ISSN 0378-4347

VOL. 278 NO. 2 DECEMBER 9, 1983

(Biomedical Applications, Vol. 29, No. 2)

THIS ISSUE COMPLETES VOL. 278

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

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JOURNAL OF CHROMATOGRAPHY

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0378-4347/83/$03.00

Printed in The Netherlands

VOL. 278, NO. 2 JOURNAL OF CHROMATOGRAPHY DECEMBER 9, 1983

(Biomedical Applications, Vol. 29, No. 2)

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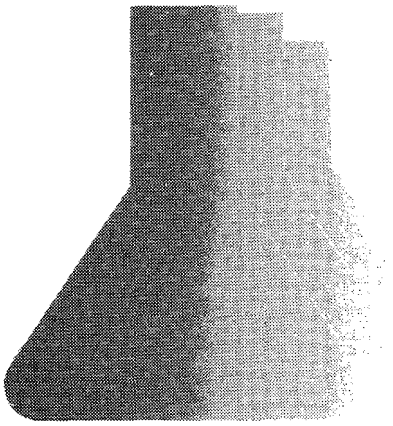
AN INTERNATIONAL SYMPOSIUM



17–19 April, 1984, Leeuwenhorst Congress Center, Noordwijkerhout, The Netherlands

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Royal Netherlands Chemical Society (KNCV), Section for Analytical Chemistry
Netherlands Biotechnology Society (NBV) under the sponsorship of the Federation of European Chemical Societies

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- Analytical methods currently available are now being adapted for practical use in biotechnological research, development and industrial production. But a large gap remains to be bridged between experts in analytical methodology and experts in biotechnology.
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Journal of Chromatography, 278 (1983) 231—244
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1879

CAPILLARY GAS CHROMATOGRAPHIC PROFILING OF TOTAL LONG-CHAIN FATTY ACIDS AND CHOLESTEROL IN BIOLOGICAL MATERIALS

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(First received May 30th, 1983; revised manuscript received July 29th, 1983)

SUMMARY

The profiling of total long-chain fatty acids and cholesterol in a variety of biological materials, using capillary gas chromatography with flame ionization detection, is described. The within-run precision and day-to-day precision for fifteen fatty acids and cholesterol in erythrocyte samples were investigated. Quantitative data on the analysis of amniotic fluid samples collected from women in the 30th to 38th week are given together with a correlation study on their lecithin/sphingomyelin and their palmitic acid/stearic acid ratios. In addition, the method was applied to lumbar cerebrospinal fluid, plasma, isolated leukemic blood cells and neuroblastoma tissue.

INTRODUCTION

Lipids serve both as metabolic fuel and membrane constituents of cells, and their determination as either the individual phospholipids, triglycerides, sterols,

0378-4347/83/$03.00

sterol esters and fatty acids is of importance to the understanding of many (patho)physiological processes.

Traditionally, determinations of free and total fatty acids have been performed by gas chromatography on polar stationary phases adsorbed to supporting materials (packed columns). Packed-column gas chromatography, however, is limited with respect to its separating power and in most cases requires the injection of a relatively large amount of the analyte, which is due to undesired adsorption properties. On the other hand, the development of capillary gas-chromatographic techniques using polar stationary phases has long been hampered by the lack of temperature resistance of such phases coated on the glass wall.

In this paper we describe a generally applicable method for the profiling of total long-chain fatty acids in biological materials using a CP-Sil-88-coated fused-silica capillary column. Using the same prepurified sample total cholesterol could be determined by its injection on an apolar stationary phase coated capillary gas chromatographic system.

MATERIALS AND METHODS

Standards and reagents

Certified cholesterol from the National Bureau of Standards, Washington, DC, U.S.A., was a generous gift from Dr. H.J.G.M. Derks of the R.I.V., Bilthoven, The Netherlands; 5β-cholestan-3α-ol (chola*) was from Steraloids, Wilton, NH, U.S.A., and tri-Sil-TBT from Pierce, Rockford, IL, U.S.A. 20:3c,ω9 was a generous gift from Dr. J. Kloeze, Unilever, Vlaardingen, The Netherlands. Other fatty acid standards were obtained from Applied Science, Oud Beyerland, The Netherlands, and Packard, Downers Grove, IL, U.S.A. All other reagents were from Merck, Darmstadt, F.R.G.

Samples

Blood samples (10 ml) were collected in EDTA-containing vacutainer tubes by venepuncture. Samples were immediately cooled in ice and centrifuged at 800 *g* for 10 min in a cooled centrifuge. Plasma was centrifuged again at 1400 *g* for 10 min. The buffy coat of the packed cells was removed as completely as possible and the red cells were washed three times with 5-ml portions of 0.15 mol/l sodium chloride solution adjusted to pH 7.4 by the addition of a saturated sodium hydrogen carbonate solution in water. After each washing the buffy coat was removed as completely as possible. The red cells were finally

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*Abbreviations used in the text, figures and tables: 14:0 = myristic acid; 15:0 = pentadecanoic acid; 16:0 = palmitic acid; 16:1c,ω7 = palmitoleic acid; 16:1tr,ω7 = palmitelaidic acid; 17:0 = margaric acid; 18:0 = stearic acid; 18:1c,ω7 = vaccenic (*cis*) acid; 18:1c,ω9 = oleic acid; 18:2c,ω6 = linoleic acid; 18:3c,ω3 = linolenic acid; 18:3c,ω6 = γ-linolenic acid; 20:0 = arachidic acid; 20:1c,ω9 = *cis*-11-eicosenoic acid; 20:2c,ω6 = *cis,cis*-11,14-eicosadienoic acid; 20:3c,ω6 = dihomo-γ-linolenic acid; 20:3c,ω9 = all *cis*-5,8,11-eicosatrienoic acid; 20:4c,ω6 = arachidonic acid; 22:0 = behenic acid; 22:5c,ω3 = all *cis*-7,10,13,16,19-docosapentaenoic acid; 22:6c,ω3 = all *cis*-4,7,10,13,16,19-docosahexaenoic acid; 23:0 = tricosanoic acid; 24:0 = lignoceric acid; 24:1c,ω9 = nervonic acid; chole = cholesterol; chola = 5β-cholestan-3α-ol; BHT = butylated hydroxytoluene (2,6-di-*tert.*-butyl-*p*-cresol).

resuspended to a haematocrit of about 50% and counted by means of a Coulter counter.

Amniotic fluid samples from patients in the 30th to 38th week were collected by amniocentesis for diagnostic purposes. After centrifuging for 10 min at 800 *g* the lecithin/sphingomyelin ratio was determined by thin-layer chromatography, and creatinine by means of an Automatic Clinical Analyzer (Dupont).

Lumbar cerebrospinal fluid samples were obtained from patients with a variety of neurological diseases, and centrifuged for 10 min, at 800 *g*.

Peripheral leukemic blood cells were isolated from patients in a leukemic phase by a Lymphoprep (Nijegaard, Oslo, Norway) density gradient. The cells were resuspended in isotonic phosphate-buffered saline pH 7.4 to a concentration of about 10^7 per 200 μl, and counted by means of a Coulter counter.

Neuroblastoma tissue was obtained from paediatric patients after (partial) surgical removal. In each case the diagnosis was confirmed by pathological anatomical examination. Tissue samples were either freeze-dried or frozen at —20°C before use.

TABLE I

RELATIONSHIP BETWEEN THE AMOUNT OF SAMPLE, ANTIOXIDANT (BHT), INTERNAL STANDARDS (17:0, 5β-CHOLESTAN-3α-OL) AND THE FINAL VOLUME FOR THE GAS CHROMATOGRAPHIC DETERMINATION OF TOTAL FATTY ACIDS AND CHOLESTEROL IN DIFFERENT KINDS OF BIOLOGICAL MATERIAL

Material	Amount (μl)	Additions			Final volume (μl)
		BHT (mg)	17:0 (μg)	Chola (μg)	
Cerebrospinal fluid	1000	0.2	2.5	5.0	20
Amniotic fluid*	1000	0.4	10.0	10.0	80
Plasma/serum	100	1.0	50.0	200.0	400
Erythrocytes**	200	1.0	50.0	100.0	400
Leucocytes***	200	0.4	10.0	10.0	80
Neuroblastoma§	1.5 mg	0.2	20.0	20.0	160

*Samples obtained between the 30th and 40th week of gestation.
**Suspension with a haematocrit of about 50% (ca. 10^9 cells per 200 μl).
***Suspension containing ± 10^7 cells per 200 μl.
§Dry tissue (ca. 10 mg wet tissue).

Transesterification of fatty acids and hydrolysis of cholesterol esters

Table I shows the amounts of biological material together with those of the internal standards and antioxidant used for the determination of the total fatty acid profile and cholesterol. Indicated amounts were pipetted or put into 15 ml Sovirel tubes using stock solutions of butylated hydroxytoluene (BHT; 10 g/l) margaric acid (17:0; 0.5 g/l) and 5β-cholestan-3α-ol (chola; 0.5 g/l) in methanol. The volumes of cerebrospinal fluid and amniotic fluid were reduced to at least 200 μl by evaporation at 40°C under a stream of nitrogen. Two milliliters of a methanol–hydrochloric acid solution, prepared by adding 50 ml

of methanol to 10 ml of a 6 mol/l hydrochloric acid solution in water, were added. The tubes were flushed with a stream of nitrogen, tightly capped and heated at 90°C for 4 h. After cooling the samples were extracted with 2 × 2 ml of hexane and the combined hexane layers evaporated to dryness at 40°C under a stream of nitrogen.

Trimethylsilylation

To the dry residues were added 150-μl aliquots of tri-Sil-TBT and the tubes heated at 80°C for 30 min in a heating block. After the addition of 4 ml of water, fatty acid methyl esters and trimethylsilylated sterols were extracted into 4 ml of hexane. The hexane layer was evaporated to dryness at 40°C under a stream of nitrogen and the residues redissolved in the indicated amounts of hexane (Table I).

Gas chromatography

Profiling of total fatty acids. Aliquots of 2 μl were automatically injected into a Hewlett-Packard Model 5880 gas chromatograph equipped with a Model 7672 A automatic injection system and a 25 m × 0.25 mm I.D. CP-Sil-88-coated (film thickness 0.25 μm) fused-silica column (Chrompack, Middelburg, The Netherlands). Helium gas flow-rate was 0.65 ml/min, split ratio 1:15, flame ionization detector temperature 300°C, and injector temperature 220°C. The oven temperature program was: 150°C, 1°C/min to 200°C, 10 min at 200°C.

Determination of total cholesterol. Amounts of 0.5—2.0 μl were automatically injected into the same gas chromatograph equipped with a 15 m × 0.20 mm I.D. cross-linked methylsilicone-coated (film thickness 0.11 μm) fused-silica column (Hewlett-Packard). Helium gas flow-rate was 0.39 ml/min, split ratio 1:15, flame ionization detector temperature 300°C, injector temperature 280°C, and oven temperature 265°C.

Identification and quantification

Fatty acid methyl esters were identified on the basis of their retention times using two different standard solutions containing even- and odd-carbon-numbered saturated fatty acids (14:0—24:0) and (mono- and poly)unsaturated fatty acids. 18:1c,ω7 and 22:5c,ω3 were identified on the basis of the erythrocyte membrane total phospholipid fatty acid profiles reported by Heckers et al. [1], who used a similar capillary gas-chromatographic system. Peak area ratios were calculated using a Tracor 812 analytical processing data system. Concentrations were calculated by the following equation:

$$\text{Concentration of fatty acid} = \frac{\text{peak area of fatty acid}}{\text{peak area of 17:0}} \times \frac{\text{amount of 17:0}}{\text{molecular weight of fatty acid}} \times \frac{1}{\text{sample size}}$$

in which the amount of 17:0 is given in g, and sample size may either have the dimension of l, g, mol of creatinine, or number of cells.

Cholesterol concentrations were calculated by comparing the peak area ratios of cholesterol and 5β-cholestan-3α-ol of samples with those of a standard containing equal amounts of both compounds.

RESULTS AND DISCUSSION

In Fig. 1 the time-dependent changes in the yields of methylated long-chain fatty acids (expressed as peak area ratio to the internal standard 17:0) and free cholesterol (expressed as peak area ratio to 5β-cholestan-3α-ol) are shown during heating in the methanol—hydrochloric acid solution at 90°C. Both erythrocytes and plasma were chosen as models to investigate the completeness of transesterification and cholesterol ester hydrolysis, as erythrocytes (composed of glycerophospholipids, sphingolipids and free cholesterol) and plasma (mainly composed of triglycerides and cholesterol esters) provide a natural source of variability in lipid class composition. From Fig. 1 it can be concluded that for both types of samples a steady state was reached after at least 3 h. For routine purposes a transesterification—hydrolysis time of 4 h was selected. On account of the presence of 16.7% of water in the methanol—hydrochloric acid mixture no dehydration or methylation of cholesterol, nor the formation of dimethylacetals from long-chain aldehydes, liberated from potentially present plasmalogens, was experienced.

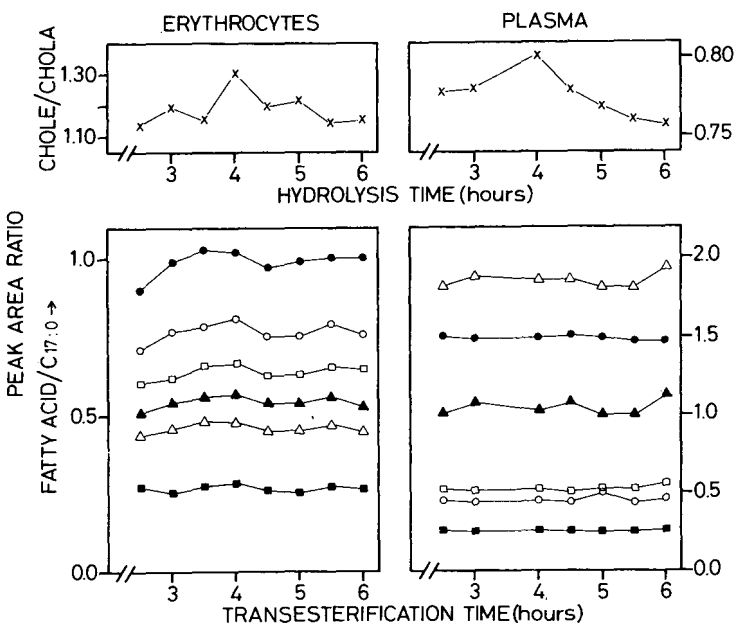

Fig. 1. Time-dependent changes in the yields of methylated fatty acids and free cholesterol for erythrocytes and plasma during heating in the methanol—hydrochloric acid solution at 90°C. Data are expressed as peak area ratios of the quantitatively most important analytes relative to their internal standards. (×), cholesterol; (•), 16:0; (○), 18:0; (□), 20:4c,ω6; (▲), 18:1c,ω9; (△), 18:2c,ω6; (■), 22:6c,ω3.

As split injection of samples containing mixtures of compounds characterized by a large range in boiling points may lead to day-to-day variation in the yields of the individual analytes on the column (split-dependent fractionation), this possible source of imprecision was studied by calculating the day-to-day coefficient of variation in peak areas obtained from the standard containing a mixture of equal amounts of even- and odd-carbon-numbered saturated fatty acid methyl esters (14:0–24:0). In Fig. 2 the carbon number

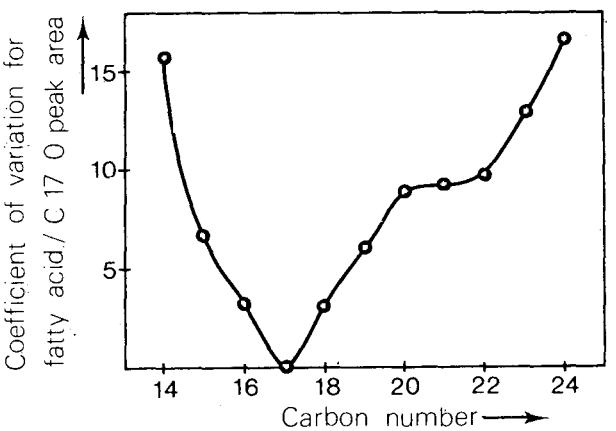

Fig. 2. Relationship between the carbon numbers of even and odd saturated fatty acid methyl esters and the day-to-day coefficient of variation of their respective gas chromatographic peak area ratios relative to 17:0. The coefficient of variation was calculated for fourteen determinations, equally divided over a period of nine months.

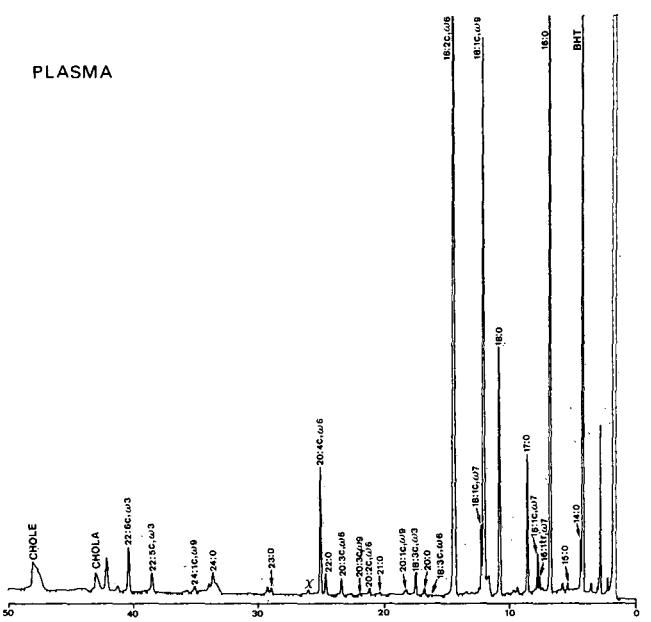

Fig. 3.

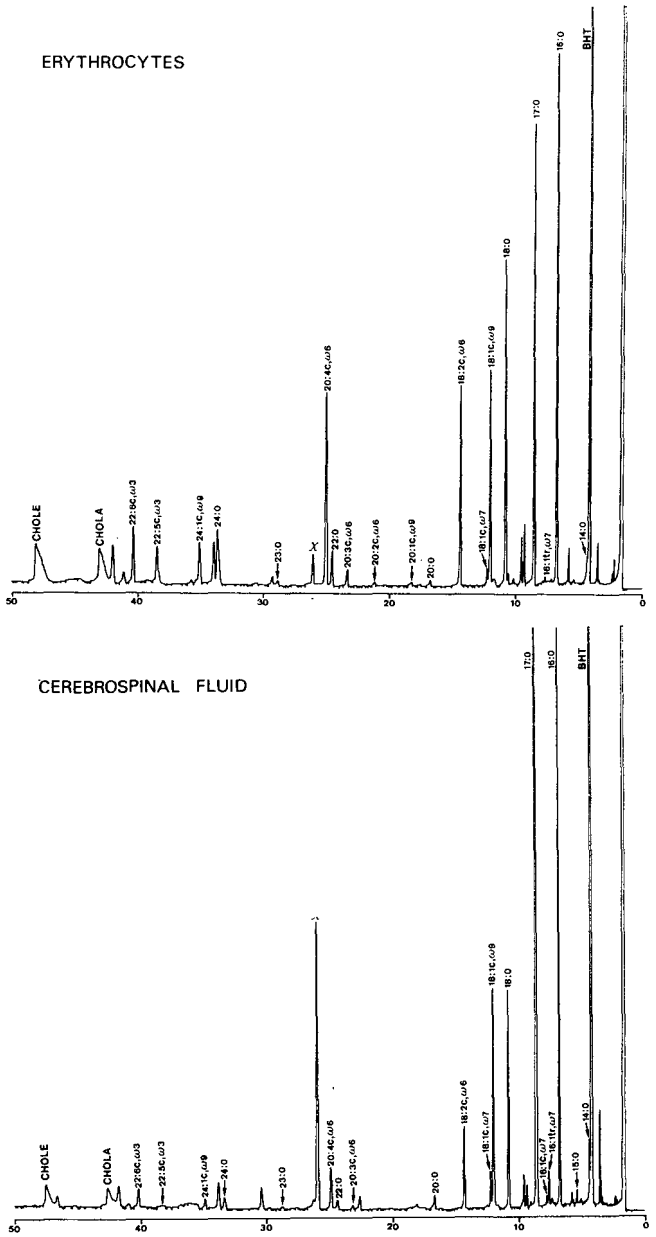

Fig. 3. Typical gas chromatograms of methylated total fatty acids from plasma, erythrocytes and cerebrospinal fluid prepared on a CP-Sil-88-coated fused-silica capillary column. In the case of plasma, 25 μg of 17:0 were added to 100 μl of sample, For erythrocytes and cerebrospinal fluid 50 and 2.5 μg of 17:0 were added to 200 μl (haematocrit ± 50%) and 1 ml, respectively. *X* = compound of unknown identity (possibly a phthalate) introduced during the clean-up procedure. Time axis in minutes.

dependent coefficients of variation of the respective peak area ratios relative to 17:0 are depicted. It can be concluded that, using 17:0 as an internal standard, notably fatty acid chain lengths smaller than 15:0 and longer than 22:0 suffer from the mentioned phenomenon. Uninvestigated improvements that may (partly) solve this source of imprecision include the use of on-column injectors or splitless injectors, and the additional employment of "low"- and "high"-boiling internal standards.

Fig. 3 shows typical gas chromatograms of methylated total fatty acids from plasma, erythrocytes and cerebrospinal fluid. The original set-up of our method, to measure simultaneously the total fatty acid profile and cholesterol in a single gas chromatographic run, could not be realized as the trimethylsilylated sterols showed poor gas chromatographic properties on the polar CP-Sil-88 stationary phase, giving rise to unacceptable quality control data. As notably polyunsaturated fatty acids are difficult to obtain in a highly purified form we decided to quantify all acids on the basis of the peak area of the internal standard 17:0, by assuming an equal ratio between the amount (expressed in g) and its peak area for each of the individual fatty acids. With respect to possible fractionation introduced by split injection, this approach

TABLE II

QUALITY CONTROL DATA ON THE DETERMINATION OF THE TOTAL FATTY ACID AND CHOLESTEROL COMPOSITION OF A FROZEN ERYTHROCYTE SUSPENSION

Compound	Within-run precision		Day-to-day precision	
	Mean* (nmol per 10^8 cells)	C.V. (%)	Mean** (nmol per 10^8 cells)	C.V. (%)
16:0	17.50	3.5	18.14	4.0
18:0	13.36	4.8	12.88	4.0
18:1c,ω9	8.36	3.0	8.40	1.1
18:1c,ω7	0.67	10.0	0.78	9.0
18:2c,ω6	9.01	1.9	8.93	3.7
20:0	0.32	7.8	0.32	11.0
20:2c,ω6	0.22	10.2	0.26	15.5
20:3c,ω6	1.16	6.5	1.03	10.3
20:4c,ω6	10.96	3.1	10.45	5.3
22:0	1.61	4.5	1.50	13.4
22:5c,ω3	1.78	4.6	1.70	8.0
22:6c,ω3	3.90	7.0	3.06	14.9
23:0	0.23	12.0	0.25	12.6
24:0	3.58	4.0	3.20	16.9
24:1c,ω9	2.35	2.9	2.23	17.6
Total fatty acids	74.94	2.3	73.13	2.1
Cholesterol	29.57	4.5	n.d.***	n.d.
Total fatty acids/ cholesterol	2.54	4.4	n.d.	n.d.

*Mean of twelve determinations.
**Mean of six determinations equally divided over a period of four months.
***n.d. = not determined.

leads to a slight underestimation of analytes with lower boiling points than 17:0 and a slight overestimation of analytes with higher boiling points.

In Table II some quality control data are presented for a 50% erythrocyte suspension prepared from a blood sample of a normal healthy adult. For the determination of the day-to-day precision, 200-μl amounts were stored at −20°C under nitrogen and subsequently analyzed over a period of four months. Due to the large quantitative differences between the individual fatty acids, relatively large coefficients of variation were calculated for the minor ones. Striking discrepancies were found between the within-run and the day-to-day coefficients of variation for 22:0, 24:0, and 24:1c,ω9. This was explained to be caused by the previously mentioned split-dependent fractionation and further by small, but significant, day-to-day changes in the relative retention times of these compounds, leading to incomplete separation from neighbouring peaks. During intensive use of the column over a period of six months a gradual decrease in its separating power and a subsequent worsening of the within-run quality control data for each of the individual fatty acids was experienced. The data shown in Table II should therefore only be considered as indicators of the quality control that can be achieved by the present method.

In Table III the total fatty acid composition of eight amniotic fluid samples obtained from women in the 34th and 35th week of gestation is shown. As the interindividual amniotic fluid volume is subject to large variation it was

TABLE III

MEAN TOTAL FATTY ACID COMPOSITION CALCULATED FOR EIGHT AMNIOTIC FLUID SAMPLES OBTAINED IN THE 34th (FOUR SPECIMENS) AND 35th (FOUR SPECIMENS) WEEK OF GESTATION

Fatty acid	Concentration				Relative amount*	
	μmol/l	C.V. (%)	mmol/mol creatinine	C.V. (%)	mol per 100 mol	C.V. (%)
15:0	1.53	21.6	18.9	38.8	1.37	80.8
16:0	82.80	69.0	907.9	61.1	46.69	25.3
16:1c,ω7	3.66	72.2	39.7	56.0	2.17	21.2
18:0	13.52	39.8	155.3	33.6	9.35	22.7
18:1c,ω9	23.04	57.8	246.9	30.8	14.96	22.9
18:1c,ω7	3.14	53.9	34.2	35.2	2.02	13.6
18:2c,ω6	13.30	51.8	146.0	26.5	9.30	34.6
20:0	0.71	10.2	8.8	32.9	0.66	78.0
20:3c,ω6	1.66	47.6	19.4	56.4	1.10	58.0
20:4c,ω6	10.27	51.5	116.9	48.9	6.44	20.8
22:0	1.57	16.1	19.0	30.0	1.37	72.4
22:6c,ω3	2.91	57.7	32.4	45.5	1.85	25.6
24:0	1.89	16.4	22.8	25.2	1.59	63.6
24:1c,ω9	1.17	30.2	14.5	43.4	1.14	87.2
Total	161.18	55.7	1782.7	44.6	100.01	—

*Trace amounts ($\leq$ 0.5 mol per 100 mol) of 23:0 were detectable in five samples, 22:5c,ω3 and 18:3c,ω6 in two samples, and 20:1c,ω9, 20:2c,ω6 and 21:0 in one sample. 14:0 and 16:1tr,ω7 were detectable in all samples, but not included in the calculations.

investigated whether expressing the fatty acid concentrations as a ratio to that of creatinine would lead to a somewhat more uniform picture. This was in some degree found to be the case, but except for the quantitatively minor acids the smallest coefficient of variation was calculated for the relative amounts.

Correlation studies using the data obtained from analyses of twelve amniotic fluid samples that were well defined with respect to gestational age, revealed high correlation coefficients between their lecithin/sphingomyelin ratios and the concentrations (in mmol/mol of creatinine) for the following fatty acids: 16:0 (correlation coefficient = 0.92), 22:6c,ω3 (0.90), 18:1c,ω9 (0.84), 20:4c,ω6 (0.81) and 18:0 (0.79). These data suggested that besides the 16:0/18:0 ratio other fatty acid/18:0 ratios might be of use as indicators of fetal lung maturity. In the left panel of Fig. 4, 16:0 and 18:0 concentrations are depicted as a function of gestational age, while the graphs shown in the right panel allow a visual comparison between the gestational age-dependent increases of the different fatty acid/18:0 ratios mentioned above. From this figure it is clear that during the last two months of gestation the 16:0/18:0 ratio undergoes the most notable increase.

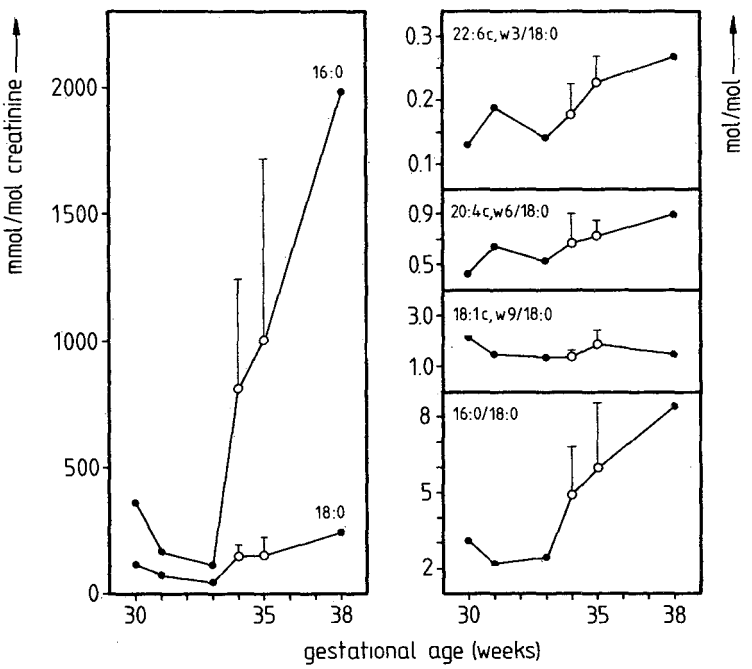

Fig. 4. Gestational age-dependent concentrations of 16:0 and 18:0 (left) and fatty acid/18:0 ratios (right) in twelve amniotic fluid samples. (○), mean ± 1 S.D. of three data.

In Fig. 5 the results of a correlation study between the lecithin/sphingomyelin ratio and the 16:0/18:0 ratio for 22 amniotic fluid samples are depicted. We found a linear relationship between the two ratios with a correlation coefficient of 0.908, which observation is somewhat different from that of Lavoinne et al. [2]. Apart from the differences in analysis time, quality control and degree of difficulty between the two methods, there is, in our opinion, no preference for one of them.

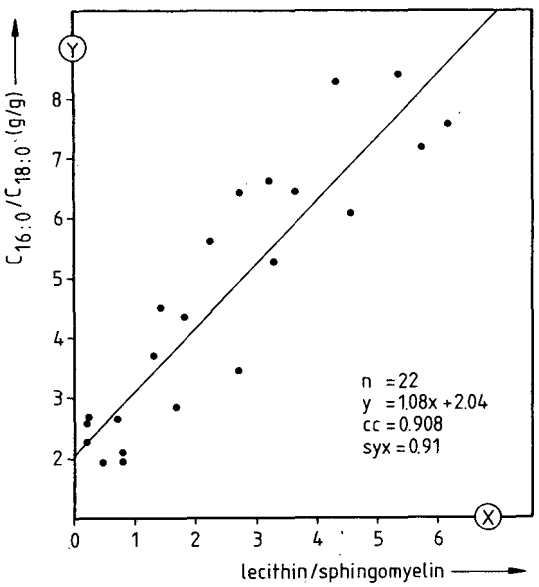

Fig. 5. Correlation between the lecithin/sphingomyelin ratio and the 16:0/18:0 (g/g) ratio for 22 amniotic fluid samples.

TABLE IV

MEAN TOTAL FATTY ACID COMPOSITION CALCULATED FOR SEVEN CEREBROSPINAL FLUID SAMPLES

Fatty acid	Concentration		Relative amount*	
	μmol/l	C.V. (%)	mol per 100 mol	C.V. (%)
15:0	0.67	9.5	2.26	24.4
16:0	8.50	15.6	27.95	15.5
18:0	4.36	18.9	14.22	11.9
18:1c,ω9	7.44	17.8	24.13	6.5
18:1c,ω7	1.01	17.2	3.30	12.8
18:2c,ω6	4.12	46.4	12.78	31.3
20:0	0.39	37.4	1.25	29.3
20:3c,ω6	0.24	8.5	0.79	19.6
20:4c,ω6	1.73	27.9	5.53	16.7
22:0	0.52	20.2	1.69	19.8
22:6c,ω3	0.93	31.2	2.01	25.6
24:0	0.44	34.8	1.39	22.1
24:1c,ω9	0.53	32.2	1.70	23.0
Total	30.86	18.4	100.00	—

*Trace amounts (≤ 0.7 mol per 100 mol) of 21:0 and 23:0 were detectable in three samples and 22:5c,ω3 in one sample. 14:0 and 16:1tr,ω7 were detectable in all samples, but not included in the calculations.

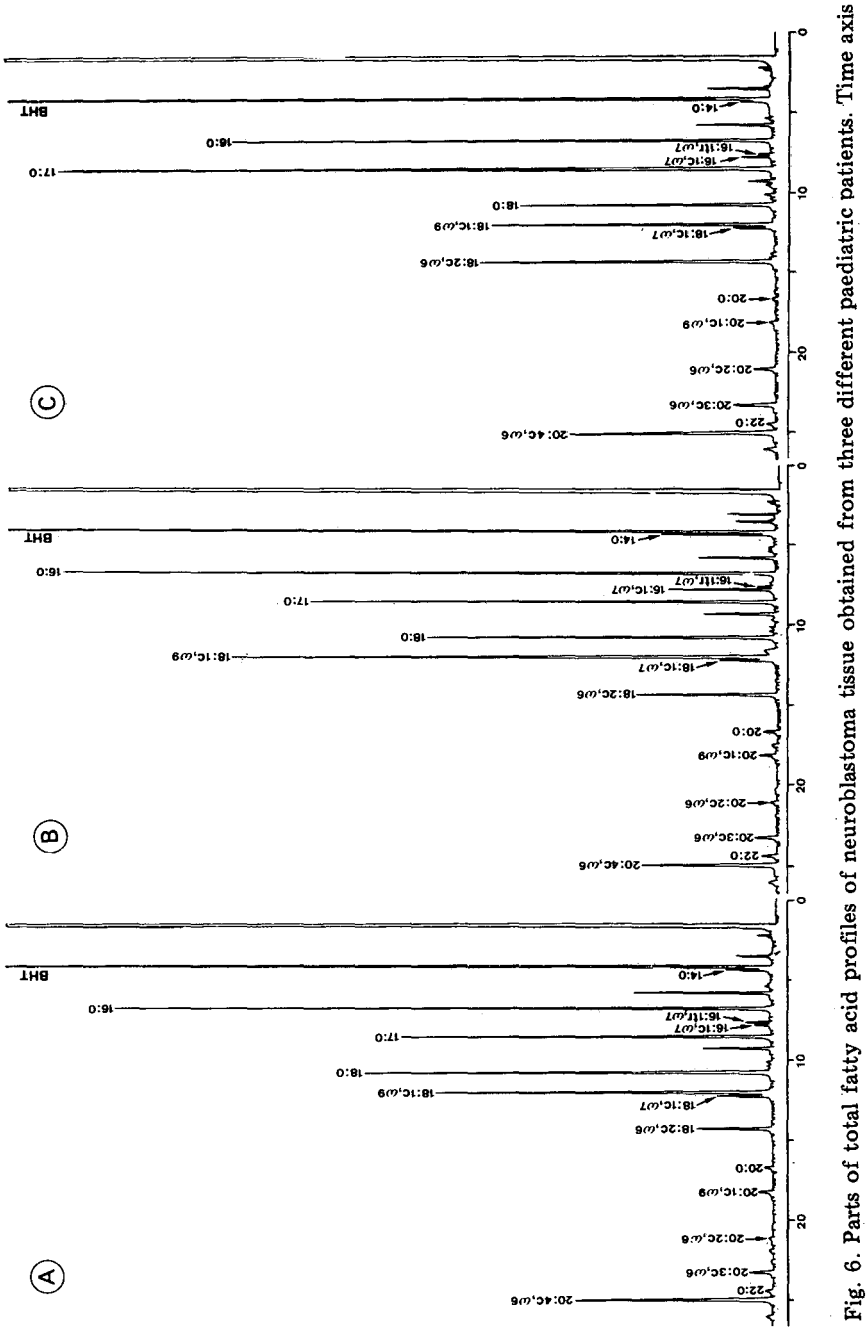

Fig. 6. Parts of total fatty acid profiles of neuroblastoma tissue obtained from three different paediatric patients. Time axis in minutes.

In Table IV the total fatty acid concentrations determined for seven cerebrospinal fluid samples of patients with various non-degenerative neurological diseases are presented. As is the case with amniotic fluid, the (patho)physiological significance of total fatty acid profiling of cerebrospinal fluid is poorly understood and has therefore not yet found its application in clinical diagnosis. The large interindividual variations in total fatty acid concentrations and their relative amounts found by others [3] and in the present study may have hampered further investigations in this field.

In Fig. 6 parts of strongly differing total fatty acid profiles prepared from neuroblastoma tissue of three different paediatric patients are shown. Recently Dawson and Golomb [4] reported fatty acid differences amongst isolated leukemia cell types, suggesting a possible application in terms of the diagnosis of analytical subtypes and the development of new therapeutic strategies. In addition, in vitro experiments revealed differences between normal and tumor cells in their preference to incorporate certain fatty acids [5], while changes in fatty acid metabolism have been observed after the induction of tumor cell differentiation [6, 7]. It is nowadays widely accepted that the phospholipid fatty acid composition influences the fluidity of the membrane bilayer, which in its turn influences, for example, membrane-associated enzymatic activity, receptor activity and transport mechanisms. Although we realize that statements on tissue samples which are poorly defined with respect to their homogeneity are at the least tentative, the observed variance in the fatty acid composition of the neuroblastoma samples may be explained by differences in fatty acid uptake and/or differences in fatty acid synthesis associated with pathologic-anatomically indistinguishable subtypes.

In conclusion, the present method has proven its usefulness for total long-chain fatty acid profiling and the determination of total cholesterol in a variety of biological materials. The results obtained from its application to a study of erythrocyte and plasma samples collected from normal healthy adults after an overnight fast will be presented in a separate paper [8].

ACKNOWLEDGEMENTS

The authors thank Dr. H.W.A. de Bruyn for providing the amniotic fluid samples and the determination of their lecithin/sphingomyelin ratios, Dr. A.W. Teelken for providing the cerebrospinal fluid samples, Dr. J.W. Smit for the isolation of leukemic blood cells, and Mr. G. Jansen for his skilful technical assistance.

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Journal of Chromatography, 278 (1983) 245—254
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1877

GAS CHROMATOGRAPHY—MASS SPECTROMETRY OF TRIMETHYLSILYL PTERIDINES*

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(Received July 29th, 1983)

SUMMARY

The trimethylsilyl derivatives of about 70 naturally occurring as well as synthetic pteridines have been investigated by glass capillary gas chromatography—mass spectrometry. On the apolar SE-52 phase used, the retention time of the compounds, tabulated in methylene units, was influenced more by the polarity than by the molecular weight. The electron-impact mass spectra of most compounds showed intense $M^{+\cdot}$ and M^{+}—15 ions and characteristic fragmentation patterns of 5,6,7,8-tetrahydropteridines, 7,8-dihydropteridines, sepia analogues and fully oxidized pteridines. The methylene units, together with the five most intense fragments tabulated, provide a good basis for the identification of these compounds in biological samples and for structure elucidation of unknown pteridines.

INTRODUCTION

Investigation of the butterfly wing pigments, at the beginning of this century, led to the identification of several compounds with the same heterocyclic ring structure, which is called pteridine (Fig. 1, A). Although the term pterin was used earlier as a collective name for the butterfly wing pigment, nowadays, according to IUPAC [1], it is used exclusively for the 2-amino-4-hydroxypteridine residue (Fig. 1, B). In the last few years, due to their important role as cofactors in various metabolic pathways, pterins have gained increasing interest in biochemistry and medicine [2—4] and therefore different methods of isolating and separating these compounds have been developed. Very promising quantitative results were reported especially with high-performance liquid chromatography [5, 6]. The inherent poor selectivity of

*Dedicated to Prof. H.-Ch. Curtius on the occasion of his 60th birthday.

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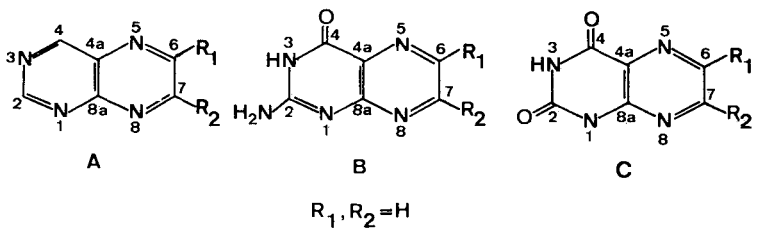

Fig. 1. Structural formulae of the investigated compounds: A = pteridine, B = 2-amino-4-hydroxypteridine (pterin), C = 2,4-dihydroxypteridine (lumazine).

chromatographic methods, however, prevents identification of unknown compounds in many cases. Mass spectrometry (MS) in combination with gas chromatography (GC) is well suited to solve such problems.

The first report on an MS analysis of acetylated and trimethylsilylated pteridines was by Kobayashi and Goto in 1970 [7], and Lloyd et al. [8] investigated the GC—MS behaviour of some pterins in 1971. Röthler and Karobath [9] presented in 1976 a mass-fragmentographic assay for biopterin and neopterin in human urine.

In the course of our work with pterins, we are often confronted with compounds which have not been known to occur in humans. In such cases, a library of reference compounds would be very helpful in structure elucidation and thus we present here the GC and MS data for 67 trimethylsilyl (TMS) derivatives. This data base has been used for pteridine analysis in various biological materials; an example can be found in ref. 10.

EXPERIMENTAL

The gas chromatograph used was a Fractovap 2900 (Carlo Erba, Milan, Italy) with a Grob-type split—splitless injector and a 20 m × 0.3 mm SE-52 glass capillary column (H. Jaeggi, Trogen, Switzerland). The injector temperature was 275°C; the carrier gas was helium at 1.2 bars. The temperature program was 3 min at 180°C to 270°C at a rate of 4°C per min. The GC—MS interface was an open split and direct coupling device with a fused-silica transfer line [11, 12]. The mass spectrometer was a VG-16F single-focusing magnetic field instrument. Electron-impact ionization with 30 eV at an ion source temperature of 200°C. Accelerating voltage was 4 kV and the scan range *m/z* 100—750 with a cycle time of 2 sec (scan time 1.4 sec). A Finnigan Incos 2000 data system was used.

Most reference compounds were generous gifts from Prof. W. Pfleiderer, University of Constance (F.R.G.), Prof. M. Viscontini, University of Zürich (Switzerland) and Dr. B. Schircks, Wettswil (Switzerland). Some 7,8-dihydropteridines were prepared by reduction of the fully oxidized compounds with dithionite and some 5,6,7,8-tetrahydropteridines by catalytic reduction [13].

Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was from Regis Chemical Co., acetonitrile from Fluka, Buchs, Switzerland.

For derivatization, a mixture, 200 μl, of acetonitrile—BSTFA (1:1, v/v) was added to 20—50 μg of the dry sample, sonicated for 30 sec and heated for 1 h at 100°C. For GC—MS, 1 μl was injected at an inlet split ratio of 1:10.

RESULTS AND DISCUSSION

Table I lists the investigated compounds which are either pteridine (A), pterin (B) or lumazine (C) derivatives by increasing methylene units together with the number of TMS groups, the molecular weights and the five most prominent ions in the mass spectra. Fig. 2 shows the total ion current chromatogram of a set of reference compounds together with the even-numbered straight chain alkanes (C_{18}—C_{28}) used for the determination of the methylene units.

Gas chromatography

From the results in Table I, the following general rules concerning the GC behaviour of TMS pteridines on SE-52 could be deduced:

(1) Lumazines have shorter retention times than pterins (Table I, Nos. 1/2, 3/15, 4/22, 14/25, 30/44, 48/60).

(2) 7-Substituted isomers elute faster than the corresponding 6-substituted isomers (see hydroxylumazines No. 3/4, xanthopterins No. 15/22 and pterin carboxylic acids No. 39/43), confirming the observation reported for 6- and 7-biopterins [9].

(3) The retention times are influenced more by polarity than by the molecular weight (MW) of the compound. Thus, leukopterin-$(TMS)_4$, MW 483 (No. 25) elutes faster than 2′-deoxysepiapterin-$(TMS)_2$, MW 365 (No. 27), since silylation of the two oxo groups in positions 6 and 7 via enolization lowers the polarity of the compound compared with No. 27 bearing a free polar imino group in position 8.

(4) Derivatives with an additional TMS group (e.g. on the exocyclic 2-amino group) elute slower than the parent compound. Here, the molecular-weight criterion gains in importance (see e.g. Nos. 2/11, 22/29 or 45/53 and 55). The increments for one TMS group, however, are not constant. Thus, in tetrahydro-6,7-dimethylpterin (Nos. 8 and 9), it is only 0.11 methylene unit whereas in xanthopterin (Nos. 22 and 29), it is found to be 1.17, reflecting the superimposed influence of the polarity upon the retention time.

(5) *erythro*-Biopterin elutes faster than *threo*-biopterin (No. 44/47), whereas opposite behaviour is found in neopterin (No. 56/60) probably due to reversed polarities.

(6) In the series of biopterin (Nos. 44, 37, 53) and neopterin (Nos. 60, 52, 62 and 56, 65), the 7,8-dihydro compounds have shorter retention times than the aromatic and the 5,6,7,8-tetrahydro species, which is another indication that the chromatographic behaviour results from a mixture of polarity and molecular-weight effects.

The described analyses have been performed over a period of about six months. Due to the ageing of the column, there can be some variation in the determination of the methylene units in that the retention times tend to become lower with time. An example of this is Fig. 2: the column has been in use for two years and the methylene units are about 0.3 lower than those in Table I.

TABLE I

METHYLENE UNITS (MU) AND ELECTRON-IMPACT (30 eV) MASS SPECTRAL DATA OF PTERIDINE TRIMETHYLSILYLETHER (TMS) DERIVATIVES

Ref No.	Type*	R_1	R_2	Compound name	No. of TMS	MU	MW	Base peak	Mass spectral data: *m/z* (relative intensity)
1	C	—H	—H	Lumazine	2	18.64	308	308	293(47),147(46),100(26),309(25)
2	B	—H	—H	Pterin	2	20.17	307	292	307(87),293(32),308(26),147(17)
3	C	—H	—OTMS	7-Hydroxylumazine	3	20.40	396	396	381(54),397(36),382(18),398(15)
4	C	—OTMS	—H	6-Hydroxylumazine	3	20.60	396	396	381(50),397(36),395(23),100(20)
5	A	—H	—H	Pteridine	2	20.77	306	291	306(78),219(31),234(22),292(14)
6	B	—CH_3	—H	6-Methylpterin	2	20.84	321	306	321(82),307(36),322(25),308(14)
7	C	—CH_3	—OTMS	6-Methyl-7-hydroxylumazine	3	20.86	410	410	395(98),411(34),396(33),397(17)
8	B	—CH_3	—CH_3	5,6,7,8-Tetrahydro-6,7-dimethylpterin	2	20.94	339	339	324(62),340(26),325(17),309(13)
9	B	—CH_3	—CH_3	5,6,7,8-Tetrahydro-6,7-dimethylpterin	3	21.05	411	411	396(90),412(36),397(32),413(16)
10	B	—CH_3	—H	5,6,7,8-Tetrahydro-6-methylpterin	2	21.06	325	325	310(50),326(27),327(10),294(9)
11	B	—H	—H	Pterin	3	21.28	379	364	379(54),365(31),147(22),292(19)
12	B	—CH_3	—H	5,6,7,8-Tetrahydro-6-methylpterin	3	21.29	397	325	397(77),382(67),310(62),326(30)
13	B	—CH_3	—CH_3	6,7-Dimethylpterin	2	21.60	335	320	335(81),321(31),336(23),322(10)
14	C	—OTMS	—OTMS	Leukolumazine	4	21.64	484	469	484(95),470 (44),395(36),485(33)
15	B	—H	—OTMS	Isoxanthopterin	3	21.69	395	380	395(67),381(49),396(43),382(32)
16	B	—H	—H	5,6,7,8-Tetrahydropterin	4	21.71	455	455	456(45),292(25),440(24),307(19)
17	B	—CH_3	—OTMS	6-Methylisoxanthopterin	3	21.71	409	394	409(35),395(34),405(14),393(13)
18	C	—OTMS	—H	7,8-Dihydro-6-hydroxylumazine	4	21.77	470	470	471(58),397(37),469(35),455(32)
19	B	—CH_3,—CH_3	—H	5,6,7,8-Tetrahydro-6,6-dimethylpterin	3	21.78	411	411	396(64),412(36),397(21),381(13)
20	B	—CHO	—H	6-Formylpterin	2	21.89	335	335	320(87),336(35),321(19),100(18)
21	B	—CH_3	—H	6-Methylpterin	3	21.93	393	378	393(75),379(47),394(30),380(20)
22	B	—OTMS	—H	Xanthopterin	3	22.15	395	380	935(83),381(61),396(42),382(30)
23	B	—H	—OTMS	Isoxanthopterin	4	22.52	467	452	380(52),467(45),453(36),395(28)
24	B	—CH_3	—OTMS	6-Methylisoxanthopterin	4	22.57	481	466	465(75),467(31),394(28),481(15)
25	B	—OTMS	—OTMS	Leukopterin	4	22.68	483	100	147(76),468(72),469(43),483(26)
26	B	—OTMS	—H	7,8-Dihydroxanthopterin	4	22.80	469	469	470(60),454(37),471(29),468(26)
27	B	—CH(OTMS)C_2H_5	—H	2′-Deoxysepiapterin	2	22.81	365	365	366(36),350(20),308(14),293(10)
28	C	—COOTMS	—H	Lumazine-6-carboxylic acid	3	23.00	424	424	409(53),425(38),410(21),426(12)
29	B	—OTMS	—H	Xanthopterin	4	23.32	467	452	467(86),453(45),468(32),454(22)
30	C	—CH(OTMS)CH(OTMS)CH_3	—H	*erythro*-Biolumazine	4	23.48	526	410	411(39),338(27),147(26),117(21)
31	B	—CH(OTMS)C_2H_5	—H	2′-Deoxybiopterin	3	23.56	437	408	437(63),409(46),422(43),438(20)
32	C	—COOTMS	—OTMS	7-Hydroxylumazine-6-carboxylic acid	4	23.69	512	512	497(47),513(32),498(31),395(20)
33	B	—CH_2OTMS	—H	6-Hydroxymethylpterin	3	23.70	409	394	409(96),395(44),410(44),396(22)
34	B	—OTMS	—NH_2	7-Aminoxanthopterin	4	23.79	482	467	482(56),100(55),466(48),468(44)
35	C	—CH(OTMS)C_2H_5	—H	7,8-Dehydro-2′-deoxysepialumazine	3	23.89	436	436	421(35),435(33),437(32),347(18)
36	B	—CH(OTMS)C_2H_5	—H	2′-Deoxysepiapterin	3	23.98	437	437	438(46),380(15),365(13),422(12)
37	B	—CH(OTMS)CH(OTMS)CH_3	—H	7,8-Dihydro-*erythro*-biopterin	4	24.09	527	410	527(49),411(30),437(29),528(23)

38	B	—C(OTMS)C(OTMS)CH_3	—H	Sepiapterin	3	24.48	453	453	336(58),452(57),309(24),438(21)
39	B	—H	—COOTMS	Pterin-7-carboxylic acid	3	24.52	423	408	423(98),409(41),424(40),147(11)
40	A	—H	—CH(OTMS)C_2H_5	2,4-Diamino-7-(1'-hydroxypropyl)pteridine	4	24.56	510	379	380(30),291(19),306(11),495(5)
41	B	—H	—CH(OTMS)C_2H_5	5,6,7,8-Tetrahydro-7-(1'-hydroxypropyl)pterin	4	24.62	513	513	512(72),382(56),514(48),498(25)
42	A	—H	—CH(NHTMS)C_2H_5	5,6,7,8-Tetrahydro-2,4-diamino-7-(1'-aminopropyl)pteridine	4	24.76	509	380	381(32),291(31),306(30),307(15)
43	B	—COOTMS	—CH_3	Pterin-6-carboxylic acid	3	24.88	423	423	408(99),409(55),424(54),425(30)
44	B	—CH(OTMS)CH(OTMS)CH_3	—H	L-*erythro*-Biopterin	4	24.88	525	409	410(46),101(35),408(34),117(25)
45	B	—CH(OTMS)CH(OTMS)CH_3	—H	(6*R*)-5,6,7,8-Tetrahydro-L-*erythro*-biopterin	3	24.90	457	238	457(27),237(10),239(7),442(6)
46	B	—CH(OTMS)CH(OTMS)CH_3	—H	7,8-Dihydro-L-*erythro*-biopterin	5	24.96	599	599	482(85),600(47),483(42),509(37)
47	B	—CH(OTMS)CH(OTMS)CH_3	—H	D-*threo*-Biopterin	4	24.98	525	409	410(37),408(25),337(21),117(18)
48	C	—CH(OTMS)CH(OTMS)CH_2OTMS	—H	D-*erythro*-Neolumazine	5	25.09	614	410	409(99),411(37),412(22),599(15)
49	B	—CH(OTMS)CH(OTMS)CH_3	—H	2-N,N-Dimethyl-L-erythro-biopterin	3	25.41	481	365	364(51),101(30),147(26),366(24)
50	B	—C(OTMS)C(OTMS)CH_3	—H	Sepiapterin	4	25.48	525	525	526(56),408(28),435(26),510(25)
51	B	—OTMS	—COOTMS	Xanthopterin-7-carboxylic acid	4	25.66	511	496	100(75),511(44),510(43),147(42)
52	B	—CH(OTMS)CH(OTMS)CH_2OTMS	—H	7,8-Dihydro-D-*erythro*-neopterin	5	26.72	615	410	337(69),409(61),615(52),411(44)
53	B	—CH(OTMS)CH(OTMS)CH_3	—H	(6*R*)-5,6,7,8-Tetrahydro-L-*erythro*-biopterin	4	25.76	529	310	529(55),117(33),530(32),311(29)
54	B	—CH(OTMS)CH(OTMS)CH_3	—H	(6*S*)-5,6,7,8-Tetrahydro-L-*erythro*-biopterin	3	25.83	457	238	457(31),222(16),239(16),117(15)
55	B	—CH(OTMS)CH(OTMS)CH_3	—H	(6*R*)-5,6,7,8-Tetrahydro-L-*erythro*-biopterin	5	26.04	601	382	601(59),600(49),409(32),602(31)
56	B	—CH(OTMS)CH(OTMS)CH_2OTMS	—H	L-*threo*-Neopterin(monapterin)	5	26.37	613	613	598(81),409(68),614(60),599(52)
57	B	—CH(OAc)CH(OAc)CH_3	—H	1',2'-Diacetyl-L-*erythro*-biopterin	2	26.43	465	465	336(86),363(85),450(60),405(54)
58	B	—C(OTMS)$CHCH_2$OTMS	—H	2'-Deoxy-3'-hydroxysepiapterin	4	26.52	525	525	435(40),526(34),527(21),510(16)
59	B	—CH(OTMS)CH(OTMS)CH_2OTMS	—H	7,8-Dihydro-*erythro*-neopterin	6	26.53	687	687	688(56),494(50),689(36),482(27)
60	B	—CH(OTMS)CH(OTMS)CH_2OTMS	—H	D-*erythro*-Neopterin	5	26.58	613	409	408(50),410(37),613(17),598(13)
61	B	—CH(OTMS)CH(OTMS)CH_3	—H	(6*S*)-5,6,7,8-Tetrahydro-L-*erythro*-biopterin	4	26.77	529	310	529(59),311(27),530(25),238(20)
62	B	—CH(OTMS)CH(OTMS)CH_2OTMS	—H	(6*R*)-5,6,7,8-Tetrahydro-D-*erythro*-neopterin	5	26.87	617	310	617(93),618(37),619(28),311(26)
63	B	—C(OTMS)C(OTMS)CH_2OTMS	—H	3'-Hydroxysepiapterin	5	27.06	613	613	614(78),615(47),598(22),616(20)
64	B	—CH(OTMS)CH(OTMS)CH_2OTMS	—H	(6*R*)-5,6,7,8-Tetrahydro-D-*erythro*-neopterin	6	27.28	689	689	382(87),690(80),688(74),691(41)
65	B	—CH(OTMS)CH(OTMS)CH_2OTMS	—H	(6*R*)-5,6,7,8-Tetrahydro-L-*threo*-neopterin	5	27.54	617	310	617(69),618(33),619(25),311(22)
66	B	—CH(OTMS)CH(OTMS)CH_2OTMS	—H	(6*S*)-5,6,7,8-Tetrahydro-D-*erythro*-neopterin	5	27.91	617	310	617(71),618(37),311(25),238(16)
67	B	—C(OTMS)$CHCH_2$OTMS	—H	2'-Deoxy-3'-hydroxysepiapterin	5	28.16	597	507	597(81),435(72),508(47),436(41)

*A = pteridine derivative, B = pterin derivative, and C = lumazine derivative (see Fig. 1 for formulae).

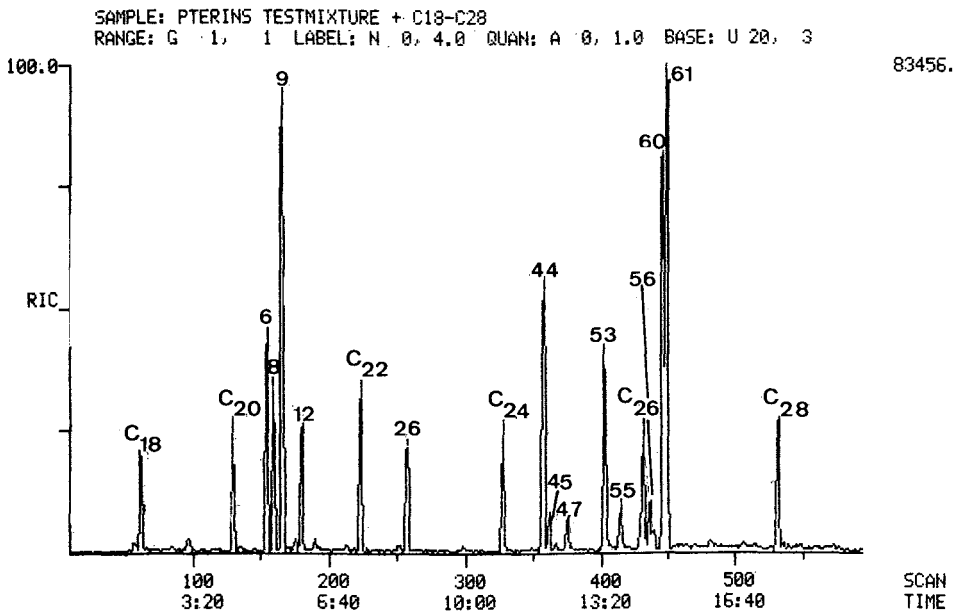

Fig. 2. Total ion current chromatogram of a pterin test mixture (different amounts of each compound) together with the even-numbered straight-chain alkanes C_{18}—C_{28} (10 ng each on the column). For peak identification see Table I.

Mass spectrometry

The mass spectra of hydroxylated and/or methylated and carboxylated pterins and lumazines are rather simple and need no further explanations. As often reported, the spectra of positional isomers differ in the relative intensities only (e.g. Table I, Nos. 3/4, 15/22, 23/29 and 39/43). In the lumazine series, $M^{\cdot+}$ forms the base peak (an exception is leukolumazine, No. 14), whereas the pterin derivatives often show the $M^{\cdot+}-15$ ion as the most intensive peak. When the pyrazine ring is partially or fully hydrated, the tendency for the molecular ion to become the base peak increases. Because of the particular biological interest in pteridines substituted in position 6 by a hydroxylated carbon chain, the fragmentation of such derivatives is discussed in more detail.

Fig. 3 shows the electron-impact mass spectra of *erythro*-neopterin (No. 60), 7,8-dihydro-*erythro*-neopterin (No. 52) and (6*R*)-5,6,7,8-tetrahydro-*erythro*-neopterin (No. 62), and the *m/z* (relative intensity) values of the five most prominent ions for the investigated compounds are given in Table I.

A parallel fragmentation pattern is found in the aromatic biopterins, biolumazines, neopterins and neolumazines (Nos. 30, 44, 47, 48, 49, 56 and 60) which all have a vicinal 1′,2′-glycol residue R_1 in position 6. The dominating fragmentation yielding the base peak involves this substituent and is accompanied by a hydrogen rearrangement. Pictorially, this reaction may be formulated by a six-membered transition state as outlined in Fig. 4 for neopterin. The primary ionization involves the double bond in position 6,7. The prerequisite is the aromatic ring system and the availability of an —OR ether function in position 2′. So 2′-deoxybiopterin (No. 31) does not show

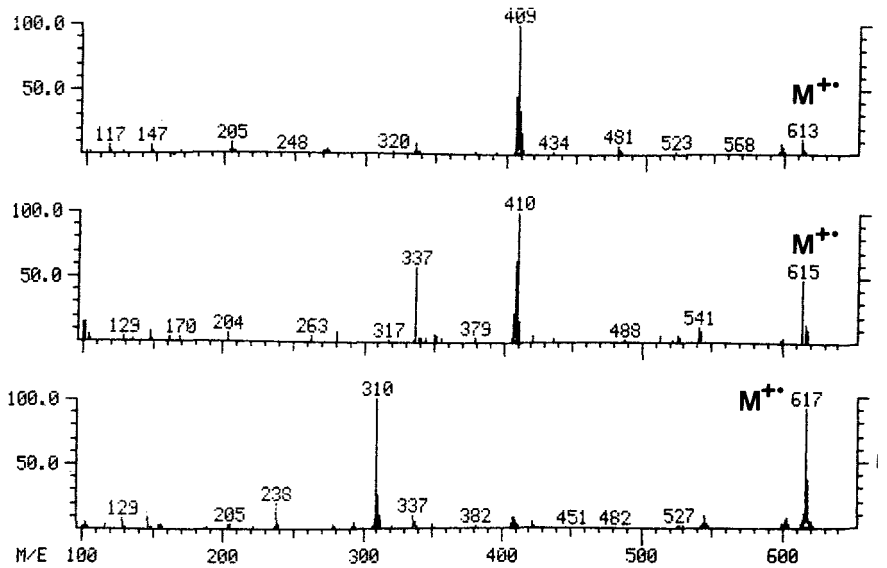

Fig. 3. Electron-impact (30 eV) mass spectra of D-*erythro*-neopterin (above), 7,8-dihydro-D-*erythro*-neopterin (centre) and (6*R*)-5,6,7,8-tetrahydro-D-*erythro*-neopterin (below).

an ion at *m/z* 409, but has its base peak at *m/z* 408 which arises by simple 1′—2′ bond cleavage. 1′,2′-Diacetyl-L-*erythro*-biopterin (No. 57) shows how sensitive the mass spectrum can be towards changes in functional groups. Dominating reactions here are acetic acid elimination (*m/z* 465→405) followed by ketene elimination yielding *m/z* 363. The rearrangement mentioned in Fig. 4 plays a minor role only: *m/z* 379, equivalent to *m/z* 409 in biopterin, has a relative intensity of 6% but the acetyl elimination (43 mass units) following yields one of the most prominent ions at *m/z* 336.

In the 7,8-dihydro compounds Nos. 37, 46, 52 and 59, the hydrogen rearrangement of Fig. 4 is suppressed. Instead, pushed by the 5,6-double bond, intense ions arise by cleavage of the 1′,2′-bond to yield *m/z* 410 and 482, respectively (Fig. 5). The same cleavage process also occurs in the 7,8-dihydro compound sepiapterin (Nos. 38 and 50) yielding the ions at *m/z* 336 and 408,

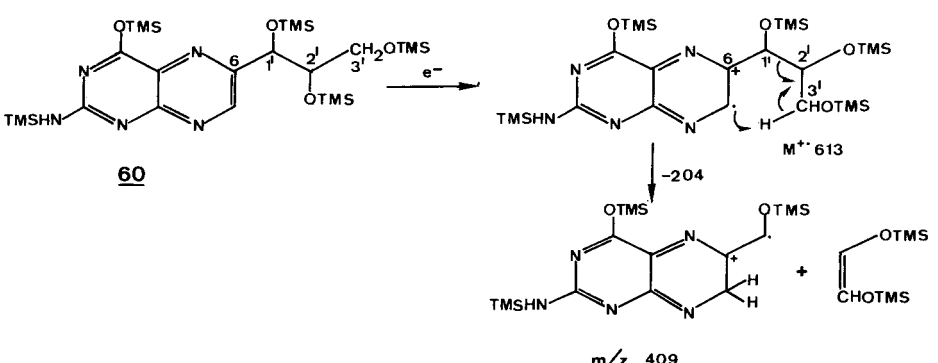

Fig. 4. Mass spectral fragmentation of neopterin yielding the base peak at *m/z* 409. Erroneously, 60 is shown in L-*threo* configuration; OTMS at C-2′ should be drawn upwards.

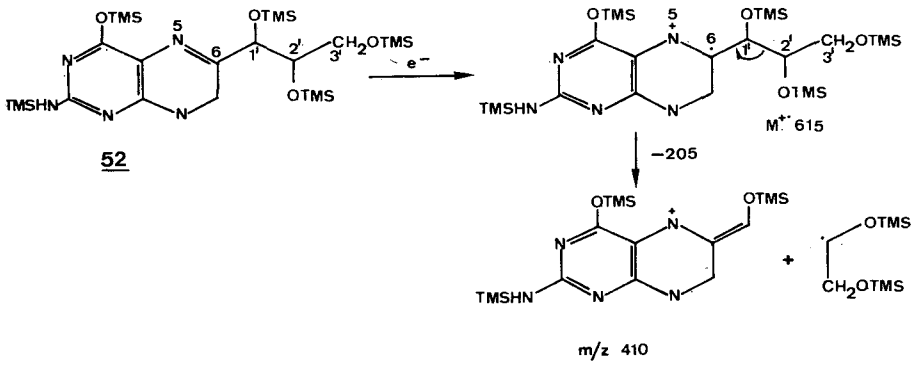

Fig. 5. Mass spectral fragmentation of 7,8-dihydroneopterin yielding the base peak at *m/z* 410. Erroneously, 52 is shown in L-*threo* configuration; OTMS at C-2′ should be drawn upwards.

respectively. The dominating fragmentation in 2′-deoxy-3′-hydroxysepiapterin (Nos. 58 and 67) is trimethylsilanol elimination ($M^{\ddagger}$—90) to yield *m/z* 435 and 507, a process which cannot be observed in the other compounds and thus might be an indication for an isolated primary alcohol function.

In the 5,6,7,8-tetrahydro compounds (Nos. 45, 53, 54, 55, 61, 62, 64, 65 and 66), due to the lack of a second double bond in ring B, the ionization can be formulated to occur primarily at the N-5 followed by the 6,1′-α-cleavage yielding very intense ions at *m/z* 238, 310 and 382 (Fig. 6).

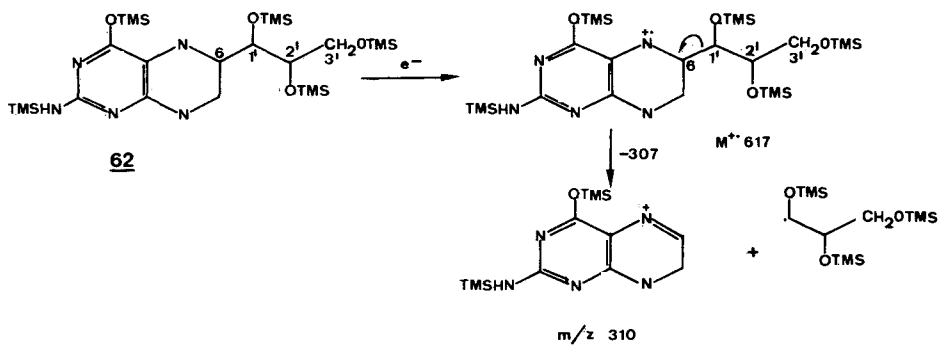

Fig. 6. Mass spectral fragmentation of (6*R*)-5,6,7,8-tetrahydroneopterin yielding the base peak at *m/z* 310. Erroneously, 62 is shown in L-*threo* configuration; OTMS at C-2′ should be drawn upwards.

It must be added that, due to the poor and non-specific fragmentation, the mass spectra of the TMS derivatives alone are not very suitable for structure elucidation. Often there remain several possibilities of interpretation and the position of the substitution cannot be determined. In combination with the GC methylene units, however, the number of possible structures can be reduced in many cases to a few or even to one only. Table II is an attempt to generalize the data from Table I, in order to have guidelines for the interpretation of unknown pteridines.

TABLE II

GUIDELINES FOR INTERPRETATION OF PTERIDINE MASS SPECTRA

Finding	Possible interpretation
Methylene unit $\leqslant 23$	Simple substituted pteridine
Methylene unit $\geqslant 23$	More complex substituted pteridine
$M^{\ddagger}$:	
Odd-numbered	Pterin
Even-numbered	Lumazine
Low intensity	Aromatic skeleton
Medium intensity	7,8-Dihydro compound
High intensity	5,6,7,8-Tetrahydro compound
Same of two compounds	Compound with lower retention time is the 7-substituted isomer
Intense ions at m/z:	
238, 310 or 382	5,6,7,8-Tetrahydropterin structure
239, 311 or 383	5,6,7,8-Tetrahydrolumazine structure
409	Aromatic pterin with vicinal glycol moiety in position 6 or 7
410	Aromatic lumazine with vicinal glycol moiety in position 6 or 7; or 7,8-dihydropterin with a 6-(1′-hydroxy) or 6-(1′-oxo) group
$M^{\ddagger}$—90	Isolated hydroxyl group in side-chain
Similar fragmentation, MW difference 72 methylene units	Same compound with an additional TMS group

ACKNOWLEDGEMENTS

We wish to express our thanks to Prof. W. Pfleiderer, Prof. M. Viscontini and Dr. B. Schircks for their generous gifts of reference compounds. We thank Dr. W.L.F. Armarego, Australian National University, Canberra, Australia, for a gift of 6,6-dimethyl-5,6,7,8-tetrahydropterin and we are grateful to Mrs. Ana Matasović for her skilful technical assistance. This work was supported by the Swiss National Science Foundation, project No. 3.266-0.82.

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Journal of Chromatography, 278 (1983) 255—263
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1850

DETERMINATION OF CATECHOLAMINES IN URINE BY ION-EXCHANGE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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(First received April 1st, 1983; revised manuscript received July 15th, 1983)

SUMMARY

A liquid chromatographic method for the determination of free urinary concentrations of epinephrine, norepinephrine and dopamine is presented. For urine samples, pre-purified by adsorption onto alumina, ion-exchange chromatography was, in terms of selectivity, found to be superior to the more widely used reversed-phase chromatography. The column eluates were monitored with an electrochemical detector utilizing a glassy carbon working electrode. The method allows determination of the concentrations in 0.5 ml of normal urine samples with a relative standard deviation below 2%.

INTRODUCTION

Simplified analytical methods for the determination of free urinary catecholamine concentrations are of great interest, and during the last decade continuous efforts have been made to improve these techniques. Liquid chromatographic methods with on-line fluorometric [1—5] or electrochemical detection [6—8] have been developed to achieve better sensitivity and selectivity than given by the previously used trihydroxyindole procedure. Urine is a complex matrix which contains many electroactive and fluorescent substances making pre-purification mandatory. In post-column trihydroxyindole methods the fluorescence of dopamine is weak [2, 4, 5]. In the case of electrochemical detection a single purification step has proved to be insufficient [6—8]. Extensive purification by adsorption onto alumina combined with cation-exchange resin [6], boric acid gel [7] or Sephadex [8] have been used. An improvement was obtained when the catecholamines were extracted using complex formation in alkaline medium between diphenylborate and the catechol group [9]. A method for direct injection of urine using pre-column sample enrichment in a micro liquid chromatographic system has

0378-4347/83/$03.00

been presented [10] and recently a paper reported that purification by alumina adsorption was sufficient when using a sodium gradient for the separation [11].

The pre-purification needed depends on the selectivity of the liquid chromatographic system used for analysis. Reversed-phase C_{18} materials have frequently been used in chromatography of catecholamines. However, for the analysis of urinary catecholamines, microparticulate strong cation-exchange columns appear to be favourable as they do not retain interfering components as is the case for the reversed-phase columns. The sample preparation could then be a simple solvent extraction and adsorption onto alumina to eliminate non-catechols and to concentrate the sample.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of an LDC mini-pump 711-47 (Riviera Beach, FL, U.S.A.) with a Touzart-Matignon pulse dampener (Vitry-sur-Seine, France), an injection valve (Rheodyne 7125, Berkeley, CA, U.S.A.) with a 60-μl loop, a stainless-steel separation column (150 × 4.5 mm I.D.) and an electrochemical detector [Bioanalytical Systems (BAS) LC 4, West Lafayette, IN, U.S.A.]. The detector was operated at +0.7 V with an Ag/AgCl reference electrode BAS RE 1 and a thin-layer cell BAS TL 5A consisting of a glassy carbon working electrode. A Cenco rotary mixer for 56 tubes (Breda, The Netherlands) was used to rotate the tubes.

