ISSN 0378-4347

VOL. 182 NO. 1 APRIL 11, 1980
(Biomedical Applications, Vol. 8, No. 1)

JOURNAL OF CHROMATOGRAPHY BIOMEDICAL APPLICATIONS

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

PUBLICATION SCHEDULE FOR 1980

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

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

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Publication. The *Journal of Chromatography* (including *Biomedical Applications* and *Chromatographic Reviews*) has 22 volumes in 1980. The subscription price for 1980 (Vols. 181–202) is Dfl. 2838.00 plus Dfl. 352.00 (postage) (total *ca.* US$ 1636.00). The subscription price for the *Biomedical Applications* section only (Vols. 181–183) is Dfl. 399.00 plus Dfl. 48.00 (postage) (total *ca.* US$ 229.25). Journals are automatically sent by air mail to the U.S.A. and Canada at no extra costs, and to Japan, Australia and New Zealand with a small additional postal charge. Back volumes of the *Journal of Chromatography* (Vols. 1 through 180) are available at Dfl. 140.00 (plus postage). Claims for issues not received should be made within three months of publication of the issue. If not, they cannot be honoured free of charge. For customers in the U.S.A. and Canada wishing additional bibliographic information on this and other Elsevier journals, please contact Elsevier/North-Holland Inc., Journal Information Centre, 52 Vanderbilt Avenue, New York, N.Y. 10017. Tel: (212) 867-9040.

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BIOMEDICAL APPLICATIONS

VOL. 8 1980

ELSEVIER SCIENTIFIC PUBLISHING COMPANY
AMSTERDAM

Printed in The Netherlands

Journal of Chromatography, 182 (1980) 1—26
Biomedical Applications

CHROMBIO. 493

COMPARATIVE DETERMINATION OF PLASMA PHOSPHOLIPIDS BY AUTOMATED GAS—LIQUID CHROMATOGRAPHIC AND MANUAL COLORIMETRIC PHOSPHORUS METHODS

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(Received October 25th, 1979)

SUMMARY

Plasma samples obtained during a prevalence study of hyperlipemia in a free-living urban population were analyzed for phosphatidylcholine, sphingomyelin and lysophosphatidylcholine content by automated high-temperature gas—liquid chromatographic (GLC) and manual colorimetric phosphorus (thin-layer chromatographic, TLC) methods. The GLC estimates were obtained from a quantitative analysis of the diacylglycerol, ceramide and monoacylglycerol moieties released from the parent phospholipids by digestion with phospholipase C, while the TLC estimates were derived by manual colorimetric phosphorus analyses of the individual phospholipid classes resolved by TLC. On samples analyzed over a two-year period the methods gave excellent correlation for the total phospholipids (r = 0.98), phosphatidylcholine (r = 0.98) and sphingomyelin (r = 0.90), but resulted in a poor agreement for lysophosphatidylcholine (r = 0.69). Comparable results were obtained for estimates of these phospholipids in plasma very low density, low density and high density lipoproteins. The between-method coefficient of variation ranged from 3 to 5% for phosphatidylcholine and from 5 to 10% for sphingomyelin. The relative error for the estimates of lysophosphatidylcholine ranged from 10 to 25%, and was due to the inclusion in the GLC estimates of a variable proportion of plasma free monoacylglycerols. Other differences between the two methods are due to various analytical errors and biases inherent in the two techniques. The within-day, within GLC, relative error averaged 1% for phosphatidylcholine, 3% for sphingomyelin and 5% for lysophosphatidylcholine. The apparent high precision and accuracy of the GLC method recommend it as an alternative to conventional direct methods of phospholipid analyses based on TLC isolation of lipid classes and colorimetric measurements of their phosphorus content. The GLC analyses of the plasma phospholipids are particularly convenient in conjunction with GLC measurements of plasma cholesterol and triacylglycerois, where a smaller throughput of samples is not a limitation and where both total amount and relative proportion of the lipids are of interest.

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INTRODUCTION

Individual neutral lipid classes of plasma can be readily separated and quantitatively estimated by conventional [1—5] and automated [6—11] high-temperature gas—liquid chromatography (GLC). The results obtained for plasma total cholesterol and total triacylglycerols compare closely to those derived by automated chemical methods (AutoAnalyzer) of analysis, when calibration is made with plasma or serum [6, 9]. These estimates may also include the neutral lipid moieties present in the phospholipids, provided they are quantitatively released by dephosphorylation with phospholipase C [2, 4, 10] or by pyrolysis [1], but extensive comparisons with chemical methods of analysis have not been made.

In the following study we have compared the results of the automated GLC and the manual phosphorus methods of analysis of phospholipids in over 100 samples of plasma and of individual lipoprotein fractions from normal subjects and patients with hyperlipoproteinemia. In general, the GLC method gives values which are within 5—10% of those obtained by the chemical assay. The differences are traced to various inherent errors and biases in the two methods of analysis.

MATERIALS AND METHODS

The synthetic neutral lipid and free fatty acid mixtures along with the tridecanoylglycerol internal standard were available in the laboratory from previous studies [9]. The synthetic samples of glycerophospholipids and sphingomyelins were purchased from Applied Science Labs., State College, Pa., U.S.A. The plasma samples of normal subjects and patients with hyperlipoproteinemia as well as the lipoprotein fractions were supplied by the Toronto-McMaster Lipid Research Clinic, Toronto, Canada. The lipoprotein fractions [very low density (VLDL), $d < 1.006$; low density (LDL), $d = 1.006$—1.063; high density (HDL_2), $d = 1.063$—1.125; HDL_3, $d = 1.125$—1.21] had been obtained by means of ultracentrifugation according to the description of Hatch and Lees [12]. Phospholipase C (α-toxin of *Clostridium welchii*, Type I) was purchased from Sigma, St. Louis, Mo., U.S.A. The TRISIL/BSA was obtained from Pierce, Rockford, Ill., U.S.A. Other reagents and solvents were of Fisher certified reagent grade and were not further purified. All glass-ware was rinsed with chloroform—methanol (2:1) prior to use.

Preparation of total lipid extracts

Total lipid extracts of plasma and of plasma lipoproteins were obtained with chloroform—methanol (2:1) essentially, as described by Folch et al. [13]. Plasma samples (0.2 ml each) were pipetted directly into 15-ml centrifuge tubes containing 3 ml of ice-cold methanol. After mixing on a Vortex mixer, 6 ml of chloroform were added to each tube and the contents were again mixed. The tubes were then left at room temperature for 30 min with occasional stirring. After adding 2.0 ml of 0.9% NaCl, the tubes were subjected to rigorous mixing and were centrifuged. The lower phases were withdrawn quantitatively to 8-ml glass vials after passing through a Pasteur pipette column of anhydrous Na_2SO_4 and were evaporated in a stream of nitrogen. To the centrifuge tube containing

residual plasma proteins and upper phase, 6 ml each of Folch lower phase were added. The tubes were stirred thoroughly for 3 min and centrifuged. The lower phases were collected into the original vials and dried. The process was repeated one more time. The extracts were not backwashed. To check the efficiency of the extraction method, the plasma samples were extracted three times with 9 ml each of chloroform—methanol—HCl (200:100:0.1). After centrifugation, the pooled extracts were washed with 1 *N* HCl, the lower phases neutralized with ammonia and washed with Folch upper phase containing NaCl. The lower phases were dried and subjected to phosphate analyses and/or thin-layer chromatography (TLC). The extent of extraction by the two methods was further ascertained by saponification of the residual protein from both extraction procedures with 1 *N* KOH, extracting the acidified reaction mixture with diethyl ether and quantitating any fatty acids recovered by GLC [14].

Preparation of neutral lipid extracts

Total neutral lipids of plasma were obtained by treatment of an isopropanol extract of plasma (19:1) with Zeolite [12], which removed phospholipids and other polar components but retained free fatty acids along with the neutral lipids in the solution. Alternatively, neutral lipids were isolated from the solvent front of the TLC plates developed with phospholipid solvents from total lipid extracts of whole plasma or of individual lipoprotein classes (see below).

Hydrolysis with phospholipase C

The plasma samples were digested with phospholipase C as previously described [2, 9]. EDTA (0.01%) plasma (0.2—0.5 ml) was added to a solution of 0.2—0.4 mg (1—2 units) of phospholipase C in 2—3 ml of 17.5 m*M* Tris buffer (pH 7.3) and 1.3 ml of 1% $CaCl_2$, and 1 ml of diethyl ether. The mixture was incubated with stirring for 2 h in tightly closed screw-cap vials at 30°. The reaction was terminated by the addition of five drops of 0.1 *N* HCl and extracting once with 10 ml of chloroform—methanol (2:1) containing 150—250 μg of tridecanoylglycerol. The solvent phases were separated by centrifuging for 10 min at 200 *g* after each extraction. The clear chloroform phase was passed through 2 g of anhydrous Na_2SO_4. The effluent was evaporated to dryness and diluted to a known volume from which aliquots were taken for the determination of the total lipid profile by GLC, and of any residual phospholipids by TLC and phosphorus determination.

Plasma lipoproteins were digested with phospholipase C under the general conditions described for whole plasma except that the Ca^{2+} concentration in the digestion medium was increased ten-fold to overcome the chelating effect of the extra EDTA present in the sedimentation media [9].

Thin-layer chromatography

For the measurement of phospholipid class composition the total lipid extracts were chromatographed on thin layers of silica gel H as described by Shaikh and Palmer [15]. The lipid extracts were applied as 2 cm wide bands to TLC plates preactivated for 1 h at 120°. The chromatograms were developed in paper-lined tanks containing chloroform—methanol—acetic acid—water (100:

45:20:7) as the developing solvent. The phospholipids were identified by comparison with known standards and were located after exposure to iodine vapour and/or spraying the plates with the acid molybdate reagent of Dittmer and Lester [16].

Total neutral lipids were recovered as a single band from the top of the 2',7'-dichlorofluorescein-sprayed TLC plates and, after elution with chloroform, were silylated and subjected to GLC examination as described below.

Analysis of phosphorus

The phospholipid contents of any thin-layer fractions were determined by the method of Bartlett [17] after direct digestion of the lipid-containing silica gel in perchloric acid—sulfuric acid (1:2) mixture. The phospholipid content of total lipid extracts was determined directly. Standard curves for phosphate were prepared daily using appropriate reagents. The error of the method was ± 1% for components making up more than 5% of the total.

Automated gas—liquid chromatography

The automated analyses of the plasma and lipoprotein total lipids were performed following preliminary dephosphorylation and trimethylsilylation as previously described [7, 9]. For this purpose a Hewlett-Packard Model 5700 automatic gas chromatograph was equipped with dual nickel columns (54 × 0.2 cm I.D.) packed with 3% OV-1 on Gas-Chrom Q (100—120 mesh) as supplied by Applied Science Labs. The peak areas were measured by means of an electronic integrator, the output of which was recorded on a punched paper tape. The tape was processed by means of a Hewlett-Packard 8900 calculator using appropriate computer programs as previously described [6, 7]. The peak areas were calculated in relation to the tridecanoylglycerol internal standard and were expressed as mg%.

Calculations

Total phospholipid content of a sample was determined by summing the areas of peaks 22—24 and 34—42, and multiplying by calibration factors derived from standard lysophosphatidylcholine (LPC), phosphatidylcholine (PC) and sphingomyelin (SPH). Due to frequent poor reproducibility of the automated integration of the peak areas in the C_{40}—C_{42} carbon number region, an alternative calculation was devised to obtain the total PC content from the areas of carbon numbers C_{36} and C_{38}, and of SPH from the area of carbon number C_{34}, all of which are easily measured, as follows. Total PC = total DG × 1.28, where total DG = corrected $(C_{36} + C_{38})/0.81$, and corrected $C_{36} + C_{38} = (C_{36} + C_{38}) - C_{34}$ ceramide; with C_{34} ceramide = $C_{34} - 0.051 \times C_{36}$. The total SPH = total ceramide × 1.28, where total ceramide = C_{34} ceramide × (0.758/0.30). The factor 0.758 is the ratio of the response factors for ceramide and diacylglycerol trimethylsilyl (TMS) ethers (0.758=0.681/0.898). The factors 0.81 and 0.30 represent the diacylglycerol and ceramide fractions, respectively, measured in the total PC and SPH from large plasma pools. The multiplication factor 1.28 converts the ceramide and diacylglycerol moieties into the corresponding phosphorylcholine derivatives.

Statistical analyses

The evaluation of the GLC procedure for phosphatidylcholine and sphingomyelin determination was modeled on a comparable study of methodology reported for total cholesterol and triacylglycerols [9]. Systematic errors were measured by the difference between average GLC values and the manual phosphorus or target values using linear regression and correlation methods [18]. Random error was estimated by the variance or standard deviation. The relative error values are averages of percentage deviations defined as: relative error = (GLC value − phosphorus value) × (100/phosphorus value). A coefficient of variation between duplicates was calculated using the formula C.V. % = $(100\ \sqrt{d^2/2})/\bar{X}$, where d is the difference between duplicates and $\bar{X}$ is the mean.

The within-day standard deviation was used as the measure of within-day variability. The overall standard deviation was used as the measure of the variability of a single determination of a quality control sample by the GLC method.

RESULTS

Molecular weight distribution of plasma phosphatidylcholines and sphingomyelins

Fig. 1 shows the carbon number profiles of the diacylglycerol moieties of the total plasma phosphatidylcholines and of the ceramides of total plasma sphingomyelins following isolation of each phospholipid class by TLC, enzymic dephosphorylation and trimethylsilylation. It is seen that the diacylglycerol moieties range in carbon number from 34 to 42, with the carbon numbers 34 and 42

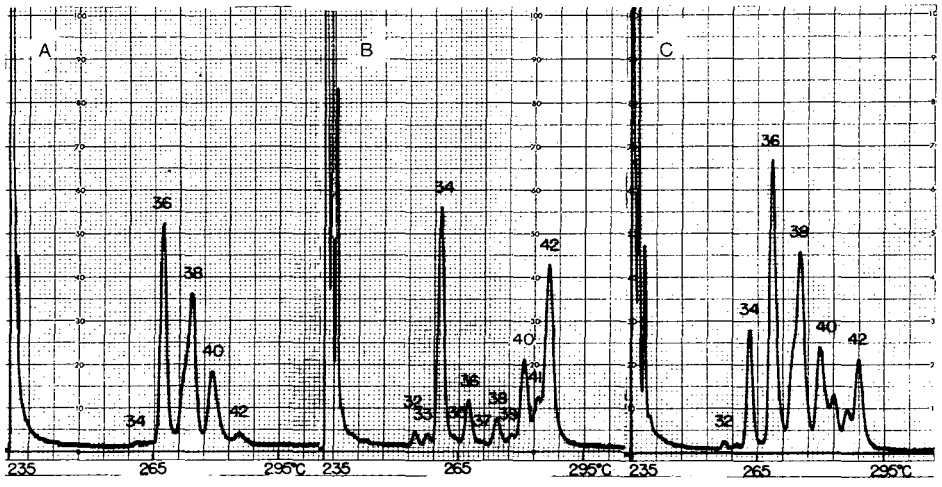

Fig. 1. GLC analyses of diacylglycerol and ceramide moieties of choline phospholipids of whole plasma following a prior TLC isolation, enzymic dephosphorylation and trimethylsilylation. A, Diacylglycerols; B, ceramides; C, mixture of 60 parts A and 40 parts B. Peaks 34—42, TMS ethers of diacylglycerols with 32—40 acyl carbons; peaks 32—42, TMS ethers of ceramides with 30—40 ceramide carbons. Temperature program as shown. Other GLC conditions are given in the text.

contributing only minor amounts (<5%). The ceramide moieties of the plasma sphingomyelins also range in carbon number from 34 to 42, but in this case the carbon numbers 34 and 42 make major contributions to the total (<30%). Dietary fats have a marked influence on the proportion of carbon numbers 36 and 38 of the diacylglycerol moieties of plasma phosphatidylcholines, but no significant changes take place in the overall carbon number range on normal diets. In general these lipid profiles hold also for the individual lipoprotein classes, but there are certain differences that require analytical attention. Both dietary and lipoprotein differences are illustrated in Table I. The samples were

TABLE I

CARBON NUMBER DISTRIBUTION OF PHOSPHATIDYLCHOLINES FROM PLASMA LIPOPROTEINS OF NORMOLIPEMIC SUBJECTS ON SATURATED AND UNSATURATED FAT DIETS

The isocaloric diets contained 20% protein, 40% carbohydrate and 40% fat. The fats had polyunsaturated:saturated fatty acid ratios of 0.25 (saturated fat) and 4 (polyunsaturated fat) [A. Kuksis et al. (1975), unpublished results]. Values are given as the mean ± S.D. for four subjects.

Carbon No.	Saturated fat			Unsaturated fat		
	VLDL (%)	LDL (%)	HDL_3 (%)	VLDL (%)	LDL (%)	HDL_3 (%)
34	1.9±0.5	3.1±0.3	2.5±0.2	1.9±0.4	3.7±0.3	2.1±0.4
36	42.2±2.6	41.1±0.9	36.4±1.2	38.7±4.1	40.4±3.9	34.6±3.6
38	38.7±2.7	37.9±1.6	40.1±0.8	43.7±2.2	40.4±2.4	42.8±1.9
40	15.1±1.0	15.4±0.4	18.3±0.6	13.5±2.9	12.6±1.4	17.2±2.8
42	2.1±0.5	2.5±0.3	2.7±0.5	2.2±0.4	2.9±0.5	3.4±0.8
36+38	80.9±2.0	79.0±1.2	76.5±1.0	82.4±	80.8±3.1	77.4±3.0

taken from a controlled dietary study lasting two weeks on each diet and involved four normolipemic individuals [Kuksis et al. (1975), unpublished results]. It is seen that the unsaturated diet caused an increase in the proportion of the diacylglycerols with carbon number 38 in the VLDL, LDL and HDL_3, while the saturated diet increased the proportion of the diacylglycerols with carbon number 36 in these lipoprotein classes. On both diets the diacylglycerols of carbon number 40 were increased in the HDL fraction over that in any other plasma lipoprotein class. Despite these variations in the carbon number proportions, the sum of the carbon numbers 36 and 38 accounted for 81±1% of total diacylglycerol species on both diets in the VLDL and LDL fractions, while in the HDL fraction, this sum accounted for 77±1% of the total on both diets. In the total plasma lipid profile the sum of carbon numbers 36 and 38 averaged 80±2% of the total diacylglycerol species and could be used as an effective basis of quantitating total plasma phosphatidylcholines when difficulties were experienced with the resolution of the higher-molecular-weight peaks or with the automatic slope sensor of the peak area integrator. Under normal conditions diacylglycerophospholipids other than phosphatidylcholines would not be expected to contribute more than a few per cent of the total glycerophospholipid of whole plasma or of any plasma lipoprotein fractions [19].

Short-term (two weeks) dietary regimens had a slight effect on the carbon number profiles of the ceramide moieties of the plasma sphingomyelins. Greater differences were seen in the ceramide profiles of the different plasma lipoproteins. Fig. 2 compares the ceramide profiles of the LDL and HDL_3 lipo-

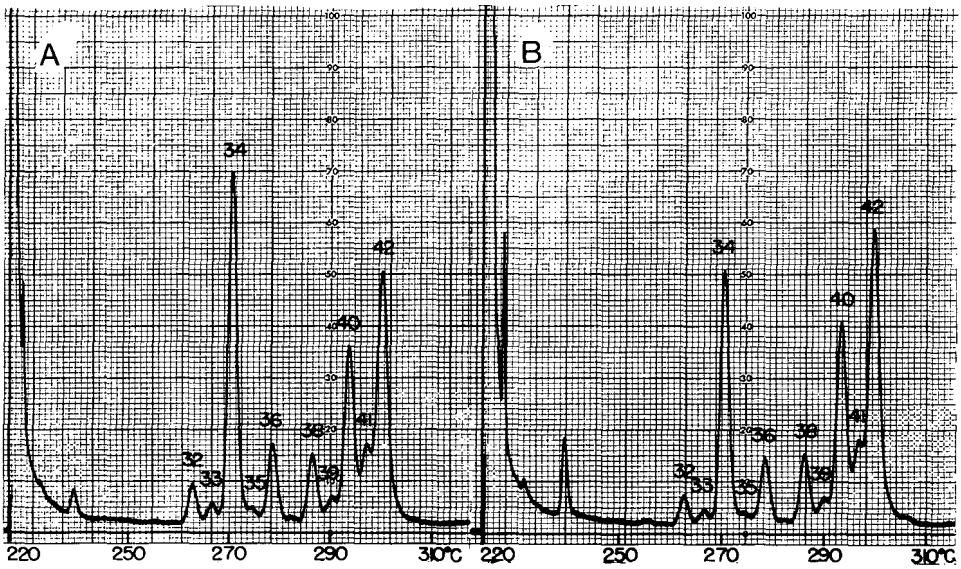

Fig. 2. GLC analyses of ceramide moieties of sphingomyelins of plasma lipoproteins of a healthy male. A, LDL; B, HDL_3. Peaks 32—42, *tert.*-butyldimethylsilyl ethers of ceramides with 30—40 ceramide carbons. Temperature program as shown. Other GLC conditions as given in the next.

proteins of a representative normolipemic subject. There is a significantly higher proportion of the longer chain ceramides in the HDL_3 than in the LDL fraction although there is no change in the overall range of the carbon numbers. Table II gives the carbon number proportions in the ceramides of the LDL and HDL_3 fractions obtained from four normolipemic subjects on free-choice diets. It is seen that the total carbon number range remains the same for all subjects in both lipoprotein classes, but that the proportion of the higher-molecular-weight species is significantly greater in the HDL_3 fraction. Carbon number 34 ceramides make up an average of 33% of the total ceramide population in the LDL and 24% in the HDL_3 fraction. These proportions can be used as a reliable basis for the calculation of the total ceramide content in these plasma lipoproteins [20]. Likewise, the total ceramide or sphingomyelin content of whole plasma could be calculated from the total lipid profile of the plasma on the basis of an average contribution of 30% for carbon number 34 to the total plasma sphingomyelin. This estimate, however, could be in serious error if the LDL and HDL proportions were significantly altered in a given plasma sample. The overall error of analysis of the isolated mixtures approaches the error of the reproducibility of the chromatographic system [6, 7, 9].

