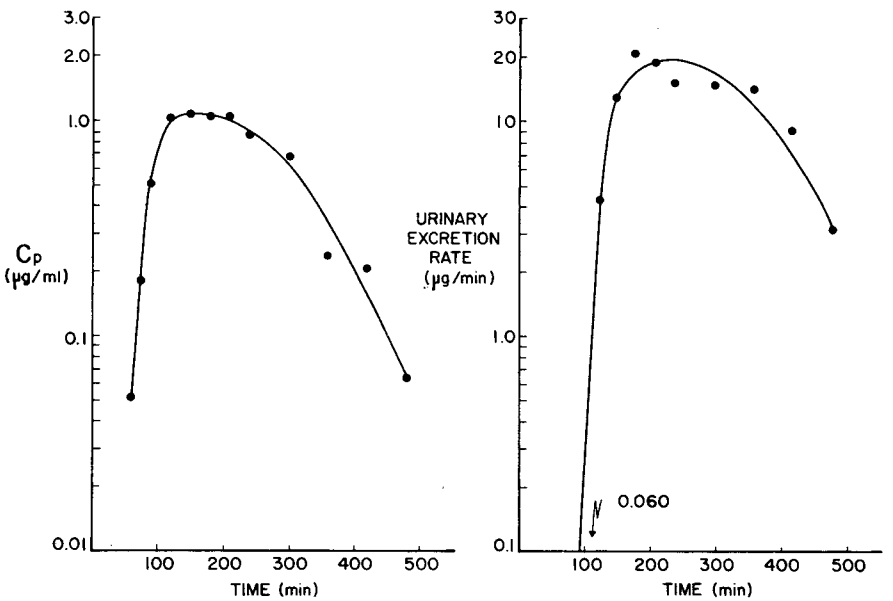


Fig. 3. Time course of azosemide urinary excretion rate (right) and concentration in serum (left) for one subject.

This assay provides an efficient and accurate method for analysis of azosemide in large numbers of biologic samples. A similar approach should be feasible with other structurally similar compounds allowing accurate determination of pharmacokinetics and of pharmacodynamic relationships.

ACKNOWLEDGEMENTS

The authors wish to acknowledge Ms. Joan Beck for her technical assistance, and Mrs. Stephanie Wooten for her secretarial assistance. This work was sup-

ported in part by the Clinical Research Center at the University of Texas Health Science Center at Dallas supported from USPHS Grant 1-MOI-RR00633, a National Institute of Health Young Investigator Award, and a grant from Merrell-National Laboratories.

REFERENCES

1 D.C. Brater, Clin. Pharmacol. Ther., 25 (1979) 428.
2 D.C. Brater, S.A. Anderson and S. Strowig, Clin. Pharmacol. Ther., 25 (1979) 435.
3 F. Kruck, W. Bablok, E. Besenfelder, G. Botzien and B. Kaufmann, Eur. J. Clin. Pharmacol., 14 (1978) 153.

Journal of Chromatography, 182 (1980) 262—266
Biomedical Applications

CHROMBIO. 527

Note

High-performance liquid chromatographic determination of indoprofen in plasma and urine

KERSTIN LANBECK and BJÖRN LINDSTRÖM*

National Board of Health and Welfare, Department of Drugs, Box 607, S-751 25 Uppsala (Sweden)

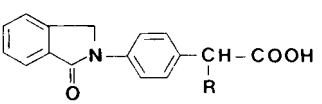
and

LARS WIBELL

Department of Medicine, University Hospital, Uppsala (Sweden)

(First received October 23rd, 1979; revised manuscript received December 7th, 1979)

Indoprofen (I) is an isoindoline derivative which has been reported to have analgesic and anti-inflammatory activity [1, 2]. Methods for its determination in plasma [3—5] and urine [3] have been described. These methods are based on gas chromatography with flame ionisation detection and involve extraction with rather large solvent volumes and derivatisation steps. The aim with the present method was to cover the same concentration range in the biological samples as the gas chromatographic methods but with a simpler sample handling procedure and avoidance of derivatisation. In the described liquid



R = CH_3 indoprofen (I)
R = C_2H_5 indobufen (II)

chromatographic method, 100 μl of plasma (or urine) samples were used to quantify indoprofen down to about 0.5 μg/ml. A homolog of indoprofen, indobufen (II), was used as internal standard.