Chemicals

Epinephrine (E) and norepinephrine (NE) (hydrogen tartrate form) were obtained from Société des Usines Chimiques (Paris, France) and Österreichische Stickstoffwerke (Linz, Austria), respectively. Dopamine (DA) hydrochloride and reduced glutathione (GSH) were from Sigma (St. Louis, MO, U.S.A.). α-Methyldopamine (MDA) hydrochloride was obtained from Merck Sharp and Dohme (Rahway, NJ, U.S.A.) and tris(hydroxymethyl)aminomethane (Tris), analytical grade, was of Fluka quality (Buchs, Switzerland). Alumina, Woelm neutral, was from Woelm Pharma (Eschwege, F.R.G.) and was prepared according to the method given in ref. 12. 3,5-Dimethylcyclohexyl sulphate (DMCHS) was supplied by the Department of Organic Chemistry, AB Hässle (Mölndal, Sweden). Tetrahydrofuran (THF) and hexane, both of HPLC grade, were purchased from Rathburn Chemicals (Walkerburn, U.K.). Disodium EDTA, methanol, ethyl acetate, sodium hydroxide, acids and buffer substances were all of analytical grade from E. Merck (Darmstadt, F.R.G.).

Analytical procedure

Fresh urine samples were adjusted to pH 2 with hydrochloric acid (5 mol/l) before freezing. Frozen samples were thawed, homogenized by shaking and centrifuged. Urine to which the internal standard MDA (4 μmol/l urine) had been added was mixed for a few seconds with two volumes of ethyl acetate followed by one volume of hexane, the organic solvents being aspirated. Aqueous phase corresponding to 0.5 ml of urine was transferred into a 4-ml

conical centrifuge tube; 50 µl of GSH (0.05 mol/l), 50 µl of EDTA (0.3 mol/l, pH 7) and 20 mg of alumina were added. Then 300 µl of Tris buffer (1 mol/l, pH 8.6), or the volume giving a pH of 8.5, were added while vortexing the tube, which was then rotated in a rotary mixer for 10—20 min. The aqueous phase was discarded and the alumina was washed three times by mixing for a few seconds with an EDTA solution (3 mmol/l, pH 7). The tube was centrifuged, whereupon the amines were eluted from the alumina by vortexing for 1 min with 250 µl of perchloric acid (0.2 mol/l). The tube was stored frozen (—20°C) and thawed just before injection of 50 µl onto the column. Each series of analyses also included reference samples of 40 pmol of the catecholamines and 2 nmol of the internal standard.

Chromatographic system

The separation column was packed with Nucleosil SA (strong cation-exchange), 5-µm average particle size, from Macherey-Nagel (Düren, F.R.G.). The packing was performed upwards at 450 bar with methanol as slurry medium and eluent (see ref. 13). The mobile phase was a citrate buffer (I = 0.15, pH 5.0) containing 7% THF. The composition of the buffer was so-

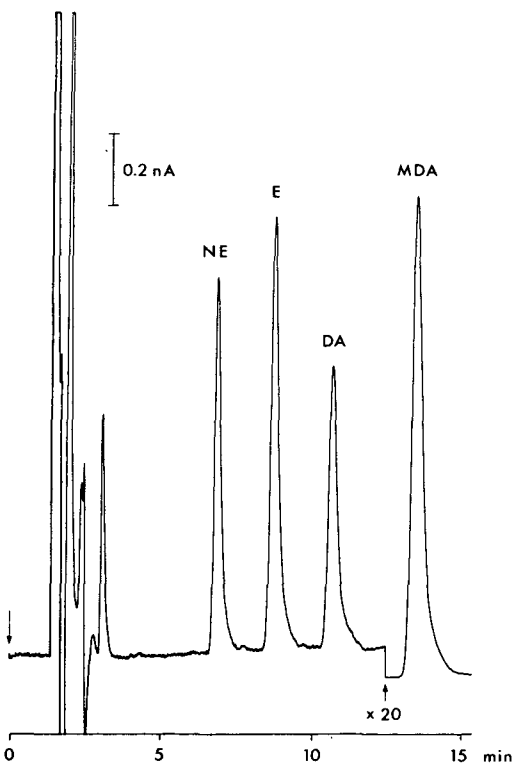

Fig. 1. Chromatogram of a reference sample, worked up according to the analytical procedure, containing 40 pmol each of norepinephrine (NE), epinephrine (E) and dopamine (DA), and 2 nmol of α-methyldopamine (MDA). A 50-µl sample was injected. Stationary phase: Nucleosil 5 SA, 5 µm. Mobile phase: citrate buffer (I = 0.15, pH 5.0) containing 7% THF. Potential: +0.7 V.

dium hydroxide (98.4 mmol/l) and citric acid (52.2 mmol/l). Deionized water, which had been passed through a Milli-Q reagent-grade water system (Millipore, Bedford, MA, U.S.A.), was used. The mobile phase was degassed by vacuum filtering through a 0.45-μm MF-Millipore filter. The flow-rate was 1.0 ml/min. The detector cell was kept in a Faraday cage to avoid disturbances.

Quantitative evaluation

The ratio between the peak height of the catecholamine and the internal standard for the sample was compared with the median value of the corresponding ratios for the reference samples.

RESULTS AND DISCUSSION

Chromatography

Ion-exchange chromatography was used for the separation of the catecholamines in urine, the mobile phase being citrate buffer with THF as organic modifier. Chromatograms from injections of a reference sample and a urine sample, worked up according to the analytical procedure, are shown in Figs. 1 and 2 respectively. If reversed-phase chromatography was used the isolation by adsorption onto alumina was insufficient. This can be seen in Fig. 3, which shows a chromatogram for the same sample as in Fig. 2.

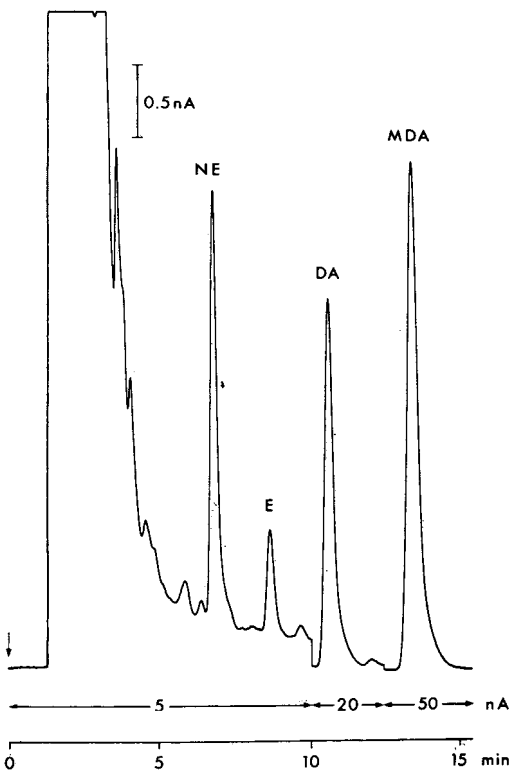

Fig. 2. Chromatogram from 0.5 ml of urine, containing 103 pmol of norepinephrine (NE), 25 pmol of epinephrine (E) and 488 pmol of dopamine (DA). The chromatographic conditions were the same as in Fig. 1.

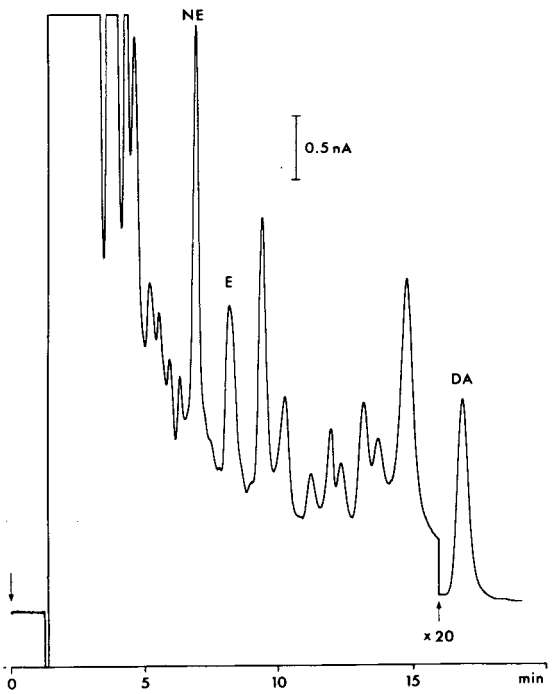

Fig. 3. Chromatogram of the same sample as in Fig. 2, injected onto a reversed-phase system. A 50-μl sample was injected. Stationary phase: LiChrosorb RP-18, 5 μm. Mobile phase: citrate buffer (I = 0.1, pH 5.0) containing 2% THF and DMCHS (4.2 nmol/l). Potential: +0.7 V.

The ion-exchange columns used have shown both efficiency and long-term stability. However, the quality of different batches of the ion-exchange packing material was found to vary. The variability of the retention properties from one batch to another could in most cases be overcome by modifying the ionic strength of the mobile phase. A few batches were not usable at all, since they either did not retain the catecholamines or retained interfering substances. An example of the latter is shown in Fig. 4, where the sample injected was the same as in Fig. 2.

Methanol was originally used as organic modifier in the mobile phase, but in some urines other sample components interfered, which resulted in falsely high values. With THF this was avoided and epinephrine was better separated from dopamine without an increase of the total elution time. Several mobile phases with varying contents of methanol, THF, acetonitrile or a combination of two of these were tested.

Detection

The effect on the detector response of varying contents of methanol or THF in citrate buffer was investigated (Fig. 5). The separation column was substituted with a PTFE coil to eliminate the difference in retention properties of the column for the various contents of organic modifier. The response decreased with increasing content of organic solvent, more rapidly

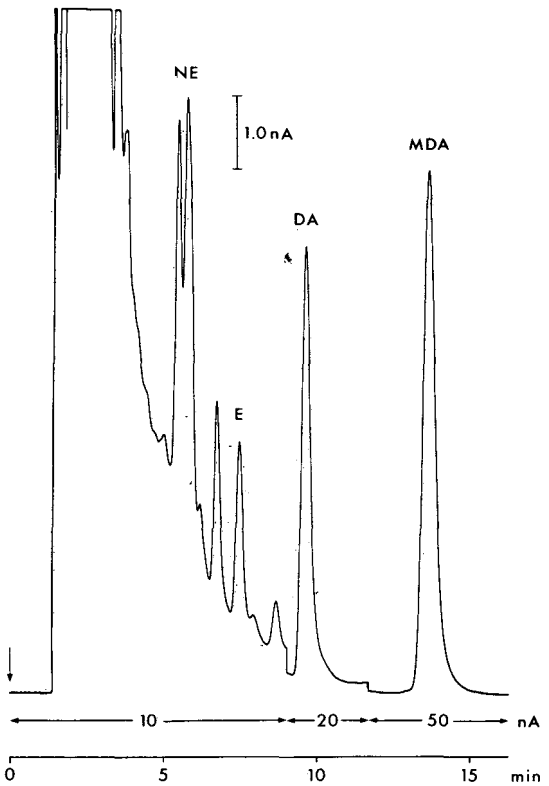

Fig. 4. Separation on an ion-exchange column retaining interfering substances. The urine sample injected and the chromatographic conditions were the same as in Fig. 2.

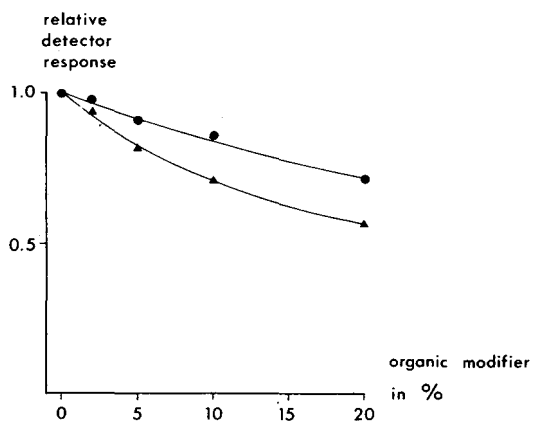

Fig. 5. Detector response versus addition of methanol or THF in citrate buffer (I = 0.1, pH 5.0) as mobile phase. (●), Methanol; (▲), THF.

for THF than for methanol. The loss of response was, however, compensated by increased column efficiency. The admixtures of methanol and THF used, 20 and 7% respectively, gave about the same response.

Accuracy, recovery and coefficient of variation

Extraction of the urine with ethyl acetate before adsorption onto alumina was not needed for most of the urines tested. However, the extraction resulted in a smaller front peak and for some urines in the removal of small interfering peaks, which is why it is recommended. Chromatograms of such a urine sample before and after extraction are shown in Figs. 6 and 2, respectively. The extraction with hexane removed the residual ethyl acetate dissolved in the aqueous phase.

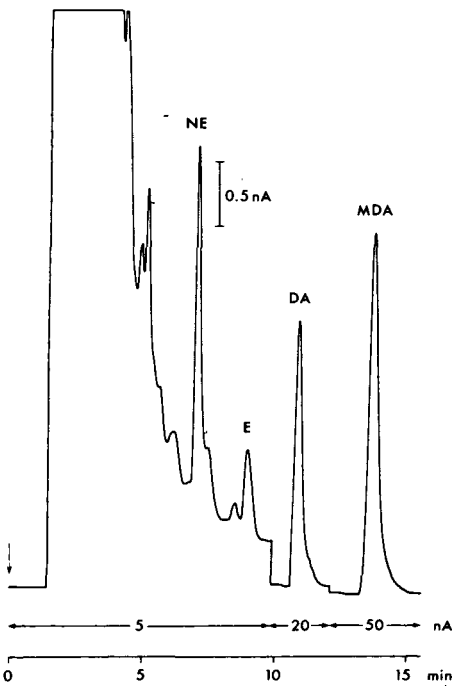

Fig. 6. Chromatogram of a sample not extracted with ethyl acetate. The urine used and the chromatographic conditions were the same as in Fig. 2.

Ten urine samples and a reference solution were monitored at two different potentials. The quotients between the peak heights of the catecholamines at the two potentials were the same in the urine samples and in the reference solution, which indicated that there were no interfering peaks.

It was observed that the internal standard MDA was less stable than the other catecholamines. In 0.01 mol/l perchloric acid solution MDA was unstable when stored in the refrigerator (+4°C). A peak that eluted at the same time as epinephrine appeared after a few days. To make sure there was no decomposition of the MDA in the worked-up samples, the MDA solution was assayed in parallel to the urine samples. No interfering peak had appeared after storing the samples for one month at −20°C.

Standard curves of norepinephrine, epinephrine and dopamine with and without urine were co-linear for each of the catecholamines, thus quantification could be performed from aqueous reference samples. The absolute re-

covery of the latter was earlier determined to be 82—92% [12]. The intra-assay coefficients of variation were 1.5% for norepinephrine, 1.1% for epinephrine and 0.6% for dopamine, when performing the analysis on twelve replicates of a urine sample.

If higher sensitivity than that obtained by the analytical procedure is required, a urine volume of 2 ml and an elution volume of 150 μl can be used without any decrease in recovery. The linearity of the method was tested for concentrations up to ten times that of an average urine sample.

Application

Urine samples collected over a 24-h period from five healthy persons were analysed. The urine from each 8-h period was collected in 10 ml of hydrochloric acid (3 mol/l), to maintain a final pH between 2 and 3, and was stored at −70°C before being analysed. The results are summarized in Table I. During the period covering sleep there was a decrease in the concentrations of epinephrine and norepinephrine, which is in agreement with results by others [14, 15]. The amounts excreted in the urine during 24 h showed great interindividual variations and are within the range of values reported for normal human urines [1, 4, 7, 8, 16].

TABLE I

MEAN URINARY EXCRETION OF CATECHOLAMINES OVER 8-h PERIODS IN FIVE HEALTHY PERSONS

Hours	Amount excreted (nmol ± S.D.)		
	NE	E	DA
07—15	69.4 ± 24	19.3 ± 11.4	399 ± 143
15—23	70.5 ± 6.2	20.9 ± 5.4	476 ± 109
23—07	39.4 ± 11.3	3.7 ± 1.8	485 ± 125

CONCLUSION

We have found this method to be a simple and accurate procedure for the determination of free urinary catecholamines. Ion-exchange chromatographic columns appeared to be more selective than reversed-phase columns and the pre-purification of urine could be simplified.

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Journal of Chromatography, 278 (1983) 265—274
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1856

RAPID ESTIMATION OF CATECHOLAMINES, OCTOPAMINE AND 5-HYDROXYTRYPTAMINE IN BIOLOGICAL TISSUES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH COULOMETRIC DETECTION

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(First received April 25th, 1983; revised manuscript received July 12th, 1983)

SUMMARY

A rapid, convenient procedure is described for the simultaneous determination of catecholamines, monohydroxyphenolamines and 5-hydroxytryptamine in biological tissues. The procedure involves homogenization of tissue in perchloric acid, addition of heparin and centrifugation followed by direct injection of the supernatant onto a C_{18} reversed-phase high-performance liquid chromatographic column. The mobile phase employed sodium dodecyl sulfate as ion pair reagent with 20% acetonitrile and 10—12% methanol as organic modifier. Eluted fractions were detected electrochemically using dual coulometric electrodes operated in screen mode. The procedure has been applied to the analysis of norepinephrine, epinephrine, dopamine, octopamine, tyramine, 5-hydroxytryptamine and tryptophan in a variety of tissues including mammalian heart and brain and insect nerve cord.

INTRODUCTION

Abnormal levels of catecholamines, their monohydroxy equivalents and 5-hydroxytryptamine (5HT) in the brain have been implicated in a number of psychiatric disorders including schizophrenia, depression, migraine, aggression, irritability and Parkinsonism [1—5]. The importance of these compounds in brain function, together with their presence in mammalian myocardium [6], has resulted in intensive efforts to provide rapid, sensitive analytical procedures with which to determine tissue levels. High-performance liquid chromatography (HPLC) with electrochemical detection (ED) has been particularly useful in this regard especially for the determination of catecholamines and 5HT [7]; however, few reports have described simultaneous determination of catecholamines, 5HT and monohydroxyphenolamines [8]. One constraint associated with simultaneous determinations of these compounds by HPLC—ED is that monohydroxyphenolamines require a higher electrode poten-

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tial than catecholamines in order to effect electro-oxidation [8]. This problem can be overcome to some extent by the use of multiple working electrodes which effectively extend the potential range of ED [9]. However, the relatively small surface of the tubular electrodes or thin-layer cells that are employed in amperometric detectors tends to result in fouling of the electrode surface at higher potentials. Coulometric detector electrodes provide a larger surface which is less susceptible to perturbations resulting from adsorption of reaction products and also confers greater redox efficiency [10]. Thus, a dual coulometric detector system offers advantages for detection of catecholamines, monohydroxyphenolamines and 5HT in a single biological sample.

The present report describes a convenient procedure for simultaneous analysis of catecholamines, 5HT and octopamine (OA) in biological tissues. The procedure involves direct inject of the sample onto a C_{18} reversed-phase HPLC column with the eluted fractions detected electrochemically using a dual-electrode coulometric detector. The utility of the described procedure is demonstrated by analysis of biogenic amine levels in rat heart and insect nerve cord and the brain of rat and mouse.

MATERIALS AND METHODS

Reagents

Norepinephrine bitartrate, epinephrine bitartrate, dopamine hydrochloride and tyramine hydrochloride were obtained from Calbiochem-Behring (La Jolla, CA, U.S.A.), and octopamine hydrochloride, 5-hydroxytryptamine creatine sulfate complex, tryptophan and sodium heparin were purchased from Sigma (St. Louis, MO, U.S.A.). Sodium dodecyl sulfate (SDS) (electrophoresis purity) was supplied by Bio-Rad Labs. (Richmond, CA, U.S.A.) and 3,4-dihydroxybenzylamine (DHBA), used as the internal standard, was donated by Bioanalytical Systems (West Lafayette, IN, U.S.A.). Solvents (HPLC grade) were obtained from Caledon Labs. (Georgetown, Canada) and all other chemicals (Baker Analyzed Grade) from CanLab (Toronto, Canada).

Catecholamine standards were prepared at 100 μg/ml in 0.1 *M* perchloric acid and working concentrations achieved by serial dilution in 0.1 *M* perchloric acid. Water used for the preparation of all solutions was deionized, distilled and re-distilled over potassium permanganate prior to use.

Animals and tissue collection

Male rats of the Sprague-Dawley strain, weighing between 220 and 330 g, were obtained from a colony maintained in the Department of Biology, University of Waterloo, on a 12 h:12 h light:dark cycle and provided with food (Purina Labs.) and water ad libitum. Rats were isolated from the colony 12 h prior to sacrifice by decapitation. Brains and hearts were excised and frozen in liquid nitrogen before being stored at −75°C.

Male mice of the C-57 strain, weighing between 20 and 27 g, were obtained from a colony maintained in the Department of Psychology, University of Waterloo, under similar conditions to those described for rats. Brain tissues were obtained and stored in a similar manner to that described for rats.

Adult male cockroaches were taken at 1–3 months after the adult moult

from a colony of *Periplaneta americana* maintained under standard conditions in this laboratory [11]. Insects were isolated 12 h prior to removal of nerve cord tissues (including thoracic and abdominal ganglia). Nerve cords were rinsed in cockroach saline [12] and transferred to a preweighed microcentrifuge tube containing 250 μl of ice cold 0.1 *M* perchloric acid and the internal standard, dihydroxybenzylamine (DHBA).

Sample preparation

Brain and heart tissues were weighed and broken in the frozen state before being placed in 1.5 ml (rat tissues) or 1.0 ml (mouse brain) of ice-cold 0.2 *M* perchloric acid containing DHBA. All tissues were homogenized by ultrasonic disintegration over ice using a Branson Sonifier Model 200 (75 W, 50% pulsed power for 2 min). Sodium heparin (rat tissues, 2000 units; mouse brain, 750 units; insect nerve cord, 200 units) was added to facilitate lipoprotein precipitation [13]. Samples were centrifuged at 40,000 *g* for 30 min and the supernatant was then diluted appropriately with water to reduce the amount of contaminating material injected onto the HPLC column.

Apparatus

Chromatography was achieved using a Spectra-Physics Model 740B solvent delivery system equipped with an extra pulse dampener. Samples were introduced through a Valco CV-6-UHPa-N60 injection valve fitted with a 50-μl sample holding loop. Separations were performed on an Ultrasphere IP C_{18} column (150 × 4.6 mm I.D., 5-μm particle size) (Beckman, Toronto, Canada) protected by a Brownlee guard column (30 × 2.1 mm I.D., 5-μm particle size) (Technical Marketing Assoc., Mississauga, Canada).

The electrochemical detection system (ESA 5100A, Bedford, MA, U.S.A.) comprises a coulometric guard cell and a solid-state analytical cell containing dual coulometric working electrodes made from porous graphite (Fig. 1). The guard cell is installed before the injector and, by operating it at a high poten-

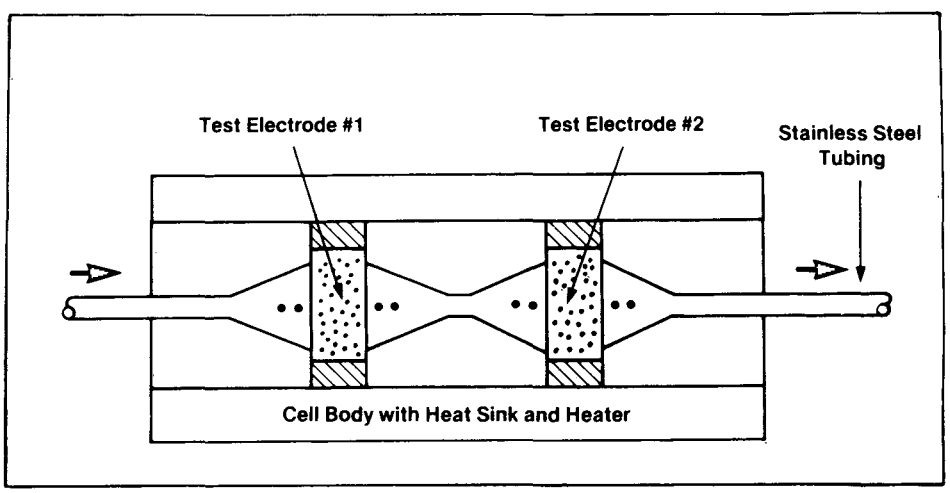

Fig. 1. Schematic of cell design of dual-electrode electrochemical detection system (reproduced with permission from ESA, Bedford, MA, Brochure No. 182-5100).

tial (0.9 V), can preoxidize mobile-phase contaminants thereby reducing the noise generated by the buffer at the analytical detector.

For detection of catecholamines in brain and heart tissues the first coulometric detector was set at 0.1 V to reduce interference by contaminating electroactive compounds at the second detector which was set at 0.3 V, the potential required for electro-oxidation of norepinephrine (NE), epinephrine (E), dopamine (DA), 5HT and the internal standard, DHBA (Fig. 2A). Detection of OA requires a higher oxidation potential [8]; therefore, for the analysis of OA the first detector was set at 0.5 V for detection of NE, E, DA, 5HT, some tryptophan (TP), and DHBA, whereas the second detector was set at 0.75 V and detected OA, tyramine (TA), 5HT and TP (Fig. 3). Signals from the coulometric detectors were recorded and integrated using an SP4270 computing integrator for detector 1 and an SP4100 computing integrator for detector 2 (Spectra Physics, San Jose, CA, U.S.A.).

Chromatography

The mobile phase employed for catecholamine and 5HT determinations in brain and heart tissues contained 0.02 *M* trichloroacetic acid (TCA), 0.075 *M* sodium phosphate, 1.5 μM EDTA with 1.5 m*M* SDS as ion-pair reagent and 20% acetonitrile and 10% methanol as organic modifier. The buffer, final pH 3.1, was continually degassed with a stream of helium and pumped at a flow-rate of 1.0 ml/min. When operating at higher potentials for the determination of OA, the mobile phase contained 2.0 m*M* SDS and 12% methanol with the remaining buffer components as described above. The flow-rate was reduced to 0.8 ml/min when OA determinations were required.

RESULTS AND DISCUSSION

Chromatographic system

A previous report [8] demonstrated that baseline separation of DA, NE, TA and OA was achieved using a 150 mm × 4.6 mm I.D. Ultrasphere RP-18 IP column packed with 5-μm particles, an acetic acid—ammonium hydroxide buffer containing octane sulfonic acid (OSA) for ion pairing and amperometric detection. Subsequently it was found that replacement of OSA with SDS provided better separation of amines, a quieter baseline at high oxidation potentials due to the lower amounts of ion-pair reagent required and, therefore, lower limits of detection for OA and TA ($\leqslant$100 pg with signal:noise ratio $\geqslant$4).

Use of the ESA coulometric detector in the present study requires a buffer system that generates low currents at both working electrodes and resulted in adoption of the TCA—phosphate buffer containing 1.5 μM EDTA; this provides currents of less than 1 μA at 0.75 V. The separation and peak symmetry obtained for various amines using TCA—phosphate buffer was further improved by the use of high concentrations of SDS (1.5—2.0 m*M*) together with high organic solvent content ($\geqslant$30% mobile phase). This improvement was achieved without any increase in the capacity factor for TA, a relatively non-polar amine. The present buffer system affords elution of 5HT within

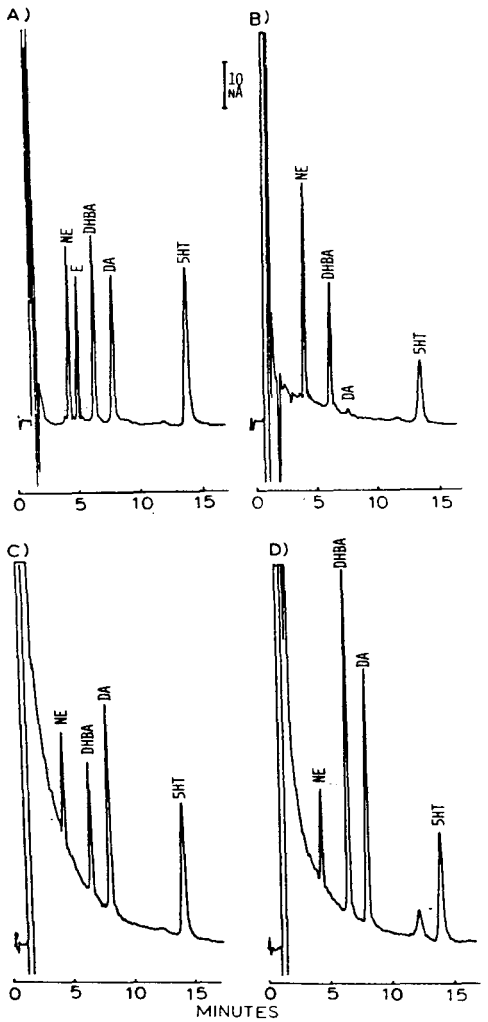

Fig. 2. Chromatograms of 1 ng of standards (A), rat heart (B), rat brain (C) and mouse brain (D) with second detector set at 0.3 V. Flow-rate 1.0 ml/min; other chromatographic conditions are defined in the text.

15 min while still retaining NE for at least 4.5 min (Fig. 2). The increased resolution obtained with high SDS concentration may be explained by its strong adsorption to the bonded-phase packing so that at 2.0 m*M* the surface coverage of the bonded-phase column particles approaches the coverage of the chemically bonded C_{18} groups. The critical micelle concentration (CMC) for SDS in a mobile phase containing 20% methanol is 2.5 m*M* [14], thus the concentration of SDS used in the present study (2.0 m*M*) for mobile phase containing 32% organic solvent is below the CMC. The high concentration of organic solvent maintained reproducible column retention of amines even when the mobile phase (2 l) was recycled for over 100 injections and also provided a fast run-time per sample even at high potentials. The direct injection of biological samples revealed few slowly eluting fractions; indeed TP,

which was detected at the 0.75-V electrode at about 20 min, demonstrated the highest capacity factor among the compounds of interest.

Sample preparation

Tissue samples were normally processed within 24 h following dissection, however, samples could be stored at −75°C for several months without any significant change in amine levels.

Several workers have described the use of dilute perchloric acid to precipitate proteins as an early step in the preparation of samples for biogenic amine determinations using HPLC—ED [15—18]. However, samples in which proteins were precipitated with perchloric acid and then centrifuged reveal a large number of early peaks when the supernatant is injected directly onto a reversed-phase column with ED. These peaks interfere with the detection and quantification of NE, thus only a few studies have exploited the convenience of the technique [9, 16—20]. The present study demonstrates that the addition of heparin to 0.2 *M* perchloric acid increases the amount of precipitation and results in a cleaner chromatogram (Figs. 2 and 3). Heparin is known to form insoluble complexes with lipoprotein molecules [13, 21] and may also co-precipitate electroactive molecules which would otherwise elute from a reversed-phase column. It is appropriate to indicate that the buffer employed in this study also contributes to a cleaner signal at the front of the chromatogram through the rapid elution of many molecules that are not retained by an ion-pair mechanism because of the high content of organic solvent.

Recovery

An internal standard, DHBA, was included in the sample to accommodate dilution of supernatant by tissue fluid. Determinations of amine recoveries with and without the addition of heparin demonstrate that heparin has no effect on the recovery of tissue amines; therefore, the amine levels reported in Tables I and II indicate 100% recovery.

Detection

The applied potential required to oxidize the biogenic amines with the coulometric detector used in the present study is considerably lower than that required for amperometric detection [8]. Many commercial amperometric detectors use a silver/silver chloride reference electrode, the half-cell potential of which is subject to drift due to diffusion of chloride ions from the reference electrode into the mobile phase [18]. By contrast, the proprietary solid-state reference electrode used in the ESA coulometric detector provides potential control through a H_2/H^+ couple and this, together with the close proximity (only 1 mm apart) of the reference and working electrodes, results in lower applied potentials, greater electrode stability and minimal uncompensated potential drop (IR) over the solution between the working and reference electrodes. Consequently, the optimized applied potential for detection of octopamine in the present study was 0.75 V whereas an amperometric detector with a silver/silver chloride reference electrode required an optimal applied potential of 0.9 V [8].

Operation of the detector in a screen mode offers increased selectivity particularly for compounds which oxidize at high applied potentials. Fig. 3

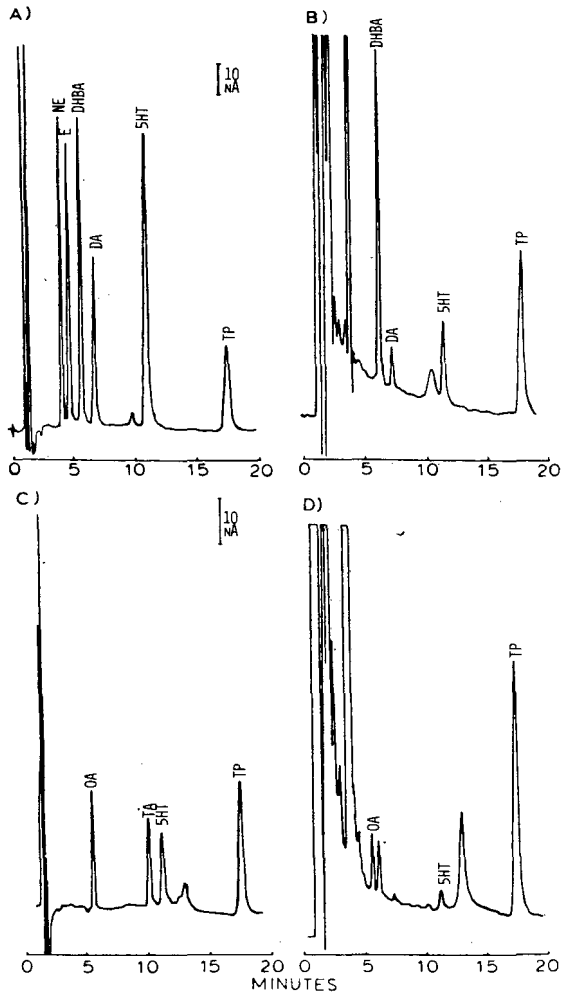

Fig. 3. Chromatograms of 1 ng of standards (A, C) and cockroach nerve cord extracts (B, D) with detector 1 set at 0.5 V (A, B), detector 2 at 0.75 V (C, D). Flow-rate 0.8 ml/min; other chromatographic conditions are defined in the text. (The peak appearing at 13.10 min on detector 2 is an unidentified contaminant originating from the injector seal material.)

illustrates the detection of NE, E, DHBA, DA, 5HT and TP at the first coulometric detector (set at 0.5 V) whereas TA, OA, 5HT and TP are detected at the second detector (set at 0.75 V). Thus, many compounds that oxidize at the lower potential are screened from the second detector. The advantages of this system are evident by reference to OA which appears at detector 2 at 5.80 min without any interference from DHBA which is retained for 6.16 min but which is totally oxidized at detector 1. Dual-electrode amperometric detector systems oxidize only a fraction of the electroactive compound and, therefore, could not provide this degree of selectivity. Fig. 3 demonstrates also that 5HT and TP undergo oxidation reactions at both detectors. The first oxidation of 5HT occurs at the 5-hydroxy group of the indole while the second reaction involves oxidation of ring nitrogen to N^+ [22, 23]. The nature of the dual detection of TP has not been elucidated.

Preoxidation of electroactive material in the mobile phase was achieved by means of a guard cell set at 0.9 V. This resulted in lowered background currents at the detector electrodes and improved the signal:noise ratio.

The coulometric detector employed in the present study should not be used at high potentials when any part of the chromatographic system contains graphite-impregnated PTFE material because this releases electroactive substances which contribute unidentified peaks to the chromatogram. An unidentified peak originating from the manual injector is evident in Fig. 3C and D at about 13 min; however, this contaminant can be separated from peaks of interest.

Biogenic amines in tissues

The levels of NE, DA and 5HT in rat heart, rat brain and mouse brain are presented in Table I. The catecholamine levels found in the heart fall within the ranges reported in previous studies [6, 16, 20] while the 5HT levels are, to the best of our knowledge, the first to be described for this organ using HPLC—ED. The biogenic amine levels reported for other tissues in Table I are also consistent with earlier studies on rat brain [17, 24, 25] and mouse brain [26, 27]. The close correlation of the data reported in Table I with those of previous investigations provides vindication of the current procedure for estimating tissue levels of catecholamines and 5HT.

By contrast to the widespread use of HPLC—ED for estimation of catecholamines, most determinations of monohydroxyphenolamines have employed radioenzymatic procedures [28]. Indeed, HPLC—ED has been applied only to the estimation of OA, TA and synephrine in plant products [29, 30] and HPLC with fluorometric detection has been used to monitor OA in ganglia of *Aplysia californa* [31]. Table II shows the levels of OA, DA, 5HT and TP obtained for nerve cords of the american cockroach *Periplaneta americana* using the HPLC—ED procedure described in this study. The levels are slightly lower than those reported by other workers using radioenzymatic techniques [32], possibly due to the greater specificity that is afforded by the present technique. In addition to providing increased specificity, the present proce-

TABLE I

CONCENTRATIONS OF NOREPINEPHRINE, DOPAMINE AND 5-HYDROXYTRYPTAMINE IN RAT HEART, RAT BRAIN AND MOUSE BRAIN ESTIMATED BY HPLC—ED

Values indicate mean ± S.D. for the number of determinations shown in parentheses.

Tissue	Amine concentration (ng/g)		
	Norepinephrine	Dopamine	5-Hydroxytryptamine
Rat heart	725.2 ± 123.2 (10)	23.1 ± 2.1 (2)	253.1 ± 73.5 (10)
Rat brain	376.4 ± 27.5 (12)	905.9 ± 69.6 (12)	340.8 ± 46.8 (12)
Mouse brain	473.1 ± 24.4 (16)	1423.7 ± 114.9 (16)	586.8 ± 54.7 (16)

TABLE II

CONCENTRATIONS OF OCTOPAMINE, DOPAMINE, 5-HYDROXYTRYPTAMINE AND TRYPTOPHAN IN COCKROACH NERVE CORD ESTIMATED BY DUAL COULOMETRIC ELECTROCHEMICAL DETECTION OF HPLC-SEPARATED FRACTIONS

Values indicate mean ± S.D. for the number of determinations shown in parentheses. Average weight of nerve cords = 8.4 ± 1.7 mg.

Amine	Amine concentrations (ng/g)	
	Detector 1 (0.5 V)	Detector 2 (0.75 V)
Octopamine	—	888.3 ± 127.8 (8)
Dopamine	311.5 ± 64.9 (8)	—
Serotonin	335.9 ± 77.4 (8)	368.8 ± 105.2 (8)
Tryptophan	4634.0 ± 1399.0 (8)	4499.2 ± 1069.8 (8)

dure enables simultaneous estimation of other biogenic amines contained within the biological sample. The sensitivity of the HPLC—ED technique described herein also compares favourably with the radioenzymatic procedure and permits OA estimates in single ganglia.

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Journal of Chromatography, 278 (1983) 275—282
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1863

DETERMINATION OF β-GALACTOSIDASE ACTIVITY IN THE INTESTINAL TRACT OF MICE BY ION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING ε-N-1-(1-DEOXYLACTULOSYL)-L-LYSINE AS SUBSTRATE

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(First received May 25th, 1983; revised manuscript received July 11th, 1983)

SUMMARY

ε-N-1-(1-Deoxylactulosyl)-L-lysine was synthesized and used as a substrate to assay β-galactosidase activity. ε-N-1-(1-Deoxylactulosyl)-L-lysine and its degradation product ε-N-1-(1-deoxyfructosyl)-L-lysine were detected by high-voltage paper electrophoresis and ion-exchange high-performance liquid chromatography. The β-galactosidase activity in different parts of the intestinal tract of germ-free and control mice was determined and compared with a β-galactosidase activity which degrades lactose at pH 8.5 and 5.0 and which corresponded with bacterial and host enzymatic activities, respectively.

INTRODUCTION

The bacterial flora which is normally present in the gastrointestinal tract protects the host against potentially pathogenic microorganisms from the environment [1]. This mechanism is called colonization-resistance (CR) [2, 3]. This CR-associated protecting microflora might be strongly reduced by treatment with certain antibiotics. In order to monitor the bacterial infection risk during treatment of immunocompromised patients, the presence of the dipeptide β-aspartylglycine in faeces was used as an indicator of the degree of reduction of CR-associated intestinal microflora [4—6]. Analysis of β-aspartylglycine is routinely performed by high-voltage paper electrophoresis. During these analyses it was noticed that two other substances accumulated in complete absence of intestinal microflora (unpublished results). These two substances appeared to be identical to the spots observed by Ersser et al. [7] in the faecal contents of a germ-free infant and several newborn infants. These two

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substances were identified as ε-N-1-(1-deoxylactulosyl)-L-lysine (lactose-lysine) and ε-N-1-(1-deoxyfructosyl)-L-lysine (fructose-lysine), which are products of the Maillard reaction [8, 9]. This reaction takes place between a reducing sugar and the amino group of an amino acid. Whenever protein is heated in the presence of reducing sugars, for instance during processing of food, the free ε-amino group of lysine in proteins may react which results in a glycosylated protein. This carbohydrate-lysine product is liberated when the protein is digested. Particularly dried milk contains relatively large amounts of lactose-lysine and fructose-lysine [10]. The latter is the adduct of glucose and lysine after Amadori rearrangement [8, 9]. Erbersdobler and co-workers [11, 12] found that fructose-lysine was only degraded by microorganisms and not by the host. This fact makes fructose-lysine a possible marker of the absence of intestinal microflora. However, since fructose-lysine originates from processed food it was assumed that there was no direct relationship between the amount of fructose-lysine in the faeces and the disturbance of the intestinal flora. The bacterial enzymatic activities which degrade fructose-lysine and lactose-lysine might be more suitable indicators of a reduced bacterial flora.

Possible targets for such carbohydrate-lysine degrading enzymes are (1) cleavage of the carbohydrate-lysine bond, (2) degradation of the lysine moiety, (3) degradation of the carbohydrate part. Preliminary studies showed that the carbohydrate-lysine bond was hardly cleaved. Therefore this activity was not suitable for our purpose. Erbersdobler et al. [11] studied the microbial breakdown of fructose-lysine and found 20—40% deamination in 10 h, which gives an estimated 2—4% breakdown in 1 h. Since it is not possible to detect the deamination products fluorometrically with *o*-phthalaldehyde (OPA) and as will be shown in this paper, the β-galactosidase activity which cleaves lactose-lysine into β-galactose and fructose-lysine (further referred to as β-galactosidase) is much higher, the latter was chosen as a possible marker for the presence of intestinal microflora. In this β-galactosidase assay the cleavage of lactose coupled to lysine is measured. In order to couple lactose and lysine, these two substances are heated together. Since lysine has two amino groups, two adducts can be formed: α-N-1-(1-deoxylactulosyl)-L-lysine (α-lactose-lysine) and ε-lactose-lysine. If the heating is prolonged, adducts with more than one lactose molecule attached to lysine can be formed and brown polymeric products occur [8, 9]. These undesired side-reactions do not occur if a relatively short reaction time is chosen. The separation of α- and ε-lactose-lysine by semipreparative ion-exchange high-performance liquid chromatography (ion-exchange HPLC) will be described. In this study the cleavage of lactose-lysine is compared to the enzymatic cleavage of free lactose (further referred to as lactase activity). The relationship between these two types of β-galactosidase activity and the intestinal microflora will be discussed.

EXPERIMENTAL

Animals

Adult germ-free C3H mice were kept under germ-free isolation conditions in sterilized plastic isolators, fed with autoclaved SRM food (Hope Farms, Woerden, The Netherlands) and supplied with autoclaved drinking water. Adult

conventional C57B1/10 mice served as controls. These mice were fed RMH food (Hope Farms) supplied with normal tap water and housed under clean, conventional conditions.

Preparation of enzyme solutions

The animals were killed with ether and the entire intestine was removed. The small intestine was divided into two equally long segments. The segments were cut into pieces and a 0.5 volume of 10 m*M* sodium phosphate, pH 7.4, was added. The mixture was homogenized with a glass rod in a test tube and sonicated for 4 min at 80 W with a Branson B12 sonicator. The tube was chilled with crushed ice during sonication. The contents of cecum and colon were treated in the same way. After sonication, the samples were centrifuged for 40 min at 6000 *g*. The supernatants were dialyzed against 10 m*M* sodium phosphate, pH 7.4, and stored at −18°C.

Chemicals

L-Lysine—HCl was obtained from Calbiochem (San Diego, CA, U.S.A.); lactose was obtained from Lamers and Indemans ('s Hertogenbosch, The Netherlands); β-alanine was obtained from Aldrich Europe (Beerse, Belgium); Whatman 3MM chromatography paper was obtained from Whatman (Maidstone, U.K.); Durrum DC6A cation-exchange resin was obtained from Durrum (Sunnyvale, CA, U.S.A.). Absolute methanol was prepared as described by Gottschalk [13]. Other chemicals were purchased from Merck (Darmstadt, F.R.G.).

Chromatographic system

The chromatographic system consisted of a solvent delivery pump (Model LC20, Pye Unicam, Cambridge, U.K.) connected to a sample loop injector (Model 7125 Rheodyne, Berkeley, CA, U.S.A.). Fluorescence was monitored with a fluorescence detector (Model 420C Waters Assoc., Milford, MA, U.S.A.). The stainless-steel column was thermostated by means of a water bath.

Synthesis of ε-lactose-lysine

One gram of L-lysine—HCl, 9.5 g of lactose and 150 ml of absolute methanol were refluxed for 4 h. Unreacted lactose was removed by filtration. The methanol was evaporated in vacuo and the residue which contained lysine, α- and ε-lactose-lysine was dissolved in 50 ml of water. Batches of 1 ml were lyophilized and further purified by semipreparative HPLC. A batch was redissolved in 100 μl of 0.3 *M* pyridine acetate buffer, pH 3.4, of which 10 μl were injected onto a 100 × 4.6 mm column packed with Durrum DC6A cation-exchange resin (particle size, 11 ± 1 μm). Elution was performed with 0.3 *M* pyridine acetate, pH 3.4. The retention times of α- and ε-lactose-lysine were established by post-column derivatization with OPA and subsequent fluorometric detection (see Fig. 1) as described by Lee et al. [14]. Next, the remaining 90 μl of the batch were injected and the fractions containing α- and ε-lactose-lysine were collected directly from the outlet of the column at the appropriate times [15]. The elution positions of α- and ε-lactose-lysine were assigned by comparison with samples isolated from faeces and with

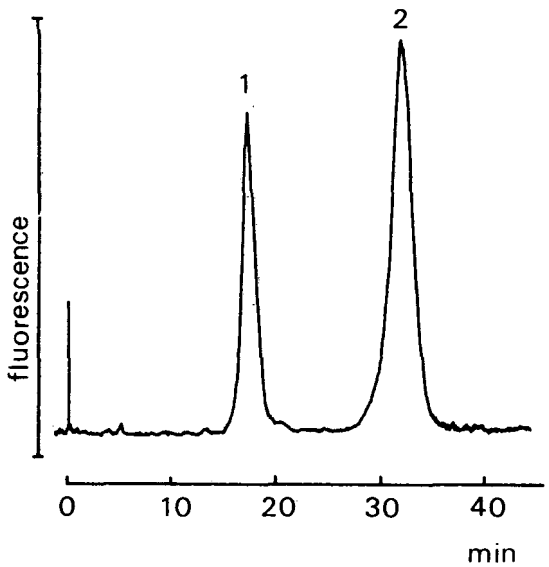

Fig. 1. Separation of α-lactose-lysine and ε-lactose-lysine on a cation-exchange column 100 × 4.6 mm with 0.3 *M* pyridine acetate, pH 3.4. Flow-rate, 0.5 ml/min; temperature, 50°C. Lysine eluted much later after the subsequent semipreparative run. 1 = α-Lactose-lysine; 2 = ε-lactose-lysine.

synthetic ε-lactose-lysine prepared from α-formyl-lysine after hydrolysis (1 *M* hydrochloric acid, 100°C, 15 min) of the protecting formyl group [16, 17]. The fractions were lyophilized and stored at −18°C in absolute methanol.

Amino acid analysis

Amino acid analyses were performed with an amino acid analyzer Liquimat III (Kontron, Zürich, Switzerland) using Pico buffers obtained from Pierce (Rockford, IL, U.S.A.).

β-Galactosidase assay

About 20 nmol of ε-lactose-lysine in methanol were dried under a stream of nitrogen; 10 μl of buffer and 10 μl of enzyme solution were added and the mixture was incubated for 1 h at 37°C. The following buffers were used: 0.1 *M* sodium acetate (pH 5.0); 0.1 *M* sodium maleate (pH 6.0); 0.1 *M* sodium phosphate (pH 7—7.5); 0.05 *M* sodium barbiturate (pH 8—9). The incubation was stopped by adding 100 μl of methanol containing 20 nmol of β-alanine as internal standard. Precipitated protein was removed by centrifugation at 6000 *g* for 15 min. A 50—100-μl aliquot of the supernatant was injected onto a 45 × 3.6 mm stainless-steel column packed with Durrum DC6A resin and eluted with pyridine—acetic acid—water (6:60:176, v/v).

Detection was performed with OPA as described by Lee et al. [14] (see Fig. 2). The amount of lactose-lysine and fructose-lysine was calculated from the peak height × peak width at half height assuming an equal specific fluorescence for both compounds. The percentage conversion of lactose-lysine to fructose-lysine in 1 h was taken as a measure for the β-galactosidase activity.

Alternatively, the incubation mixture was applied to Whatman 3MM chroma-

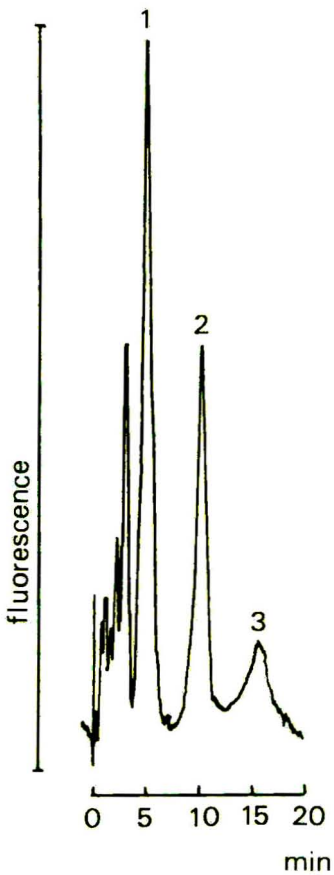

Fig. 2. Chromatogram obtained after injection of a β-galactosidase incubation mixture onto a 45 × 3.6 mm cation-exchange column. Mobile phase: pyridine—acetic acid—water (6:60:176, v/v). Flow-rate, 0.4 ml/min; temperature, 50°C. In front of lactose-lysine some free amino acids elute which are the result of proteolytic activity in the intestinal enzyme preparations. 1 = Lactose-lysine; 2 = β-alanine (internal standard); 3 = fructose-lysine.

tography paper and subjected to high-voltage paper electrophoresis at pH 3.5 for 1 h at 3000 V as described by Welling [6]. The spots were stained with ninhydrin and the β-galactosidase activities were estimated from the intensities of the lactose-lysine and fructose-lysine spots (see Fig. 3).

Lactase assay

Lactase activities were determined as described by Dahlquist [18].

RESULTS

Synthesis of lactose-lysine

After refluxing lactose and lysine, amino acid analysis showed that the reaction mixture contained 2.3% α-lactose-lysine, 7.3% ε-lactose-lysine and 90.4% lysine. Since no colour factor of lactose-lysine is known, the colour

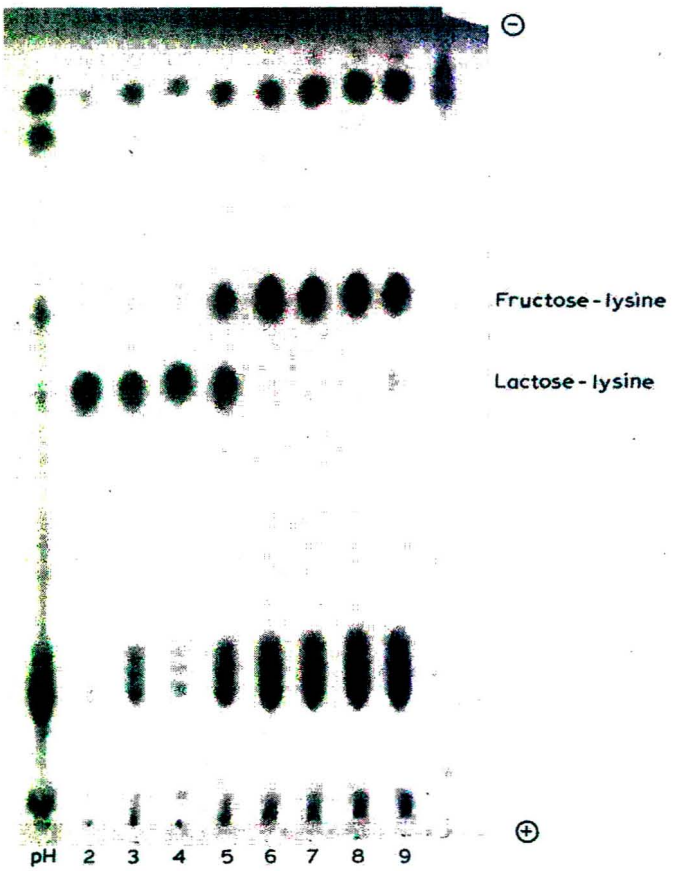

Fig. 3. High-voltage paper electrophoresis at pH 3.5 for 1 h at 3000 V. β-Galactosidase incubation mixtures at different pH values after overnight incubation. Because of complete degradation of lactose-lysine at pH 6—8, a shorter incubation time (1 h) was chosen.

factor of lysine was also used for lactose-lysine in this calculation. Amino acid analysis of lactose-lysine samples purified by HPLC showed no contamination.

Lactase and β-galactosidase activities

Influence of pH. pH—activity curves were made from a small intestinal sample and from a cecal sample obtained from conventional mice. Lactase in the cecum had an optimum pH of around 8, whereas lactase in the small intestine showed an optimum pH of 6.0. In the small intestine microorganisms do not contribute to any major extent to the production of disaccharidases such as lactase [19]. Therefore it was concluded that the lactase activity in the small intestine which showed a pH optimum of 6 was host-derived. The lactase in the cecum with an optimum pH of around 8 is more likely to be of bacterial origin since it was absent in the germ-free animals and the conventional cecum is known to contain large amounts of bacteria. The β-galactosidase (lactose-lysine cleaving) activity showed a broad pH optimum from pH 6 to 8. In various parts of the intestinal tract of germ-free and conventional mice this activity was determined at pH 7.5.

TABLE I

β-GALACTOSIDASE AND LACTASE ACTIVITIES IN DIFFERENT PARTS OF THE INTESTINAL TRACT OF ADULT GERM-FREE AND CONTROL MICE

Animals	Activity*	Small intestine		Cecum	Colon
		Upper	Lower		
Germ-free	β-Galactosidase	—	—	—	—
	Lactase pH 5.0	+	+	—	—
	Lactase pH 8.5	—	—	—	—
Control	β-Galactosidase	+	—	++	+
	Lactase pH 5.0	++	+	+	—
	Lactase pH 8.5	—	—	++	+

*Lactase: —, less than 0.01 U/ml; ++, more than 0.1 U/ml. β-Galactosidase: —, less than 2.5% degradation per hour; ++, more than 25% degradation per hour; +, intermediate values.

Distribution of activities in different parts of the gastrointestinal tract of germ-free and conventional mice. The β-galactosidase activity, given as percentage conversion per hour of lactose-lysine to fructose-lysine, in the upper small intestine, the lower small intestine, the cecum and colon of control mice was 9%, 0%, 28% and 13%, respectively. In the small intestine and colon of germ-free mice no β-galactosidase activity was found, while with an enzyme preparation from the cecum less than 2.5% of lactose-lysine was converted to fructose-lysine.

The results are summarized and compared with lactase activities in Table I. β-Galactosidase and lactase at pH 8.5 show a similar distribution pattern and are mainly present in cecum and colon, whereas lactase at pH 5.0 is mainly present in the small intestine. The latter activity was the only lactase or β-galactosidase activity found in the germ-free animals.

DISCUSSION

The semipreparative HPLC method used to purify lactose-lysine is essentially the same as that used by Weinstein et al. [15], except that we used as commercially available column packing. With slight modifications this method can be used for rapid purification of many amino acids or peptide-like substances. Since a volatile buffer is used, desalting is not necessary.

The use of lactose-lysine as substrate in the β-galactosidase assay has the advantage that it occurs naturally in diets which contain processed food, which is always the case with patients, and therefore reflects an in vivo situation. The amino group of lysine allows sensitive detection with amino acid reagents like OPA. Maximum sensitivity, however, was not achieved because of quenching of the fluorescence, probably due to the pyridine in the mobile phase. Attempts to separate lactose-lysine and its degradation product fructose-lysine in a reversed-phase HPLC system were not successful, probably because of the very similar hydrophobic part of the two molecules.

The results indicate that the β-galactosidase activity, determined in this way,

is not present in germ-free animals and therefore might be used as a marker for the presence of bacteria. The distribution of this β-galactosidase resembles lactase at pH 8.5, which is most likely also of bacterial origin. Examination of more samples is needed to establish these facts more firmly.

ACKNOWLEDGEMENTS

We thank E. Schwander and P. Jekel for performing the amino acid analyses, W. Hofstra for providing intestinal contents of germ-free mice, G. Helmus for assistance with the high-voltage paper electrophoresis, P. Wietzes for technical assistance with the HPLC and G. Groen, R. van der Zee and Dr. J.J. Beintema for helpful discussions.

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Journal of Chromatography, 278 (1983) 283—289
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1849

A GAS CHROMATOGRAPHIC ASSAY FOR THE DETERMINATION OF 5,6-DIHYDROFLUOROURACIL AND 5-FLUOROURACIL IN HUMAN PLASMA

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(First received January 26th, 1983; revised manuscript received July 12th, 1983)

SUMMARY

A gas chromatographic assay for the determination of 5-fluorouracil (5-FU) and 5,6-dihydrofluorouracil (FDHU) is described. The selectivity and sensitivity of the method allows the determination of both 5-FU and FDHU in 200 μl of plasma. Diphenylsuccinimide and chlorouracil were used as external and internal standard, respectively. The assay including the extraction shows a good linearity in the range 0—5000 ng/ml plasma for 5-FU as well as for FDHU. 5-FU and FDHU plasma concentrations of a number of patients with breast cancer treated with 5-FU were determined in order to demonstrate the usefulness of the method.

INTRODUCTION

Although 5-fluorouracil (5-FU) has been used in clinical oncology for more than two decades, the literature [1, 2] offers only a few data about one of its quantitatively most important metabolic products, 5,6-dihydrofluorouracil

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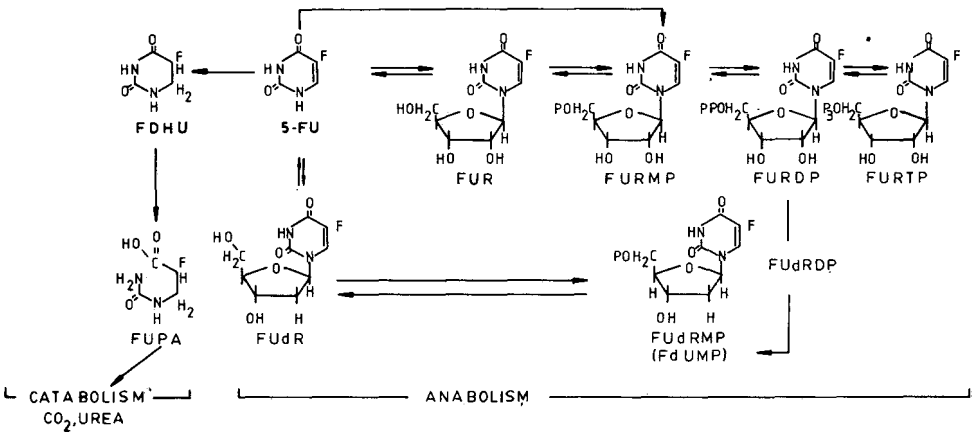

Fig. 1. Metabolic pathways of the 5-FU metabolism.

(FDHU). Most effort has been put into the determination of products of the anabolic pathway of 5-FU metabolism [3—6], since these products show cytostatic activity.

Determination of FDHU might be of great importance in 5-FU studies. From Fig. 1 it can be deduced that saturation in the catabolic pathway may lead to non-linearity in 5-FU pharmacokinetics, as suggested in the literature [1, 2].

An overflow towards the anabolic pathway might result in relatively higher antineoplastic activity. Besides this, a practical point demands attention. Plasma levels of FDHU can be monitored more easily than intracellular concentrations of metabolites from the anabolic pathway like 5-fluorouridine (FUR), 5-fluoridine-5′-monophosphate (FURMP), 5-fluoridine-5′-diphosphate (FURDP), 5-fluorodeoxyuridine-5′-diphosphate (FUdRDP), 5-fluorodeoxyuridine-5′-monophosphate (FdUMP) and 5-fluorodeoxyuridine (FUDR) (Fig. 1).

Since pharmacokinetic studies require the determination of the time course of several disposition phenomena like drug absorption, distribution, elimination and metabolism, the frequent measuring of drug and metabolite concentrations in plasma is the most obvious way to obtain an insight into these phenomena.

The low concentrations to be measured in pharmacological studies require selective and sensitive analytical techniques. At present high-performance liquid chromatography (HPLC) and gas chromatography (GC) are the methods of choice for routine measurements. FDHU shows neither fluorometric nor electrochemical activity and owing to its low molar absorptivity, GC with nitrogen-selective detection is preferred to HPLC. In spite of the selective detection an extraction procedure is required.

EXPERIMENTAL

Apparatus

A gas chromatograph (Model 420, Packard Becker, Delft, The Netherlands) equipped with a nitrogen—phosphorus detection system (Model 18-789 A,

Hewlett-Packard, Avondale, PA, U.S.A.) and a flat bed recorder (BD 7, Kipp & Zn., Delft, The Netherlands) were used for the experiments. SCOT OV-275 capillary columns with a length of 7 m were prepared according to ref. 7. A ball-valve solid-sample injector as described in the literature [8] was used. The inlet and detector temperatures were set at 245°C and 300°C, respectively, while FDHU was chromatographed at an oven temperature of 195°C and 5-FU at 215°C. Helium was used for both carrier gas (12 ml/min) and make-up gas (30 ml/min).

Reagents

All reagents were of analytical grade (J.T. Baker Chemicals, Deventer, The Netherlands). Ethyl acetate was distilled twice; chloroform was washed twice with distilled water; methanol was used without any purification. 5-FU and FDHU were kindly supplied by Hoffmann-La Roche (Mijdrecht, The Netherlands). Diphenylsuccinimide (DPS) was synthesized by Chemische Industrie Katwijk (Katwijk, The Netherlands) and purified by recrystallization from tetrahydrofuran (THF). Chlorouracil (CU) was purchased from Calbiochem (Los Angeles, CA, U.S.A.).

The stock solutions of 5-FU (100 mg/l methanol), FDHU (10 mg/l ethyl acetate), DPS (250 mg/l ethyl acetate) and CU (100 mg/l methanol) were stored at 4°C.

Extraction

Polythene tubes were chosen to avoid loss of compound (5-FU and FDHU) by adsorption onto the surface [9, 10]. In order to remove interfering compounds, 0.2 ml of plasma with 100 ng of CU as internal standard was extracted twice with 3 ml of chloroform. Subsequently the plasma fraction was extracted twice with 3 ml of ethylacetate under vigorous shaking on a whirlmixer for 20 sec. In order to obtain a good phase separation, the mixture was centrifuged at 1000 *g* for 5 min. The ethyl acetate fractions were collected in a tube containing the external standard (DPS, 250 ng), to correct for injection volume variations, and dried under a gentle nitrogen stream at ambient temperature. The residue was dissolved in 100 μl of ethyl acetate and aliquots of 10 μl were brought onto the needle of the solid-sample injection system.

Patients and drug administration

Five hospitalized female patients, aged 57—72 years, treated for breast cancer with 5-FU (500 mg/m^2 intravenously), were sampled via an indwelling intravenous catheter in the arm opposite to that of 5-FU application. Blood samples, collected in heparinized polythene tubes, were taken at 0, 0.04, 0.08, 0.25, 0.5, 1.0, 1.5, 3.0, 4.5, 6.0, 7.5, 9.0, 12.0 and 24 h after drug administration. The samples were centrifuged at 1000 *g* within 1 h after sampling; the plasma was decanted and stored at —15°C until analysis.

RESULTS AND DISCUSSION

Chromatography of FDHU

The GC conditions were optimized with respect to temperature and

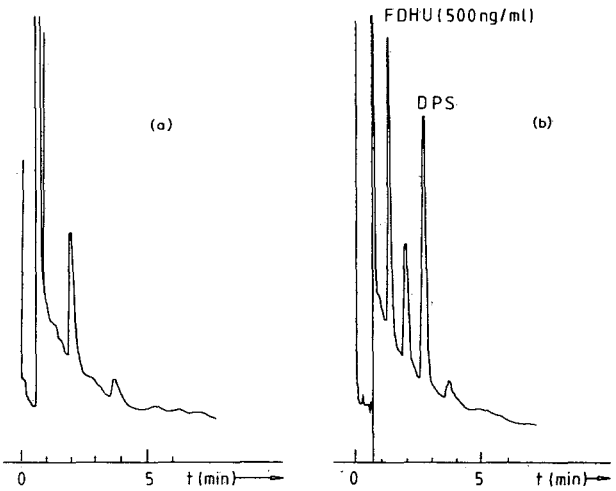

Fig. 2. (a) Chromatogram of an extract of blank plasma. (b) Chromatogram of an extract of plasma spiked with FDHU and DPS.

flow-rate. At 195°C an adequate separation of FDHU and DPS (external standard) was combined with a reasonable time of analysis. Under these conditions the peak shape is good (asymmetry factor, measured at 0.1 of peak height, smaller than 1.2), allowing a quantification based on peak height.

The detection limit based on a signal-to-noise ratio of 3:1 was determined to be 500 pg for FDHU. Fig. 2a and b shows the chromatograms of an extract of blank plasma and plasma spiked with FDHU, respectively. From the figures it can be seen that concentrations down to 100 ng/ml plasma can be determined easily.

Chromatography of 5-FU

If only 5-FU has to be determined, the column temperature can be increased to 215°C. At this temperature the retention times of 5-FU and CU amount to about 3 and 6 min, respectively, allowing a high throughput of samples. In spite of an adequate separation of FDHU, DPS, 5-FU and CU under these conditions, it is not possible to determine all components in one single run because of interfering peaks of the plasma matrix. Although 5-FU and FDHU can be determined in one single run by applying a temperature program, we prefer two isocratic runs for routine measurements.

Linearity and precision of the method

Quantification of FDHU. The linear dynamic range of the method was investigated by injecting different amounts of FDHU, directly as well as after extraction from spiked plasma. From Fig. 3, showing the relation between relative peak area of FDHU/DPS and FDHU concentrations, the recovery of the extraction can be calculated as the ratio of the slopes of the two calibration curves after direct injection and after extraction, respectively; this was about 60%. The coefficient of variation for the assay ranges from 6.6% (n = 3) at a level of 100 ng/ml to 1.6% at 5 μg/ml (n = 3).

Quantification of 5-FU. The calibration curve for 5-FU (Fig. 4) was con-

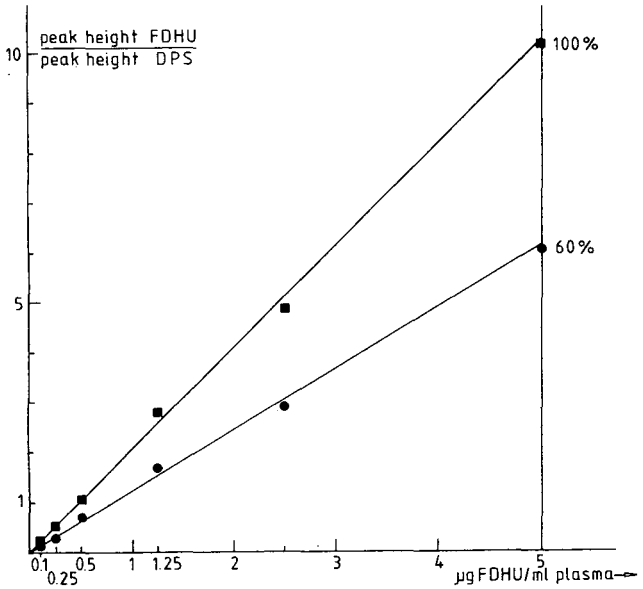

Fig. 3. Calibration curve for FDHU obtained after extraction from plasma (lower curve). The upper curve corresponds to 100% (direct injection).

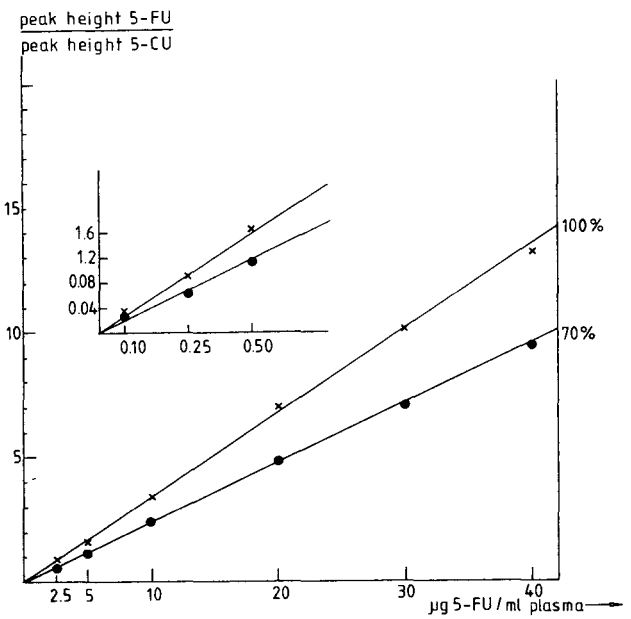

Fig. 4. Calibration curve for 5-FU obtained after extraction from plasma (lower curve). The upper curve corresponds to 100% (direct injection).

structed as described for FDHU. The recovery of 5-FU extracted from plasma amounted to about 70%, the absolute limit of detection being about 1 ng, resulting in measurable concentrations down to 150 ng 5-FU per ml plasma. The coefficient of variation of the assay ranges from 11% at a level of 100 ng/ml to 1.5% at 40 μg/ml.

In the case of the intravenous bolus injection of 5-FU it can be generally stated that samples taken just after administration have to be diluted to determine the 5-FU. These diluted samples cannot be used for determining FDHU. Plasma samples taken 15 min after 5-FU infusion can be used for the determination of both FDHU and 5-FU in one extract.

The described assay for 5-FU in plasma samples was compared with a method using a packed column, described in the literature [11]. There appeared to be a good correlation at levels higher than 2 μg/ml (correlation coefficient 0.988, slope 0.927, intercept —0.550, n = 46). However, at levels below 2 μg/ml the correlation was poor (correlation coefficient 0.768, slope 0.861, intercept —0.527, n = 12), which could be explained by an irreversible adsorption of 5-FU to the support material of the packed column. For this reason we prefer capillary columns. Owing to the wide range of physicochemical properties, relatively short capillaries can be used. Comparison of the developed method with an HPLC assay [12] shows a good correlation (correlation coefficient 0.994, slope 1.082, intercept —0.9962, n = 25).

Examples of FDHU plasma concentration—time curves of four patients treated with 5-FU are given in Fig. 5. Remarkably, a fifth patient had no detectable FDHU in the plasma at all. As can be seen, FDHU plasma profiles cannot be readily fitted by using common pharmacokinetic models.