TABLE II

CARBON NUMBER DISTRIBUTION OF SPHINGOMYELINS FROM LOW AND HIGH DENSITY LIPOPROTEINS OF NORMOLIPEMIC SUBJECTS ON FREE-CHOICE DIETS

Subjects as in Table I. Kuksis et al. (1975), unpublished results. Underlined values are specifically mentioned in the text. Values are given as per cent of total.

Carbon	Subject 1		Subject 2		Subject 3		Subject 4		Average	
No.	LDL	HDL_3	LDL	HDL_3	LDL	HDL_3	LDL	HDL_3	LDL	HDL_3
32	3.1	2.7	4.3	3.5	2.7	1.8	2.5	1.7	3.2	2.4
33	1.8	1.4	2.3	1.6	1.6	1.0	1.6	0.9	1.8	1.2
34	31.4	24.3	33.1	26.0	33.7	22.7	35.0	24.3	33.3	24.3
35	1.3	0.9	1.5	1.1	1.2	0.8	2.0	0.6	1.5	0.8
36	7.5	6.5	7.3	6.8	6.6	5.7	8.6	6.9	7.5	6.5
37	0.5	0.7	0.9	0.5	0.6	0.4	0.6	0.1	0.7	0.4
38	6.0	6.4	5.4	5.4	4.9	5.5	4.6	4.2	5.2	5.4
39	2.0	1.9	2.1	2.1	1.8	1.8	1.2	0.9	1.8	1.7
40	16.1	18.9	14.6	17.7	15.7	20.2	12.4	15.3	14.7	18.0
41	5.6	5.5	6.2	6.2	5.4	5.9	4.8	6.3	5.5	6.0
42	24.4	30.3	21.9	28.9	24.7	32.7	26.5	38.6	24.4	32.6
43	0.4	0.8	0.6	0.4	0.9	1.5	0.2	0.1	0.5	0.7

Interference from free mono- and diacylglycerols and ceramides

Fig. 3 shows the GLC profiles of the plasma neutral lipids and of the corresponding total lipids following dephosphorylation with phospholipase C. It is seen that the neutral lipids of a normolipemic subject contain very little free diacylglycerol (peaks 36 and 38) or free ceramides (peak 34). Furthermore, there is very little overlap between the longer chain diacylglycerols and ceramides, and the shorter chain cholesteryl esters (peak 41). There is also very little free monoacylglycerol (peaks 22 and 24) in the neutral lipid chromatograms. A marginal increase takes place following phospholipase C hydrolysis of the plasma lysophosphatidylcholines, which, however, is not complete (see below). In addition, the monoacylglycerol peaks are split up into two or three components due to a partial resolution of the saturated and unsaturated species and of the positional isomers (*sn*-1(3)- and *sn*-2-enantiomers). The unsaturated monoacylglycerols migrate ahead of the saturated ones, as do the *sn*-2-isomers, when compared to the *sn*-1(3)-isomers, although not to the same extent. As a result the quantitation of the monoacylglycerols by GLC is erratic and accurate estimates of lysophosphatidylcholines may be difficult to obtain. Table III gives the results of quantitative estimation of free diacylglycerols and monoacylglycerols in the plasma of a representative number of normolipemic and hyperlipemic subjects selected at random from an urban population. It is seen that the free diacylglycerols (peaks 36 and 38) range from 2 to 59 mg% over a triacylglycerol range of 65—2032 mg% and are related closely to the total lipid and especially the total triacylglycerol level of the plasma, $r = 0.96$ and $r = 0.95$, respectively. The correlation with phosphatidylcholine, however, was relatively poor ($r = 0.71$). Since the elevation of free diacylglycerols accompanying elevated total triacylglycerols is usually associated with an increase in plasma phospholipids, the plasma free diacylglycerols would not be expected to

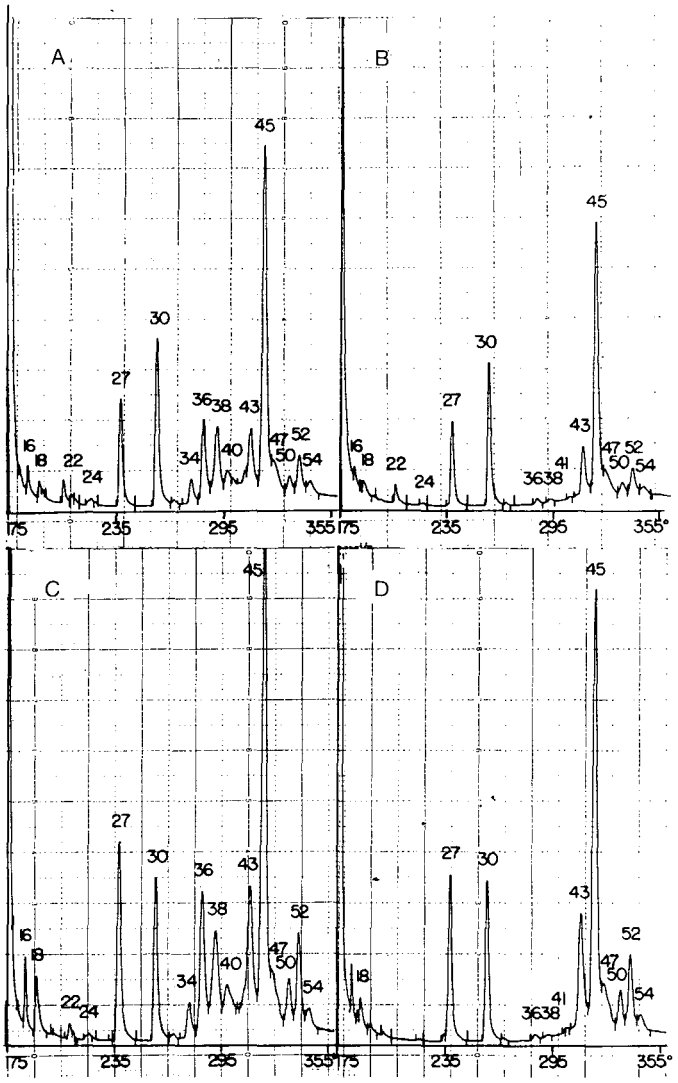

Fig. 3. GLC profiles of corresponding total and neutral plasma lipids of a normolipemic and a hyperlipemic subject. A, Total lipids of normolipemic subject; B, neutral lipids of normolipemic subject; C, total lipids of hyperlipemic subject; D, neutral lipids of hyperlipemic subject. Peaks 16 and 18, TMS esters of free fatty acids with 16 and 18 acyl carbons; peaks 22—24, di-TMS ethers of monoacylglycerols with 16 and 18 acyl carbons; peak 27, TMS ether of cholesterol; peak 30, tridecanoylglycerol (internal standard); peak 34, TMS ether of palmitoylsphingosine; peaks 36—42, TMS ethers of diacylglycerols with a total number of 34—40 acyl carbons; peaks 43—47, cholesteryl esters of fatty acids with a total number of 16—20 acyl carbons; peaks 48—54, triacylglycerols with a total number of 48—54 acyl carbons. Sample size 1 μl of an approximately 1% solution in silylation mixture. Attenuation: 100 times full sensitivity. Temperature program as shown. Other GLC conditions as given in the text.

TABLE III

ESTIMATES OF FREE MONOACYL- AND DIACYLGLYCEROL CONTENT IN PLASMA OF NORMOLIPEMIC AND HYPERLIPEMIC SUBJECTS

Plasma samples	Lipid classes* (mg%)						
	MG	DG	TG	TC	PC	(MG/TG) x 100	(DG/TG) x 100
Normal							
1	2	4	121	202	184	1.6	3.3
2	1.9		65	170	120	2.9	
3	2	2	92	167		2	2.4
4	2	4.4	162	220	172	1.2	2.7
5	2	2	69	189	163	2.8	2.8
6	0.9	2	89	214		1.0	2.2
7	2	3.0	105	157	132	1.9	2.8
8	2.1	1.8	95	194		2.2	1.9
9	2	6.0	161	234	189	1.2	3.7
10	2	4.2	77	149	155	2.5	5.4
11	3	2.7	104	192	195	2.8	2.6
12	2	2	254	113	115	0.8	0.8
*Hyperlipemic***							
1	2	7.0	106	312	192	1.8	6.6
2	2	2.4	89	300	193	2.2	2.7
3	2	3.5	73	330	272	2.7	4.7
4	4	8.0	126	258	216	3.2	6.3
5	5	7.3	114	368	294	4.4	6.4
6	2.8	7.4	169	382	176	1.6	4.3
7	2.8	19.1	640	282	281	0.4	3.0
8	3.7	6.0	236	367	237	1.5	2.5
9	5	16.1	262	463	304	1.9	6.1
10	3	11.4	231	300	238	1.2	4.9
11	2	9.5	243	275	209	0.8	3.9
12	2	24.7	348	327	253	0.5	7.0
13	2.1	13.1	266	490	494	0.2	4.9
14	2	21.8	328	253	195	0.6	6.6
15	2	10.9	294	268	204	0.7	3.7
16	5.4	5.2	179	188	153	3.0	2.9
17	2	59.4	2008	387	517	0.01	2.9
18	6.7	9.2	423	210	184	1.6	2.2
19	9.2	42.5	851	175	209	1.1	5.0
20	13	58.1	1828	424	415	0.7	3.2
21	8	52.8	2032	365	427	0.4	2.6
22	2	9.6	194	217	166	0.1	4.9
*Intralipid****							
1	3.2	10.1	148	257	227	0.2	6.8
2	2	1.6	121	140	105	1.6	1.3
3	3	11.1	242	240	104	1.2	4.6
4	2.0	12.4	392	397	253	0.5	3.2

*MG, peaks 22 + 24; DG, peaks 34 + 36 + 38; TG, TC and PC as described in the text.

**As by phenotyping algorithm in the Manual of Laboratory Operations, Lipid Research Clinics Program, Vol.1, Lipid and Lipoprotein Analysis, NHLI, NIH, Bethesda, Md., 1974.

***Fasting samples taken 16 h after infusion of moderate doses of Intralipid.

contribute significantly (more than 5%) to the estimate for total phosphatidylcholine, as determined on the basis of the diacylglycerol released by phospholipase C.

Only the hyperlipemic samples contained a measurable peak with carbon number 34, which could have represented either free ceramide or free diacylglycerol, or both. However, even if the entire peak was attributed to ceramides, this would not alter the GLC estimate of plasma sphingomyelin significantly. These findings and their interpretation are in agreement with previous work [21], which had indicated very low levels of free ceramides in human plasma.

Table III also gives estimates for the plasma free monoacylglycerol content, which could falsely inflate the estimates for plasma lysophosphatidylcholine based on the monoacylglycerol released by phospholipase C. It is seen that the free monoacylglycerol levels range from zero to about 13 mg%, with the highest levels of monoacylglycerols again being found in the samples containing the highest total lipid and triacylglycerol levels, although the overall correlation was not as good ($r = 0.62$) as for free diacylglycerols. Very few of the hyperlipemic samples contain more than 5 mg% free monoacylglycerol. These findings also are in agreement with previous work [19]. It is shown below (see Table VI) that the total monoacylglycerol levels measured following phospholipase C digestion of plasma lysophosphatidylcholine range from 5 to 20 mg% and therefore its plasma levels are seriously compromised by the presence of free monoacylglycerols in the blood. It may also be noted that the region of the chromatogram which contains the free monoacylglycerols frequently also contains other unidentified substances at a low but variable level.

A similar examination of the total and neutral lipid profiles of the major plasma lipoprotein classes (results not shown) revealed comparable low levels of free diacylglycerols, in proportion to the triacylglycerol content of the fraction [19]. Clearly, the small amounts of free diacylglycerols and free ceramides are not likely to influence the estimation of the plasma phosphatidylcholines and sphingomyelins based on their neutral lipid moieties released by phospholipase C, either in whole plasma or in isolated fractions of plasma lipoproteins.

GLC of plasma glycerophospholipids and sphingomyelins

Table IV shows the reproducibility of the quantitative estimates for total phosphatidylcholine and sphingomyelin in whole plasma of normolipemic young adults [10] analyzed over a short period of time. A total of 17 samples was examined in quadruplicate and the results calculated separately for the carbon numbers corresponding to the major ceramides and diacylglycerols. The peak areas of C_{36} and C_{38} were estimated most precisely with an average coefficient of variation of less than 1%. The peak areas of C_{34} and C_{40} were also estimated with a relatively high precision as indicated by an average coefficient of variation of less than 2%. The peak areas for C_{41} and C_{42} were estimated much less precisely with the coefficients of variation ranging from 5 to 10%, and some values exceeding 50%. Much of this variation was due to the variable contribution of the cholesteryl myristate peak as well as an incomplete resolution of the long-chain diacylglycerols and ceramides emerging in this region of the chromatogram. The values derived for total diacylglycerols and ceramides on the basis of calculations from the more precisely measured peaks, as de-

TABLE IV

REPRODUCIBILITY OF QUANTITATIVE ESTIMATES OF INDIVIDUAL CARBON NUMBERS ON REPEAT ANALYSIS OF NORMOLIPEMIC PLASMA SAMPLES

Values are given in mg % (mean ± S.D. of quadruplicate analyses).

Plasma	Carbon numbers of diacylglycerols and ceramides						Total phospholipids*	
samples	34	36	38	40	41	42	PC	SPH
1	9.27±0.03	36.16±0.08	38.38±0.23	13.76±0.17	8.77±0.08	4.12±0.16	106.6	23.8
2	10.49±0.04	42.13±0.10	39.93±0.09	12.18±0.09	11.07±0.06	3.84±0.04	117.1	26.7
3	10.62±0.25	48.01±1.95	40.24±2.03	12.99±1.09	11.40±1.21	4.34±0.74	127.2	26.1
4	10.28±0.03	42.63±0.12	43.17±0.10	14.11±0.10	10.68±0.07	4.31±0.07	123.4	25.9
5	9.67±0.05	39.05±0.17	40.81±0.15	14.61±0.16	10.21±0.05	4.06±0.34	114.6	24.6
6	9.24±0.09	39.06±0.21	39.06±0.10	14.02±0.18	8.35±0.05	3.85±0.21	112.5	23.2
7	8.78±0.10	37.75±0.26	34.70±0.23	10.98±0.14	8.85±0.07	3.49±0.51	104.2	21.9
8	7.48±0.23	33.40±0.12	34.17±0.57	11.49±2.97	7.67±0.14	4.69±0.05	98.1	18.5
9	8.37±0.11	37.93±0.08	38.85±0.46	15.24±0.12	8.15±0.12	4.40±0.14	111.7	20.6
10	9.20±0.19	45.46±0.21	43.12±0.16	15.66±0.46	8.71±1.23	4.95±1.29	129.7	22.0
11	7.06±0.11	34.37±0.07	34.46±1.01	15.87±0.02	6.21±3.23	4.10±1.10	100.9	17.0
12	8.74±0.47	39.71±0.09	42.26±0.11	15.46±3.79	8.64±0.96	4.94±0.39	119.5	21.5
13	9.30±0.34	45.56±0.06	45.08±0.11	16.00±0.09	8.74±0.13	4.66±0.06	132.9	22.3
14	8.52±0.57	37.60±0.22	39.80±0.78	16.68±0.33	8.18±0.09	4.59±0.45	112.4	21.1
15	8.16±0.53	37.81±0.24	37.65±0.18	12.44±3.80	4.97±3.23	3.28±1.71	109.4	19.9
16	7.84±0.68	35.78±1.40	36.94±2.24	14.03±1.22	6.63±2.65	3.51±2.02	105.9	19.2
17	7.63±0.54	38.30±0.23	40.69±0.86	11.13±3.32	7.59±0.60	3.07±0.49	116.4	18.2
Average							114.3±9.9	21.9±2.9

*Total phosphatidylcholine and sphingomyelin estimated as explained in the text.

scribed under Methods, show coefficients of variation averaging about 1% and 2%, respectively. The estimates for total phosphatidylcholine (114.3±9.9 mg%) and sphingomyelin (21.9±2.9 mg%) are significantly lower than the values (122.6 and 33.9 mg%, respectively) calculated from the data reported by Noel et al. [22] for these phospholipids from a much older population.

Table V gives the reproducibility of a decaplicate estimation of the peak

TABLE V

REPRODUCIBILITY OF QUANTITATIVE ESTIMATES OF INDIVIDUAL CARBON NUMBERS ON REPEAT PROCESSING OF PLASMA SAMPLES OF INCREASING LIPID CONTENT

Values are given in mg % (mean ± S.D. of decaplicates). A and B represent replicate GLC analyses only.

Plasma samples*	Ceramide and diacylglycerol carbon numbers			Total phospholipids**	
	34	36	38	PC	SPH
LRC 1A	13.51±0.19	50.75±0.75	54.68±0.82	150.089±2.34	34.93±0.54
LRC 1B	13.40±0.59	49.60±2.67	53.22±2.83	147.59 ±6.62	34.95±1.53
LRC 2A	17.78±0.60	59.38±1.97	64.22±4.29	172.58 ±8.15	47.40±1.91
LRC 2B	16.93±0.33	59.22±0.81	62.66±0.78	171.58 ±2.19	44.32±1.16
LRC 3	24.46±0.81	74.22±1.45	78.38±1.18	209.50 ±3.06	66.17±2.37

*LRC 1—3 represent control samples prepared by adding known amounts of VLDL or LDL to a common plasma pool.

**Total phospholipid estimated as explained in the text.

areas of carbon numbers C_{34}—C_{38} in a series of control pools prepared by adding VLDL or LDL to a common plasma pool to yield samples of increasing total lipid content. Each replicate constituted an independent digestion, extraction, dilution with the internal standard and derivatization of an aliquot of the plasma. It is seen that the precision of estimation of the completely resolved peaks is again very good. The coefficients of variation for the estimates of total phosphatidylcholine and sphingomyelin range from 1 to 4%. Furthermore, the values derived for these phospholipids by GLC for the various concentration levels compare closely to those expected from the knowledge of the composition of the lipoprotein fractions combined to obtain the synthetic plasma samples [Breckenridge (1975), unpublished results].