MATERIALS AND METHODS

Standards

Indoprofen (I) and indobufen (II) were kindly donated by Pharmitalia, Carlo Erba, Milan, Italy.

*To whom correspondence should be addressed.

Instrumental

The liquid chromatograph consisted of an M6000 pump, a U6K injector and an M440 filter UV detector (Waters Assoc., Milford, Mass., U.S.A.). The 280 nm filter was used in the detector. A radial compression separation system (RCSS, Waters Assoc.) was used, consisting of a Radial-PAK A column, 10 cm × 1.3 cm, with octadecylsilane bonded to silica, (10 μm) and mounted in an RCM-100 radial compression module. The eluent was a mixture of 55% acetonitrile and 45% 0.01 *M* phosphate buffer (pH 3) operated at a flow-rate of 1.5 ml/min at room temperature (20—22°).

Plasma assay

To a plasma sample (100 μl) in a 10-ml screw-capped tube were added 100 μl of internal standard solution (10 μg/ml of II in methanol), 1 ml of 0.1 *M* HCl and 4 ml of diethyl ether. The extraction was carried out on a shake board for 10 min and the tube was then centrifuged for 5 min at 500 *g* (Wifug XI).

The ether phase was transferred to a new tube, extracted with 2 ml of 0.5 *M* NaOH (shake board for 10 min) and centrifuged (500 *g*). The aqueous phase (ca. 1.5 ml) was then transferred to another tube to which 0.5 ml of 2 *M* HCl and 4 ml diethyl ether were added and extracted as before. The organic phase was transferred to a conical tube and the solvent evaporated under a stream of nitrogen. The residue was dissolved in 200 μl of the eluent and an aliquot (20 μl) was injected into the liquid chromatograph.

Urine assay (free indoprofen)

A 100-μl sample of urine was added to 1 ml of 0.2 *M* acetate buffer (pH 5), and from here on the same procedure was followed as in the plasma assay described above.

Urine assay (free and conjugated indoprofen)

A 100-μl volume of urine was added to 100 μl of 0.2 *M* acetate buffer (pH 5) and 1 mg of β-glucuronidase. The mixture was incubated at 37° overnight. After acidification with 1 ml of 0.1 *M* HCl the same scheme was followed as above. Since the concentrations of I in urine reach higher levels than in plasma, two calibration graphs were used. In the higher range the concentration of internal standard solution was 100 μg/ml.

RESULTS AND DISCUSSION

The extraction of indoprofen (I) from an aqueous phase (pH = 1) to diethyl ether was close to quantitative using the proportions described in the method. The same was true for the back extraction from ether into sodium hydroxide solution. On injection of a series of samples that had been extracted once with ether only, the base-line became rather unstable due to substances with long retention times. In order to reduce these interfering substances in the samples to an acceptable level, it was necessary to do an extraction into an alkaline aqueous phase from the first ether extract; after acidification this was followed by another ether extraction. This extra purification step allowed determinations of I down to about 0.5 μg/ml in both plasma and urine. Further purification steps were considered unnecessary.

When the fraction of free I in urine was determined the pH was adjusted to 5. This was done because at pH 1, but not at pH 5, the conjugate of I will be co-extracted to some extent into the ether phase. The conjugate will then be hydrolysed to I during the extraction with the sodium hydroxide solution, thus introducing an error into the determination of I. The extraction yield of I was found to be slightly lower at pH 5. It was also possible to determine I after a single extraction with ether since the conjugate does not interfere with I in the chromatographic system. However, the peak resulting from compounds slightly or not retained will be rather large, making it difficult to resolve the peak of I. Figs. 1 and 2 show typical chromatograms obtained when using the method described.