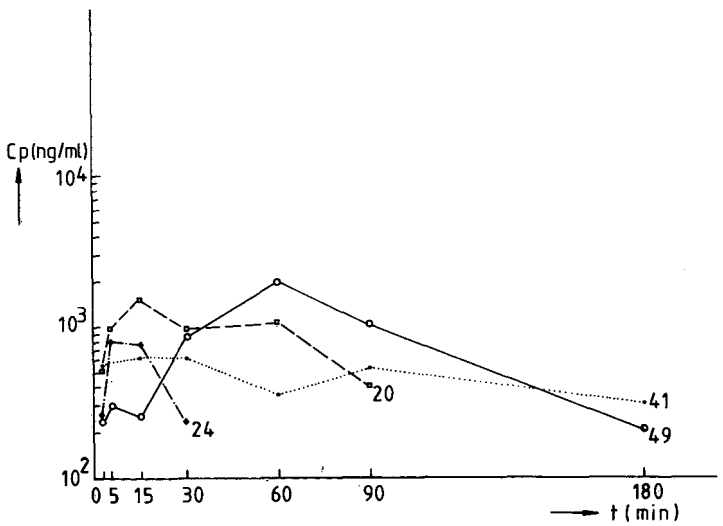

Fig. 5. Pharmacokinetic profiles of four patients treated with 5-FU (dose: 500 mg/m² intravenously).

CONCLUSION

The GC assay developed for the determination of 5-FU and FDHU in plasma can be used routinely for monitoring both compounds in plasma samples from patients treated with 5-FU.

ACKNOWLEDGEMENTS

We are grateful to Hoffmann-La Roche B.V. for furnishing us with FDHU and 5-FU. Our thanks also go to Prof. Dr. D.D. Breimer for his cooperation in this study, which was financially supported by the Koningin Wilhelmina Fonds and the Maurits and Anna de Kock Stichting.

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Journal of Chromatography, 278 (1983) 291—299
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1874

QUANTIFICATION OF AMITRIPTYLINE, NORTRIPTYLINE, AND 10-HYDROXY METABOLITE ISOMERS IN PLASMA BY CAPILLARY GAS CHROMATOGRAPHY WITH NITROGEN-SENSITIVE DETECTION

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(First received April 24th, 1983; revised manuscript received July 18th, 1983)

SUMMARY

A selective, sensitive method for the determination of amitriptyline and its metabolites is described. This method involves liquid—liquid extraction and capillary gas chromatography with nitrogen-sensitive detection. The detection limits of amitriptyline, nortriptyline, 10-hydroxy(*E*)amitriptyline, 10-hydroxy(*E*)nortriptyline, and 10-hydroxy(*Z*)nortriptyline were slightly less than 0.5 ng/ml in 1.0-ml plasma samples. The coefficients of variation for within-run and between-run analyses of samples containing 100 ng/ml were less than 12% and 9%, respectively. The method offers rapid analysis of individual isomers, increased sensitivity over high-performance liquid chromatographic methodology and the conveniences of the gas chromatographic technique.

INTRODUCTION

In recent years the tricyclic antidepressants (TCAD) have been widely used for the treatment of endogenous depression. Two of the most prescribed TCADs are amitriptyline (Ami) and its active desmethyl metabolite, nortriptyline (Nor). Amitriptyline metabolism in humans yields nortriptyline via N-demethylation, or 10-hydroxyamitriptyline (*E* or *Z* isomers) via hydroxylation. Nortriptyline, in turn, can be metabolized to 10-hydroxynortriptyline (*E* or *Z* isomers). These structures and metabolic pathways are illustrated in Fig. 1.

Plasma levels of amitriptyline and nortriptyline have been measured clinically in attempts to establish a therapeutic range of concentration in plasma [1]. However, correlations between steady-state plasma level and clinical response have been questioned [2, 3]. Low correlation can be attributed

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to many factors. Among these are activities of unmeasured metabolites, inaccuracies in measurements, differences in specimen collection and handling, and heterogeneity of response to drug.

Recently, studies have shown that 10-hydroxy metabolites of amitriptyline and nortriptyline possess pharmacological activities similar to their parent compounds in inhibiting norepinephrine and serotonin uptake in vitro. However, these metabolites have been shown to possess different degrees of pharmacological activity [4, 5]. If these 10-hydroxy metabolites exhibit pharmacological activity in vivo, the quantitation of these metabolites should be included in studies involving efficacy versus plasma levels.

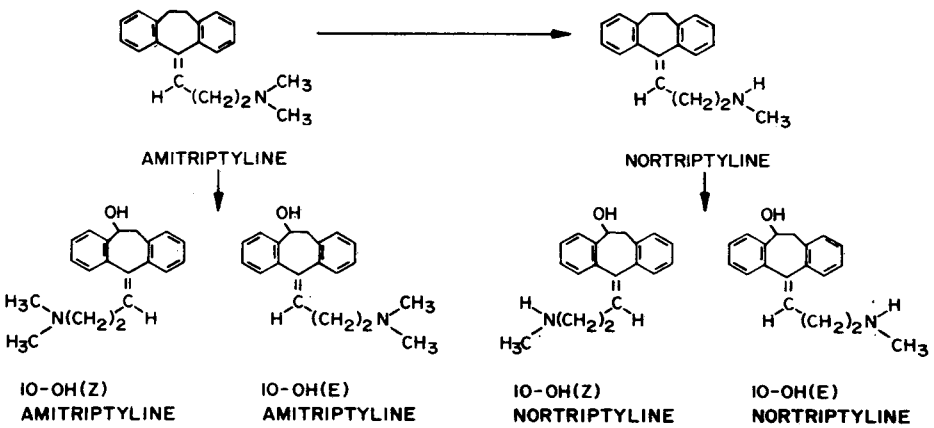


Fig. 1. Metabolic pathways of amitriptyline.

Methods have been developed to quantitate 10-hydroxy metabolites of amitriptyline and nortriptyline. Initial gas chromatographic (GC) methods [6—10] involved dehydration of these metabolites to unsaturated products having suitable GC properties since the isomers tail and are poorly resolved on conventional packed columns. In the case of 10-hydroxyamitriptyline, this dehydration of both *E* and *Z* isomers yielded the drug cyclobenzaprine (CAS-6202-23-9). These initial methods lacked the ability to distinguish and quantitate the individual isomers of 10-hydroxyamitriptyline or 10-hydroxynortriptyline. On the other hand, these individual isomers have been quantitated by recently developed high-performance liquid chromatography (HPLC) methodology [11—15]. Limits of sensitivity of these methods are approximately 5—10 ng/ml using 1—3 ml of serum.

In order to utilize the sensitivity of nitrogen—phosphorus detection and alternatively, the convenient interfacing for mass spectrometry afforded by GC methodology, we developed a GC method using fused-silica capillary columns. In this method we report an assay that offers greater sensitivity, superior resolution, and rapid analysis of amitriptyline, nortriptyline, and the 10-hydroxy isomers in physiological samples.

EXPERIMENTAL

Materials

Amitriptyline {10,11-dihydro-5-[3-(dimethylamino)propylidene]-5H-dibenzo-[*a*,*d*]cycloheptene hydrogen chloride} (CAS-549-18-8) and nortriptyline {10,11-dihydro-5-[3-(methylamino)propylidene]-5H-dibenzo[*a*,*d*]cycloheptene hydrogen chloride} (CAS-894-71-3) were obtained from the United States Pharmacopeial Convention (USPC). The hydroxy metabolites, 10-hydroxy-(*E*)amitriptyline {(*dl*)-(*E*)-10,11-dihydro-5-[3-(dimethylamino)propylidene]-5H-dibenzo[*a*,*d*]cyclohepten-10-ol} (CAS-64520-05-4), 10-hydroxy(*E*)nortriptyline {(*dl*)-(*E*)-10,11-dihydro-5-[3-(methylamino)propylidene]-5H-dibenzo[*a*,*d*]-cyclohepten-10-ol hydrogen maleate} (CAS-47132-16-1), and 10-hydroxy-(*Z*)nortriptyline {(*dl*)-(*Z*)-10,11-dihydro-5-[3-(methylamino)propylidene]-5H-dibenzo[*a*,*d*]cyclohepten-10-ol oxalate ethanolate} (CAS-47132-19-4) were synthesized by a previous method [16] and were gifts from Merck Sharp and Dohme (Rahway, NJ, U.S.A.). The 10-hydroxy(*Z*)amitriptyline isomer was unavailable. The two internal standards, protriptyline [5-(3-methylamino-propyl)-5H-dibenzo[*a*,*d*]cycloheptene hydrogen chloride] (CAS-1225-55-4) and chlorprothixene [2-chloro-9-(3-dimethylaminopropylidene)thioxanthene] (CAS-113-59-7) were also purchased from the USPC.

Reagents

Hydrochloric acid and sodium hydroxide were analytical grade. Hexane, 2-butanol, methanol, water and *n*-butyl acetate were HPLC solvent grade.

Standards

Stock standards of each compound were prepared as 1 mg free base per ml of methanol. All other working standards (1, 10, 100 ng/μl methanol) were prepared from these stock standards. A working solution containing both internal standards was diluted with water to obtain a concentration of 0.5 ng/μl.

Extraction procedure

For each analysis, 1 ml of plasma or serum was placed into a silanized 15-ml culture tube and a 100-μl aliquot of the internal standard solution containing protriptyline and chlorprothixene was added to produce a concentration of 50 ng/ml of plasma. Next, the solution was adjusted to pH 14 by addition of 1 ml of 4 *M* sodium hydroxide and was vortex-mixed for 10 sec. This mixture was extracted with 8 ml of hexane—2-butanol (98:2, v/v), mixed for 2 min, and centrifuged for 2 min. The hexane—2-butanol (upper phase) was transferred to a second silanized 15-ml culture tube. One ml of 0.001 *M* hydrochloric acid was added to the hexane—2-butanol that contained the extracted drug. This solution was mixed for 2 min and centrifuged for 2 min. Then the organic layer was aspirated and discarded. Using a borosilicate Pasteur pipette, the acid phase was transferred to a 15-ml conical-tipped tube containing 0.5 ml of 4 *M* sodium hydroxide and the solution was mixed for 10 sec. Next, a 100-μl aliquot of *n*-butyl acetate was added

to this mixture. This solution was mixed for 2 min and centrifuged for 2 min. Then most of the aqueous phase was withdrawn and discarded using a borosilicate Pasteur pipette. A fraction of the *n*-butyl acetate phase (0.5—8.0 μl) was removed and injected into the gas chromatograph.

Equipment

The gas chromatograph was a Hewlett-Packard 5710 equipped with a 18740B capillary injector and a 18789A nitrogen—phosphorus detector (Hewlett-Packard, Palo Alto, CA, U.S.A.). A Hewlett-Packard 3390A integrator was used that printed peak height values after each run.

The column was a 15 m × 0.32 mm I.D. Durabond® capillary column with DB-5 stationary phase (J & W Scientific, Rancho Cordova, CA, U.S.A.). The DB-5 stationary phase is a non-extractable bonded phase equivalent to SE-54. Injector and detector temperatures were 200°C and 300°C, respectively. The fused-silica injection port insert was silanized before use. After injection in splitless mode, the split was opened 40 sec later. The column temperature raised from 120°C to 250°C at 32°C/min upon injection. Helium (carrier) pressure to the column was 69 kPa resulting in a column linear velocity of 48 cm/sec. Detector gas flow-rates were hydrogen, 3 ml/min; air, 50 ml/min; and helium (make-up), 30 ml/min. Detector voltage offset was adjusted to 10% at attenuation 32 when the oven temperature was 250°C.

Quantitation

The internal standards, protriptyline and chlorprothixene, were incorporated into the specimens to aid in quantitation. Concentrations for specific compounds (amitriptyline, nortriptyline, or the 10-hydroxy metabolites) were determined from standard curves for each compound. These curves were derived from analyses of standard concentrations (10, 25, 50, 100, 250, 500, and 1000 ng/ml) added to drug-free plasma. The ratios of the peak height of each compound to that of protriptyline were plotted versus the standard concentrations of each compound. These standard curves were linear across the entire concentration range for all compounds.

Precision

To evaluate the precision of this method, within-run and between-run coefficients of variation (C.V.) were calculated. For both calculations, data were used from two concentrations (10 ng/ml and 100 ng/ml) of amitriptyline, nortriptyline and each 10-hydroxy metabolite in plasma. To estimate the within-run C.V., concentrations were calculated from six or seven assays of each of these two plasma samples using a single standard curve for each compound.

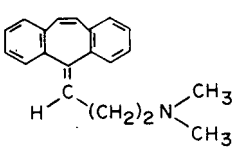
To calculate the between-run C.V., seven tubes of each of the two concentrations were frozen for analysis in seven subsequent runs. A standard curve was constructed for each run. Concentrations were determined in duplicate, and mean values from each of the seven runs were used to calculate the between-run C.V.

Efficiency

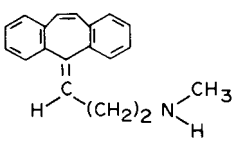
The analytical efficiency of the method was calculated using the internal standard, chlorprothixene. Reference standards were prepared containing known amounts of amitriptyline, nortriptyline, each of the 10-hydroxy metabolites and protriptyline in conical-tipped tubes. A known amount of chlorprothixene (50 ng) was added to each. Then *n*-butyl acetate was added to these reference standards to obtain a final volume of 100 μl. Plasma samples were prepared for extraction containing amounts of each drug identical to the reference standards. Samples of each concentration were extracted and reconstituted with 100 μl of *n*-butyl acetate containing 50 ng of chlorprothixene. Sample peak height ratios of each compound to chlorprothixene were compared to the peak height ratios from the reference standards to determine the percentage analytical efficiency.

Dehydration

To obtain dehydration of the 10-hydroxy metabolites, each metabolite was treated at room temperature for 2 h with 12 *M* hydrochloric acid to obtain the products illustrated in Fig. 2. The solutions were adjusted to pH 14 with 4 *M* sodium hydroxide, extracted and analyzed as above.



DEHYDRATED FORM OF
10-OH AMITRIPTYLINE



DEHYDRATED FORM OF
10-OH NORTRIPTYLINE

Fig. 2. Structures resulting from dehydration of 10-hydroxy metabolites of amitriptyline and nortriptyline.

RESULTS AND DISCUSSION

Since the TCAD and metabolites had terminal amines (secondary or tertiary), the extraction under basic conditions eliminated acidic materials while the subsequent acidic extraction eliminated neutral organic impurities. The final partitioning of drug into *n*-butyl acetate following alkalinization allowed injections to be made directly from this solvent. This technique obviated evaporation of samples to dryness, which requires a significant amount of time, introduces undesirable variation, and results in sample loss [17]. Chromatograms with the retention times of each of the compounds are displayed in Fig. 3 along with a chromatogram from drug-free plasma.

Standardization

Two internal standards, protriptyline and chlorprothixene, were added to all samples to normalize extraction efficiency in the quantitation of each compound. The former, a secondary amine, and the latter, a tertiary amine, were used due to their structural similarities to nortriptyline and amitrip-

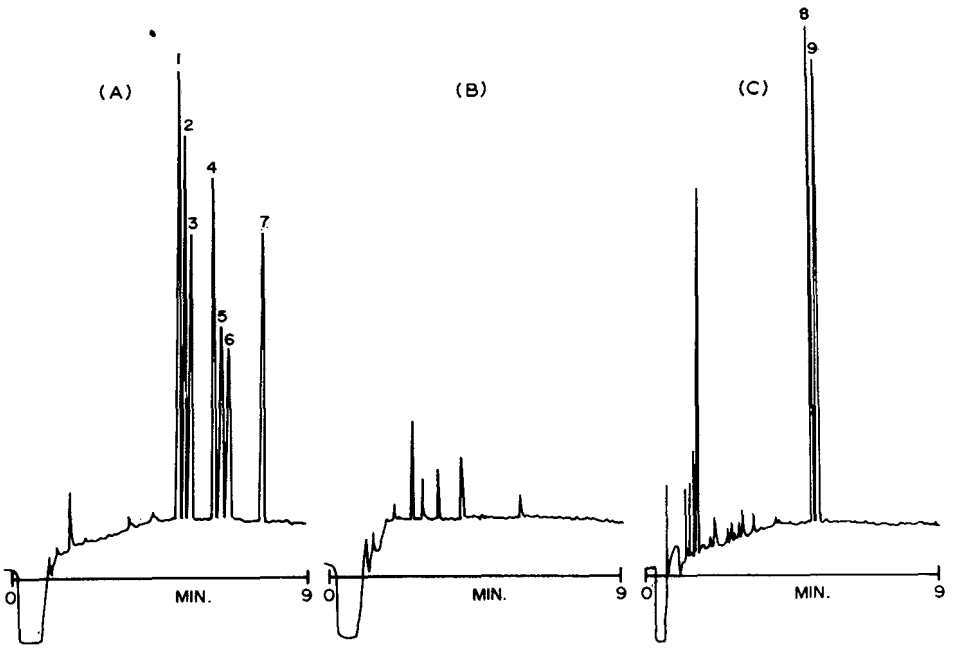


Fig. 3. Chromatograms of (A) amitriptyline, metabolites, and internal standards protriptyline and chlorprothixene; (B) drug-free plasma extracts; (C) dehydrated products from 10-hydroxyamitriptyline and -nortriptyline. Peaks: 1 = amitriptyline (5.05 min); 2 = nortriptyline (5.16 min); 3 = protriptyline (5.40 min); 4 = 10-hydroxy(*E*)amitriptyline (6.17 min); 5 = 10-hydroxy(*E*)nortriptyline (6.37 min); 6 = 10-hydroxy(*Z*)nortriptyline (6.50 min); 7 = chlorprothixene (7.66 min); 8 = dehydrated form of 10-hydroxyamitriptyline (5.35 min); 9 = dehydrated form of 10-hydroxynortriptyline (5.41 min).

tyline, respectively. We have found that protriptyline was the better internal standard for quantitation of all compounds as assessed by the standard curve linearity. Each line was fitted using least-squares regression analysis. Typical standard curves, using protriptyline as the internal standard are shown in Fig. 4.

Dehydration

Each of the 10-hydroxy metabolites has been shown to dehydrate readily in a strong acidic environment. A concentration of 0.01 *M* hydrochloric acid or greater produced detectable quantities of the dehydrated compounds in the chromatogram, as shown in Fig. 3. Note that the dehydrated products of 10-hydroxynortriptyline elute with protriptyline and interfere with the quantitation of every compound. On the other hand, we have found no detectable formation of dehydrated products with 0.001 *M* hydrochloric acid as used in our method. This allows individual quantitation of the 10-hydroxy isomers.

Precision

The precision of this method was determined using within-run and between-run C.V. of plasma samples containing 10 and 100 ng/ml of each com-

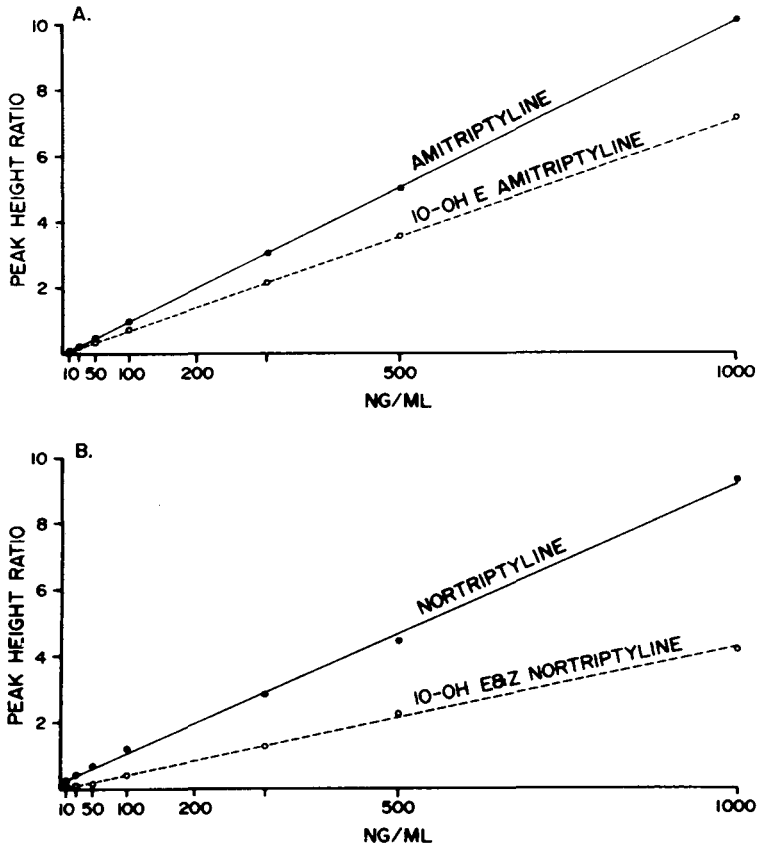


Fig. 4. Standard response curves for (A) amitriptyline and 10-hydroxy metabolite; (B) nortriptyline and 10-hydroxy metabolites.

TABLE I

WITHIN-RUN AND BETWEEN-RUN COEFFICIENTS OF VARIATION (%)

Concentration (ng/ml)	Ami	Nor	10-Hydroxy-(*E*)ami	10-Hydroxy-(*E*)nor	10-Hydroxy-(*Z*)nor
10*	10.0	17.0	13.8	14.0	10.0
100*	11.3	5.2	4.7	4.9	4.2
10**	21.8	17.0	17.8	14.0	20.0
100**	8.9	4.9	5.7	6.0	7.1

*Relative standard deviations of seven spiked samples at each concentration which were analyzed using a single standard curve for each compound.

**Relative standard deviations of means of duplicate spiked samples analyzed and standardized on seven different days.

pound, shown in Table I. The within-run C.V. values represent the variability of several samples using a single standard curve. Note that at the lower concentration, which approaches the limit of detection of HPLC methods, the maximum C.V. value was only 17.0%. At a typical therapeutic concentration, 100 ng/ml, the C.V. values were approximately 5% with the exception of amitriptyline.

The between-run C.V. values represent the variability of the means of duplicate samples determined using a different standard curve for each pair of samples. The C.V. values were 21.8% or less for a concentration of 10 ng/ml plasma and 8.9% or less for a concentration of 100 ng/ml plasma. This increase in relative variation includes variability contributed by the multiple standard curves and by sample-to-sample variation.

Efficiency

In calculating the analytical efficiency of our method, the peak height ratios of reference standards represented 100% extraction efficiency. Peak height ratios of extracted plasma samples, expressed as percentages of ratios from reference standards, are listed as analytical efficiencies in Table II. The average extraction efficiency from 10—1000 ng/ml plasma was greater than 80% for all compounds except for amitriptyline, which was 73%. These values indicate a reasonably efficient and consistent extraction throughout the entire concentration range. It is of interest to note that the extraction of amitriptyline was the least efficient as well as the most variable. No correction for analytical efficiency was applied in routine quantitation other than that implicit in standardization.

TABLE II

EXTRACTION EFFICIENCIES

Extracted spiked samples were compared to non-extracted reference standards.

Concentration (ng/ml)	Recovery (%)					
	Ami	Nor	10-Hydroxy-(*E*)ami	10-Hydroxy-(*E*)nor	10-Hydroxy-(*Z*)nor	Pro
10	81	92	90	88	112	122
25	60	73	80	90	90	84
50	76	82	81	83	80	89
100	64	80	71	103	106	96
250	79	83	82	79	80	87
500	72	87	89	88	92	85
1000	78	97	82	92	91	98
Mean	73	85	82	89	94	94
S.D.	8.0	8.0	6.3	7.6	10.9	13.3

Sensitivity

The detection limit of drug in plasma was somewhat less than 0.5 ng/ml. The peak heights at this concentration were greater than five times the baseline peak—peak noise for each compound.

In conclusion, we have developed a method to resolve and quantitate amitriptyline and metabolites by capillary GC. Advantages of our method include rapid analysis of individual isomers, increased sensitivity over HPLC, acceptable precision, and the conveniences of the GC technique including the potential for direct interfacing with mass spectrometers.

ACKNOWLEDGEMENTS

We thank Dr. Robert J. Madden for his technical assistance, Dr. John M. Yancey for his excellent suggestions with the statistics involved in this paper, and Drs. Edward L. Englehardt and David C. Remy of Merck Sharp and Dohme for the generous gift of the 10-hydroxy metabolites.

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Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1866

COMPARISON OF GAS CHROMATOGRAPHIC—ELECTRON-CAPTURE DETECTION AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE DETERMINATION OF BUTOFILOLOL IN BIOLOGICAL FLUIDS

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(First received April 25th, 1983; revised manuscript received July 18th, 1983)

SUMMARY

Two selective and sensitive methods for the quantitative analysis of butofilol in human plasma and urine are discussed. The first method is a gas chromatographic assay with electron-capture detection using extraction with toluene, several clean-up procedures and derivatization. The second method is based on high-performance liquid chromatography and a single extraction with dichloromethane. The two assay methods were applied to the determination of the same human plasma samples after administration of a single, oral 200-mg dose of butofilolol. A good correlation between the results (inter-laboratory comparison) is obtained, validating both techniques.

INTRODUCTION

Butofilolol (CAFIDE®), a new β-adrenoceptor blocking agent (Fig. 1), was found to be highly effective for the treatment of hypertension [1, 2]. Animal pharmacokinetic studies were performed in the baboon using intravenous and oral administration of the ^{14}C-labelled drug. Absorption was complete but, due to an extensive first-pass hepatic metabolism, relatively low plasma levels of the parent drug were observed after oral administration [3]. Therefore human pharmacokinetic investigations required a very sensitive and selective analytical procedure for the quantitative determination of the parent drug in plasma and urine.

First, a gas chromatographic (GC) assay with electron-capture detection (ECD) of the diheptafluorobutyrate derivative of butofilolol was developed. However, this method required several clean-up procedures and was time consuming. A more simple and rapid method was developed, using a single-step extraction with high-performance liquid chromatographic (HPLC) separation.

The GC–ECD and HPLC methods are described below. The precision and reproducibility of each procedure were evaluated. The two methods were applied to the analysis of butofilolol in identical plasma samples collected from a volunteer after a single, 200-mg oral administration of the drug. Comparison of the results allowed evaluation of the respective advantages and disadvantages of the two methods.

EXPERIMENTAL

Reagents

Organic solvents used were toluene, dichloromethane and methanol. All solvents were analytical grade and were obtained from Merck (Darmstadt, F.R.G.). Toluene was glass-distilled prior to use. The other solvents were used without prior distillation.

Heptafluorobutyric anhydride (Merck) was used for derivatization before GC–ECD analysis. The reagent was freshly purified by distillation over excess phosphorus pentoxide for 3 h, then kept under anhydrous conditions until use. Trimethylamine solution (1.2 *M* in toluene) was freshly prepared before each experiment from recrystallized trimethylammonium chloride (Merck).

The inorganic reagents were all prepared in distilled water. Sodium hydroxide, sodium bicarbonate and dipotassium hydrogen phosphate were obtained from Merck.

Standard solutions

Butofilolol as maleate (CM 6805 a) and its internal standard (CM 6859) (Fig. 1) were obtained from Sanofi Research/Center of Montpellier (France). Standard solutions of these compounds were freshly prepared each day at suitable dilution in phosphate buffer, pH 7.4.

Glassware

All glassware used in the extraction and derivatization procedures was washed with sulphochromic acid, rinsed with deionized water and finally dried

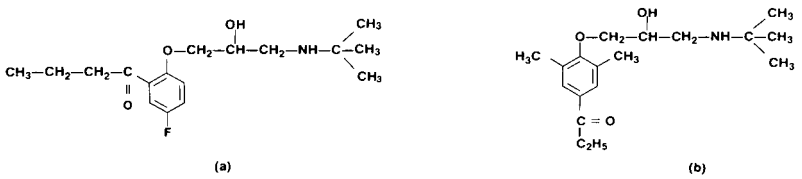

Fig. 1. Molecular structures of: (a) butofilolol and (b) its internal standard.

at 60°C. For the GC—ECD procedure, glassware was silanized with 2% dimethyldichlorosilane (Fluka, Paris, France) in toluene, rinsed with toluene and then methanol and dried at 60°C.

Sample preparation

GC—ECD procedure. A 1-ml sample of plasma or urine was added to a glass-stoppered 10-ml centrifuge tube containing 1 ml of an aqueous solution of the internal standard (500 ng) and 0.2 ml of 1.0 *M* carbonate buffer, pH 12 (1 *M* sodium carbonate—0.5 *M* sodium hydroxide). After shaking with 6 ml of toluene for 15 min and centrifuging at 2500 *g* for 5 min, 5 ml of the organic phase were transferred to another tube containing 4 ml of 0.1 *M* phosphate buffer, pH 2.0. This tube was shaken and centrifuged under the same conditions. Toluene was discarded and a 3-ml aliquot of the acidic phase was transferred to another tube, made alkaline (pH 12) with 0.2 ml of carbonate buffer (1 *M* Na_2CO_3—5 *M* sodium hydroxide) and re-extracted with 6 ml of toluene. After centrifugation, 5 ml of the toluene phase were transferred into a small glass tube and evaporated to dryness under a stream of nitrogen.

The dry residue was reconstituted in 1 ml of toluene and 0.1 ml of 1.5 *M* trimethylamine, then derivatized with 50 μl of heptafluorobutyric anhydride for 5 min in an ice-bath. Then excess of reagents was removed by clean-up with 3 ml of 0.5 *M* phosphate buffer, pH 6.0. The phases were separated by centrifugation, and 1–5 μl of the toluene phase were injected.

HPLC procedure. A 1-ml sample of plasma or urine was spiked with internal standard and made alkaline under the same conditions as described for the GC—ECD procedure in the first extraction step. Dichloromethane was used as organic solvent instead of toluene. Tubes were vortexed for 1 min and centrifuged at 2500 *g* for 15 min. A 5-ml aliquot of the organic layer was transferred to another tube. Extraction of the sample was repeated a second time under the same conditions, and a second 5-ml aliquot of the dichloromethane phase was separated. Then, the combined organic phase was evaporated to dryness under a stream of dry nitrogen at room temperature. The residue was dissolved in 50 μl of the mobile phase and injected.

Apparatus and chromatographic conditions

For GC—ECD analysis, a Hewlett-Packard 5710 A gas chromatograph equipped with a ^{63}Ni electron-capture detector was used. The glass column (180 × 0.4 cm I.D.) was filled with 3% OV-17 on Chromosorb W AW DMCS (100–120 mesh). The chromatograph was operated isothermally with the oven, detector and injection-port temperatures maintained at 230, 300 and 250°C,

respectively. The carrier gas was 10% methane in argon at a flow-rate of 30 ml/min. The chromatograph was connected to a recorder with a scale range of 1 mV (Sefram type 1.10 PE; Paris, France).

The mass spectrometric analysis of the butofilolol derivative was performed using a gas–liquid chromatograph coupled on-line to a mass spectrometer (Ribermag 10-10 B) operating in the chemical-ionization mode with ammonia as reagent gas.

HPLC analyses were performed using a 6000A pump, a U6K sample injector, a UV filter (313 nm) spectrometer M 440 equipped with an 8-μl capacity flow cell (all from Waters). A 250-mm steel column was used, packed with a monomolecular layer of octadecyltrichlorosilane, chemically bonded to Porasil beads with an average particle size of 10 μm (Bondapak C_{18}, Waters). The mobile phase was a mixture of methanol–water–Pic B 7 (Waters) (60:40:1) with a flow-rate of 2 ml/min.

All materials used throughout the analyses, especially those used for the GC–ECD procedure, were made of glass to avoid interaction between biological material and plasticizers [4].

Biological sampling

Blood (about 7 ml) from one patient receiving a single 200-mg oral dose of butofilolol was collected on heparinized glass centrifuge tubes. The plasma samples were immediately separated by centrifugation, then stored at −20°C until analyzed.

Calibration and quantitation

Quantitation of butofilolol was achieved on the basis of a calibration curve. Standard curves were run daily by spiking 1.0 ml of blank plasma with a known amount of internal standard and increasing amounts of butofilolol. A least-squares regression between concentration and peak-height ratios of the drug to the internal standard was calculated.

RESULTS AND DISCUSSION

GC–ECD method

The use of toluene as extraction solvent at pH 12 provided less interfering substances in the chromatogram in comparison to heptane, benzene or ethyl ether.

Due to the probable steric hindrance of the *tert.*-butyl radical on the nitrogen atom and the acyl radical in the *ortho*-position of the side chain of butofilolol, the derivatization step was critical and needed the use of a catalyst to improve the yield of the reaction. The acylation of butofilolol and its internal standard with heptafluorobutyric (HFB) anhydride provided derivatives with excellent properties for GC analysis and high response to electron-capture detection.

The reaction was improved using trimethylamine (TMA) in toluene at 0°C in an ice-bath. Under these conditions, the derivatization was optimal after 5 min. The structures of the HFB derivatives were confirmed by mass spectrometry with chemical ionization (Fig. 2). The molecular ion of the

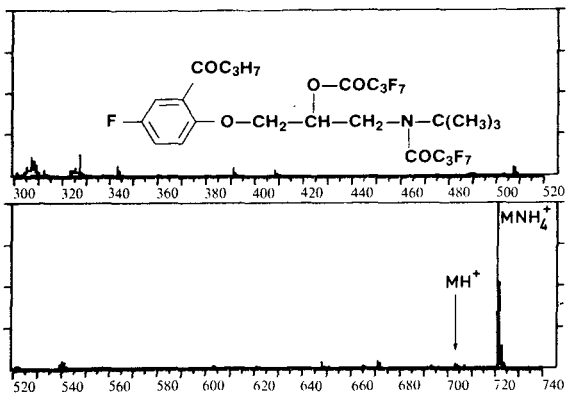

Fig. 2. Mass spectrum of the HFB derivative of butofilolol.

parent drug derivative observed at *m/e* 721 was consistent with the derivatization product proposed.

Attention should be paid during extraction and derivatization steps when handling solutions of the drug in its basic form in solvents of low polarity such as toluene. Indeed, adsorption on glass walls was observed, and the use of pre-treated glassware was necessary. Consequently, all glassware was silanized to avoid irreproducible losses by adsorption.

The overall recovery (extraction from plasma and derivatization) was estimated to be 85—90%. This yield was constant over the concentration range to be considered (10—2000 ng/ml). Usually, the calibration graphs were made in the concentration range 25—500 ng/ml with 500 ng of internal standard. A least-squares regression analysis of the fit between the peak-height ratios of the sample substance and the internal standard versus amounts of substance added was applied. An example of such a regression line was: $y = 0.03349x - 0.02203$, with a correlation coefficient, r, of 0.9999, where y corresponds to the peak-height ratio and x to the drug concentration. The calibration curves were linear within the range used. The limit of detection of butofilolol was estimated to be 20 ng/ml.

Examples of chromatograms obtained after analysis of blank plasma and spiked plasma are illustrated in Fig. 3. Under the chromatographic conditions used, the two peaks corresponding to butofilolol and internal standard derivatives were well resolved. No interfering peaks appeared in the analysis of plasma samples from several patients.

The results of the reproducibility are reported in Table I. The coefficient of variation ranged from 13.1 to 1.5%. The average reproducibility of the assay over the concentration range studied was 6.3%.

HPLC method

Examples of HPLC chromatograms are shown in Fig. 4. No interfering peaks due to endogenous compounds were observed.

A typical standard curve obtained responded to the following equation: $y = (0.0047 \pm 0.00001)x + (0.15 \pm 0.10)$, where y corresponds to the peak-height ratio and x to the drug concentration, with a correlation coefficient, r,

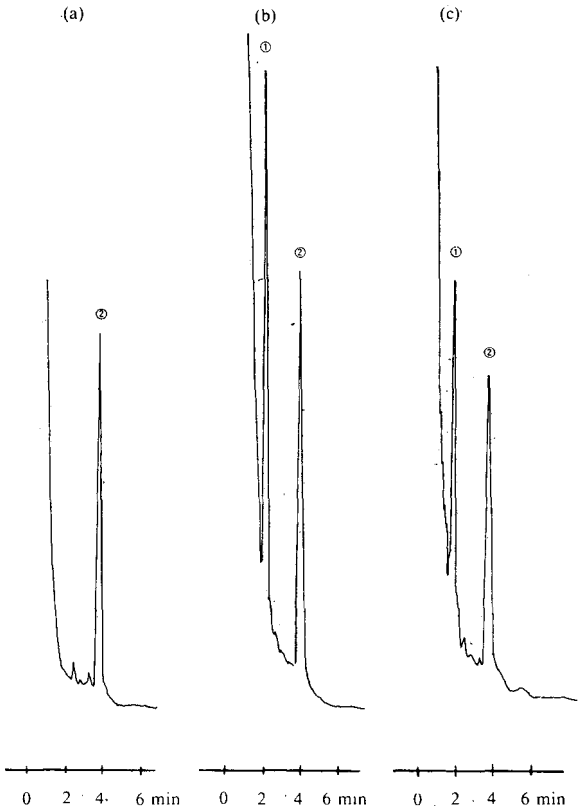

Fig. 3. GC—ECD chromatograms from human plasma samples: (a) blank control; (b) spiked with 250 ng of butofilolol and 250 ng of internal standard; and (c) plasma obtained from a patient receiving a 100-mg oral dose of butofilolol. 1 = Internal standard; 2 = butofilolol.

TABLE I

PRECISION AND ACCURACY OF THE ANALYSIS OF BUTOFILOLOL USING THE GAS CHROMATOGRAPHIC—ELECTRON-CAPTURE METHOD

Theoretical concentration of butofilolol (ng/ml)	Measured concentration of butofilolol (ng/ml)*	Coefficient of variation (%)
50	54.2 ± 7.1	13.1
100	98.8 ± 5.9	5.9
200	190 ± 9.3	4.9
500	463 ± 7.0	1.5

*Values are the mean ± the standard deviation (n = 7).

of 0.9995. These data indicated that the peak-height ratio at the origin was not significantly different from zero. This confirmed the absence of endogenous interfering peaks and the linearity of the detector response within the concentration range studied.

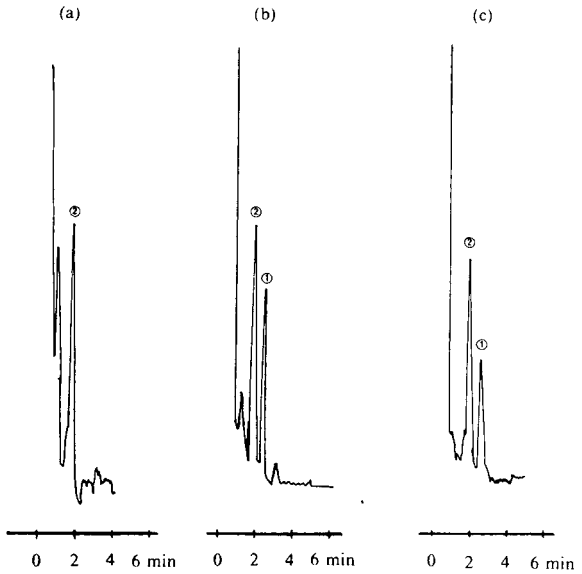

Fig. 4. Typical HPLC chromatograms of human plasma: (a) before administration (control); (b) spiked with 100 ng/ml of butofilolol; (c) plasma obtained from a patient receiving a 200-mg oral dose of butofilolol. 1 = Butofilolol; 2 = internal standard.

Extraction recovery was calculated using the peak-height ratio obtained after direct injection of pure solutions and after extraction. The extraction recovery decreased from 85% for the lowest concentration (20 ng/ml) to 67% for the highest one (2000 ng/ml).

The recovery of the drug from the entire procedure was determined from spiked plasma samples (n = 10) at several concentrations (Table II). Mean results appeared to be very close to the theoretical concentrations, showing suitable recovery and accuracy. The standard deviation was at a minimum of 3.1% for the 2000 ng/ml level. The limit of detection was about 20 ng/ml.

TABLE II

PRECISION AND ACCURACY OF THE ANALYSIS OF BUTOFILOLOL USING THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD

Theoretical concentration of butofilolol (ng/ml)	Measured concentration of butofilolol (ng/ml)*	Coefficient of variation (%)
20	23.5 ± 3.4	14.3
50	48.6 ± 6.4	13.2
100	117.3 ± 10.6	9.0
200	191.4 ± 12.2	6.4
500	501.8 ± 43.9	8.7
1000	998.0 ± 46.0	4.6
2000	2000.0 ± 62.0	3.1

*Values are the mean ± the standard deviation (n = 10).

Comparison of GC—ECD and HPLC methods

The applicability of the two analytical methods was assessed and compared using the same set of human plasma samples. Fig. 5 summarizes the results obtained for the samples collected after a single, 200-mg oral administration of butofilolol. A fit for the data from Fig. 5 was obtained by linear regression. The equation obtained was $y = 1.148x - 32.2$, where y = concentration obtained after the GC-ECD analysis and x = concentration obtained after HPLC analysis; the correlation coefficient, r, was 0.991 ($p < 0.001$) ($n = 14$). This good correlation indicated that the two methods gave very similar results.

An example of the time course of the butofilolol concentration in plasma in one subject was shown in Fig. 6. The maximum plasma level of about 350 ng/ml was reached 1.25 h after administration, showing the rapid absorption of the drug from the gastrointestinal tract. The plasma concentration—time

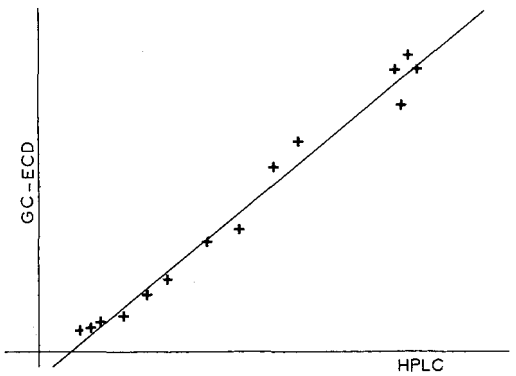

Fig. 5. Least-squares regression line between butofilolol concentrations measured in the same samples by the two different methods.

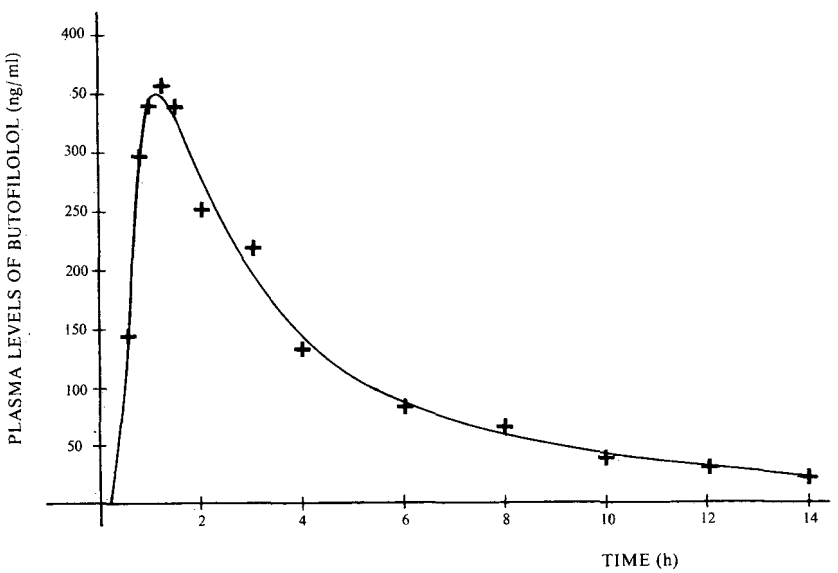

Fig. 6. Plasma profile of butofilolol in a patient following a single, 100-mg oral dose.

curve was adequately fitted to an open two-compartment pharmacokinetic model. The apparent elimination half life of the drug was about 2.4 h. This figure demonstrates that the two methods are sensitive enough for quantitative determination of butofilolol in biological samples. The limit of detection of butofilolol was similar for the two methods (20 ng/ml).

The inter-laboratory comparison of butofilolol plasma levels was very satisfactory considering that two different techniques were used on the same samples. The sensitivity and precision of the two methods were in the same order of magnitude. However, the GC—ECD method requires back extraction, derivatization and several clean-up procedures, and was also time consuming. The HPLC technique appears to be easier for human pharmacokinetic and drug monitoring studies of butofilolol.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. R. Roncucci and Dr. W. Cautreels for their valuable help in the preparation of the manuscript.

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Journal of Chromatography, 278 (1983) 311—319
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1854

SIMULTANEOUS HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF OMEPRAZOLE AND ITS SULPHONE AND SULPHIDE METABOLITES IN HUMAN PLASMA AND URINE

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(First received April 15th, 1983; revised manuscript received July 13th, 1983)

SUMMARY

Omeprazole, a substituted benzimidazole which suppresses gastric acid secretion, and its sulphone and sulphide metabolites were simultaneously measured in human plasma and urine using a selective, reversed-phase, high-performance liquid chromatographic method with a sensitivity of 5 ng/ml for omeprazole, 30 ng/ml for omeprazole sulphone, and 50 ng/ml for omeprazole sulphide. The coefficients of variation for within-day assays were 4.4, 7.5, and 17.5%, respectively. In a pilot pharmacokinetic study, 40 mg of omeprazole (encapsulated enteric-coated granules) were administered to two healthy volunteers. Peak plasma concentrations for omeprazole of 240 and 520 ng/ml, and for omeprazole sulphone of 320 and 400 ng/ml, were reached between 3 and 4 h post-dose. Omeprazole concentrations fell rapidly with apparent half-lives of about 40 min, and concentrations of both omeprazole and the sulphone metabolite were below the minimal detectable level by 6—8 h. Omeprazole sulphide could not be detected in this study.

0378-4347/83/$03.00

INTRODUCTION

Substituted benzimidazoles are novel inhibitors of gastric acid secretion in animals and man [1, 2]. They appear to act by selectively inhibiting the hydrogen/potassium ($H^+ + K^+$)-ATPase [3] in the secretory canalicular membrane of the parietal cell [4]. This ($H^+ + K^+$)-ATPase is thought to be involved in gastric hydrogen ion transport from the cell to the lumen [5]. One of these substituted benzimidazoles, omeprazole, is currently being investigated in man. This drug is effective in the control of gastric acidity in Zollinger–Ellison syndrome patients not responding satisfactorily to histamine H_2-receptor antagonists [6]. At present there are no published methods for measuring this drug in biological samples.

This report describes a high-performance liquid chromatographic (HPLC) assay for the determination of omeprazole in human plasma and urine, which is also suitable for the simultaneous measurement of the sulphone and sulphide metabolites. This method allowed analysis of plasma and urine samples obtained from pilot pharmacokinetic studies in two volunteers.

EXPERIMENTAL

Reagents

Pure samples of omeprazole (H168/68, 5-methoxy-2-{[(4-methoxy-3,5-dimethyl-2-pyridinyl)-methyl] sulphoxide}-1H-benzimidazole), omeprazole sulphone (H168/66, 5-methoxy-2-{[(4-methoxy-3,5-dimethyl-2-pyridinyl)-methyl] sulphone}-1H-benzimidazole), omeprazole sulphide (H168/22, 5-methoxy-2-{[(4-methoxy-3,5-dimethyl-2-pyridinyl)-methyl]sulphide}-1H-benzimidazole), and internal standard (H168/24, 5-methyl-2-{[(4-methoxy-3,5-dimethyl-2-pyridinyl)-methyl] sulphoxide}-1H-benzimidazole) were provided by Astra Pharmaceuticals (North Ryde, Australia) (Fig. 1). The HPLC mobile phase contained analytical grade methanol, triethylamine, phosphoric acid, and glass-distilled water.

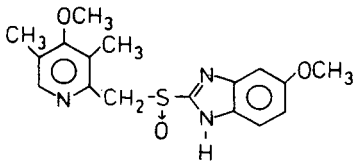
Instrumentation

A constant-flow high-pressure liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) was used for all assays. This consisted of a solvent delivery system (Model 6000A), a universal injector (Model U6K), and a variable-wavelength ultraviolet absorbance detector at 302 nm (Model 480). The reversed-phase plastic column was obtained prepacked (Rad Pak A; C_{18} 10-μm particles, 100 mm × 8 mm I.D.; Waters Assoc.) and was housed in a radial compression module (RCM-100; Waters Assoc.) which maintained external column pressure at approximately 370 kPa.

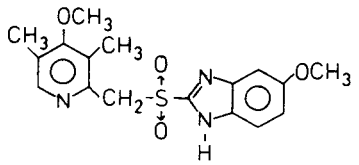
Calibration standards

Samples of pooled drug-free plasma were spiked with a mixture of pure omeprazole, omeprazole sulphone, and omeprazole sulphide (1000 ng/ml, 500 ng/ml, and 500 ng/ml respectively) dissolved in polyethylene glycol 400. Plasma was buffered by adding 4 μl of 1.0 *M* sodium carbonate per

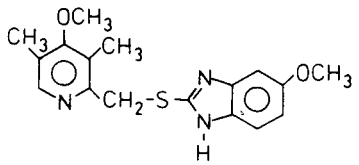
Omeprazole



Omeprazole-sulphone



Omeprazole-sulphide



Internal Standard H168/24

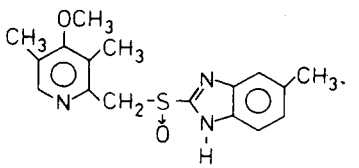


Fig. 1. Structural formulae of omeprazole, omeprazole metabolites and internal standard.

ml of plasma, and plasma standards were obtained by double dilutions with drug-free plasma. The concentrations of these standards were 500 ng/ml, 250 ng/ml, and 125 ng/ml for omeprazole and 250 ng/ml, 125 ng/ml, and 62.5 ng/ml for omeprazole sulphone and omeprazole sulphide. Similarly, drug-free urine (buffered with 4 μl of 1.0 *M* sodium carbonate per ml of urine) was spiked with pure omeprazole (10 μg/ml), omeprazole sulphone (5 μg/ml) and omeprazole sulphide (5 μg/ml). Urine standards were then prepared by dilution. The final concentrations were 5 μg/ml, 2.5 μg/ml and 1.25 μg/ml for omeprazole, and 2.5 μg/ml, 1.25 μg/ml, and 0.625 μg/ml for omeprazole sulphone and omeprazole sulphide. All standards were stored at −20°C. Calibration curves were prepared by plotting the relationship between the peak height ratio of omeprazole, omeprazole sulphone, or omeprazole sulphide to the internal standard (H168/24).

Analytical recovery

Recoveries of omeprazole, omeprazole sulphone and omeprazole sulphide were estimated by comparing the peak height of an extracted plasma sample containing a known amount of the substance with the peak height of an aqueous solution containing the same amount of each compound.

Extraction of plasma

Internal standard (H168/24, 40 μg/ml, 50 μl), and dichloromethane (10 ml) were added to 1.0 ml of plasma in a 30-ml glass tube. After vortex mixing (60 sec) and centrifugation (1500 *g*, 10 min) the organic layer was carefully transferred to a second tube and evaporated under a gentle stream of nitrogen at 45°C. The residue was reconstituted in 100 μl of the chromatographic mobile phase, and 40 μl were injected into the liquid chromatograph. The samples were chromatographed on the day of extraction.

Extraction of urine

The internal standard (H168/24, 40 μg/ml, 250 μl) and dichloromethane (10 ml) were added to 1.0 ml of urine in a 30-ml glass tube, and the sample was treated as described for plasma. Reconstitution and injection were also the same as for plasma.

Chromatography

The mobile phase was methanol—water (60:40) containing 1% triethylamine, and was adjusted to pH 7 with phosphoric acid (85%, v/v). The flow-rate was 3 ml/min at a back pressure of approximately 340 kPa. The approximate retention times were omeprazole 3.6 min, omeprazole sulphone 3.1 min, omeprazole sulphide 7.7 min, and internal standard 4.9 min.

Patient sampling

Two healthy male volunteers (25 and 22 years, 74 and 73 kg) were given single oral doses of omeprazole (40 mg) as two 20-mg capsules containing enteric-coated granules. Food and beverages were witheld for 8 h prior to and 3 h after dosage. Venous blood samples (10 ml) were withdrawn immediately before dosage, and at intervals up to 24 h. Each sample was collected in a heparinized tube, cooled for at least 5 min, and then centrifuged for 10 min. The plasma phase was transferred to plastic tubes containing 20 μl of 1.0 *M* sodium carbonate, and stored at −20°C. Urine was also collected pre-dose and serially to 4, 8, 12, and 24 h. Urine samples were buffered after collection by the addition of 4 μl of 1.0 *M* sodium carbonate per ml of urine, and stored at −20°C.

Pharmacokinetic calculations and statistical analysis

Coefficients of variation were calculated from the ratio of values of the standard deviation to the mean. Correlation coefficients of calibration curves were determined by least-squares regression analysis. Plasma omeprazole concentration versus time data were subjected to non-linear least-squares regression analysis [7].

RESULTS AND DISCUSSION

Chromatograms of drug-free plasma, spiked plasma, and plasma from a volunteer who had received omeprazole are shown in Fig. 2. Those of blank, spiked, and volunteer urine are shown in Fig. 3. These chromatograms show distinct, well-resolved peaks for omeprazole, omeprazole sulphone, omeprazole

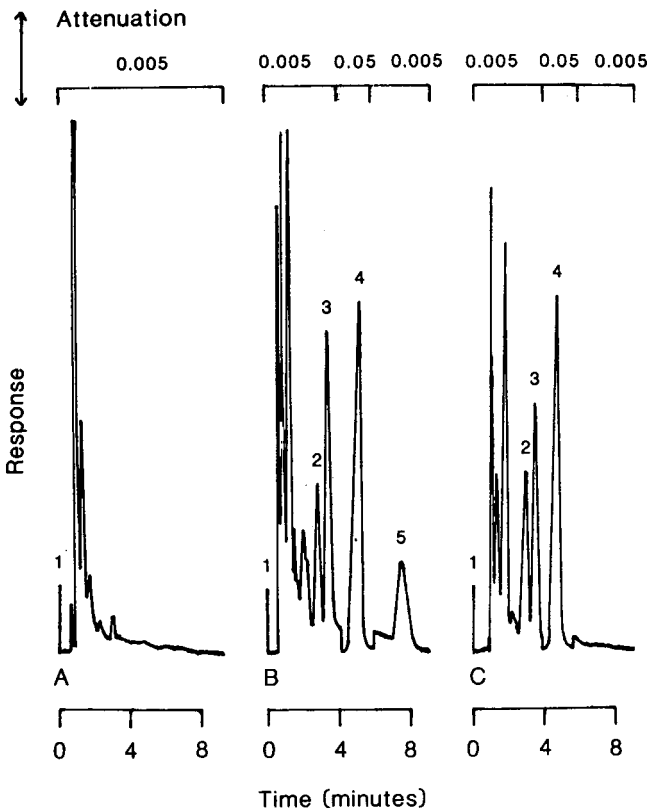


Fig. 2. Chromatogram of (A) a blank plasma extract, (B) a spiked plasma extract (omeprazole concentration = 125 ng/ml, omeprazole sulphone and omeprazole sulphide concentrations = 62.5 ng/ml), and (C) an extract of plasma sample from a patient who received a single dose of omeprazole (omeprazole concentration = 93 ng/ml and omeprazole sulphone concentration = 69 ng/ml). 1 = Injection; 2 = omeprazole sulphone; 3 = omeprazole; 4 = internal standard, H168/24; 5 = omeprazole sulphide.

sulphide, and internal standard. The minimum detectable level (defined as three times baseline noise) in plasma was 5 ng/ml for omeprazole, 30 ng/ml for omeprazole sulphone and 15 ng/ml for omeprazole sulphide.

The chromatogram of blank plasma shows a small endogenous component with a retention time similar to that for the metabolite, omeprazole sulphone. This peak was never seen to exceed 3—4% of full-scale deflection on the highest detector sensitivity used (× 0.005 a.u.f.s.), and did not interfere in the determination of omeprazole. Measurements of omeprazole sulphone in concentrations less than 30 ng/ml were unreliable due to its presence. However, it did not appreciably affect the accuracy or reproducibility of the determinations above this concentration (Table I).

Calibration curves of peak height ratios (i.e. peak height of drug to peak height of internal standard) against concentrations of omeprazole, omeprazole sulphone, and omeprazole sulphide, all showed linearity for both plasma and urine ($r \geqslant 0.999$ for all substances in plasma and urine). Analytical re-

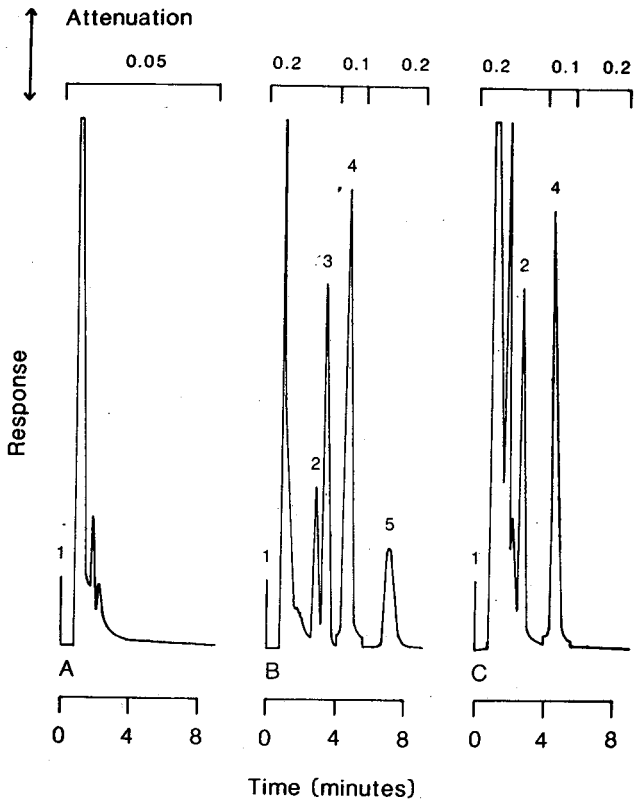


Fig. 3. Chromatogram of (A) a blank urine extract, (B) a spiked urine extract (omeprazole concentration = 5 μg/ml, omeprazole sulphone and omeprazole sulphide concentrations = 2.5 μg/ml), and (C) an extract of a urine sample from a patient who received a single dose of omeprazole (omeprazole sulphone concentration = 6.1 μg/ml). 1 = Injection; 2 = omeprazole sulphone; 3 = omeprazole; 4 = internal standard, H168/24; 5 = omeprazole sulphide.

coveries from plasma were 95% for omeprazole, 99% for omeprazole sulphone, and 82% for omeprazole sulphide.

The within-day, and day-to-day precision of the assay was determined for each of the substances by replicate assays of aliquots of the same sample (Table I). There was little variability in the assays for omeprazole and omeprazole sulphone, with coefficients of variation below 8%. By contrast the results for replicate assays of the sulphide metabolite were more variable at the concentrations tested. The accurate determination of sulphide levels is therefore limited to concentrations above 50 ng/ml. There was no appreciable deterioration of substances in either plasma or urine with storage at −20°C for two months.

The assay method was applied to the analysis of samples obtained from a pilot pharmacokinetic study in two healthy volunteers. The plasma concentration—time profiles of omeprazole and omeprazole sulphone are shown for each subject in Fig. 4 and 5. In no samples were the plasma levels of ome-

TABLE I

COEFFICIENTS OF VARIATION FOR WITHIN-DAY AND DAY-TO-DAY ASSAYS FOR OMEPRAZOLE, OMEPRAZOLE SULPHONE AND OMEPRAZOLE SULPHIDE IN HUMAN PLASMA AND URINE

Compound	Mean drug concentration (n = 5)	Coefficient of variation (%)	
		Within-day	Day-to-day
Plasma			
Omeprazole	104.7 ng/ml	4.4	2.7
Omeprazole sulphone	77.5 ng/ml	7.5	5.5
Omeprazole sulphide	47.5 ng/ml	17.5	23.4
Urine			
Omeprazole	1.25 μg/ml	1.7	2.7
Omeprazole sulphone	0.64 μg/ml	1.8	1.8
Omeprazole sulphide	0.63 μg/ml	2.0	3.3

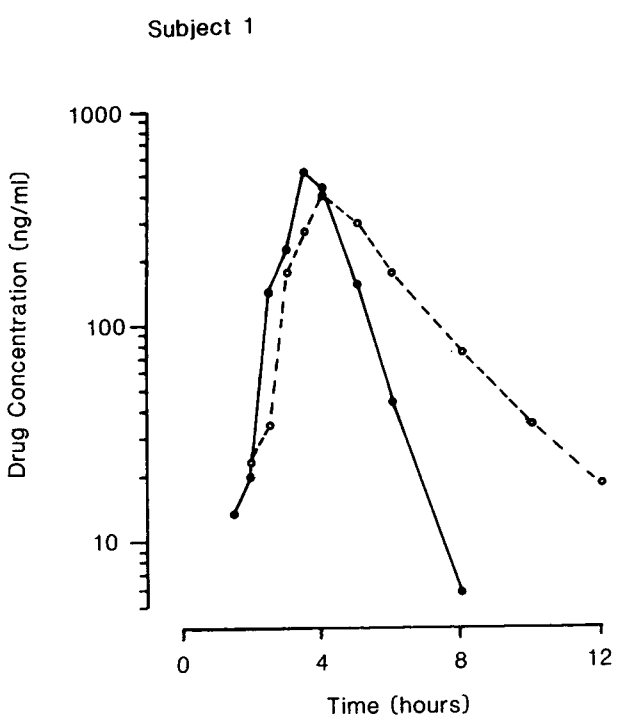


Fig. 4. Plasma levels of omeprazole (•) and omeprazole sulphone (○) following 40-mg oral dosage (subject 1).

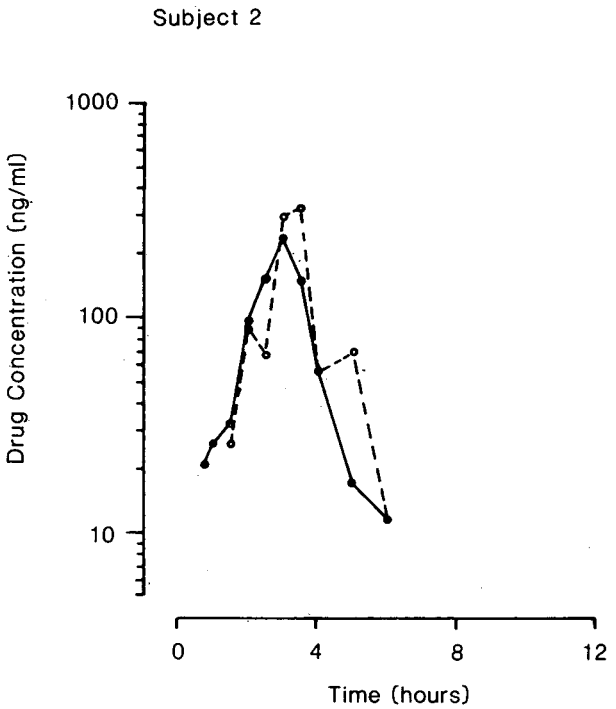


Fig. 5. Plasma levels of omeprazole (•) and omeprazole sulphone (○) following 40-mg oral dosage (subject 2).

TABLE II

OMEPRAZOLE AND OMEPRAZOLE SULPHONE PHARMACOKINETIC PARAMETERS CALCULATED FOR SUBJECTS 1 AND 2, AFTER 40-mg ORAL DOSAGE

Pharmacokinetic parameters*	Subject 1	Subject 2
Omeprazole		
T_{lag} (h)	1.8	1.7
C_{pk} (ng/ml)	519.0	239.6
T_{pk} (h)	3.5	3.0
k_a (h^{-1})	0.81	1.27
β (h^{-1})	1.05	1.06
$t_{½\beta}$ (h)	0.66	0.65
$AUC_{0\to\infty}$ (ng h ml^{-1})	1024.3	429.4
Omeprazole sulphone		
C_{pk} (ng/ml)	400.6	317.7
T_{pk} (h)	4.0	3.5
β (h^{-1})	0.42	1.08
$t_{½\beta}$ (h)	1.64	0.64

*T_{lag} = lag time; C_{pk} = peak plasma concentration; T_{pk} = time to reach peak plasma concentration; k_a = absorption rate constant; β = elimination rate constant; $t_{½\beta}$ = elimination phase half-life; $AUC_{0\to\infty}$ = area under plasma concentration—time curve from time 0 to infinity.

prazole sulphide in excess of the minimum detectable concentration for this metabolite [in subsequent chronic studies this metabolite has been identified at low concentrations in human plasma (unpublished observations)]. The resultant pharmacokinetic parameters for omeprazole and omeprazole sulphone are summarised in Table II. After an initial lag, absorption of omeprazole was rapid with peak levels being reached at approximately 3.25 h. Thereafter, omeprazole plasma levels fell monoexponentially with a short apparent elimination half-life of 39 and 40 min. Plasma levels of omeprazole could be followed for 5—6 half-lives before they fell below the minimum detectable concentration of 5 ng/ml by 8 h post-dose.

Omeprazole undergoes rapid metabolism in part to omeprazole sulphone, the levels of which follow a similar pattern to that seen with omeprazole. This metabolite was eliminated with apparent half-lives of 98 and 38 min.

Neither omeprazole nor omeprazole sulphide were detected in any urine samples; 2.3% and 2.5% of the dose were recovered in urine as omeprazole sulphone.

In summary, the proposed method is sufficiently sensitive, selective and precise for the quantitation of omeprazole and its sulphone and sulphide metabolites in human plasma and urine.

ACKNOWLEDGEMENT

This work was supported in part by the National Health and Medical Research Council of Australia.

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Journal of Chromatography, 278 (1983) 321—328
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1860

DETERMINATION OF A NEW ANTIBACTERIAL AGENT (AT-2266) AND ITS METABOLITES IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received May 5th, 1983; revised manuscript received June 30th, 1983)

SUMMARY

A high-performance liquid chromatographic method has been developed which enables accurate determination of a new synthetic antibacterial agent, AT-2266, and its metabolite, M-2, in plasma, and AT-2266 and its five metabolites, M-1, M-2, M-3, M-4 and M-5, in urine. AT-2266 is extracted as ethyl carbamate with chloroform containing 1% ethyl chloroformate and assayed on a liquid chromatograph equipped with an ultraviolet detector at 340 nm.

Accurate determinations are possible over a concentration range of 0.1—10 μg/ml AT-2266 in plasma, and 1—500 μg/ml AT-2266 in urine. The coefficient of variation at the 2 μg/ml level of AT-2266 is 1.9% (n = 6). The minimum detectable concentrations of AT-2266 in plasma and urine are 0.01 μg/ml and 0.1 μg/ml, respectively, and those of other metabolites are similar to those of AT-2266. Plasma levels and urinary excretion of AT-2266 in a man following single oral administration (400 mg) have also been determined.

INTRODUCTION

The compound 1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-1,8-naphthyridine-3-carboxylic acid, AT-2266 ([1], Fig. 1), is a new potent synthetic antibacterial agent.

After oral dosing of AT-2266 in humans the unchanged drug and a metabolite, M-2 (Fig. 1), were detected in plasma, and four other metabolites, M-1, M-3, M-4 and M-5, were also detected in minute quantities together with the unchanged drug and M-2 in urine [2]. In order to study the pharmacokinetics of AT-2266, simple and selective assay methods for unchanged drug and its metabolites are necessary.

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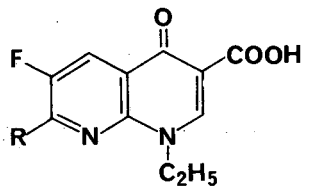
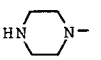
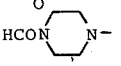
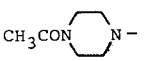
AT-2266 and its Metabolites



Compound	R-
AT-2266	
M-1	H_2N-
M-2	
M-3	
M-4	
M-5	$NH_2CH_2CH_2NH-$

Internal Standard

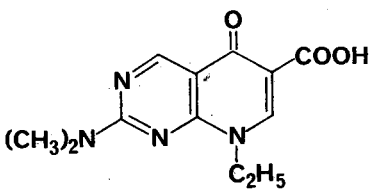


Fig. 1. Chemical structures of AT-2266, its metabolites and the internal standard.

This paper describes a high-performance liquid chromatographic (HPLC) method with a good reproducibility and specificity for determining AT-2266 and its metabolites in plasma and urine.

EXPERIMENTAL

Chemicals and reagents

AT-2266, M-1 (7-amino-1-ethyl-6-fluoro-1,4-dihydro-4-oxo-1,8-naphthyridine-3-carboxylic acid), M-2 [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(3-oxo-1-piperazinyl)-1,8-naphthyridine-3-carboxylic acid], M-3 [1-ethyl-6-fluoro-7-(4-formyl-1-piperazinyl)-1,4-dihydro-4-oxo-1,8-naphthyridine-3-carboxylic acid], M-4 [7-(4-acetyl-1-piperazinyl)-1-ethyl-6-fluoro-1,4-dihydro-4-oxo-1,8-naphthyridine-3-carboxylic acid] and M-5 [7-(2-aminoethylamino)-1-ethyl-6-fluoro-1,4-dihydro-4-oxo-1,8-naphthyridine-3-carboxylic acid] were synthesized in our laboratories [1]. 8-Ethyl-5,8-dihydro-2-dimethylamino-5-oxopyrido(2,3-*d*)-pyrimidine-6-carboxylic acid was used as the internal standard [3]. The chemical structures of these compounds are shown in Fig. 1. Ethyl chloroformate was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). All other chemicals used were of analytical-reagent grade.

Standard solutions

The compounds were dissolved in methanol (50—100 μg/ml) in a 50—60°C water bath, and diluted with methanol. The internal standard was dissolved in the minimum volume of 0.1 *M* sodium hydroxide and diluted with 0.2 *M* phosphate buffer (pH 7.4) to concentrations of 1 μg/ml for plasma and 20 μg/ml for urine. These standard solutions were stored in a refrigerator (about 4°C).

Instrumentation

HPLC was carried out using a Waters Model ALC/GPC 204 liquid chromatograph equipped with a dual-delivery pump (Model 6000A), an automatic sampler (Model 710B), an ultraviolet (UV) detector (Model 440) with a 340-nm-wavelength filter and a recorder with integration (Data Module, Model 730). The stainless-steel column (30 cm × 4 mm I.D.) packed with μBondapak C_{18} (particle size 10 μm) was used (Waters Assoc.). The following three mobile phases were used at a flow-rate of 1.5 ml/min: (1) methanol—0.1 *M* citric acid—acetonitrile (9:5:1), (2) methanol—0.1 *M* citric acid—acetonitrile (6:5:1), (3) methanol—5% acetic acid—acetonitrile (6:10:1).

Assay procedure for AT-2266 and M-2 in plasma

To 1 ml of plasma sample were added 1 ml of the internal standard solution (1 μg) and 3 ml of chloroform containing 1% of ethyl chloroformate in a glass-stoppered 15-ml centrifuge tube. The tube was shaken for 10 min and centrifuged for 10 min at 2000 *g*. The organic layer (2 ml) was transferred to another tube and evaporated to dryness under a gentle stream of air at 50°C. The residue was dissolved in 200 μl of methanol, and a 20-μl aliquot of the solution was injected into the liquid chromatograph with mobile phase 1.

Assay procedure for AT-2266 and its metabolites in urine

AT-2266, M-2 and M-5 (procedure U-1). To 1 ml of urine sample were added 1 ml of the internal standard solution (20 μg) and 3 ml of chloroform containing 1% ethyl chloroformate in a glass-stoppered 15-ml centrifuge tube. The tube was shaken and centrifuged. The organic layer (2 ml) was evaporated to dryness. The residue was dissolved in 2 ml of methanol, and a 30-μl aliquot of the solution was injected into the liquid chromatograph with mobile phase 2.

M-1, M-3 and M-4 (procedure U-2). To 1 ml of urine sample were added 1 ml of the internal standard solution (20 μg) and 3 ml of chloroform in a glass-stoppered 15-ml centrifuge tube. The tube was treated as described in the above procedure U-1, and the liquid chromatograph was used with mobile phase 3.

Calibration curves

The drug solutions, used in the construction of the calibration curve, were prepared by diluting the stock solution with methanol, pipetted into glass-stoppered 15-ml centrifuge tubes and evaporated to dryness. The amounts used for plasma calibration curve were in the range of 0.1—10 μg/ml AT-2266 and 0.03—2.5 μg/ml M-2. The tubes were treated according to the assay procedure. Peak-height ratios of AT-2266 and M-2 to the internal standard were measured and plotted against the amount of AT-2266 and M-2 seeded.

The amounts used for urine calibration curve (procedure U-1) were in the range of 1—500 μg/ml AT-2266, 1—125 μg/ml M-2 and 1—20 μg/ml M-5. Other minor metabolites, M-1, M-3 and M-4, were added in the same range 1—20 μg/ml (procedure U-2).