Fig. 4 shows a series of plots obtained for the estimates of the various carbon numbers of ceramides and diacylglycerols in a total of 137 A and B pairs of plasma samples analyzed over a period of one year without the knowledge of the sample identity. The correlation coefficients between the diacylglycerol peaks are: C_{36}, 0.95; C_{38}, 0.96; C_{40}, 0.96; C_{41}, 0.93 (103 pairs of data only); and C_{42}, 0.97 (33 pairs of data only). The correlation is best for the larger and more completely resolved peaks. The reproducibility is especially high for the diacylglycerol peaks C_{36} and C_{38}, which are used in the indirect estimation of the total phosphatidylcholine content. However, the correlation coefficient for the ceramide peak C_{34} used in the computation of total sphingomyelin content is also satisfactory (r = 0.88). The legend to Fig. 4 gives the slopes and intercepts of each regression line. In general these values are of the same order as

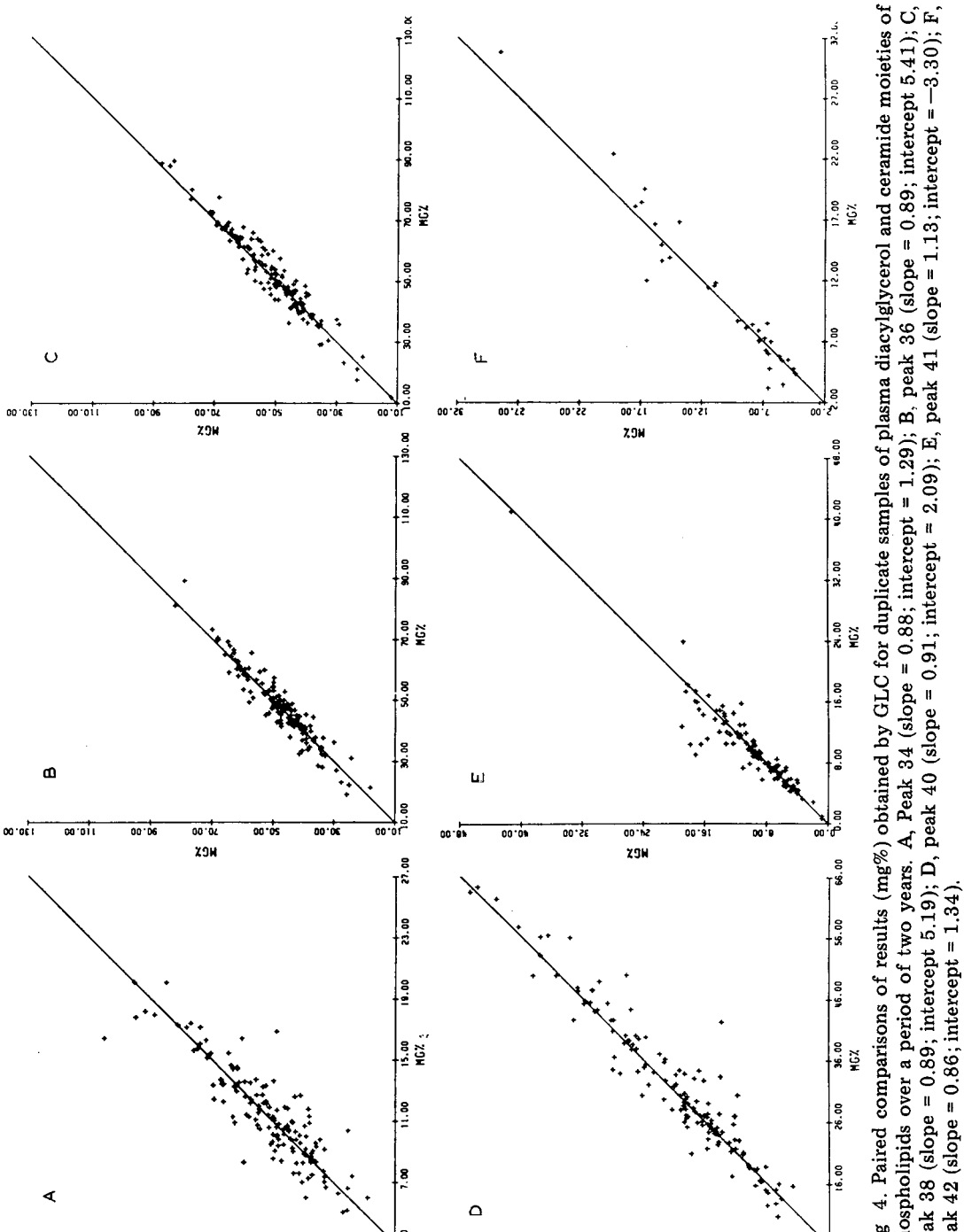

Fig 4. Paired comparisons of results (mg%) obtained by GLC for duplicate samples of plasma diacylglycerol and ceramide moieties of phospholipids over a period of two years. A, Peak 34 (slope = 0.88; intercept = 1.29); B, peak 36 (slope = 0.89; intercept 5.41); C, peak 38 (slope = 0.89; intercept 5.19); D, peak 40 (slope = 0.91; intercept = 2.09); E, peak 41 (slope = 1.13; intercept = −3.30); F, peak 42 (slope = 0.86; intercept = 1.34).

those recorded for replicate analyses of the same sample over short periods of time.

Fig. 5 shows the paired comparisons for the total phosphatidylcholine (PC) and sphingomyelin (SPH) content as calculated from the C_{36} + C_{38} and C_{34} peaks, respectively. Again excellent correspondence is obtained between the independent GLC analyses carried out on stored samples over a period of one year. The correlation coefficient (r) for the phosphatidylcholine is 0.96 and for sphingomyelin it is 0.88. The latter value is the same as that for peak C_{34} from which the sphingomyelin values were calculated. A fair correlation is also observed between the ratios PC:SPH in the paired samples, r = 0.80. The legend to Fig. 5 gives the actual slopes of the various linear relationships along with the corresponding intercepts. The correlations of the C_{40}—C_{42} peaks to each other

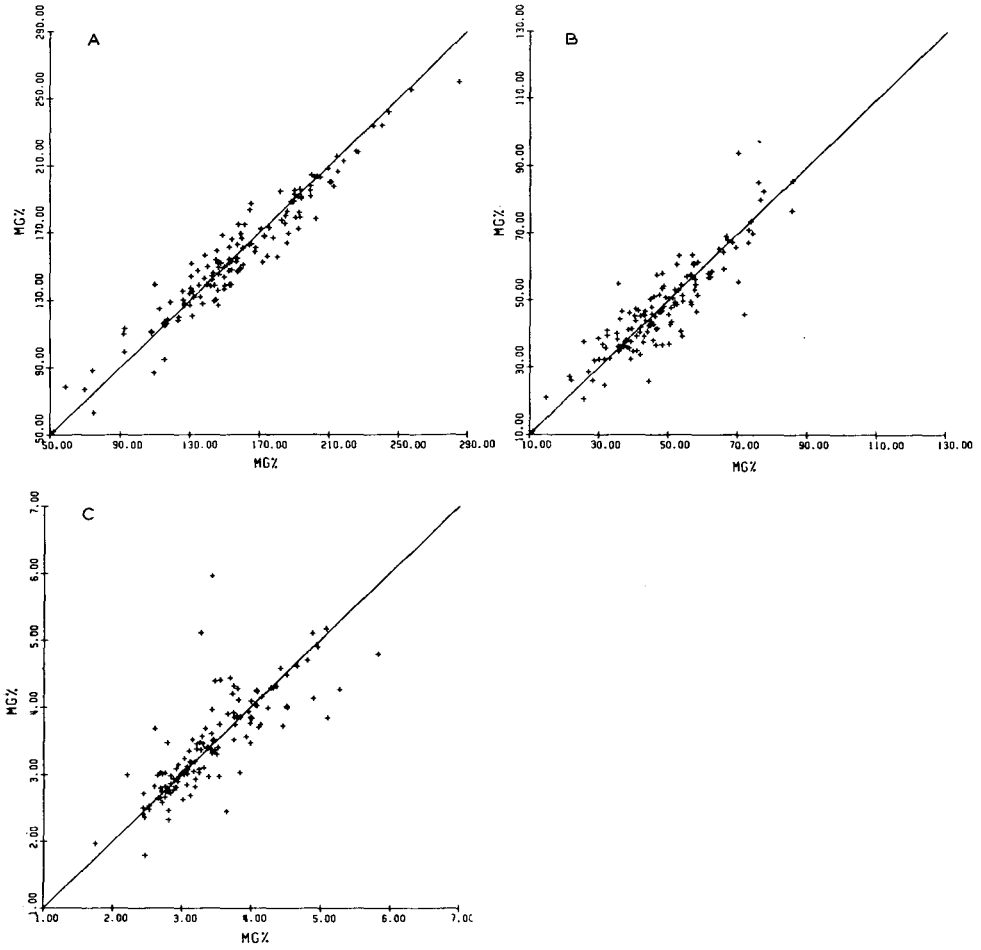

Fig. 5. Paired comparisons of results (mg%) obtained by GLC for duplicate samples of diacylglycerol and ceramide moieties of plasma phospholipids over a period of two years. A, PC (slope = 0.89; intercept = 16.33); B, SPH (slope = 0.82; intercept = 5.17); C, PC:SPH (slope = 0.86; intercept = 0.73).

and to the total phosphatidylcholine values were much poorer and indicated that the latter peak areas were not separately recorded in a manner sufficiently reproducible for a precise measurement of the molecular species of the phospholipids in these plasma samples. Poor correlations were also obtained for the levels of the lysophosphatidylcholines calculated from the total monoacylglycerol content in the A and B pairs of plasma samples (results not shown).

Comparative studies with manual phosphorus (TLC) methods

Table VI gives the statistical results of parallel analyses of 58 samples of

TABLE VI

COMPARISON OF GLC AND TLC ANALYSIS OF PHOSPHOLIPIDS IN WHOLE PLASMA AND ISOLATED LIPOPROTEIN CLASSES

Values are given as mean ± S.D.

Lipid classes*	No. of samples (*n*)	Automated GLC (mg %)	Manual phosphorus (mg %)
Total plasma			
PC	58	176.7±84.1	165.2±80.9
SPH	58	46.1±14.4	47.0±17.0
Total	58	222.8±92.4	212.3±94.6
PC:SPH	58	3.9± 1.27	3.5± 0.83
VLDL, LDL, HDL			
PC	23	70.9±30.1	72.9±29.7
SPH	23	18.9±11.6	23.9±12.0
Total	23	89.8±36.9	96.9±37.0
PC:SPH	23	5.24±3.24	3.6± 1.7

*Abbreviations as given in the text.

whole plasma and 23 samples of VLDL, LDL and HDL_3 subfractions of it by the GLC and TLC methods. The values are generally in good agreement. Thus, the average value derived for total plasma phosphatidylcholine for the entire population was 177 mg% (GLC) and 165 mg% (TLC), while the corresponding values for sphingomyelin were 46 mg% and 47 mg%. The somewhat lower TLC value for phosphatidylcholine is due to a partial hydrolysis of the phosphatidylcholines upon storage of the plasma samples, an essentially complete agreement being obtained for the sum of the phosphatidyl and lysophosphatidylcholines by the two methods. Furthermore, the estimates for the total plasma phospholipids of both normolipemic and hyperlipemic subjects obtained by the GLC method corresponded to the range calculated from the data reported by Phillips and Dodge [23]; for example, 141—182 mg% (phosphatidylcholine) and 36—38 mg% (sphingomyelin) for a normolipemic population of a corresponding age, assuming molecular weights of 783 and 740, respectively, for the two phospholipid classes.

However, part of the difference is also due to the possible inclusion in the estimates for phosphatidylcholine of the estimates for phosphatidylethanolamine, which is present in plasma to about 4% of the total lipid phosphorus [19].

Fig. 6 shows a series of plots correlating the estimates for plasma phospha-

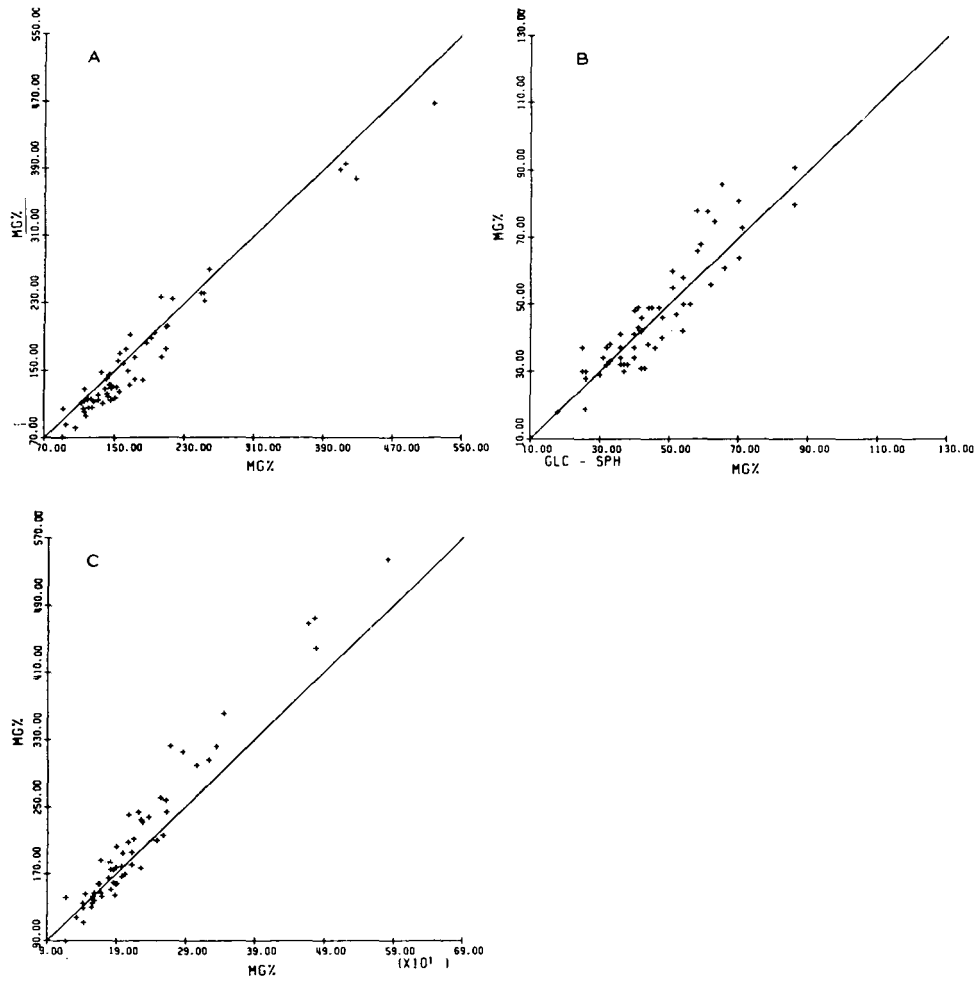

Fig. 6. Comparison of results (mg%) obtained by GLC (ordinate) and manual phosphorus (TLC) (abscissa) methods for whole plasma from subjects with a wide range of total lipids. A, PC (slope = 0.94; intercept = —1.01); B, SPH (slope = 1.06; intercept = —1.91); C, total (slope = 1.00; intercept = –10.04).

tidylcholines, sphingomyelin and total phospholipids as derived by the GLC and the manual phosphorus methods. For this purpose the phosphorus determinations were made on the individual phospholipid classes separated from plasma total lipid extracts by TLC, while the GLC estimates of the choline-containing phospholipids are based on the quantitation of the C_{36} + C_{38} and C_{34} peaks, representing the diacylglycerol and ceramide moieties, respectively. The estimates are derived from parallel analyses of a total of 62 samples of normolipemic and hyperlipemic plasmas. An excellent correlation is obtained for the phosphatidylcholine (r = 0.98) and for total plasma phospholipids (r = 0.98), with only slightly less effective agreement between the sphingomyelin analyses (r = 0.90). These correlations are somewhat inferior to those realized for du-

TABLE VII

COMPARISON OF ESTIMATES FOR PLASMA LYSOPHOSPHATIDYLCHOLINE AS OBTAINED BY GLC AND MANUAL PHOSPHORUS (TLC) METHODS

Plasma samples	GLC				TLC			
	LPC* (mg%)	PC (mg%)	LPC+PC (mg%)	LPC×100/(LPC+PC)	LPC* (mg%)	PC (mg%)	LPC+PC (mg%)	LPC×100/(LPC+PC)
Normal								
1	9	144	153.2	5.8	18.2	144.7	162.9	11.1
2	12	262	274.3	4.3	22.6	218.5	241.1	9.3
3	30	104	134.2	22.3	39.9	114.3	152.2	26.2
4	12	121	133.1	9.0	21.5	105.8	127.4	16.9
5	12	135	147.6	8.1	23.6	112.2	135.8	17.4
6	8	132	149.2	5.0	17.3	114.9	132.2	13.1
7	6	116	121.7	4.9	11.2	100.9	112.2	10.0
8	9	132	141.3	6.3	15.5	120.8	136.3	11.3
9	9	124	133.6	6.7	14.7	106.3	121.1	12.1
10	9	156	155.1	6.2	17.0	124.9	141.9	11.9
11	9	142	151.5	5.9	18.0	122.3	140.4	12.8
12	12	231	243.4	4.9	19.8	201.9	221.7	8.9
13	12	137	148.9	8.0	19.9	110.6	130.5	15.2
14	9	167	176.7	5.1	14.6	132.9	147.5	9.8
15	12	149	161.1	7.4	21.7	116.1	137.9	15.8
16	9	118	127.1	7.1	19.4	96.3	115.7	16.7
17	12	139	151.6	7.9	24.4	127.6	152.0	16.0
18	9	142	151.7	5.9	19.7	118.7	138.3	14.2
19	8	146	153.8	4.8	16.5	128.4	144.9	11.4
20	8	182	189.6	4.0	17.5	139.2	156.7	11.1
21	6	151	157.1	3.8	14.1	116.8	130.9	10.8
22	9	165	174.4	5.2	19.4	150.3	169.7	11.4
23	12	146	158.3	7.6	16.0	113.6	129.2	5.8
24	12	145	147.8	8.1	24.9	128.6	153.5	16.2
25	23	106	128.3	17.5	31.2	82.1	113.3	27.5
26	12	161	172.9	6.9	24.3	132.7	157.0	15.4
27	8	146	153.9	4.9	18.0	131.8	149.8	12.0
28	5	115	119.5	3.7	7.9	104.3	112.3	7.0

*Hyperlipemic***								
1	15	192	207	7.2	21.8	171.2	193	11.2
2	12	193	205	5.8	17.9	176	193.9	9.2
3	8	216	223.5	3.3	15.9	189.1	204.9	7.7
4	12	249	251	4.7	23.5	242.0	265.6	8.8
5	12	281	293	4.0	20.5	242.3	262.9	7.8
6	9	237	246	3.6	18.7	237.2	255.9	7.3
7	15	259	274	5.5	28.5	269.8	298.3	9.5
8	9	211	220	4.0	15.5	203.3	218.8	7.1
9	14	209	222.5	6.1	13.8	182.9	196.8	7.0
10	30	409	439	6.8	23.8	390.2	414.0	5.7
11	23	253	275.3	8.1	19.9	235.3	255.2	7.8
12	15	473	485	3.1	23.4	371.5	395.0	5.9
13	8	195	202.5	3.7	13.8	165.8	179.6	7.7
14	12	204	216	5.5	12.7	166.3	178.9	7.1
15	27	517	544	5.0	23.9	468.8	492.7	4.8
16	14	209	222.5	6.1	11.6	176.1	187.7	6.2
17	14	415	428.5	3.1	19.7	397.3	416.9	4.7
18	31	427	458	6.7	24.5	378.5	403	6.1
*Intralipid****								
1	8	227	234.5	3.2	16.2	194.5	210.7	7.7
2	6	105	111	5.4	11.0	115.1	126.1	8.7
3	3	104	107	2.8	5.9	70.4	76.4	7.8
4	12	253	265	4.5	18.9	232.6	251.5	7.5

*LPC, sum of peaks 22 and 24; other lipid classes estimated as described in the text.
**Hyperlipemic samples as in Table II.
***Fasting samples of plasma 16 h after receiving moderate doses of Intralipid.

plicate analyses of the same sample by either method alone. The good agreement between the estimated and measured levels of plasma phosphatidylcholine indicates that the sum of C_{36} + C_{38} peaks represents an essentially constant proportion (81±1%) of total species of plasma phosphatidylcholine on normal diets, as already noted in Table I. The estimation of the total plasma sphingomyelin on the basis of the single ceramide peak is somewhat less effective. This is due largely to the greater scatter of the values arising from a measurement of a relatively small peak area in the total GLC profile. When sufficiently large area is recorded for the ceramide peak (C_{34}), a more precise and an apparently accurate account of the total ceramide or sphingomyelin content is obtained because this peak constitutes an essentially constant proportion (30%) of the total plasma sphingomyelin under normal dietary conditions, even though the total amount of sphingomyelin in the plasma may vary.