The eluent mixture used in the liquid chromatograph consisted of phosphate buffer (pH 3) and acetonitrile. At pH 7, I eluted much faster and a lower acetonitrile concentration could be used, but at this pH there was a considerable increase in background interference. In order to construct a calibration graph a series of plasma samples to which had been added 0.5—10

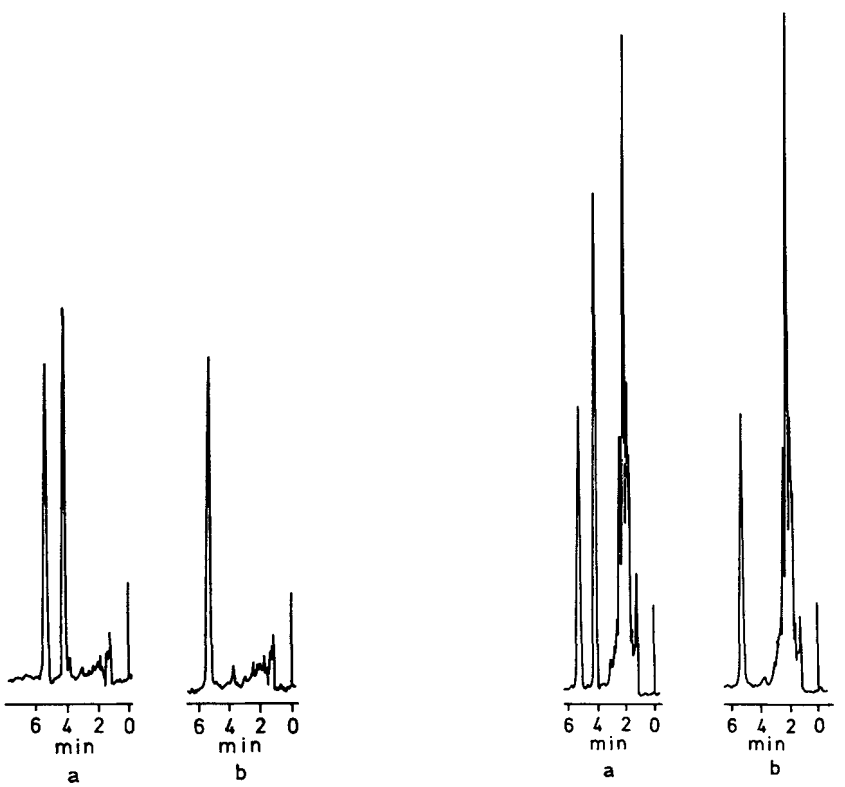


Fig. 1. (a) Chromatogram obtained on analysis of a plasma sample containing 10 μg/ml of indoprofen. The peaks at 4 min and at 5 min 15 sec correspond to indoprofen and the internal standard, respectively. (b) Chromatogram obtained on analysis of a blank plasma sample.

Fig. 2 (a) Chromatogram obtained on analysis of a urine sample containing 15 μg/ml of indoprofen. Indoprofen retention time was 4 min. Internal standard retention time was 5 min 15 sec. (b) Chromatogram obtained on analysis of a blank urine sample.

μg/ml of I were analysed according to the method described. The peak height ratios (I/II) were plotted against the concentration of I. The graph was linear and passed through the origin. The precision was determined to be 1.5% (n = 10) and 3.6% (n = 10) at the concentrations of 5 and 1 μg/ml of plasma, respectively. The reproducibility in the determination of total indoprofen in urine after enzyme hydrolysis of the conjugate was determined by repeated analysis of a sample from a patient (n = 10). This sample contained 35 μg/ml of I after hydrolysis (4 μg/ml before) and the variation was 1.9%. The absolute recovery of I from plasma and urine was 90% and 93%, respectively. The absolute recovery of I from urine, with pH 5 in the first extraction step, was 87%.