Stability of AT-2266 and its metabolites

The stability of AT-2266 and its metabolites in methanol, plasma and urine was examined. The methanol solutions (50 μg/ml) were stored in a refrigerator (about 4°C), and determined (0—28 days). The drugs in plasma (1 μg/ml) or urine (50 μg/ml) were also determined when incubated at 37°C (0—24 h) or stored at −20°C (0—90 days). The stability of the internal standard in 0.2 *M* phosphate buffer (pH 7.4) was also examined for three months.

Drug administration to human and determination of plasma levels and urinary excretion

AT-2266 was administered orally at a dose of 400 mg to a man. Heparinized blood samples were drawn by venipuncture, and the plasma samples were kept frozen until analysis. The urine samples were collected for 24 h after dosing and also kept frozen until analysis.

RESULTS AND DISCUSSION

Extraction of AT-2266

AT-2266 is an amphoteric compound which is not extractable when non-polar solvents (e.g. *n*-hexane, ether) are used. The pH dependence of the extractability of AT-2266 with chloroform as shown in Fig. 2 shows that only 47% of the compound is extracted at pH 7.0—8.0 when equal volumes of the organic and aqueous phases are used. Therefore, the following extractive derivatization procedure was adopted. AT-2266 was extracted as ethyl carbamate by shaking with chloroform containing 1% of ethyl chloroformate in satisfactory yields (Fig. 2). M-5 was similarly derivatized. This procedure could be utilized for the simple and rapid derivatization of primary and secondary amine [4] or for the separation of tertiary amine from primary and secondary

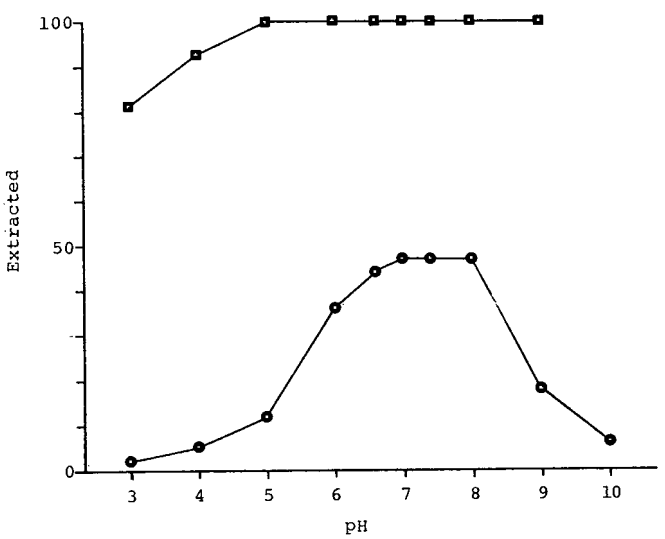


Fig. 2. pH dependence of extraction of AT-2266 (•) and AT-2266 ethyl carbamate (▪). Equal volumes of organic phase and buffered solution (pH 3—10) were used. Organic phase: (•) chloroform, (▪) chloroform containing 1% ethyl chloroformate.

amine [5]. Other metabolites and the internal standard were extracted as the intact form. Moreover, the separation was markedly improved by this procedure. For example, the separation (R_S) of AT-2266 and M-2 was 0.85 and 3.40 before and after derivatization, respectively, when using mobile phase 1.

Determination of AT-2266 and M-2 in plasma

Typical chromatograms of control plasma and plasma containing 1 μg/ml AT-2266 and 0.3 μg/ml M-2 are shown in Fig. 3. With the assay procedure, the peaks of those compounds were found to be separated from those due to control plasma and solvent.

The calibration curve obtained with 0.1—10 μg of AT-2266 in 1 ml of plasma was rectilinear and passed through the origin. The coefficients of

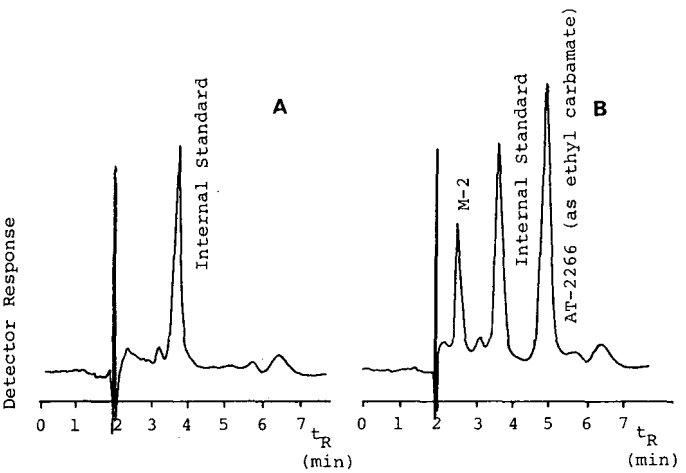


Fig. 3. Typical chromatograms of AT-2266 and its metabolite, M-2, in plasma. (A) Control plasma; (B) AT-2266 and M-2 corresponding to plasma concentrations of 1 and 0.3 μg/ml, respectively. HPLC conditions as described in the text.

variation at 2 and 0.5 μg/ml levels of AT-2266 were 1.9 and 2.8%, respectively. The minimum detectable concentration was 0.01 μg/ml. The calibration curve obtained with 0.03—2.5 μg of M-2 in 1 ml of plasma was also rectilinear. The coefficient of variation was 5.0% at the 0.5 μg/ml level, and the minimum detectable concentration was 0.01 μg/ml. Determinations of AT-2266 and M-2 in plasma obtained on different days were reproducible from day to day.

Determination of AT-2266 and its metabolites in urine

Simultaneous determination of AT-2266 and its five metabolites in urine was possible, but was too time-consuming to be suitable in practice (one chromatogram takes at least 40 min). Therefore, we separated the assay method into two procedures. One was the determination of AT-2266, M-2 and M-5 (procedure U-1), and the other was the determination of M-1, M-3 and M-4 (procedure U-2), which were excreted in minute quantities (less than 1% of the dose).

Fig. 4 shows a typical chromatogram of a urine sample containing AT-2266,

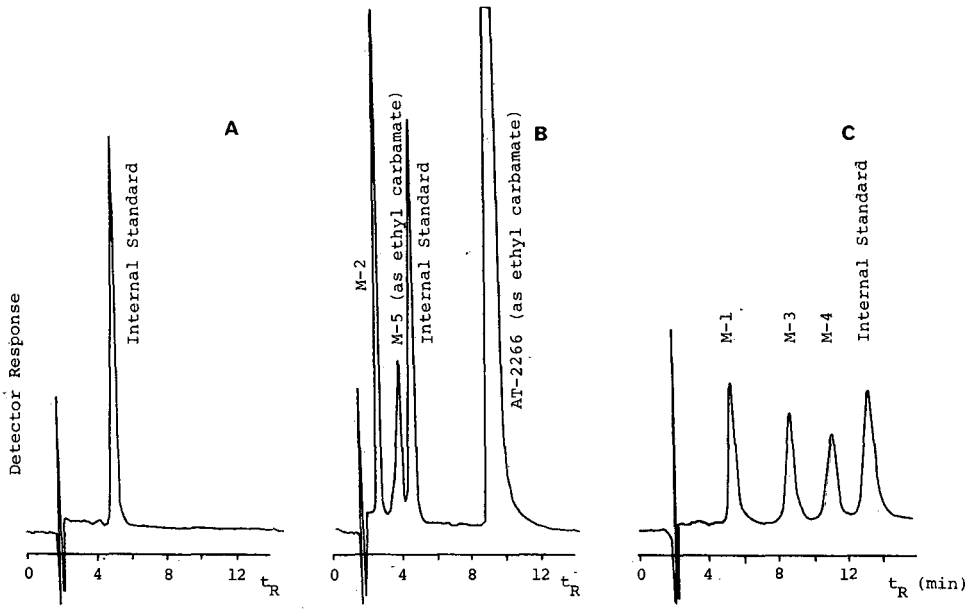


Fig. 4. Typical chromatograms of AT-2266 and its metabolites in urine. (A) Control urine; (B) AT-2266, M-2 and M-5 corresponding to urine concentrations of 51, 13 and 5 μg/ml, respectively; (C) M-1, M-3 and M-4 corresponding to urine concentrations of 5 μg/ml.

M-2 and M-5, and a chromatogram of control urine treated according to procedure U-1.

The calibration curve obtained with 1—500 μg of AT-2266 in 1 ml of urine was a straight line over 500-fold concentration range and passed through the origin. The coefficients of variation at 100 and 20 μg/ml levels of AT-2266 were 1.1 and 1.4% (n = 6), respectively. The minimum detectable concentration was 0.1 μg/ml. The calibration curves of M-2 and M-5 in urine were linear and passed through the origin. The precisions and minimum detectable concentrations were similar to those of AT-2266. Determinations of AT-2266, M-2 and M-5 in urine obtained on different days were reproducible from day to day.

A typical chromatogram of urine containing M-1, M-3 and M-4 treated according to procedure U-2 is shown in Fig. 4. The calibration curves with the same concentration range of 1—20 μg/ml were linear and passed through the origin. The coefficients of variation were 4.2, 2.3 and 3.4% (n = 3) at 5 μg/ml levels of M-1, M-3 and M-4, respectively.

Stability of the compounds

AT-2266 and its metabolites were stable in methanol for at least 28 days when stored in a refrigerator (about 4° C), and the compounds were stable in plasma and urine for at least 24 h at 37° C and 90 days at −20° C. The internal standard was also stable for at least three months in 0.2 M phosphate buffer (pH 7.4) in a refrigerator.

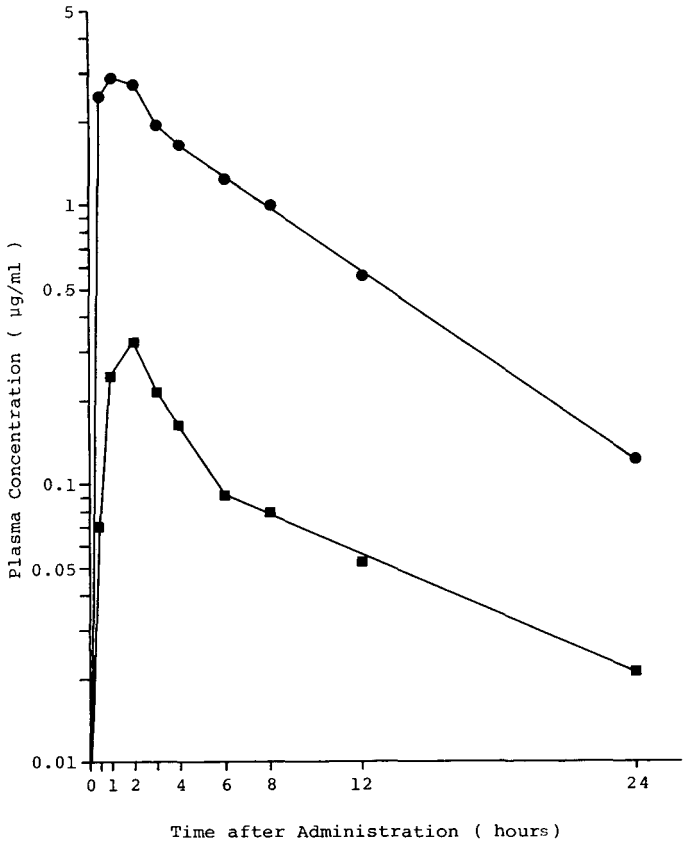


Fig. 5. Plasma levels of AT-2266 (•) and its metabolite, M-2 (▪), in a man following single oral administration at a dose of 400 mg of AT-2266.

TABLE I

24-h URINARY EXCRETION OF AT-2266 AND ITS METABOLITES IN A MAN FOLLOWING SINGLE ORAL ADMINISTRATION OF 400 mg OF AT-2266

Percentage of dose						
AT-2266	M-1	M-2	M-3	M-4	M-5	Total
54.26	0.27	8.11	0.13	0.29	0.99	64.05

Plasma levels and urinary excretion of AT-2266 in man

Plasma levels of AT-2266 and M-2 in a man after single oral dose of 400 mg of AT-2266 are shown in Fig. 5. Plasma levels of the unchanged drug were maximal 1 h after dosing with a level of 2.86 µg/ml, followed by a biphasic decrease with an apparent terminal elimination half-life of 5.3 h. Plasma levels of M-2 were about one-tenth those of the unchanged drug, and the curve was similar to that of the unchanged drug.

Urinary excretion of the unchanged drug and M-2 for 24 h after dosing were

found to be 54.26 and 8.11% of the given dose, respectively, and those of other metabolites were less than 1% of the dose (Table I).

CONCLUSION

We have developed a selective HPLC method for the determination of AT-2266 and its metabolites in plasma and urine. The high specificity and simplicity of the method would permit pharmacokinetic and bioavailability studies in both man and experimental animals.

ACKNOWLEDGEMENT

The authors are grateful to Dr. H. Nishimura, Director of this laboratory, for his support of this work.

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Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1867

DETERMINATION OF NALOXONE IN BLOOD BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received May 9th, 1983; revised manuscript received July 18th, 1983)

SUMMARY

A rapid, sensitive, selective and reproducible reversed-phase high-performance liquid chromatographic method is described for the quantification of naloxone in small blood samples of premature infants. Naloxone and the internal standard, naltrexone, are extracted from alkalinized blood into diethyl ether and subsequently back extracted into 0.05% (v/v) phosphoric acid before chromatographing on a reversed-phase system. The mobile phase comprises 85 parts of acetonitrile and 15 parts of 0.06% (v/v) triethylamine in an aqueous phosphoric acid solution at pH 5 and is pumped at 1.5 ml/min. The retention times of naloxone and naltrexone were observed to be 5.4 and 7.5 min respectively. Ultraviolet detection at 214 nm enabled a limit of detection of 1 ng to be achieved. The reproducibility of the method was good at both 100 ng (C.V. = 3.4%; n = 9) and 10 ng (C.V. = 5.1%; n = 6). The high sensitivity and speed with which this assay can be performed makes it especially useful for the estimation of naloxone in small volumes (0.3—0.6 ml) of blood. It is thus particularly valuable for the determination of naloxone blood concentration—time profiles in premature infants where the minimization of the volume of blood collected is of paramount importance.

INTRODUCTION

Naloxone is a pure narcotic antagonist with no agonist activity which is commonly used to reverse narcotic-induced respiratory and cardiovascular depression following surgery and narcotic overdose [1]. In obstetric practice, parenteral administration of naloxone to mothers shortly before delivery or to

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0378-4347/83/$03.00

the neonate after birth [2, 3] has been reported to reverse neonatal depressant effects of pethidine, acquired transplacentally during parturition.

Several analytical methods for the determination of naloxone in biological fluids have been described previously, including the detection by scintillation counting of radioactively labelled naloxone after its administration [4], ultraviolet (UV) spectrophotometry [5], radioimmunoassay [6] and gas–liquid chromatography (GLC) [5, 7, 8]. Only the radioimmunoassay procedure [6] and the GLC method, employing an electron-capture detector (ECD–GLC) [8], had sufficient sensitivity to enable quantification of naloxone in biological fluids after conventional doses in man. However, the radioimmunoassay [6] lacked specificity in that the antiserum reacted, not only with naloxone, but also with its reduction product naloxol. The more specific ECD–GLC method [8] suffered from the disadvantage that the perfluoroalkyl ester derivatives of naloxone and the internal standard, naltrexone, were unstable and, therefore, peak height ratios varied with time.

In view of the growing use of naloxone in premature infants and adults, a rapid, sensitive, selective and reproducible high-performance liquid chromatographic (HPLC) method has been developed for the quantification of naloxone in blood. This method involves the use of the structurally related compound, naltrexone, as internal standard (Fig. 1) and a relatively simple extraction procedure prior to chromatography.

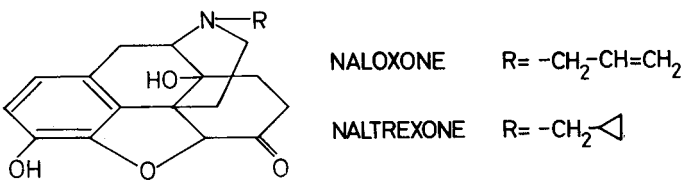

Fig. 1. Structures of naloxone and internal standard, naltrexone.

EXPERIMENTAL

Reagents and materials

Naloxone hydrochloride and naltrexone hydrochloride were gifts from Endo Labs. (Garden City, NY, U.S.A.). Aqueous standard solutions of these compounds were stored at 4°C. Triethylamine and phosphoric acid (Ajax Chemicals Unilab, Syndney, Australia) and acetonitrile (Waters Assoc., Milford, MA, U.S.A.) were used without further purification. Diethyl ether (Anaesthetic Grade, BP) was freshly distilled. Carbonate buffer, 1 *M* (pH 10.0), was prepared by dissolving 5.3 g of sodium carbonate and 4.2 g of sodium hydrogen carbonate in 100 ml distilled water. Glass tubes, cleaned in 2% (v/v) Extran 300 (Merck, Rahway, NJ, U.S.A.) were used in all steps of the analysis.

HPLC instrumentation

A Varian Aerograph (Palo Alto, CA, U.S.A.) Model 8500 HPLC pump equipped with a 100-μl loop injection system (Valco, Houston, TX, U.S.A.) and a 30 × 0.39 cm I.D. μBondapak phenyl column (10 μm) (Waters Assoc.)

was used. Naloxone and naltrexone were detected at 214 nm by means of an Altex Model 160 fixed-wavelength ultraviolet detector equipped with a zinc lamp (Altex Scientific, Berkeley, CA, U.S.A.). A single-pen recorder (Cole-Parmer, Chicago, IL, U.S.A.) with input set of 10 mV and chart speed of 20 cm/h was used.

Selection of mobile phase

An aqueous solution of naloxone hydrochloride was initially chromatographed to select a suitable mobile phase. Binary mixtures comprising varying proportions of either acetonitrile and distilled water, acetonitrile and 0.05% (v/v) phosphoric acid in distilled water or acetonitrile and 0.06% (v/v) triethylamine in distilled water acidified to pH 5.0 with phosphoric acid (triethylamine phosphate solution) were initially investigated as potential mobile phases. All aqueous components of the mixtures were passed through a 0.45-μm HA filter (Millipore, Bedford, MA, U.S.A.) prior to mixing with acetonitrile. The binary mixture which gave a desirable retention time and peak shape for naloxone was then adjusted to provide satisfactory resolution between naloxone and the internal standard, naltrexone.

The mobile phase used for quantification of naloxone in blood was acetonitrile—0.06% (v/v) triethylamine phosphate solution, pH 5 (85:15). The flow-rate was set at 1.5 ml/min resulting in an inlet pressure of approximately 34.5 bar. All chromatographic separations were carried out at ambient temperature.

Collection of biological samples

Blood samples were obtained at various times (up to 4 h) after the administration of a single intravenous dose of naloxone hydrochloride to premature apnoeic infants. Blood samples (0.3—0.6 ml) were collected by heel-prick into 1-ml heparinized plastic capillary collecting tubes (Walter Sarstedt, Edwards Labs., Sydney, Australia). A maximal aliquot of each blood sample was accurately measured and transferred to an extraction tube. The blood was then diluted with an equal volume of distilled water before freezing (—22°C). Analyses for naloxone were carried out on whole blood because sample volumes in these subjects were necessarily minimal. A sample of blood was obtained from each subject, before drug administration, to serve as control.

Extraction procedure

Diluted blood (0.6—1.2 ml) together with 100 ng of naltrexone hydrochloride (100 ng per 100 μl) as internal standard, and 250 μl of 1 *M* carbonate buffer (pH 10.0) were vortexed with 5 ml diethyl ether for 1 min. After centrifugation for 5 min at 1000 *g*, the ethereal layer was transferred to a 7-ml glass tube with a tapered base containing 100 μl of 0.05% (v/v) phosphoric acid, pH 2.35. Both naloxone and naltrexone were then back-extracted to this acidic aqueous phase by vortexing for 1 min. After further centrifuging about 80—90 μl of the phosphoric acid extract were injected directly onto the HPLC column.

Calibration and reproducibility

Control blood samples were supplemented with known quantities of

naloxone hydrochloride ranging from 1 ng to 200 ng (0.9—180 ng naloxone base) and analysed as outlined above. A calibration curve was constructed by calculating the naloxone:naltrexone peak height ratio and plotting this ratio against the amount of naloxone added to the sample. Naloxone concentrations in the unknown blood samples were determined by referring the observed peak height ratios to a calibration curve established on the same day.

The reproducibility of the method was assessed by performing the assay on replicate blood samples which contained either 100 ng (n = 9) or 10 ng (n = 6) of naloxone hydrochloride (equivalent to 90 ng and 9 ng of naloxone base, respectively).

RESULTS AND DISCUSSION

With reversed-phase HPLC, the chromatographic behaviour is usually quite predictable from the pH of the eluent and the pK_a and partition coefficient of the eluate. When naloxone was chromatographed on a reversed-phase column using a non-acidified mobile phase, it was strongly retained. A capacity factor (k') of 2 was obtained with an eluent of acetonitrile—water (85:15). When the mobile phase was acidified, a similar capacity factor was obtained with an eluent of acetonitrile—0.05% (v/v) phosphoric acid (10:90). However, broad and tailing naloxone peaks were obtained with these two systems. Asymmetric peaks are reported to be a recurrent problem in the separation of many weak organic bases [9]. This is generally attributed, at least in part, to ion-exchange or adsorption phenomena with unreacted silanol groups on the solid support. The slow kinetics of desorption from these sites are responsible for the broad and tailing peaks.

Triethylamine has frequently been incorporated into the eluent to mask the surface silanols and to competitively inhibit the adsorption of weak organic bases [9]. In this study, the incorporation of triethylamine in the eluent similarly improved the peak shapes and, consequently, the resolution of naloxone and the internal standard, naltrexone (Fig. 3b). However, improvement in naloxone peak shape was observed only when the eluent concentration of triethylamine was equal to or exceeded 0.06% (v/v). At triethylamine concentrations of less than 0.06%, a broad and tailing naloxone peak was observed. Concentrations in the range 0.06—0.2% (v/v) gave no further improvement in peak shape. This observation is presumably due to the limited adsorption capacity of the unreacted silanols which are then essentially completely occupied [10]. Thus, 0.06% (v/v) triethylamine was included in all subsequent analyses.

Lowering the pH of the triethylamine phosphate solution to 2.5 resulted in asymmetric peaks of naloxone. The peaks improved with increasing mobile phase pH up to 7.5. However, column life is shortened at elevated pH, thus pH 5 was selected as a satisfactory compromise.

The chromatographic parameters, k', α (selectivity factor) and R (resolution) for naloxone and naltrexone are summarized in Table I for mobile phases containing various proportions of acetonitrile in triethylamine phosphate solution. A mobile phase comprising acetonitrile—triethylamine phosphate solution (85:15) provided good resolution of naloxone and naltrexone. It is

TABLE I

EFFECT OF COMPOSITION OF THE MOBILE PHASE ON CHROMATOGRAPHIC PARAMETERS OF NALOXONE AND NALTREXONE

Mobile phase (acetonitrile—triethylamine phosphate solution)*	Capacity factor**		Selectivity factor (α)	Resolution*** (R)
	k'_1	k'_2		
40:60	3.6	4.2	1.17	0.20
50:50	3.7	4.6	1.24	0.22
60:40	3.8	4.8	1.26	0.38
70:30	3.9	5.1	1.31	0.45
85:15	4.0	5.8	1.44	0.60
90:10	6.0	—§	—	—

*0.06% triethylamine in distilled water, adjusted to pH 5.0 with phosphoric acid.
**Subscripts 1 and 2 refer to naloxone and naltrexone, respectively.
***Resolution calculated as quotient of retention time difference to average peak width.
§ Determination neglected because the naloxone peak was unacceptably broad.

interesting to note the unexpected observation that increasing the content of acetonitrile increased the retenton times of naloxone and naltrexone and improved the resolution between the compounds.

Since the mobile phase was rich in organic solvent, thus it was necessary to wash the column with water in order to avoid column pressure build-up. When another column from a different batch was obtained from the same manufacturer, the acetonitrile—triethylamine phosphate solution composition ratio had to be adjusted to 55:45 in order to obtain comparable retention and resolution.

Although the ultraviolet absorption peak of naloxone occurs at 284 nm, detection at this wavelength did not give satisfactory sensitivity. The recent availability of low-wavelength ultraviolet detectors fixed at 214 nm and of higher-purity acetonitrile (with UV cut-off at 190 nm) have enabled a new analytical approach for naloxone with excellent sensitivity. Detection at 214 nm increased the sensitivity of the assay approximately ten-fold relative to that at 284 nm. However, an excellent signal-to-noise ratio using the Altex Model 160 fixed-wavelength detector, allowed the use of 0.001 a.u.f.s. sensitivity setting for blood samples. At this setting, the signal-to-noise ratio obtained when quantifying 1 ng of naloxone hydrochloride (equivalent to 0.9 ng of naloxone base) was 10:1.

A typical chromatogram of extracted blank blood together with a chromatogram of blank blood to which 50 ng of naloxone hydrochloride and 100 ng of naltrexone hydrochloride had been added is shown in Fig. 2a and b. The retention times of naloxone and naltrexone were 5.4 and 7.5 min, respectively. The calibration curves of peak height ratio versus amount of naloxone added to the samples were linear over the concentration range from 0.9 ng to 180 ng of naloxone base ($Y = 0.0355X + 0.0134; r = 0.9994$). Separate calibration curves were established on each day that patient samples were analysed.

The analytical recovery was determined by comparing the chromatographic peak heights obtained from the analysis of nine replicate blank blood samples

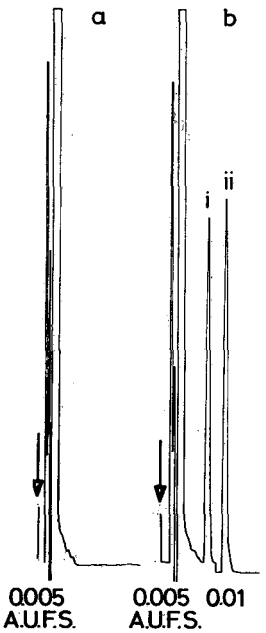

Fig. 2. Chromatograms of extracts of (a) blank blood and (b) blank blood spiked with 50 ng of naloxone hydrochloride (i) and 100 ng of internal standard, naltrexone hydrochloride (ii). The retention times of i and ii are 5.4 and 7.5 min, respectively. The arrows indicate the time of injections.

to which had been added 100 ng of naloxone hydrochloride, with the peak heights resulting from the direct injection of 100 ng of naloxone hydrochloride contained in aqueous standards. The overall recovery of naloxone from blood was 78 ± 3.2% (n = 9). There was no effect on extraction recovery or on peak height ratio when different volumes of blood were extracted. The reproducibility of the method is good as indicated by the coefficients of variation at 100 ng (3.4%; n = 9) and at 10 ng (5.1%; n = 6).

Fig. 3 shows a typical chromatogram obtained by the analysis of a blood sample collected from an apnoeic, preterm infant who had received 0.4 mg of naloxone hydrochloride intravenously. Also shown is the chromatogram from the analysis of a pre-dose blood sample. An endogenous peak was also observed in these blood samples (Fig. 3). However, no interference occurred with peaks of naloxone or naltrexone in these samples or those from other subjects participating in the study. A typical blood naloxone level—time profile is shown in Fig. 4.

The selectivity of the assay was further investigated by examining the retention characteristics of several drugs commonly administered to women in premature labour or to premature neonates. The drugs examined included betamethasone, salbutamol, diazepam, ritodrine, pethidine, folic acid, theophylline, vitamins D and K, gentamycin, ampicillin and frusemide. None of these drugs interfered with the analysis of naloxone.

In summary, this HPLC method is sensitive, selective and reproducible. It

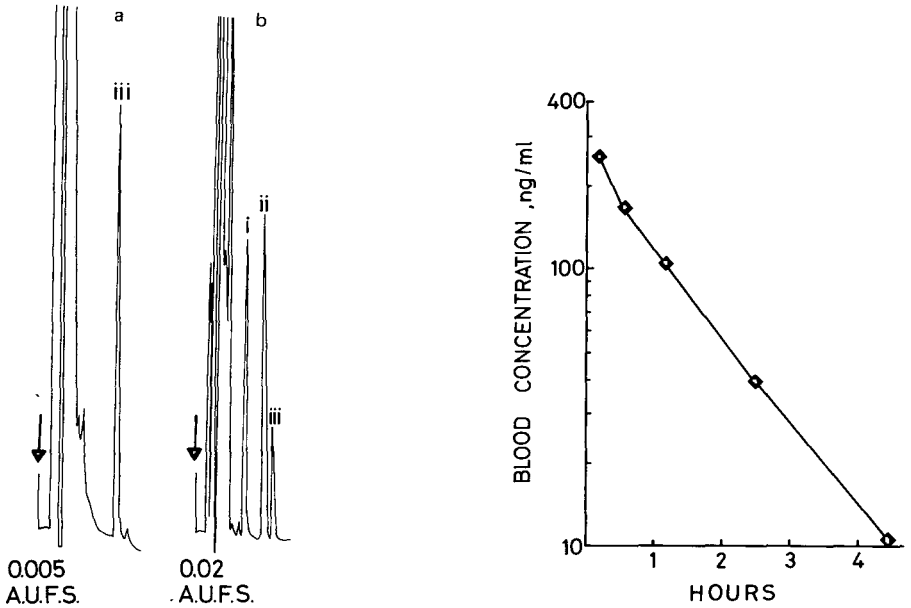

Fig. 3. Chromatograms of extracts of blood from preterm infant (a) just prior to dosing and (b) 35 min after dosing with 0.4 mg of naloxone HCl intravenously. Peaks: i, naloxone; ii, naltrexone; and iii, endogenous peak. The arrows indicate the time of injections.

Fig. 4. Blood naloxone concentration—time profile in a preterm infant (weight = 0.83 kg) after the intravenous administration of 0.4 mg of naloxone HCl.

can be rapidly performed — ten blood samples can be assayed in 90 min — since it does not involve a time-consuming evaporation step prior to chromatographic analysis and no derivatization is necessary. The assay therefore represents an improvement over the less specific radioimmunoassay method [3] and the more complex ECD—GLC method [8] which requires multiple extractions, followed by evaporation and derivatization. The HPLC method reported here has proven particularly useful for pharmacokinetic studies of naloxone in preterm infants and the results of those studies will be reported elsewhere.

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Journal of Chromatography, 278 (1983) 337—345
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1871

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC QUANTITATION OF TRIMETHOPRIM, SULFAMETHOXAZOLE, AND N^4-ACETYLSULFAMETHOXAZOLE IN BODY FLUIDS

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(First received February 8th, 1983; revised manuscript received July 20th, 1983)

SUMMARY

We describe a rapid, precise and simple procedure for the quantitative determination of trimethoprim, sulfamethoxazole, and N^4-acetylsulfamethoxazole in body fluids by reversed-phase high-performance liquid chromatography. This method utilizes antipyrine as an internal standard with the compounds detected by dual-wavelength monitoring at 225 nm and 254 nm after a single-step extraction. Precision, sensitivity, and accuracy of this assay are within the range of clinical utility; the coefficient of variation is $\leqslant 3\%$, sensitivity < 0.5 μg/ml for all compounds, and recovery $> 97\%$. The short time for performance and small sample size makes the assay ideal for clinical drug monitoring and pharmacokinetic studies.

INTRODUCTION

Trimethoprim [2,4-diamino-5(3,4,5-trimethoxybenzyl)pyrimidine] (TMP) in combination with sulfamethoxazole [N'-(5-methyl-3-isoazolyl)sulfanilamide]

0378-4347/83/$03.00

(SMX), often referred to as Co-Trimoxazole, is a widely used antibacterial agent which has excellent in vitro activity against a variety of aerobic gram-negative bacilli [1, 2].

A major metabolite of SMX is its corresponding N^4-acetylated sulfonamide: N^4-acetylsulfamethoxazole (acetyl-SMX). Acetylated sulfonamides have been associated with toxic reactions [3], particularly with deteriorating renal function [4, 5]. Crystalluria due to the low solubility of the acetylated metabolites has also been reported [6, 7].

It has been suggested that the monitoring of TMP and SMX is necessary to adjust dosing intervals to maintain therapeutic blood concentrations [8, 9] particularly in patients with changing renal function. A rapid, reliable, precise and accurate assay of TMP, SMX, and acetyl-SMX is desirable to limit toxic reactions; such an assay would also aid in devising individualized dosing regimens, and would facilitate clinical pharmacokinetic studies.

There have been several methods developed for the quantitation of TMP in body fluids: microbiological [10], gas–liquid chromatography [11], and ion-pair chromatography [8]. Sulfonamide concentrations are usually determined by a colorimetric assay based on the Bratton–Marshall reaction [12]. N^4-Acetylated metabolites have been quantitated with the same reaction, but with certain modifications [13].

High-performance liquid chromatography (HPLC), a reliable and rapid analytical tool in clinical laboratories has several advantages over conventional methods described above. HPLC techniques for the individual determination of TMP and SMX have been described [14, 15]. A normal-phase HPLC method for the simultaneous determination of TMP, SMX, and acetyl-SMX [16] and a reversed-phase HPLC method in which two determinations are necessary for quantitating TMP, SMX, and acetyl-SMX have also been reported [17]. Gochin et al. [18] describe a simultaneous determination of TMP, SMX, and acetyl-SMX by reversed-phase HPLC; however, this method requires 1.0 ml of serum and an extraction of serum and urine. These methods use only single-wavelength monitoring; absorbance ratios with dual-wavelength monitoring ensure greater degree of confidence in the specificity of the assay [19, 20]. We describe here a reversed-phase HPLC method using dual-wavelength monitoring for the simultaneous determination of TMP, SMX, and acetyl-SMX. The mobile phase is a simple aqueous buffer–solvent mixture, and the sample is prepared by a simple one-step precipitation procedure. The method is rapid and selective and its precision, reproducibility, accuracy, linearity, recovery and sensitivity are validated or documented. TMP was also measured with a normal-phase HPLC assay [21] and the values were compared with the reversed-phase technique.

EXPERIMENTAL

Materials

We used a high-pressure liquid chromatographic pump which could deliver a mobile phase at a constant flow-rate up to 31 MPa (Model Constrametric I, Laboratory Data Control, Riviera Beach, CA, U.S.A.). A sample injection loop with a loop filler port (Model 70-10 and Model 70-11, Rheodyne, Berkeley,

CA, U.S.A.) was fitted to the pump. We used a 10-μm, 30 cm × 3.9 mm I.D. Waters Assoc. (Milford, MA, U.S.A.) μBondapak C_{18} reversed-phase column (ASI 28N-S/N 44802, Analytical Sciences, Santa Clara, CA, U.S.A.). The mobile phase was monitored in series with a fixed-wavelength UV detector (UV III) at 254 nm and a variable-wavelength detector (Spectro Monitor III) at 225 nm. Both were purchased from Laboratory Data Control. A dual-pen recorder set at 0.5 cm/min (Omniscribe Recorder, Model B5217-5, Houston Instruments, Austin, TX, U.S.A.) received signals from both detectors. The flow-rate was 1.0 ml/min.

TMP, SMX, and acetyl-SMX (Lot 150, 754017, and RR-319-3, respectively) were kindly donated by Dr. R. Cleeland of Hoffmann-La Roche (Nutley, NJ, U.S.A.). Stock solutions of TMP and SMX were stored at −70°C at 100 μg/ml in distilled water and 1.9 mg/ml in 0.1 *M* sodium hydroxide, respectively. The acetyl-SMX stock solution was stored at −70°C as a 1.0 mg/ml solution in methanol–water (60:40, v/v).

Antipyrine (Aldrich, Milwaukee, WI, U.S.A.) was dissolved in acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) at 100 μg/ml, and was used as an internal standard and was stored at 5°C. It was stable under these conditions for three months.

The mobile phase consisted of methanol–0.067 *M* phosphate buffer (35:65, v/v). The phosphate buffer was made with 97% potassium phosphate, KH_2PO_4 (Mallinckrodt, Paris, KY, U.S.A.) and 3% sodium phosphate, Na_2HPO_4 (MCB Manufacturing Chemists, Cincinnati, OH, U.S.A.) and the pH was adjusted with phosphoric acid to 3.5. The buffer is stable for two weeks at 22°C. The mobile phase is prepared daily with Omnisolv glass-distilled methanol (MCB Manufacturing Chemists) and buffer by filtering through a 0.22-μm filter (Type GS, Millipore, Bedford, MA, U.S.A.) and degassed in vacuo.

Methods

Aliquots of 50 μl of standards, controls (stored at −70°C) and samples were added to 50 μl of acetonitrile containing the internal standard. The mixture was mixed vigorously for 15 sec on a Vortex mixer and centrifuged at approximately 10,000 *g* (Eppendorf Centrifuge Model No. 5412) for 2 min. A 20-μl aliquot of the supernatant was injected into the column. Absorbance unit full scale (a.u.f.s.) was changed between the TMP peak and SMX peak from 0.05 to 0.2. Then a.u.f.s. was changed to 0.1 after elution of the SMX peak. Peak heights were measured and the ratio of the drug (or metabolite) peak height to internal standard peak height was calculated. These ratios showed a direct linear correlation with the drug concentration of known standards. Drug concentrations of unknowns were calculated from standard curves obtained by least-squares regression.

Linearity

The stock solution of TMP was diluted to 20, 10, 5.0 and 2.5 μg/ml with distilled water. Similarly SMX was diluted to 190, 95, 47.5 and 23.75 μg/ml and acetyl-SMX was diluted to 100, 50, 25 and 12.5 μg/ml. Standards containing these concentrations were assayed and peak height ratio to the internal standard calculated. The standard curve was analyzed by linear regression

analysis to determine linearity. Acetyl-SMX standards were stable at 5°C for at least one month, and TMP and SMX were stable at −70°C for at least four months.

Precision

Stock solutions were diluted with normal pooled human sera, aliquoted and stored at −70°C. Two concentrations for both TMP and SMX were used and one concentration for acetyl-SMX. Seven aliquots at each concentration were assayed in one batch (within-assay precision); then an aliquot at each concentration was assayed daily over a four-month period (between-assay precision).

Accuracy

Known amounts of TMP, SMX, and acetyl-SMX were dissolved in normal human sera and assayed twice in an encoded fashion.

Recovery

Identical amounts of TMP, SMX and acetyl-SMX were dissolved in distilled water or human sera and assayed. Drug peak heights in serum were compared to drug peak heights in distilled water. Human sera, urine and cerebrospinal fluid (CSF) samples from patients not receiving TMP or SMX were also assayed.

Sensitivity

Working standards were serially diluted and assayed until a concentration was reached in which the peak height was twice the background noise.

Specificity

Peak heights at 225 nm were routinely used to determine concentrations. However, the ratio of peak height at 225 nm to peak height at 254 nm was constant: 4.25 for TMP, 0.85 for SMX, and 1.30 for acetyl-SMX. A value greater than 2 standard deviations (S.D.) from this ratio indicated an interfering substance.

Method comparison

A total of 312 serum samples were assayed for TMP by the reversed-phase HPLC method described here, and by the normal-phase HPLC described by Siber et al. [21].

RESULTS

Representative chromatograms of serum samples before and after Co-Trimoxazole administration are depicted in Fig. 1. Retention times of TMP, SMX, and acetyl-SMX are 4.6, 8.0, and 14.8 min, respectively. The retention time of the internal standard is 11.0 min.

A standard curve was calculated using aqueous solutions of five concentrations for TMP, SMX, and acetyl-SMX. Peak height ratios (described in Methods) were plotted versus concentrations. The squares of the correlation coefficients (r^2) were 0.99945, 1.0000, 0.99941 for TMP, SMX, and acetyl-

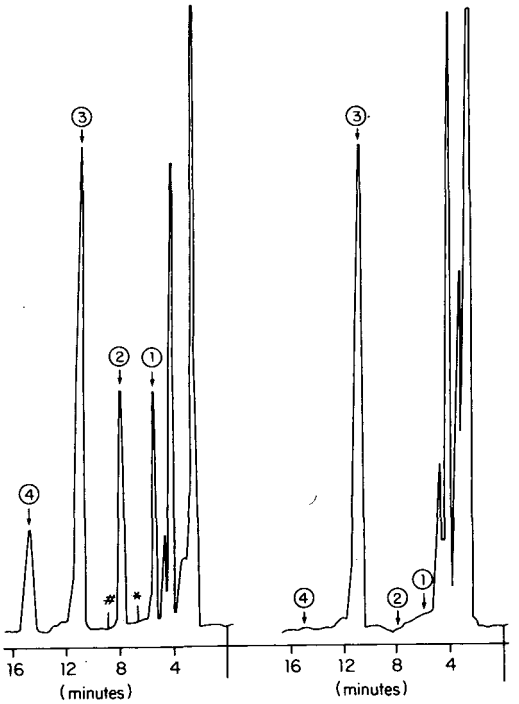

Fig. 1. Representative chromatogram of serum containing TMP, 1; SMX, 2; internal standard, 3; and acetyl-SMX, 4. Left, serum was obtained after the patient received Co-Trimoxazole with the absorbance measured at 225 nm; right, before drug administration. *Indicates a change of a.u.f.s. from 0.05 to 0.2; # indicates a change of a.u.f.s. from 0.2 to 0.1.

TABLE I

WITHIN-RUN REPRODUCIBILITY OF REVERSED-PHASE QUANTITATION OF TMP, SMX AND N-ACETYL SMX

Concentrations are given in μg/ml.

	TMP	SMX	Acetyl-SMX
Number (n)	7	7	10
Mean value	1.76	40.20	56.1
Standard deviation	0.053	0.40	0.95
Coefficient of variation (%)	3.0	1.0	1.7
Number (n)	7	7	
Mean value	9.56	185.8	
Standard deviation	0.22	1.19	
Coefficient of variation (%)	2.2	0.6	

SMX, respectively. Because of these excellent correlations, only three concentrations (including 0 μg/ml) were used to determine standard curves in routine analysis. Slopes of the peak height ratio to the standard curves (n = 30) were 0.0791 ± 0.0043, 0.0055 ± 0.0003, and 0.0055 ± 0.0003 for TMP, SMX, and

TABLE II

PRECISION OF THE QUANTITATION OF TMP, SMX AND ACETYL-SMX ON SUCCESSIVE DAILY DETERMINATIONS

Concentrations are given in μg/ml.

	TMP	SMX	Acetyl-SMX
Number (n)	41	41	40
Mean value	1.89	38.97	54.23
Standard deviation	0.11	0.70	1.79
Coefficient of variation (%)	5.8	1.8	3.3
Number (n)	41	41	
Mean value	9.80	188.40	
Standard deviation	0.24	2.87	
Coefficient of variation (%)	2.4	1.4	

acetyl-SMX, respectively. The y-intercepts did not differ significantly from zero in all cases ($p > 0.05$).

Precision analysis of seven identical aliquots (n = 10 for acetyl-SMX) of TMP, SMX, and acetyl-SMX are summarized in Table I. The coefficients of variation of quantitation of TMP < 3.0% and < 2.0% for SMX and acetyl-SMX indicate good reproducibility within therapeutic ranges. Precision analysis of day-to-day variation in assays is depicted in Table II. There was no detectable change in concentrations of aliquots stored at −70° C for four months.

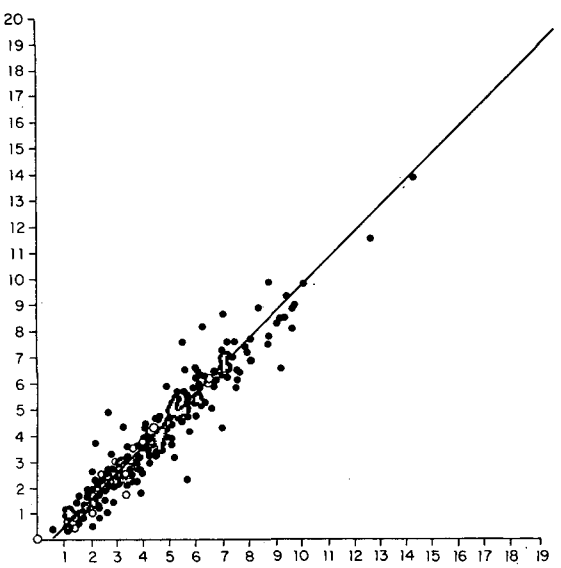

Fig. 2. Comparison of TMP quantitation by two techniques. The y-axis represents TMP quantitated by the reversed-phase method. The x-axis represents TMP quantitated by normal-phase HPLC [21]. TMP concentration is in μg/ml. The parameters of the regression line are: slope = 1.0293, y-intercept = −0.533, correlation coefficient = 0.9611 with 312 samples. Open circles represent more than one point having the same value.

TABLE III

ACCURACY OF REVERSED-PHASE QUANTITATION OF TMP, SMX AND ACETYL-SMX ON REPLICATE DETERMINATIONS (n = 14)

Concentrations are given in μg/ml.

TMP			SMX			Acetyl-SMX		
Actual concn.	Measured concn.	Percent difference	Actual concn.	Measured concn.	Percent difference	Actual concn.	Measured concn.	Percent difference
20	20	0	190	181.2	4.6	100	95.1	4.9
20	19.3	3.5	190	179.3	5.6	100	96.9	3.1
15	15.3	2.0	142.5	142.6	>0.1	75	75.7	0.9
15	15.1	0.7	142.5	137.7	4.7	75	72.7	3.1
10	9.8	2.0	95.0	92.1	3.0	50	46.7	6.6
10	10.0	0	95.0	90.6	4.6	50	47.9	4.2
7.5	7.4	1.3	66.5	65.5	1.5	35	34.9	0.3
7.5	7.4	1.3	66.5	65.1	2.1	35	34.6	1.1
5	5.1	2.0	47.5	47.0	1.1	25	24.6	1.6
5	4.9	2.0	47.5	47.0	1.1	25	25.0	0
2.5	2.4	4.0	114.0	111.2	3.3	60	59.4	1.0
2.5	2.3	8.0	114.0	112.1	2.5	60	59.4	1.0
1.0	1.0	0	74.0	74.0	0	30	20.1	3.0
1.0	1.0	0	74.0	73.8	0.3	30	29.4	2.0
Mean percent difference		1.9	Mean percent difference		2.45	Mean percent difference		2.34
Range of percent difference		0—8.0	Range of percent difference		0—5.6	Range of percent difference		0—6.6

Accuracy studies, depicted in Table III, demonstrate good accuracy over a wide range of concentrations for TMP, SMX, and acetyl-SMX. The mean percent difference between actual and observed values was < 3.0% for all three compounds.

Recovery studies comparing spiked sera to aqueous standards of TMP, SMX, and acetyl-SMX showed an average recovery of 97.3%, 99.4%, and 100.3%, respectively. Percent recovery was constant throughout the concentration range. Normal human sera, urine and CSF displayed no peaks on the chromatogram at the retention times of the indicated compounds.

Using a 20-μl injection, the sensitivity of detection (defined in Methods) of TMP, SMX, and acetyl-SMX is 0.05 μg/ml, 0.2 μg/ml and 0.5 μg/ml, respectively.

Out of 523 patient samples 63 samples were obtained from patients with renal failure. Of these, 2.5% had interference with SMX and internal standard peaks. Interference was present with acetyl-SMX in 6.1% of the samples. Interference with the TMP peak occasionally occurred in routine analysis; however, slight adjustment of pH or the percentage of methanol eliminated the interfering substances from the TMP peak, (when adjusting pH or percentage of methanol. the TMP standards must be reinjected to determine their retention time and for quantitation).

Comparison of TMP serum levels quantitated by reversed-phase HPLC and a normal-phase HPLC method [21] are depicted in Fig. 2. The correlation coefficient (r^2) was > 0.96 with a slope of 1.029.

TMP, SMX, and acetyl-SMX levels in serum, urine and CSF could be determined within 30 min after obtaining the samples.

DISCUSSION

A reliable, rapid and accurate assay is necessary for the routine clinical analysis of drugs and/or metabolite concentrations. The method described here meets these criteria. The monitoring by dual wavelength ensures specificity; when interfering substances are present, small changes in percentage of methanol or pH will separate interfering compounds from the TMP, SMX, acetyl-SMX peaks. The reversed-phase method negates the need of an extraction step, and the use of expensive organic solvents, which are used in normal-phase chromatography.

This method can be scaled down by using 10 μl of sample and 10 μl of internal standard. This allows quantitating the antibiotics in serum samples obtained by fingertip puncture or heel prick methods. To date, 500 samples have been assayed with the same column. A pre-column of large pore C_{18} μBondapak (37—50 μm) was used to increase the useful life span of the analytical column; however, after 500 injections, increases in back pressure and slight changes in peak shapes indicated column deterioration. Daily washing of the column was carried out with 30% methanol in water before and after. This method will allow the clinician to adjust the dose of Co-Trimoxazole to maintain therapeutic body fluid concentrations of TMP and SMX; it also allows quantitation of serum acetyl-SMX concentrations. Avoiding high acetyl-SMX concentrations may minimize toxicity.

ACKNOWLEDGEMENTS

Supported in part by grant GM 26337 and by the National Institutes of Health.

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Journal of Chromatography, 278 (1983) 347—357
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1872

RAPID, SENSITIVE AND FULLY AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY WITH FLUORESCENCE DETECTION FOR SULMAZOLE AND METABOLITES

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(First received March 15th, 1983; revised manuscript received July 20th, 1983)

SUMMARY

Sulmazole {2-[(2-methoxy-4-methylsulfinyl)phenyl]-3H-imidazo [4,5-b] pyridine; AR-L 115 BS} and two metabolites (sulfide, sulfone) were quantified from directly injected body fluids (plasma, urine, bile) after high-performance liquid chromatographic separation. No internal standard is needed, which is particularly advantageous when fluorescence detection is established. After automated pre-column enrichment on Corasil C_{18} (37—50 μm), the parent compound and biotransformation products could be backflushed and chromatographed on ODS-Hypersil (5 μm) with a mixture of 0.075 mol/l phosphate buffer—acetonitrile (2:1), an elution rate of 2.0 ml/min and fluorimetric detection (λ_{ex} = 330 nm; λ_{em} = 370 nm). A hydroxylated metabolite of sulmazole which occurs preferentially in urine (and bile) can be quantified in the above-mentioned solvent system diluted 1:1 with water, but with different fluorescence characteristics (λ_{ex} = 345 nm; λ_{em} = 515 nm). The assay was linear in the range 8—1000 ng/ml. The lower limit of detection was about 8 ng/ml or 80 pg with coefficients of variation between 0.4 and 5.8% for sulmazole.

INTRODUCTION

Sulmazole* is a new potent cardiotonic drug which combines a positive inotropic and vasodilatory action in animals [1—4] and man [5—8]. For optimal drug therapy and pharmacokinetic investigations it is necessary to set up a rapid, simple, selective and practicable assay for the pharmacologically active species in body fluids. In general it is also desirable to detect, when possible, simultaneously occurring plasma metabolites to relate any as yet

*Commercial name: Vardax®. Manufacturer: Dr. Karl Thomae GmbH, Biberach/Riss, F.R.G.

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unknown (side) effects to the amounts of metabolites in the body or to assess deviations caused by drug interactions.

The present paper describes a high-performance liquid chromatographic (HPLC) assay utilising the principle of "alternated pre-column enrichment" which we have described elsewhere [9]. This assay is applicable for routine drug monitoring in pharmacokinetic or clinical investigations. Concentrations of sulmazole and two less-active plasma metabolites, AR-L 114 BS (sulfone of sulmazole) and AR-L 113 BS (sulfide of sulmazole), are simultaneously detected.

EXPERIMENTAL

Reagents

The amount of 13.8 g disodium hydrogen phosphate dihydrate, molecular weight (MW) 177.99 (Merck No. 6580) and 9.1 g of sodium dihydrogen phosphate, MW 136.0 (Merck No. 4873) were each dissolved in 2000 ml of deionized water. The pH was adjusted to 6.8 with 85% phosphoric acid (Merck No. 573). Acetonitrile LiChrosolv (Merck No. 30) was used as organic solvent. The chromatographic cocktail contained one part of each buffer solution and one part of acetonitrile. Reversed-phase materials for the HPLC columns were Bondapak C_{18} Corasil, 37—50 μm (Waters No. 27248) and ODS-Hypersil, 5 μm (Shandon No. 580X 5).

Reference compounds of sulmazole (Lot No. FNTK 775), and metabolites AR-L 114 BS and AR-L 113 BS, and the hydroxylated metabolite M2 were from Dr. K. Thomae, Biberach, F.R.G. (Fig. 1).

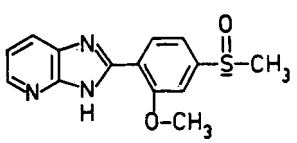

Fig. 1. Formulae of sulmazole and metabolites.

HPLC equipment

A diagram of the equipment is given in Fig. 2. A single piston pump, Model 410 (Kontron, Switzerland) was used as pump A delivering deionized water

with a flow-rate of 1.5 ml/min. A constant flow pump, Model 600/200 (Gynkotek, F.R.G.) was used as pump B. This pump delivered the eluent for backflush elution of the enriched material from the pre-columns (4 cm × 0.46 cm I.D.; Bischoff, F.R.G.; dry-packed with C_{18} Corasil, particle size 37—50 μm) to the analytical column (12.5 cm × 0.46 cm I.D.; Bischoff; packed with ODS-Hypersil; Shandon, U.K.; particle size 5 μm). A guard column (2 cm × 0.46 mm I.D.; Bischoff) filled with 5-μm ODS-Hypersil was used to protect the analytical column. The composition of the eluent was 0.075 mol/l phosphate buffer—acetonitrile (2:1; v/v) pH 6.8 with a flow-rate of 2.0 ml/min. Samples were injected by a WISP 710 B autosampler (Waters, U.S.A.). An SM-2 (Gynkotek) column switching module was used for the column switching technique.

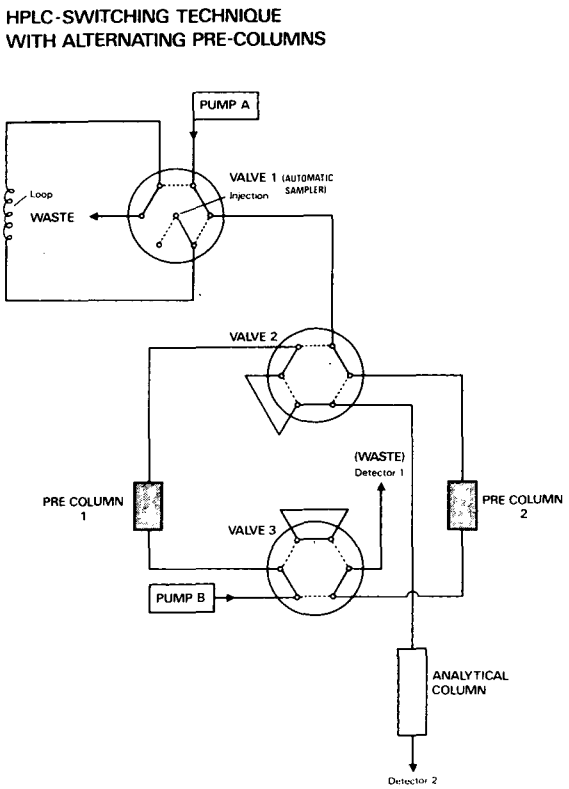

Fig. 2. HPLC column switching technique with alternated pre-column sample enrichment [9].

The compounds were detected with a fluorescence spectrophotometer, Model 650-10 LC (Perkin Elmer, F.R.G.). Wavelengths for sulmazole and metabolites were 330 and 370 nm, and for the hydroxylated metabolite 345 and 515 nm. The detector was adjusted to 5 nm slit width for excitation and emission; output 10 mV, range 1; multiplier gain, response and sensitivity mode normal. Signals were recorded with an integrator/plotter Model 3388 A (Hewlett-Packard, U.S.A.).

Alternated pre-column enrichment system [9]

An autosampler (WISP 710 B, Waters or Model SS 100, Perkin-Elmer), designated valve 1, is connected with a six-way Rheodyne valve (valve 2) which delivers alternately, depending on its switching position, a sample by means of pump A to one of the two pre-columns. These two pre-columns are mounted parallel with another valve (valve 3) which distributes the chromatographic eluent of a second pump (pump B) alternately to one of the pre-columns (backflushing). Both valves are simultaneously activated by a programmable time function of the integrator. This assembly of valves 2 and 3 is now commercially available (e.g. Gynkotek, Switching Module 2).

The samples of pure body fluids are directly injected (5—50 μl) by an autosampler into the enrichment pre-column system and washed with water (2—4 min) in order to guarantee separation of the additional water-soluble components such as salts and proteins. These components are flushed into waste under the described conditions. After this loading and enrichment step, the enrichment pre-column system is switched in the eluent stream of pump B, initiated by a programmable internal time event function of the integrator. Now the enriched material is backflushed onto the analytical column in order to obtain proper separation of the components. A second enrichment pre-column (Fig. 2) is mounted such that when one pre-column is loaded with a sample from the autosampler, the second pre-column, which had been loaded before, is in the backflush mode. With the assembly of a set of two pre-columns which are used in an alternated mode, we save time for the analysis. The working cycle of the pre-columns thus consists of an equilibration step, i.e. flushing with the eluent of pump A for 1—2 min in order to equilibrate the reversed-phase material, followed by the loading or enrichment step with an additional flushing period with water for another 2—4 min. After backflushing the enriched material with the eluent of pump B, the pre-column is again switched back into the eluent stream of pump A which delivers water to re-equilibrate the pre-column for the next sample injection.

Technical recommendations for alternating column switching

For the successful operation of alternating column switching from biological fluids, several practical points should be observed:

(1) The steel capillaries from the autoinjector to the enrichment columns should be of an inner diameter (I.D.) of 0.5 mm instead of 0.25 mm, whereas the capillaries from the pre-columns to the analytical column should be of 0.25 mm I.D.

(2) Do not use commercially available inlet frits in capillaries where biological material is transported, since they might block the solvent stream.

(3) Special attention should also be paid to the metal sieves for the pre-columns, which should not be too fine. Sieves of at least 18 μm pore size are recommended with an additional glass fibre filter.

(4) In order to guarantee a homogeneous distribution of the administered plasma on top of the pre-column, crossed grooves should be filed onto the surface of the end fitting which closes the top of each pre-column. This is important to allow immediate distribution of the arriving plasma bolus over the whole top surface of the enrichment column. Without a solvent distribution

device in the connecting nut, plasma may block the frit, because it is focused only to a small area on the sieve.

(5) The analytical column should be protected by a 2—4 cm guard column in order to increase its lifetime.

Collection of samples

Blood samples should be withdrawn with heparinized syringes (e.g. heparinized Monovette, Sarstedt, F.R.G.). Freshly prepared plasma can be immediately injected in the HPLC system. Frozen plasma samples should be brought to ambient temperature, thoroughly shaken and centrifuged in order to prevent blocking of the HPLC capillaries.

Urine or bile can be directly injected (5—10 μl).

RESULTS AND DISCUSSION

Chromatograms

Typical chromatograms of sulmazole and metabolites from directly injected plasma are shown in Fig. 3. The integrator starts drawing the chromatograms immediately on backflush of the enriched drugs from the pre-columns to the analytical column. Therefore the recorded apparent retention times do not reveal the whole time needed for one chromatogram. The time for washing the pre-columns after the plasma injection must be added, in our case 2 min, to obtain the real time needed for a chromatographic run. Thus, sulmazole and metabolites can be chromatographed fully automatically from body fluids in about 6 min. Apparent retention times from plasma are: sulmazole, 1.37 min;

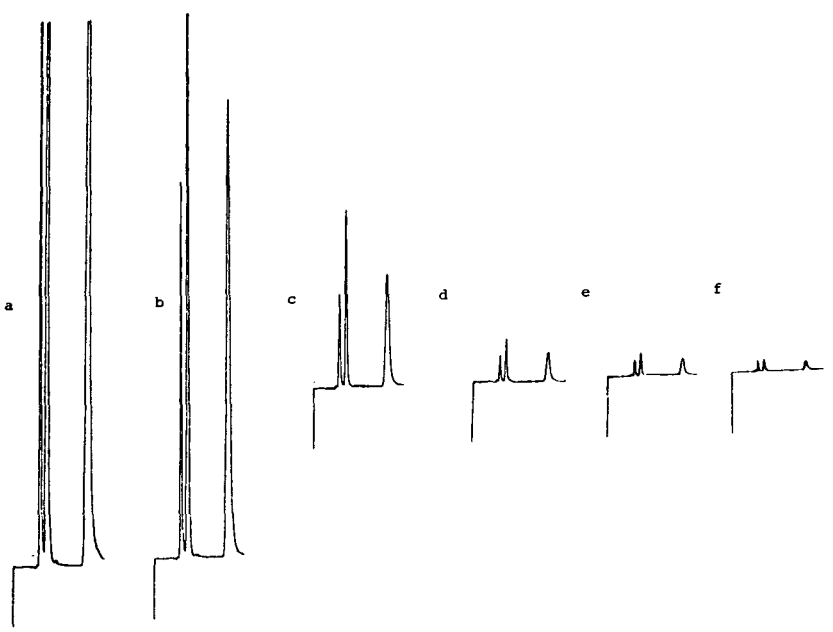

Fig. 3. HPLC chromatogram after automated pre-column sample enrichment of identical concentrations of sulmazole (I) and sulfone and sulfide metabolites (II and III) from 10 μl of directly injected plasma: 1000 ng/ml (a), 500 ng/ml (b), 125 ng/ml (c), 32 ng/ml (d), 16 ng/ml (e), 8 ng/ml (f).

AR-L 114 BS, 1.75 min; AR-L 113 BS, 3.82 min.

Chromatograms from directly injected samples of urine, bile and plasma from a pharmacokinetic investigation are shown in Figs. 4—7. It can be demonstrated that the fluorescence detection enables excellent chromatograms to be obtained from body fluids without any interference from the biological matrix.

Stability in body fluids

Sulmazole and metabolites are stable in various body fluids at 20°C and body temperature [11].

Calibration

Stock solutions of 1 mg/ml and 0.01 mg/ml in ethanol were prepared and kept in the deep freezer. Appropriate aliquots were taken for plasma standards.

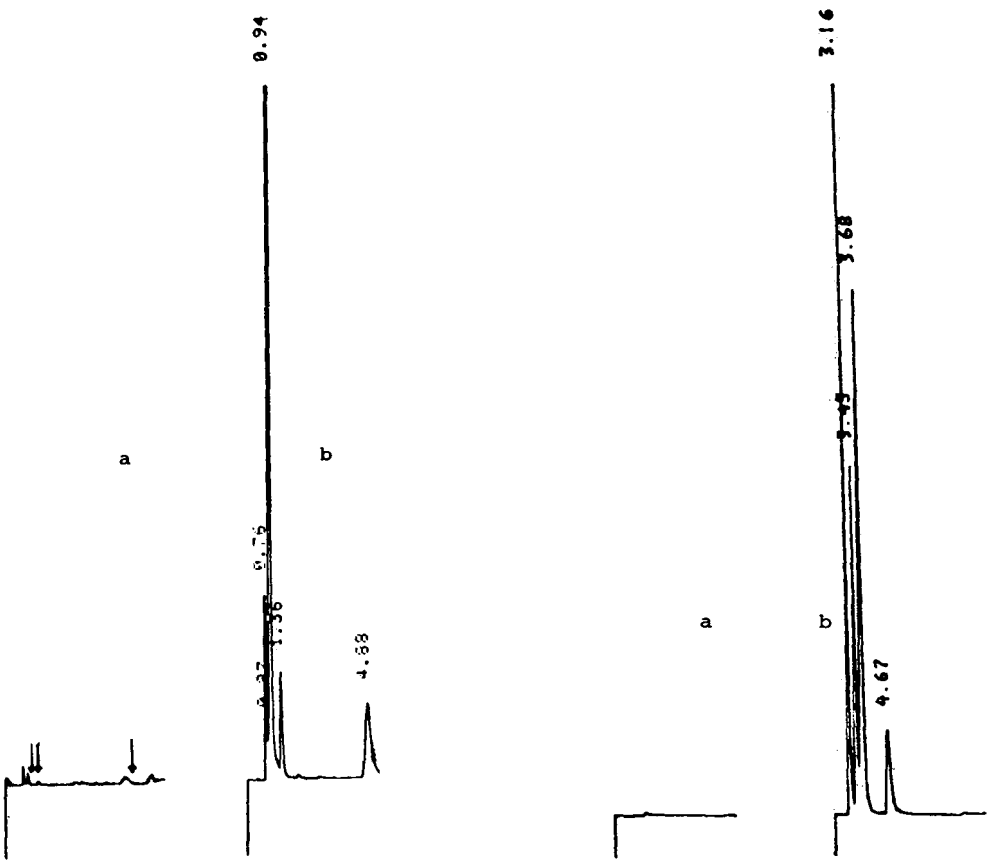

Fig. 4. HPLC chromatograms after automated pre-column sample enrichment of 5 µl blank human urine (a) and 5 µl of human urine (b) after administration of sulmazole (solvent system: 850 ml eluent + 150 ml water). Apparent retention time of 1.36 min represents parent compound.

Fig. 5. HPLC chromatograms after automated pre-column sample enrichment of blank rat bile (a) and rat bile (b) after administration of sulmazole (5 µl injected). Apparent retention time of 3.68 min represents parent compound (solvent system: 1000 ml eluent + 250 ml water).

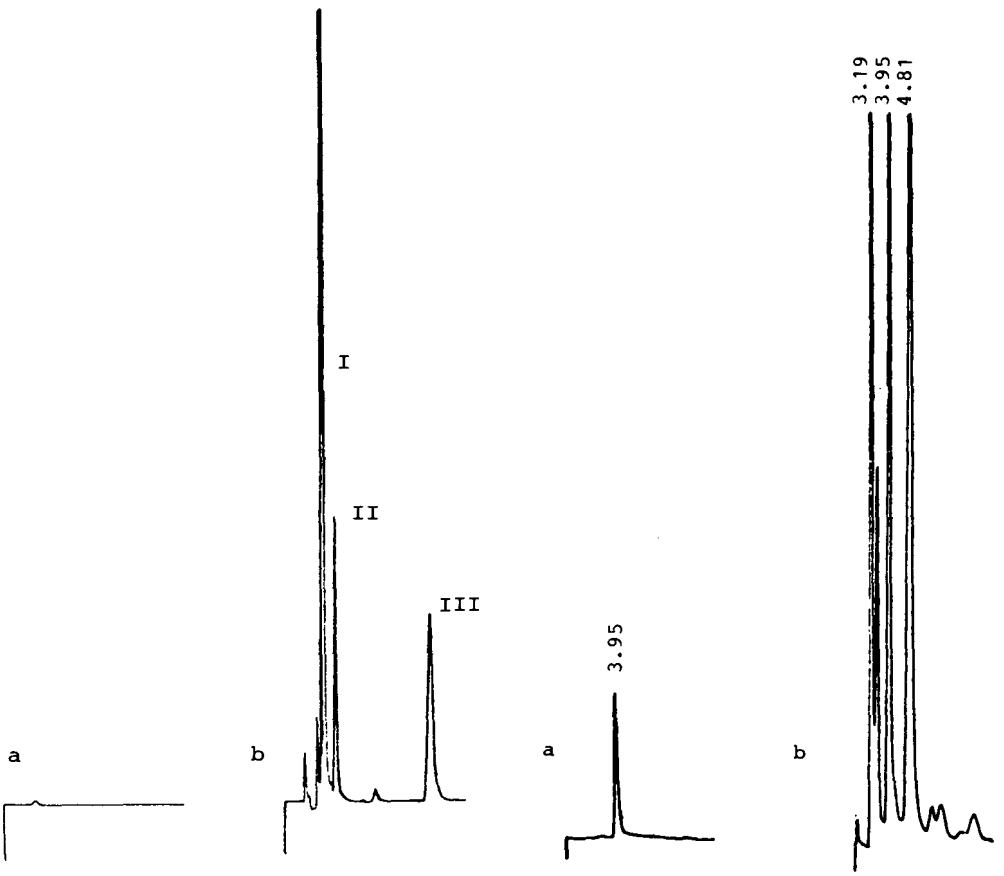

Fig. 6. HPLC chromatograms after automated pre-column sample enrichment of blank human plasma (a) and human plasma (b) after administration of sulmazole. Concentrations: I = 1745 ng/ml sulmazole, II = 137 ng/ml sulfon, and III 223 ng/ml sulfide.

Fig. 7. HPLC chromatograms after automated pre-column sample enrichment of 10 µl blank rat bile (a) and 10 µl of pooled rat bile (b) 0—2 h after intraduodenal administration of sulmazole. Retention time of 4.81 = metabolite M2 (= 6-hydroxy AR-L 115 BS). λ_{ex} = 345 nm; λ_{em} = 515 nm. Eluent = solvent system for pump B, diluted 1:1 with deionized water.

Concentrations of sulmazole and metabolites, AR-L 114 BS and AR-L 113 BS, were automatically calculated and recorded by calibrating the printer/plotter computer where peak area was used as a measurement of drug and metabolite concentration. The calibration curves were constructed from five replicate measurements of concentrations between 7.8 and 1000 ng/ml plasma (Table I), unless otherwise stated.

Machine standard

Pooled human plasma spiked with known concentrations of the three compounds was used as external and instrument standard. The standard or control samples were run between unknown plasma samples to assess precision and accuracy of the system during continuous chromatography (Table I).

TABLE I

CONCENTRATION—PEAK AREA CALIBRATION DATA OF AR-L 115 BS (SULMAZOLE) AND METABOLITES AR-L 114 BS AND AR-L 113 BS AFTER HPLC SEPARATION AND FLUORIMETRIC DETECTION FROM DIRECTLY INJECTED BODY FLUIDS

Volume injected = 10 μl.

CONCENTRATION (NG/ML)	AR-L 115 BS (VARDAX) AREA UNITS	C.V. %	METABOLITE AR-L 114 BS AREA UNITS	C.V. %	METABOLITE AR-L 113 BS AREA UNITS	C.V. %
1000	3567 3358 3314 3299 3275	0.35	8536 8460 8311 8221 8093	2.14	11948 11599 11345 11213 11168	2.82
500	1642 1629 1637 1634 1645	0.39	4074 4036 3999 3947 3937	1.45	5520 5444 5468 5369 5319	1.47
250	800 807 800 812 819	1.01	1948 1944 1928 1933 1912	0.74	2614 2625 2550 2540 2547	1.58
125	398 404 414 406 420	2.12	943 934 949 938 898	2.15	1276 1236 1231 1227 1235	1.60
62.5	197 195 210 216 223	5.8	465 467 455 466 468	1.13	633 609 607 647 643	2.99
31.3	788* 775 794	1.3	233 229 230 228 232	0.9	314 314 313 314 324	1.46
15.6	381* 377 386	1.2	110 110 110 115 121	4.34	177 161 185 151 -	9.11
7.8	226* 227 241	3.6	61 55 57 61 63	6.23	- - - - -	-
Y = a . X r = CORRELATION COEFFICIENT	a = 0.2991 r = 0.9995		a = 0.1215 r = 0.9996		a = 0.0886 r = 0.9992	

*Volume injected = 50 μl.

Precision and linearity

The coefficient of variation (C.V.) for the within-day precision of the automated assay for sulmazole, calculated on the basis of five repetitive injections per concentration, was 0.35% (1000 ng/ml), 0.4% (500 ng/ml), 1.0% (250 ng/ml), 1.3% (31 ng/ml), 1.2% (16 ng/ml) and 3.6% (8 ng/ml).

Between-day precision, characterized by the coefficient of variation (C.V. %) after five replicates in each concentration over a period of twelve months was 2.8% (1000 ng/ml), 1% (500 ng/ml) and 2% (100 ng/ml).

Pre-column enrichment — extraction efficiency

It is desirable that the HPLC assay measures total (i.e. free and bound) drug and metabolites. This requires that the pre-column strips all bound drug from plasma proteins in addition to adsorbing free drug. The total amount of drug in plasma is the sum of free drug and protein-bound drug, where the extent of reversible protein binding at equilibrium is dependent on the affinity of the drug molecule to the plasma proteins.