Table VII compares the estimates for plasma lysophosphatidylcholine derived by the phospholipase C—GLC and the manual phosphorus—TLC methods of analysis. It would appear that the GLC method underestimates the lysophosphatidylcholine level by about 50%. The correlation coefficient for the two estimates is also relatively poor (r = 0.69). Since the GLC analyses of the samples were performed on fresh samples while the phosphorus analyses were done on samples that had been stored for 1—2 years, it is likely that the discrepancies are due to variable hydrolysis of the phosphatidylcholine during storage. This possibility is supported by the observation that the sum of phosphatidylcholine and lysophosphatidylcholine showed excellent agreement between the two methods (r = 0.98; slope = 1.08; intercept = 1.57). Analyses of samples performed by both methods at the same time gave comparable results (data not shown). Furthermore, when appropriately calibrated, the GLC estimates of the lysophosphatidylcholine levels were within the range (5—10% of the total lipid phosphorus) reported in the literature [19, 22], while those obtained by the phosphorus analysis on the stored samples were significantly higher. There is a possibility, however, that the GLC values could have been lower due to an incomplete digestion of the plasma lysophosphatidylcholine by phospholipase C [24, 25]. The possibility that much of the discrepancy between the two methods was due to partial hydrolysis during the time intervening between the analyses is further attested to by the relatively good agreement between the values of lysophosphatidylcholine expressed as percentages of the sum of lysophosphatidylcholine plus phosphatidylcholine measured by the two methods (r = 0.81).

The lysophosphatidylcholines were largely absent from the plasma lipoprotein fractions prepared in the present experiments as examined by both methods. Previous studies [19, 22], however, had reported small and variable amounts of lysophosphatidylcholine in various preparations of HDL, which, however, could have been due to contamination with albumin and/or lipoproteins of higher density. It is therefore obvious that relatively accurate estimates of plasma phosphatidylcholine may be obtained by direct GLC analysis when appropriate periods of enzyme digestion are selected along with appropriate correction and calibration factors of peak areas, as claimed previously on the basis of much more limited data [2].

Fig. 7 shows a series of plots correlating the results of some 69 parallel anal-

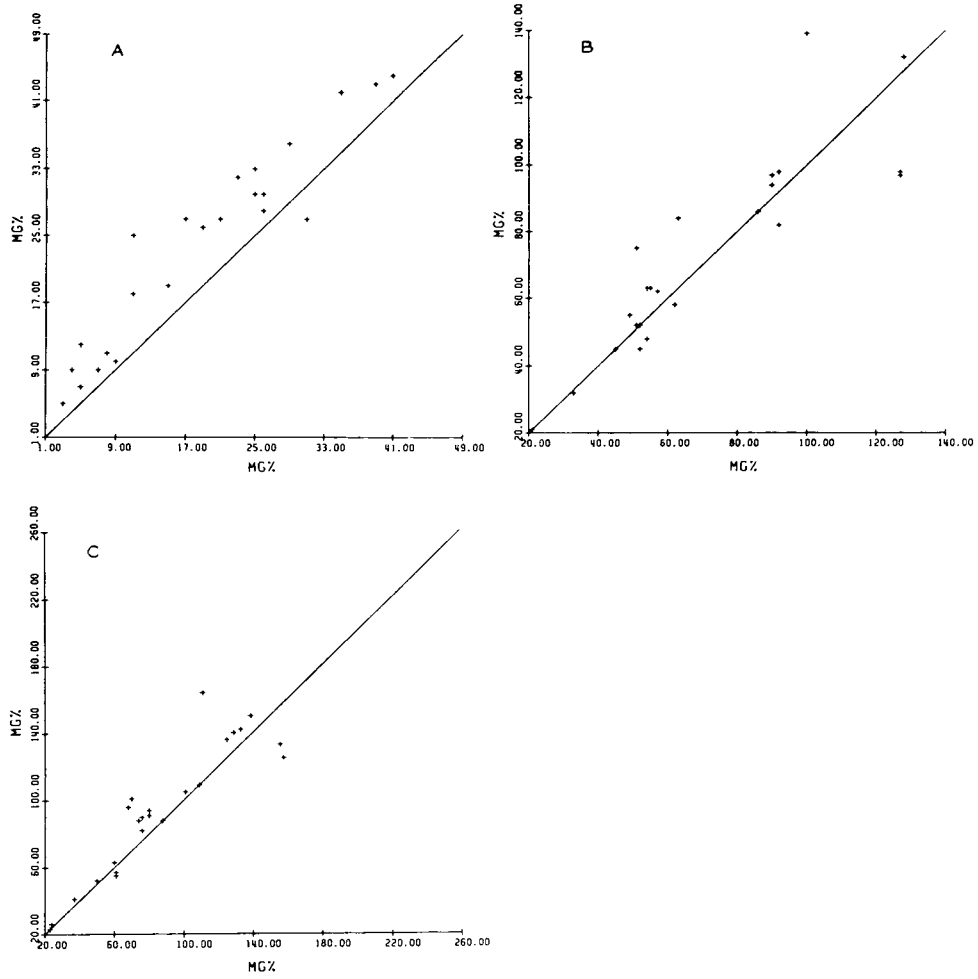

Fig. 7. Comparison of results (mg%) obtained by GLC (ordinate) and manual phosphorus (TLC) (abscissa) methods for plasma lipoproteins from subjects with a wide range of total lipids. A, SPH of VLDL, LDL and HDL (slope = 0.98; intercept = 5.29); B, PC of VLDL, LDL and HDL (slope = 0.87; intercept = 11.47); C, total PL of VLDL, LDL and HDL (slope = 0.90; intercept = 16.18).

yses of the phosphatidylcholine and sphingomyelin content of isolated plasma lipoproteins. Because of the relatively small number of parallel analyses made on the individual lipoprotein classes, the values for the different comparisons have been plotted on the same graph. Again the GLC estimates of the phosphatidylcholines are based on the total amount and relative proportions of peaks C_{36} and C_{38} for diacylglycerols and of peak C_{34} for ceramides. As can be seen the agreement is reasonably close for the phosphatidylcholines (r = 0.88) and total choline-containing phospholipids (r = 0.89), with the discrepancies approximating the reproducibility of duplicate estimates for the same sample with either method alone, when executed at a comparably low level of sample concentration. Surprisingly, the correlation was better for the estimates of the

sphingomyelin of the lipoproteins (r = 0.95). The linear regression plot for the phosphatidylcholines gave a slope of 0.87 with an intercept value of 11.47. The corresponding slope for the sphingomyelins was essentially unity (0.98) with an intercept value of 5.29, which represented a significant underestimation of this phospholipid by the GLC method, or an overestimation by the manual phosphorus method. The slope and intercept values for the total phospholipid comparison in the VLDL, LDL and HDL fractions were 0.90 and 16.18, respectively. An examination of the limited number of parallel comparisons carried out with the individual lipoprotein classes revealed that the best correlations were obtained for the phosphatidylcholines of the VLDL and LDL fractions and for the sphingomyelins of the LDL fraction. The correlations for the sphingomyelins of the VLDL and HDL fractions and the phosphatidylcholines of the HDL fractions were less perfect, but there was no obvious explanation for the differences in the estimates obtained by the two methods. Both methods were clearly measuring the same components with about the same precision. It should be noted that the GLC estimates for total plasma sphingomyelins were in good general agreement with those obtained by the manual phosphorus method (Table VI, Fig. 6). Obviously, the GLC method can be used for a reliable estimation of both the total choline-containing phospholipids of plasma, and of the individual phosphatidylcholine and sphingomyelin classes. The agreement between the manual phosphorus method and the automated GLC technique could be further improved by including in the GLC estimates also the small amounts of diacylglycerols likely to be released by phospholipase C from plasma phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol [19]. These contributions, however, are uncertain at the moment because of the lack of knowledge about the molecular species composition of the minor phospholipids of plasma. Theoretically a correction should also have been made for the free plasma diacylglycerols, the molecular species of which were known. These corrections, however, would not have seriously affected the correlations described.

There was very little free monoacylglycerol or lysophosphatidylcholine associated with the major plasma lipoproteins, as they presumably remained in the $d < 1.21$ fraction in complex with albumin [19].

DISCUSSION

We had previously shown that either pyrolysis [1] or hydrolysis with phospholipase C [2] results in a reproducible conversion of the phospholipids to neutral lipids to permit a GLC estimation of the sum of the diacylglycerophospholipids and sphingomyelins of plasma along with the free cholesterol, cholesteryl esters and triacylglycerols. The present study confirms these observations and also demonstrates that precise and accurate estimates may separately be obtained for the phosphatidylcholines and sphingomyelins provided certain peak areas are quantitatively measured and certain assumptions about the composition of the molecular species of the plasma phospholipids are valid. Although we have now documented the major assumptions and extrapolations, there is a need for a further discussion of this part of the work.

Validity of analytical basis

The present GLC estimates of the phosphatidylcholine and sphingomyelin content of whole plasma and of the individual lipoprotein classes is based upon a reproducible separation, identification and quantitation of selected molecular species of diacylglycerols and ceramides released from these phospholipids by phospholipase C. The selected reaction conditions result in an essentially complete hydrolysis of the plasma phosphatidylcholines and sphingomyelins. A TLC examination of the reaction products has failed to yield any measurable phosphorus for the corresponding undigested phospholipids. The hydrolysis of standard lysophosphatidylcholine was about 90% complete, while the other minor plasma diacylglycerophospholipids (ethanolamine, serine and inositol phosphatides) were hydrolyzed to about 20—25% of their content. These findings are supported by previous reports in the literature which have indicated a comparable extent of hydrolysis of these phospholipids either separately or in mixture with other phospholipids [26—28].

The range of the carbon numbers of the diacylglycerols released from the plasma phosphatidylcholines remains the same on normal diets, although the proportions of the individual carbon numbers change with the source of the dietary fat. These changes in the diacylglycerol proportions are confined essentially to carbon numbers C_{36} and C_{38} which together still account for 81±1% of the total. Since the latter peak areas can be measured with great precision under all experimental conditions, it is possible and practical to use them to estimate the total phosphatidylcholine content of the plasma or any lipoprotein fraction, when estimates of individual molecular species are not necessary. There are no known changes in the carbon numbers of the ceramides with diet. It is therefore practical to use the carbon number C_{34}, which accounts for 30±1% of the total sphingomyelin, as a reliable indicator of the content of spingomyelin in total plasma. Because of the change in the carbon number proportions among the lipoproteins, the sphingomyelin content of HDL_3 must be estimated by assigning 24±1% of the total peak area to peak C_{34}. The finding that the HDL_3 sphingomyelins have a somewhat higher molecular weight than those of other lipoproteins of plasma is in agreement with earlier observations on their fatty acid composition [19], although from different subjects. We have recently confirmed these findings in samples collected from the same subjects [20].

Recoveries of plasma phospholipids

The relative recoveries of the diacylglycerols and ceramides in the GLC profiles were of the order anticipated on the basis of the known proportion of the corresponding phospholipids in human plasma [19, 23]. The absolute recoveries of the phospholipids from whole plasma by the GLC method were calculated by comparison with the values obtained by the manual determination of the phosphorus in the various phospholipid fractions isolated by TLC from the corresponding plasma samples. It was seen that the means and standard deviations for total phospholipids, total phosphatidylcholine and sphingomyelin corresponded closely, as did the phosphatidylcholine:sphingomyelin ratios. The TLC method gave somewhat lower values for phosphatidylcholine and hence total phospholipid because of the partial hydrolysis of the phosphatidylcholine

upon storage of plasma samples. Correlation plots of the values obtained by the GLC procedure and the manual phosphorus analysis method showed points falling on both sides of the ideal correlation line with about equal frequency. The average values obtained for total plasma lysophosphatidylcholine by the GLC method (4 mg%) when corrected for the presence of about 8 mg% of free monoacylglycerol, were only slightly below the range (5—8 mg%) calculated from the data reported by Phillips and Dodge [23], assuming a molecular weight of 524. The values measured by TLC on the stored samples averaged 19 mg%. Clearly, the GLC estimates for plasma phosphatidylcholine and sphingomyelin are in the range previously reported by different laboratories [19, 22, 23], while the estimates for the lysophosphatidylcholine are somewhat lower due to incomplete hydrolysis by phospholipase C [24, 25] and because of possible incomplete extraction of the released monoacylglycerols [29].

Recovery of lipoprotein phospholipids

Likewise, the relative recoveries of the diacylglycerols and ceramides in the GLC profiles of the lipoproteins were of the order anticipated for the corresponding phospholipids in the appropriate fractions of human plasma [19, 22]. The average absolute values derived from specific paired comparisons showed good general agreement, comparable to that obtained for total plasma lipid analyses, while the correlation plots of the values obtained by the two methods showed points falling on both sides of the ideal correlation line with nearly equal frequency. An exception was provided by the HDL fraction, which failed to yield truly identical estimates by the two methods for sphingomyelin. It is possible that higher values derived by the phosphorus analysis for the HDL sphingomyelin were due to some lipid phosphorus compound which overlapped with sphingomyelin in the TLC system employed. Alternatively, some species of sphingomyelin in the HDL fraction may have resisted either the digestion with phospholipase C or solvent extraction to a greater extent than those in the other lipoproteins. The various lipoprotein fractions examined in the present study were essentially free of lysophosphatidylcholine, but small amounts of this minor phospholipid have been reported in plasma lipoprotein fractions prepared by other laboratories [19, 22].

Influence of neutral lipids on GLC quantitation of phospholipids

The relative and absolute recoveries of the different phospholipid classes are influenced to a variable although minor extent by the presence of neutral glycerolipids and low-molecular-weight cholesteryl esters in the total lipid extracts of whole plasma or of the lipoprotein fractions. We have estimated that the free plasma diacylglycerol content ranges from 1 to 2% of the total neutral lipid, which corresponds to about 4% of the total plasma lipid as claimed by Skipski et al. [30]. Furthermore, the content of free diacylglycerols was found to be proportional to the total triacylglycerol content of the sample, including lipoprotein fractions. However, increased plasma triacylglycerol levels were usually associated with increased amounts of phosphatidylcholine, so that the relative contribution of free diacylglycerols to the diacylglycerols arising from hydrolysis of phosphatidylcholine remained about the same in all instances ($< 2\%$). Furthermore, since the free diacylglycerols were made up of at least two major

carbon numbers (C_{36} and C_{38}) of about equal proportions, they contributed less than 2% to the total peak area of the corresponding diacylglycerols derived from the plasma phosphatidylcholine. The plasma free ceramide levels (0.4% of total) were too low to affect significantly the estimates of sphingomyelin based on the release of the bound ceramides by phospholipase C.

The contamination of the longer chain diacylglycerol and ceramide peaks with the peaks due to the short-chain cholesteryl esters (largely cholesteryl myristate) was much more significant and was mainly responsible for the poor reproducibility of the C_{40}—C_{42} peak areas measured from the retention time window. The overlap was especially bad in samples with high proportions of cholesteryl esters, when shoulders or poorly defined peaks with flat tops were recorded. As a result it was frequently impossible to measure the contributions of the C_{40}—C_{42} peaks to the total lipid profile during the fast programming rates. It was therefore necessary to calculate the total diacylglycerol and ceramide peak area from precise measurements of a few peaks as explained above. This difficulty may be eliminated or minimized by using capillary GLC columns [J.J. Myher and A. Kuksis (1979), unpublished results].

The free plasma monoacylglycerols represent largely C_{16} and C_{18} fatty acid esters just like those that are released from plasma lysophosphatidylcholines by digestion with phospholipase C. As a result there may occur a significant overestimation of the plasma lysophosphatidylcholine levels by the GLC method unless correction is made for the content of free monoacylglycerols. Although apparently correct estimates for lysophosphatidylcholine can be obtained, this correction is not entirely sound because of the frequent appearance of other unknown lipid components in this part of the plasma lipid profile. Since both free monoacylglycerols and lysophosphatidylcholines are largely absent from the plasma lipoproteins, the quantitation of these components has not been further pursued.

Despite the limitations discussed above, this study shows that with precise peak area integration the GLC method can provide plasma phospholipid estimates approaching those obtained by direct measurements of phosphorus on isolated phospholipid classes. A statistical evaluation of the data demonstrates that the quantitative GLC estimates for total plasma and lipoprotein phosphatidylcholines and sphingomyelins are of about the same order of accuracy and precision as those demonstrated for the plasma cholesterol and triacylglycerols [9]. The GLC analyses of phospholipids are recommended in conjunction with plasma neutral lipid analysis, which results in a more efficient utilization of the required specialized equipment and analytical expertise.

ACKNOWLEDGEMENTS

These studies were supported by funds from the Heart and Lung Institute, NIH-NHLI-72-917, Bethesda, Md., U.S.A., the Ontario Heart Foundation, the Province of Ontario Health Research Grant PR 51, and the Medical Research Council of Canada.

The plasma samples of young adults were kindly provided by Dr. Ann Hedlin of the Department of Physiology, University of Toronto, while the plasma lipoprotein fractions from subjects on controlled experimental diets

were obtained by Dr. O. David Taunton of the Lipid Research Clinic, Baylor University and Methodist Hospital, Houston, Texas, U.S.A.

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Journal of Chromatography, 182 (1980) 27—33
Biomedical Applications

CHROMBIO. 506

GAS CHROMATOGRAPHIC DETERMINATION OF THE FATTY ACID PATTERN OF RED CELL MEMBRANE PLASMALOGENS IN HEALTHY CHILDREN

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(First received July 20th, 1979; revised manuscript received November 29th, 1979)

SUMMARY

A gas chromatographic technique for the determination of the fatty acid pattern of the phosphatidylcholine (PC) and phosphatidyl ethanolamine (PE) plasmalogen and non-plasmalogen fractions in human red cell membranes has been developed. The coefficient of variation lies between 5 and 10%. The technique has been applied to blood samples of healthy Belgian school children (8—10 years old). PE contains 47% plasmalogen and 53% non-plasmalogen form, whereas PC occurs only in its non-plasmalogen form.

The fatty acid pattern of the various fractions and subfractions has been determined.

INTRODUCTION

Appropriate methods for the determination of the fatty acid (FA) pattern of the various phospholipid fractions, using thin-layer chromatography (TLC) and gas—liquid chromatography (GLC) have been developed during the last twenty years [1—3] and have been applied to human red blood cells [4].

Nelson [4] has reviewed reports concerning the FA pattern of the major erythrocyte phospholipids in healthy adults, but for children, only little and mainly incomplete data exist. There has also been little investigation on the occurrence and FA pattern of the individual phospholipid subfractions.

In human red cells, phosphatidyl ethanolamine (PE) and phosphatidylcholine (PC) are possibly present in two subfractions, a plasmalogen and a non-plasmalogen form [4]. A separation method for these two forms, using two-dimensional TLC, has been reported by Owens [5]. A solution of 5 m*M* $HgCl_2$ in water is sprayed on the TLC plate after development of the first dimension. This is not recommended for subsequent determination of the FA pattern of both subfractions, as they are rich in polyunsaturated FA.

In this study, an appropriate TLC—GLC technique for the determination of the FA pattern of red cell membrane plasmalogens is reported.

EXPERIMENTAL

Materials

Organic solvents pro analysis grade were obtained from Merck (Darmstadt, G.F.R.). Sephadex G-25, coarse, was from Pharmacia (Uppsala, Sweden), TLC "Redi Coats", Supelcosil 42A^R (silica gel with 10% magnesium silicate), from Supelco (Bellefonte, Pa., U.S.A.), and TLC silica gel H from Merck. The phospholipid standards for TLC were from Sigma (St. Louis, Mo., U.S.A.), the methyl esters of FA from Merck, Fluka (Buchs, Switzerland), Applied Science Labs. (State College, Pa., U.S.A.), Sigma and Supelco. Boron trifluoride—methanol (14% w/v), and the BHT antioxidant (2,6-di-*tert.*-butyl-4-methylphenol) were both from Merck.

Blood collection

After the children had fasted overnight, 10-ml blood samples were taken and placed in ice-cooled heparinised tubes (100 I.U./ml blood), then immediately centrifuged for 30 min at 3000 *g* and 4°, thus separating the red cells.

Preparation of erythrocyte "ghosts"

This was carried out according to the method of Hanahan and Ekholm [6]. An isotonic Tris buffer 310 imOsm (ideal milliosmolar) pH 7.6 and an hypotonic Tris buffer 20 imOsm pH 7.6 were used as washing and hemolysing media, respectively.

Extraction of the total lipids

One volume of the ghost suspension was extracted twice, using 5.5 volumes isopropanol (+ 100 mg/l BHT) and 3.5 vol. chloroform [7].

Purification of the lipid extract

The total lipid extract was purified essentially free of all non-lipid components by partition column chromatography using sephadex G-25, coarse [1]. The lipids were eluted with a chloroform—methanol (19:1) mixture, saturated with water.

Separation of the phospholipids by TLC

Two-dimensional TLC on Supelcosil-42A was carried out to separate the phospholipids. For the first dimension a mixture of chloroform—methanol—ammonium hydroxide (65:25:5, v/v) was used and for the second dimension chloroform—methanol—acetone—acetic acid—water (30:40:10:10:5, v/v) [8]. The phospholipid spots were detected by spraying the plate with a non-destructive Rhodamine 6G solution (0.001% in water), and by inspection of the plate in day light.