Since I is excreted to a large extent as conjugate in urine, the stability of the conjugate was investigated when exposed to the conditions of the method. To urine containing both I and the conjugate of I was added hydrochloric acid in an amount corresponding to the concentration used in the method. Samples of this urine were then analysed immediately and after 5, 10, 20 and 60 min. No significant change in the concentration of I could be detected.

Fig. 3 shows a mean plasma concentration curve of I obtained from three subjects that had been given 200 mg of indoprofen orally. The plasma samples drawn from the subjects were analysed using the described method. The plasma concentrations and $t_{½}$ values were in good agreement with earlier findings [3, 5].

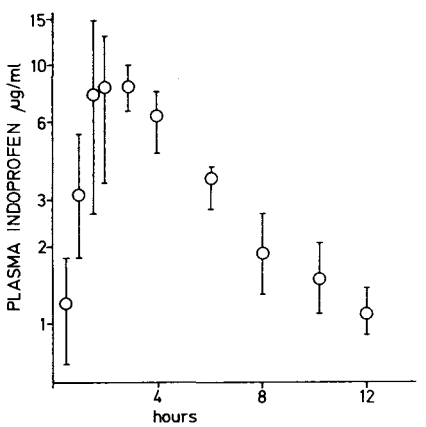


Fig. 3. Mean plasma time—concentration curve obtained from three subjects who had been given 200 mg of indoprofen orally.

ACKNOWLEDGEMENT

The skilful technical assistance of Mr. Jan Levin is acknowledged.

REFERENCES

1 G. Nannini, P.N. Giraldi, G. Molgora, G. Biasoli, F. Spinelli, W. Logemann, E. Dradi, G. Zanni, A. Buttinoni and R. Tommasini, Arzneim.-Forsch., 23 (1973) 1090.
2 A. Buttinoni, A. Currica, J. Franceschini, V. Mandelli, G. Orsini, N. Passerini, C. Turba and R. Tommasini, Arzneim.-Forsch., 23 (1973) 1100.
3 G.P. Tosolini, A. Forgione, E. Moro and V. Mandelli, J. Chromatogr., 92 (1974) 61.
4 G.P. Tosolini, E. Moro, A. Forgione, M. Ranghieri and V. Mandelli, J. Pharm. Sci., 63 (1974) 1072.
5 R.V. Smith, D.V. Humphrey and H. Escalona-Castillo, J. Pharm. Sci., 66 (1977) 132.

Journal of Chromatography, 182 (1980) 267—268
Biomedical Applications

CHROMBIO. 512

Book Review

Analysis of drugs and metabolites by gas chromatography—mass spectrometry, by B.J. Gudzinowicz and M.J. Gudzinowicz, Marcel Dekker, New York, *Vol. 4 (Central nervous system stimulants)*, 1978, XI + 458 pp., price SFr. 125.00, ISBN 0-8247-6614-8, *Vol. 5 (Analgesics, local anesthetics, and antibiotics)*, 1978, XI + 541 pp., price SFr. 140.00, ISBN 0-8247-6651-2, *Vol. 6 (Cardiovascular, antihypertensive, hypoglycemic, and thyroid-related agents)*, 1979, XI + 446 pp., price SFr. 106.00, ISBN 0-8247-6757-8.

The authors have made an attempt to survey the field of drug analysis by gas chromatography—mass spectrometry (GC—MS) and have produced a compilation of Homeric proportions. Like the Augean stables, these books would have benefitted from the tidying hand of a Hercules. Although Vols. 4—6 in this series contain a substantial amount of useful information, much of it is buried in a mire of less than useful information.

Vols. 4 and 5 lean too heavily on GC assays with little coverage of GC—MS. Worse yet, some of the detail on GC techniques is unacceptable for books advertised to be about GC—MS [e.g., the lengthy discussion of electrolytic conductivity detection (Vol. 4, pp. 99—100)]. Some material is totally unrelated to instrumental methods of analysis [e.g., a list of melting points of amine dinitrophenyl hydrazones (Vol. 4, p. 33)]. Vol. 6 comes much closer to matching content with series title and makes reference to papers of much more recent vintage than do Vols. 4 and 5.