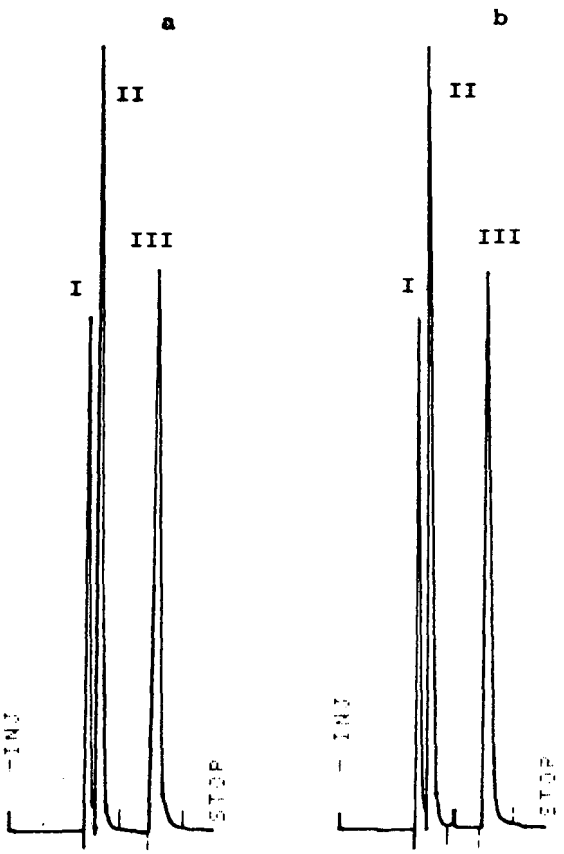

Fig. 8. HPLC chromatograms after automated pre-column sample enrichment of sulmazole (I) and sulfone and sulfide metabolites (II and III) in spiked water (a) and in human plasma (b) of 1000 ng/ml of each compound. Conditions see Fig. 7.

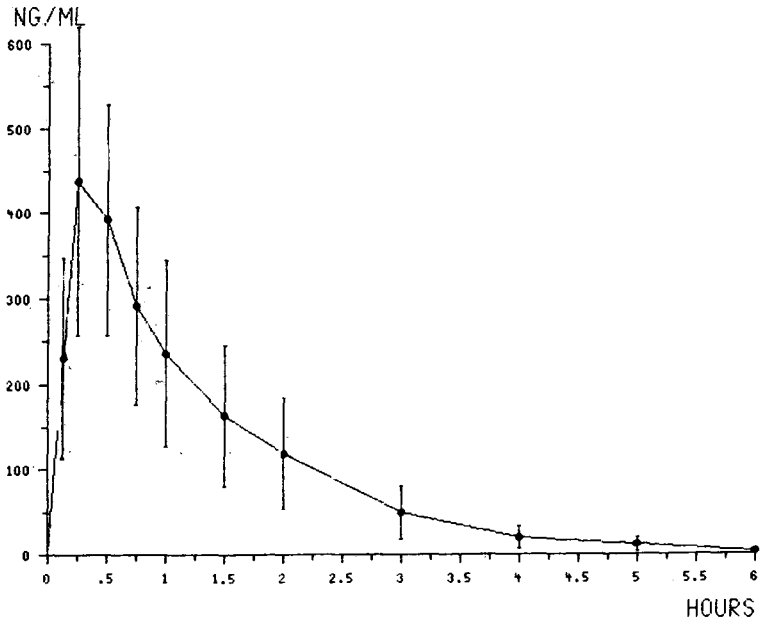

Fig. 9. Time course of sulmazole in humans (n = 8) after oral administration of a 50 mg solution.

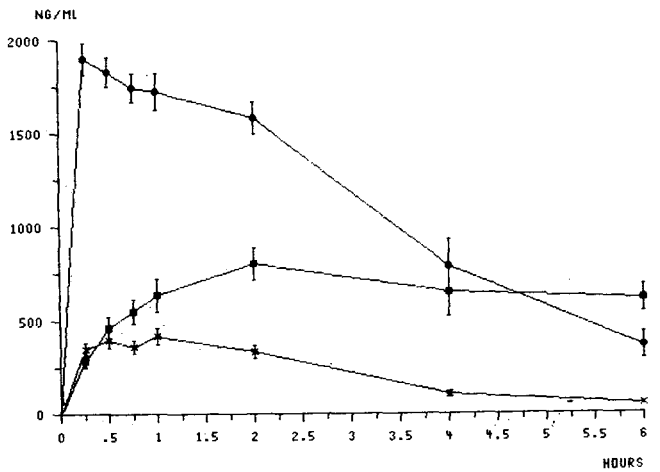

Fig. 10. Time course of sulmazole (•) and sulfone (▪) and sulfide (×) metabolites after oral administration of a 15 mg/kg aqueous solution to rats (n = 5; mean ± S.D.).

It is therefore important to check the partition characteristics of drug between binding sites on plasma proteins and on the lipophilic reversed-phase material under the conditions of the HPLC assay. The lipophilic alkyl brushes of the reversed-phase modified silica matrix should interact with the drug—protein complex and should adsorb free and protein-bound drug simultaneously to the reversed-phase matrix of the enrichment column.

In the case of the pre-column enrichment technique in automated HPLC

from directly injected plasma, it is therefore important to confirm that the binding of the reversed-phase matrix competes effectively for the plasma protein binding. This can be achieved by comparing the peak signals of spiked buffer and spiked plasma injections of the same total drug concentrations (Fig. 8).

The data show that protein-bound and free sulmazole and metabolites are quantitatively partitioned from plasma onto the pre-columns, indicating that their binding to the reversed-phase matrix is effectively infinitely greater than plasma protein interactions under these specific conditions.

Plasma level

The time course in plasma of sulmazole and metabolites, detected with the above described HPLC methodology is depicted in Figs. 9 and 10.

CONCLUSIONS

In addition to the previously reported HPLC assay of sulmazole with classical sample pre-treatment [10], we have developed an HPLC methodology which works without any manual sample pre-treatment step prior to HPLC analysis. High-performance liquid chromatographic separation with automatic alternated pre-column enrichment technique and fluorimetric detection is able to quantify sulmazole and two plasma metabolites, AR-L 114 BS (sulfone of sulmazole) and AR-L 113 BS (sulfide of sulmazole), in one HPLC run from directly injected plasma, urine or bile. Metabolite M2, as one representative of a hydroxylated sulmazole species, was detectable in rat bile and in dog urine [11].

The method is easy to handle, needs no sample pre-treatment, has a high precision and accuracy, is ideally suited for pharmacokinetic drug monitoring and thus can be immediately used for a fast individual dose titration.

ACKNOWLEDGEMENTS

The excellent technical assistance of Mr. K. Beschke and Mr. M. Schwilk is greatly appreciated.

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Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1873

SIMULTANEOUS DETERMINATION OF ASTEMIZOLE AND ITS DEMETHYLATED METABOLITE IN ANIMAL PLASMA AND TISSUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received March 28th, 1983; revised manuscript received June 27th, 1983)

SUMMARY

A high-performance liquid chromatographic procedure has been developed for the determination of astemizole and its primary metabolite in plasma and animal tissues. Both compounds and the internal standard were extracted from alkalinized plasma with heptane—isoamyl alcohol and analyzed using a reversed-phase column and UV monitoring at 254 nm. The detection limits for both compounds were 1 ng/ml of plasma and 5 ng/g of tissue and extraction recoveries were sufficiently high (71—84%). The method was applied to plasma and tissue samples from dogs after repeated oral administration, and to plasma samples from a volunteer taking a 300-mg oral dose of the drug. The results were compared with those obtained by a formerly developed radioimmunoassay.

INTRODUCTION

Astemizole, 1-[(4-fluorophenyl)methyl]-N-{1-[2-(4-methoxyphenyl)ethyl]-4-piperidinyl} -1H-benzimidazol-2-amine (I, Fig. 1), is a new, orally very potent and long-acting histamine H_1 receptor antagonist, devoid of central, sedative and peripheral anticholinergic effects [1]. Astemizole is rapidly absorbed after oral administration, both in man and experimental animals. It undergoes extensive first-pass metabolism and is slowly eliminated, mainly with the faeces [2]. Main metabolic pathways are oxidative O-demethylation, aromatic hydroxylation at the benzimidazole moiety and oxidative N-dealkylation at the piperidine nitrogen, yielding desmethylastemizole (II, Fig. 1) as the major metabolite [2].

Besides radioactivity measurements, drug concentrations in plasma have mostly been measured by a previously described radioimmunoassay (RIA) procedure [3].

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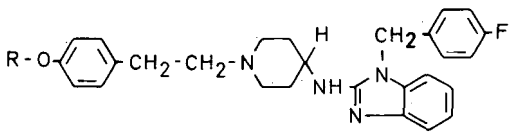

Compound	R
I	CH_3
II	H
III	CH_2-CH_3

Fig. 1. Chemical structure of astemizole (I), desmethylastemizole (II) and the internal standard (III).

The present paper describes a selective high-performance liquid chromatographic (HPLC) method that enables the measurement of both the unaltered drug and its demethylated metabolite in animal plasma and tissues. The method was used to gain further information about the pharmacokinetics of the drug in experimental animals and to allow the validation of the existing RIA procedure.

EXPERIMENTAL

Reagents

Astemizole (R 43 512), desmethylastemizole (R 44 271), 1-[(4-fluorophenyl)methyl]-N-{1-[2-(4-hydroxyphenyl)ethyl]-4-piperidinyl}-1H-benzimidazol-2-amine and the internal standard (R 44 180), 1-[(4-fluorophenyl)-methyl]-N-{1-[2-(4-ethoxyphenyl)ethyl]-4-piperidinyl}-1H-benzimidazol-2-amine (III, Fig. 1), were obtained from the Life Sciences Products Division of Janssen Chimica (Beerse, Belgium) and were of analytical grade.

Spectrophotometric grade acetonitrile, methanol and *n*-heptane were used (Uvasol; E. Merck, Darmstadt, F.R.G.). All other reagents were of analytical grade.

Standard solutions

Stock solutions, corresponding to 0.1 mg/ml of methanol, were prepared for compounds I, II and III. Standard solutions were obtained by diluting the stock solution of I and II to concentrations ranging from 0.02 to 20 μg/ml of methanol. To spike the samples with the internal standard, the stock solution of III was further diluted to 1 μg/ml.

Extraction procedure

Plasma. Two millilitres of plasma (unknown samples, drug-free plasma, or plasma standards containing known amounts of the drugs) were transferred to 15-ml glass centrifuge tubes, spiked with 0.1 μg of the internal standard. The solutions were made alkaline with 2 ml of a 0.05 *M* borax (sodium borate decahydrate) solution, and 4 ml of a heptane—isoamyl alcohol mixture (95:5, v/v) were added. The tubes were carefully rotated for 10 min (10 rpm, Cenco rotary mixer) and then centrifuged (5 min, 1000 *g*). The upper organic layer

was transferred to a second centrifuge tube by means of a disposable pasteur pipet and the plasma was extracted again with 4 ml of the heptane—isoamyl alcohol mixture. The combined organic layers were back-extracted with 3 ml of 0.05 *M* sulphuric acid and removed after centrifugation. The remaining acidic phase was made alkaline with concentrated ammonia and extracted twice with 2-ml aliquots of the heptane—isoamyl alcohol mixture. The combined organic layers were finally evaporated to dryness under a gentle stream of nitrogen in a water bath at 55° C.

Tissue samples. Aliquots of the various tissues, previously ground in a Waring commercial blender, were further homogenized (1:4, w/v) in distilled water by means of an Ultra-Turrax TP 18/10 homogenizer. Two millilitres of the resulting homogenates were then extracted as described above.

Calibration procedure

Using the standard solutions of astemizole and its demethylated metabolite, samples of blank control plasma (2 ml) were spiked with both compounds at concentrations ranging from 1 to 100 ng/ml, and with the internal standard at a fixed concentration of 50 ng/ml. These calibration samples were then taken through the extraction procedure described above.

In the same way, standard curves for both astemizole and the metabolite were prepared in blank control animal tissue homogenates (1:4, w/v). Depending on the type of study, the samples were spiked with either high (10 μg/g) or low (0.25 μg/g) concentrations of the internal standard.

Apparatus

The liquid chromatograph used consisted of a Spectra-Physics SP 8700 solvent delivery system, equipped with an SP 8750 organizer module fitted with a 50-μl loop Valco valve injector, and an SP 8300 selectable-wavelength UV—visible detector operating at 254 nm. The separations were achieved using a reversed-phase column (15 cm × 2.1 mm I.D.) packed with 5-μm particle-sized RSiL C18HL (Alltech Europe) by the balanced density procedure by means of an air-driven liquid pump (Haskel). The samples were eluted with acetonitrile—water (50:50) at a constant flow-rate of 0.6 ml/min. To suppress the ionization of the basic functions of the investigated compounds, 0.05% diethylamine was added to the solvent system. Area integrations, peak height measurements, calculations and plotting of the chromatograms were carried out by a Spectra-Physics Model SP 4100 computing integrator.

High-performance liquid chromatography

The various extraction residues were redissolved in 50 μl of methanol by vigorous vortexing, and aliquots as large as possible were injected onto the HPLC column.

Calculations

Ultimate sample concentrations were calculated by determining the peak area ratios of astemizole or desmethylastemizole, related to the internal standard, and comparing these ratios with the standard curves obtained after analysis of the calibration samples.

RESULTS

The recoveries of the extraction procedure for astemizole and desmethylastemizole (100 ng) from 2-ml control plasma or tissue homogenate samples are summarized in Table I.

TABLE I

EXTRACTION RECOVERIES FOR ASTEMIZOLE AND DESMETHYLASTEMIZOLE FROM BIOLOGICAL SAMPLES

Compound	Percentage recovery* (mean ± S.D., n = 5)	
	Plasma	Tissue
Astemizole (I)	84 ± 3	75 ± 4
Desmethylastemizole (II)	73 ± 4	71 ± 4

*Percentage recovery = recovery of the extraction procedure, obtained after analysis of 100 ng of the appropriate compounds added to 2 ml of control plasma or tissue homogenate.

Retention times under the described chromatographic conditions were 5.8, 1.9 and 8.3 min for compounds I, II, and III, respectively. Fig. 2 shows that no interfering peaks occurred at these retention times and that all compounds eluted as separated symmetrical peaks, although the detection of desmethylastemizole (II) was somewhat hindered by the broad solvent front caused by substances originating from the plasma. Linear relationships (r = 0.999) were found when the ratio of the peak ara of astemizole and desmethylastemizole to the peak area of their internal standard were plotted on the y-axis against various concentrations of either astemizole or desmethylastemizole on the x-axis. The different correlation coefficients and mathematical expression of the standard curves for I and II in both plasma and tissue calibration samples are summarized in Table II.

The accuracy and precision of the procedure was ascertained by adding different amounts of both compounds to drug-free plasma and analyzing four samples of each concentration with the method described. The results are summarized in Tables III and IV. The detection limits were 1 ng/ml plasma or 5 ng/g tissue for both investigated compounds.

The method described has been used to measure plasma levels of I and II in a male volunteer, taking a 300-mg oral dose, representing 10—30 times the therapeutic dose. The plasma concentration—time profile is depicted in Fig. 3. The method has also been utilized successfully in the analysis of plasma and tissue samples from dogs, after chronic oral treatment with astemizole at a dose level of 10 mg/kg. Some results are given in Fig. 4.

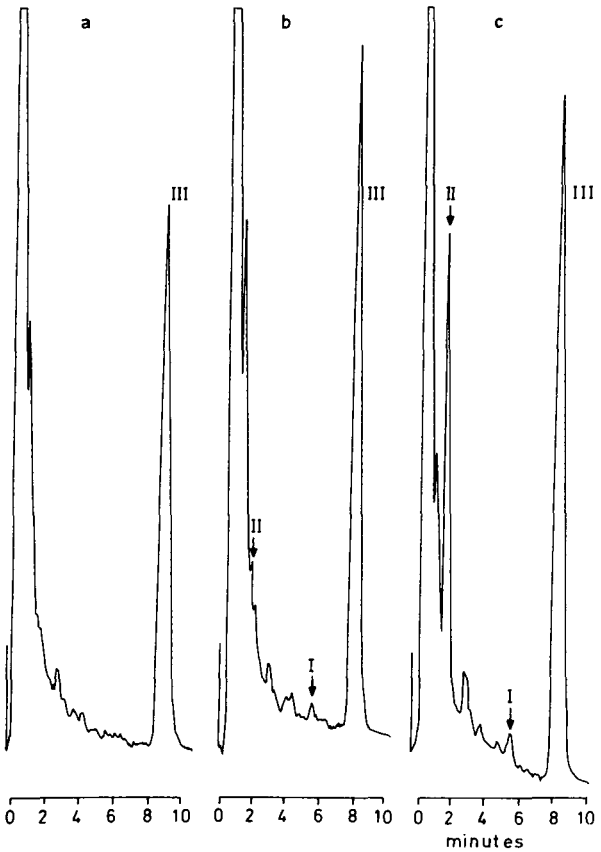

Fig. 2. Chromatograms of extracts from (a) blank control plasma, (b) blank control plasma spiked with 1 ng/ml astemizole and desmethylastemizole, and (c) plasma from a volunteer, 24 h after oral intake of 300 mg of astemizole. I = Astemizole (1.0 and 1.3 ng/ml for chromatograms b and c); II = desmethylastemizole (1.0 and 18.0 ng/ml for chromatograms b and c); III = internal standard (50 ng/ml for all chromatograms). Chromatographic conditions were as indicated in the text.

TABLE II

STANDARD CURVES FOR ASTEMIZOLE (I) AND DESMETHYLASTEMIZOLE (II) IN BIOLOGICAL SAMPLES

Compound	Sample	Internal standard concentration	Range (ng/ml or ng/g)	Regression equation ($Y = aX + b$)*		Correlation coefficient	
				a	b	r	n
I	Plasma	50 ng/ml	1—100	0.024	—0.004	0.9999	8
	Tissue	250 ng/g	5—500	0.0047	—0.008	0.9995	10
II	Plasma	50 ng/ml	1—100	0.035	—0.017	0.9999	8
	Tissue	250 ng/g	5—500	0.0069	—0.013	0.9994	10

*Y = peak area ratio (astemizole/internal standard, or desmethylastemizole/internal standard); X = astemizole or desmethylastemizole concentration (ng/ml or ng/g).

TABLE III

ACCURACY AND PRECISION OF THE HPLC METHOD FOR THE DETERMINATION OF ASTEMIZOLE IN PLASMA SAMPLES

Theoretical astemizole plasma concentration (ng/ml)	Observed astemizole plasma concentration (ng/ml, mean ± S.D., $n = 4$)	C.V.* (%)	Accuracy (%)
1	1.21 ± 0.16	13.1	121.0
2.5	2.56 ± 0.28	11.0	102.4
5	4.85 ± 0.29	5.9	97.0
10	9.30 ± 0.37	4.0	93.0
25	25.0 ± 0.6	2.4	100.0
50	50.6 ± 1.0	1.9	101.2
100	99.8 ± 3.0	3.0	99.8

*C.V. = coefficient of variation

TABLE IV

ACCURACY AND PRECISION OF THE HPLC METHOD FOR THE DETERMINATION OF DESMETHYLASTEMIZOLE IN PLASMA SAMPLES

Theoretical desmethyl-astemizole plasma concentration (ng/ml)	Observed desmethyl-astemizole plasma concentration (ng/ml, mean ± S.D., $n = 4$)	C.V.* (%)	Accuracy (%)
1	0.90 ± 0.17	18.6	90.0
2.5	2.63 ± 0.38	14.3	105.2
5	4.19 ± 0.20	4.8	83.8
10	10.1 ± 0.5	5.3	101.0
25	25.0 ± 0.5	1.8	100.0
50	50.6 ± 1.0	2.0	101.2
100	99.9 ± 3.3	3.3	99.9

*C.V. = coefficient of variation.

DISCUSSION

The extractability of both compounds from plasma was tested in recovery experiments using different alkaline buffer systems with several heptane—isoamyl alcohol mixtures as the solvent. The maximum extraction recovery was obtained at pH 9 using heptane—isoamyl alcohol (95:5, v/v) and amounted to over 80% and 70% for astemizole and desmethylastemizole, respectively.

In separate experiments, all efforts were made to improve the sensitivity of the method. UV absorbance detection appeared to be more sensitive than fluorescence or electrochemical detection and, although astemizole possesses two absorbance maxima (ϵ_0 = 10 300 at 255 nm and ϵ_0 = 12 300 at 287 nm), 254 nm was selected because of the better intrinsic sensitivity and the more general accessibility of fixed-wavelength detectors compared to variable-wavelength types.

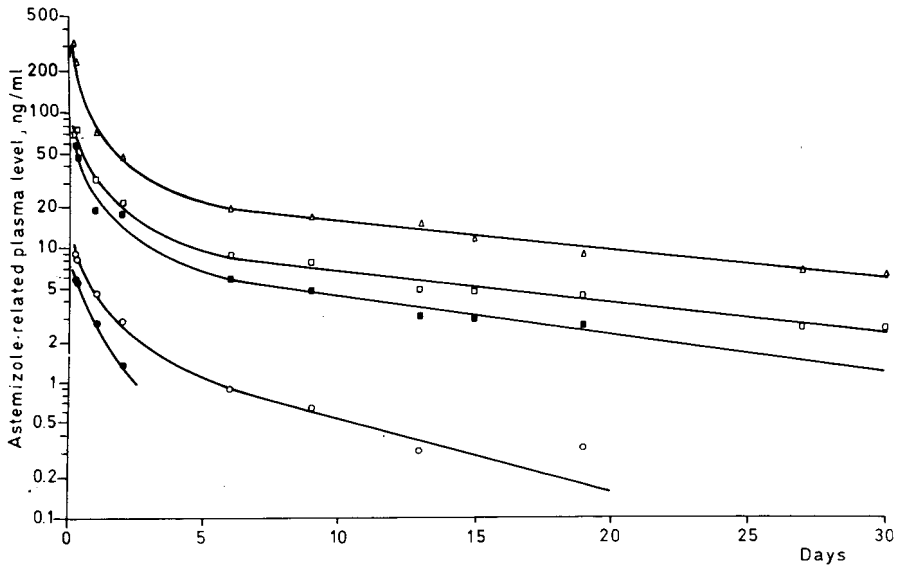

Fig. 3. Astemizole-related plasma levels in a healthy volunteer after ingestion of 300 mg of the drug. (•), Unchanged astemizole (I), as determined by the presented HPLC method; (▪), desmethylastemizole (II), as determined by the presented HPLC method; (○), unchanged astemizole, as determined by RIA after the most selective extraction procedure; (□), astemizole and all its phenolic metabolites, as determined by RIA after a single extraction procedure; (△) astemizole and all related metabolites, as determined by direct RIA.

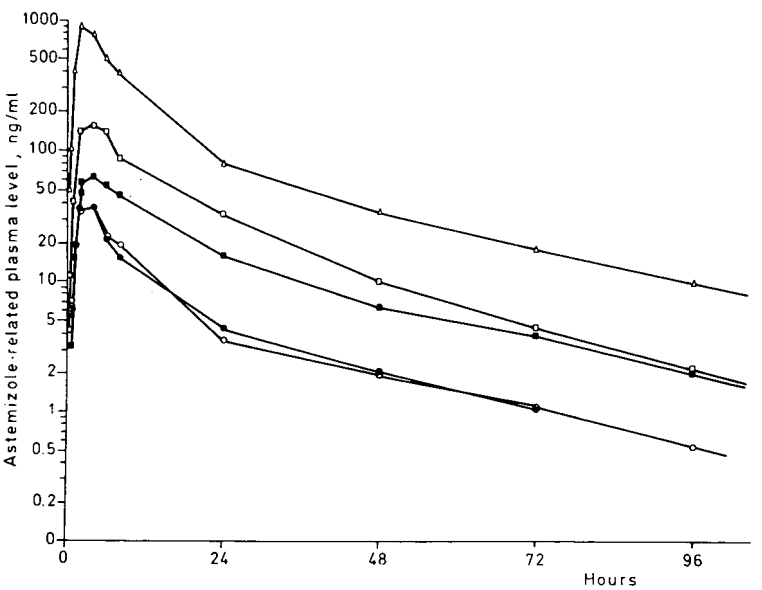

Fig. 4. Mean astemizole-related plasma levels in four dogs, orally treated with the drug at a dose level of 10 mg/kg. (•), Unchanged astemizole (I), as determined by the presented HPLC method; (▪), desmethylastemizole (II), as determined by the presented HPLC method; (○), unchanged astemizole, as determined by RIA after the most selective extraction procedure; (□), astemizole and all its phenolic metabolites, as determined by RIA after a single extraction procedure; (△) astemizole and all related metabolites, as determined by direct RIA.

Although a detection limit of 1 ng/ml could be reached, this proved to be insufficient for monitoring therapeutic plasma levels of astemizole, and a more sensitive RIA procedure has been previously developed for this purpose [3]. In order to compare the RIA and HPLC methods for astemizole, samples from experiments in dogs as well as samples from a volunteer taking a 300-mg oral dose, were measured using both assay methods (Figs. 3 and 4). It can be seen that, despite the lack of specificity of the RIA method, there is a striking parallelism between the results obtained for unchanged astemizole (Fig. 3) and for desmethylastemizole (Figs. 3 and 4), as determined by each method. Notwithstanding that very similar results were obtained for the plasma levels of I by both procedures in dogs (Fig. 4), there was a significant overestimation by RIA of the unchanged astemizole plasma levels in man (Fig. 3). This was most probably due to the presence of the N-dealkylated metabolite, 1-[(4-fluorophenyl)-methyl]-N-(4-piperidinyl)-1H-benzimidazol-2-amine, which partially interfered in the RIA procedure and which was found to be proportionally more important in man [2]. The latter compound, being a secondary amine with less lipophylic properties, was only extracted to a lower extent, and eluted with the solvent front under the described chromatographic conditions. Hence, the compound as well as all polar metabolites, found by Meuldermans et al. [2], will not interfere in the HPLC procedure presented.

ACKNOWLEDGEMENTS

The authors wish to thank Mrs. C. Verellen and Mr. H. Vermeer for the preparation of the manuscript.

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Journal of Chromatography, 278 (1983) 367—378
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Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1876

AUTOMATED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF ANTIPYRINE AND ITS MAIN METABOLITES IN PLASMA, SALIVA AND URINE, INCLUDING 4,4'-DIHYDROXY-ANTIPYRINE

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(Received May 17th, 1983)

SUMMARY

A rapid, selective and sensitive method was developed for the determination of antipyrine and its main metabolites in plasma, saliva and urine by an automated high-performance liquid chromatographic system. Using a MOS-Hypersil reversed-phase column with a phosphate buffer—acetonitrile mobile phase, baseline separation of antipyrine, its metabolites 3-hydroxymethylantipyrine, norantipyrine and 4-hydroxyantipyrine, and the internal standard, phenacetin, was achieved within 6 min. Factors regarding the accuracy and precision of the method and the stability of phase I metabolites during sample preparation are discussed, taking into account certain drawbacks of previously published methods.

Based on the same chromatographic system a method was developed for the assay of 4,4'-dihydroxyantipyrine in urine. This compound is an important metabolite of antipyrine in the rat, representing 12.6 ± 1.8% of the administered dose (n = 18).

INTRODUCTION

Antipyrine is commonly used as a model compound to study the influence of disease, drugs and environmental factors on hepatic drug-metabolizing enzyme activity in vivo [1, 2]. In such studies this activity was mostly assessed on the basis of plasma or saliva elimination kinetics.

Recently, the value of the antipyrine test has been improved by also assessing the antipyrine metabolite profile [3], since the formation of the main metabolites of antipyrine was shown to be regulated by different forms of cytochrome *P*-450 [4—8]. In rats, the formation of 3-hydroxymethylantipyrine (HMA) is associated with the phenobarbital (PB) type of cytochrome

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P-450, that of 4-hydroxyantipyrine (OHA) with the methylcholanthrene (MC) type, while norantipyrine (NORA) is probably also formed by an MC-inducible type of cytochrome *P*-450. Hence the assessment of rates of formation of antipyrine metabolites enables the quantitation of different drug-oxidizing enzymes in one test [9].

Aromatic ring hydroxylation was recently found to be an additional pathway in the biotransformation of antipyrine in man and rat [10]. Of these aromatic ring hydroxylated products, 4,4′-dihydroxyantipyrine (DOHA) appears to be a relatively important metabolite, since it represents 11—18% of the dose in rats and 3—6% in man [11]. 4′-Hydroxyantipyrine (pOHA) was found to be a minor metabolite of antipyrine, representing 2—4% of the dose in man and less than 1% in the rat [12]. For investigations in man, only HMA, NORA and OHA are relevant, since the amounts of the other metabolites do not exceed 5% of the administered dose [12, 13]. In rats however, DOHA is also an important metabolite [11].

In this study, an automated high-performance liquid chromatographic (HPLC) method for the determination of antipyrine in plasma, saliva and urine, and its metabolites HMA, NORA and OHA in urine is described, taking into account certain drawbacks of previously published methods [3, 14—20]. Special emphasis is put on the stability of phase I metabolites during sample preparation. Furthermore, an HPLC method was developed for the determination of DOHA in urine.

MATERIALS AND METHODS

Chemicals

Antipyrine (AP) was purchased from Brocacef (Maarssen, The Netherlands). 4-Hydroxyantipyrine (OHA), norantipyrine (NORA) and 3-hydroxymethylantipyrine (HMA) were synthesized according to previously described methods [21—24]. 4,4′-Dihydroxyantipyrine (DOHA) was a gift from Dr. Schüppel and co-workers, Institut für Pharmakologie und Toxikologie der Technischen Universität Braunschweig (F.R.G.). Organic solvents were obtained from Baker Chemicals (Deventer, The Netherlands) and limpet acetone powder type I (glucuronidase—sulphatase) from Sigma (St. Louis, MO, U.S.A.).

Instrumentation and chromatographic conditions

The liquid-chromatographic system (Waters Assoc., Milford, MA, U.S.A.) consisted of an M-45 pumping device, a WISP 710B automatic sample injector and an M-440 UV detector at 254 nm. The chromatographic data were processed by a Hewlett-Packard 3390 reporting integrator.

Columns (100 × 3.0 mm) were packed with spherical 5 μm MOS-Hypersil (Shandon, Southern Instruments, Astmoor, U.K.). Evaporation of extraction solvents was achieved under reduced pressure by a Büchler vortex evaporator.

The eluent for the separation of antipyrine and its metabolites, HMA, NORA, and OHA (assays A, B and C) consisted of a mixture of 0.02 *M* phosphate buffer, pH 7.2, and acetonitrile (100:10) containing sodium pyrosulphite 2 g/l. For the assay of DOHA in urine (assay D) the eluent consisted of a mixture of 0.02 *M* phosphate buffer, pH 6.5, and acetonitrile (100:8).

The eluent for the determination of antipyrine in blood, plasma and saliva (assay E) consisted of a mixture of 0.0067 *M* phosphate buffer, pH 7.2, and acetonitrile (100:18). For all eluents, flow was set at 2 ml/min, resulting in a pressure of about 135 bars.

Calibration graphs

Calibration graphs were prepared by spiking blank samples with antipyrine or the metabolites to be measured and carrying the samples through the analytical procedure. Evaluation of chromatograms was based on peak area ratios as calculated by the internal standard procedure of the HP-3390 reporting integrator. Calibration graphs for antipyrine in human plasma and saliva ranged from 1.0 to 20.0 μg/ml and in rat blood from 2.5 to 50.0 μg/ml; in urine for antipyrine from 2.5 to 30.0 μg/ml, for HMA and DOHA from 12.5 to 100.0 μg/ml and for NORA and OHA from 12.5 to 150.0 μg/ml.

Analytical procedures

Assay A: HMA and AP in urine. To 0.50 ml of urine, 0.50 ml of 0.2 *M* acetate buffer pH 4.5 and 10 mg of limpet acetone powder were added. After incubation of this mixture for 3 h at 37° C, 10 μg of phenacetin (as internal standard in 50 μl of ethanol), 200 mg of sodium chloride and 0.10 ml of 4 *M* sodium hydroxide were added successively and mixed on a whirlmixer for 15 sec. The mixture was extracted with 7 ml of dichloromethane and the organic layer was collected and evaporated to dryness with a vortex evaporator. The residue was disssolved in 0.20 ml of methanol and diluted with 0.50 ml of eluent. Of this solution 15 μl were injected into the HPLC system.

Assay B: NORA and OHA in urine. To 0.50 ml of urine, 0.50 ml of 0.5 *M* acetate buffer pH 4.5 containing 40 mg of $Na_2S_2O_5$ and 10 mg of limpet acetone powder were added. After incubation and addition of internal standard (see above) the mixture was extracted with 5 ml of dichloromethane—*n*-pentane (3:7, v/v) by mixing on a whirlmixer for 15 sec. After centrifugation the organic phase was collected and evaporated in a vortex evaporator under reduced pressure at room temperature. The residue was dissolved in 0.20 ml of methanol and diluted with 0.50 ml of 0.01 *M* acetate buffer containing 12.5 mg of $Na_2S_2O_5$ (freshly prepared). Of this solution 15 μl were injected into the HPLC system.

Assay C: simultaneous assay of HMA, NORA, AP and OHA. Sample handling was identical to that described for the assay of NORA and OHA (assay B), except that after enzymatic hydrolysis the mixture was saturated with NaCl (200 mg) and extracted with 5 ml of chloroform—ethanol (9:1, v/v).

Assay D: DOHA and OHA in urine. A mixture of 0.25 ml of urine and 0.10 ml of concentrated hydrochloric acid was heated in a boiling water-bath for 90 min. Adjustment to pH 4—5 was achieved by addition of 0.20 ml of 4 *M* sodium hydroxide and 200 mg of sodium acetate. The solution was saturated by adding 200 mg of sodium chloride. After addition of the internal standard phenacetin (10 μg in 50 μl of ethanol), the mixture was extracted with 7 ml of chloroform—ethanol (9:1). The organic phase was collected and evaporated to dryness in a vortex evaporator under reduced pressure at 40° C. The residue was dissolved in 0.10 ml of methanol and diluted with 0.25 ml of 0.01 *M*

hydrochloric acid containing 1 mg of $Na_2S_2O_5$ to ensure stability of OHA. Of this solution 25 μl were injected into the HPLC system.

Assay E: antipyrine in blood plasma and saliva. To 0.50 ml of blood, plasma or saliva 0.10 ml of 4 *M* sodium hydroxide and 4 μg of phenacetin (as internal standard in 50 μl of ethanol) were added. After extraction with 5 ml of dichloromethane—*n*-pentane (1:1, v/v) on a whirlmixer for 15 sec, the organic layer was collected and evaporated to dryness under reduced pressure (Büchler vortex evaporator) or by standing overnight at room temperature. The residue was dissolved in 0.20 ml of methanol and diluted with 0.50 ml of eluent. Of this solution 15 μl were injected into the HPLC system.

RESULTS AND DISCUSSION

Hydrolysis of metabolite conjugates

The metabolites of antipyrine are excreted in urine mainly as conjugates [11]. In man, the major conjugation route is glucuronidation. HMA is conjugated to an extent of 30—75%, while the remainder is excreted as free metabolite [25]. OHA and NORA are glucuronidated almost completely, about 2% being conjugated to sulphate or excreted as free metabolite [11]. In rats, sulphation is the major conjugation reaction [26].

Since these conjugates are very stable, it would be desirable to determine them directly without cleavage. Selective extraction and separation of such hydrophilic compounds, however, is very difficult, while reference compounds are not available. Thus far separation and quantitation of the intact conjugation has only been achieved by thin-layer chromatography with radiolabelled compounds [11, 27] or by an off-line combination of liquid chromatography and field desorption mass spectrometry [28]. Therefore a hydrolysis step has to be included when antipyrine metabolites are to be determined routinely by commonly used HPLC detectors like UV spectrophotometry.

Hydrolysis under strong acidic conditions [17] was not satisfactory for the assay of HMA and NORA, since NORA decomposes under these conditions, while HMA glucuronide is only hydrolysed up to 70%. Therefore, enzymatic hydrolysis with glucuronidase—sulphatase was preferred for the assay of HMA, NORA and OHA, since it resulted in complete cleavage of their conjugates [3, 16]. Addition of sodium pyrosulphite (16 mg/ml) was necessary to prevent decomposition of NORA [16] and OHA [3].

Tabarelli-Poplawski and Uehleke [29] reported that OHA irreversibly binds to protein, resulting in a loss of 0.25 μg/mg protein. Our experiments also indicated that small amounts of OHA and NORA were bound to the hydrolysing enzyme powder. As a result, calibration graphs showed negative intercepts, though good linearity was obtained between 10 and 200 μg/ml and slopes were the same with and without enzyme incubation. The loss of NORA and OHA may be less significant if purified enzymes are used, though Eichelbaum et al. [16] reported decreased recoveries of OHA and NORA when more than 10,000 Fishman units or purified *Helix pomatia* enzyme were used per ml of urine.

For the assay of DOHA conjugates, only acidic hydrolysis as described by Böttcher et al. [17] gave satisfactory results, since DOHA decomposed readily

at pH 4.5, which is required for enzymatic hydrolysis. OHA conjugates were also hydrolysed quantitatively with this method [17]. The optimal hydrolysis time was 90 min (boiling water-bath). Continuation of hydrolysis after 90 min resulted in slightly decreased DOHA recoveries, while OHA recovery remained the same (Fig. 1).

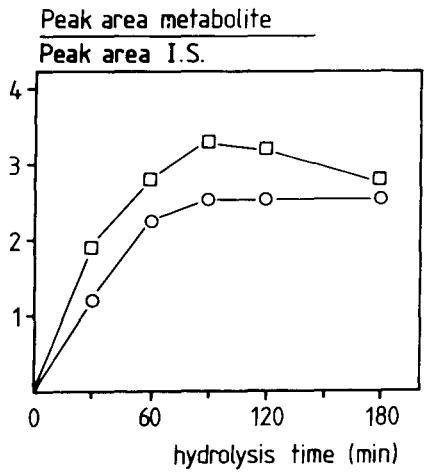

Fig. 1. Peak area ratios of DOHA (□) and OHA (○) after acidic hydrolysis as a function of time. Each point represents the mean of three determinations.

Stability of antipyrine metabolites

One of the most difficult problems in the determination of antipyrine metabolites is the relative instability of NORA. Some authors claim that NORA adsorbs to glass and that silanisation of glassware solves this problems [3, 14, 16]. A more likely explanation of higher recoveries after silanisation of glassware is that NORA dissolves in the silanised layer because of its lipophilicity and is thus protected from further decomposition. However, back-extraction from this layer will be incomplete, and will depend on the thickness of the layer. As a consequence silanisation of glassware leads to irreproducible results and non-linear calibration graphs of NORA.

A better approach to obtain reproducible results is to take the causes of NORA loss into account, i.e. volatility and decomposition due to pH influences and oxidation. NORA is volatile at room temperature [27] and readily disappears when evaporation to dryness of the extraction solvent is achieved by a stream of air or nitrogen. This problem was solved by evaporation of the extraction solvents under reduced pressure (e.g. Büchler vortex evaporator) or by addition of 0.25 ml of methanol before evaporation and stopping the evaporation when about 0.1 ml of solvent (methanol) remains. In both cases evaporation should take place without heating.

Loss of NORA due to oxidation in aqueous media (e.g. urine) can be prevented by addition of sodium pyrosulphite at concentrations of 16 ml/ml [16]. Our observations confirm this result and further indicate that NORA decomposes at pH $>$ 6 (Fig. 2) as well as at pH $<$ 2. OHA is also unstable at pH $>$ 6. Oxidation of OHA can be prevented by adding sodium pyrosulphite

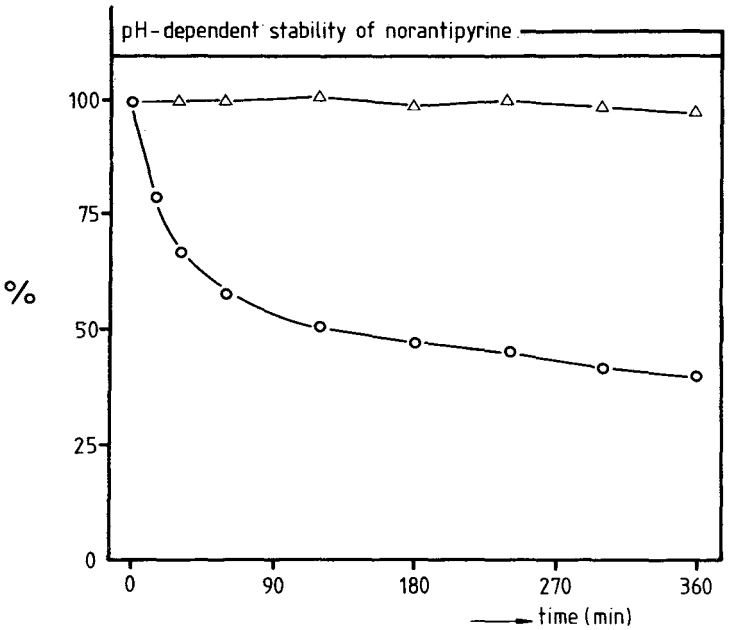

Fig. 2. Stability of NORA in solutions of different pH: (△), in 0.01 *M* acetate buffer pH 4.5 containing sodium pyrosulphite 16 mg/ml; (○), in eluent (0.02 *M* phosphate buffer pH 7.2 containing 10% acetonitrile) containing sodium pyrosulphite 16 mg/ml. Each point represents the mean of three determinations.

[3]. As a result, a solution of OHA and NORA in 0.01 *M* acetate buffer at pH 4.5 containing 16 mg/ml sodium pyrosulphite was found to be stable for at least 6 h (for NORA this is shown in Fig. 2). By addition of an equal volume of 0.5 *M* acetate buffer containing 40 mg/ml sodium pyrosulphite to 0.5 ml of urine, the same results were obtained during enzymatic hydrolysis for 3 h at 37°C.

DOHA is unstable in aqueous solutions at pH > 2. Stability of DOHA at pH 4.5 was not improved by addition of sodium pyrosulphite.

So far, no stability problems have been observed for HMA. The parent compound antipyrine is volatile (J. Böttcher and H. Bässmann, personal communication). Therefore loss of antipyrine may occur when evaporation of extraction solvents takes place at temperatures above 30°C.

Extraction procedures

For extraction of the phase I metabolites of antipyrine from urine, obtained after enzymatic hydrolysis (HMA, NORA and OHA), the systems described by Danhof et al. [3], Eichelbaum et al. [16] and Böttcher et al. [17] were compared. The extraction recoveries obtained with different systems are shown in Table I. NORA, OHA and unchanged antipyrine gave a sufficient extraction yield with all three systems at pH 4.5, whereas NORA and OHA were not extracted at pH > 10. For extraction of HMA, saturation of the aqueous layer was necessary, while pH had little influence. The highest recoveries were obtained when chloroform—ethanol (9:1) was used. The recovery of the

internal standard phenacetin was higher with increasing lipophilicity. Considering the low selectivity of the single-step extraction procedures [16, 17], several other compounds were co-extracted (Fig. 3), resulting in considerably higher background in the chromatograms than after the more selective extraction procedures [3]. This may lead to overestimation of peak areas (Table II) and shortening of column life-times. Interference of other drugs or metabolites may also occur.

We therefore recommend the more selective dual extraction procedure described by Danhof et al. [3] whenever antipyrine is given simultaneously with other drugs. When antipyrine is given alone, the less selective extraction described by Böttcher et al. [17] may be preferred, since it requires only one extraction step and one chromatographic run. Eichelbaum's method [16] gives lower HMA recoveries (Table I), while the chromatographic background is similar. For extraction of DOHA after acidic hydrolysis, the hydrolysate had to be saturated with sodium chloride. Optimal pH for extraction of DOHA was pH 4—5 [17]. This pH adjustment was achieved by adding 0.20 ml of 4 *M*

TABLE I

EXTRACTION RECOVERIES OF ANTIPYRINE AND METABOLITES FROM HUMAN URINE

Extraction solvents	pH	NaCl saturation	Percentage recovery ± S.D. (n = 5)			
			HMA	NORA	OHA	AP
Dichloromethane (DCM)	>10	+	54 ± 3	n.d.*	n.d.	100 ± 1
DCM—*n*-pentane (3:7)	4.5	—	n.d.	90 ± 2	90 ± 2	98 ± 1
DCM—isopropanol (9:1)	4.5	+	58 ± 3	88 ± 2	87 ± 2	98 ± 1
Chloroform—ethanol (9:7)	4.5	+	96 ± 2	82 ± 3	99 ± 1	99 ± 1

*n.d. = not detected.

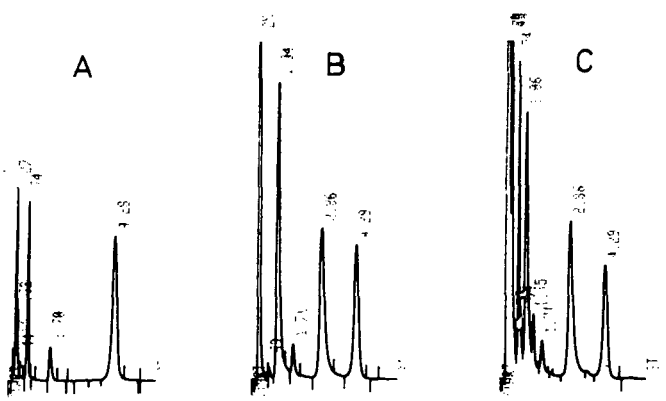

Fig. 3. Chromatograms of the same urine sample of a human volunteer, obtained after different extraction procedures. A, B and C correspond to the assay methods as described in Materials and methods section. Retention times (in min): 0.74 = HMA, 1.04 = NORA, 1.71 = AP, 2.86 = OHA, 4.29 = internal standard.

TABLE II

ACCURACY AND PRECISION OF THE ANALYSIS OF ANTIPYRINE AND ITS METABOLITES. COMPARISON OF OBTAINED CONCENTRATIONS (μg/ml ± S.D., $n = 5$) FROM THE SAME HUMAN URINE SAMPLE AFTER DIFFERENT ASSAY METHODS*

Extraction solvents	Method	HMA	NORA	OHA	AP
Dichloromethane (DCM)	A	16.0 ± 0.6	n.d.**	n.d.	2.9 ± 0.1
DCM—*n*-pentane (3:7)	B	n.d.	48.6 ± 1.2	54.7 ± 0.9	2.9 ± 0.1
Chloroform—ethanol (9:1)	C	22.5 ± 1.5	51.7 ± 1.5	55.9 ± 0.9	2.8 ± 0.1

*For corresponding chromatograms, see Fig. 3.
**n.d. = not detected.

sodium hydroxide and 200 mg of sodium acetate to the hydrolysate. After extraction with 7 ml of chloroform—ethanol (9:1) the recovery of DOHA was 90 ± 2%, which is in agreement with previous results [17]. Since both acidic hydrolysis and extraction (Table I) of OHA are satisfactory with the procedure described for DOHA, OHA can be quantified simultaneously with DOHA.

Dissolution of residues

After evaporation of the organic solvents, the residues are usually dissolved in eluent before injection. However, at the pH of the eluent (7.2) OHA and NORA are unstable (Fig. 2). Furthermore, NORA, OHA and phenacetin (internal standard) dissolve slowly in the eluent.

To overcome these problems, we dissolved the residues in 0.20 ml of methanol and diluted then with 0.50 ml of 0.01 *M* acetate buffer pH 4.5 containing sodium pyrosulphite (25 mg/ml). Under these conditions, all metabolites and phenacetin readily dissolved and were stable for at least 6 h, except DOHA. The residue obtained in the DOHA assay could not be dissolved in eluent (pH 6.5) due to instability of DOHA at pH > 2. Therefore the residue was dissolved in 0.10 ml of methanol and diluted with 0.25 ml of 0.01 *M* hydrochloric acid. This medium provided sufficient stability of DOHA: after 3 h more than 95% was left. Since only 15—25 μl of these solutions were injected, neither methanol nor the difference in pH caused any change in column equilibrium.

Chromatographic system

Recently three HPLC systems have been described that provide baseline separation of HMA, NORA, OHA and unchanged antipyrine [3, 16, 18]. The method described by Kahn et al. [18] is time-consuming, since it includes a derivatization step and a gradient elution, resulting in an injection cycle of 36 min. Eichelbaum et al. [16] described a straight-phase system in which we find it difficult to keep the composition of the mobile phase constant, due to volatility of ammonia. Therefore NORA retention times were not constant with time. The reversed-phase system described by Danhof et al. [3] was preferred, since it requires no derivatization, an isocratic run takes no longer than 10 min and the mobile phase (0.05 *M* phosphate buffer pH 6.5

containing acetonitrile, 100:5) is very stable. Improvement was achieved by using MOS-Hypersil instead of LiChrosorb RP-2, resulting in a decrease of pressure from 200 to 100 bars at a flow-rate of 1.5 ml/min. At an eluent pH of 7.2, an excellent separation was achieved between HMA, NORA, OHA, unchanged antipyrine and phenacetin, while an increase in the acetonitrile content in the eluent from 5% to 10% shortened the run time to 6 min (Fig. 4). Typical chromatograms of the metabolites of antipyrine after extraction from a human urine sample (methods A, B and C) are shown in Fig. 3.

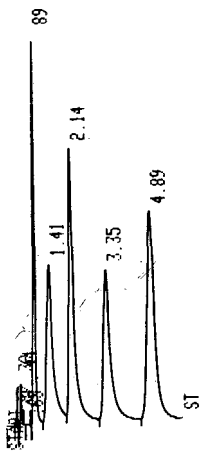

Fig. 4. Chromatogram obtained after injection of a standard solution containing HMA (retention time = 0.89 min), NORA (1.41), AP (2.14), OHA (3.35) and phenacetin (4.89). Abbreviations and conditions given in Materials and methods section.

The chromatographic systems for all assays described in this paper were identical except for small changes in composition of the eluents. In the assay of antipyrine in blood, plasma and saliva, acetonitrile content was 18%, resulting in very short retention times and excellent resolution (Fig. 5).

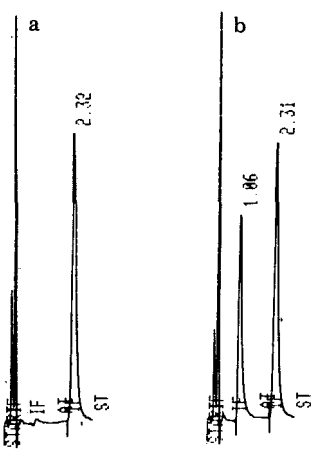

Fig. 5. Chromatograms obtained after extraction of blank plasma (a) and of plasma containing 4.8 μg/ml antipyrine (b) (retention time = 1.06 min). Both samples contained the internal standard phenacetin (retention time = 2.31 min).

Since antipyrine and phenacetin are not readily oxidized, sodium pyrosulphite was omitted from the eluent. As to the assay of DOHA, separation was optimal with the eluent at pH 6.5 containing 8% acetonitrile. Fig. 6 shows a typical chromatogram of a rat urine sample carried through the analytical procedure D as described. Finally the life-time of the pump seals was markedly increased by lowering the molarity of the phosphate buffer from 0.05 to 0.02 *M* in the metabolite assays and to 0.0067 *M* in the assay of antipyrine in blood, plasma and saliva. These decreased molarities proved to be sufficient to maintain constant pH.

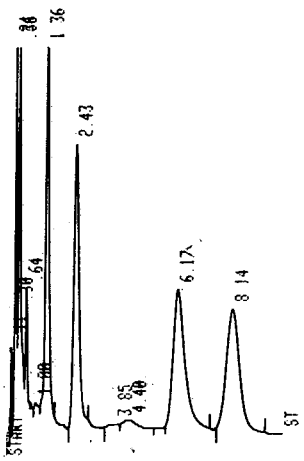

Fig. 6. Chromatogram obtained with assay method E of rat urine. Retention times (in min): 2.43 = DOHA, 4.40 = AP, 6.17 = OHA and 8.14 = internal standard. Other antipyrine metabolites: 1.36 = HMA (not fully hydrolysed) and 3.85 = NORA (partly decomposed).

Accuracy and precision

Linear calibration graphs with correlation coefficients better than 0.995 were obtained for all assay procedures, with standard deviations of 3—5% in the lower concentration ranges and 1—3% in the higher ones (n = 5). A calibration graph for DOHA in urine is shown in Fig. 7. Table II shows the concentrations (μg/ml ± S.D.) of antipyrine and its metabolites in the same human urine sample as assayed by the methods A, B and C. Corresponding chromatograms are shown in Fig. 3. The urine sample was a 0—24 h sample from a male volunteer who had received 500 mg of antipyrine intravenously, resulting in metabolite concentrations of 25—35% of the highest calibration graph concentration.

All assay methods showed good reproducibility, with standard deviations of 2—3% (n = 5), except for HMA after dichloromethane extraction (4%). The latter was due to the low extraction recovery of this method and may be improved by repeated extraction. Concentrations of NORA and especially HMA seemed to be higher with the one-step extraction than after two separate extractions (Table II). This difference was attributed to increased peak areas, caused by interference of co-extracted compounds that were not sufficiently separated from the peaks to be measured (Fig. 3C), despite baseline separation of the metabolites (Fig. 4). Therefore the precision of the selective dual extraction method described by Danhof et al. [3] was found to be better than that of

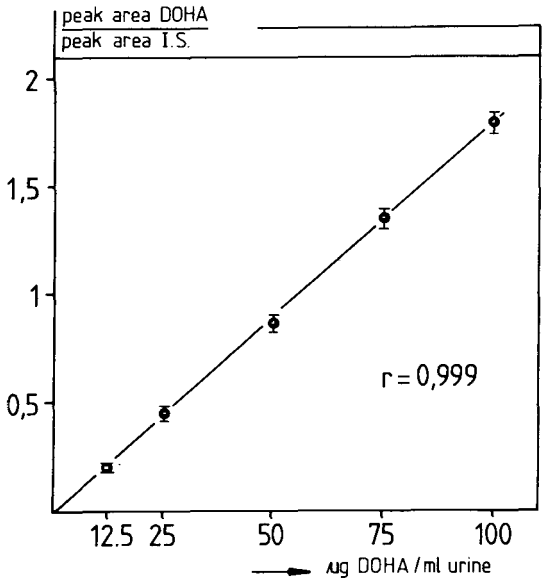

Fig. 7. Calibration graph of 4,4'-dihydroxyantipyrine (DOHA) in urine in a concentration range of 12.5—100.0 µg/ml. Each point represents the mean ± S.D. of five observations.

the single-step extraction method. The lowest detectable concentration for all compounds was about 400 ng/ml. Detection limits were easily improved to 25 ng/ml by increasing the injection volume from 15 to 200 µl.

CONCLUSIONS

The phase system described in this paper for separation of antipyrine and its metabolites is an improvement on previously described methods, since it is highly reproducible and stable, while run times are short. The same system, with small changes in eluent composition can be used for all assays. In the assay procedures no derivatization step is included, though hydrolysis of metabolite conjugates is still required. For HMA, NORA and OHA enzymatic hydrolysis is to be preferred, while for DOHA acidic hydrolysis gives better results.

With the new assay for DOHA, it is now possible to quantify another important metabolite of antipyrine in urine with a rapid and simple HPLC method. In male Wistar rats (n = 18), DOHA formation accounted for 12.6 ± 1.8% of the administered dose (10 mg of antiyprine) [7, 8]. These results are in good agreement with those obtained by Böttcher et al. [17].

Improved stability of metabolites during the assay made all assays suitable for automatic injection. Automatic injection enabled the assay of the samples of twelve experiments with one chromatographic system in one day, using eight blood or saliva samples and one urine sample in each experiment. For human experiments a dose of 2 mg/kg antipyrine is sufficient to allow reliable determination of antipyrine in plasma or saliva and of antipyrine and its metabolites in urine, while for rat experiments 10 mg/kg is the minimum dose. The procedure described in this paper is quite suitable for detailed studies on antipyrine metabolism in man [30] and rat [7, 8].

ACKNOWLEDGEMENTS

This work was partly supported by a grant from the Dutch Prevention Fund, The Hague, The Netherlands. The authors wish to thank Dr. H. Bässmann and Dr. J. Böttcher for fruitful discussions.

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Journal of Chromatography, 278 (1983) 379—385
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1878

METHOD OF ANALYSIS OF THE NEW CARDIOTONIC AGENT, MDL 19,205, IN PLASMA AND URINE AND ITS APPLICATION IN A DOG PHARMACOKINETIC STUDY

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(First received February 9th, 1983; revised manuscript received July 29th, 1983)

SUMMARY

A high-performance liquid chromatographic method has been developed for the analysis of plasma and urine concentrations of a new cardiotonic agent, MDL 19,205 (I). This procedure was utilized to study the pharmacokinetics of I in beagle dogs. The results of the dog study show that the compound is completely and rapidly absorbed. Plasma concentrations fell in a monoexponential manner with a half-life of about 1.3 h which was unaffected by dose in the range 3—30 mg/kg. Urinary excretion of unchanged I accounts for about one-half of the dose and is essentially complete in 24—48 h.

INTRODUCTION

MDL 19,205 (I) 4-ethyl-1,3-dihydro-5-(4-pyridinyl-carbonyl)-2H-imidazol-2-one, is a new non-catechol, non-glycoside cardiotonic agent which is currently undergoing clinical evaluation at Merrell Dow Pharmaceuticals for the treatment of congestive heart failure. The structure of the compound is shown in Fig. 1. Pharmacological studies in both anesthetized and conscious dogs have shown that it produces a dose-dependent increase in cardiac contractile force which was accompanied by relatively small increases in heart rate and minor decreases in blood pressure [1, 2]. These effects were not altered by

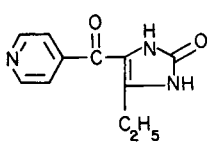

Fig. 1. Structure of MDL 19,205 (I).

α- or β-adrenergic receptor blockade, catecholamine depletion produced by reserpine or by bilateral carotid sinus denervation [2].

In order to facilitate the studies of bioavailability and pharmacokinetics in man of this compound, a sensitive and reliable analytical procedure was needed to measure plasma and urine concentrations of I. This communication describes a reversed-phase high-performance liquid chromatographic (HPLC) method developed for this purpose and the application of the assay to a pharmacokinetic study in the beagle dog.

EXPERIMENTAL

Materials

Methanol and acetonitrile were HPLC grade (J.T. Baker), ethyl acetate was glass-distilled (Burdick and Jackson Labs.) and the water was glass-distilled. I and the internal standard (MDL 82,261, 4-(3,4-dimethoxybenzoyl)-1,3-dihydro-5-methyl-2H-imidazol-2-one) were supplied by the Merrell Research Center. Standard solutions of I and the internal standard (I.S.) were prepared in methanol.

Sample preparation

Plasma samples (0.5 ml) were analyzed in duplicate by adding 1 μg of I.S., diluting to 2 ml with water and precipitating the proteins with 2 ml of acetonitrile. This mixture was then mixed, centrifuged and the supernatant decanted into an extraction tube containing 9 ml of water-saturated ethyl acetate. Ten ml of the organic layer was then transferred and evaporated to dryness under a stream of nitrogen. The residue was redissolved in 750 μl of methanol, diluted with 1.25 ml of distilled water and 400 μl injected into the liquid chromatograph.

Internal standard (10 μg) was added to 40-μl aliquots of urine samples and 50 μl of methanol. This solution was then diluted with 1.9 ml of mobile phase and 400 μl injected into the liquid chromatograph.

Chromatography was performed with a Waters Assoc. Model 6000A pump and Model 440 UV detector (340 nm wavelength) and a WISP 710B injector. The column was a DuPont Zorbax C-8 (25 cm × 4.6 mm I.D., 6 μm particle size) with a mobile phase of 37.5% methanol in distilled water (degassed) at a flow-rate of 1.5 ml/min. The retention time of I was ca. 5.4 min and that of I.S. ca. 7.4 min. Peak areas of I and I.S. were calculated using the CALS chromatographic data system (Computer Inquiry Systems). The peak area of I expressed as a percent of the I.S. peak area, was plotted against the I standard concentrations. The equation of the power-fitted linear regression line, determined from the standards, was then used to calculate the concentration of I in the unknown samples.

Animal experiment

Six male beagle dogs (three groups of two dogs) were dosed with 3 mg/kg intravenously, 3 mg/kg orally by gavage and 30 mg/kg orally by gavage in a three-way crossover. Blood samples were taken periodically, plasma obtained and frozen until assayed. Periodic urine samples were also obtained and frozen

until assayed. One animal which was difficult to handle was dropped from the study and not replaced.

Pharmacokinetics

Area under the plasma concentration curve (AUC) was calculated by trapezoidal rule. Half-life of elimination ($t_{½}$) was calculated as

$$t_{½} = \frac{0.693}{-\beta \times 2.303}$$

where β was the terminal slope of the $\log_{10}$ plasma concentration vs. time curve.

Total body clearance was calculated as the ratio of the administered dose (adjusted for bioavailability) to AUC. Renal clearance was determined as the ratio of the cumulative amount excreted to AUC. Apparent volume of distribution, V_d, was calculated as the ratio of total body clearance to the elimination rate [3].

RESULTS AND DISCUSSION

The recovery of I from plasma was determined by comparing the peak areas of extracted standards to the peak areas of pure standards injected. The mean recovery was 61.6% with a range of 57.9 to 70.4% over the concentration of 0.2 to 30 μg/ml.

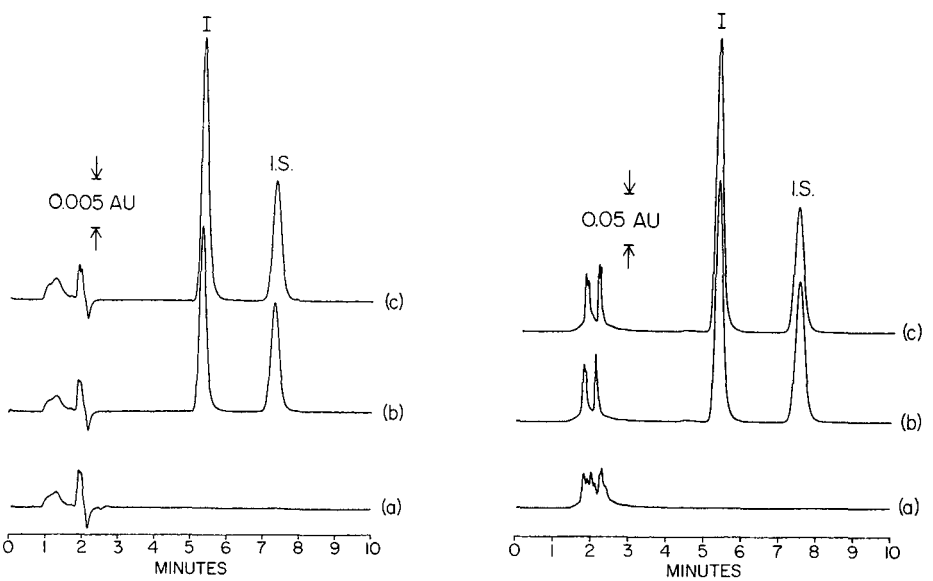

Fig. 2. Typical chromatograms in plasma: (a) extracted blank plasma; (b) extracted plasma from a dog dosed with I; (c) 5 μg/ml standards extracted from plasma.

Fig. 3. Typical chromatograms in urine: (a) blank urine; (b) urine from a dog dosed with I; (c) 250 μg/ml standards in urine.

TABLE I

SUMMARY OF WITHIN DAY ACCURACY AND PRECISION DATA WITH I (n = 6) IN PLASMA

Unknown concentration* (μg/ml)	Concentration found (μg/ml)	Standard deviation	Standard error	Coefficient of variation (%)	Percent of expected
0.25	0.255	0.028	0.011	10.71	102.0
1.00	1.073	0.094	0.038	8.72	107.3
5.00	4.573	0.274	0.112	5.98	91.5
8.75	9.100	0.494	0.202	5.43	104.0
15.00**	15.607	1.092	0.488	7.00	104.0
23.75	23.814	0.638	0.260	2.68	100.3

*Zero values were always zero.
**n = 5.

TABLE II

SUMMARY OF WITHIN-DAY ACCURACY AND PRECISION DATA WITH I (n = 6) IN URINE

Unknown concentration* (μg/ml)	Concentration found (μg/ml)	Standard deviation	Standard error	Coefficient of variation (%)	Percent of expected
9.43	10.459	0.638	0.261	6.10	110.9
33.00	37.257	0.702	0.287	1.88	112.9
94.28	102.431	4.239	1.731	4.14	108.6
330.00	346.300	11.655	4.758	3.37	104.9
1178.00	1112.942	45.031	18.384	4.05	94.5
3300.00	3128.501	199.195	81.321	6.37	94.8

*Zero values were always zero.

TABLE III

SUMMARY OF ACCURACY AND PRECISION DATA WITH I IN PLASMA OVER SIX DAYS

Unknown concentration* (μg/ml)	n	Concentration found (μg/ml)	Standard deviation	Standard error	Coefficient of variation (%)	Percent of expected
0.10	12	0.114	0.020	0.006	17.14	114.0
0.25	15	0.270	0.050	0.013	18.48	108.0
0.75	12	0.739	0.070	0.020	9.48	98.5
1.00	16	1.017	0.092	0.023	9.07	101.7
3.00	12	3.091	0.236	0.068	7.64	103.0
5.00	16	4.692	0.279	0.070	5.94	93.8
6.25	12	6.397	0.464	0.134	7.25	102.4
8.75	16	9.108	0.488	0.122	5.36	104.1
11.25	12	11.589	0.859	0.248	7.41	103.0
15.00	16	15.320	0.981	0.245	6.40	102.1
18.75	12	19.514	1.455	0.420	7.46	104.1
23.75	16	24.067	0.823	0.206	3.42	101.3

*Zero values were always zero.

TABLE IV

SUMMARY OF ACCURACY AND PRECISION DATA WITH I IN URINE OVER SIX DAYS

Unknown concentration* (μg/ml)	*n*	Concentration found (μg/ml)	Standard deviation	Standard error	Coefficient of variation (%)	Percent of expected
9.43	16	11.174	0.818	0.205	7.32	118.5
16.50	12	19.169	1.115	0.322	5.82	116.2
33.00	16	37.896	1.269	0.317	3.35	114.8
66.00	12	73.337	2.593	0.749	3.58	111.1
94.28	16	99.113	4.190	1.048	4.23	105.1
165.00	12	171.066	6.576	1.898	3.84	103.7
330.00	15	337.670	12.695	3.278	3.76	102.3
660.00	12	633.839	24.467	7.063	3.86	96.0
1178.00	16	1089.758	40.741	10.185	3.74	92.5
2640.00	12	2458.589	113.567	32.784	4.62	93.1
3300.00	16	3167.027	168.651	42.163	5.33	96.0

*Zero values were always zero.

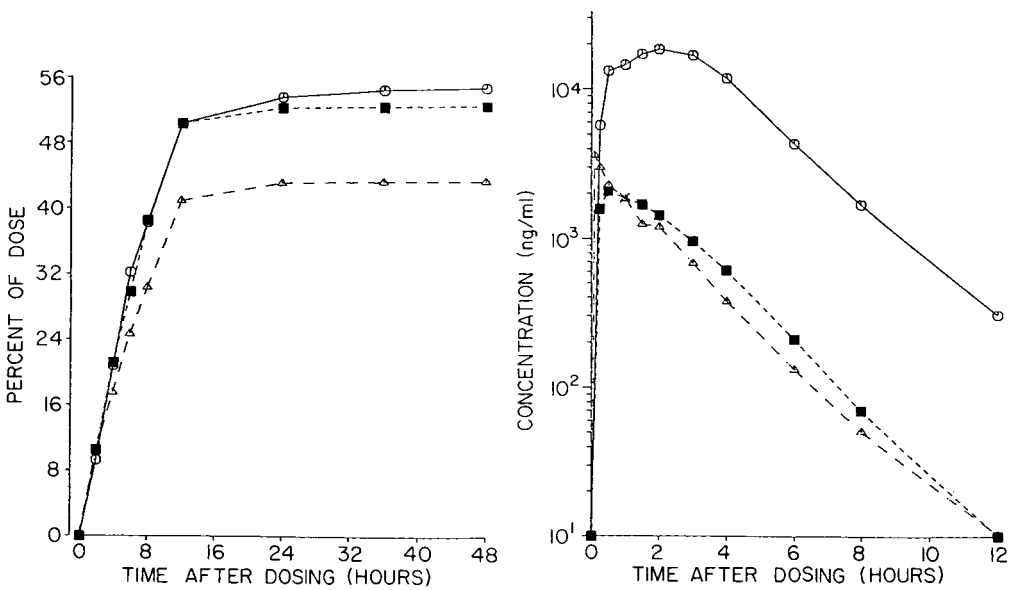

Fig. 4. Plasma concentrations in male beagle dogs following an acute dose of I: (△) 3 mg/kg intravenous; (■) 3 mg/kg oral solution; (⊙) 30 mg/kg oral solution. Points are mean of five determinations.

Fig. 5. Cumulative excretion of I in the urine of male beagle dogs following an acute dose of I: (△) 3 mg/kg intravenous; (■) 3 mg/kg oral solution; (⊙) 30 mg/kg oral solution.

Typical chromatograms obtained from plasma and urine analyses are shown in Figs. 2 and 3, respectively. As the assay is presently configured, the limit on quantitation of I in plasma is ca. 40 ng/ml; in urine the limit appears to be ca. 1—5 μg/ml.

TABLE V

PHARMACOKINETIC SUMMARY OF I DATA IN THE BEAGLE DOG

Administration	Dog*	AUC (μg h/ml)	Peak concn. (μg/ml plasma)	Time to peak concn. (h)	$t_{1/2}$ (h)	F** Bioavailability (%)	Cumulative percent recovery in urine	Clearance (ml/min/kg)			V_d (l/kg)
								Total	Renal	Extrarenal	
3 mg/kg intravenously	81-106	8.80	—	—	1.46	—	40.7	5.68	2.31	3.37	0.72
	81-107	4.98	—	—	0.83	—	54.0	10.03	5.42	4.62	0.72
	81-108	5.56	—	—	1.21	—	24.0	8.99	2.15	6.83	0.94
	81-109***	—	—	—	—	—	—	—	—	—	—
	81-111	6.06	—	—	1.40	—	47.0	8.24	3.88	4.37	1.00
	Mean	6.35	—	—	1.23	—	41.4	8.24	3.44	4.80	0.85
	S.D.	1.69	—	—	0.28	—	12.8	1.85	1.53	1.46	0.15
3 mg/kg orally	81-106	8.08	2.52	0.25	1.47	99.3	44.1	6.14	2.37	3.41	0.78
	81-107	5.67	2.68	0.5	1.25	109.3	57.4	9.65	5.07	4.58	1.05
	81-108	5.86	1.60	1.5	1.20	94.3	44.9	8.04	3.83	4.21	0.84
	81-109	5.40	2.81	1.0	1.05	***	57.8	10.83	5.35	5.47	0.98
	81-111	7.77	2.56	0.5	1.58	130.0	56.0	8.37	3.60	4.77	1.14
	Mean	6.56	2.43	0.8	1.31	108.2	52.0	8.61	4.12	4.49	0.96
	S.D.	1.27	0.48	0.5	0.21	15.8	6.9	1.77	1.08	0.76	0.15
30 mg/kg orally	81-106	98.26	16.69	3.0	1.76	116.0	23.6	5.90	1.20	4.70	0.90
	81-107	89.79	29.92	1.0	1.23	179.9	64.7	10.02	3.60	6.42	1.07
	81-108	85.13	19.34	3.0	1.40	136.9	63.9	8.04	3.75	4.29	0.97
	81-109	71.52	17.82	2.0	1.12	***	58.8	10.68	4.11	6.57	1.04
	81-111	85.52	19.90	2.0	1.53	142.3	61.1	8.31	3.57	4.75	1.10
	Mean	86.04	20.73	2.2	1.41	143.8	54.4	8.59	3.25	5.35	1.02
	S.D.	9.69	5.29	0.8	0.25	26.6	17.4	1.87	1.16	1.07	0.08

*Individual animal number.
**Based on plasma AUC.
***Pharmacokinetics for intravenous data not computed for this dog due to unusual plasma level profile.

The composite power-fitted least-squares regression equation for standards in plasma was $Y^{1.0121} = 32.8259\ X - 0.0008$, with a correlation coefficient (r) of 0.9979 over the range of the assay. In urine, the line was $Y^{0.9523} = 0.2608\ X - 0.0001$, with $r = 0.9986$.

The within-day accuracy and precision data are shown in Tables I and II for plasma and urine, respectively. The corresponding day-to-day data are shown in Tables III and IV. These data indicate that the assay as described is sufficiently accurate and precise for use in pharmacokinetic studies.

This method was then utilized in a dog pharmacokinetic study. The mean plasma concentrations of I in male beagle dogs following single doses of either 3 mg/kg intravenously, 3 mg/kg orally or 30 mg/kg orally are shown in Fig. 4 and the cumulative urinary excretion data are shown in Fig. 5. The summary of the pharmacokinetic variables is found in Table V.