Separation of PE_{total} and PC_{total} in plasmalogen and non-plasmalogen form

The scrapings of the PE_{total} and PC_{total} spots were extracted with chloroform—methanol (2:1) + 100 mg/l BHT and hydrolysed with 90% acetic acid for 18 h at 37° under an atmosphere of nitrogen [9]. After elimination of the excess acetic acid by evaporation (Rotavapor, Büchi) of the binary azeotrope

with carbon tetrachloride (30—35°, under vacuum), the hydrolysis products were separated by TLC on silica gel H with chloroform—methanol—water (70:30:4, v/v) [10]. The phospholipid spots were detected with the Rhodamine 6G solution in day light.

Determination of the FA pattern of both subfractions

The PE, LPE (lyso-PE), PC and LPC (lyso-PC) spots were scraped off, extracted with chloroform—methanol (2:1, v/v) + 100 mg/l BHT, transmethylated with boron trifluoride—methanol and then extracted with pentane [11]. The FA methyl esters were separated by GLC using a column filled with 5% EGSS-X on Chromosorb W HP (100—200 mesh). Gas chromatographic conditions: the temperature was raised from 130° to 210° at 3°/min. Dual columns (2 m × 1/8 in. I.D.) were used to compensate for column bleeding. The flow-rates were: nitrogen, 15 ml/min; hydrogen, 28 ml/min and air, 400 ml/min. A Perkin-Elmer F17 gas chromatograph with a dual flame ionization detection system was used. Peaks were identified by their relative retention times versus that of the internal standard ($C_{17:0}$) and quantitated with a Hewlett-Packard Model 3380A digital integrator.

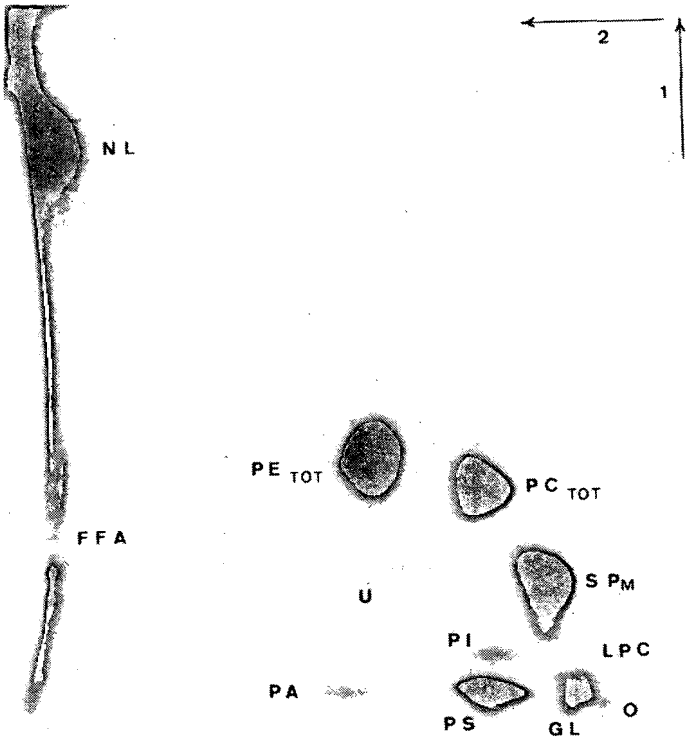

Fig. 1. Two-dimensional TLC of red cell membrane phospholipids. NL, neutral lipids; FFA, free fatty acids; U, unknown; PE_{tot}, phosphatidyl ethanolamine (total); PC_{tot}, phosphatidylcholine (total); SP_M, sphingomyelin; LPC, lyso-PC; PI, phosphatidylinositol; PS, phosphatidylserine; PA, phosphatidic acid; GL, globoside and O, origin. Conditions are as described in Methods.

RESULTS

The reproducibility of the complete technique was investigated. The coefficient of variation of each FA lies between 5 and 10% (n = 6). No oxidation of the polyunsaturated FA could be detected during the whole procedure.

A two-dimensional thin-layer chromatogram of human red cell membrane phospholipids is shown in Fig. 1. A complete separation of the various phospholipid fractions was obtained. The PE_{total} and PC_{total} spots contain plas-

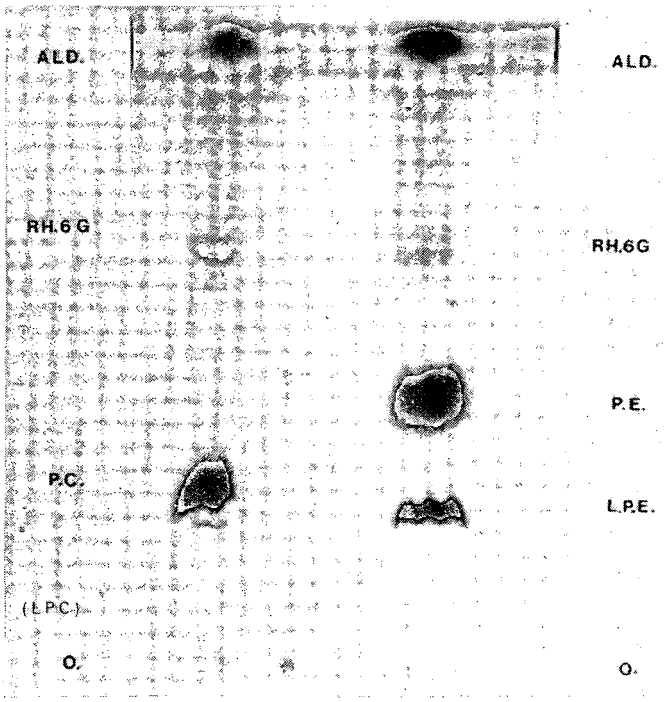

Fig. 2. Separation of PE_{total} and PC_{total} into plasmalogen and non-plasmalogen subfractions by TLC on silica gel H. ALD, aldehydes; RH6G, Rhodamine 6G and antioxidant BHT; PE, phosphatidyl ethanolamine; LPE, lyso-PE; PC, phosphatidylcholine; LPC, lyso-PC and O, origin. Conditions are as described in Methods.

TABLE I

PLASMALOGEN AND NON-PLASMALOGEN CONTENT OF TOTAL PE AND PC FRACTIONS IN RED CELL MEMBRANES OF HEALTHY CHILDREN

Conditions are as described in Methods. Results are mean ± S.D. with n = 6.

	Plasmalogen (%)		Non-plasmalogen (%)	
	girls	boys	girls	boys
PE_{total}	47.6 (±1.4)	47.3 (±1.2)	52.4 (±1.3)	52.7 (±1.4)
PC_{total}	0.0 (±0.0)	0.0 (±0.0)	100.0 (±0.0)	100.0 (±0.0)

malogen and non-plasmalogen forms. A further separation, after acid hydrolysis, was obtained by one-dimensional TLC as shown in Fig. 2. A spot equivalent to LPC (i.e. the plasmalogen form of PC) was never found.

After TLC, phosphorus determination of the PE, LPE, PC and LPC spots was carried out, using the Bartlett method [12]. To determine the LPC spot, a known area of the TLC plate with a R_F value corresponding to a reference LPC spot, was scraped off and its phosphorus content quantitated. The results for healthy Belgian school children (8—10 years old) are shown in Table I. The FA patterns of the PE_{total}, PE, LPE, PC_{total} and PC spots were each determined for the same healthy boys and girls. No sex-dependent differences were detected. The GLC separation of the FA methyl esters of the PE plasmalogen and non-plasmalogen forms are shown in Fig. 3a and b. The antioxidant BHT used in the extraction has an identical retention time to the palmitoleic acid methyl ester, hence the latter can not be quantitated. The FA compositions of PC_{total}, PE_{total} and subfractions in the red cell membranes of healthy girls are shown in Table II (n = 8). Identical

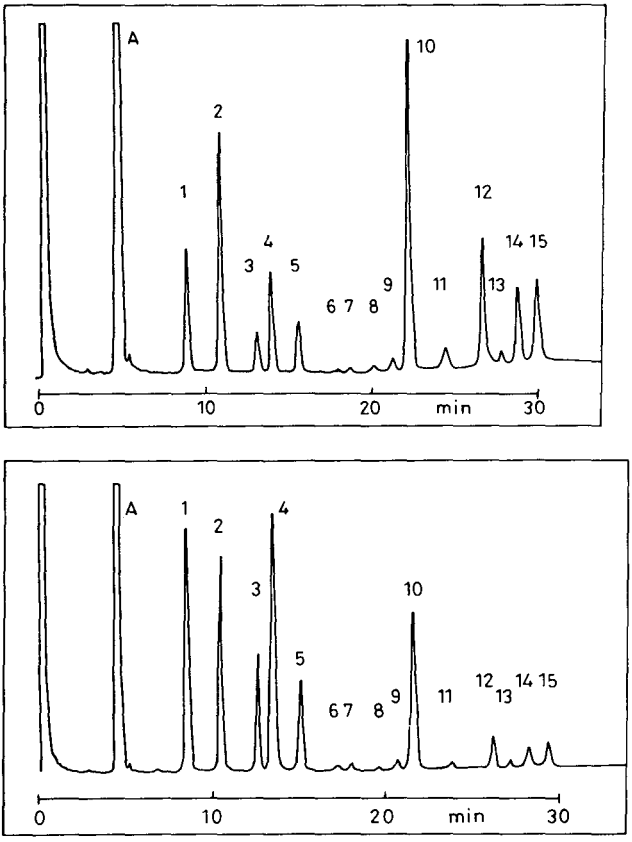

Fig. 3. Gas chromatograms of (a) the PE plasmalogen and (b) non-plasmalogen form of red cell membranes of healthy children. The column packing used was 5% EGSS-X and conditions are as described in Methods. Peaks: A, BHT derivative; 1, $C_{16:0}$; 2, $C_{17:0}$; 3, $C_{18:0}$; 4, $C_{18:1\omega9}$; 5, $C_{18:2\omega6}$; 6, $C_{18:3\omega3}$ + $C_{20:0}$; 7, $C_{20:1\omega9}$; 8, $C_{20:2\omega6}$; 9, $C_{20:3\omega6}$; 10, $C_{20:4\omega6}$; 11, $C_{20:5\omega3}$; 12, $C_{22:4\omega6}$; 13, $C_{22:5\omega6}$; 14, $C_{22:5\omega3}$; 15, $C_{22:6\omega3}$.

TABLE II

FATTY ACID COMPOSITION OF TOTAL PC, PE AND SUBFRACTIONS IN RED CELL MEMBRANES OF HEALTHY GIRLS

Conditions are as described in Methods. Results are mean ± S.D. with $n = 8$.

Fatty acid	PE_{total}	$PE_{non\text{-}plasmalogen}$	$PE_{plasmalogen}$	PC_{total}
$C_{16:0}$	16.8 ± 1.0	21.7 ± 1.4	7.7 ± 2.1	34.9 ± 2.5
$C_{18:0}$	8.9 ± 1.2	10.3 ± 0.7	2.0 ± 1.1	12.1 ± 0.7
$C_{18:1\omega9}$	19.1 ± 0.6	24.4 ± 1.1	9.2 ± 1.0	19.4 ± 0.5
$C_{18:2\omega6}$	7.0 ± 1.0	9.5 ± 1.1	3.8 ± 0.7	22.0 ± 1.5
$C_{18:3\omega3}$	0.4 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
$C_{20:1\omega9}$	0.6 ± 0.1	0.7 ± 0.1	0.4 ± 0.1	0.3 ± 0.1
$C_{20:2\omega9}$ $\omega6$	0.4 ± 0.1	0.5 ± 0.2	0.3 ± 0.2	0.4 ± 0.1
$C_{20:3\omega6}$	1.4 ± 0.2	1.6 ± 0.3	1.4 ± 0.3	1.9 ± 0.4
$C_{20:4\omega6}$	24.0 ± 0.5	17.7 ± 0.6	36.3 ± 1.5	4.9 ± 0.9
$C_{20:5\omega6}$	1.2 ± 0.3	0.7 ± 0.1	2.0 ± 1.0	0.4 ± 0.1
$C_{22:4\omega6}$	7.7 ± 0.7	4.5 ± 0.4	14.6 ± 1.4	0.5 ± 0.2
$C_{22:5\omega6}$	1.2 ± 0.2	1.0 ± 0.2	1.7 ± 0.3	0.5 ± 0.3
$C_{22:5\omega3}$	4.8 ± 0.4	2.6 ± 0.3	9.4 ± 0.7	0.6 ± 0.3
$C_{22:6\omega3}$	6.6 ± 1.0	4.6 ± 0.8	11.0 ± 1.8	1.8 ± 0.8

results were found for healthy boys of the same age group ($n = 6$). Since the FA pattern of the PC_{total} is not significantly different from that of the $PC_{non\text{-}plasmalogen}$ and a spot for $PC_{plasmalogen}$ was never detected, we conclude that there is no plasmalogen subfraction in the red cell membranes of 8—10 year old healthy children.

DISCUSSION

After determination of the phosphorus content of the various PL fractions in red cell membranes, it appears that for healthy children the PE_{total} spot contains 47% plasmalogen and 53% non-plasmalogen form. PC occurs only in its diacyl form. For healthy adults, Farquhar [13] found 67% plasmalogen form in PE and 10% in PC, Williams et al. [14] reported 52% and 4%, Cohen and Derksen [10] 46% and 3%, and Hill et al. [15] 36% and 4%, respectively. These variations are probably due to the different analytical methods used and to difficulties in achieving a clean separation of the PL components present, especially those techniques using column chromatography instead of TLC. The FA pattern of the total PC and PE fractions of erythrocyte membranes in healthy adults has been described in detail [10, 13, 14, 16—18], whereas the FA composition of their subfractions has only been analysed by a few investigators [10, 14].

As far as we know, no data are available for the FA pattern of the erythrocyte PL subfractions of children. Kobayashi et al. [19] have determined the FA composition of the major red cell PL fractions in healthy children of 10 years old, but no further separation into the subfractions has been carried out. Our results showing the FA pattern of the total PE and PC fractions are in good agreement with theirs. The FA compositions of the PE subfractions

of healthy children are comparable with those described for healthy adults [10, 14].

ACKNOWLEDGEMENTS

Dr. I. Mandelbaŭm (Medical Chemistry Department of the Saint Pierre Hospital, Brussels) is thanked for his most valuable advice. This work is supported by the "Belgische Vereniging voor Strijd tegen Mucoviscidose" and the Medical Foundation, Koningin Elisabeth.

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CHROMBIO. 503

DETERMINATION OF 5-METHYLTETRAHYDROFOLIC ACID IN PLASMA AND SPINAL FLUID BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY, USING ON-COLUMN CONCENTRATION AND ELECTROCHEMICAL DETECTION

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(First received August 15th, 1979; revised manuscript received November 22nd, 1979)

SUMMARY

Chromatographic conditions for the determination of 5-methyltetrahydrofolic acid in plasma and spinal fluid are described, involving simple pretreatment of the sample. Electrochemical detection was used. The linear range of the method is more than 10^3. Recovery from plasma and spinal fluid is 100%, and the detection limit of the method is $2 \cdot 10^{-9}$ *M*, sufficient for the detection of endogenous plasma and spinal fluid levels. The detection conditions are discussed. Endogenous concentrations of the compound in plasma and spinal fluid were determined and correlated with a folate bioassay. Plasma concentrations have been shown after the administration of leucovorin which is used in anticancer therapy.

INTRODUCTION

Folic acid analogues play an important role in several biochemical processes [1]. In man, 5-methyltetrahydrofolic acid (5-MeTHF) is the main analogue found to be present in plasma [2]. Its concentration in plasma is important for the determination of folate deficiency in patients [3]. Folate activity in serum is determined by microbiologic assay [4] or radiometric analysis using a labeled drug [5]. For the treatment of malignancies 5-MeTHF plays a role as the active

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metabolite of leucovorin which is used in the prevention of methotrexate toxicity [6—9]. Its concentration may be important to estimate the optimal antitumor effect [10,11]. In test samples 5-MeTHF has been separated from other folate analogues by high-performance liquid chromatography (HPLC) [12]. In plasma and spinal fluid the determination of 5-MeTHF is usually carried out by microbiological assay [13].

For the analysis of body fluids, a sensitive HPLC method is expected to be fast and selective and to have a wide linear dynamic range. Generally, bioassays are hampered by interference due to the presence of folates and antibiotics. As 5-MeTHF is very sensitive to oxidation [14], relatively large amounts of antioxidant must be added to the sample. Since the endogenous plasma concentrations lie between 10^{-9} *M* and 10^{-8} *M*, the determination of 5-MeTHF demands high efficiency of the chromatographic system. For this purpose a sensitive electrochemical detection was investigated. Among the electrochemical properties of folate analogues reduction has been reviewed [15]. However, oxidation at a glassy carbon electrode had not been previously reported. For easily oxidizable compounds this detection method is sensitive and selective.

A two-column concentration system [16] in combination with this detection method may have the following advantages: (1) the large ratio of antioxidant concentration, added to the sample medium, with respect to the small concentration of 5-MeTHF gives rise to a serious separation problem, but this is considerably diminished by the first separation step; (2) the possibility of injecting large sample volumes improves the lower detection limit; (3) extra sample clean-up of compounds interfering in the chromatogram, by elution from the concentration column, is included.

EXPERIMENTAL

Chromatographic equipment and columns

The liquid chromatograph has been described before [16]. Ion-exchange columns (Partisil SAX) were obtained from Whatman (Maidstone, Great Britain) and reversed-phase columns from Chrompack (Middelburg, The Netherlands). The particle size was 10 μm for both materials and the column dimensions were 25 cm × 4.6 mm I.D. and 4.6 cm × 3 mm I.D., respectively.

Detection system

An electrochemical detector with a large-surface electrode was used [17]. A glassy carbon electrode (finally polished with diamond powder) with a surface of 2.65 cm^2 was used. This material (grade GC-10, obtained from Tokai Carbon, Tokyo, Japan) has a porosity of 0.2—0.4%. Polyester spacers were used with a thickness of 87 μm and 25 μm. The polyester film (Melinex, type S) was obtained from ICI Holland (Rotterdam, The Netherlands). The electrode potential was held at +0.3 V vs. Ag/AgCl/0.001 *M* Cl^-. The detection system is commercially available from Kipp Analytica (Emmen, The Netherlands).

Before analyzing samples with concentrations just above the detection limit the electrode was allowed to equilibrate overnight at constant potential. Early plasma peaks in the chromatogram and foreign eluent, coming form the void volume of the concentration column, were

vented in order to avoid possible electrode "poisoning". For simultaneous measurement of other folate analogues (e.g. during drug treatment) a UV detector (Schoeffel Instruments, Westwood, N.J., U.S.A., Model 770) was placed in series upstream from this detector.

For the measurement of the coulometric yield in the detection cell an infusion pump was used (Infors, Basle, Switzerland, Type 5003).

Number of electrons, involved in the oxidation reaction

The number of electrons which are transferred per molecule in the oxidation reaction was calculated by Faraday's law [17] at a coulometric yield of 1.0 and was found to be 1.45.

Reagents and standards

All reagents were of analytical grade. Demineralised water was used for the preparation of all solutions, except for the concentration eluent. For the concentration eluent a solution of 0.015 *M* citrate in 0.015 *M* phosphate buffer (pH 4.95) was used. Sodium azide, 0.5—1 mg/l, was used in order to prevent the growing of micro-organisms. The analytical eluent consisted of 0.05 *M* sodium phosphate buffer (pH 4.95)—methanol (4:1, v/v) and contained 0.001 *M* sodium chloride for the electrochemical detection. For the deproteinization of samples a solution containing 10% (w/w) of trichloroacetic acid (TCA) in 0.1 *N* HCl was used. A solution of 1% of L-(+)ascorbic acid was freshly prepared each day. 5-MeTHF was obtained from Sigma (St. Louis, Mo., U.S.A.). The samples were standardized by means of the formula $\epsilon = A \cdot W/Q$ where ϵ = molar extinction, A = peak area, W = eluent flow-rate and Q = amount of compound. From Gupta and Huennekens [18] a value for ϵ of $31.7 \cdot 10^3$ AU$\cdot$mol$^{-1}\cdot l\cdot$cm^{-1} is obtained for pH 7. This value was corrected by us for the difference in pH. By this calculation the purity of the commercially availably samples was found to be only about 60%.

Sample treatment

Sample treatment consisted of the following two steps: protection against oxidation and a deproteinization/centrifugation step.