Numerous analytical approaches are presented but not discussed. They appear as a loosely organized string of long abstracts. There is essentially no critical discussion of the assay methods (some of the GC work should have been excluded). The various chapters deal with pharmacological classes of drugs; however, there is no discernable orderly progression within each class. There are too many massive tables of marginally relevant information. Unimportant details are reported to excess while critical details are sometimes omitted. What can best be described as sloppy prose will offend some and confuse others.

Despite these criticisms, this series is basically a valuable one. The information in our review copies has helped us on a number of occasions. These volumes provide structural formulae for virtually all of the drugs and metabolites covered and present a reasonable amount of information on the metabolism and even pharmacokinetics of drugs. If the publisher would guide

the Gudzinowicz team with proper editing (restraint on content, evenness and relevance of detail, tightness and precision of language) the entire series would condense into a several volume set which could be the standard reference for workers in drug analysis and metabolism.

Merck Sharp & Dohme Research Laboratories, P.O. Box 2000, Rahway, N.J. 07065 (U.S.A.)

W.J.A. VANDENHEUVEL and J.S. ZWEIG

JOURNAL of ANALYTICAL and APPLIED PYROLYSIS

VOL. 1, NO. 1 JOURNAL OF ANALYTICAL AND APPLIED PYROLYSIS JUNE 1979

CONTENTS

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

7158

Two New Titles:

COMPLEX HYDRIDES AND RELATED REDUCING AGENTS IN ORGANIC SYNTHESIS

by **A. HAJÓS,** *Research Institute for Pharmaceutical Chemistry, Budapest, Hungary*

Studies in Organic Chemistry 1

This book provides an up-to-date, extensive survey of complex hydrides and related reducing agents now available for chemical reductions in organic synthesis. The text is laid out so as to make the selection of reducing agents as simple and coherent as possible by the inclusion of an exhaustive index. Moreover, at the beginning of each chapter the important properties of each reagent and the methods for its preparation are discussed.

CONTENTS: 1. Introduction. 2. Alkali metal and alkaline-earth metal hydrides. 3. Borane and its derivatives. 4. Aluminium hydride and its derivatives. 5. Metal borohydrides. 6. Metal aluminium hydrides. 7. Silanes. 8. Organotin hydrides. 9. Transition metal hydrides. 10. Analytical procedures for the determination of complex hydrides and related reducing agents. 11. Techniques of selective reductions with complex hydrides. 12. Mechanistic aspects of reductions with complex hydrides. Addenda. References. Author Index. Subject Index.

1979 400 pages US $76.00/Dfl. 156.00 ISBN: 0-444-99791-1

NEW TRENDS IN HETEROCYCLIC CHEMISTRY

R. B. MITRA, N. R. AYYANGAR, V. N. GOGTE, *National Chemical Laboratory, Poona, India,* **R. M. ACHESON,** *Queen's College, Oxford and* **N. CROMWELL,** *University of Nebraska, Lincoln, U.S.A.* (Editors)

Studies in Organic Chemistry 3

This book consists of 18 invited review papers prepared by 34 internationally respected authorities in heterocyclic chemistry. Based on the current research projects of the authors, topics covered include synthetic heterocyclic chemistry and its stereochemical, theoretical, physicochemical, and spectroscopic aspects. Nitrogen, phosphorus and sulphur heterocycles from small ring to complex polycyclic systems have been dealt with in the various reviews. Especially noteworthy is the fact that much attention has been paid to newer areas of heterocyclic chemistry. This overall survey of the present state of heterocyclic chemistry and indications for its future will be of wide interest to organic chemists and biochemists in universities and industry.

1979 xii + 407 pages US $75.50/Dfl. 155.00 ISBN: 0-444-41816-4

ELSEVIER

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

52 Vanderbilt Ave
New York, N.Y. 10017

7147

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

GENERAL INFORMATION

(A leaflet *Instructions to Authors* 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 reviews, see page 2 of cover under Submission of Papers.