These data indicate that I undergoes complete and rapid absorption in the dog. Plasma levels decline in a monoexponential manner with a half-life of about 1.3 h which was unaffected by dose. The apparent volume of distribution of about 1 l/kg would suggest extensive distribution approaching that of body water.

The compound is rapidly excreted and approximately one-half of the dose is recovered in the urine as unchanged I within 48 h (the majority of which is recovered in the first 24 h).

In conclusion, an HPLC method of analysis of plasma and urine concentrations of MDL 19,205 has been developed and applied in a pharmacokinetic study in beagle dogs. This method will be utilized in a human pharmacokinetic study which will be published shortly.

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Journal of Chromatography, 278 (1983) 387—395
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1865

MEASUREMENT OF AMPHOTERICIN B IN SERUM OR PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received June 28th, 1983)

SUMMARY

A high-performance liquid chromatographic method for the analysis of amphotericin B in 25 μl of serum or plasma is described. The procedure involves the addition of the internal standard, *p*-nitrobenzyloxyamine, to the sample followed by a precipitation of protein with acetonitrile. The supernatant is directly injected into a chromatograph attached to a reversed-phase μBondapak (Waters) column containing C_{18} packing. The mobile phase is a 60 : 40 mixture of a sodium acetate buffer (10 m*M*, pH 7.0)—acetonitrile, and we employ a flow-rate of 1.5 ml/min and a detection wavelength of 405 nm. Total analysis time per sample is 10 min. Coefficients of variation were found to be less than 4% for concentrations less than 2 mg/l. Analytical recoveries were between 75 and 80%. No drug or drug metabolite interference was found. The method will be used to study pharmacokinetic and pharmacodynamic data in a pediatric population.

INTRODUCTION

Amphotericin is an antifungal compound which was isolated in 1956 from a soil actinomycete, *Streptomyces nodosus*, found in the Orinoco river area of Venezuela [1]. It exists in two forms, A and B; the latter, being more active, is used clinically. The basic moiety of amphotericin B (AMB) is aminodesoxyhexose, an aminomethyl pentose. It is active and useful clinically against a wide range of fungi including *Candida* species, *Histoplasma capsulatum*, *Cryptococcus neoformans*, *Coccidioides immitis*, *Blastomyces dermatitidis*, *Paracoccidioides brasiliensis*, *Sporotrichum schenckii*, *Aspergillus*, *Mucor*, and

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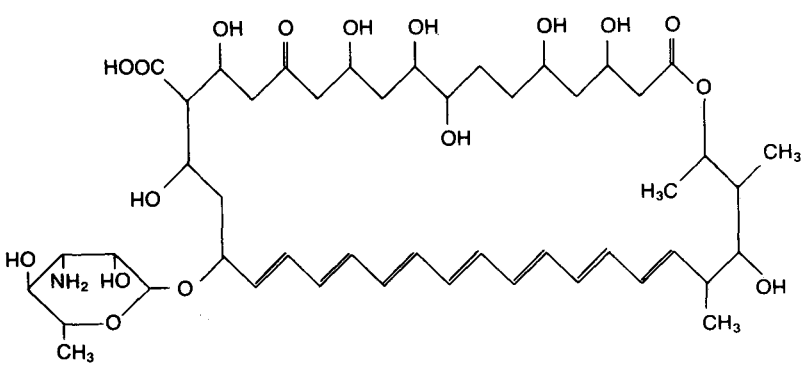
0378-4347/83/$03.00

Rhizopus. It is fungicidal against most of these fungi at concentrations of 1.0 mg/l or less [1].

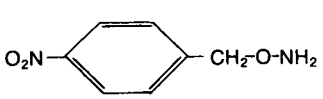
There is a limited amount of pharmacokinetic data on AMB, especially in children. The intravenous injection of 0.5—1.0 mg/kg yields peak plasma concentrations of 1.5—2.0 mg/l. There is no universal agreement on the method of intravenous administration. It is, however, common practice to treat fungal infections on the basis of a total dose of AMB. In general, a daily dose of 0.5—1.0 mg/kg will be administered for as many days as it takes to attain the desired total dose. AMB is perhaps the only antimicrobial agent which is frequently administered on the basis of a total cumulative dose rather than on the basis of a daily dose administered for an arbitrary period of time. Dosing on the basis of attaining plasma concentrations at least two- to three-fold in excess of the amount required to kill the specific fungi would seem to be a more rational approach. An accurate and reproducible analytical assay for AMB is necessary to permit this approach to dosing.

The most frequent and clinically significant side-effect of AMB therapy is nephrotoxicity [2]. Some degree of renal functional impairment occurs in almost all treated patients, and this is often a limiting factor to the total amount of drug which can be administered. Whether such renal dysfunction can be predicted and prevented by the monitoring of AMB serum concentrations, followed by appropriate dose adjustments, has not yet been established. Investigation of this possibility again requires the availability of an accurate and reproducible AMB assay.

The chemical properties of AMB make it a difficult drug to analyze. It has a high molecular weight (924.1), is poorly soluble in water and organic solvents,



Amphotericin B



p-Nitrobenzyloxyamine

Fig. 1. Structures of amphotericin B and the internal standard, *p*-nitrobenzyloxyamine.

and is sensitive to light, heat, air and pH below 6 or above 9 [3]. Only a few procedures exist for the measurement of AMB. These include colorimetry [4], microbiological assays [5], and high-performance liquid chromatography (HPLC) [4, 6]. The inherent problem with the colorimetric method is the absorption of hemoglobin at 405 nm. Lengthy analysis time and poor specificity and sensitivity are shortcomings with the bioassay. One HPLC method employs a sample volume of 1 ml, omits use of an internal standard, and requires many steps (deproteinization, centrifugation, filtration) prior to chromatography [4]. This paper [4] reports the superiority of the HPLC method over the bioassay procedure. A standard deviation of 3.3% (HPLC) compared to 32.6% (bioassay) was found. Another HPLC method incorporates an internal standard, but reports a fairly large coefficient of variation of 18% within a range of 0.08—10.0 mg/l [6].

We describe a rapid, accurate and sensitive micro procedure for the analysis of AMB in 25 μl of serum or plasma using *p*-nitrobenzyloxyamine (PNBA) as the internal standard. This method is capable of measuring AMB within the generally accepted, clinically significant range of 0.5—2.0 mg/l [1]. The chemical structure of this non-aromatic heptane antibiotic and that of PNBA are shown in Fig. 1.

EXPERIMENTAL

Apparatus

A Perkin-Elmer Series 2/2 high-performance liquid chromatograph (Norwalk, CT, U.S.A.) equipped with a Rheodyne 7105 loop injector valve was used for the analysis. Chromatography was performed on a reversed-phase μBondapak 30 × 0.4 cm stainless-steel column, particle size 10 μm (Waters Assoc., Milford, MA, U.S.A.). The detector used was a Waters Model 441 spectrophotometer with 405-nm filter and adjusted to full-scale absorbance of 0.005 absorbance units. An Omniscribe B-5000 strip chart recorder (Houston Instruments, Austin, TX, U.S.A.) was used. Ultraviolet and visible scans of the drug and the internal standard were obtained using a PMQII (Carl Zeiss, Oberkochen, F.R.G.) spectrophotometer.

Reagents and standards

Acetonitrile and methanol were purchased from Anachemia (Mississauga, Canada). AMB was obtained from Sigma (St. Louis, MO, U.S.A.), while PNBA was purchased from Regis Chemical (Morton Grove, IL, U.S.A.). Dimethyl sulfoxide and sodium acetate were provided from Fisher Scientific (Fairlawn, NJ, U.S.A.). The mobile phase is sodium acetate buffer (10 m*M*, pH 7.0)—acetonitrile (60 : 40).

A stock standard solution of AMB (500 mg/l) was prepared in dimethyl sulfoxide while a working standard solution (2 mg/l) was prepared by dilution of the stock solution with the mobile phase. Control samples were prepared in drug-free sera to give concentrations of 0.5, 1.0 and 2.0 mg/l AMB. These solutions are stable for many months at −20°C. A 1 g/l solution of PNBA was prepared in water and stored at 4°C. Solutions of PNBA could not be prepared in sodium acetate buffer or acetonitrile due to lack of solubility.

TABLE I

CHROMATOGRAPHIC CONDITIONS

Parameter	Conditions
Column	μBondapak C_{18}
Mobile phase	Sodium acetate buffer (10 m*M*, pH 7.0)—acetonitrile (60 : 40)
Wavelength	405 nm
Flow-rate	1.5 ml/min
Temperature	Ambient temperature
Internal standard	*p*-Nitrobenzyloxyamine
Chromatography time	5 min
a.u.f.s.	0.005
Sample volume	25 μl

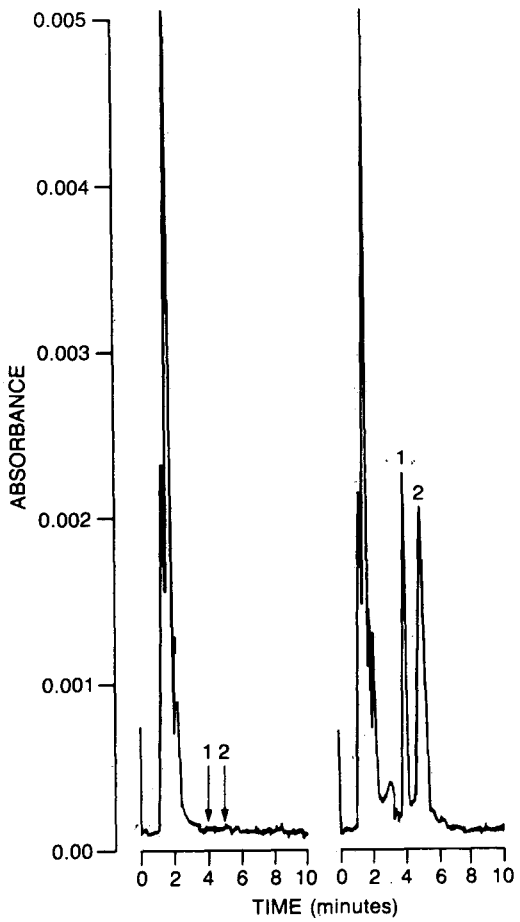


Fig. 2. Typical chromatograms of (left) a blank serum and (right) a serum sample containing 1 mg/l AMB. Peak 1 = internal standard, peak 2 = AMB.

Extraction procedure and conditions of analysis

Add 25 μl of internal standard (1 g/l PNBA in water) to 25 μl of serum or plasma in a 6 × 50 mm glass test tube. Then acetonitrile (50 μl) is added to the solution which is vortexed for 15 sec. Centrifuge at 6500 *g* for 2 min. Inject 80 μl into the chromatograph. Table I lists the chromatographic conditions used in the analysis. Typical chromatograms for a serum sample and a serum blank are shown in Fig. 2.

RESULTS AND DISCUSSION

Analytical variables

Detector wavelength. Fig. 3 shows the absorbance scan for AMB and PNBA. AMB was found to have three absorbance maxima in the ultraviolet–visible spectrum. Initially a variable-wavelength spectrophotometer (Perkin-Elmer) was utilized for measurement of absorbance at the absorbance maximum of 384 nm. However, use of a fixed line source mercury lamp spectrophotometer and a wavelength of 405 nm provided better sensitivity, an improved signal-to-basesline noise ratio, and permitted use of a smaller sample volume. Both carbamazepine and phenytoin co-eluted with AMB and interfered with the measurement of AMB at 384 nm but not at 405 nm.

Column choice and column temperature. Studies using the 5- or 8-mm Radial Pak A columns (10 × 0.8 cm, Waters) resulted in poor band symmetry and provided undesirably broad peaks for AMB. Good peak symmetry with minimal band spreading were obtained with the μBondapak C_{18} columns. The retention time of AMB varied minimally with column temperature changes between 25°C and 40°C.

Standard and internal standard. No difference was noted between the pharmaceutical (Squibb Canada, Montreal, Canada) and chemical (Sigma) preparations of AMB. Both of these solutions contained a small peak with a retention time at approximately 3 min which probably corresponds to ampho-

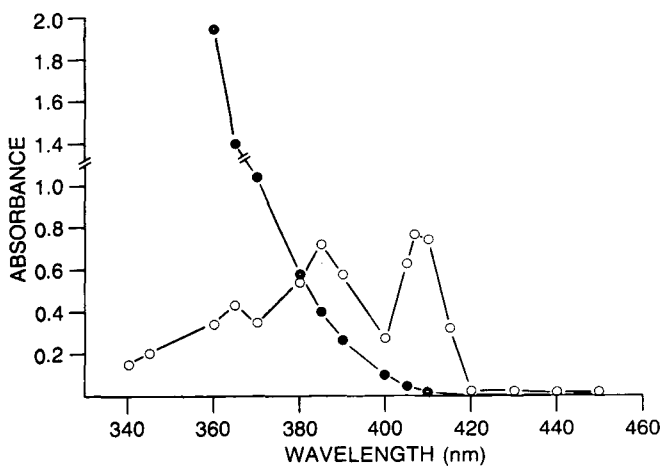


Fig. 3. Absorbance scan of 10 mg/l amphotericin B (○) and 2000 mg/l *p*-nitrobenzyloxyamine (●) in mobile phase.

tericin A. Amphotericin A is present as a trace contaminant in AMB preparations [7]. For purposes of quantitation of AMB, this contamination of the AMB standard was ignored.

Composition of the mobile phase. The optimal separation of AMB and PNBA was obtained when the buffer had a pH of 7.0 and a ratio of buffer to acetonitrile of 60 : 40. A buffer pH of 4.0, while having little effect on the retention time of the internal standard, significantly lengthened the retention time of AMB. Also, at this acidic pH amphotericin A coeluted with PNBA. Increasing the acetonitrile content shortened the retention time and improved the peak shape of AMB.

Method of calculation

The retention times of AMB and PNBA were determined by the daily injection of these compounds into the chromatograph. AMB was identified by its retention time relative to that of PNBA. A daily mean factor, based on the peak height ratios of AMB (A) to PNBA (B) observed in the three control sera, was derived. For example, in a control sample containing 2 mg/l AMB the calculation is as follows:

$$\frac{A}{B} \times \frac{1}{2} = \text{peak height ratio obtained for 1 mg/l AMB} = \text{factor.}$$

The average of the three resulting values produced the mean factor which was employed to calculate the controls and the unknown sample by the following formula:

$$\frac{\text{AMB peak height}}{\text{PNBA peak height}} \times \frac{1}{\text{mean factor}} = \text{AMB concentration in mg/l.}$$

Linearity

Injection of known amounts of AMB and PNBA demonstrated a linear relationship between peak height and concentration (Fig. 4). The serum concentration that corresponds to a full-scale recorder deflection is 3.9 mg/l AMB. This is an ample range for clinical interest. The sensitivity of the assay, i.e. peak height corresponding to twice the baseline noise, was found to be 0.1 mg/l.

Recovery

AMB was added to drug-free plasma to provide concentrations of 0.5, 1.0 and 2.0 mg/l. Recovery was determined by the replicate analysis (n = 10) of each of these three plasma pools. The peak heights obtained for AMB and PNBA were compared with the peak heights obtained by direct injection of working standard solutions. The results are summarized in Table II.

Precision

The between-day precision was assessed by analysing the three plasma pools for twenty working days. The coefficient of variation was equal or less than 4.0 at serum concentrations of 0.5—2.0 mg/l AMB (Table III).

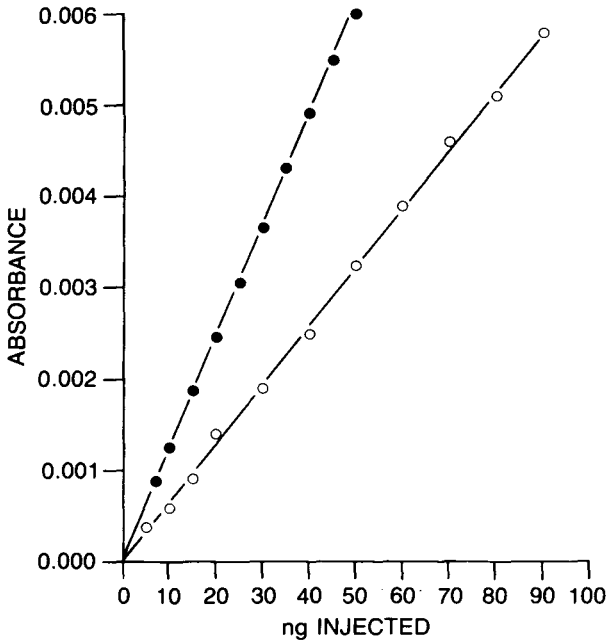


Fig. 4. Relative response curve for amphotericin B (○) and *p*-nitrobenzyloxyamine (●).

TABLE II

RECOVERY STUDIES

Compound	Concentration (mg/l)	Recovery (%) (n = 10)
AMB	0.5	80
	1.0	75
	2.0	78
PNBA	1000	95

TABLE III

BETWEEN-DAY PRECISION STUDIES

Compound	Concentration (mg/l)	C.V. (%) (n = 20)
AMB	0.5	3.4
	1.0	4.0
	2.0	3.1

Selectivity

Twenty sera obtained from pediatric patients known not to be receiving AMB were assayed without the presence of the internal standard. No interfering peaks were observed in the chromatograms. Further assessment of

TABLE IV

SPECIFICITY STUDIES

Antineoplastic	Antibiotic	Other
5-Fluorouracil	Gentamicin	Phenobarbital
Cytarabine	Ampicillin	Primidone
Cyclophosphamide	Cloxacillin	Carbamazepine
Methotrexate	Penicillin G	Ethosuximide
Vincristine	Trimethoprim	Phenytoin
Daunorubicin	Sulfamethoxazole	Theophylline
Allopurinol	Tobramycin	Folic acid
Thioguanine	Cephalothin	Diazepam
6-Mercaptopurine	Chloramphenicol	Furosemide
Cisplatin	Amikacin	Acetazolamide
Adriamycin		Metoclopramide
Dactinomycin		Acetaminophen
Flucytosine		Vidarabine
		Acetylsalicylic acid
		Dimenhydrinate
		Nabilone
		Chlorpromazine

TABLE V

STABILITY STUDIES

Days	Storage temperature		
	25° C	4° C	−20° C
	Concentration (mg/l)		
1	0.7	0.7	0.7
14	0.6	0.7	0.7
24	0.5	0.7	0.7

selectivity was determined by the direct injection of the aqueous solutions of the pure compounds listed in Table IV. None were found to interfere in the described method.

Stability studies

AMB was added to several drug-free plasma pools to give a concentration of 0.7 mg/l. Aliquots from these pools were stored in the dark at 25° C, 4° C and −20° C. Samples stored at each of these temperatures were analyzed on days 1, 14 and 24. The results are shown in Table V. As can be seen, AMB is stable when stored at either 4° C or −20° C for 24 days.

Future clinical application

We have described an accurate and reproducible procedure for the measurement of AMB. This will permit important pharmacokinetic and pharmacodynamic studies to be undertaken on this antifungal agent in the pediatric population. Based upon the monitoring of serum concentrations of AMB, we

hope to be able to rationalize our treatment schedules. We will also be able to investigate the possible relationship between nephrotoxicity and serum concentrations of AMB. If such a relationship exists, it may be possible to predict and hence prevent the occurrence of this side-effect.

ACKNOWLEDGEMENT

This work was supported in part by Research Grant PR922 from the Ontario Ministry of Health.

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Journal of Chromatography, 278 (1983) 397—405
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1861

QUANTITATION OF DIGITOXIN AND THE BIS- AND MONODIGITOXOSIDES OF DIGITOXIGENIN IN SERUM

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(Received May 17th, 1983)

SUMMARY

A specific assay is described for measuring the concentration of digitoxin and the bis- and monoglycosides of digitoxigenin in serum. The procedure includes: (1) addition of a tracer amount of tritium labeled parent compound to the serum in order to measure percentage recovery; (2) solvent extraction to separate polar and non-polar metabolites; (3) reversed-phase thin-layer chromatography of the non-polar fraction to separate digoxigenins from digitoxigenins; (4) thin-layer chromatography to isolate digitoxin, and the bis- and monoglycosides of digitoxigenin; and (5) use of an ^{125}I-radioimmunoassay to determine the concentration of the glycosides. Each of these three glycosides was administered intravenously to a normal subject, and the concentration of parent compound was measured in the serum at various times.

INTRODUCTION

In order to study the pharmacokinetics of digitoxin it is necessary to have a method which is sensitive enough for its quantitation at the concentrations found in serum after a single dose and specific enough to separate it from its many metabolites. Several methods have been described for the measurement of digitoxin in serum and urine. Assays based on Na^+, K^+-ATPase inhibition [1], red blood cell ^{86}Rb uptake [2], and competitive protein binding [3] have, in general, been replaced by radioimmunoassays (RIA) [4]. Although these methods are sufficiently sensitive to measure concentrations of digitoxin in biological fluids, they do not distinguish digitoxin from its metabolites.

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0378-4347/83/$03.00

Therefore, a variety of chromatographic methods have been devised. These include a double isotope dilution derivative assay [5], gas—liquid chromatography (GLC) [6—8], high-performance liquid chromatography (HPLC) [9], column chromatography [10], and thin-layer chromatography (TLC) [11,12]. The double isotope dilution derivative assay and HPLC methods are generally not sensitive enough to directly measure the digitalis glycosides in serum, but HPLC has been combined with RIA for the quantitation of digoxin and its metabolites [13]. GLC requires three additional chromatographic steps and the preparation of derivatives and therefore is not suitable for the assay of large numbers of samples. Radiolabeled digitoxin has also been administered to man, and column or TLC techniques used to separate the parent drug from its metabolites [14,15]. However, the use of labeled material in human subjects is undesirable.

Pharmacokinetic studies which use non-specific methods to measure digitoxin are difficult to interpret due to the presence of metabolites in the serum. In some conditions, such as renal failure, patients have an increased proportion of metabolites, especially water-soluble metabolites [16,17]. Therefore, the pharmacokinetic parameters for digitoxin may be in error, and the percentage of digitoxin excreted may be overestimated when a non-specific assay is employed. Conflicting data obtained in azotemic subjects using non-specific assays for digitoxin have been summarized by Vöhringer and Rietbrock [18].

The method described here is sufficiently sensitive and specific to obtain pharmacokinetic information following the administration of a single dose of non-radioactive digitoxin or its metabolites, the bis- and monoglycosides of digitoxigenin. The method involves solvent extraction followed by TLC separation of the non-polar metabolites. The concentration of the parent compound is then measured using ^{125}I-RIA. The efficiency of the procedure is determined for each sample by addition of a tracer amount of the tritiated parent compound prior to extraction.

EXPERIMENTAL

Materials

The following unlabeled compounds were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A.: digoxin (DG), digoxigenin bisdigitoxoside (bis-DG), digoxigenin monodigitoxoside (mono-DG), digoxigenin (genin-DG), digitoxin (DT), digitoxigenin bisdigitoxoside (bis-DT), digitoxigenin monodigitoxoside (mono-DT), digitoxigenin (genin-DT), and dihydrodigitoxin (DTH). [^{3}H]Digitoxin (13.8 Ci/mmol) and [^{3}H]digitoxigenin monodigitoxoside (34.8 Ci/mmol) were obtained from New England Nuclear, Boston, MA, U.S.A. [^{3}H]Digitoxigenin bisdigitoxoside (12.6 Ci/mmol) was obtained from Burroughs Wellcome, Research Triangle Park, NC, U.S.A. (through the courtesy of Dr. Ronald Cresswell). Each compound was purified by TLC prior to use. TLC plates (Si 250 TLC precoated, 250 μm) were obtained from J.T. Baker, Phillipsburg, NJ, U.S.A., and reversed-phase plates (KC_{18} precoated, 200 μm) from Whatman, Clifton, NJ, U.S.A.

Extraction and chromatography of serum samples

A flow diagram of the analytical procedures is presented in Fig. 1. After collection, blood samples were centrifuged and the serum was stored frozen in glass vials at −20°C until assayed. A 2-ml aliquot of each serum sample was pipetted into a screw top centrifuge tube and ^{3}H-labeled parent compound (approximately 2×10^5 dpm) in 0.1 ml of phosphate-buffered saline [0.15 *M* sodium chloride, 0.01 *M* dipotassium hydrogen phosphate, 5% bovine serum albumin (Miles Laboratories, Kankakee, IL, U.S.A.) pH 7.4] was added. An aliquot of ^{3}H-labeled parent compound was also added to each of five scintillation vials containing 10 ml of scintillation fluid {5 g of 2,5-diphenyloxazole (PPO), 200 mg of 1,4-bis[2(4-methyl-5-phenyloxazolyl)]-benzene (dimethyl POPOP), 100 g of naphthalene and dioxane to 1 l}. The serum was then extracted three times with 5-ml aliquots of dichloromethane (CH_2Cl_2). Each time, samples were mixed for 10 min on a Labquake® and then centrifuged to separate the water-soluble and dichloromethane-soluble fractions. The dichloromethane fractions were transferred with a Pasteur pipet to a centrifuge tube and the dichloromethane evaporated with nitrogen. The sides of each centrifuge tube were washed three times with 2 ml of dichloromethane and the solvent evaporated each time to concentrate the glycosides in the tip. The concentrate was dissolved in approximately 0.7 ml of chloroform—methanol (3:1) and spotted 2.5 cm from the bottom edge of a reversed-phase TLC plate. The

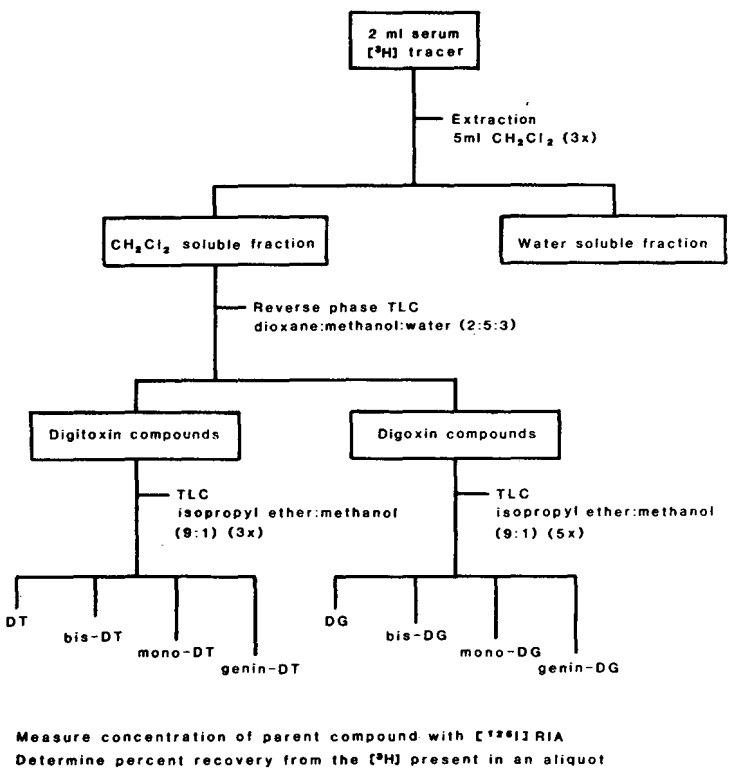

Fig. 1. Analytical procedures.

plates had been scored in 2.2 cm wide lanes separated by 0.7 cm lanes. Standard solutions of digoxin, digitoxin, and several of their metabolites (approximately 5 μg each) were spotted in either outside lane of the plates. The plates were then placed in a chromatography tank equilibrated with dioxane—methanol—distilled water (2:5:3) and developed until the solvent front had moved to a height of 19 cm. After drying, the lanes of the plate containing the standards were cut off with a glass cutter and sprayed with a freshly mixed chloramine-T—trichloroacetic acid reagent [3% chloramine-T (Eastman Organic Chemicals, Rochester, NY, U.S.A.)—25% ethanolic solution of trichloroacetic acid (1:4)] and heated in an oven for 8 min at 110°C. The digitalis compounds were visualized and marked under long-wavelength ultraviolet light. The area in each lane corresponding to the digitoxin compounds was identified based on the standards, and was scraped into one centrifuge tube. The silica gel was pulverized and the glycosides were eluted three times from the silica using 4 ml of ethanol. The pooled eluants were evaporated and the sides of the tubes washed down to concentrate them in the tip. This initial chromatographic step was necessary to separate digoxin and digoxin metabolites (which have been reported to be metabolites of digitoxin) from digitoxin and metabolites retaining the digitoxin steroid nucleus.

The eluate corresponding to the digitoxin compounds area was transferred as described previously to silica gel TLC plates which had been dried overnight at 40°C. The plates were developed three times to the top of the plate with isopropyl ether—methanol (9:1) in a pre-equilibrated tank. They were dried between runs and standards were visualized as previously described. The area of each lane corresponding to the parent glycoside was scraped from the plate and eluted from the silica with three washings of 4 ml of ethanol. After evaporating the ethanol with nitrogen, the glycoside was dissolved in phosphate-buffered saline. A 0.1-ml aliquot was pipetted in duplicate from the test tube into a counting vial containing 10 ml of scintillation fluid and counted to 2% error. Quenching was corrected for using an external standard. The concentration of each compound was then determined with a digitoxin RIA. All serum samples were also assayed directly using the RIA.

The percentage recovery was calculated for each chromatographed sample by dividing dpm of ^{3}H recovered after chromatography by the total dpm added to each serum sample prior to extraction. Since ^{3}H tracer contributed to the total radioimmunoassayable parent compound after chromatography, the amount of digitalis glycoside present as ^{3}H-labeled compound was calculated from its specific activity and the dpm present and subtracted from the total assayable compound. RIA was used to calculate specific activity for each ^{3}H-labeled compound after each was chromatographed to greater than 97% purity. The concentration of non-radioactive material was then corrected for percentage recovery.

Digitoxin radioimmunoassay

The concentration of digitoxin and the bis- and monodigitoxosides of digitoxigenin was measured using ^{125}I-labeled digitoxin tracer (3-O-succinyl-digitoxigenin L-tyrosine) and antiserum from Becton-Dickinson, Orangeburg, NY, U.S.A. The assay procedure used was a modification of the one provided

with the Becton-Dickinson RIA kit. Digitoxin standards in the range of 3—60 pmol/ml were prepared in phosphate-buffered saline and run with each assay. Samples with concentrations above this range were diluted with phosphate-buffered saline. Duplicate 0.025-ml volumes of standard or sample were mixed with 1.0 ml of ^{125}I-labeled digitoxin tracer and then 0.1 ml of antibody was added. Each 10-ml vial of antibody was diluted with 3.5 ml of phosphate-buffered saline prior to use. After a 30-min incubation, 0.5 ml of a charcoal suspension [0.15 *M* sodium chloride, 3.6 m*M* sodium barbital, 3.6 m*M* sodium acetate, 0.03% Dextran T-70, and 1.25% Norit-A charcoal (J.T. Baker, Phillipsburg, NJ, U.S.A.)] was added. Following centrifugation at 1000 *g*, 1 ml of supernatant was pipetted into a tube for gamma counting. Samples were counted to a maximum error of 2%. Counts per min were plotted as a function of log of digitoxin concentration, and the concentration of digitoxin, and the bis- and monodigitoxosides of digitoxigenin was calculated from this standard curve. This was possible since the bis- and monodigitoxosides of digitoxigenin demonstrated an affinity for the digitoxin antibody indistinguishable from that of digitoxin.

Standard solutions of bis-DT, mono-DT, genin-DT, DTH, and DG were assayed in order to determine their binding affinities for the digitoxin antibody relative to that of digitoxin. Standard curves were prepared for each and compared to the digitoxin standard curve. The relative binding affinities were calculated from the concentration of each compound compared to digitoxin required to displace 50% of the labeled digitoxin bound in the absence of any unlabeled compound.

It was important that the composition of the sample and standard tubes be as similar as possible. Therefore, all serum samples from a subject following each drug administration were assayed at the same time. Since 0.025 ml of serum sample was required for each assay tube for the serum samples which were assayed directly, a 0.025-ml aliquot of serum from the same subject drawn prior to drug administration was added to each standard tube. If dilution of serum samples was necessary it was done with the same blank serum. The samples assayed after extraction and chromatography were reconstituted in the same phosphate-buffered saline used to prepare the standards, and, therefore, no adjustments were necessary.

Twenty standard samples of digitoxin at concentrations of 0, 3.3, 6.7, 13.1, 23.4 and 39.4 pmol/ml were prepared and assayed by the RIA to permit determination of the accuracy, precision, and sensitivity of the assay.

Human studies

The general utility and specificity of the method is illustrated by the following experiment. A normal subject, aged 23 years, was given a single intravenous dose of digitoxin after giving informed consent. After one month he was given the bis-DT and one month later the mono-DT. The dosing sequence was 1 mg of digitoxin (Crystodigin, Eli Lilly, Indianapolis, IN, U.S.A.), then 0.83 mg of bis-DT, and then 0.66 mg of mono-DT (i.e. 1308 nmol of each). Each intravenous dose was diluted to approximately 15 ml with sterile saline and injected intravenously over a 10-min period. Blood samples were drawn prior to injection and at approximately 5, 15, 30, 45 min and 1, 2, 4, 6, 8, 12 h and 2,

4, 6, 8, 12, 16, 24 and 28 days after injection. Exact sampling times were recorded.

RESULTS

While the initial reversed-phase chromatographic step separated the digitoxin from the digoxin classes of compounds, a second chromatographic step was necessary to separate individual compounds within the digitoxin class of compounds. The R_F values for both TLC systems for digoxin, digitoxin and the primary non-polar metabolites are given in Table I.

The mean (± S.D.) percentage recovery after extraction and chromatography was 58.3 ± 12.1% for digitoxin, 52.9 ± 10.8% for bis-DT, and 52.3 ± 3.8% for mono-DT. Approximately 5% of the ^{3}H-tracer remained in the serum after extraction. Therefore, losses occurred primarily during the TLC steps.

The digitoxin RIA standard curve was linear over the range 3—50 pmol/ml. All standard curves were prepared using digitoxin standards since there was little difference in the affinity (on a molar basis) of digitoxin, bis-DT, and mono-DT, for the digitoxin antibody. The relative binding affinities were 1.0, 0.94, and 0.96, respectively. Dihydrodigitoxin and digoxin were significantly less reactive with the digitoxin antibody than digitoxin, with relative binding affinities of 17.5 and 46.9, respectively.

The accuracy and precision of the ^{125}I-RIA were determined at five digitoxin concentrations (Table II). All but one of the mean values were within 2 pmol/ml of the calculated concentrations. The minimum detectable concentration was 1.6 pmol/ml.

The serum concentration versus time profiles for digitoxin, bis-DT and mono-DT following the administration of each separately as intravenous doses are presented in Fig. 2. The data obtained from the direct assay of the serum, and the data resulting from the extraction and chromatography are

TABLE I

TLC R_F VALUES FOR DIGITOXIN AND ITS METABOLITES

Compound	R_F × 100		
	TLC 1*	TLC 2**	
		3×***	5×***
Digitoxin	24	24	—
Bis-DT	30	41	—
Mono-DT	36	58	—
Genin-DT	43	79	—
Digoxin	54	—	14
Bis-DG	61	—	23
Mono-DG	69	—	40
Genin-DG	74	—	52

*TLC 1: reversed-phase system, dioxane—methanol—water (2:5:3).
**TLC 2: silica gel TLC, isopropyl ether—methanol (9:1).
***Plates were developed three or five times.

TABLE II

ACCURACY AND PRECISION OF [^{125}I] RIA FOR DIGITOXIN

Concentration (pmol/ml)		Mean deviation from theoretical value
Theoretical	RIA*	
3.34	3.77 ± 0.09 (6.4)	+ 0.4
6.67	7.99 ± 0.24 (5.2)	+ 1.3
13.07	12.43 ± 0.85 (6.8)	— 0.6
26.36	31.48 ± 2.22 (7.1)	+ 5.1
39.44	40.40 ± 1.95 (4.8)	+ 1.0

*Mean of 20 samples ± S.D. (coefficient of variation).

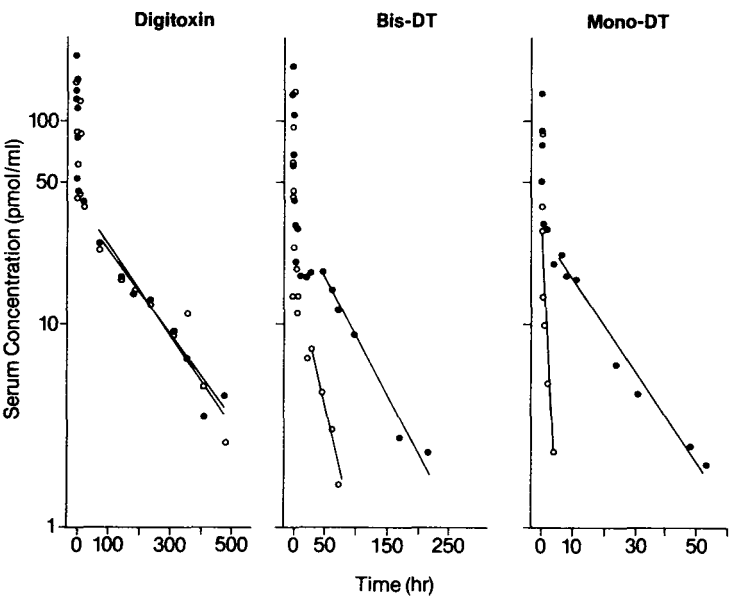

Fig. 2. Digitoxin, bis-DT and mono-DT serum concentrations as a function of time following the intravenous administration of equimolar doses of each. Concentrations measured directly in serum using RIA (•) and concentrations measured using a specific assay (○) are presented.

both given to illustrate the differences between the specific and the non-specific assays. As can be seen from the figure there is little difference between the two assays when digitoxin was measured in the serum of the one subject studied. However, with bis-DT and mono-DT there appear to be significant concentrations of metabolite(s) which also react with the antibody. This would result in a considerable overestimate of the concentration of the parent compound if only total serum was assayed.

DISCUSSION

The method described was specifically designed to measure the pharmacokinetics of digitoxin and two of its metabolites, bis-DT and mono-DT, since there is interest in these latter two compounds as potential therapeutic agents. However, the method could also be applied to digitoxigenin as the digitoxin antibody readily reacts with this compound. The relative binding affinity for digitoxigenin was 1.16. This method in conjunction with a digoxin RIA could also be applied to digoxin, bis-DG, mono-DG and genin-DG. Since digitoxin is not a metabolite of digoxin the first chromatographic step would not be required. Five rather than three developments would be necessary to achieve adequate separation in the second chromatographic step (Table I). It would also be necessary to use a [^{3}H] digoxin tracer with high specific activity in order to minimize its contribution to the digoxin RIA.

The above assay was specific and sufficiently sensitive to allow measurement of non-radioactive parent drug for several half-lives. The use of a tritium tracer as an internal standard to permit corrections for recoveries to be made was essential due to the relatively low and quite variable recovery. This method also separates and allows identification of metabolites of the parent compound.

Digoxin and/or its metabolites have been reported to account for 7.5% of the daily excreted radioactivity following digitoxin administration, but to comprise only 2% of the metabolites in the serum of subjects on a maintenance dose of digitoxin [19,20]. Increased hydroxylation of digitoxin has been reported in patients with impaired renal function [17] but in another study this increase was not found [16]. If assay of the serum samples with a digoxin RIA indicates that digoxin and/or its metabolites are not present to a significant degree, the first chromatographic step may be omitted. The fact that there is minimal cross-reactivity between digoxin and the digitoxin antibody also increases one's confidence in omitting the first chromatographic step.

Dihydrodigitoxin has not been found to be a significant metabolite of digitoxin in man, but increased serum concentrations have been reported in azotemic patients [21]. Most TLC solvent systems, including this one, are unable to separate the dihydro compounds from their precursors. Since the Becton-Dickinson digitoxin ^{125}I-RIA antibody does not significantly cross-react with the dihydro compounds, it was assumed that concentrations of the dihydro compounds did not become sufficiently high so as to interfere. An RIA using an antibody which is specific for dihydrodigoxin but does not cross-react with digoxin has been described [22] and a similar assay may be developed to quantitate the dihydrodigitoxin compounds. A gas chromatographic—mass spectroscopic technique has also been reported for measuring dihydrodigitoxin in serum samples and could be used to quantitate these compounds after chromatography [21].

The method reported here is most useful for pharmacokinetic studies where it is necessary to separate the parent digitalis compound from its metabolites in serum. When digitoxin was administered to a subject, the half-life and area under the serum concentration—time curve calculated from data based on the specific assay were not very significantly different from those determined

by RIA of the total serum (7.3 vs. 4.9 days and 8.4 vs. 8.0 nmol h/ml). However, when bis-DT or mono-DT were administered their half-lives were much shorter and area considerably smaller when calculated from data based on the specific assay (23 vs. 56 h and 667 vs. 2417 pmol h/ml for bis-DT, and 1.6 vs. 18 h and 57 vs. 611 pmol h/mol for mono-DT). This suggests that there were significant levels of metabolite(s) that cross-react with the digitoxin RIA antibody. The water-soluble metabolites which were measured by RIA in the aqueous phase after the dichloromethane extraction were found to be present to a greater extent when bis-DT and mono-DT were given than with digitoxin. It appears that the fewer digitoxose sugars on the glycoside the shorter its half-life, and the more extensive the metabolism, especially the formation of water-soluble metabolites. These water-soluble metabolites are less active [23] and are present in the serum of subjects given mono-DT for many hours after mono-DT is no longer measurable.

ACKNOWLEDGEMENTS

Supported by grants from the Burroughs Wellcome Company, The Flinn Foundation, Phoenix, AZ, and grant HL24559 from the National Heart, Lung and Blood Institute, National Institutes of Health. We are indebted to Catherine Abelli and Edith Makler for secretarial assistance.

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Journal of Chromatography, 278 (1983) 406—411
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1857

Note

Determination of D-glucaric acid by high-performance liquid chromatography

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In 1963 Marsh [1] established D-glucaric acid (GA) to be a normal constituent of human urine and demonstrated an oxidative metabolic pathway of D-glucuronolactone in mammals.

Not only can hepatic cytochrome *P*-450 mixed function oxidase enzyme systems be induced, but many compounds have an inducing effect on the formation of glucuronic acid from glucose and galactose, too [2—6]. The amount of urinary D-glucaric acid seems to correlate with the amount of enzyme-inducing drugs [3, 5, 7]. Many drugs, environmental chemicals and food additives can induce hepatic microsomal enzyme activities [4, 8—11]. The daily urinary excretion of GA has been applied as a useful test to assess the hepatic microsomal drug-metabolizing enzyme activity [10, 12—14] and the enzyme induction by xenobiotics as well [15].

The methods most used for the quantitative determination of GA in urine are still the enzymic assays modified from the method of Marsh [1], which are based on the β-glucuronidase inhibitory effects of boiled solutions of GA, but the results may vary, since many factors in the assay itself may influence the β-glucuronidase activity [16]. Ishidate et al. [17] in 1965 published an ion-exchange chromatographic method, which was modified by Tokola et al. [18] in 1975. Some gas—liquid chromatographic methods for lactone-forming organic acids have also been introduced. In all of them a volatile derivative of the acid is formed. The carboxyl groups are usually converted into methyl esters or trimethylsilyl esters, or the hydroxyl groups into trimethylsilyl ethers [16, 19—22].

The separation of GA and its lactones from aqeuous solution by high-performance liquid chromatography (HPLC) is presented in this paper. Some possibilities for the analysis of GA in urine are also investigated.

0378-4347/83/$03.00

EXPERIMENTAL

Apparatus

A Waters Model 6000 A liquid chromatography pump was used together with a Waters Lambda-Max Model 480 LC spectrophotometer detector and injector (Waters Assoc., Milford, MA, U.S.A.) and a Goerz Servogor S recorder (Goerz Electro, Vienna, Austria). The column was a self-packed Spherisorb-NH_2, particle size 5 μm (228 × 5 mm I.D.).

Reagents

D-Saccharic acid monopotassium salt (Sigma No. S-0250), D-saccharic acid 1,4-lactone (Sigma No. S-0375) and glycolic acid (Sigma No G-1884) were all from Sigma (St. Louis, MO, U.S.A.). Acetonitrile, HPLC-grade, was from Rathburn Chemicals (Walkerburn, U.K.). Orthophosphoric acid and 1-octanol were from E. Merck (Darmstadt, F.R.G.), absolute ethanol was from Oy Alko (Rajamäki, Finland).

Urine samples

Twenty-four-hour urine samples for glucaric acid were collected from a healthy female subject.

Sample preparation

Under acidic and neutral conditions GA is always in equilibrium with its intramolecular esters, the lactones, including D-glucaro-1,4-lactone, D-glucaro-3,6-lactone and D-glucaro-1,4-3,6-dilactone [18]. The equilibrium depends on pH and temperature [15]. In basic solution (pH $\leqslant$ 8) GA is totally in the form of free acid.

Aqueous standards (in concentrations of 0.5—3.0 mg/ml) were made alkaline by addition of 1 *M* sodium hydroxide to pH 8. Aqueous standards and urine samples were made acidic by 1 *M* hydrochloric acid to pH 2.5.

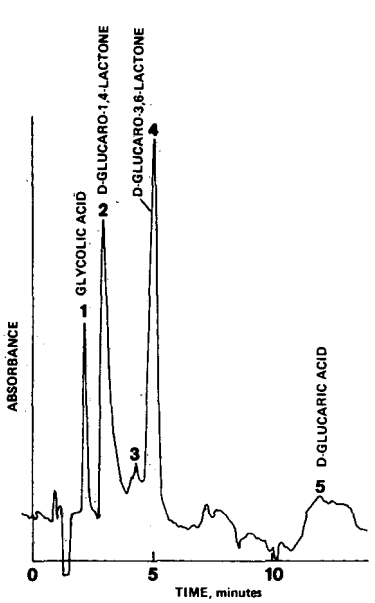
Urine samples and standards were hydrolyzed by boiling in water for 1 h, cooled to room temperature and centrifuged (2000 *g* for 5 min). Glycolic acid was used as internal standard in a final concentration of 0.5 mg/ml. Urine samples were concentrated by vacuum distillation to 1/10 of the volume. To prevent foaming a few drops of 1-octanol were added.

HPLC procedure

The mobile phase was 0.01 *M* phosphoric acid (pH 2.5)—acetonitrile (75:25, v/v) and the flow-rate 2 ml/min at room temperature. The mobile phase was filtered through a Millex-HV filter unit, 0.45 μm (Millipore) and degassed ultrasonically (Bransonic 220). It took several hours to stabilize the column with the buffer—acetonitrile eluant, so the column was eluted at a minimum flow-rate overnight. Standards and concentrated urine samples were injected into the column. The eluate was monitored at 220 nm.

RESULTS

Fig. 1 shows the HPLC separation of GA and its lactones at pH 2.5 with the



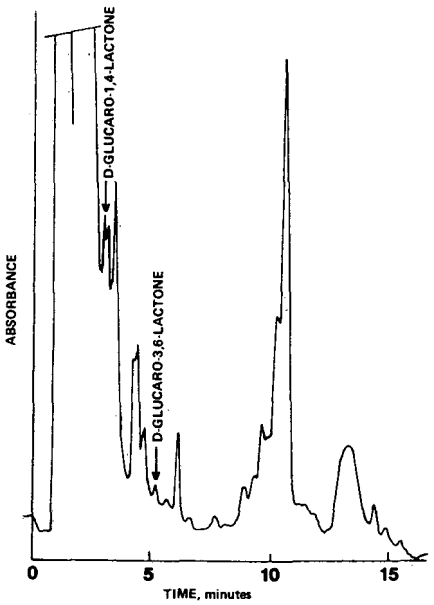


Fig. 1. HPLC separation of D-glucaric acid from its lactones after hydrolysis at 100°C and pH 2.5 for 1 h. Glycolic acid was used as the internal standard. Conditions: column Spherisorb-NH_2; mobile phase acetonitrile—0.01 *M* phosphoric acid (25:75, v/v); ultraviolet detection at 220 nm; flow-rate 2 ml/min; 0.01 a.u.f.s; chart speed 600 mm/min.

Fig. 2. HPLC chromatogram of concentrated normal human urine by ultraviolet detection (220 nm). Conditions as in Fig. 1.

Spherisorb-NH_2 column used in the weak anion-exchange mode. The internal standard (glycolic acid) elutes first (peak 1), and next D-glucaro-1,4-lactone (peak 2), which is well separated from the internal standard. It was identified with a pure D-glucaro-1,4-lactone solution. A small peak 3, obviously D-glucaro-1,4-3,6-dilactone, is not totally separated from peak 4, obviously D-glucaro-3,6-lactone (according to Fiedler et al. [16]). D-Glucaric acid elutes last. The separation of all compounds takes less than 13 min.

Table I summarizes the chromatographic behavior and the composition by area (%) of GA and its lactones and the internal standard glycolic acid.

TABLE I

HPLC EVALUATION OF SEPARATED COMPOUNDS IN D-GLUCARIC ACID ANALYSIS ON SPHERISORB-NH_2 COLUMN AT pH 2.5

Compound	Retention time (min)	Capacity factor (k')	Composition by area (%)
Glycolic acid	2.1	0.50	—
D-Glucaro-1,4-lactone	2.9	1.07	30
Unknown	4.4	2.14	5
D-Glucaro-3,6-lactone	5.1	2.64	40
D-Glucaric acid	12.1	7.64	25

TABLE II

THE PRECISION OF A SERIES OF D-GLUCARIC ACID SAMPLES AT A CONCENTRATION OF 2 mg/ml

	D-Glucaro-1,4-lactone/ internal standard	D-Glucaro-3,6-lactone/ internal standard
	1.77	2.66
	2.20	2.70
	2.05	2.72
	1.95	2.79
	2.21	2.77
Mean	2.04	2.73
S.D.	0.18	0.05
C.V. (%)	9.0	1.9

In basic solution (pH $\leqslant$ 8) GA is totally in acidic form, and so the peak of GA obtained is quite broad. Thus, it is better to calculate the total GA content in acidic standards from the relationship between the peak area of D-glucaro-1,4-lactone and the internal standard.

The precision of a series of GA samples is shown in Table II.

In the standard curve the relationship between the total amount of D-glucaric acid and the peak area of D-glucaro-1,4-lactone divided by the peak area of the internal standard (glycolic acid) showed a linear response over the concentration range 0.5—3 mg/ml.

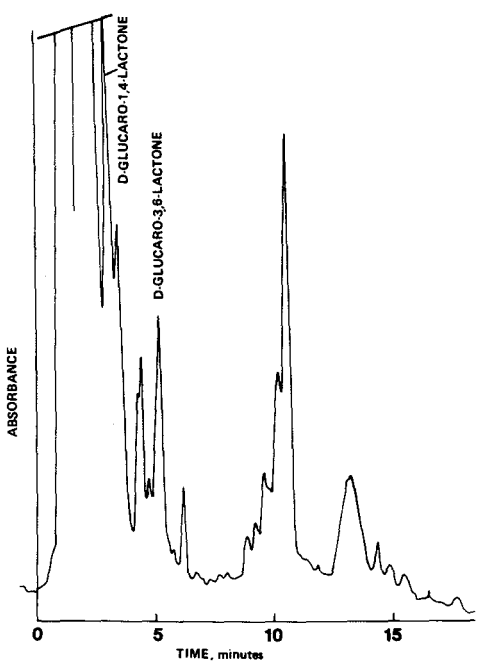


Fig. 3. HPLC chromatogram of concentrated normal human urine, when the D-glucaro-lactones are qualitatively identified by co-elution with their standard solution.

The detectable amount of GA in aqueous solution is 2—4 μg at 220 nm.

The chromatogram of a urine sample obtained from a healthy subject is shown in Fig. 2.

Fig. 3. is a chromatogram of the same urine sample, in which the D-glucaro-1,4-lactone and the 3,6-lactone have been qualitatively identified by co-elution with the standard solution of lactones.

DISCUSSION

The results show that GA and its lactones can be analyzed quantitatively in aqueous solution by HPLC on the weak anion-exchange column Spherisorb-NH_2. The column should be used in the anion-exchange mode to separate the compounds with ionizable protons. In the straight phase they presumably form salts with the amino groups in the column packing. The amino column might be preserved for longer with ammonium acetate—acetonitrile or ammonium acetate—methanol as eluant, but this was not checked. The amino column has earlier been found to be suitable for the analysis of some organic acids [23, 24]. Possibly the D-glucaro-3,6-lactone could be used for quantification of GA, too, because it separates well and has a rather low variation coefficient.

Baseline noise is quite disturbing at wavelengths as low as 220 nm. In the blank, however, it remained satisfactory. By preparing a colored derivative, the measurements could be made at a visible wavelength.

By this method GA can only be qualitatively demonstrated in urine, because the peaks of D-glucarolactones do not separate well enough to allow quantitative determination. The urine samples should first be purified. The internal standard glycolic acid stays under the matrix, and normal urine contains a little of it [25].

Certain organic acids in urine have been analyzed by HPLC [26, 27] but D-glucaric acid was not included.

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Journal of Chromatography, 278 (1983) 412—417
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1852

Note

Flat-bed isoelectric focusing of high-density lipoproteins

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(First received March 3rd, 1983; revised manuscript received July 12th, 1983)

The subfractionation of high-density lipoproteins (HDL) by isoelectric focusing (IEF) has been described by several authors [1—16], who reported a remarkable heterogeneity within this lipoprotein class. Nevertheless, no reliable classification of the observed subclasses could be achieved, since these results did not correspond to each other in respect to number of subfractions, p*I* values and relative amounts. As these differences may be due in part to the methodological approach used, a new method for a rapid and reproducible subfractionation of HDL by IEF was developed.

METHODS AND MATERIALS

Normolipidemic human serum and the same serum after removal of very-low-density and low-density lipoproteins (VLDL and LDL, respectively) by polyanion precipitation were investigated. Freshly drawn fasting venous blood was allowed to clot for 2 h at room temperature. Then it was centrifuged at 900 *g* and 4°C for 20 min. Polyanion precipitation was performed using dextran sulphate (MW 500,000; Pharmacia, Uppsala, Sweden) and mag-

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nesium chloride (Merck, Darmstadt, F.R.G.) exactly as descibed by Kostner et al. [17], and the supernate (DX-HDL) was used for IEF.

IEF was carried out in an LKB multiphor chamber (LKB, Bromma, Sweden). The 1% agarose (Agarose EF; LKB) slab gel (24 × 11 cm), which was prepared according to an application note of LKB [18], was 0.5 mm thick and contained 3% Ampholine, pH 4.0–6.5. Running conditions were 10°C, 4 W, 80 min. In preliminary studies the samples were applied either near the cathode or near the anode to make sure that the subfractions really reached their isoelectric point at the end of the run. Since the region of pH 6.0 was found to be free of lipoproteins, this area was later chosen for application of samples. After the IEF run, the detection of HDL subfractions was achieved by immediate polyanion precipitation of lipoproteins in the agarose gel slab as described by Seidel [19]. pH Values were measured with a surface electrode (LKB) immediately after the IEF run.

In order to show the apoprotein content of the IEF subfractions, immunoelectrophoresis in the second dimension was carried out after IEF. For this purpose, a strip (5 × 60 mm) which contained freshly focused sample (serum or DX-HDL) was cut out of the IEF gel, placed on a 7 × 7 cm glass plate and immediately covered with 1% agarose in Tris buffer, pH 8.6, which contained 4% antiserum against apolipoprotein A-I (Behring, Marburg, F.R.G.). Running conditions for the immunoelectrophoresis were 20°C, 12 h, 30 V.

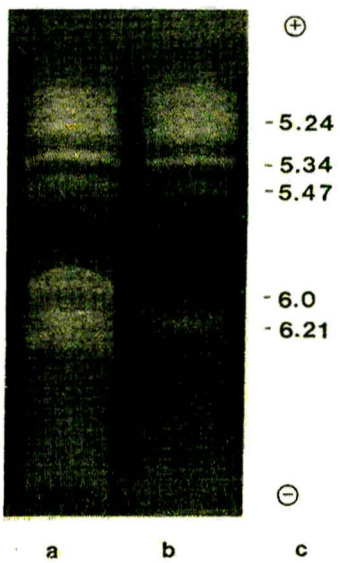

Fig. 1. Precipitation pattern of HDL after IEF. (a) Whole serum of a normolipemic male, age 37. (b) Supernatant after removal of VLDL and LDL by polyanion precipitation. (c) p*I* Values of subfractions obtained.

RESULTS

Representative patterns of lipoprotein subfractions of whole serum and DX-HDL after IEF with subsequent precipitation are shown in Fig. 1. These

patterns resulted regardless of whether the samples had been applied near the cathode or near the anode. Three groups of bands can be visualized at pH 5.47, 5.34 and around pH 5.24. It is likely that the subfractions at pH 5.47 and 5.24 consist of two and three subunits, respectively. The main difference between the pattern of whole serum and DX-HDL is the absence in the latter of bands at the application spot and near the cathode in the pH range 6.1—6.4. They contain apoprotein B (unpublished results) and are mostly removed by precipitation of VLDL and LDL by dextran sulphate. A remaining diffuse band at pH 6.21 is unidentified. According to preliminary results it contains neither apoprotein B nor apoprotein A-I or A-II. The band pattern of both samples in the range between pH 5.2 and 5.5 showed excellent agreement. It represents the subfractions of HDL. This HDL pattern has apparently not been influenced by the precipitation of VLDL and LDL with dextran sulphate.

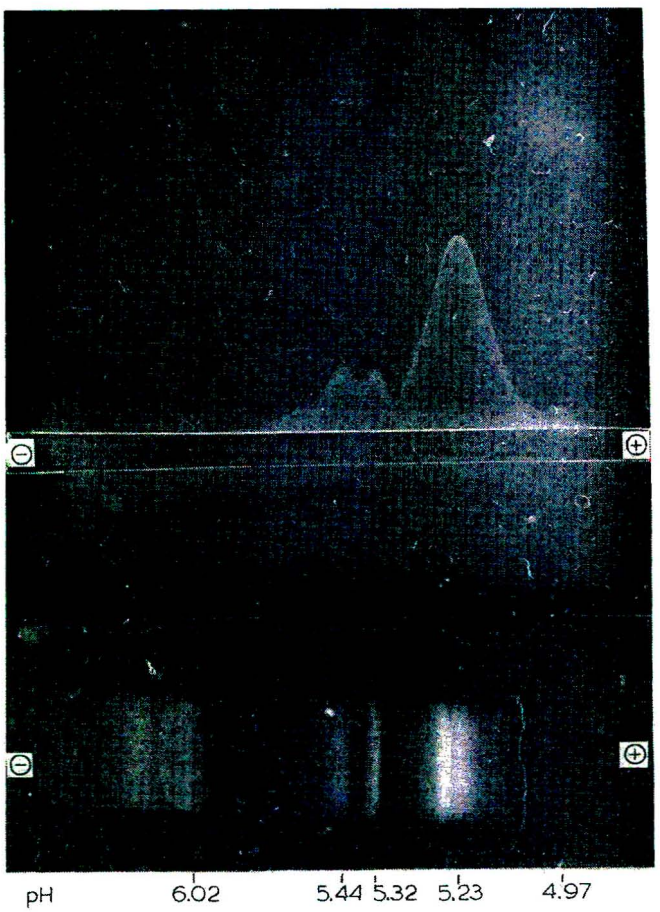

Fig. 2. Above: crossed immunoelectrophoresis after IEF of the supernatant of DX-HDL of VLDL/LDL precipitation using antilipoprotein A-I. Below: precipitation pattern of the same sample (normolipemic female, age 25).

The aim of the immunoelectrophoresis in the second dimension was to show that the bands which can be precipitated in the agarose slab after IEF indeed contain lipoproteins. For this purpose we employed antiserum against apoprotein A-I, the major apolipoprotein of HDL. Fig. 2 shows the precipitation pattern of the subfractions of DX-HDL. It was photographed together with a parallel agarose strip from the same IEF run in which HDL subfractions had been precipitated as described below. Again, one major and two minor peaks were observed, which correspond in location to the regions of the HDL bands precipitated in the original IEF plate. A fourth little peak is found at the anodic side (pH 4.97) of the large peak, and corresponds to a very weak band in the precipitation pattern. It is likely that the major peak observed is composed of more than one reaction peak; the lack of shoulders corresponding to the precipitation pattern may be explained by diffusion of the narrow bands during the time which passed between IEF and second-dimension electrophoresis (about 30 min).

DISCUSSION

IEF has rarely been used as an analytical tool for the subfractionation of HDL. A reason for this may have been the inconsistent and discrepant results in the literature which in our view may be due to the method applied by the majority of previous investigators, i.e. vertical IEF in a sucrose gradient. This procedure is time-consuming, may cause problems in reproducibility and does not allow measurement of the pH in the medium itself. Flat-bed IEF, in contrast, overcomes the disadvantages and furthermore offers the possibility of application of samples near the cathode as well as near the anode, which allows the exclusion of focusing artifacts which result in false apparent p*I* values.

Our results show that IEF in flat-bed agarose can clearly and rapidly separate HDL into at least three subfractions whose p*I* values are well reproducible. For rapid visual detection, these subfractions can be precipitated in the IEF agarose. The precipitated bands correspond well to the peak pattern produced by crossed immunoelectrophoresis with antiserum against apolipoprotein A-I which proves that the observed bands indeed represent HDL subfractions.

Number and p*I* values of the subfractions obtained do not agree with the results of other investigators. The main difference is the narrow pH range (pH 4.9—5.5) in which the HDL subfractions were focusing, while most authors found ranges between pH 4.0 and 6.0 [2, 9, 14—17]. Only Kostner et al. [6] and Eggena et al. [3] describe ranges somewhat similar to ours (pH 4.6—5.1 and 4.7—5.4, respectively) but their results differ from ours in the number of subfractions (Eggena et al.) as well as in the p*I* values of individual subfractions (Kostner et al., Eggena et al.). A possible reason for these discrepancies may be that most authors measured the pH values at room temperature and not at the temperature at which the IEF had been carried out [2, 5, 8, 9, 11—14]. Furthermore, it remained unexplored to what extent the sucrose content of the focusing solutions affected the determination of pH values. Since increasing temperatures decrease pH values, and sucrose

or glycerol also influence pH measurements, both parameters must be taken into account [20]. Our method, in contrast, allows measurement of the pH right on the surface of the cooled agarose and is not influenced by sucrose. Since the subfractions are found with identical p*I* values, regardless of whether the sample was applied near the anode or near the cathode, this focusing technique appears to be more reliable. It is of interest to note that the pH range in which we observed focusing of the intact HDL subfractions closely matches the p*I* range of delipidized aproprotein A-I (p*I* = 5.60) and apoprotein A-II (p*I* = 4.88) [21].

Of the two kinds of sample which we investigated (whole serum and DX-HDL) the latter is, in our view, the more appropriate for further studies, since LDL and VLDL are removed and the remaining HDL particles are apparently not altered by the addition of dextran sulphate. This advantage of DX-HDL over whole serum gains relevance especially in hypertriglyceridemia where one may find a considerable distortion of the IEF pattern of whole serum by interfering triglyceride-rich lipoproteins (unpublished observation).

Since HDL has several metabolic origins and functions, the present method may be a helpful tool for further studies of lipid metabolism and of lipoprotein disorders. It seems to be specially useful for the subfractionation of HDL in situations where the number of samples to be analyzed is very large, such as for clinical screening and epidemiological studies, or where the available amount of sample is very limited (e.g. studies in infants and small laboratory animals).

ACKNOWLEDGEMENTS

The authors wish to thank Professor Dr. W. Schwartzkopff, Head, Lipid Clinic, Department of Medicine, Free University, Berlin, for his continuing interest and support. We also wish to thank Mrs. M. Schmidt for excellent technical assistance.

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Journal of Chromatography, 278 (1983) 418—423
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1858

Note

Simple and rapid method for the determination of caffeine in urine using Extrelut-1 columns

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(First received April 18th, 1983; revised manuscript received July 7th, 1983)

Caffeine (1,3,7-trimethylxanthine) is consumed by the population mainly as an ingredient of coffee (average 83 mg/cup) [1], cola beverages and tea. A smaller amount comes from the use of caffeine in pharmaceuticals. Indeed, besides its diuretic effect caffeine acts as a central nervous and cardiac muscle stimulant, as well as a smooth muscle relaxant [2], the usual therapeutic dose in man being 200 mg. Based on its stimulant properties, however, caffeine-containing formulations can also be used as doping agents in sportsmen. Thus, the quantity of caffeine ingested can be highly significant, especially for those seeking to improve their athletic performance.

In order to detect the abuse of caffeine in human sports, a simple and rapid method for the quantitative determination of caffeine in urine is presented in this paper. Gas chromatography with nitrogen selective detection (GC—NPD) and column extraction with Extrelut® columns are used.

EXPERIMENTAL

Materials

Caffeine was obtained from Merck (Darmstadt, F.R.G.) and the internal standard mepivacaine hydrochloride was supplied by Astra (Södertälje, Sweden). Stock solutions were prepared in double-distilled water. The concentration of the internal standard used was 100 $\mu g\ ml^{-1}$.

Extrelut-1 columns and Extrelut refilling bags were purchased from Merck.

The ammonium buffer was prepared by adjusting a saturated ammonium chloride solution to pH 9.5 with ammonium hydroxide.

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Gas chromatography

A Varian 3700 gas chromatograph with a nitrogen-selective detector was used and connected to a Varian CDS 111 integrator. The glass column (200 × 0.25 cm I.D.) was packed with 3% OV-7 on Chromosorb W HP. Nitrogen was used as carrier gas at a flow-rate of 25 ml min^{-1}. Column, injector and detector temperatures were kept at 195°C, 230°C and 330°C, respectively.

Analytical procedure

Urine (2 ml) was pipetted into a reaction tube and made alkaline (pH 9.3) by adding 0.2 ml of ammonium buffer. After adding 0.1 ml of the internal standard solution, the contents of the tube were briefly vortexed and 1 ml pipetted in duplicate on top of Extrelut-1 columns.

After 5 min, each column was eluted with 6 ml of methylene chloride—methanol (9:1). The eluate was collected in a conical reaction tube and evaporated under nitrogen at 40°C. The residue was redissolved in 0.2 ml of ethyl acetate and 1 µl was injected into the gas chromatograph.

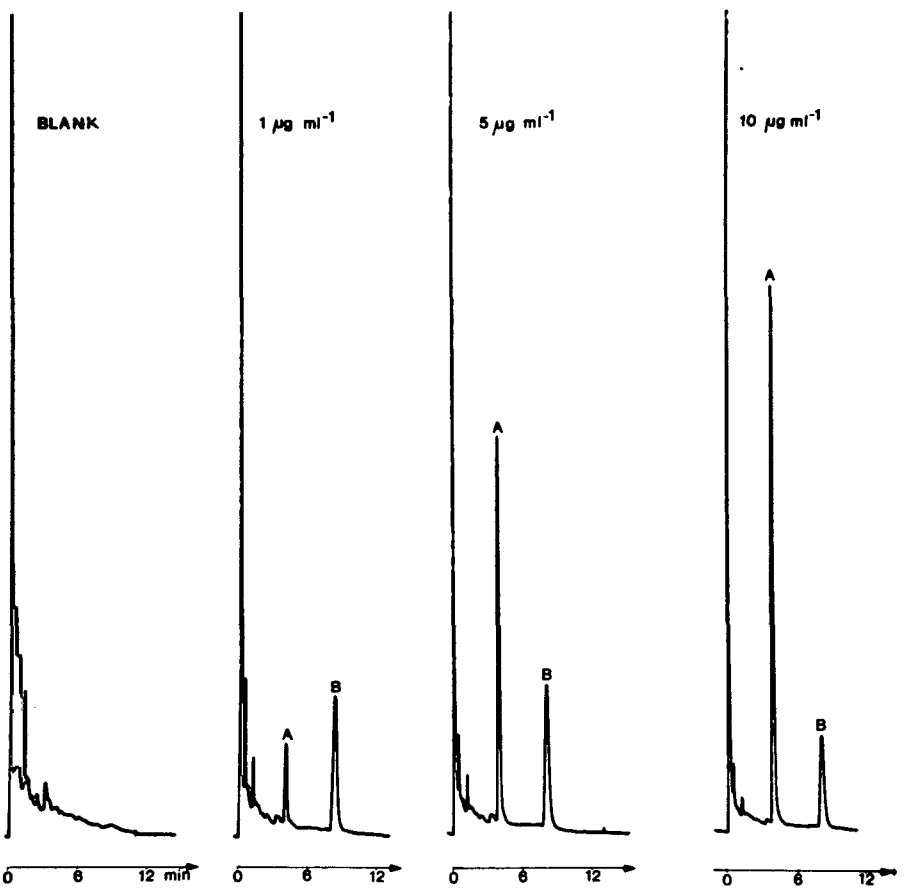

Fig. 1. Chromatograms obtained from horse urine spiked with caffeine (A = caffeine, B = mepivacaine).

RESULTS AND DISCUSSION

Although caffeine is currently mentioned in drug screening procedures [3—6] there is a scarcity of published methods suitable for the routine quantitative determination of caffeine in urine.

Drugs such as caffeine as well as their metabolites must be extracted from body fluids before they can be determined by chromatographic procedures. The determination of caffeine in plasma using GC with NPD and conventional liquid—liquid extraction as proposed by Cohen et al. [7] requires an analysis time of at least 25 min. A recently published method [8] for the GC estimation of caffeine in the urine of dogs needs a 10-ml sample and two extraction periods of 20 min each. Liquid chromatographic (LC) determinations of caffeine in serum require a sample preparation of 30—40 min [9—12] with a high volume ratio (extraction solvent/sample) or special techniques like multidimensional LC allowing the direct injection of biological fluids without pretreatment [13].

Nevertheless, it is well known that Extrelut provides a means of achieving a rapid and precise extraction without the problems of emulsion formation and consumption of high volumes of solvent [14—16].

Under the chromatographic conditions described previously, caffeine and the

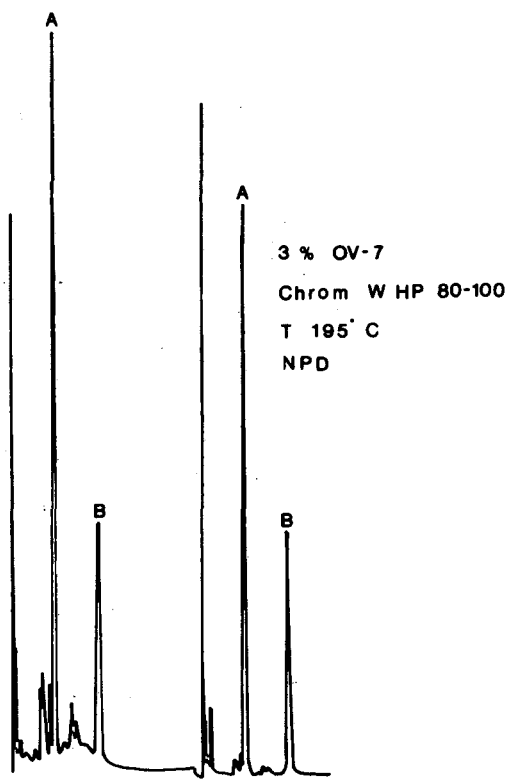

Fig. 2. Chromatograms from extracts of urine from coffee drinkers (A = caffeine, B = mepivacaine).

internal standard mepivacaine gave sharp peaks with retention times of 4.35 and 8.74 min, respectively. Typical chromatograms obtained by processing control blank and spiked horse urine containing different amounts of caffeine are presented in Fig. 1. The principal human caffeine metabolites [17] (theophylline, theobromine, 1-methylxanthine, 3-methylxanthine, 7-methylxanthine, paraxanthine and 1-methyluric acid) did not interfere under these conditions. Processing urine of coffee drinkers as described in the analytical procedure resulted in chromatograms as illustrated in Fig. 2.

The influence of the pH on the elution recovery was studied by adding caffeine in a final concentration of 20 μg ml^{-1} to horse urine, brought to different pH values. Each urine sample (1 ml) was analysed in triplicate. The column residue was redissolved in 200 μl of a methanolic internal standard solution (mepivacaine 100 μg ml^{-1}) and 1 μl was injected and quantified.