To 0.9 ml plasma or spinal fluid, 0.1 ml of a solution containing 1% ascorbic acid was added. The mixture was allowed to equilibrate for 3 min. During vigorous mixing 1 ml TCA solution [10% (w/w) in 0.1 *N* HCl] was added. After centrifugation at 2000 *g* for 5 min 1 ml of the clear supernatant could be injected.

In order to prevent oxidation by dissolved oxygen during sample pretreatment an antioxidant is necessary. For this purpose the following compounds were tested: sodium sulfite, dithioglycol (1,2-ethanedithiol), 2-mercaptoethanol, cysteine·HCl and ascorbic acid. For a one-column separation on either a reversed-phase (RP-8, Merck, Darmstadt, G.F.R.) or an ion-exchange (Partisil SAX) column sodium sulfite gave the best peak. However, the addition of sulfite was found to be insufficient for stabilizing 5-MeTHF. The other agents, except ascorbic acid, contained impurities which were detected by the electrochemical detector. Ascorbic acid was therefore the best choice for stabilization. Because the recovery from plasma was found to decrease when using lower

amounts of ascorbic acid, only a freshly prepared solution was used. For storage fresh plasma samples were frozen immediately and stored at −20°; when thawed ascorbic acid was added immediately. The deproteinizated samples were injected within 30 min. After two hours at ambient temperature the concentration of 5-MeTHF in this mixture was found to decrease by about 5%, but could be less by keeping the samples in ice.

Quantitation

To determine the concentration of 5-MeTHF, its peak height in the chromatogram is measured. Homogenized plasma containing 5-MeTHF was divided into samples of 1 ml. Each day, the concentration of 5-MeTHF was measured in two samples in order to correct for changes in peak height.

RESULTS

Chromatographic procedure

A reversed-phase column (RP-8, Merck) was tested for a combined concentration and first separation step, followed by an extra separation on an ion exchanger (Partisil SAX) as described earlier for the analysis of methotrexate [16]. Column dimensions and the composition of the analytical eluent were similar. A concentration eluent volume of 7 ml was chosen, which was enough to produce a good sample clean-up during concentration, while still small enough not to cause elution from the concentration column.

Detection conditions

Although folate analogues show high UV absorption at wavelengths between 280 and 300 nm, UV detection did not appear to be useful for the detection of endogenous plasma levels of 5-MeTHF. This is demonstrated in Fig. 1 where the same separation is shown with UV detection and with electrochemical detection, indicating the increased sensitivity of the latter.

In order to investigate the detection signal as a function of eluent flow-rate in a static way the oxidation current resulting from a solution containing 5-MeTHF, flowing directly through the detector, was measured.

By means of a precise infusion pump the flow-rate was increased stepwise. 5-MeTHF was previously purified by elution on the two-column system. A plot of measured current against flow-rate is given in Fig. 2. At the lowest flow-rates a coulometric yield of 1.0 is reached. This yield is the fraction of all the molecules that react at the electrode [17]. The actual yield was calculated for various flow-rates of the 5-MeTHF containing eluent (dashed line). A flow-rate of 120 ml/h was used for the determination of 5-MeTHF. A coulometric yield of 12% can be determined from Fig. 2.

Detection limit, linear dynamic range, recovery

The method has a detection limit of $2 \cdot 10^{-9}$ *M* (0.9 ng/ml) for both plasma and spinal fluid. The standard deviation in peak height is 7.5% at $2 \cdot 10^{-9}$ *M* ($n = 7$) and 3.5% at concentrations higher than $3 \cdot 10^{-9}$ *M*. The upper limit of the linear dynamic range was measured to be at least 10^{-5} *M*. The linear dynamic range is more than 10^3. In the static measurement the linear range was lower

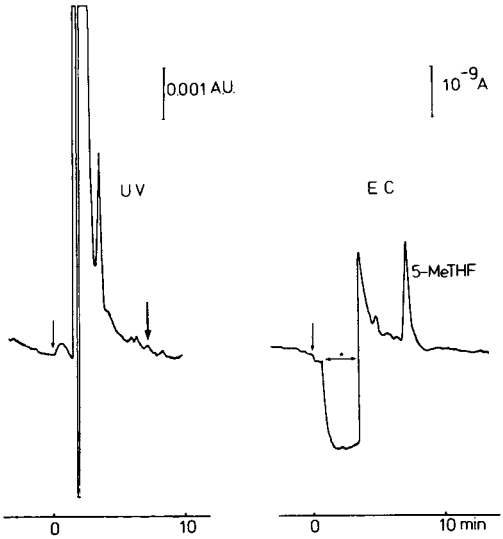

Fig. 1. Chromatogram of a plasma sample for the two-column system using UV detection at a wavelength of 290 nm and electrochemical (EC) detection at a working electrode potential of + 0.3 V. Injection volume, 1 ml; concentration of 5-MeTHF $5 \cdot 10^{-9}$ M; asterisk indicates venting just upstream from EC detector.

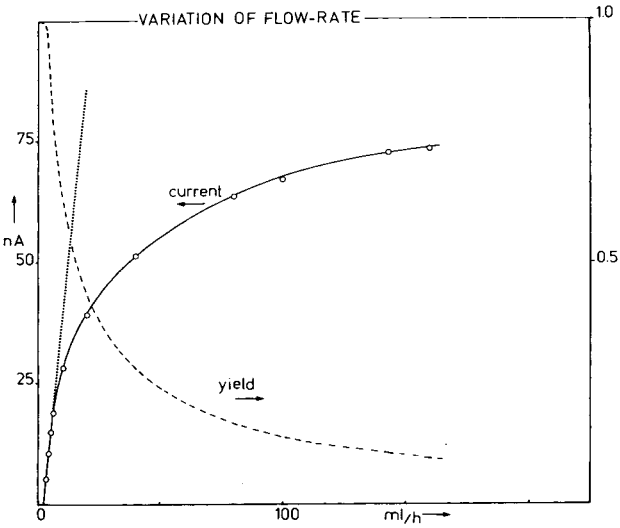

Fig. 2. Static measurement of oxidation current against flow-rate for a solution containing $1.3 \cdot 10^{-7}$ M 5-MeTHF (solid line). Working electrode potential + 0.3 V, liquid film thickness 87 μm. The current vs. flow-rate at a coulometric yield of 1.0 is represented by the dotted line; the coulometric yield vs. flow-rate by a dashed line.

and can be explained by a higher accumulation of reaction products which have to be resorbed from the electrode surface. Under the conditions described under Sample treatment the recoveries from plasma and spinal fluid were found to be 100% over the measured concentration range.

Correlation with total-folate assay

In clinical analysis for the determination of folate deficiency in patients the measurement of the concentration of 5-MeTHF is often based on the growth of *Lactobacillus casei* [4]. The concentrations of patient samples, obtained by this method, are plotted against concentrations obtained by the HPLC method for both plasma (n = 83) and spinal fluid (n = 49) (Fig. 3). Statistical data are given in the legend to Fig. 3. The diffuse pattern can be explained by the interference of the other compounds present in the sample effecting bacteria growth. The systematic deviation from the line connecting equal values is probably due to standardization of the bioassay using solutions of folic acid instead of 5-MeTHF [4].

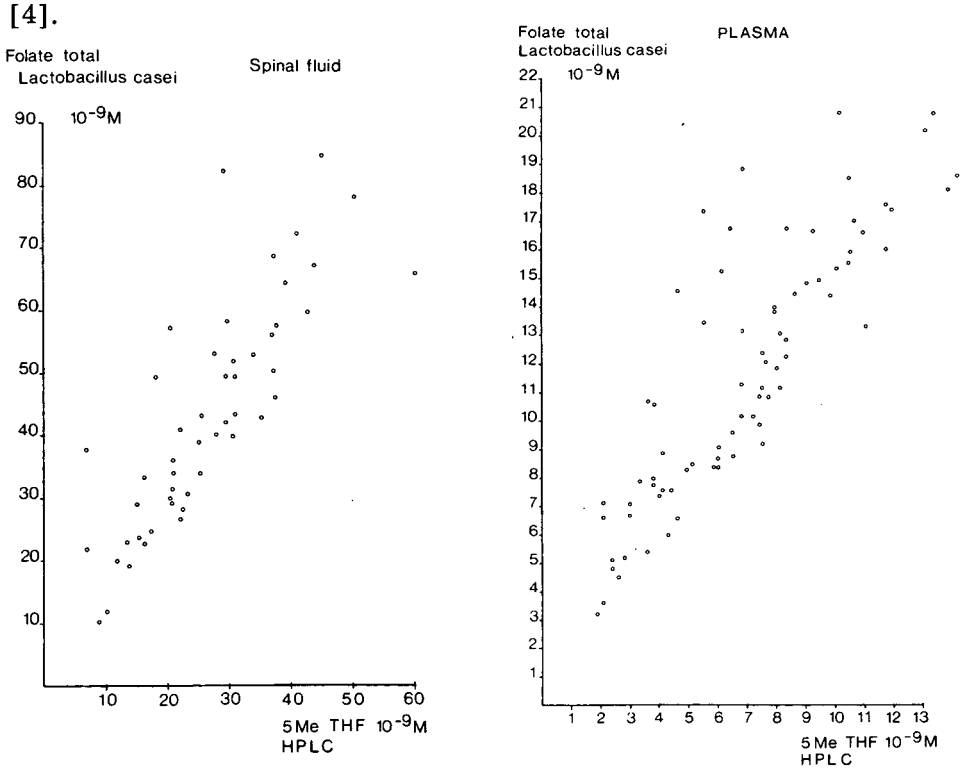

Fig. 3. Correlation of concentrations of 5-MeTHF with total folate measurements using the growth of *Lactobacillus casei* for plasma (n = 83) and spinal fluid (n = 49) samples; computer generated regression lines are represented by $y = 3.45 \cdot 10^{-9}\ M + 1.31 \cdot 10^{-9}\ M \cdot x$ and $y = 8.95 \cdot 10^{-9}\ M + 1.30 \cdot 10^{-9}\ M \cdot x$, respectively, where y = ordinate and x = abscissa; regression coefficient for both cases = 0.83.

Measurement during administration of folates

When folic acid, leucovorin (Ca-folinate) and methotrexate were present in the plasma they showed a sensitivity of less than 0.1% of that of 5-MeTHF under the described conditions. Relatively high concentrations of these other folates can be determined simultaneously by UV detection. The analytical column did not show a good separation of these compounds, and by altering the pH this selectivity could not be substantially improved.

5-MeTHF concentrations can be measured simultaneously with leucovorin or folic acid concentrations. The combination of folic acid and 5-MeTHF in rat plasma is shown in Fig. 4.

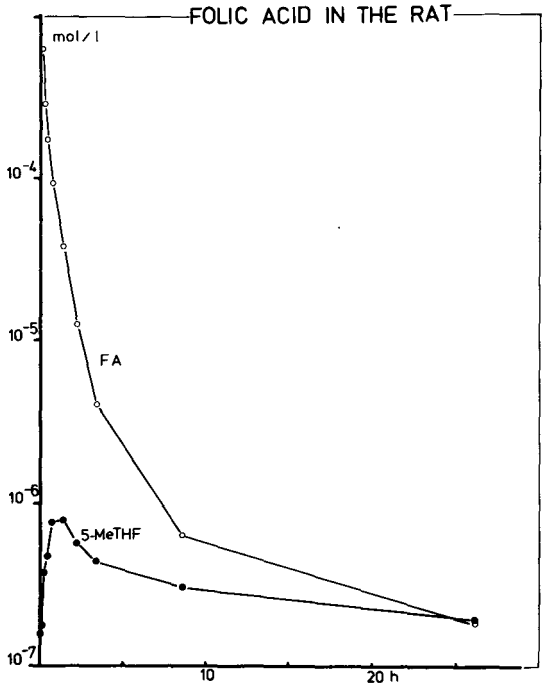

Fig. 4. 5-MeTHF plasma concentrations (EC detection at + 0.3 V) after intraperitoneal administration of folic acid (FA) (UV detection at 280 nm) in the rat (dose 90 mg/kg).

Plasma concentrations in man

Plasma concentrations of 5-MeTHF were measured after oral administration of 15 mg leucovorin (Ca-folinate) in two volunteers. The plasma concentration vs. time profile is shown in Fig. 5, indicating a rapid conversion of leucovorin to 5-MeTHF. The latter compound was shown to have a half-life of 3.5 h in the plasma of these volunteers.

Measurement in urine samples

After addition of ascorbic acid to a human urine sample the concentration of 5-MeTHF was found to increase by about 70% in 8 h. This phenomenon can be explained by the stimulated chemical generation from other folates present in urine or the breakdown of endogenous folates to 5-MeTHF. When no ascorbic acid was added the concentration fell rapidly. Because of these fluctuations determination of 5-MeTHF in urine was less reliable.

DISCUSSION

One-column system

When deproteinized plasma was injected directly into a reversed-phase column or an ion-exchange column a large, unretained, tailing peak was seen from

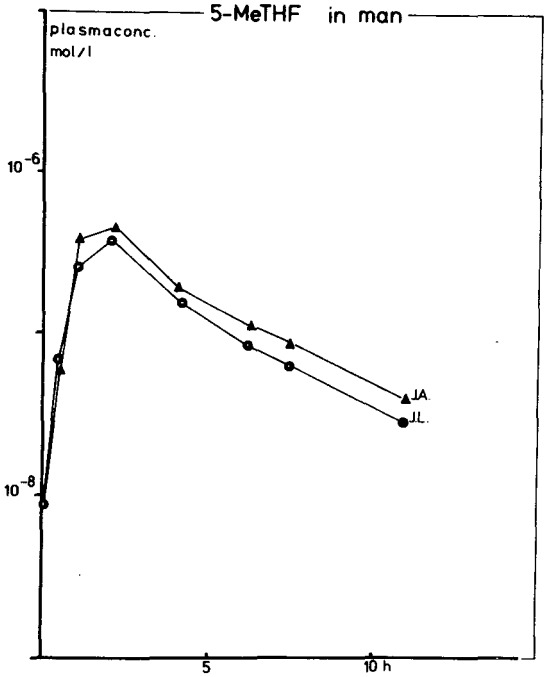

Fig. 5. 5-MeTHF plasma concentration vs. time curves after oral intake of 15 mg leucovorin in two healthy volunteers, 26 (J.A., ▲) and 27 (J.L., ●) years of age.

both the ascorbic acid and the TCA. TCA could be extracted just before injection by diethyl ether, but the concentration of 5-MeTHF in the aqueous layer also decreased considerably. This can be explained by the formation of ion-pairs which are extracted in the organic layer and/or by destruction of the compound.

Concentration eluent volume

Tap water was also investigated as the concentration eluent used for the methotrexate determination [16]. In Fig. 6 the peak height is presented as a function of the concentration eluent volume. It can be concluded that tap water is not useful here, due to elution of 5-MeTHF from the concentration column. After the addition of citrate the retention was considerably increased. However, the growth of micro-organisms in this medium was a serious problem. The addition of 2-hydroxy-1-isopropyl-4-methylbenzene (thymol) had a favourable effect in retarding this growth but appeared to decrease the retention of 5-MeTHF during concentration. This was not the case after the addition of 0.5—1 mg/l sodium azide, which was found to be the most useful for this purpose. In Fig. 6 an upper limit of 12 ml is seen for the concentration eluent.

Choice of electrode potential

The peak height as a function of electrode potential is shown in Fig. 7, indicating a half-wave potential of + 0.3 V (vs. Ag/AgCl/0.001 *M* Cl^-). When the potential increases, a higher sensitivity can be expected, but a lower selectivity

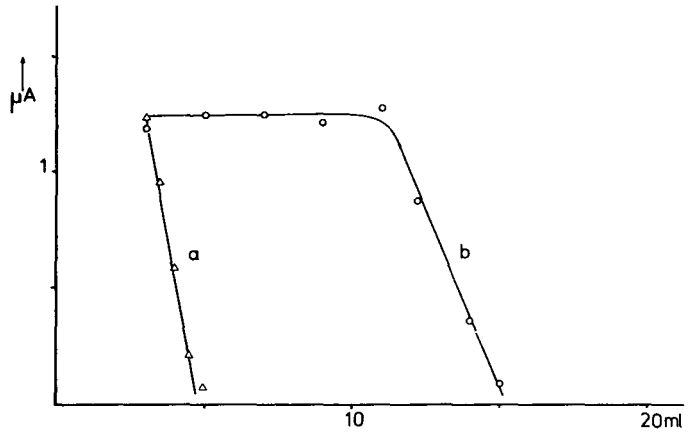

Fig. 6. Effect of citrate on retention during the concentration step. The peak height versus the volume of the concentration eluent for two compositions of concentration eluent is given: (a) tap water; (b) a solution of 0.015 *M* sodium citrate in 0.015 *M* phosphate buffer (pH 4.9), containing 0.5—1 mg/l sodium azide.

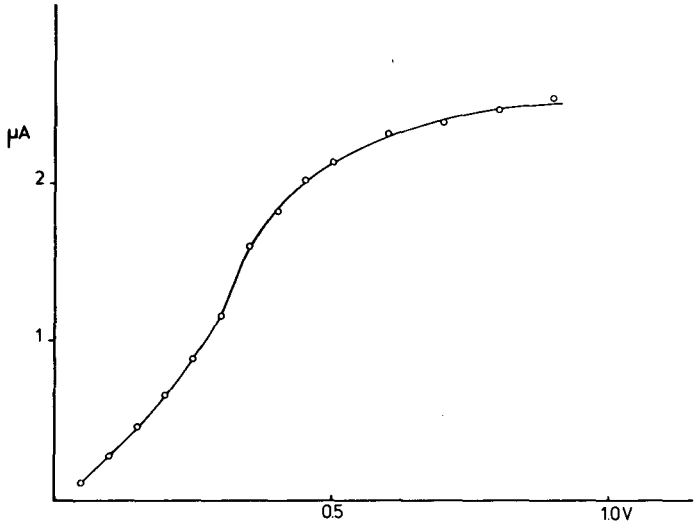

Fig. 7. Maximal peak height in μA vs. working electrode potential (vs. Ag/AgCl/0.001 *M* Cl^-) after injection of $1.8 \cdot 10^{-9}$ mole 5-MeTHF.

towards other oxidizable plasma or spinal fluid components occurs. The difference in the measured chromatogram found by an increase in potential from + 0.3 to + 0.5 V is demonstrated in Fig. 8. A potential of + 0.3 V appeared to be optimal with respect to sensitivity and selectivity.

Quantitation — peak height versus peak area

Whether peak height or peak area is taken as a measure for 5-MeTHF depends on the electrochemical conditions. In voltammetric detection the signal is proportional to the concentration in the detection cell. In order to reduce the decrease in concentration near the electrode surface only a small electrochemical

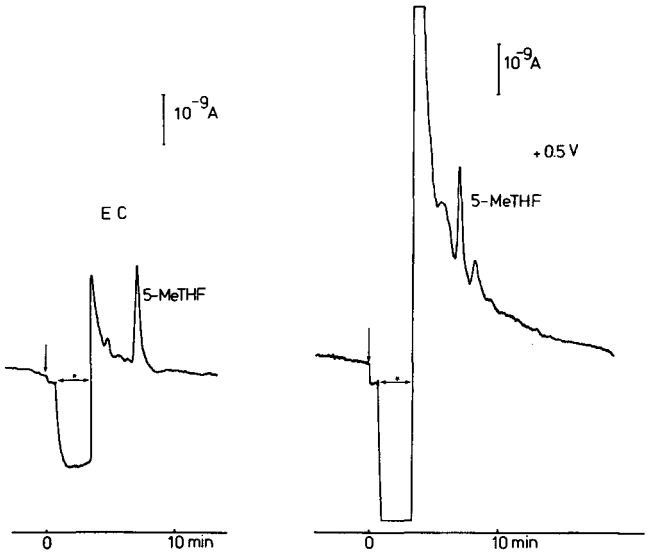

Fig. 8. Effect of electrode potential on detection selectivity for a plasma sample containing 5-MeTHF ($5 \cdot 10^{-9}$ *M*). This figure should be compared with Fig. 1; the asterisks indicate venting period. Chromatograms shown at + 0.3 V and + 0.5 V.

conversion is allowed. In this case the peak height is used for quantitation. However, when the electrochemical reaction or adsorption/desorption processes of reagents and products are rate-limiting an increase in sensitivity is not expected by facilitating transport. From Fig. 2 it can be concluded that under the present chromatographic conditions the voltammetric character dominates. So the peak height should be taken for quantitation. In agreement with these findings dynamic measurement showed more variation of peak area than of peak height when the flow was varied.