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.

Title. The title of the paper should be concise and informative. Since titles are widely used in information retrieval systems, care should be taken to include the key words. The title should be followed by the authors' full names, academic or professional affiliations, and the address of the laboratory where the work was carried out. If the present address of an author is different from that mentioned, it should be given in a footnote. Acknowledgements of financial support are *not* to be made in a footnote to the title or name of the author, but should be included in the Acknowledgements at the end of the paper.

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. One original and two photocopies are required. Attention should be given to any lettering (which should be kept to a minimum) and to spacing on axes of graphs in order to ensure that numbers etc. remain legible after reduction. Axes of a graph should be clearly labelled. The figures should preferably be of such a size that the same degree of reduction can be applied to all of them. Photographs should have good contrast and intensity. Sharp, glossy photographs are required to obtain good halftones. References to the illustrations should be included in appropriate places in the text using arabic numerals. 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. The numbers should appear in the text at the appropriate places in square brackets. In the reference list, periodicals [1], books [2], multi-author books [3] and proceedings [4] should be cited in accordance with the following examples:

1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 H. C. S. Wood and R. Wrigglesworth, in S. Coffey (Editor), *Rodd's Chemistry of Carbon Compounds, Vol. IV, Heterocyclic Compounds*, Part B, Elsevier, Amsterdam, Oxford, New York, 2nd ed., 1977, Ch. 11, p. 201.
4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

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". The *Journal of Chromatography; Journal of Chromatography, Biomedical Applications* and *Chromatographic Reviews* should be cited as *J. Chromatogr.*

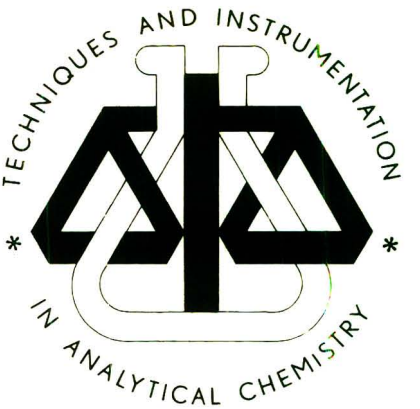
Proofs. One set of proofs will be sent to the author to be carefully checked for printer's errors. Corrections must be restricted to instances in which the proof is at variance with the manuscript. "Extra corrections" will be inserted at the author's expense.

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.

Evaluation and Optimization of Laboratory Methods and Analytical Procedures



A Survey of Statistical and Mathematical Techniques

D.L. MASSART, A. DIJKSTRA *and* L. KAUFMAN.

with contributions by S. Wold, B. Vandeginste *and* Y. Michotte

Techniques and Instrumentation in Analytical Chemistry - Volume 1

This book provides detailed treatment, in a single volume, of formal methods for optimization in analytical chemistry. It is a comprehensive and practical handbook which no analytical laboratory will want to be without.

All aspects of optimization are discussed, from the simple evaluation of procedures to the organization of laboratories or the selection of optimal complex analytical programmes. Quantitative discrete analysis as well as qualitative and continuous measurement techniques are evaluated.

The book consists of 30 chapters divided into 5 main parts.The main sections are: Evaluation of the Performance of Analytical Procedures, Experimental Optimization, Combinatorial Problems, Requirements for Analytical Procedures, and Systems Approach in Analytical Chemistry.

This work will be of practical value not only to those involved with optimization problems in analytical chemistry, but also to those in related fields such as clinical chemistry or specialized fields such as chromatography. Because it discusses the application of many mathematical techniques in analytical chemistry, this book will also serve as a general introduction to the new field of Chemometrics.

Oct. 1978 xvi + 596 pages US $57.75/Dfl. 130.00 ISBN 0-444-41743-5

ELSEVIER

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

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

52 Vanderbilt Ave
New York, N.Y. 10017

7113