Fig. 3 indicates that the elution recovery of caffeine in the pH range 7—10 is practically not influenced by the urinary pH. The recoveries are close to 90%. In addition, the influence of the pH on the elution of mepivacaine was also studied using caffeine as internal standard. The peak ratios, ΔMepiv./ΔCaff., (mean of three determinations) as indicated in Fig. 3 show that the extractability of mepivacaine using Extrelut-1 columns is not substantially influenced by the urinary pH in the pH range 8—10.

A standard curve was obtained by adding different amounts of caffeine to horse urine and treating the samples in quadruplicate according to the procedure. The calibration graph is linear in the range 0—20 μg ml^{-1} caffeine in urine (σ = 0.9995).

Replicate determinations of caffeine in a human urine sample produced a

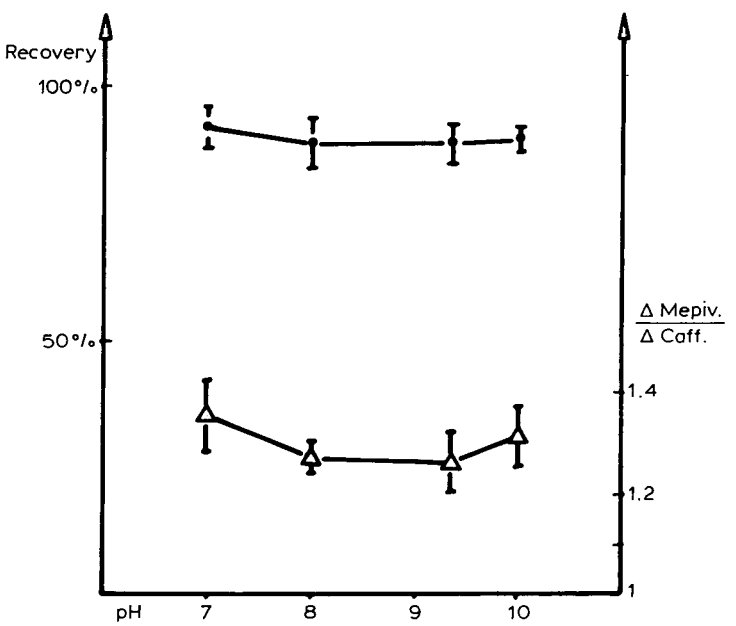

Fig. 3. Influence of the pH on the elution recovery (%) of caffeine (•) and on the extractability measured by peak ratios of mepivacaine (△).

relative standard deviation of 1.5% (1.71 ± 0.026 μg ml^{-1}, n = 7). Starting with 1 ml of urine, the lowest concentration which allowed quantitative detection in routine analysis was 0.4 μg ml^{-1} corresponding to about 1.75 ng injected. This assay is considerably more sensitive than a previously published GC-NPD procedure [7] in which a 1-ml plasma sample was required to produce a sensitivity of 10 ng injected. Notwithstanding the difference in biological matrix between plasma and urine, this improvement in detection limit can be partly attributed to a decrease in background noise seen with Extrelut-1 columns. Indeed, in agreement with our experience [14], there are reports in many papers that Extrelut eluates are cleaner than the corresponding liquid—liquid extracts. Moreover, progress has been made by introducing glass columns instead of polyethylene columns and altering the composition of the packing material of the refilling bags. It should be clear that comparable results can be obtained by using selfprepared columns filled with equivalent amounts of Extrelut.

Following this method, the urinary caffeine content of a great consumer of coffee was monitored during a 60-h period (Fig. 4). Based on the caffeine content of both brewed and instant coffee as suggested by Burg [17], the intake was estimated at 700 mg/day. The amount excreted over the 60-h period (18.2 mg) is in agreement with values from other studies [18, 19], indicating that only approximately 1% of caffeine is excreted unchanged.

In order to establish an excess level of caffeine for sporting competitions,

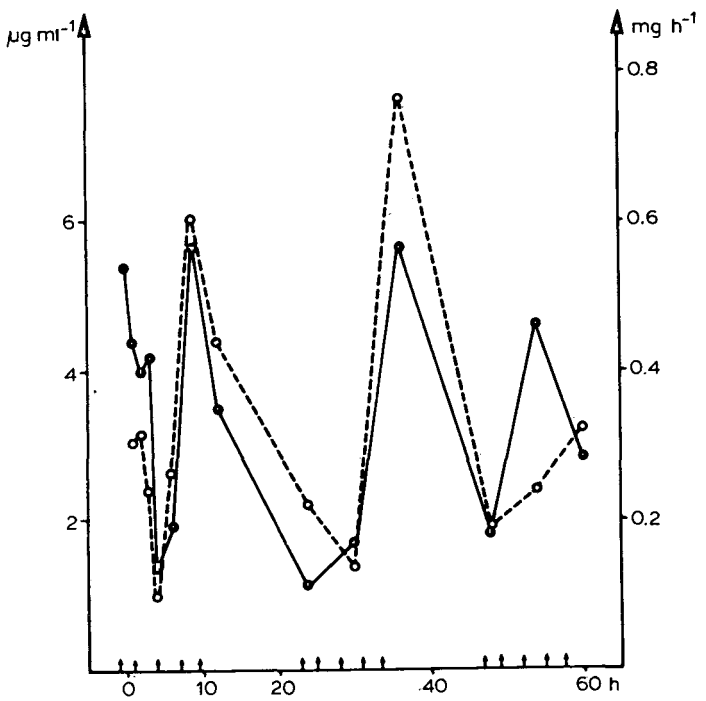

Fig. 4. Monitoring of the urinary excretion of caffeine in a great consumer of coffee. Excretion expressed as μg ml^{-1} (•) and mg h^{-1} (○). The intakes of coffee (approximately 140 mg) are marked by arrows.

further work with respect to the urinary caffeine content of both coffee drinkers and sportsmen is currently being investigated.

The results reported here indicate that the quantitative determination of caffeine in urine of athletes can easily and rapidly be done with low solvent consumption using Extrelut-1 columns.

ACKNOWLEDGEMENTS

The authors wish to thank Mrs. G. Demey, Mrs. D. Dhaenens and Mr. N. Desmet for their technical assistance. The gift of some Extrelut-1 columns by Merck Belgium is greatly appreciated. We are grateful to Dr. M. Moss of Racecourse Security Services Labs. (Newmarket, U.K.) for a gift of caffeine metabolites.

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Journal of Chromatography, 278 (1983) 424—428
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1859

Note

Gas—liquid chromatographic determination of methohexital in plasma or whole blood with electron-capture detection of the pentafluorobenzyl derivative

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(First received April 15th, 1983; revised manuscript received July 11th, 1983)

Methohexital (methohexitone, Brietal®) is an ultra-short-acting barbiturate used for induction of anaesthesia or as an intravenous sedative [1]. Quantitative analysis of methohexital in biological samples has been done by means of ultraviolet spectrophotometry [2], gas—liquid chromatography (GLC) with flame ionization detection [3] and GLC with nitrogen-selective detection [4, 5]. Only the last method has sufficient sensitivity and selectivity for pharmacokinetic studies.

Analysis of barbiturates as pentafluorobenzyl derivatives, with electron-capture detection (ECD), has not become a standard method, perhaps because of the tedious work-up and derivatization procedures reported [6]. We therefore wish to describe a simple and rapid GLC—ECD method for methohexital in biological samples.

EXPERIMENTAL

Reagents and chemicals

Methohexital was supplied by Eli Lilly Sweden (Stockholm, Sweden). Hexobarbital was of European Pharmacopoeia quality. The barbiturates were dissolved in 6.7 m*M* phosphate buffer (pH 7.4) and the appropriate stock solutions were then prepared by dilution with distilled water. Pentafluorobenzyl (PFB) bromide was purchased from Aldrich-Europe (Beerse, Belgium) and triethylamine was Eastman synthetic grade (Rochester, NY, U.S.A.). Toluene (8325; Merck, Darmstadt, F.R.G.), methanol (6009; Merck), cyclohexane (9666; Merck) and absolute ethanol (Svensk Sprit, Stockholm, Sweden) were used without further purification.

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Instrumentation

A Varian (Palo Alto, CA, U.S.A.) 3700 gas chromatograph equipped with a ^{63}Ni electron-capture detector and a Model 8000 autosampler was used. The glass column (150 × 0.2 cm I.D.) was packed with 3% OV-17 on 100—120 Supelcoport. The oven temperature was 188°C and the injector and detector temperatures were 240°C and 250°C, respectively. Nitrogen (30 ml/min) was used as carrier gas. Mass spectra were obtained, under similar gas chromatographic conditions, on a Finnigan (Sunnyvale, CA, U.S.A.) 4510 quadrupole instrument operated in the chemical ionization mode.

Method

To 1.0 ml samples of plasma or haemolysed (frozen and thawed) whole blood containing methohexital (0—16.0 μg/ml = 0—61.2 μ*M*) were added 0.5 ml of internal standard solution (hexobarbital 10.0 μg/ml = 42.3 μ*M* in water), 0.5 ml of 0.25 *M* hydrochloric acid and some 0.04 g of sodium chloride. The samples were extracted with 3 ml of toluene on a Hook and Tucker rotamixer and the solvent layers were separated by centrifugation. The toluene layer was transferred to another tube and extracted with 2 ml of 0.01 *M* sodium hydroxide. The aqueous phase was separated, acidified with 1 ml of 0.25 *M* hydrochloric acid and extracted with 3 ml of toluene. The solvent was evaporated on a sand bath (50 ± 5°C) under a stream of dry air.

The residue was taken up in 0.1 ml of 0.5 *M* triethylamine in methanol and 5 μl of pentafluorobenzyl bromide were added. The mixture was heated for 10 min on a 56 ± 1°C water bath. The volatiles were evaporated on the sand bath under a stream of dry air. The residue was taken up in a few drops of absolute ethanol and diluted to 4 ml with cyclohexane. By means of the autosampler, 1 μl was injected into the gas chromatograph.

The extraction recovery was determined by adding methohexital to 1 ml of plasma or whole blood (final concentration 2.0 μg/ml), freezing and thawing the whole blood and extracting by the standard procedure with 3.0 ml of toluene. To 2.0 ml of the toluene phase 0.5 ml of hexobarbital in methanol (10 μg/ml) was added as external standard. The solvent was evaporated and the residue derivatized and assayed. A mixture of methohexital—hexobarbital (2:5) served as reference. Four samples of each, plasma, blood and reference, were used.

RESULTS AND DISCUSSION

Work-up

For plasma samples containing >1 μg/ml methohexital the re-extraction step can be omitted. The recovery of methohexital from plasma was 93 ± 1% and from haemolysed whole blood 88 ± 5%.

Derivatization procedure

The derivatization conditions are those given by Walle [7], who did not, however, work with biological samples. The reaction was tried out by running the recovery experiment as described but adding hexobarbital pentafluorobenzyl derivative at the end as an external standard. With reaction times

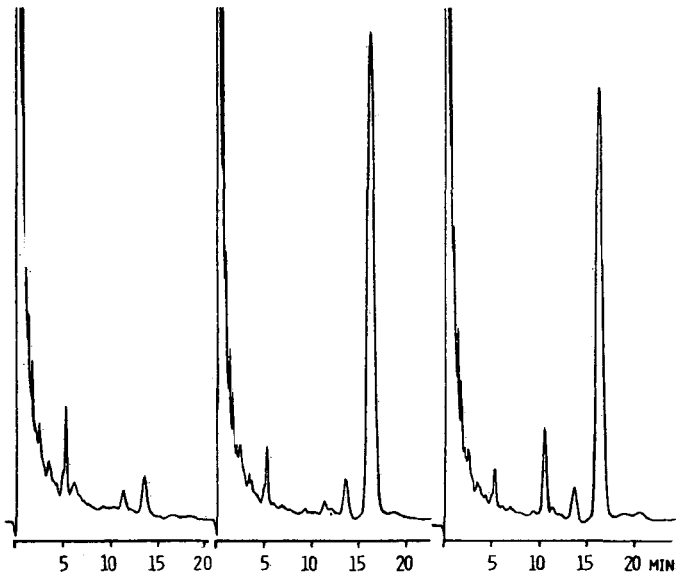

Fig. 1. Chromatograms of plasma samples from a patient undergoing minor surgery. From left to right: a blank sample taken before anaesthesia; a blank sample with internal standard added; and a sample taken 10 min after induction of anaesthesia with intravenous methohexital sodium 1.25 mg/kg. The methohexital concentration of the last sample is 0.75 μg/ml.

exceeding 5 min the relative height of the methohexital peak vs. the standard peak did not change. Instead, with protracted reaction times (> 30 min) extraneous peaks interfering with the desired ones began to appear.

The derivatives are stable in solution for at least a month at room temperature. Typical chromatograms are shown in Fig. 1.

On-column methylation with 0.2 *M* trimethylanilinium hydroxide in methanol [8] was also tried. The methyl derivative of hexobarbital was not stable, however, but decomposed in a way previously described for N,N-dimethylphenobarbital [9].

Mass spectra

The methane chemical ionization mass spectra confirmed the putative structures of the methohexital and hexobarbital PFB derivatives. The pseudomolecular ions $[M+H]^+$ appeared as expected at *m/z* 443 and 417, respectively. Fragmentation, in the form of cleavage of the PFB group from the barbiturate [10], was observed for the hexobarbital derivative only.

Standard curve, precision and sensitivity

Standard curves drawn on analysis of duplicate samples containing 0.125, 0.250, 0.500, 1.00, 2.00 and 4.00 μg/ml methohexital were linear (generally r = 0.977—0.999 on a four-point standard curve). With high sample concentrations, 8.00 or 16.00 μg/ml, the final solution of derivative had to be diluted four- to five-fold before injection. Otherwise these points would drop below the straight line. "Unknown" samples showing methohexital concentrations above 4 μg/ml should be re-run after similar dilution.

Analysis of eight samples spiked with 1.00 μg/ml methohexital (3.83 μM) gave a mean value of 1.005 μg/ml (3.84 μM) with a standard deviation of 0.065 μg/ml (0.25 μM), which gives a relative S.D. of 6.5%.

The lowest plasma methohexital concentration which can be satisfactorily quantitated is around 0.1 μg/ml (0.4 μM).

CONCLUSIONS

Several attempts have been made to determine barbiturates in biological samples by pentafluorobenzylation and ECD [6]. The derivatization of pentobarbital with pentafluorobenzyl bromide in aqueous sodium carbonate solution required prolonged heating of the reaction mixture [10]. Extractive alkylation of phenobarbital from saliva worked well but removal of excess reagent required the use of a pre-column venting system [11]. Pentobarbital and phenobarbital form di-pentafluorobenzyl derivatives. The N-methylated barbiturates methohexital and hexobarbital form mono derivatives and, in

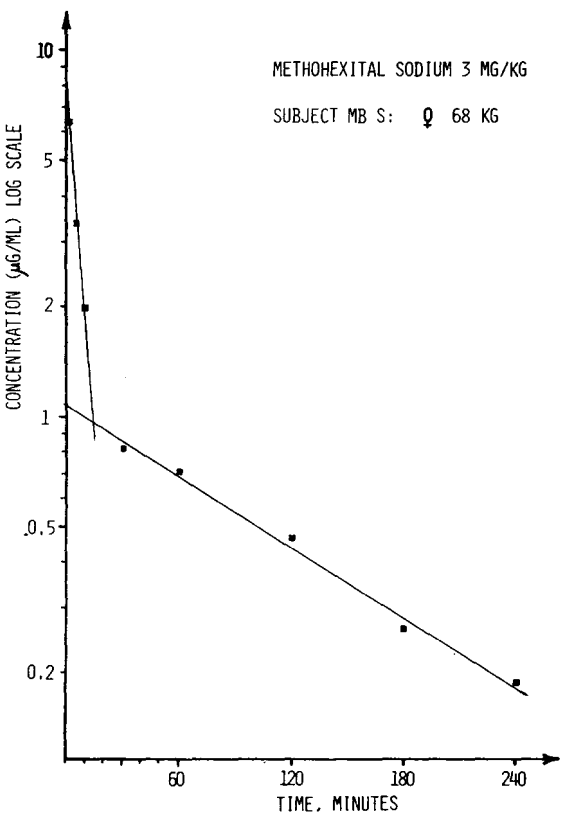

Fig. 2. Plasma methohexital concentration curve from a patient under methohexital—halothane—nitrous oxide anaesthesia. Three mg/kg methohexital sodium (Brietal), corresponding to 2.76 mg/kg free acid, was given as an intravenous bolus dose. The apparent volume of distribution (V_d = 2.6 l/kg) and the terminal half-life (β = 95 min) were calculated from the five-point regression line.

the triethylamine—methanol system, the reaction is easily driven to completion with a moderate excess of reagent. This makes our method feasible for multi-sample analysis and its usefulness in pharmacokinetic work is exemplified in Fig. 2.

ACKNOWLEDGEMENTS

We thank Dr. Claes Lindberg, AB Draco, Lund, Sweden for recording the mass spectra. Our thanks are also due to Miss Ulla Jönsson for skilful technical assistance.

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Journal of Chromatography, 278 (1983) 429—433
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1869

Note

Measurement of moclobamide, a new monoamine oxidase inhibitor, by gas chromatography with nitrogen-selective detection

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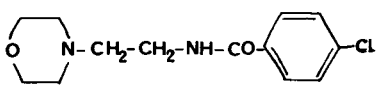
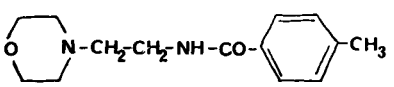
Moclobamide [*p*-chloro-N-(2-morpholinoethyl)benzamide, Fig. 1] is a new monoamine oxidase (MAO) inhibitor. Its inhibitory effect on MAO in the brain is reversible and specific for the A form of the enzyme. An open evaluation suggested the drug had a rapid onset of antidepressant effect and low toxicity [1]. Its antidepressant efficacy has since been confirmed in a double-blind comparison with amitriptyline [2].



A



B

Fig. 1. The structures of moclobamide (A) and the internal standard (B).

To evaluate both the pharmacokinetics of moclobamide and the relationship between plasma concentrations and antidepressant efficacy, we have developed an analytical method based on gas chromatography (GC) with nitrogen-selective detection.

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EXPERIMENTAL

Materials

Moclobamide (Ro 11-1163) and Ro 11-9506 (Fig. 1) were obtained from Hoffmann-La Roche (Basle, Switzerland). The latter was used as the internal standard. Ethyl acetate, HPLC grade was obtained from Waters Assoc. (Milford, MA, U.S.A.).

All glassware was soaked overnight in a 5% solution of phosphate-free detergent, Lipsol (Lip. Ltd. Shipley, West Yorkshire, U.K.), thoroughly rinsed in tap water then finally rinsed with glass-distilled water.

Extraction procedure

Samples of 0.5 or 1 ml of plasma were diluted to 5 ml with glass-distilled water in 20-ml glass tubes. Unknown samples, four plasma standards and a quality control were included in each extraction. The internal standard solution (Ro 11-9506 in ethanol) was added to give a concentration of 250 ng per ml of sample. After mixing, the samples were alkalinized with 0.5 ml of 5 *M* sodium hydroxide, and 5 ml of ethyl acetate were added. The samples were shaken for 10—15 min on a horizontal shaker and centrifuged for 5 min at 1400 *g*. A maximum aliquot of the solvent phase was transferred to clean tubes containing 1 ml of 0.1 *M* hydrochloric acid. The plasma phase was re-extracted with a further 5 ml of ethyl acetate and a maximum aliquot of the solvent was added to the first extract. The combined solvent phases were extracted with the acid by shaking for 10—15 min followed by centrifugation as before. The acid phase was removed to clean 5-ml glass tubes, alkalinized with 0.3 ml of 5 *M* sodium hydroxide, and extracted by gentle rotation (10—15 min) with 2 ml of ethyl acetate. After centrifugation the solvent phase was transferred to 5-ml V-shape tubes. The solvent was evaporated under air at 37°C and the

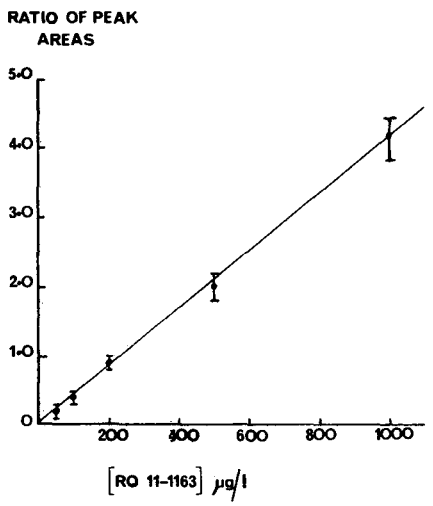

Fig. 2. Plasma standard curve for moclobamide (Ro 11-1163). Calculated linear regression line was $y = 0.0042x - 0.0053$, $r = 0.9994$. The error bars represent ± 1 S.D. and the number of determinations at each concentration was 5.

samples were stored at −4°C. Prior to analysis, samples were reconstituted in 10 μl of ethanol. Aliquots of 1—5 μl were injected into the gas chromatograph depending on the expected concentration.

Gas chromatography

The analysis was carried out on a Hewlett-Packard Model 5710A gas chromatograph. Gas chromatographic separation was carried out on a silanised glass column (2 m × 2 mm I.D.) packed with a 3% OV-101 on Gas-Chrom W HP (80–100 mesh).

Nitrogen was used as carrier gas at a flow-rate of 20 ml/min. The injection port was maintained at 260°C and the oven was held initially at 220°C for 2 min and then programmed to 240°C at 4°C/min. Moclobamide and the internal standard eluted after 5.2 and 4.5 min, respectively. Peak areas were integrated using a Hewlett-Packard 3380A integrator. The peak area ratio of moclobamide/internal standard was calculated for each sample and standard curves were constructed using linear regression analysis (Fig. 2).

Precision studies

Drug-free plasma (Blood Bank) was used to prepare standards to which known amounts of moclobamide were added. These were used each run to prepare standard curves and to evaluate the day-to-day precision of the assay.

Single-dose experiments

Five depressed patients were given 50 mg of moclobamide. Samples were taken over 8 h via an indwelling heparinized cannula, the plasma separated and stored frozen until analysed. The half-life of elimination for each patient was calculated by linear regression analysis of the terminal plasma concentrations.

Multiple-dose experiments

Blood samples were obtained from ten patients participating in a double-blind comparative trial of moclobamide versus amitriptyline [2]. Samples were obtained weekly (prior to the 0800 or 1200 dose) over a four-week period, the plasma separated and stored frozen until analysed.

RESULTS AND DISCUSSION

There has not been many published studies concerning the measurement of monoamine oxidase inhibitors in plasma since platelet MAO activity is easily measured and serves as an indication of whether the dosage is sufficient to elicit a clinical response. Platelet MAO is the B form of the enzyme and moclobamide is specific for the A form. To look at either pharmacokinetics or clinical efficacy, the actual drug concentrations in plasma must be determined for moclobamide.

Gas chromatography has been used previously to measure concentrations of MAO inhibitors in biological media [3, 4], and combined gas chromatography—mass spectrometry has also been used [5]. In the latter case, the plasma concentrations of phenelzine were extremely low (< 10 ng/ml), hence the better sensitivity achieved using the combined technique was required.

TABLE I

REPRODUCIBILITY OF THE ASSAY FOR MOCLOBAMIDE CONCENTRATIONS IN PLASMA

n = 10 at all concentrations.

Expected concentration (μg/l)	Found concentration (μg/l)	± S.D.	± C.V. (%)
50	54.8	8.6	15.7
100	103.1	9.8	9.5
200	207.6	13.9	6.7
500	508.8	34.5	6.8
1000	999.2	62.1	6.2

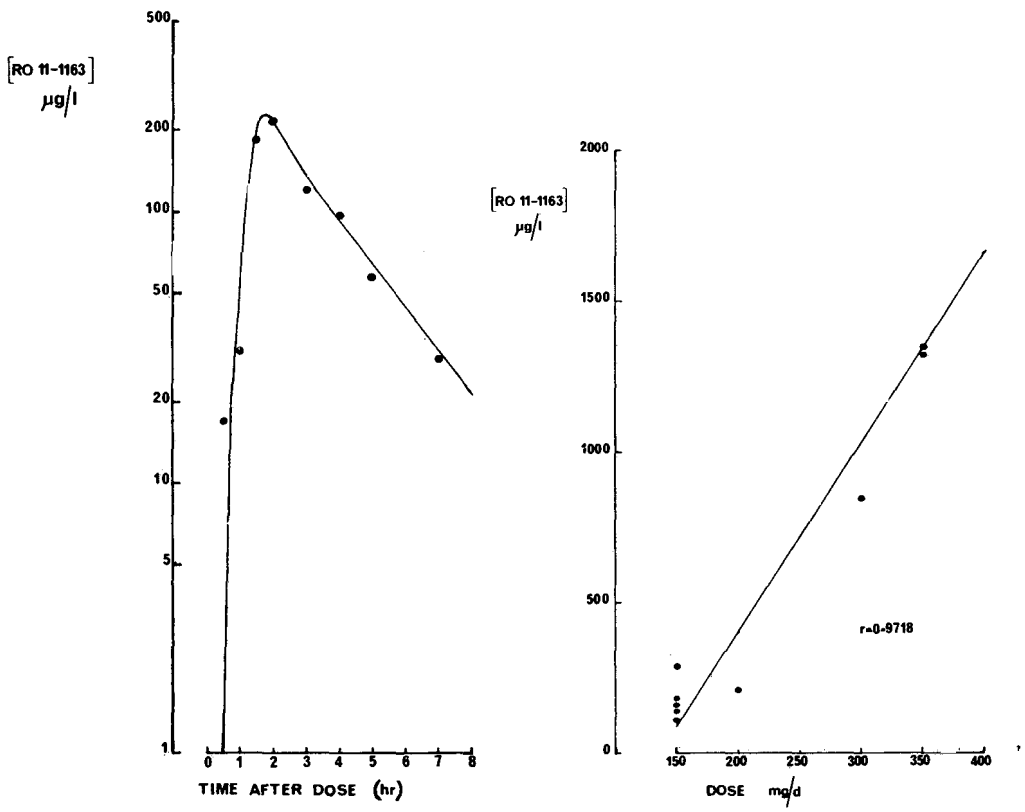

Fig. 3. Mean plasma concentrations of moclobamide (Ro 11-1163) following a single oral dose of 50 mg in five depressed patients.

Fig. 4. Steady-state plasma concentrations versus daily dose of moclobamide (Ro 11-1163) in ten depressed patients.

Adequate sensitivity for moclobamide was achieved using GC with nitrogen-selective detection.

Ethyl acetate was found to be a satisfactory solvent for extracting moclobamide but a three-step extraction procedure was found necessary to provide clean extracts for analysis. The average recovery for moclobamide through the procedure was 70% and the sensitivity was 10 μg/l. The second ethyl acetate extraction could be omitted when analysing steady-state concentrations as the sensitivity would still be adequate. The reproducibility over the concentration range 50—1000 μg/l is shown in Table I.

The assay is suitable for the measurement of plasma concentrations following either a single oral dose or after multiple doses. This is demonstrated in Figs. 3 and 4. Following a 50-mg single dose in five depressed patients, a mean peak concentration of 365 μg/l was reached at 1.7 h post-dose. The mean elimination half-life was 1.5 h. Pharmacokinetic data obtained for another MAO inhibitor, phenelzine, was similar to that for moclobamide [6]. A fuller treatment of the pharmacokinetics will be published separately.

Little published data is available on the metabolism of moclobamide. Urinary excretion studies show that only 0.4% of the dose is excreted as unchanged drug, that the metabolite pattern is complex and the major metabolites are as yet unidentified [7].

The steady-state concentrations achieved by patients treated over four weeks with varying dosages of moclobamide were measured. A linear relationship between the dosage administered and the steady-state plasma concentrations achieved was obtained (Fig. 4). High concentrations (1000 μg/l) were achieved with the highest doses, and considerable interindividual variability in the plasma concentrations was observed at each dose.

The method as described is simple to perform, has the required sensitivity for single-dose kinetic analysis and is reproducible over the range required for steady-state plasma concentration analysis.

ACKNOWLEDGEMENTS

The authors would like to thank Roche Products Pty. Ltd. (Sydney, Australia) for financial support, Ms. Jane Spiers for technical assistance and Dr. A. Fulton for collection of the single-dose blood samples.

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Journal of Chromatography, 278 (1983) 434—438
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1875

Note

Improved gas—liquid chromatographic method for measuring fenfluramine and norfenfluramine in heparinised plasma

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(First received May 9th, 1983; revised manuscript received July 18th, 1983)

Fenfluramine, a phenylethylamine derivative, is widely prescribed as an anorectic agent in a range of formulations, including the hydrochloride Ponderax. Previous gas—liquid chromatographic (GLC) methods for measuring fenfluramine and its active N-demethylated metabolite, norfenfluramine [1] have been reported in plasma and urine; however, these methods have either involved derivatisations and electron-capture detection [2], lacked sensitivity or proved difficult to reproduce in our laboratory [3].

Therapeutic monitoring of plasma levels is not only a check of patient compliance, but may also avert reported dose-related side effects [2, 4, 5]. The assay described is currently being applied in a long-term study involving the measurement of steady-state plasma levels of fenfluramine and norfenfluramine in post jaw-wiring obese patients. Blood samples are drawn in an outpatient clinic setting and it is hoped that data obtained will provide information on a therapeutic range for fenfluramine and its metabolite.

MATERIALS AND METHODS

Reagents

All reagents were analytical grade and aqueous solutions prepared using glass-distilled water. The extracting solvent diethyl ether (May and Baker, Melbourne, Australia) was redistilled in glass and *n*-butyl acetate (Mallinckrodt, Australia) was first washed three times with 20% (v/v) pure water (to remove residual acetic acid) and distilled twice in glass. The aqueous solutions, 0.5 mol/l sulphuric acid and 5 mol/l sodium hydroxide, were washed three times with 10% (v/v) diethyl ether before use. The internal standard was N,N-di-

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ethylaniline (BDH). Fenfluramine hydrochloride and norfenfluramine base were kindly donated by Servier (Melbourne, Australia).

Standards

A standard solution containing (DL)fenfluramine (10.8 μmol/l) and norfenfluramine (12.3 μmol/l) was prepared in 0.5 mol/l sulphuric acid. A standard curve was constructed using a concentration range of 0.1 to 2.0 μmol/l. The internal standard was diluted into 0.5 mol/l sulphuric acid to give a final concentration of 13.4 μmol/l.

Preparation of glassware

All procedures relating to the assay were carried out in glassware which had firstly been rinsed in a pyrogen-free detergent solution, rinsed with distilled water and dried. Secondly, the glassware was rinsed with methanol and, before drying, rinsed with ethyl acetate. This latter procedure was found essential to avoid interfering peaks in the final GLC trace.

Chromatography

The separation was performed on a Perkin-Elmer Sigma 3 gas chromatograph equipped with a nitrogen detector. The column (2 m × 2 mm I.D.) was packed with Chromosorb W-AW (80—100 mesh) coated with 10% potassium hydroxide and 10% Carbowax 20 M. The gas flow-rates for nitrogen, air and hydrogen were 60, 110 and 10 ml/min, respectively. The detector and the injection port temperatures were adjusted to 200°C and the column oven temperature to 130°C. The column was conditioned for 3 h at 150°C prior to use.

Assay technique

Aliquots (2 ml) of heparinised plasma were added to 0.4 ml of 0.5 mol/l sulphuric acid in glass-stoppered extraction tubes. In the case of the standard solutions the plasma was drawn from patients who were not receiving fenfluramine. This plasma had been previously shown to contain no compounds which interfered with the peaks of interest on the final GLC trace. The acid in these standard solutions contained known concentrations of fenfluramine and norfenfluramine ranging from 0.1 to 2.0 μmol/l. Aliquots (100 μl) of the internal standard solution were added to each tube and then basified with 1.0 ml of 5 mol/l sodium hydroxide. After briefly vortexing each tube, 2.0 ml of diethyl ether were added, the tubes capped, shaken for 10 min and centrifuged at 1000 *g* for 10 min. The phases were separated by pipetting the organic layer into a second glass-stoppered centrifuge tube. Back extraction was carried out by adding 1.0 ml of 0.5 mol/l sulphuric acid to this organic phase and shaking for 10 min. Tubes were again centrifuged for 10 min at 1000 *g*. The organic phase was aspirated to waste and the aqueous phase basified with 0.5 ml of 5 mol/l sodium hydroxide before adding 0.2 ml of *n*-butyl acetate. The tubes were vortexed for 30 sec and centrifuged for 3 min at 1000 *g*. The aqueous layer was carefully pipetted to waste and 5-μl aliquots of the organic layer subjected to GLC.

The performance of the assay was monitored by assaying a plasma stock containing 0.4 μmol/l fenfluramine and 0.5 μmol/l norfenfluramine. Hence,

coefficients of variation were estimated by comparing eight determinations made in a single assay run, and five determinations made on a weekly run basis.

The limits of detection were determined by preparing further dilutions into plasma of the stock solution of fenfluramine and norfenfluramine, and assaying these solutions as described above. A peak height-to-noise ratio of 2:1 was accepted as the minimum detectable level.

The recoveries of fenfluramine and norfenfluramine through the assay were determined by comparing the peak heights (mean of five injections) of a stock solution of the two compounds (0.4 and 0.5 μmol/l, respectively) prepared in *n*-butyl acetate, with the corresponding peak heights of six samples, containing the same concentrations of the two compounds, prepared in plasma and carried through the assay procedure described above.

RESULTS

The retention times of fenfluramine, norfenfluramine and internal standard (N,N-diethylaniline), were 4.1, 4.8 and 6.0 min, respectively (Fig. 1). The peak height of norfenfluramine was consistently about 60% of that of the parent

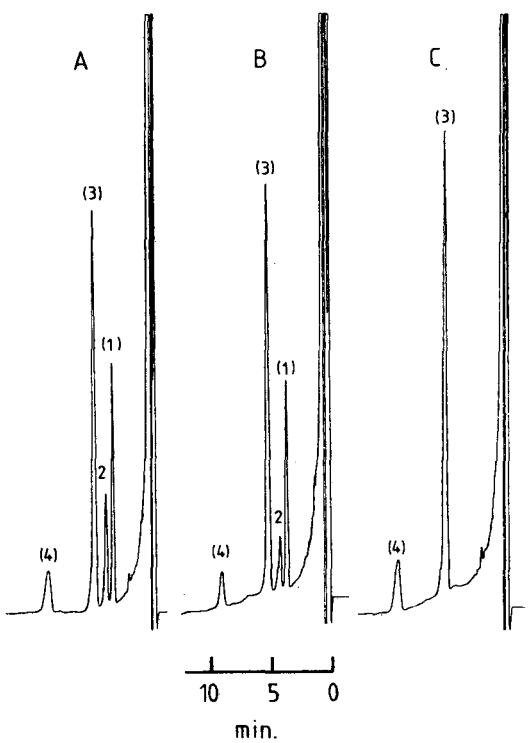

Fig. 1. Sample GLC traces showing fenfluramine (1); norfenfluramine (2); N,N-diethylaniline, internal standard (3); and an unidentified plasma peak (4), with retention times of 4.1, 4.8, 6.0 and 9.3 min, respectively. (A) Blank plasma to which fenfluramine (0.4 μmol/l) and norfenfluramine (0.5 μmol/l) had been added; (B) typical sample from a patient receiving fenfluramine; and (C) sample from an obese patient not receiving fenfluramine. The internal standard (3) is shown in each case.

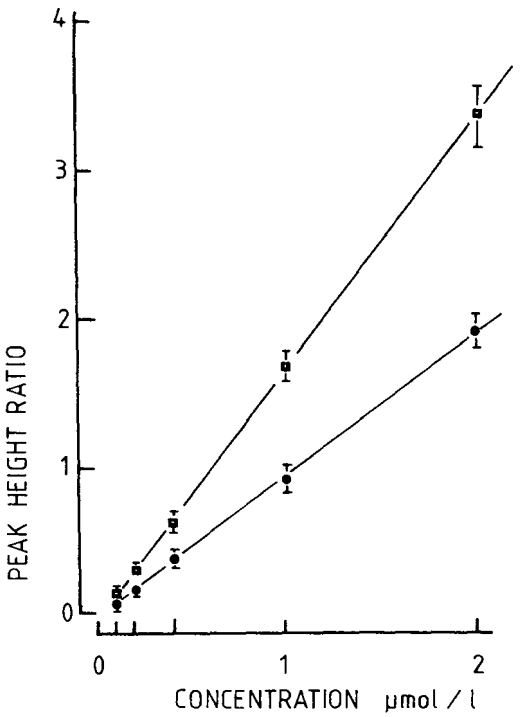

Fig. 2. Standard curves for fenfluramine (▪) and norfenfluramine (•) showing the peak height ratios [i.e., sample peak (mm) : internal standard (mm)] over a concentration range of 0.1 to 2.0 µmol/l. Values are means (± S.E.M.) of determinations made in four different assay runs. The correlation coefficients (r^2) for fenfluramine and norfenfluramine were 1.0000 and 0.9993, respectively.

compound, fenfluramine. The ratios of the peak heights of fenfluramine and of norfenfluramine to the internal standard were linear over the concentration range of 0.005 µmol/l (the detection limit of the assay) to at least 2.0 µmol/l. However, as shown in Fig. 2, the concentration range routinely employed for estimating patient sample levels was 0.1 to 2.0 µmol/l. The method was standardised by assaying in each assay run a plasma sample which had been previously spiked with fenfluramine (0.4 µmol/l) and norfenfluramine (0.5 µmol/l), divided into 2.5-ml aliquots and stored at −20°C. These estimations indicated coefficients of variation of 2.0% and 5.9% between assays (n = 5), and 2.0% and 4.5% within a single assay (n = 8) for fenfluramine and norfenfluramine, respectively. Further, the recoveries through the assay at the same concentrations were 65% and 82% for fenfluramine and norfenfluramine, respectively.

Amphetamine, the structurally related compound from which fenfluramine was derived, and methylamphetamine, when assayed by this method were found to interfere with norfenfluramine and the internal standard in the final GLC trace. The retention times for amphetamine and methylamphetamine were 5.0 and 5.5 min, respectively.

DISCUSSION

The method presented here for the assay of fenfluramine and norfenfluramine in plasma represents an improvement over previously published methods as it employs nitrogen detection with GLC. The methodology is less complex than some of the previous methods as no derivatisation is required. Hence, the assay run time, the resolution of peaks and the assay sensitivity have been improved over other methods.

The performance of this sensitive assay has proved to be dependent on the purity of the reagents used and the cleanliness of the glassware employed as described above. The time spent initially in decontaminating reagents and glassware has proved valuable in terms of the continuing satisfactory performance of the assay.

The method is currently being applied to plasma samples from obese patients in an outpatient clinic setting. The aim of this on-going study is to attempt to establish a dose–response relationship and a possible therapeutic range for fenfluramine and norfenfluramine. Hence, although the method is equally applicable to measurement of these two compounds in urine, such levels are of little value in terms of the above aim since it has been shown [3, 6] that, like many drugs, urinary excretion of fenfluramine and norfenfluramine is very dependent on the volume and pH of the urine. Such levels could, however, be utilized to monitor pharmacodynamics or compliance.

Of the 50 patient samples assayed so far, none have shown any interfering compounds on the final GLC trace, as evidenced by the presence of other peaks or shoulders on expected peaks.

The inclusion of amphetamine was based on its structural and pharmacological (in terms of its anorectic properties) relationship to fenfluramine. Methyl amphetamine [2] and amphetamine [6] have also been used as internal standards in previous methods. Hence, in the unlikely event that their fenfluramine assay were applied to a sample containing amphetamine and/or methylamphetamine, a false result would be obtained. The present method would at least recognise, although not resolve, the presence of the amphetamine compounds. Further, with the appropriate choice of standards, the assay could be equally applied to the measurement of amphetamine and methylamphetamine in patient samples.

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Journal of Chromatography, 278 (1983) 439—444
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1848

Note

Rapid reversed-phase high-performance liquid chromatographic assay of diflunisal in biological fluids

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Diflunisal (2′,4′-difluoro-4-hydroxy-3-biphenylcarboxylic acid) (Fig. 1) is a prostaglandin synthetase inhibitor [1] and a potent analgesic and anti-inflammatory drug whose chief use is in the treatment of rheumatoid arthritis and osteoarthritis [2—4]. The bioavailability is almost 100% after oral administration; after doses of 250—500 mg once or twice a day it is reported to be eliminated from the blood circulation with a half-life of 10–12 h [5], which is longer than that of other salicylates. However, recent reports claim that diflunisal, like salicylic acid itself, has a limited capacity in the elimination processes [6, 7]. Suspected intoxication may therefore arouse greater concern even though the therapeutic index is several times that of aspirin [8].

Different methods for assaying diflunisal in biological fluids have been published. For some years the methods of Tocco et al. [7] were used, but the gas chromatographic, radiometric and fluorimetric techniques described are time-consuming and laborious, or lack specificity. High-performance liquid chromatographic methods with UV detection have since been reported [9—11] and they are all quicker and simpler. However, the methods of Van Loenhout et al. [9] and Balali-Mood et al. [11] have the disadvantage of using other analgesics (naproxen and flufenamic acid, respectively) as internal standards, which may unintentionally confound their therapeutic use Wåhlin-Boll et al. [10] used 6-methoxy-2-naphthylacetic acid as internal standard, which is not a drug. Furthermore, this method [10] employed a water-immiscible organic sample clean-up unlike the procedure of Balali-Mood et al. [11], in which a potential of column pressure build-up was accepted due to injection of a sample supernatant obtained from an incomplete acetone precipitation of plasma proteins.

0378-4347/83/$03.00

This report deals with a new high-performance liquid chromatographic assay of diflunisal in plasma and urine and a pilot study carried out to judge its practical application in conjunction with the development of the method.

MATERIALS AND METHODS

Materials and reagents

Diflunisal and 1-hydroxy-2-naphthoic acid (internal standard) (Fig. 1) were supplied from the Synthesis Laboratories of Dumex. All other chemicals were of analytical reagent grade. Standard solutions of diflunisal and internal standard were prepared separately; concentrations were 10 mg and 1.5 mg per 100 ml methanol, respectively. As an auxiliary to the extraction agent (see below) 10 g of concentrated sulphuric acid were added to an aqueous solution of 14.2 g disodium sulphate per l. The water was glass distilled. The mobile phase consisted of volume fractions of 50% of methanol, 33.3% of 0.1 *M* phosphoric acid, and 16.7% of tetrahydrofuran. To a mixture of 1 l, about 9 ml of 1 *M* aqueous sodium hydroxide were added until a pH value of 3.0 was reached. The mobile phase was filtered and placed in an ultrasonic bath before use.

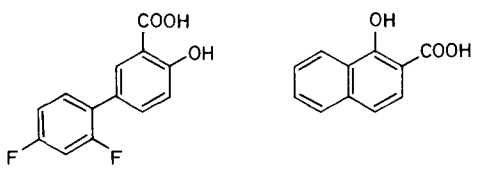

Fig. 1. The chemical structure of diflunisal (left) and plasma internal standard 1-hydroxy-2-naphthoic acid (right).

Instruments and equipment

Chromatographic separations were performed on a Waters Model 6000A constant-flow solvent delivery system, a Waters U6K injection unit and a Waters Model 440 UV detector equipped with a 254-nm filter. Detector response was monitored with a Hewlett-Packard Model 3380 integrator. A water-bath was used to maintain a column temperature of 35°C. A stainless-steel column (Knauer, Taunus, F.R.G.), 25 cm × 4 mm I.D., was filled with 5-μm Spherisorb ODS (Phase Separations, Queensferry, U.K.) using a slurry technique.

Analytical procedure

A 1-ml volume of the auxiliary extraction solution was added to samples of 100 μl (or another suitable volume) of serum, plasma or urine. Except for urine analysis, 20 μl of the internal standard solution were then added and the mixture was shaken vigorously. After adding 3 ml of diethyl ether and gently rotating for 3 min the tubes were centrifuged at 10°C. Then 2-ml portions of the organic phase were transferred to conical tubes and evaporated under nitrogen jet streams at ambient temperature. The residues were dissolved in 200 μl of the mobile phase and 50-μl aliquots were injected onto

the column. The mobile phase was delivered at a rate of 1 ml/min and the column was maintained thermostatically at 35°C.

Calibration of the method was performed by means of the peak areas (or peak area ratios) stemming from diflunisal (and the internal standard) in standards. The peak area ratios were only used in the analysis of serum and plasma diflunisal. The standards were prepared as drug-free samples to which were added known amounts of the compound(s).

Quantification of diflunisal was possible from the peak areas (or peak area ratios) of the samples by reference to a linear standard curve determined by least-squares linear regression.

RESULTS AND DISCUSSION

Method development

UV detection was selected because of previous reports of its usefulness [9—11].

The chromatographic phases were the next factors to be examined. Van Loenhout et al. [9] described ion pair elution involving methanol and tetramethylammonium as the ion pairing agent, the latter alkalinized with Tris [tris(hydroxymethyl)aminomethane]. However, this system had a tendency to peak tailing and a remarkably low detector response. In keeping with this the selective principle of ion pair elution was tried with other systems, such as tetrabutylammonium hydrogen sulphate buffered to pH 9 by means of aqueous borate and modified with methanol. However, the chromatographic performance was still not considered satisfactory, because of severely tailing peaks.

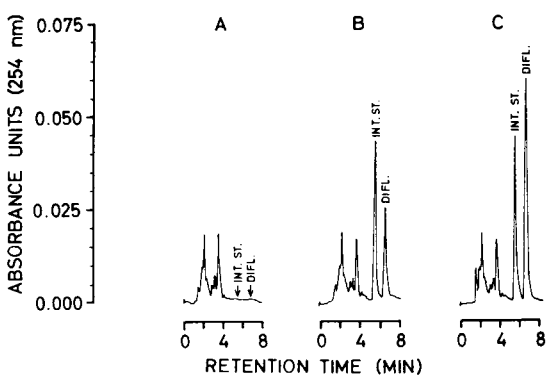

Fig. 2. Representative chromatograms of (A) blank serum extract; (B) serum standard extract of 10 μg/ml diflunisal and 0.3 μg/ml internal standard (the peaks correspond to approximately 0.1 and 1.4 μg, respectively); and (C) volunteer serum extract containing about 20 μg/ml diflunisal (the peak of diflunisal corresponds to approximately 2.8 μg).

Chromatography was then performed at an acid pH on a C_{18} column, which improved the peak shape. The final choice of mobile phase was a mixture of tetrahydrofuran, an aqueous phosphate buffer solution, and methanol, which at room temperature was found to be useful for the analysis of serum and plasma diflunisal (Fig. 2) but not for urine. Analysis of diflunisal in urine

was accomplished by increasing the column temperature from ambient to 35°C. This brought about a more efficient retention of the urinary diflunisal peak compared to the unresolved front at the beginning of the chromatogram (Fig. 3). The elevated temperature was maintained during the serum and plasma analyses, because of improved chromatography (Fig. 2). The number of theoretical plates was about 7000 for diflunisal and about 9000 for the plasma internal standard. The capacity factors were 3.3 and 2.7, respectively, and the separation factor appeared to be 1.2.

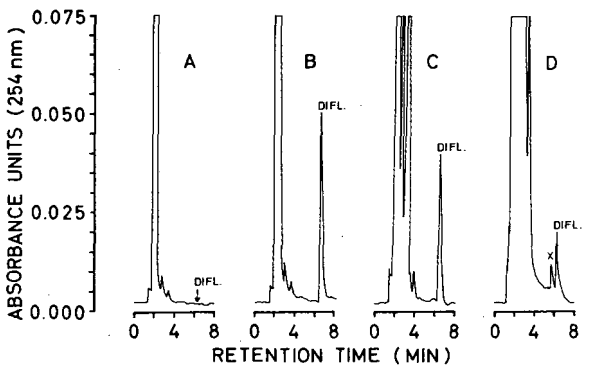

Fig. 3. Representative chromatograms of (A) blank urine extract; (B) urine standard extract of 10 μg/ml diflunisal (the peak corresponds to approximately 2.4 μg); (C) volunteer urine extract of approximately 8 μg/ml diflunisal (the peak corresponds to approximately 2 μg); and (D) volunteer urine extract showing unidentified peak (X), which appeared 48 h after administration (see text). The peak of diflunisal corresponds to approximately 0.1 μg. Extraction volume of urine was in case D ten times the normal, i.e. 1000 μl.

The extraction procedure and the choice of a possible internal standard were the final factors to be explored. Ion suppression by acidification and shifting of the partition equilibrium in favour of the analyte was found to be profitable for the extraction procedure. The final extraction technique was accomplished by adding an aqueous solution of sulphuric acid and sodium sulphate to the sample before extraction with diethyl ether.

The selection of a proper internal standard can be a matter of great import to the assay precision, especially when analysing routine samples. The choice for the serum and plasma assay was 1-hydroxy-2-naphthoic acid (Fig. 1). The impact of the same functional groups as found in diflunisal proved beneficial to the extraction and to the reversed-phase chromatography (Fig. 2). With regard to the urine assay the compound mentioned was left out because an interfering peak turned up in the chromatograms of urine samples from the two days following dosing (see application study below).

The extraction yield of diflunisal and the internal plasma standard were estimated as the ratios of the slopes of the calibration curves applying to processed and unprocessed (i.e. absolute standards) compounds. Calculation of the plasma yields provided a mean of 72% for diflunisal and 90% for the internal standard. For the urine assay the extraction yield of diflunisal was found to be essentially complete. In all cases the S.E.M. was approximately 1% with $n = 10$. The linearity of the standard curves (range 0—20 μg/ml)

was satisfactorily verified for both plasma and urine by the determination coefficient, which appeared to be $r^2 = 0.99$ with a coefficient of variation of less than 1% ($n = 10$) in both cases. The detection limit (signal-to-noise ratio of 2) was found to be approximately 1 ng of diflunisal per 1 ml of sample.

Freezing to −20°C did not cause deterioration of diflunisal in serum, plasma or urine. The assay demonstrated no interference with the following other analgesics: salicylic acid, phenacetin and paracetamol. When applied to unprocessed compounds, the capacity factors were 1.2, 0.9 and 0.6, respectively.

Quality control samples were included in each analytical series in order to estimate the precision of the method. These pools were prepared by mixing drug-free sample material and diflunisal to an appropriate concentration and dividing into 100-µl aliquots. The pools were stored at −20°C until analysis. From ten consecutive runs the inter-assay analytical variation appeared to be approximately 7% (relative standard deviation) at a concentration level of 10 µg/ml serum.

A pilot application study

In order to demonstrate the usefulness of the method, analyses have been performed on serum and urine samples obtained from a healthy volunteer, who had ingested a 250 mg tablet of Diflonid® (Dumex).

The serum concentration—time relationship found (Fig. 4) indicates an anomaly within the 7—24 h time interval. However, the overall elimination rate from the circulation was within the normal range: in the time interval 5—30 h the elimination half-life was about 10 h. Consistent with the anomaly indicated, the curve of the urinary excretion rate (Fig. 4) decreases about 10 h after medication, i.e. within the same period.

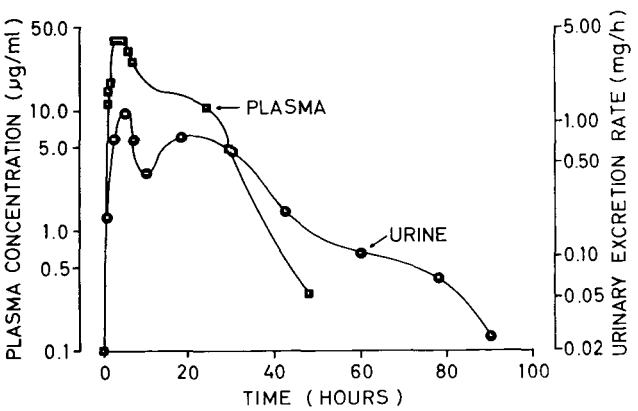

Fig. 4. Plasma concentration profile (■) and mean urinary excretion rate (●) of diflunisal after a single oral administration of 250 mg of Diflonid to a healthy volunteer. The mean urinary excretion rate for each collection period was related to the midpoints of the time intervals.

An unidentified compound with a retention time of 5.9 min (i.e. capacity factor of 2.9) occurred in chromatograms of urine samples collected after 48 h (Fig. 3). The finding of an extra peak seems to agree well with the observation of Balali-Mood et al. [11]. The size of the peak appeared intact relative to diflunisal after enzymatic hydrolysis with β-glucuronidase and sulphatase. Furthermore, treatment with 70% aqueous perchloric acid [7] did not give any clarification, instead it generated new interfering peaks in the chromatograms. The peak could be a metabolite of diflunisal, the data suggesting either the ether glucuronide or that of an unknown, similar to a previous report [11].

CONCLUSION

A rapid and sensitive method for the determination of diflunisal in serum, plasma and urine is presented. Reversed-phase liquid chromatography was applied to diflunisal after a single extraction step. The assay is simple and quick, well suited for routine purposes such as processing large numbers of samples.

ACKNOWLEDGEMENTS

The author thanks T.K. Nielsen for valuable technical assistance and M. Maibøll for preparation of the manuscript.

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Journal of Chromatography, 278 (1983) 445—451
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1851

Note

Quantification of quinine in human serum by high-performance liquid chromatography

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(First received April 11th, 1983; revised manuscript received July 12th, 1983)

The emergence of strains of *Plasmodium falciparum* resistant to chloroquine and other antimalarial drugs has resulted in a renewal of interest in the use of quinine to treat severe cases of falciparum malaria. Because of the side effects of quinine physicians should consider serum quinine concentrations when using this drug. Therefore, a rapid and accurate method for assaying quinine in biological fluids is desirable.

The most common methods for the determination of quinine levels in biological fluids are based on fluorescence procedures. Brodie and Udenfriend [1] described a protein precipitation method using metaphosphoric acid followed by testing of the supernatant with fluorescence detection. Cramer and Isaksson [2] reported a benzene extraction procedure of alkalinized plasma with fluorescent measurement of a sulphuric acid layer. Recently, gas chromatographic (GC) methods have been developed for the quantification of quinine. Bonini et al. [3] reported a sensitive and selective GC procedure for the determination of quinine added to drug-free urine and blood. Furner et al. [4] described a gas chromatographic—mass spectrometric (GC—MS) method for the quantification of quinine in human urine with a detection limit of 5 ng of quinine.

The aim of the present work was to develop a rapid, sensitive and selec-

0378-4347/83/$03.00

tive method for the determination of quinine in human serum using high-performance liquid chromatography (HPLC). The method was subsequently used to quantify quinine in serum collected from children. Reversed-phase ion-pair chromatography was used with pentanesulphonic acid as the counter-ion. A comparison was made between the HPLC method and the widely used fluorescence procedure of Cramer and Isaksson [2]. A comparison of capillary and venous blood levels of quinine in these children was also made.

MATERIALS AND METHODS

Chemicals and standards

HPLC grade acetonitrile, methanol and 1-pentanesulphonic acid were obtained from Waters Assoc. (Sydney, Australia). All aqueous solutions were prepared using deionized glass-distilled water. A stock solution of quinine dihydrochloride was prepared containing 100 μg of quinine base per ml of water and stored at 4°C in an amber glass bottle. Working solutions were prepared by appropriate dilution of the stock solution with water or drug-free serum.

Instrumentation and chromatographic conditions

The liquid chromatograph consisted of a Pye Unicam LC-XPD pump, a WISP Model 710B autosampler (Waters Assoc.), and a Whatman Partisil 10 ODS 2, particle size 10 μm (250 × 4.6 mm I.D.) reversed-phase column, protected by a LiChrosorb RP-18 guard column, particle size 10 μm (30 × 4.6 mm I.D.) (Brownlee Labs., Santa Clara, CA, U.S.A.). The detector was a Schoeffel Model FS 970 fluorometer operated at an excitation wavelength of 350 nm with a 418-nm emission cut-off filter. The fluorometer sensitivity was set at 5.6, the range at 0.2 or 0.5 μA full scale with a time constant of 6.0 sec. Peak areas were measured using a Pye Unicam DP 88 integrator. The mobile phase consisted of methanol—acetonitrile—water (30:30:40, v/v) with 0.005 *M* 1-pentanesulphonic acid (pH 3.75) pumped at a flow-rate of 1.5 ml/min (back pressure approximately 140 bar) at ambient temperature.

A Farrand Model 801 spectrofluorometer equipped with xenon lamp and 10-nm slit widths was used for the method of Cramer and Isaksson [2]. The fluorescence intensity was monitored at the excitation wavelength of 350 nm with emission wavelength set at 450 nm.

Analytical procedure for HPLC

To precipitate serum proteins a 100-μl sample (serum standards, biological sample) was mixed with 100 μl of acetonitrile in a 1.5-ml polypropylene centrifuge tube. After mixing for 15 sec, the proteins were separated by centrifugation at 12,000 *g* for 5 min. An aliquot (10 μl) of this supernatant was injected. Serum samples were tested in duplicate. Standard curves for quinine concentrations expressed as the free base were determined using peak areas.

Recovery and reproducibility

Quinine recovery was determined at concentrations of 1.25, 2.5, 5.0, 7.5

and 10 μg quinine per ml of serum by comparing peak areas with areas obtained by direct injection of pure standards. Within-day reproducibility was evaluated by analysis of serum standards (n = 5) containing 5.0 and 10.0 μg quinine per ml. Day-to-day reproducibility was determined by assaying serum standards containing 1.25, 2.5, 5.0, 7.5 and 10 μg quinine per ml on seven occasions.

Stability and storage of quinine

To assess the stability of frozen samples, 0.5-ml aliquots of serum standards were stored at −15°C in polypropylene centrifuge tubes for seven months and subsequently assayed by the HPLC method.

Patients

The subjects in this study consisted of 29 hospitalized children (15 males and 14 females) with an age range of one month to ten years. Blood was collected by venipuncture, centrifuged and the serum was stored at −15°C in polypropylene or glass tubes until analysed. Finger-prick capillary blood was collected in microtainers (Becton and Dickinson) with gentle squeezing of the finger to maintain blood flow.

Comparison of HPLC and fluorescence methods

The HPLC method developed in this study and the method of Cramer and Isaksson [2] were compared by quantifying the quinine in sera from seventeen children after administration of 10 mg/kg quinine intramuscularly. The HPLC method was also applied for comparison of venous and capillary serum quinine levels from twelve children after administration of 3 mg/kg intramuscular quinine.

RESULTS AND DISCUSSION

HPLC analysis of quinine

The minimum volume of serum required for the HPLC method was 10 μl. The speed of the method was such that three standards and an unknown sample could be assayed within 30 min. The limit of detection was 0.3 μg quinine per ml of serum (3 ng per 10 μl injected). This sensitivity should be able to be increased by injecting larger sample volumes.

Endogenous substances in serum were found not to interfere with the assay. A typical chromatogram of a patient's serum sample is shown in Fig. 1a. Chromatograms of the patient's drug-free serum and spiked serum containing 5.0 μg quinine per ml are shown in Fig. 1b and Fig. 1c, respectively. The retention time for quinine was 4.2 min with an unidentified compound appearing at 2.8 min. Pentane sulphonic acid was an essential component of the mobile phase for the separation and elution of quinine from the column.

Calibration curves (range: 1.25—10.0 μg quinine per ml of serum) prepared on seven different days were linear with correlation coefficients of not less than 0.996. The range studied encompassed the therapeutic range of quinine of 3.0—7.0 μg/ml [5]. Analysis of quinine standards in serum and water yielded identical standard curves. The recovery of quinine was excellent with

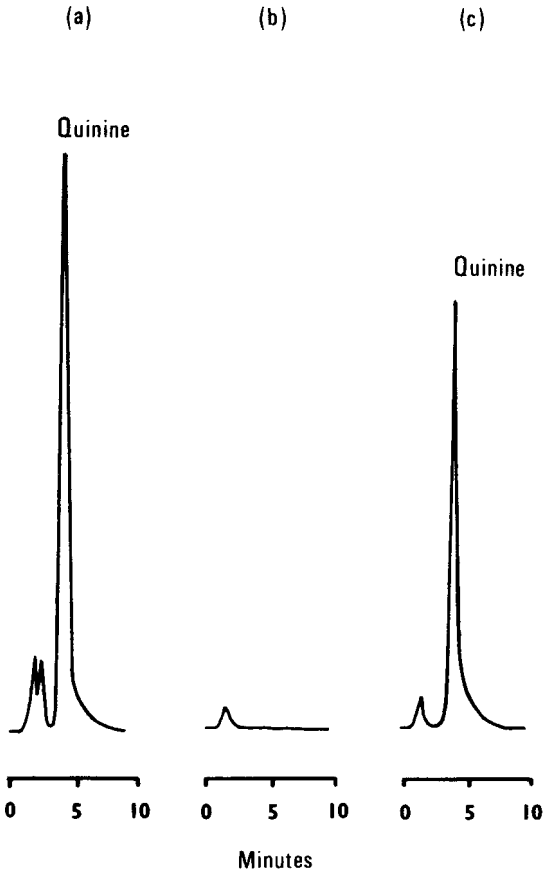

Fig. 1. Chromatograms of a patient's serum sample 6 h after receiving quinine (a), a serum sample from the same patient before receiving quinine (b), and human drug-free serum spiked with 5 μg/ml quinine (c).

TABLE I

REPRODUCIBILITY OF QUININE ESTIMATION IN SERUM

	Concentration (μg/ml)	No. of samples	Coefficient of variation (%)
Within-day	5.0	5	5.4
	10.0	5	3.0
Day-to-day	1.25	7	9.4
	2.5	7	7.7
	5.0	7	7.1
	7.5	7	7.0
	10.0	7	7.1

a mean recovery of spiked serum of 99± 2.5% over the range studied. Within-day and day-to-day coefficients of variation averaged 4.2% and 7.7%, respectively (Table I).

The principal advantages of our HPLC method over GC procedures [3, 4] are ease of sample preparation, avoidance of solvent extraction and speed of analysis. Furthermore, the GC–MS equipment used by Furner et al. [4] is expensive to buy and maintain, and requires a highly skilled operator.

None of the following drugs interfered with the determination of quinine in serum: amodiaquine, chloroquine, chloramphenicol, Fansidar®, Maloprim® and mefloquine. However, quinidine, the diastereoisomer of quinine, has a retention time similar to that of quinine and interfered with quinine assays in the present study.

Stability of serum quinine samples

There was no significant difference in quinine concentration of spiked serum samples tested before and after storage at −15°C for seven months.

TABLE II

COMPARISON OF SERUM QUININE LEVELS MEASURED BY HPLC AND FLUORESCENCE METHODS

Quinine levels are given in μg/ml.

Patient No.	Quinine at 2 h*		Quinine at 4 h		Quinine at 2 h	Quinine at 4 h
	Fluor.**	HPLC	Fluor.	HPLC	Fluor./HPLC	Fluor./HPLC
1	9.6	7.6	7.4	6.4	1.26	1.16
2	6.6	6.3	5.2	4.2	1.05	1.24
3	7.2	5.1	5.3	3.4	1.41	1.56
4	7.7	5.2	5.6	3.9	1.48	1.44
5	13.5	7.3	8.8	6.4	1.85	1.38
6	4.6	4.0	3.7	2.6	1.15	1.42
7	10.1	7.1	7.4	5.6	1.42	1.32
8	6.3	4.6	6.5	3.4	1.37	1.91
9	9.3	6.5	10.2	5.9	1.43	1.73
10	9.5	5.9	8.5	5.1	1.61	1.67
11	10.9	8.3	9.8	8.8	1.31	1.11
12	11.2	11.1	10.4	10.5	1.01	0.99
13	14.1	13.4	13.0	11.9	1.05	1.09
14	7.9	7.7	8.9	6.1	1.03	1.46
15	12.5	6.2	10.4	4.8	2.02	2.17
16	6.6	5.6	4.3	4.0	1.18	1.08
17	8.1	8.5	7.7	7.6	0.95	1.10
Mean ± S.D.	9.2± 2.7	7.1± 2.4	7.8± 2.5	5.9± 2.6	1.33± 0.30	1.40± 0.33
Paired *t*-test					$t = 4.38$	$t = 5.13$

*h represents hours after receiving quinine.
**Fluor. = fluorescence method of Cramer and Isaksson [2].

Comparison of quinine concentration measured by HPLC and fluorescence method

The fluorescence method yielded on average 37% higher quinine values than those obtained by HPLC (Table II). The serum levels of quinine measured by the two methods were statistically different ($P < 0.001$). This difference may be due to interference by metabolites of quinine in serum in the fluorescence method. In previous analyses of quinidine [6—10] determined by fluorescence methods spurious estimates of quinidine were obtained and were attributed to interference from metabolites and other components of plasma.

The difference between HPLC and the fluorescence method for the measurement of quinine suggests that HPLC is more selective. Consequently, the HPLC method would be a more appropriate method for monitoring quinine levels in man, particularly as quinine has a narrow therapeutic index.

Capillary and venous serum quinine levels

Because of the capability of the HPLC method to analyse small serum volumes, we investigated the collection of capillary serum for monitoring quinine levels. Capillary and venous serum quinine concentrations for twelve patients are presented in Table III. Capillary serum quinine concentrations average 63% of venous serum quinine concentrations. The lowered levels of serum quinine measured in capillary blood compared with that measured in venous blood may be attributed to dilution of blood with tissue fluids. Also, squeezing the finger may have caused a reduction in plasma protein in capillary blood compared to the plasma protein level in venous blood.

TABLE III

COMPARISON OF CAPILLARY AND VENOUS SERUM QUININE LEVELS

Patient No.	Serum quinine concentration (μg/ml)		Capillary/venous
	Capillary (2 h*)	Venous (2 h)	
1	1.3	2.0	0.65
2	0.8	0.8	1.00
3	1.2	1.7	0.71
4	1.3	3.6	0.36
5	1.0	3.0	0.33
6	2.6	4.0	0.65
7	0.9	2.7	0.33
8	1.2	1.7	0.71
9	2.4	4.1	0.59
10	0.8	1.0	0.80
11	0.7	0.8	0.88
12	2.2	3.7	0.59
Mean ± S.D.	1.4±0.7	2.4±1.3	0.63±0.21
Paired *t*-test			$t = 4.52$

*h = hours after receiving quinine.

As approximately 70% of total quinine is bound to plasma protein [11], any reduction of plasma protein concentration in blood could result in lower quinine levels. The use of capillary blood obtained by gently squeezing the finger, therefore, was not suitable for measuring quinine concentrations for clinical assessment.

CONCLUSION

In summary, a simple, rapid and accurate HPLC procedure for the estimation of quinine in serum has been developed, suitable for the routine monitoring of quinine levels in patients. A commonly used fluorescence method gave significantly higher concentrations of quinine than the HPLC procedure. This difference may be related to greater selectivity of the HPLC.

ACKNOWLEDGEMENTS

This paper is published with the approval of the Director-General of Army Health Services, Australian Army. The authors wish to thank CPL Brian Mundy for his technical assistance and Dr Sam Walters for his clinical assistance. The authors are grateful to Mr Brian Love, Department of Pharmacy, University of Sydney, NSW, for constructive criticism of the manuscript.

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Journal of Chromatography, 278 (1983) 452—457
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1855

Note

Rapid high-performance liquid chromatographic method for the measurement of hymecromon and its conjugates in blood plasma or serum at concentrations attained during therapy

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(First received April 22nd, 1983; revised manuscript received July 14th, 1983)

Hymecromon (Fig. 1, I = 7-hydroxy-4-methyl-2-H-benzopyran-2-one) is a choleretic and biliary antispasmodic drug which is metabolized in the human body mainly via glucuronidation of the 7-hydroxy function. At present only a few methods are available for the simultaneous qualitative or quantitative analysis of xenobiotics together with their glucuronide and sulphate conjugates. The wide range of polarities between the parent compounds and the highly polar conjugates makes the use of a single chromatographic separation procedure difficult.

Methods currently used to separate conjugates are reversed-phase chromatography [1], liquid—liquid counter-current partition [2], gas chromatography [3], ion-exchange chromatography [4] and ion-pair reversed-phase chromatography which has proved successful in separating steroid conjugates [5], the glucuronide and sulphate conjugates of harmol, phenolphthalein, 4-nitrophenol [6], and most recently of morphine and its 3- and 6-glucuronides [7].

Pharmacokinetics of hymecromon have received only scant attention. Assay methods currently employed include gel filtration, solvent extraction after enzymatic cleavage of the conjugates, and fluorimetry [8]. These techniques are slow and not sufficiently selective and precise.

The principle aim of this study was to investigate whether a simple isocratic reversed-phase system could be found to separate hymecromon and its major metabolites (Fig. 1). Since the compounds under investigation are ionizable in the pH range 2—8 the possibility of influencing the retention by ion-pair

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formation was studied. The developed technique is sensitive, selective, and simple to perform; an average of twenty plasma samples can be analyzed routinely during an 8-h working day.

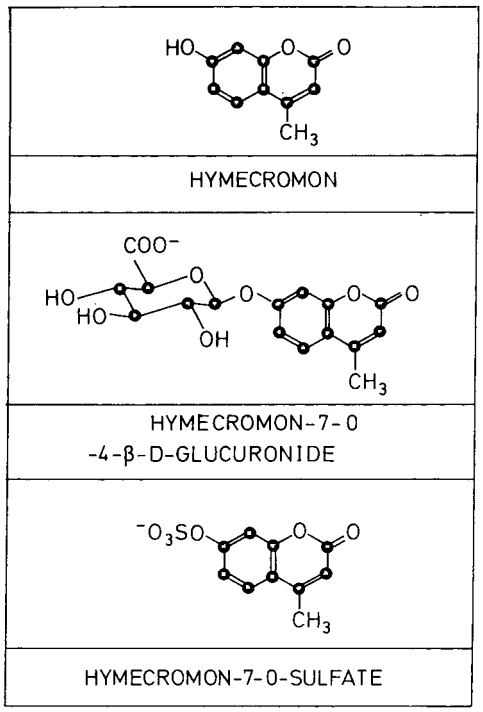

Fig. 1. Structural formulae of hymecromon and its major metabolites.

EXPERIMENTAL

Chemicals

Hymecromon was obtained from Lipha Arzneimittel (Essen, F.R.G.). Methanol (HPLC grade reagent No. 8402) was purchased from Baker (Gross-Gerau, F.R.G.). Water used for high-performance liquid chromatographic (HPLC) separations was a pyrogen-free product of Fresenius (Bad Homburg, F.R.G.). Tetrabutylammonium bromide was obtained from EGA (Steinheim, F.R.G.), 4-methylumbelliferyl-β-D-glucuronide, 4-methylumbelliferyl-sulphate from Sigma (Taufkirchen, F.R.G.) and Perchlorsäure/Perchlorat No. 9431 from Merck (Darmstadt, F.R.G.).

Sample preparation

A 100-μl volume of serum (or plasma) was deproteinized by mixing with the same volume of the perchloric acid—perchlorate mixture, vigorously shaken for 30 min in an Eppendorf Mischer 5432, and centrifuged for 15 min in an Eppendorf centrifuge 5412; 10 μl of the clear supernatant was injected into the column. By addition of a constant amount of hymecromon-7-O-

sulphate it was confirmed that the recovery was the same from one patient sample to the next. So the use of an internal standard could be avoided for routine procedures.

Serum standard solutions

Serum standards containing 0.1—50 μg of compounds I—III were prepared by dissolving the compounds in appropriate volumes of drug-free serum. The further processing was the same as described under sample preparation.

Liquid chromatography

The HPLC system used was a Waters M 45 volume delivery system combined with a U6K universal injector, and a Waters 440 dual-channel ultraviolet detector with 254-nm and 280-nm filters. A stainless-steel column was packed with a stable reversed-phase stationary phase consisting of porous silica beads (mean diameter 10 μm) coated with a chemically bonded monolayer of octadecylsilane (μBondapak C_{18}, Waters Assoc.).

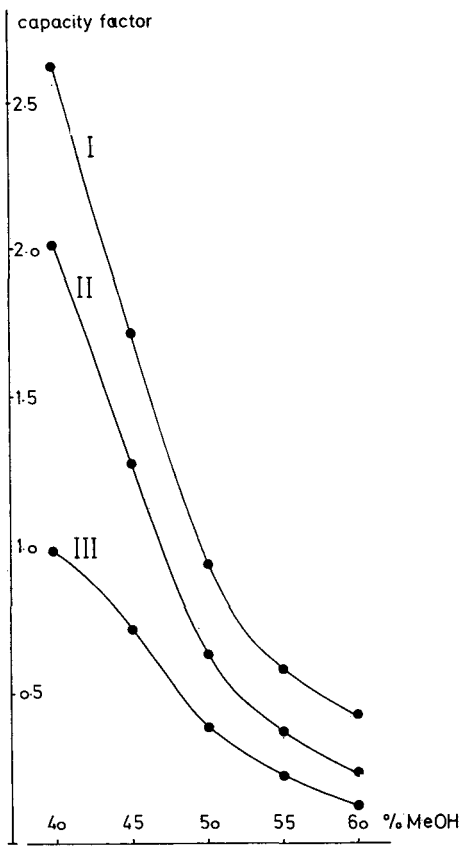

Fig. 2. Capacity factors of hymecromon, hymecromon-5-O-4-β-D-glucuronide and hymecromon-7-O-sulphate in different solvent compositions.