CONCLUSION

The method described is useful as a rapid, sensitive and specific determination of 5-MeTHF in plasma and spinal fluid. The selectivity for 5-MeTHF related to other folates in patient samples was obtained by carefully selected detection conditions rather than by separation properties of the analytical column.

In general, this phenomenon should be studied more extensively when considering optimal detection conditions in electrochemical detection. In the determination of methotrexate and 7-hydroxymethotrexate [11,16] interference of 5-MeTHF is excluded by elution during the concentration step. The application of columns with a smaller inner diameter should be investigated for analogous analytical problems. By using micropacked columns with inherent lower eluent flow-rates the detection limit would be decreased by an increase in detector sensitivity.

The behaviour of 5-MeTHF in the body after administration of leucovorin during anticancer therapy is under investigation.

ACKNOWLEDGEMENTS

Mr. J. Arends is acknowledged for participating in the pharmacokinetic study. The microbiological determinations were carried out in the Neurology Laboratory led by Mr. J.C.N. Kok. We thank Miss R. Dirks for the sample analysis. Dr. H. Poppe is thanked for revision of the manuscript. This work has been made possible by a grant of the Netherlands Cancer Society (Koningin Wilhelmina Fonds), project No. UKCN 1976-1.

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QUANTITATIVE ANALYSIS OF OXEPINAC IN HUMAN PLASMA, URINE AND SALIVA BY GAS CHROMATOGRAPHY—MASS FRAGMENTOGRAPHY

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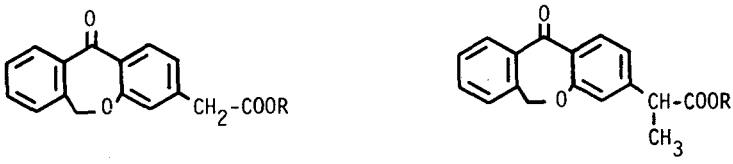
(First received July 16th, 1979; revised manuscript received November 14th, 1979)

SUMMARY

A sensitive and specific method is described for the quantitative analysis of 6,11-dihydro-11-oxo-dibenz[*b*,*e*]oxepin-3-acetic acid (oxepinac) in human plasma, urine and saliva. Oxepinac and internal standard are extracted from acidified plasma, urine or saliva, converted to the corresponding *n*-propyl esters and analysed by gas chromatography—mass fragmentography using selected ion monitoring. The method is accurate and precise over the range 100 μg/ml to 1.0 ng/ml. The method has been applied to the analysis of plasma, urine and saliva from healthy volunteers receiving therapeutic doses of oxepinac.

INTRODUCTION

The compound 6,11-dihydro-11-oxo-dibenz[*b*,*e*]oxepin-3-acetic acid, oxepinac (also DD-3314; I in Fig. 1), is currently being developed as a new anti-inflammatory agent [1, 2]. The determination of plasma levels and urinary excretion of the drug in man has become more important, because they afford valuable information concerning the bioavailability of the drug and its therapeutic and toxic thresholds. It was expected, from the known metabolism of oxepinac in animals [3], that the plasma levels in man would be in the range 0.4—1.5 μg/ml following an initial oral dose of 12.5 mg of oxepinac. Thus a sensitive and specific analytical method for oxepinac in plasma and urine had to be developed. After conversion of oxepinac to its *n*-propyl ester (II in Fig. 1), satisfactory separation by gas chromatography and sufficient sensitivity was available from detection with selected ion monitoring in gas chromatography—mass fragmentography. Saliva is a more convenient fluid to obtain than blood for studies on drug disposition. In order to study the correlation between plasma and salivary concentrations, we attempted to determine the saliva



I R = H

II R = CH_3-CH_2-CH_2-

III = H

IV = CH_3-CH_2-CH_2-

Fig. 1. Structural formulae of oxepinac (I) and DD-3505 (III), and of their *n*-propyl esters (II and IV, respectively).

levels of oxepinac after an oral dose in man. Saliva levels in man could also be detected by this analytical method.

EXPERIMENTAL

Material and reagents

All solvents and reagents were of analytical reagent grade and were used without further purification. Oxepinac (I) and DD-3505 (III) were synthesized in the Research Institute of Daiichi Seiyaku Co.

Gas chromatography—mass fragmentography

An Hitachi Model RMU-6MG mass spectrometer equipped with a gas chromatograph was used. The gas chromatographic conditions for oxepinac *n*-propyl ester (II) were as follows: a glass column (1 m × 3 mm I.D.) containing 2% OV-17 on gas-Chrom Q (80—100 mesh); the temperatures of the oven, the injection port and the separator were 270°, 310° and 310°, respectively. The carrier gas (helium) flow-rate was 30 ml/min in all instances. Mass spectrometric conditions in all instances were as follows: ionization voltage, 30 eV; target current, 100 μA; ion source temperature, 200°; multiplier potential, 2 kV. For selected ion monitoring, a multiple ion detector was employed. The following ion *m/e* focusing was used: *m/e* 310 for oxepinac *n*-propyl ester (II) and *m/e* 324 for DD-3305 *n*-propyl ester (IV).

Analytical procedure

To 1 ml of plasma, 0.1 ml of the internal standard solution (corresponding to 5 μg of DD-3305) and 0.5 ml of 1 *N* hydrochloric acid were added. The solution was extracted with exactly 5 ml of isopropyl ether by shaking for 15 min on an automatic shaker. After centrifugation, exactly 4.5 ml of the organic phase were transferred to a glass tube. The solvent was heated to 40° in a water-bath and removed by a gentle stream of nitrogen. The residue was dissolved in 1 ml of *n*-propanol saturated with hydrogen chloride. The mixed solution was heated at 60° for 1 h. After the reaction, *n*-propanol was evaporated under reduced pressure. To the residue, 1 ml of 3% sodium bicarbonate solution and 5 ml of chloroform were added. The tube was carefully shaken for 2 min. The organic phase, after being transferred to a test-tube, was heated to 40° in a water-bath and removed by a gentle stream of nitrogen.

The residue was then dissolved in 200 μl of *n*-propanol. A volume of 1 or 2 μl of this solution was injected into the gas chromatographic colum of the gas chromatography—mass fragmentography system.

To 1 ml of urine sample, stored at −20°, 0.1 ml of the internal standard solution (corresponding to 20 μg of DD-3505) and 0.5 ml of 1 *N* hydrochloric acid were added and extracted with 5 ml of isopropyl ether; the extract was processed in the same manner as described for the plasma sample.

For the analysis of the conjugate in urine, 1 ml of 2 *N* sodium hydroxide and 0.1 ml of internal standard solution were added to 1 ml of urine. The mixture was heated at 80° for 1 h. After cooling, 2 ml of 4 *N* hydrochloric acid were added, and the mixture subsequently processed in the same manner as described above.

To 2 ml of saliva, 0.1 ml of the internal standard solution (corresponding to 0.5 μg of DD-3505) and 0.5 ml of 1 *N* hydrochloric acid were added, and then processed in the same manner as described for plasma samples.

RESULTS AND DISCUSSION

Internal standard

DD-3505, 2-(6,11-dihydro-11-oxodibenz[*b*,*e*]oxepin-3-yl)propionic acid (III) was used for internal standard (I.S.). On the gas chromatography—mass spectrogram of the *n*-propyl ester of I.S. (IV), the fragment ion *m/e* 310, which was the selected monitoring ion for analysis of the *n*-propyl ester of oxepinac (II), was almost not detected (Figs. 2 and 3). The *n*-propyl esters of oxepinac (II) and I.S. (IV) had almost the same retention times under the gas chromatographic conditions used.

Conjugates

After the administration of oxepinac, large amounts of conjugated metabolites (mainly the acyl glucuronide of oxepinac) are excreted in the urine. The acyl glucuronide of oxepinac was completely converted into free oxepinac by alkaline hydrolysis. When the urinary conjugate was to be analyzed, the urine was first treated with alkali and then the total oxepinac, comprising free and conjugated oxepinac, was analysed. The acyl glucuronide decomposed easily

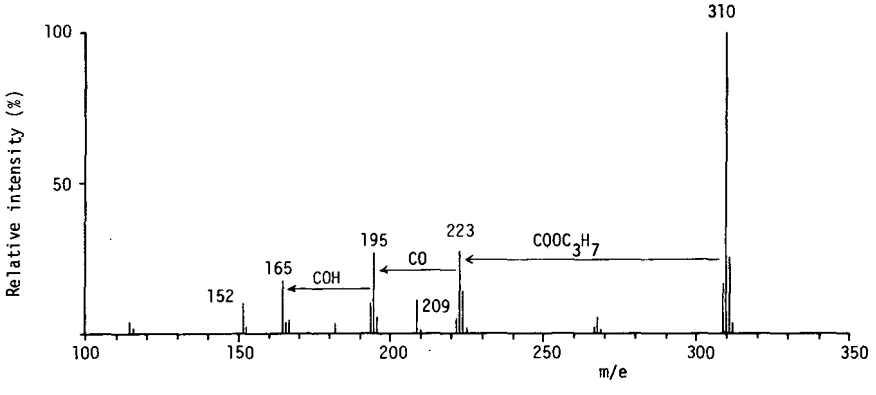


Fig. 2. Mass spectrum of oxepinac *n*-propyl ester (II).

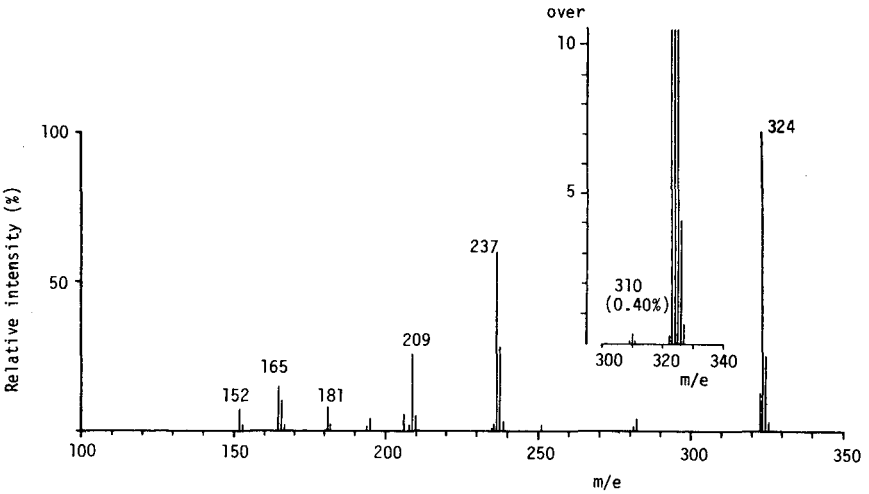


Fig. 3. Mass spectrum of DD-3505 *n*-propyl ester (IV).

and partly regenerated free oxepinac, even in mild neutral conditions, which may lead to errors in the analysis of free oxepinac. In acidic solution, decomposition of the conjugates can be disregarded for a few hours. All samples should therefore be kept deep-frozen until required for analysis.

Extraction from biological material

Isopropyl ether was found to be suitable for the extraction of oxepinac from plasma, urine or saliva. The partition coefficient of oxepinac ($C_{\text{isopropyl ether}}/C_{\text{aqueous}}$) is greater than 30 at pH 1.9 (0.1 *N* hydrochloric acid). The rates of extraction of oxepinac (I) and DD-3505 (III) with isopropyl ether from aqueous solution under acidic conditions were 98.26% and 97.26%, respectively. As solutions of oxepinac are light-sensitive, all operations must be carried out taking sufficient care to shield the solutions from light.

Derivative formation

Oxepinac (I) is a carboxylic acid and is easily converted into its *n*-propyl ester by treatment with *n*-propanol saturated with hydrogen chloride gas. The reaction kinetics are shown in Fig. 4. The reaction conditions of heating at 60° for 1 h were adequate and were chosen for the derivatization of oxepinac (I) and DD-3505 (III).

Despite the hazards associated with its use, *n*-propanol saturated with hydrogen chloride was chosen for the esterification of oxepinac (I). The mass spectrum of the methyl and ethyl esters of oxepiac gave a pattern analogous to that of the *n*-propyl ester, but when *m/e* 282 and *m/e* 296, which were the molecular ions of the methyl and ethyl esters, were monitored, these esters were found to be unsuitable for this analysis since the *m/e* 282 and *m/e* 296 ions were superimposed with the ions from the column coating. Such interference was not encountered with *m/e* 310 and *m/e* 324 ions, which were the molecular ions of the *n*-propyl esters of oxepinac and DD-3505, respectively.

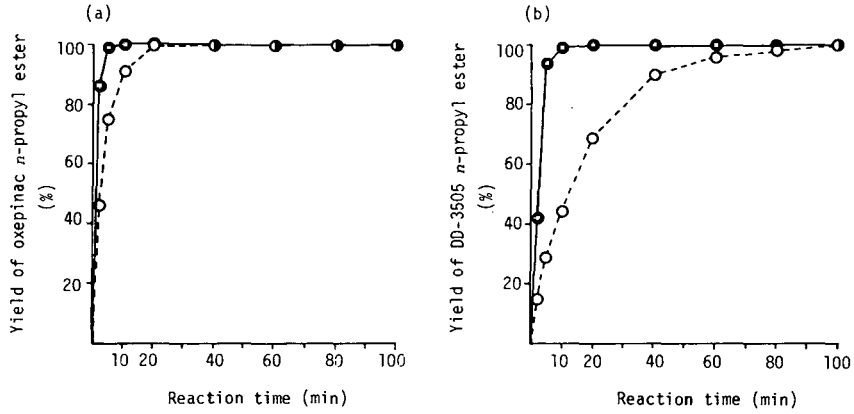


Fig. 4. Effect of temperature on the formation of (a) oxepinac *n*-propyl ester and (b) DD-3505 *n*-propyl ester. Reaction temperatures: •. 60°; ○, room temperature.

Accuracy and specificity

Typical mass fragmentograms obtained from human plasma containing oxepinac and blank plasma are given in Fig. 5. In the mass fragmentograms of extracts from blank plasma, blank urine and blank saliva, the obscured peak did not appear. Oxepinac was detected specifically. The concentration of oxepinac was determined from the peak height ratio of oxepinac *n*-propyl ester (II) to internal standard *n*-propyl ester (IV), and from response factor obtained by analysing, in parallel with the unknown samples, blank plasma, blank urine, or blank saliva to which had been added oxepinac as well as internal standard. The peak height ratio was linear over the range 1—1000 ng/ml in plasma, urine or saliva with a correlation factor of 0.997%. The overall recovery of oxepinac in this procedure was about 93%. For the lowest concentration, the precision is ±5% (n = 5) and the results are accurate to within 7%. This analytical method was so sensitive, that we could also detect the concentration of oxepinac in saliva and plasma-water in man after administrating of oxepinac.

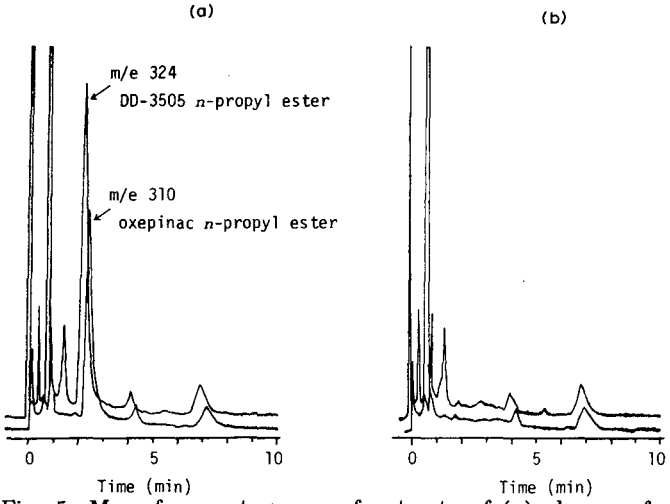


Fig. 5. Mass fragmentograms of extracts of (a) plasma of a human subject treated with oxepinac and (b) blank human plasma.

Application of the method

The utility of these methods was demonstrated by applying them to clinical experiments with human volunteers receiving single or multiple doses of oxepinac. In a typical experiment, healthy volunteers were given a tablet containing 25 mg of oxepinac. The average peak plasma level of five volunteers taking a 25-mg dose was 2.31 μg/ml. The average urinary excretion of oxepinac and acyl glucuronide during the 24 h after administration was 6.01 mg and 14.00 mg, respectively. The average peak saliva level of five volunteers taking a 100-mg dose was 25.8 ng/ml. Representative results are shown in Figs. 6—8.

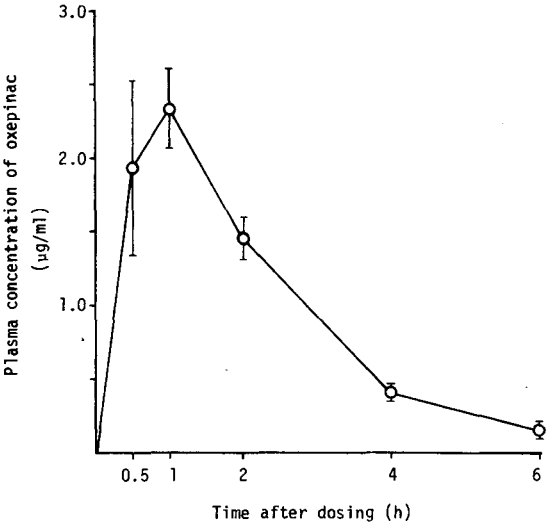


Fig. 6. Plasma levels of oxepinac in human subjects after an oral dose of 25 mg ($n = 5$).

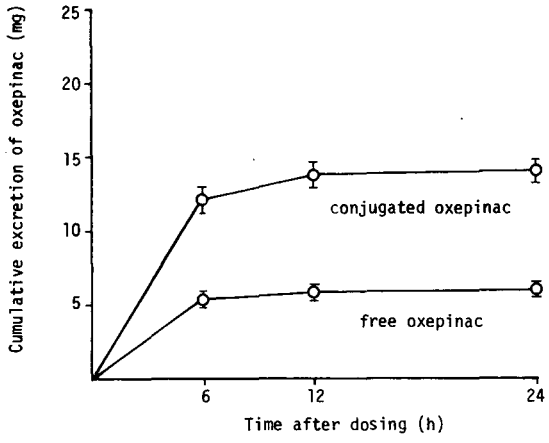


Fig. 7. Cumulative urinary excretion of free and conjugated oxepinac in human subjects after an oral dose of 25 mg ($n = 5$).

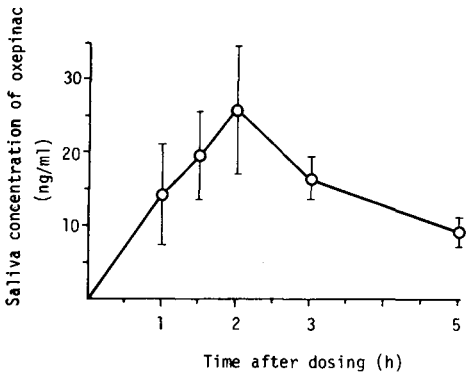


Fig. 8. Saliva levels of oxepinac in human subjects after an oral dose of 100 mg ($n = 5$).

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Journal of Chromatography, 182 (1980) 55—62
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CHROMBIO. 491

SIMULTANEOUS QUANTITATION OF CHLORMETHIAZOLE AND TWO OF ITS METABOLITES IN BLOOD AND PLASMA BY GAS—LIQUID CHROMATOGRAPHY

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(First received August 22nd, 1979; revised manuscript received October 31st, 1979)

SUMMARY

A simple and rapid gas chromatographic method for the quantitation of chlormethiazole and two of its pharmacologically active metabolites, 5-acetyl-4-methylthiazole and 5-(1-hydroxyethyl)-4-methylthiazole, in plasma and blood is described. The total analysis time is less than 20 min for a single sample. The method requires 50—500 μl of plasma or 1 ml of blood. The compounds are detected with a nitrogen—phosphorus detector. An internal standard technique is used for the quantitation. Calibration data are linear over the range 32—2376 ng of chlormethiazole and a similar range of the metabolites in plasma. The method may be used for pharmacokinetic studies.

INTRODUCTION

Chlormethiazole [5-(2-chloroethyl)-4-methylthiazole, CTZ] is an anticonvulsant with sedative and hypnotic properties. It has been used extensively in the treatment of withdrawal symptoms of alcoholism. Recently, several authors have recommended its use in obstetrics as a sedative and hypnotic during labour [1, 2], and as a sedative—anticonvulsant in the treatment of eclampsia and severe pre-eclampsia [3—5]. The disposition kinetics of chlormethiazole in mother and infant in the perinatal period are under investigation in this laboratory.