Analysis was performed isocratically with a mobile phase consisting of aqueous 0.01 *M* tetrabutylammonium bromide pH 4.7 and methanol (60:40, v/v). The mobile phase was prepared daily, filtered through a Millipore 5-μm filter, and degassed by sonification. The column was conditioned with 150 ml of the mobile phase, and run overnight with the same, using a flow-rate of 0.1 ml min^{-1}. During analysis the flow-rate was maintained at 1.8 ml min^{-1}.

RESULTS AND DISCUSSION

Ion-pair reversed-phase HPLC using an isocratic solvent composition was found to provide a reliable method to separate the compounds studied. Solvent compositions between 40% and 60% methanol, using the liquid chromatographic conditions mentioned above, resulted in the clean separation of hymecromon from its glucuronide and sulphate conjugates (Fig. 2). The system containing 40% methanol is useful in estimating the concentrations of the drug and its conjugates in biological fluids, in pharmacokinetic studies, and in clinical situations without interference from endogenous materials (Fig. 3).

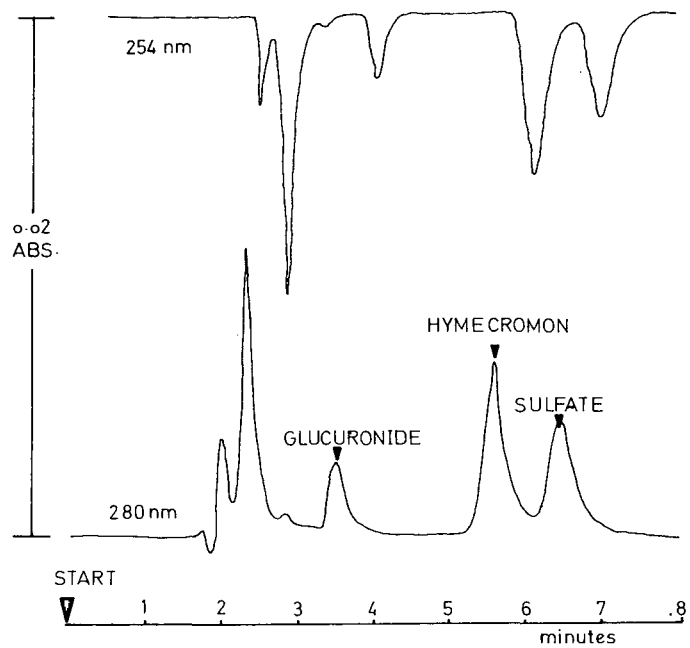

Fig. 3. Liquid chromatogram from plasma containing 100 mg l^{-1} hymecromon, 50 mg l^{-1} glucuronide, and 75 mg l^{-1} sulphate.

Quantitation

Standard curves for compounds I—III in plasma have been prepared by analyzing standard plasma concentrations according to the method described above. Peak areas were plotted against concentrations, and linear regression analysis was performed on the data.

Recovery

For serum containing 10 mg l^{-1} the total recovery was 87.6 ± 1.4%.

Reproducibility

The reproducibility of the assay was convenient. For fifteen independent determinations over a period of three weeks, the coefficient of variation was less than 7.5% for 5—40 mg l^{-1}. The intra-assay coefficient of variation measured from replicate analyses of standard solutions prepared in serum containing hymecromon at concentrations of 2.0, 10.0 and 20.0 mg l^{-1} were 2.8%, 2.5% and 2.1%, respectively.

Limit of sensitivity

The limit of accurate measurement of the assay was 0.2 mg l^{-1}; the intra-assay coefficient of variation at this concentration was 7.5%. This is adequate when it is considered that the average plasma concentration in specimens obtained from patients receiving 200 mg of this compound intravenously is about 5—40 mg l^{-1}.

Application

A typical plasma concentration profile of compounds I—III in man is shown in Fig. 4. Detailed pharmacokinetic analysis on normal subjects, and those suffering from various well-defined hepatic diseases will be published elsewhere.

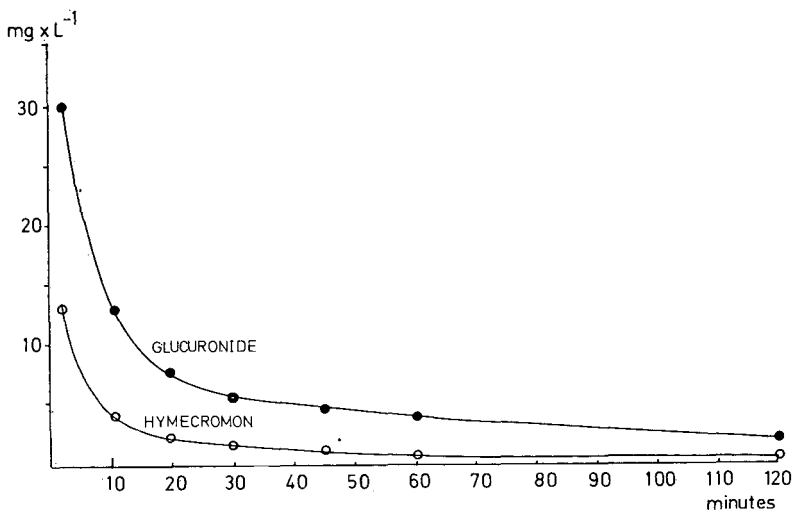

Fig. 4. Plasma concentration profile of hymecromon and the glucuronide conjugate in a human subject following intravenous administration of 200 mg of hymecromon.

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Journal of Chromatography, 278 (1983) 458—463
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1862

Note

Determination of methyprylon and its dehydro metabolite, 5-methylpyrithyldione, in plasma by high-performance liquid chromatography

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(Received June 16th, 1983)

Since the introduction in 1956 of methyprylon (Noludar®) (Fig. 1, I) into clinical practice as a non-barbituarate, sedative hypnotic, reports describing its pharmacokinetics [1—8] have varied considerably as to the half-life of elimination of this drug. One reason for these inconsistencies was the lack of an assay with suitable specificity to quantitate the parent drug in the presence of its metabolites (Fig. 1, II—V) [9—11]. These methods included colorimetry [9], thin-layer chromatography with UV spectrometry [11—13], and gas chromatography with flame ionization detection (GC—FID) [14]. More recently, GC—FID assays with conventional packed columns [7—15], and capillary columns [16], have been reported for the simultaneous assay of methyprylon and its metabolites in overdose cases. However, these assays are not sufficiently sensitive ($\geqslant$ 1—2 μg/ml) to determine the drug and its major plasma metabolite, 5-methylpyrithyldione (II), following single oral doses of methyprylon.

The present work describes the development of a normal-phase, high-performance liquid chromatographic (HPLC) assay with UV detection (214 nm) for the determination of methyprylon and its principle plasma metabolite (II). The assay was applied to the determination of methyprylon elimination in beagle dogs [17], and is herein described for the measurement of methyprylon (I) and 5-methylpyrithyldione (II) in man following a single oral 300-mg dose of methyprylon (Noludar).

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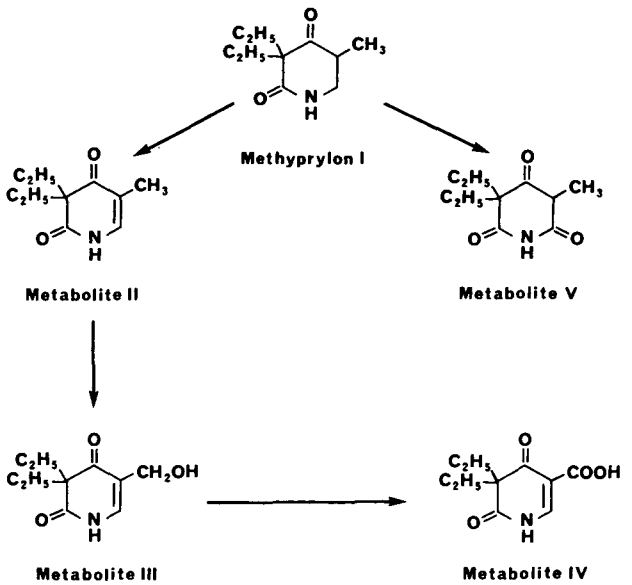

Fig. 1. Biotransformation of methyprylon in dog and man [10, 11].

EXPERIMENTAL

Reagents

All reagents were of analytical (ACS) grade purity, including concentrated hydrochloric acid, diethyl ether, and methanol. HPLC solvents were obtained from Burdick & Jackson Labs., Muskegon, MI, U.S.A. All aqueous solutions were prepared in double-distilled water.

Methyprylon (I) (3,3-diethyl-2,4-dioxo-5-methylpiperidine), and metabolites III (3,3-diethyl-2,4-dioxo-5-hydroxymethyl-1,2,3,4-tetrahydropyridine) and IV (3,3-diethyl-2,4-dioxo-5-carboxy-1,2,3,4-tetrahydropyridine) were obtained from Hoffmann-La Roche, Nutley, NJ, U.S.A. Metabolites II (3,3-diethyl-2,4-dioxo-5-methyl-1,2,3,4-tetrahydropyridine, 5-methylpyrithyldione) and V (3,3-diethyl-2,4,6-trioxo-5-methylpiperidine) were synthesized according to the methods of Dickson [14]. The internal standard, VI (3,3-diethyl-2,4-dioxo-1,2,3,4-tetrahydropyridine, pyrithyldione), was obtained from Aldrich, St. Louis, MO, U.S.A.

Standard solutions

Drug-free plasma, obtained from the Michigan Red Cross, was supplemented with varying concentrations of methyprylon and II according to the following procedure. Individual stock solutions were prepared to contain 200 μg of I and 20 μg of II per ml of distilled water. Appropriate aliquots of each solution were then added to 1.0 ml of plasma to obtain concentrations of 0.1, 1.0, 2.5, 5.0, and 10.0 μg/ml of methyprylon and 0.01, 0.1, 0.5, and 1.0 μg/ml of II. After vortexing, these standards were processed using the scheme described under Sample preparation, the results of which were used to construct calibration curves for both I and II.

Sample preparation

Blood samples were collected in heparinized vacutainers and immediately centrifuged and the plasma was stored at —20°C until processed. Prior to analysis, the samples were thawed at room temperature and centrifuged again to remove precipitated proteins. To 1.0 ml of sample or standard were added 100 μl of 0.1 *N* hydrochloric acid, 50 μl of internal standard (12.0 μg of VI per ml of distilled water), and 10 ml of diethyl ether. The mixture was vortexed for 20 sec and the layers separated by centrifugation at 1500 *g* for 5 min. The ether layer was transferred to a 15-ml conical centrifuge tube and evaporated under a stream of nitrogen at 40°C; all tubes were then stored at —20°C until analyzed. At such time, the sample extract was dissolved in 50 μl of methanol and 25—40 μl aliquots were injected into the chromatograph, as described below.

Instrumental parameters

HPLC analyses was performed on a system consisting of a Model 6000A pumping module, a Model U6K injector, and a Model 450 variable-wavelength UV detector (Waters Assoc.). Separations were obtained on a μPorasil column (10 μm, 3.9 mm I.D. × 30 cm, Waters Assoc.) using a mobile phase composed of hexane—tetrahydrofuran—methanol (72:6:2) at a flow-rate of 2.0 ml/min. Chromatographic peaks were detected at 214 nm and recorded on a single-channel strip chart recorder (Houston Instruments). The injection of 2.0 μg of methyprylon, 0.2 μg of II, and 0.3 μg of internal standard per 25 μl resulted in peaks of nearly 50% full-scale response at a sensitivity of 0.04

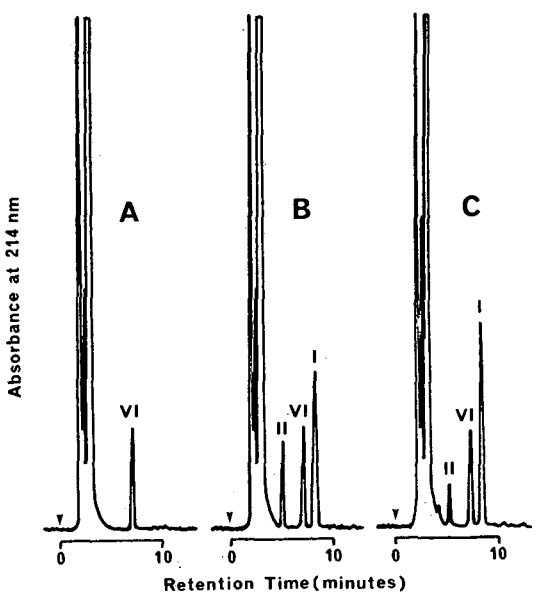

Fig. 2. (A) Chromatogram of drug-free plasma processed as described under Sample preparation. (B) Chromatogram of plasma supplemented with I (1.0 μg/ml) and II (0.1 μg/ml). (C) Chromatogram of plasma taken 1 h after oral administration of 300 mg of methyprylon (Noludar).

a.u.f.s. The chart speed was 0.067 inch/min. Fig. 2 shows representative chromatograms obtained using these conditions.

RESULTS

Statistical validation

The intra-assay linearity and precision of the method was determined over the plasma concentration ranges of 0.1—10.0 μg/ml and 0.01—1.0 μg/ml for I and II, respectively (Table I). Triplicate samples of each concentration were analyzed and peak height ratios calculated (peak height of I or II to peak height of VI). Calibration curves were then constructed by weighted linear ($1/y$) regression analysis of peak height ratio vs. concentration data.

For methyprylon, a correlation coefficient of 0.998 was obtained with an average coefficient of variation (C.V.) of 9.4% over the concentration range studied. Recovery, determined by comparing peak heights of processed vs. unprocessed standards, averaged 84—86%, with a lower limit of sensitivity of 0.1 μg/ml using 1.0-ml samples. For metabolite II, a correlation coefficient of 0.999 was obtained with an average C.V. of 8.4%. Recovery averaged somewhat higher than that of the parent compound, ranging between 88 and 90%, with a sensitivity limit of 0.01 μg/ml.

TABLE I

INTRA-ASSAY STATISTICAL EVALUATION

Compound	Concentration added (μg/ml)	Mean concentration found ± S.D. (n = 3)	Coefficient of variation (%)
Methyprylon*	0.1	0.095 ± 0.021	22.1
	1.0	1.11 ± 0.08	7.2
	2.5	2.40 ± 0.13	5.4
	5.0	5.23 ± 0.35	6.7
	10.0	9.83 ± 0.53	5.4
		Mean =	9.4
Metabolite II**	0.01	0.010 ± 0.002	20.2
	0.10	0.11 ± 0.01	5.6
	0.50	0.48 ± 0.02	3.8
	1.00	1.01 ± 0.04	4.2
		Mean =	8.5

*$Y = 0.481X + 0.004$ ($r = 0.998$).

**$Y = 2.718X + 0.010$ ($r = 0.999$), where X = concentration of I or II and Y = peak height ratio.

Selectivity

The selectivity of the assay for I and II was demonstrated by their resolution from the other three metabolites of methyprylon, III—V (Fig. 3). While the extraction of compounds III—V using the described procedure was not evaluated in this study, earlier reports have described the extraction of methyprylon and its metabolites from urine [14].

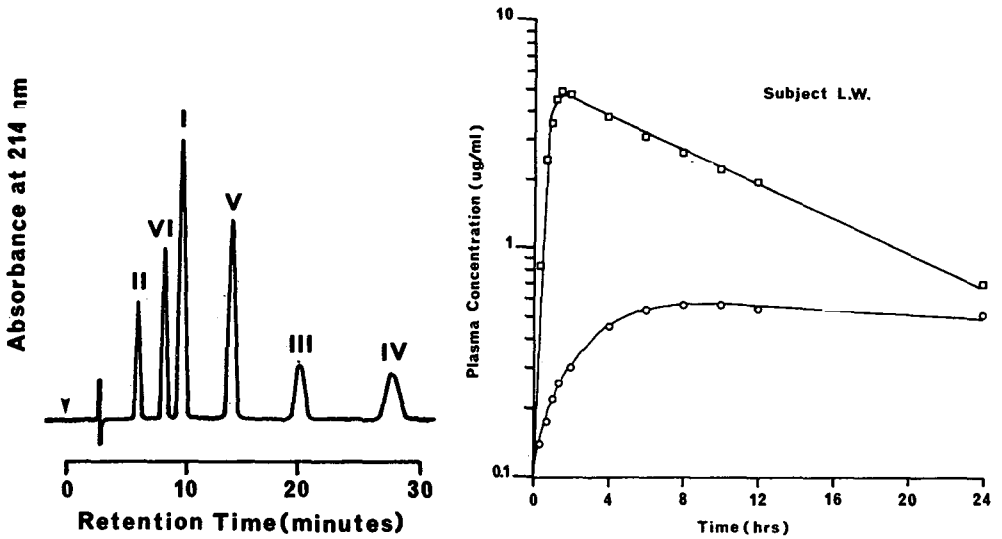

Fig. 3. Chromatogram of authentic standards I—VI obtained as described under Instrumental parameters.

Fig. 4. Plasma concentration vs. time profile for methyprylon (□) and its major plasma metabolite II (○) in a single, healthy, human volunteer (L.W.), following a single 300-mg dose of methyprylon (Noludar).

Preliminary biological applications

The assay was applied to the determination of concentrations of I and II following a single 300-mg dose of methyprylon (Noludar) in a single healthy subject (Fig. 4). A peak concentration of 4—5 μg of I per ml of plasma was determined 2—3 h post dosing, declining to 0.8 μg/ml at 24 h. Concentrations of II demonstrated a maximum plateau of approximately 0.5 μg/ml from 7 to 24 h. The pharmacokinetic profile of I in ten subjects will be the subject of a future publication [18].

DISCUSSION

During the initial development of this assay, several reversed-phase HPLC systems were investigated, including that developed by Kabra et al. [19] for the screening of sedative hypnotics in serum [19]. However, due to the similarity in lipophilic character between methyprylon and II, acceptable separation of these two compounds could not be achieved, even when ion pairing reagents (e.g. heptane sulfonic acid) were added to the mobile phase(s). The normal-phase system described herein results in baseline separation of both I and II in plasma, with retention times of 8.5 and 5.0 min, respectively.

Methyprylon displays two UV absorption maxima, at 298 and 214 nm, while metabolite II displays maxima at 307 and 218 nm. Since the molar absorptivity of methyprylon at 214 nm is approximately ten-fold greater than that at 298 nm (ϵ = 550 at 214 nm), the lower wavelength was chosen for quantitation. Although its absorptivity is low, compared to metabolite II (ϵ = 5100 at 214 nm), the lower limit of detection of this method was never-

theless sufficient to be acceptable for clinical pharmacokinetic studies.

The internal standard used in this assay, namely pyrithyldione or persedon, was chosen from several candidates based on its retention time relative to the other two compounds (7.0 min) and because of its ready availability. The dihydro derivative of pyrithyldione, with a retention time of 13.7 min, could also be used, the only disadvantages being its relatively low UV absorbancy and commercial unavailability.

ACKNOWLEDGEMENTS

The authors wish to acknowledge Ms. Joan Wittenburg for her excellent technical assistance, Dr. Peter Gwilt for supplying pharmacokinetic data, and Ms. Linda Mondrella and Ms. Martha Meek for the preparation of this manuscript. This work was supported, in part, by a grant from Hoffman-La Roche, Inc.

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Journal of Chromatography, 278 (1983) 464—468
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1864

Note

Micro high-performance liquid chromatographic procedure for the quantitation of serum propylthiouracil

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(Received June 8th, 1983)

Propylthiouracil (PTU) has been used for many years in the treatment of hyperthyroidism (Graves' disease). The drug is thought to prevent synthesis of thyroid hormones by disruption of thyroid peroxidase catalyzed reactions [1–3].

Existing methodologies for measuring serum levels of PTU have a number of disadvantages. Colorimetric determinations generally require excessive volumes of blood to achieve adequate sensitivity [4] and lack specificity in differentiating between the parent drug and metabolites [5, 6]. More recent methods have employed high-performance liquid chromatography (HPLC). These methods [7, 8] appear to have the necessary sensitivity and selectivity for determining PTU; however, they still require relatively large sample volumes for analysis. In addition, neither method incorporates an internal standard throughout the entire procedure. We report here an HPLC method that requires only 100 μl of serum, making it particularly well suited for pediatric patients and routine therapeutic drug monitoring. Quantitation is based on reference to an internal standard incorporated in the extraction solvent. The method is fast simple, and reproducible.

MATERIALS AND METHODS

Chromatography

All assays were performed on a Perkin-Elmer Series 2 liquid chromatograph equipped with an LC-75 UV/VIS variable-wavelength detector and interfaced with a Sigma 10 data system that electronically integrated the peak areas (all

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from Perkin-Elmer, Norwalk, CT, U.S.A.). A Perkin-Elmer 10 μm HC ODS/SIL-X reversed-phase column was used for the chromatography. The oven temperature was maintained at 40°C, the flow-rate was 1.6 ml/min, and the detection wavelength was 272 nm.

Reagents

PTU was obtained from Sigma (St. Louis, MO, U.S.A.). 8-Chlorotheophylline (8-CT), used as the internal standard, was obtained from ICN Pharmaceuticals (Plainview, NY, U.S.A.). The mobile phase consisted of 3% acetonitrile in a mixture of 100 m*M* monosodium phosphate and 10 m*M* pentanesulphonic acid (Eastman Kodak, Rochester, NY, U.S.A.). The solution was thoroughly mixed and degassed prior to use. All other solutions and reagents were HPLC or analytical grade.

Stock standards of PTU and 8-CT were individually prepared in methanol to yield a final concentration of 1.0 g/l. Working PTU standards were prepared in drug-free serum with appropriate dilutions from the stock solution to yield concentrations ranging from 0.1 to 10.0 mg/l. Chloroform—isopropanol (1:1), containing 0.4 mg/l of the internal standard (8-CT), was used as the extraction solvent.

Procedure

A 100-μl volume of standard, control or patient serum was placed in a 1.5-ml eppendorf centrifuge tube. Then 1 ml of extraction solvent was added. The tubes were vortexed for 1 min and then centrifuged for 5 min in a Brinkman table-top microcentrifuge. The organic phase was transferred to a 10 × 13 mm disposable glass test tube and evaporated to dryness at 40°C under nitrogen. Each dried sample was immediately reconstituted with 50 μl of distilled water. Since PTU is photosensitive, samples were stored in the dark to prevent decomposition prior to injection (10 μl).

RESULTS AND DISCUSSION

Under these conditions, PTU and 8-CT had retention times of 4.75 and 7.20 min, respectively. A typical chromatogram obtained from the analysis of a serum blank containing the internal standard is shown in Fig. 1A. Fig. 1B illustrates a chromatogram obtained from drug-free serum to which 1.0 mg/l PTU was added, and Fig. 1C is a chromatogram obtained from a patient sample in which the determined concentration of PTU was 2.0 mg/l.

PTU was added to drug-free serum to yield concentrations of 0.1 to 10 mg/l. Concentrations and peak areas were linearly related over this range as shown in Fig. 2. Recovery studies were carried out by preparing identical PTU concentrations in serum and distilled water, and then comparing the peak areas calculated during analysis. The results indicated virtually 100% recovery of PTU from serum.

Within-run precision was evaluated by processing ten aliquots of a prepared standard serum pool containing 2.5 mg/l PTU. The mean ± standard deviation was 2.50 ± 0.09 mg/l, with a coefficient of variation of 3.6%. The stability of samples kept frozen at −20°C was evaluated by analyzing aliquots of prepared

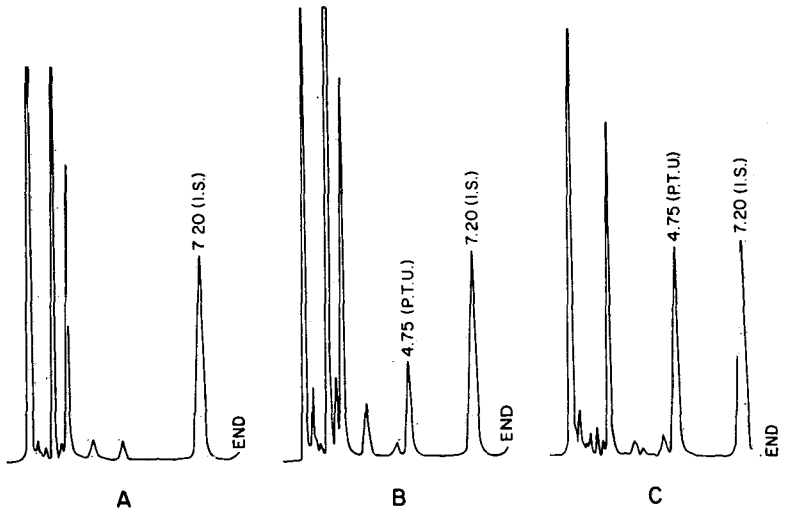

Fig. 1. Typical chromatograms of (A) blank serum containing internal standard, (B) drug-free serum reconstituted with 1.0 mg/l PTU, (C) patient's serum determined as 2.0 mg/l PTU. Retention times: PTU 4.75 min; internal standard (8-CT) 7.20 min. The peaks eluting prior to PTU are unidentified serum extractants.

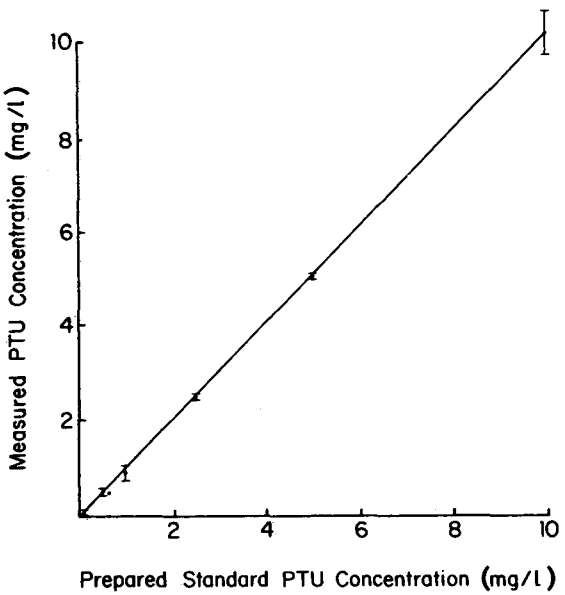

Fig. 2. PTU linearity. Mean (•) and range at each concentration (n = 5).

serum containing known concentrations of PTU. These samples were found to be stable for a period of at least eight weeks (Table I). Six randomly chosen patient samples were also re-analyzed six months later. The results indicated no loss of determined PTU (Table II).

Previously published HPLC methods [5, 7—9] have one or more of the following disadvantages. Three [5, 7, 8] require relatively large (1—5 ml)

TABLE I

STABILITY OF FROZEN PTU SERUM SAMPLES*

Week	Prepared concentrations	
	1 mg/l	5 mg/l
0	0.90	4.80
1	0.88	4.90
2	1.05	4.80
4	0.88	5.14
8	1.20	4.91
Mean ± S.D.	0.98 ± 0.14	4.91 ± 0.14

*All samples frozen at −20°C.

TABLE II

SIX-MONTH COMPARISON OF FROZEN PATIENT SAMPLES*

Patient No.	Determined concentration (mg/l)	
	Initial analysis	Re-analysis
1	1.00	1.24
2	2.40	2.78
3	0.66	0.82
4	0.70	0.71
5	2.87	2.80
6	1.04	0.95

*All samples frozen at −20°C.

volumes of serum or plasma. In addition, all of these procedures require extensive sample extraction, preparation or protein precipitation. Our method requires approximately 7 min for sample preparation, including 5 min for centrifugation. Two methods [5, 8] used chloroform as the extractant, although PTU is practically insoluble in chloroform. An internal standard was not incorporated in two methods [5, 7] and one [8] incorporated an internal standard after extraction, which may result in dilutional error. Two methods [7, 9] monitored PTU at 254 nm and 280 nm, respectively. PTU exhibits its maximum absorption at 272 nm. At this wavelength, the absorbance is more than twice that observed at 254 nm and greater than that observed at 280 nm.

A procedure for PTU determination utilizing a radioimmunoassay technique [10] requires extensive sample pretreatment, is liable to specimen deterioration, and necessitates overnight incubation. Sample deterioration is not a problem with our procedure, and unlike a recent HPLC method [9] does not require a lengthy deproteinization step.

Theophylline, acetaminophen and salicylate, drugs which might be concurrently administered to children for other purposes, were found not to interfere with the quantification of PTU. The method presented here provides

an accurate, simple and fast means of quantifying serum levels of PTU. The small volume of serum required makes this methodology particularly well suited for pediatric patients and routine therapeutic monitoring in those facilities equipped with HPLC.

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Journal of Chromatography, 278 (1983) 469—474
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1868

Note

Ion-exchange high-performance liquid chromatography in drug assay in biological fluids

III. Propranolol, nadolol and prazosin

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(First received May 6th, 1983; revised manuscript received July 18th, 1983)

The successful application of cation exchangers for the high-performance liquid chromatographic (HPLC) determination of ethmozin [1] and verapamil [2] in biological fluids has been described previously. In the present work we use such sorbents to assay three other cardiovascular drugs: propranolol (a widely used β-adrenoceptor blocker), nadolol (a new β-adrenoceptor blocker) [3] and prazosin (a relatively new antihypertensive agent) [4]. There are several methods for propranolol assay in biological fluids using HPLC [5—13], mainly on reversed-phase columns. For nadolol several methods are known: fluorimetry [14], gas chromatography—mass spectrometry [15], HPLC with electrochemical detection [16] and thin-layer chromatography [17]. Some methods for prazosin determination in plasma based on HPLC have also been described [18—22].

We present here a new method applicable to all three drugs using nearly identical chromatographic conditions. With previously published methods for ethmozin [1] and verapamil [2] we now have a universal assay method for five cardiovascular drugs based on cation-exchange HPLC. With fluorescence detection our method provides sensitivity high enough for pharmacokinetic purposes. The extraction procedure does not consume much time.

EXPERIMENTAL

Apparatus and columns

Altex 110A pump with Model 210 injection valve (Altex Scientific,

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Berkeley, CA, U.S.A.) and Model FS-970 fluorescence detector (Schoeffel, Westwood, NJ, U.S.A.) were used. The column (250 × 4.6 mm I.D.) was packed with Partisil 10-SCX (10-μm particle size) from Altex Scientific. The precolumn (40 × 3.2 mm I.D.) was packed by us with the same sorbent. The recorder used was Omniscribe model B5217 (Houston Instrument, Austin, TX, U.S.A.) with 10 mV full scale deflection.

Reagents and standards

The mobile phase was prepared from acetonitrile (LiChrosolv®; E. Merck, Darmstadt, F.R.G.), double-distilled water, diethylamine (pure grade; E. Merck) and orthophosphoric acid (chemical pure grade; Reachim, U.S.S.R.). Amyl alcohol and diethyl ether of pure grade (Reachim) were washed with 0.1 mol/l sulphuric acid and distilled. Pentane of pure grade, potassium hydroxide, sulphuric acid of analytical grade (all from Reachim) were used as received. The glassware was siliconized with AquaSil (Pierce, Rockford, IL, U.A.A.).

A 5% solution of propranolol (Germed, G.D.R.) was used as a standard for this drug. Nadolol and prazosin standards were kindly supplied by Squibb (U.K.) and Orion Pharmaceutica (Finland), respectively. Glaucin (4,5,7,8-tetramethylaporphine hydrochloride; Tatchimpharmpreparaty, Kasan, U.S.S.R.) was used as an internal standard. All standard solutions were prepared in double-distilled water and stored at 4°C.

Extraction procedure

Prazosin. The sample of serum, saliva or urine (1 ml) was placed into the glass-stoppered Pyrex tube, and 0.1 ml of 1 μg/ml glaucin solution was added. The sample was alkalinized with 0.2 ml of 2 mol/l potassium hydroxide and was extracted with 5 ml of diethyl ether for 1 min using a vortex mixer. After centrifutation at 500 *g* for 10 min, the upper layer was transferred into a conical tube and extracted with 0.1 ml of 0.05 mol/l sulphuric acid for 1 min. After brief centrifugation an aliquot of a lower acidic layer was injected onto the column.

Propranolol and nadolol. To the 1-ml sample of biological fluid, 0.1 ml of glaucin solution (50 ng/ml in the case of serum and saliva, and 1 μg/ml in the case of urine) was added followed by 0.1 ml of 1 mol/l potassium hydroxide and 7 ml of pentane—amyl alcohol mixture, (19:1) and (4:1) for propranolol and nadolol, respectively. The tube was vortexed for 20 sec and centrifuged at 500 *g* for 2 min. The upper layer was transferred into a conical tube and extracted with 0.3 ml of 0.05 mol/l sulphuric acid for 20 sec. After centrifugation at 500 *g* for 2 min, an aliquot of the lower layer was injected onto the column.

Chromatographic conditions

The mobile phase was acetonitrile—water—diethylamine—85% orthophosphoric acid (20:80:0.2:0.15, v/v) and was degassed under vacuo before being used. The flow-rate was 2 ml/min, and the column temperature was ambient. Excitation wavelengths and emission filter data are presented in Table I. Detector sensitivity was set at 3.0, and the time constant was 0.5 sec.

TABLE I

RETENTION TIMES OF DRUGS AND THE INTERNAL STANDARD; EXCITATION AND EMISSION WAVELENGTHS FOR THE FLUORESCENCE DETECTION

Drug	Retention time (min)	Excitation wavelength (nm)	Emission filter
Propranolol	4.8	225	Without filter
Nadolol	6.0	205	Without filter
Prazosin	6.2	246	370 cut-off
Glaucin	9.5	—	—

Quantitation

The internal standard method was used for the determination of the drug concentrations in serum, saliva and urine. Peak height ratios of drug to glaucin were plotted versus the concentrations of drug added to the blank samples of biological fluids, and calibration graphs for propranolol and prazosin in serum, saliva and urine, and for nadolol in serum and urine, were thus obtained. The recovery was estimated as a ratio of the peak heights produced by the same amount of each drug after the analysis of the extract of the biological fluid spiked with it and of the standard solution.

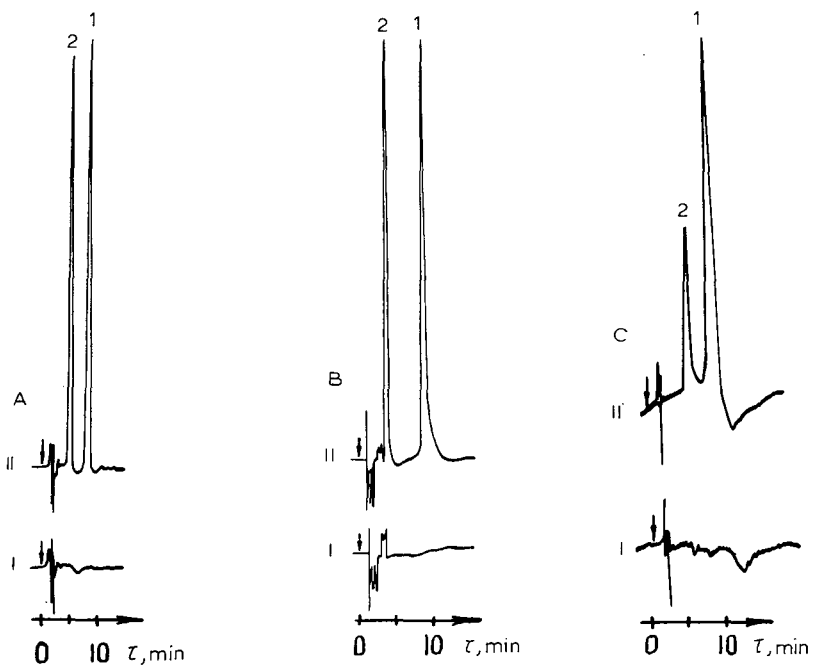

Fig. 1. Chromatograms of the extracts of biological fluid samples taken from patients before (I) and after (II) drug administration. A, Propranolol in serum. Peaks: 1 = glaucin (internal standard); 2 = propranolol (75 ng/ml found). Sensitivity range, 0.1 a.u.f.s. B, Nadolol in urine. Peaks: 1 = glaucin; 2 = nadolol (800 ng/ml found). Sensitivity range, 0.5 a.u.f.s. C, Prazosin in saliva. Peaks: 1 = glaucin; 2 = prazosin (10 ng/ml found). Sensitivity range, 0.05 a.u.f.s.

RESULTS AND DISCUSSION

The conditions described provided a rapid background separation of the drugs and internal standard. In Table I the retention time data are collected. Fig. 1 illustrates the determination of propranolol in serum (A), nadolol in urine (B) and prazosin in saliva (C) of patients treated with these drugs. In each case, the chromatogram marked I is a result of the analysis of the same biological fluid extract received before drug administration and shows the absence of interfering peaks.

TABLE II

CALIBRATION GRAPH DATA

Drug	Biological fluid	Concentration range (ng/ml)	Calibration graphs equation*	r
Propranolol	Serum	5—200	$y = 2.24x + 0.03$	0.980
	Urine	100—1000	$y = 2.37x + 0.01$	0.999
	Saliva	10—200	$y = 2.12x + 0.02$	0.985
Nadolol	Serum	20—500	$y = 0.48x + 0.05$	0.981
	Urine	1000—10,000	$y = 0.45x + 0.02$	0.997
Prazosin	Serum	2—50	$y = 6.95x + 0.02$	0.999
	Urine	2—50	$y = 7.48x + 0.03$	0.999
	Saliva	2—50	$y = 6.20x + 0.07$	0.989

*y = peak height ratio of drug to standard; x = drug concentration (ng/ml).

TABLE III

ACCURACY OF THE DRUG DETERMINATION

Drug	Biological fluid	Concentration (ng/ml)	Coefficient of variation (%) ($n = 5$)
Propranolol	Serum	20	5.7
		100	3.2
	Urine	200	2.1
		700	5.4
	Saliva	20	3.5
		100	3.7
Nadolol	Serum	40	7.3
		300	12.1
	Urine	2000	2.6
		7000	8.1
Prazosin	Serum	2	3.2
		20	1.6
	Urine	2	4.6
		20	1.5
	Saliva	2	12.6
		20	4.6

The minimal detectable levels of drugs are not higher than 1 ng/ml (peak-to-noise ratio 5:1). The recoveries were about 85%, 75%, 60% and 80% for propranolol, nadolol, prazosin and glaucin, respectively. The recovery of prazosin can be made higher by adding the second ether extraction.

In Table II the calibration graphs data are presented. The graphs were linear for all drugs in each biological fluid as was confirmed by the high correlation coefficients. The y-axis intercepts in the regression equations do not significantly differ from zero.

TABLE IV

DRUGS WHICH DO NOT INTERFERE WITH THE DETERMINATION OF PROPRANOLOL, NADOLOL AND PRAZOSIN

Amobarbital sodium	Lidocaine
Ampicillin	Methyldopa
Aspirin	Mexiletin
Atenolol	Nitroglycerine
Caffeine	Oxazepam
Carbamazepine	Phenobarbital
Chlorpropamide	Procainamide
Diazepam	Quinidine
Digoxin	Reserpine
Ephedrine	Sulfamethoxazole
Furosemide	Theophylline
Hydrochlorthiazide	Verapamil*
Isosorbide dinitrate	

*The metabolites of this drug cause interference.

Fig. 2. Chromatogram of the propranolol (peak 1), prazosin (peak 2) and glaucin (peak 3) standard mixture.

The data showing the within-day accuracy of the assay are presented in Table III. They were obtained from five parallel determinations of each drug in each biological fluid at two different levels. The day-to-day variability was higher, so it is preferable to determine a calibration coefficient each day using blank samples of biological fluids.

We have also studied the interference on our assay method of some other commonly used drugs listed in Table IV. It was established that none of them interfered in the determination of propranolol, nadolol or prazosin. Only in the case of verapamil it was found that metabolites of this drug [norverapamil, 2-(3,4-dimethoxyphenyl)-2-propylamino-3-methyl butyronitrile and 2-(3,4-dimethoxyphenyl)-2-isopropyl-6-azaheptanitrile] when present in plasma and urine at high levels after oral administration of verapamil [2] interfered with the peaks of drugs studied here. Intact verapamil did not interfere with them.

Fig. 2 demonstrates the possibility of the co-determination of propranolol and prazosin. Their peaks and that of glacuin are completely separated. However, nadolol interferes with prazosin under these conditions.

Thus, the method presented can assay propranolol, nadolol and prazosin with high sensitivity and accuracy in serum, urine and saliva using the same mobile phase, cation-exchange column and internal standard. It is now being applied with success to the pharmacokinetic study of these drugs.

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Journal of Chromatography, 278 (1983) 475—477
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1853

Letter to the Editor

Simple and rapid determination of fenoprofen in plasma using high-performance liquid chromatography

Sir,

Fenoprofen calcium has been shown to be a useful anti-inflammatory agent in osteoarthritics and rheumatoid arthritics.

The concentration of this drug in plasma has been measured by gas—liquid chromatography (GLC) [1, 2], but the GLC methods require somewhat large sample size, lengthy clean-up procedures and derivatisation. Recently, high-performance liquid chromatography (HPLC) has been utilized for the quantitation of fenoprofen in human plasma or serum [3—5]. Although these methods are accurate and reproducible, they have the disadvantages of time-consuming extractions and/or large sample requirements. Therefore, a rapid and simple HPLC assay with a small plasma sample volume was developed for the measurement of fenoprofen in plasma that did not require a complex prior extraction. The method described here involves a simple protein precipitation technique using 0.1 ml of plasma and estimating the amount of drug present with HPLC. The minimum detectable concentration of fenoprofen in plasma was 2.5 μg/ml using 0.1 ml of plasma.

The plasma sample (0.1 ml) was deproteinized by the addition of 0.5 ml of methanol which contained diphenylamine (1.5 μg) as the internal standard. After shaking for 1 min, the mixture was centrifuged at 1500 *g* for 10 min. The upper layer (100 μl) was injected into the HPLC column. The HPLC system consisted of a solvent pump (Model 6000A, Waters Assoc.), a reversed-phase HPLC column (Nucleosil $5C_{18}$, 15 cm × 4 mm I.D., Macherey-Nagel, Düren, F.R.G.), and a variable-wavelength UV detector set at 240 nm (Model UVIDEC-100-II, Japan Spectroscopic Co.). The mobile phase was acetonitrile—0.35 *M* acetic acid solution (60:40, v/v) and was passed at a flow-rate of 1.0 ml/min.

Separation of fenoprofen and internal standard was satisfactory since retention times were about 4 and 6 min, respectively, under the experimental conditions proposed. No interference by normal-plasma components was noted.

Linear regression indicated excellent linearity with a correlation coefficient

0378-4347/83/$03.00

of 0.9999, a slope of 0.0572, and an intercept of −0.0387 in the range 2.5–100.0 μg/ml of human plasma.

The precision of the assay, as determined by the coefficient of variation, was ⩽2.0%. In addition, the assay was quite accurate even at plasma concentrations as low as 5.0 μg/ml (bias = 2.0%) and was ⩽1.6% for all other concentrations. The recovery of fenoprofen was ⩾91.5%. Table I shows the precision and accuracy for assay of fenoprofen assessed at five concentrations.

TABLE I

VARIABILITY OF FENOPROFEN CONCENTRATION IN HUMAN PLASMA SAMPLES

n = 5 in all cases.

Concentration added (μg/ml)	Concentration measured (μg/ml)	C.V.* (%)	Bias** (%)	Recovery*** (%)
5.0	5.1 ± 0.1†	2.0	2.0	91.5
10.0	9.6 ± 0.1	1.0	−4.0	96.3
25.0	24.3 ± 0.4	1.6	−2.8	97.4
50.0	50.8 ± 0.7	1.4	1.6	97.1
100.0	99.8 ± 0.7	0.7	−0.2	98.2

*Coefficient of variation (%).
**100 × (measured concentration − added concentration)/added concentration.
***Plasma versus water.
†Mean ± standard deviation.

Fenoprofen is extensively metabolized to fenoprofen glucuronide and 4-hydroxyfenoprofen glucuronide and is excreted in the urine within 24 h [6, 7]. The elution time of the major metabolite, 4-hydroxyfenoprofen (2 min), was sufficiently different from fenoprofen, but since the human plasma components interfered with the peak of 4-hydroxyfenoprofen, this metabolite could not be determined by this method.

The simplicity and short extraction procedure are advantageous in the analysis of a large number of samples often encountered in pharmacokinetics and bioavailability studies. The small plasma volume needed for the analysis is also an important consideration in such studies. Moreover, this HPLC method is superior to others, since it allows higher precision and accuracy with a small volume of plasma.

The analytical method is suitable for bioavailability and pharmacokinetic studies in human subjects.

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(First received April 14th, 1983; revised manuscript received June 28th, 1983)

Journal of Chromatography, 278 (1983) 478—479
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1870

Book Review

Advances in thin layer chromatography. Clinical and environmental applications, edited by J.C. Touchstone, Wiley, New York, Chichester, Brisbane, Toronto, Singapore, 1982, XVII + 521 pp., price £45.50, ISBN 0-471-09936-8.

This volume represents the proceedings of the Second Biennial Symposium on Thin-Layer Chromatography (TLC) held in Philadelphia, PA, U.S.A., in December 1980. Forty-one papers written by authors mainly from the U.S.A. (with the exception of one Hungarian and one German author) constitute this volume. As is the tradition with symposia proceedings, the individual contributions were not arranged into logical wholes. Individual papers take the form either of reviews or of original papers.

The most sympathetic feature of this book is the fact that it reflects current trends in the area of TLC, mainly in high-performance procedures, reversed-phase separations and automation. Similarly, the clinico-chemical applications and applications to the environmental analysis indicate the areas where interest is focused today; seven chapters are devoted to mycotoxins and other toxins, five papers deal with steroids and bile acids, four papers deal with lipids, three papers are about pesticides, etc. Of the individual chapters one feels obliged to mention at least some nicely written chapters like those by Roos (about steroids), Rogers (about the influence of solvents on chromatographic selectivity) and Filthuth (on radioscanning in TLC).

The editor of the volume, J.C. Touchstone, was motivated by efforts to publish the book with a minimum delay. This, unfortunately, resulted in some negative aspects; for instance, many reviews are not documented by references at all, or the references are limited to only a few items. What is such a review good for? Also some of the original papers have the character of a preliminary note and are of little use to the reader. Few authors compared methods of their own with other chromatographic techniques like high-performance liquid chromatography or gas chromatography. One of the weakest points of the book is the Index. A number of compounds are not included at all, while other items are repeated, and numerous entries for TLC techniques are also omitted. One can pardon, perhaps, the minor errors which occurred because of the speed with which this volume was published. However, citing the journal, "J. High

Resolut. Chromatogr. Chromatogr. Commun.", as "High Performance Radial Chromatogr. Chromatogr. Commun." (p. 32) represents undesirable creativity.

It can be concluded that the positive features of the book overweigh the drawbacks, and one can foresee that it will find potential buyers from the area of biomedical applications even outside the participants of the above meeting.

Journal of Chromatography, 278 (1983) 480—481
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1944

Book Review

Literature guide to the GLC of body fluids, by A.V. Signeur, IFI/Plenum, New York, Washington, London, 1982, IX + 385 pp., price US$85.00, ISBN 0-306-65203-X.

The enormous interest in biomedical analysis which has appeared during recent years inspired Signeur to work out a bibliography of papers that deal with gas chromatography of body fluids. In comparison with other bibliographies, the arrangement of entries in this case is such that the papers are ordered alphabetically according to the name of the first author; the orientation of the type of compounds analyzed is obtained from an extensive Subject Index. This way of listing the references has its advantages and disadvantages. One advantage can be seen in the abolition of problems arising from the classification of individual papers into chapters when more types of compounds are analyzed side-by-side. Here, the Subject Index enables the reader to find the requested information rapidly. A natural prerequisite for this is a fairly perfect subject index. Unfortunately, in this particular case, the authors have compiled the Subject Index only of data available from Chemical Abstracts (C.A.). Here, when a large number of compounds are analyzed side-by-side in a single paper, not all compounds are listed. Thus, if, for instance, a reader is looking for a paper that deals with the presence of 2-methyltetrahydrofuran in diabetic patients, he will not find it, though the particular paper is listed in the book (4561, Zlatkis et al.).

The main entries under which a particular type of compound falls are another problem. Under some main entries, e.g., "Barbiturates", a note appears stating that other compounds can be found listed under specific names. There are, however, occasions where such a note is missing. For instance, under "Antidepressants" only nine references are listed even though the number of gas chromatography papers actually included is higher by about one order. Another example is portrayed by the amino acids. There are only three references at "Alanine" although this amino acid has been assayed in 36 other papers which are found under the main entry "Amino acids".

With respect to the information available from C.A., the Subject Index has been prepared with great care; however, some inaccuracies could not be avoided. One of these is the fact that some compounds are listed under different synonyms, e.g., aspirin — acetylsalicylic acid, adrenaline — epinephrine, cortisol — tetrahydrocortisone, etc. Also, a few key entries are missing: "metabolic profiling" can serve as an illustrative example. On the other hand, the importance of some entries such as "Blood" and "Urine" is doubtful.

In spite of some minor inaccuracies the evaluation of the book as a whole is positive. Based on the C.A. data, the authors have arranged a bibliographic survey on the analysis of body fluids by gas chromatography, covering the period from the late fifties to the beginning of 1981. This survey summarizes 4561 references. The book can be warmly recommended to all interested in this field.

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CHROMBIO. 1945

Book Review

Biological/biomedical applications of liquid chromatography IV, edited by G.L. Hawk (associate editors R.F. Hutton, G. Johnston and Ch. Mol), Marcel Dekker, New York, Basel, 1982, XVII + 367 pp., price SFr. 156.00, ISBN 0-8247-1842-0.

The fourth volume of this monograph represents a collection of 25 papers presented at the 4th Liquid Chromatography Symposium held in Amsterdam, The Netherlands, April 28 and 29, 1981 [for reviews of previous volumes see J. Chromatogr., 181 (1980) 516; 183 (1980) 260; and 233 (1982) 442].

Most of the papers have kept the form of original contributions or short reviews from the areas of clinical chemistry, drug analysis and chromatography of peptides and proteins. One paper is devoted to general techniques (selective chemically bonded phases). The topic of the book is rather wide ("biological" applications) and therefore it is possible to find contributions that would not be expected here, e.g., peptide synthesis and analysis of plant material. The subject of the symposium and concomitantly the contents of these Proceedings reflect current trends in the biomedical field — always the most interesting feature of this category of publication. Attention in comparison to the preceding volumes is focused on chromatography of peptides and proteins and on drug monitoring. Contributions from the area of clinical chemistry deal mainly with catecholamines and their metabolites. The paper of Van Gennip et al. is quite interesting from the point of view of clinical practice. (Why then is Wadman et al. reported as the author in the running title?) This paper deals with the screening of inborn errors of purine and pyrimidine metabolism. In the present volume the Editors attempted to arrange the individual papers according to the categories of the compounds separated, helping to constitute a clear view of the whole volume.

Unfortunately, the quality of the book is devaluated by the Index which contains so many errors as is rarely seen. It appears that no proofing has been done on this particular part of the book. Otherwise, it is difficult to explain that the G heading is doubled and that letters are missing, e.g., "Des-Tyr--endorphin" or " -endorphin", or entire words ("kynurenic "). For obscure reasons several entries refer to a footnote when there is none in the Index, e.g., "cefaloridine[b]" and "cefazolin[b]". Quite enjoyable are the entries which refer to a blank page ("bufotenine 256" and "dimethyltryptamine 256"). There are also situations where the entries refer to a page where the particular compound is not spoken of at all; or, on the contrary, there are no references to a page where a compound is dealt with. It is very likely that these drawbacks are on account of the poor desk-editorial work and that they are not the fault of the Editors; however, they devaluate considerably the work done by the Editors.

Related articles published in *Journal of Chromatography*, Vols. 268 and 269

Volume 268

Author Index

Subject Index

Erratum

J. Chromatogr., 277 (1983) 427—432
Page 431, text line 6, "300 mg" should read "600 mg".

Vol. 278, No. 2

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

MEETINGS

INTERNATIONAL SYMPOSIUM ON HPLC IN THE BIOLOGICAL SCIENCES, MELBOURNE, AUSTRALIA, FEBRUARY 20–22, 1984

An International Symposium on HPLC in the Biological Sciences is being organised to bring together Australian and International researchers involved in this rapidly growing field. This International Symposium is sponsored by the Australian Academy of Science and held under the auspices of the Australian Biochemical Society, the Endocrine Society of Australia, the Royal Australian Chemical Institute and the Australian Society for Medical Research. The organising committee, chaired by Dr. M.T.W. Hearn, St. Vincent's School of Medical Research, intend this important occasion to involve a stimulating combination of invited papers, presented oral communications and poster sessions. Manuscripts from the oral and poster sessions will be published following review, in a proceedings volume.

The three-day scientific programme will include oral presentations and poster sessions covering the following topics: Column Technology and Separation Mechanisms (Reversed Phase Chromatography, Ion-Exchange Chromatography, and Size-Exclusion Chromatography); Microscale and Macroscale Isolation of Biologically Active Substances; HPLC of Nucleotides, Polynucleotides, RNA and DNA fragments; HPLC in Polypeptide and Protein Analysis and Purification; Drug Analysis by HPLC; HPLC of Saccharides, Lipids and Steroids; and Special Topics (Multi-dimensional Chromatography, Data Handling, and Others to be announced).

The themes of this symposium will appeal to participants from a wide range of scientific backgrounds and will undoubtably include analytical chemists, biochemists, biochemical scientists, toxicologists, pharmacologists, environmental scientists and biotechnologists.

For further information, contact: The Secretary, Mrs. S. Tregellas, International Symposium on HPLC in the Biological Sciences, c/o St. Vincent's School of Medical Research, 41 Victoria Parade, Fitzroy 3065, Victoria, Australia.

20th INTERNATIONAL SYMPOSIUM ON ADVANCES IN CHROMATOGRAPHY, NEW YORK, NY, APRIL 16–19, 1984

The 20th International Symposium on Advances in Chromatography will be held April 16–19, 1984, at the Sheraton Centre in New York, NY, U.S.A. The scope of the meeting will cover papers and informal discussion groups by outstanding researchers from throughout the world in all fields of chromatography. In particular, new developments in gas, liquid and high-performance thin-layer chromatography will be included. There will also be a commercial exhibition of the latest instrumen-

tation and books. Participation in the symposium will be on the basis of invited papers as well as unsolicited contributions. Authors desiring to present papers must submit 200-word abstracts by December 1, 1983. Complete manuscripts of accepted authors will be due on April 16, 1984, at the meeting. Special, separate, intensive two-day short courses in (a) Capillary Chromatography, (b) HPLC, (c) Gas Chromatography–Mass Spectrometry, and (d) Computer Chromatography, will be available on Saturday and Sunday, April 14–15, just prior to the meeting. All correspondence pertaining to the symposium, short courses and exhibition space should be directed to: Dr. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. Tel.: (713) 749-2623.

2nd SYMPOSIUM ON THE ANALYSIS OF STEROIDS, SZEGED, HUNGARY, JUNE 12–14, 1984

The above symposium is being sponsored by the Analytical Division of the Hungarian Academy of Sciences and the Chemical Works of Gedeon Richter Ltd. Plenary lectures by acknowledged international authorities on the field of steroid analysis will highlight new developments.

Topics and scope: 1. Determination of steroids in biological samples: Clinical steroid analysis; Analytical methodology for studying the biosynthesis and metabolism of steroids as well as the pharmacokinetic study of steroid drugs; Determination of steroids in samples of plant and animal origin. 2. Industrial and pharmaceutical steroid analysis; Methods for the purity testing of steroids; Analysis of pharmaceutical dosage forms including stability assays; Analysis of the intermediates of steroid syntheses. The scope of the Symposium includes the discussion of methodological problems from the fields of all the main groups of steroids (hormones, sterols, vitamins D, bile acids, cardiac glycosides, sapogenins, alkaloids, etc.) but the detailed discussion of biochemical, diagnostical and drug stability problems etc., as well as the structure elucidation of steroids, will not be included.

The symposium language will be English.

For further information, contact: Professor S. Görög, Chairman of the Organizing Committee, c/o Hungarian Chemical Society, Anker köz 1, H-1061 Budapest, Hungary.

ADVANCES IN LIQUID CHROMATOGRAPHY, SZEGED, HUNGARY, SEPTEMBER 10–14, 1984

The Biological Research Center of the Hungarian Academy of Sciences, the Chromatography Committee of the Hungarian Academy of Sciences and the Hungarian Chemical Society will organize the above conference.

The conference will consist of two parts: (I) 4th Annual American–Eastern European Symposium on Liquid Chromatography; and (II) International Symposium on Thin-Layer Chromatography with Special Emphasis on Overpressured Layer Chromatography.

The scope will cover invited papers, oral presentations and posters in all fields of liquid chromatography. There will also be a commercial exhibition of the latest instrumentation, stationary phases, chemicals and books.

All correspondence concerning the Conference should be directed to: Dr. Huba Kalász, Department of Pharmacology, Semmelweis University of Medicine, P.O. Box 370, Budapest 1445, Hungary; or Dr. E. Tyihák, Research Institute for Plant Protection, P.O. Box 102, Budapest 1525, Hungary.

CALENDAR OF FORTHCOMING EVENTS

Jan. 16–17, 1984 Amsterdam, The Netherlands	**Short Course on Low Dispersion Liquid Chromatography** Contact: LDLC Workshop Office, Department of Analytical Chemistry, Free University of Amsterdam, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. Tel.: (020) 548-5379.
Jan. 19–20, 1984 Amsterdam, The Netherlands	**Workshop on Low Dispersion Liquid Chromatography** Contact: LDLC Workshop Office, Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. (Further details published in Vol. 276, No. 1.)

Feb. 20–22, 1984 Melbourne, Australia	**International Symposium on HPLC in the Biological Sciences** Contact: The Conference Secretary, Mrs. S. Tregellas, International Symposium on HPLC in the Biological Sciences, c/o St. Vincent's School of Medical Research, 41 Victoria Parade, Fitzroy 3065, Victoria, Australia.
Feb. 28–29, 1984 Edinburgh, U.K.	**Advances in Chromatography: Clinical and Pharmaceutical Applications** Contact: Royal Society of Chemistry, Burlington House, London W1V 0BN, U.K. Tel.: 01-734 9971.
March 5–10, 1984 Atlantic City, NJ, U.S.A.	**35th Pittsburgh Conference and Exposition on Analytical Chemistry and Applied Spectroscopy** Contact: Linda Briggs, Pittsburgh Conference, 437 Donald Road, Dept. J-005, Pittsburgh, PA 15235, U.S.A.
April 8–13, 1984 St. Louis, MO, U.S.A.	**187th National Meeting of the American Chemical Society** Contact: Meetings Department, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.
April 10–13, 1984 Munich, G.F.R.	**9th Conference on Biochemical Analysis (BIOCHEMISCHE ANALYTIK 84) & ANALYTICA 84 Exhibition** Contact: Secretary General, Dr. Rosmarie Vogel, Abteilung für Klinische Chem und Klinische Biochemie in der Chirurgischen Klinik Innenstadt der Universitä München, Nussbaumstrasse 20, D-8000 München 2, G.F.R. Tel.: (089) 15 30 3 Telex: 5 216 018 bird d. (Further details published in Vol. 276, No. 1.)
April 16–18, 1984 Neuherberg, F.R.G.	**3rd International Workshop on Trace Element Analytical Chemistry in Medicine and Biology** Contact: Dr. P. Schramel, Gesellschaft für Strahlen- und Umweltforschung, Institut für Angewandte Physik, Physikalisch-Technische Abteilung, Ingolstädter Landstrasse 1, D-8042 Neuherberg, F.R.G.
April 16–19, 1984 Exeter, U.K.	**Royal Society of Chemistry Annual Congress** Contact: Royal Society of Chemistry, Burlington House, London W1V 0BN, U.K. Tel.: 01-734 9971.
April 16–19, 1984 New York, NY, U.S.A.	**20th International Symposium on Chromatography** Contact: Professor A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A.
April 17–19, 1984 Noordwijkerhout, The Netherlands	**Analytical Methods and Problems in Biotechnology – An International Symposium** Contact: W.A. Scheffers, Symposium Analytical Methods and Problems in Biotechnology, Delft University of Technology, Laboratory of Microbiology, Julianalaan 67A, NL-2628 BC Delft, The Netherlands. Tel.: (015)-782411. (Further details published in Vol. 276, No. 2.)
April 29–May 4, 1984 Rio de Janeiro, Brazil	**12th International Congress of Clinical Chemistry, 7th Latin American Congress of Clinical Biochemistry & 12th Brazilian Congress of Clinical Analysis** Contact: 12th International Congress of Clinical Chemistry, Rua Vicente Licinio 95, Tijuca, 20270 Rio de Janeiro, RJ, Brazil.
May 9–11, 1984 Dourdan, France	**4th Weurman Flavour Research Symposium** Contact: J. Adda, Laboratoire de Recherches sur les Arômes, 17 rue Sully, 21034 Dijon Cedex, France.

May 15–18, 1984 Ghent, Belgium	**5th International Symposium on Mass Spectrometry in Life Sciences** Contact: Prof. Dr. A. De Leenheer, Symposium Chairman, Laboratoria voor Medische Biochemie en voor Klinische Analyse, Harelbekestraat 72, B-9000 Ghent, Belgium. Tel.: (091) 21.89.51.
May 20–25, 1984 New York, NY, U.S.A.	**8th International Symposium on Column Liquid Chromatography** Contact: Professor Cs. Horváth, Mason Laboratory, Yale University, P.O. Box 2159, Yale Station, New Haven, CT 06520, U.S.A. (Further details published in Vol. 272, No. 2.)
June 10–14, 1984 Toronto, Canada	**International Symposium on Drug Quality Assurance in the Hospital** Contact: Organizing Committee, International Symposium on Drug Quality Assurance in the Hospital, Toronto General Hospital, Public Relations, Bell Wing 1-602, 101 College Street, Toronto, Ontario M5G 1L7, Canada.
June 12–14, 1984 Szeged, Hungary	**2nd Symposium on the Analysis of Steroids** Contact: Professor S. Görög, Chairman of the Organizing Committee of the 2nd Symposium on the Analysis of Steroids, c/o Hungarian Chemical Society, H-1061 Budapest, Anker köz 1, Hungary.
June 18–21, 1984 Ronneby, Sweden	**International Symposium on Liquid Chromatography in the Biomedical Sciences** Contact: Swedish Academy of Pharmaceutical Sciences, P.O. Box 1136, S-11181 Stockholm, Sweden. (Further details published in Vol. 276, No. 2.)
June 20–22, 1984 St. Andrews, U.K.	**Quality Control of Packaging: Food, Beverages, Pharmaceuticals and Cosmetics** Contact: Royal Society of Chemistry, Burlington House, London W1V 0BN, U.K. Tel.: 01-734 9971.
July 29–Aug. 3, 1984 Washington, DC, U.S.A.	**36th National Meeting of the American Association for Clinical Chemistry** Contact: American Association for Clinical Chemistry, 1725 "K" Street, NW, Washington, DC 20006, U.S.A.
Aug. 21–24, 1984 Colombo, Sri Lanka	**Analytical Chemistry in Development** Contact: Centre for Analytical Research and Development, Department of Chemistry, University of Colombo, Colombo, Sri Lanka; or, Trace Analysis Research Centre, Chemistry Department, Dalhousie University, Halifax, N.S. B3H 4J1, Canada.
Aug. 26–31, 1984 Philadelphia, PA, U.S.A.	**188th National Meeting of the American Chemical Society** Contact: A.T. Winstead, American Chemical Society, 1155 16th Street, NW, Washington, DC 20036, U.S.A.
Aug. 26–Sept. 1, 1984 Cracow, Poland	**EUROANALYSIS V – 5th European Conference on Analytical Chemistry** Contact: Professor Zygmunt Kowalski, Secretary-General, Euroanalysis V, Academy of Mining and Metallurgy, Mickiewicza 30, 30-059 Kraków, Poland. (Further details published in Vol. 261, No. 3.)
Sept. 2–6, 1984 Hradec Králové, Czechoslovakia	**4th International Symposium on Isotachophoresis – ITP 84** Contact: ITP 84, Dr. Z. Prusik, C.Sc., Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Flemingovo nam. 2, CS-166 10 Praha 6, Czechoslovakia. (Further details published in Vol. 272, No. 2.)

Sept. 23–28, 1984 Philadelphia, PA, U.S.A.	**11th Annual Meeting of the Federation of Analytical Chemistry and Spectroscopy Societies** Contact: R.F. Hirsch, Division of Analytical Chemistry, American Chemical Society, 304 Beach Wood, Orange, NJ 07050, U.S.A.
Oct. 1–5, 1984 Nürnberg, G.F.R.	**15th International Symposium on Chromatography** Contact: Gesellschaft Deutscher Chemiker, Abteilung Fachgruppen, Postfach 90 04 40, Varrentrappstrasse 40–42, D-6000 Frankfurt (Main), G.F.R.
Oct. 8–10, 1984 Tarrytown, NY, U.S.A.	**3rd International Symposium on Capillary Chromatography** Contact: Dr. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A.
Oct. 24–26, 1984 Montreux, Switzerland	**3rd Workshop on LC–MS and MS–MS** Contact: Prof. Dr. R.W. Frei, Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. (Further details published in Vol. 276, No. 1.)
Nov. 19–24, 1984 Barcelona, Spain	**EXPOQUIMIA 84 – Salón Internacional de la Quimica** Contact: EXPOQUIMIA, Feria de Barcelona, Barcelona 4, Spain.
Nov. 22–24, 1984 Barcelona, Spain	**14th Annual Symposium on Analytical Chemistry of Pollutants** Contact: 3rd International Congress on Analytical Techniques in Environmental Chemistry/EXPOQUIMIA, Av. Reina Ma. Christina, Palacio No. 1, Barcelona 4, Spain. Tel.: 223.31.01; telex: 50458 FOIMB-E.
Nov. 22–24, 1984 Barcelona, Spain	**3rd International Congress on Analytical Techniques in Environmental Chemistry** Contact: 3rd International Congress on Analytical Techniques in Environmental Chemistry/EXPOQUIMIA, Av. Reina Ma. Christina, Palacio No. 1, Barcelona 4, Spain. Tel.: 223.31.01; Telex: 50458 FOIMB-E.
Feb. 25–March 1, 1985 New Orleans, LA, U.S.A.	**36th Pittsburgh Conference and Exposition on Analytical Chemistry and Applied Spectroscopy** Contact: Linda Briggs, Pittsburgh Conference, 437 Donald Road, Dept. J-005, Pittsburgh, PA 15235, U.S.A.
April 28–May 3, 1985 Miami Beach, FL, U.S.A.	**189th National Meeting of the American Chemical Society** Contact: Meetings Department, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.
July 1–5, 1985 Edinburgh, Scotland, Great Britain	**9th International Symposium on Column Liquid Chromatography** Contact: J.H. Knox, Department of Chemistry, University of Edinburgh, Edinburgh EH9 3JJ, Scotland, Great Britain.
Sept. 8–13, 1985 Chicago, IL, U.S.A.	**190th National Meeting of the American Chemical Society** Contact: Meetings Department, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.
Sept. 9–13, 1985 Manchester, Great Britain	**30th International Congress of Pure and Applied Chemistry** Contact: The Royal Society of Chemistry, Burlington House, London W1V 0BN, Great Britain.

NEW BOOKS

Biochemical research techniques: a practical introduction, edited by J.M. Wrigglesworth, Wiley, Chichester, New York, Brisbane, Toronto, Singapore, 1983, *ca.* 272 pp., price £ 19.50, US$ 39.95, ISBN 0-471-10323-3.

Progress in pesticide biochemistry and toxicology, Vol. 3, edited by D.H. Hutson and T.R. Roberts, Wiley, Chichester, New York, Brisbane, Toronto, Singapore, 1983, X + 454 pp., price £ 54.50, ISBN 0-471-90053-2.

Selected methods for the small clinical laboratory (Selected Methods of Clinical Chemistry, Vol. 9), edited by W.R. Faulkner and S. Meites, Raven Press, New York, 1982, 330 pp., price US$ 54.39.

Clinical chemistry self-assessment, edited by M.A. Brewster, Raven Press, New York, 1982, 176 pp., price US$ 20.40.

Quantitative approaches to drug design (Proc. 4th European Symp. on Chemical Structure–Biological Activity: Quantitative Approaches, Bath (U.K.), Sept. 6–9, 1982; *Pharmacochemistry Library*, Vol. 6), edited by J.C. Dearden, Elsevier, Amsterdam, Oxford, New York, 1983, X + 296 pp., price US$ 63.75 (U.S.A. and Canada), Dfl. 150.00 (rest of world), ISBN 0-444-42200-5.

Drug metabolism and distribution, edited by J.W. Lamble, Elsevier, Amsterdam, New York, 1983, XII + 184 pp., price US$ 17.50, £ 8.50, ISBN 0-444-80510-9.

PUBLICATION SCHEDULE FOR 1983

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

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INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 264, No. 3, pp. 491-494. A free reprint can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For review articles, see page 2 of cover under Submission of Papers.

Submission. Every paper must be accompanied by a letter from the senior author, stating that he is submitting the paper for publication in the *Journal of Chromatography.* Please do not send a letter signed by the director of the institute or the professor unless he is one of the authors.

Manuscripts. Manuscripts should be typed in double spacing on consecutively numbered pages of uniform size. The manuscript should be preceded by a sheet of manuscript paper carrying the title of the paper and the name and full postal address of the person to whom the proofs are to be sent. Authors of papers in French or German are requested to supply an English translation of the title of the paper. As a rule, papers should be divided into sections, headed by a caption (*e.g.,* Summary, Introduction, Experimental, Results, Discussion, etc.). All illustrations, photographs, tables, etc., should be on separate sheets.

Introduction. Every paper must have a concise introduction mentioning what has been done before on the topic described, and stating clearly what is new in the paper now submitted.

Summary. Full-length papers and Review articles should have a summary of 50-100 words which clearly and briefly indicates what is new, different and significant. In the case of French or German articles an additional summary in English, headed by an English translation of the title, should also be provided. (Short communications and Notes are published without a summary.)

Illustrations. The figures should be submitted in a form suitable for reproduction, drawn in Indian ink on drawing or tracing paper. Each illustration should have a legend, all the *legends* being typed (with double spacing) together on a *separate sheet.* If structures are given in the text, the original drawings should be supplied. Coloured illustrations are reproduced at the author's expense, the cost being determined by the number of pages and by the number of colours needed. The written permission of the author and publisher must be obtained for the use of any figure already published. Its source must be indicated in the legend.

References. References should be numbered in the order in which they are cited in the text, and listed in numerical sequence on a separate sheet at the end of the article. Please check a recent issue for the layout of the reference list. Abbreviations for the titles of journals should follow the system used by *Chemical Abstracts.* Articles not yet published should be given as "in press", "submitted for publication", "in preparation" or "personal communication".

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EVALUATION OF ANALYTICAL METHODS IN BIOLOGICAL SYSTEMS

Part A: Analysis of Biogenic Amines

edited by GLEN B. BAKER *and* RONALD T. COUTTS, *Neurochemical Research Unit, Department of Psychiatry, and Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Canada*

TECHNIQUES AND INSTRUMENTATION IN ANALYTICAL CHEMISTRY, 14

This is the first volume of a new multi-volume work entitled "Evaluation of Analytical Methods in Biological Systems" that will evaluate the various analytical techniques and approaches that can be used in a particular bioanalytical field. This volume provides an overview of techniques for the analysis of catecholamines, 5-hydroxytryptamine (serotonin), 'trace amines' and histamine in biological sytems.

The authors describe the basic principles underlying the various techniques and discuss their advantages and disadvantages relative to other available methods, thus making this a valuable reference both for those experienced in analysis of biogenic amines who are considering using a new technique, and to those entering the field for the first time.

1982 xvi + 308 pages
US $70.25 (In USA & Canada); Dfl. 165.00 (Rest of World)
ISBN 0-444-42110-6

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