Ethical considerations dictate that the minimum volume of blood be withdrawn from a patient for the purpose of drug analysis. This is especially true in the field of perinatal medicine since the mothers are subjected to additional blood loss at delivery and the total blood volume of infants is relatively small.

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Therefore, a sensitive method of drug analysis requiring a minimum volume of blood or plasma is of paramount importance.

Two metabolites of CTZ, 5-acetyl-4-methylthiazole (AMT) and 5-(1-hydroxyethyl)-4-methylthiazole (HEMT), have been reported to be pharmacologically active [6] and plasma levels of them in humans have been documented [7]. Therefore, it is desirable to follow the time course of production and elimination of these compounds. Additionally, in seeking a relationship between plasma concentration and pharmacological effect, it is necessary to quantitate these two metabolites as well as chlormethiazole.

Several methods [7—10] have been reported for the quantitation of chlormethiazole in biological fluids. These methods use gas—liquid chromatography [8—10] or mass fragmentography [7, 8]. Only the mass fragmentographic method reported by Nation et al. [7] allows simultaneous quantitation of chlormethiazole and its two metabolites, AMT and HEMT. All of these methods use relatively large volumes of plasma or blood and involve a time-consuming evaporation step prior to chromatographic analysis. This paper describes a simple and rapid gas chromatographic assay for the quantitation of chlormethiazole and two of its metabolites in plasma and blood.

EXPERIMENTAL

Reagents and materials

Chlormethiazole ethanedisulphonate and bromomethiazole [5-(2-bromoethyl)-4-methylthiazole, BTZ] ethanedisulphonate were gifts from Astra Chemicals (Sydney, Australia). The two metabolites of chlormethiazole, 5-acetyl-4-methylthiazole and 5-(1-hydroxyethyl)-4-methylthiazole, were synthesized according to the method described by Moore et al. [11]. The diethyl ether (anaesthetic grade, B.P.) used for extraction was freshly distilled in glass every morning. All other reagents were of analytical grade and were used without further purification. All the glassware was cleaned with a chromic acid mixture, rinsed with distilled water, and oven dried.

Extraction from blood or plasma

Aliquots of plasma (50—500 μl) or blood (1 ml) were pipetted into 15-ml glass centrifuge tubes. In the case of plasma volumes smaller than 500 μl, sufficient distilled water was added to bring the total volume up to 500 μl. Distilled water (700 μl) was added to the tubes containing aliquots of whole blood since this resulted in more uniform mixing. After the addition of an aqueous solution of the internal standard (2.5 μg of bromomethiazole ethanedisulphonate per 25 μl), 200 μl of water, 100 μl of phosphate buffer (pH 7.0, 0.1 *M*), and 5 ml of ether, each tube was closed with a PTFE-lined screw-cap. The tubes were shaken on a vortex mixer for 2 min. After centrifugation at 1500 *g* for 5 min, the organic phase was transferred by disposable pipette to another centrifuge tube containing 500 μl of HCl (1 *M*). The mixture was shaken and centrifuged as described above. The organic phase was aspirated off and discarded. Following the addition of 100 μl of 10 *M* NaOH, the mixture was extracted with 1 ml of ether as described previously. After centrifugation, 5—10 μl of the ethereal extract were injected into the gas chromatograph. Fig. 1 gives a schematic outline of the extraction procedure.

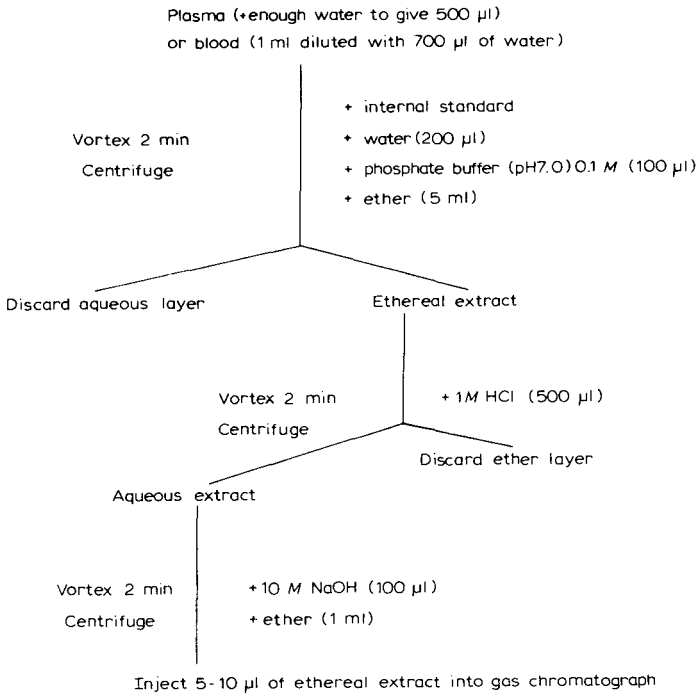

Fig. 1. Schematic diagram for the extraction of CTZ, AMT and HEMT from blood and plasma.

Chromatography

The chromatographic system consisted of a Hewlett-Packard Model 5730A gas chromatograph equipped with a Hewlett-Packard Model 18789A nitrogen—phosphorus detector. A glass column (1.5 m × 3 mm I.D.) silylated with 3% N,O-bis-(trimethylsilyl)trifluoroacetamide in toluene and packed with 5% OV-7 (Pierce Chemicals, Rockford, Ill., U.S.A.) on Gas-Chrom Q (100—120 mesh, Applied Science Labs., State College, Pa., U.S.A.) was used. The temperature of the injection port and detector was maintained at 200° and the oven at 145°. The nitrogen carrier gas flow-rate was 30 ml/min and the hydrogen and air flow-rates were 3 ml/min and 75 ml/min, respectively. The detector voltage (16—22 V) was adjusted to give a 10% recorder deflection at an attenuation setting of 32.

Calibration and reproducibility

Aqueous solutions totalling 200 µl and containing known quantities of chlormethiazole ethanedisulphonate (equivalent to 32—2376 ng of the chlormethiazole base), AMT (37—2760 ng) and HEMT (29—2160 ng) were added to blank plasma samples (50—500 µl) in such a way that samples containing low levels of CTZ contained high levels of the metabolites, and vice versa. This was to mimic the clinical situation, in which, soon after starting infusion, CTZ concentrations are high while concentrations of the metabolites are low, and after cessation of infusion, the converse may be true. The samples were assayed for CTZ, AMT and HEMT as described earlier. Calibration curves were constructed by plotting

the ratios of the peak heights of CTZ, AMT and HEMT to that of BTZ (the internal standard) versus the amounts of added CTZ, AMT and HEMT, respectively.

To check the reproducibility of the analytical procedure, three different plasma calibration curves (each composed of 5 or 6 data points) were constructed on three different days using human plasma samples from five different sources: a healthy non-pregnant female and four healthy pregnant women near term. Calibration curves (each composed of three data points) using blood (1 ml) from two healthy pregnant women near term were similarly prepared on two different days over the range 316.8—3960 ng of CTZ, 368—4600 ng of AMT and 288—3600 ng of HEMT.

Drug disposition study

Chlormethiazole ethanedisulphonate (Hemineurin®, Astra Chemicals) was administered by intravenous infusion to a pregnant patient near term (age 21 years, weight 70 kg) at a rate of 800 mg/h for the first 1.42 h and then at a rate of 120 mg/h for the next 6.28 h. Informed consent was previously obtained. Blood was withdrawn at intervals into disposable syringes (Monoject, Sherwood Medical Industries, Deland, Fla., U.S.A.) via a cannula inserted in an antecubital vein of the arm not receiving the infusion, and placed in plastic tubes containing 100 units of ammonium heparin and separation granules (Disposable Products, Sydney, Australia). The plasma was separated and stored frozen until time of analysis.

RESULTS AND DISCUSSION

Under the chromatographic conditions described above, the retention times of AMT, HEMT, CTZ and BTZ were 4, 4.6, 6 and 9 min, respectively. Fig. 2 shows chromatograms of the extracts of plasma samples (500 μl) from a pregnant patient drawn immediately prior to commencement of chlormethiazole therapy, and at 0.38 and 4.92 h after the start of the infusion. Chromatograms obtained from extracts of blood were similar. The CTZ, AMT, HEMT and BTZ peaks were satisfactorily resolved from each other and were not interfered with by peaks of endogenous compounds in plasma or blood. In extracts of samples collected 1 h or more after commencement of therapy, there was a relatively small peak which chromatographed just before the AMT. This peak may represent another metabolite of CTZ as the patient was not taking any other medication. The identity of this compound is not known.

A summary of the composite calibration data in plasma is presented in Fig. 3. The calibration curves are linear (r = 0.9997, 0.9996 and 0.9993 for CTZ, AMT and HEMT, respectively) and pass through the origin. Similar results were obtained from blood analyses (r = 0.9985, 0.9974 and 0.9993 for CTZ, AMT and HEMT, respectively). The linearity of the calibration curves demonstrates also the reproducibility of the analysis as the calibration data were obtained on different days using samples from different sources. Slopes of the linear regression lines of the plasma calibration data from three different days are presented in Table I. The method allows quantitation of 32—2376 ng of CTZ, 37—2760 ng of AMT and 29—2160 ng of HEMT in plasma, and of 316.8—3960 ng of CTZ,

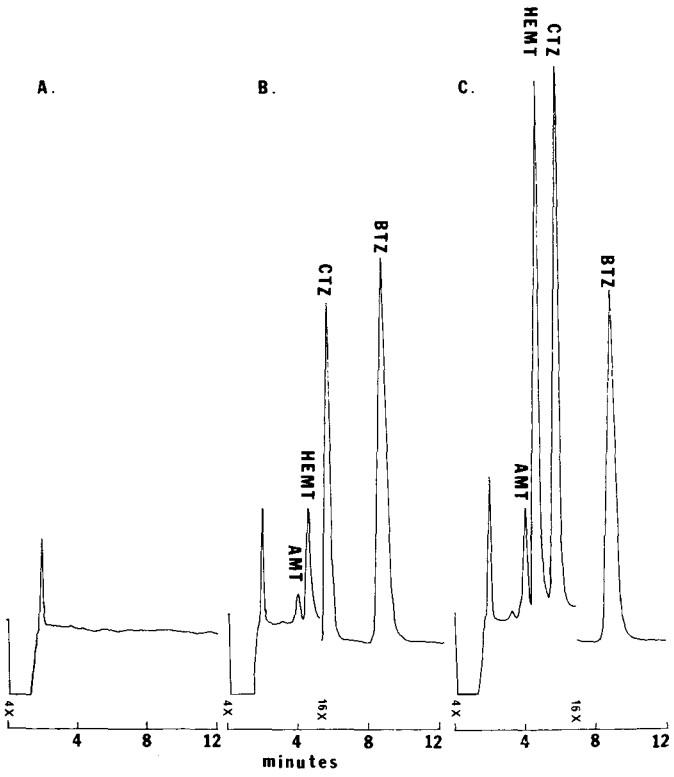

Fig. 2. Chromatograms of extracts of plasma samples (500 μl) from a pregnant patient near term. (A) Immediately prior to commencement of CTZ therapy. (B) 0.38 h after commencement of therapy (AMT, 61.4 ng/ml; HEMT, 191 ng/ml; and CTZ, 1759 ng/ml). (C) 4.92 h after commencement of therapy (AMT, 252 ng/ml; HEMT, 982 ng/ml; and CTZ, 753 ng/ml). Chromatographic conditions are given in text.

368—4600 ng of AMT and 288—3600 ng of HEMT in blood. The signal-to-noise ratio for 32 ng of CTZ was 25:1, which suggests that the method may be applicable to the analysis of considerably smaller amounts.

The major problem encountered in the development of this method was that of combining a chromatographic and detection system that provides high sensitivity and adequate separation between AMT, HEMT, CTZ and the solvent front. With conventional flame ionization detection, the solvent front corresponding to 10 μl of ether will override both the AMT and HEMT peaks and also interfere with the CTZ peak when relatively low levels of the compounds are involved. Sensitivity to low-molecular-weight substances such as CTZ, containing only six carbon atoms, is also low. The use of selective detectors can significantly reduce the interference by the solvent front. Electron-capture detection should give adequate sensitivity for CTZ and BTZ, but the metabolites, AMT and HEMT, which do not contain halogens, will not give adequate response. These problems were overcome by the use of a nitrogen—phosphorus detector (NPD). With the use of an NPD, ether only appeared as a short negative signal which did not interfere with the analysis, and high sensitivity was also achieved with the nitrogen-containing compounds involved.

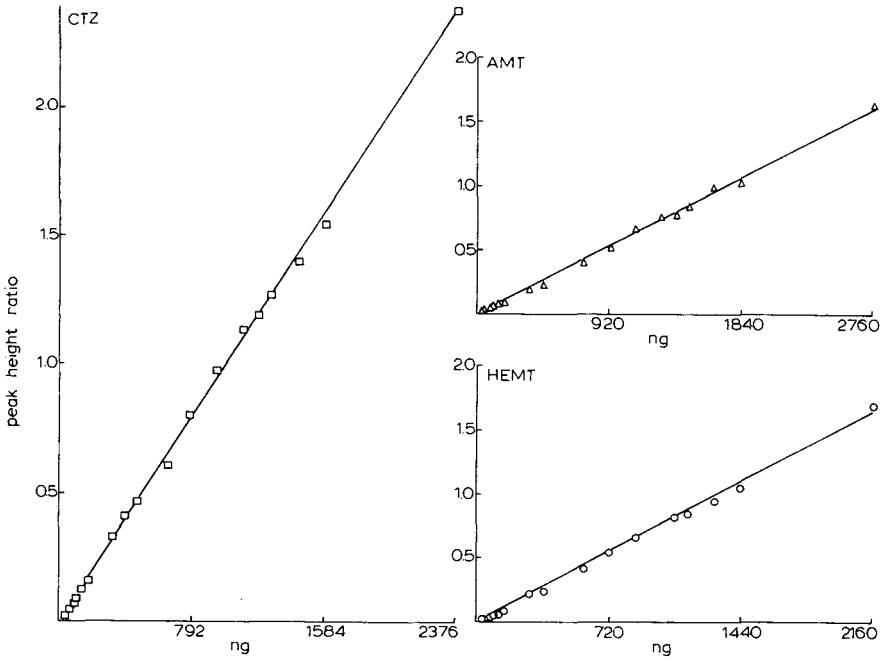

Fig. 3. Calibration data in plasma.

TABLE I

SLOPES OF THE LINEAR REGRESSION LINES OF THE PLASMA CALIBRATION DATA FROM THREE DIFFERENT DAYS

	Slope × 10^4		
	CTZ	AMT	HEMT
Day 1	9.92	5.68	7.43
Day 2	10.01	5.70	7.44
Day 3	10.05	5.95	7.73

One disadvantage of an NPD is the relatively narrow range of linear detector response. If the final ethereal extract was concentrated to about 10 μl and the entire volume chromatographed, then the calibration data showed progressive negative deviation from linearity as the amount of the compounds was increased. Obviously, higher sensitivity can be achieved by either injecting a larger volume or concentrating the ethereal extract. For example, when the entire extract was concentrated down to 10 μl and injected, the signal-to-noise ratio was approximately 40:1 for 3.2 ng of CTZ. Conversely, quantities of the compounds beyond the upper limits of the calibration curves can be analyzed by either injecting smaller volumes or diluting the final ethereal extract. When injecting only 1% (10 μl) of the final 1-ml ethereal extract, the amounts of the compounds on column were in the linear range of detector response.

The use of various silicone phase column packings (OV-1, OV-7, OV-17, OV-25 and OV-225) was investigated. All these stationary phases gave adequate

separation of AMT, HEMT, CTZ and BTZ, with OV-7 and OV-225 giving the best peak shapes. A column packed with OV-7 was chosen since cyano-substituted silicone packings such as OV-225 are incompatible with the NPD.

The extraction of CTZ and its metabolites from plasma and blood using either hexane, ether or dichloromethane was investigated. Ether was found to be most suitable as it gave adequate and consistent extraction of all the compounds. Hexane and dichloromethane also gave adequate extraction of CTZ and BTZ. But the less polar hexane resulted in poor and non-reproducible extraction of HEMT and the same applied to extraction of AMT with the more polar dichloromethane. Also, use of dichloromethane may cause a reversible loss of detector sensitivity. The acid wash of the first ethereal extract is necessary to remove neutral compounds which interfere with the analysis. The selectivity of the extraction procedure and chromatographic system is demonstrated by the chromatograms in Fig. 2.

Reproducible quantitation of CTZ, AMT and HEMT was achieved by using BTZ, the bromo analog of CTZ, as the internal standard over the range of plasma volumes 50—500 μl and for 1 ml of blood. The ability to analyze very small volumes of plasma enables clinical monitoring and pharmacokinetic studies to be carried out in newborns as only micro volumes of blood can be sampled.

By using this method, the plasma levels of CTZ, AMT and HEMT were followed for 30 h in the perinatal period in a woman who required CTZ for sedation. The plasma concentration—time profile is presented in Fig. 4. These data

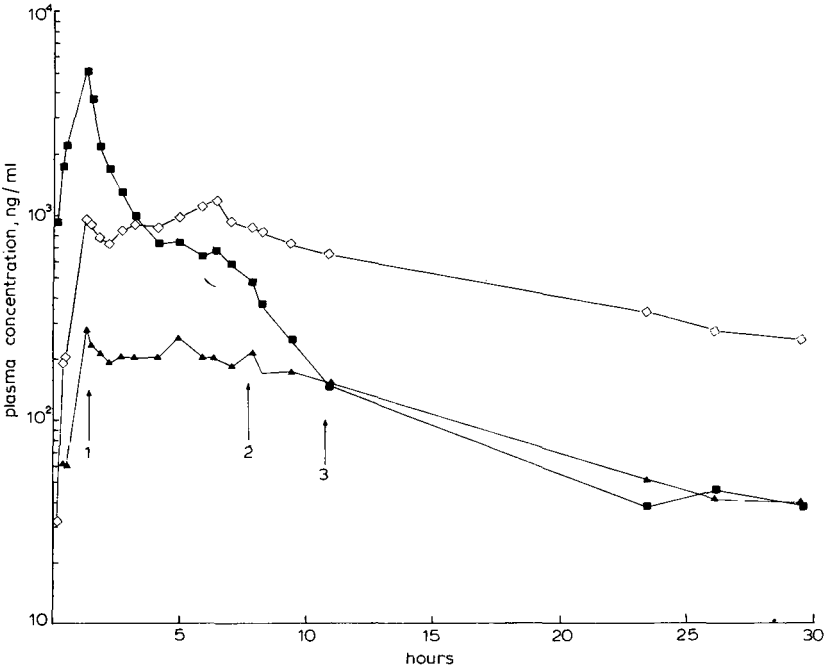

Fig. 4. Plasma concentration—time profiles of CTZ (▪), AMT (▲) and HEMT (◇) in a woman in the perinatal period. CTZ was administered by intravenous infusion. Infusion commenced at a rate of 800 mg of chlormethiazole ethanedisulphonate per hour, changed to 120 mg per hour at (1) and stopped at (2). Childbirth occurred at (3).

were amenable to pharmacokinetic analysis and this will be reported elsewhere.

This chromatographic method is rapid and precise for the quantitation of CTZ, AMT and HEMT in plasma and blood. Analysis can be performed with 50—500 μl of plasma or 1 ml of blood. The sample preparation procedure is relatively simple requiring no evaporation step. The total analysis time for a single sample is less than 20 min.

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DETERMINATION OF METHIMAZOLE IN PLASMA USING GAS CHROMATOGRAPHY—MASS SPECTROMETRY AFTER EXTRACTIVE ALKYLATION

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(First received June 26th, 1979; revised manuscript received September 14th, 1979)

SUMMARY

A gas chromatography—mass spectrometry method for quantitation of the thyreostatic agent methimazole in plasma is described. The drug was transferred from the plasma sample and derivatized in one step by extractive alkylation. Either of two alkylating agents benzyl-chloride or pentafluorobenzyl bromide were used. Deuterium-labelled methimazole was used as internal standard. The precision of the method at the level of 5 ng methimazole per ml plasma was 6%.

INTRODUCTION

Methimazole (1-methylimidazole-2-thiol) is a drug used in the treatment of hyperthyroidism. It is also generated in the body from carbimazole (ethyl-3-methyl-2-thioxo-4-imidazoline-1-carboxylate), another drug which is more widely used in the same indication. Their structures are shown in Fig. 1. Methods for the determination of methimazole in rat urine [1] and plasma [2—4] utilizing gas and liquid chromatographic techniques have been described. Only one of these methods described is sensitive enough to reach those levels of methimazole in plasma which result from standard oral therapy. But even this method lacks the sensitivity to monitor the lowest therapeutic levels.

The extraction of methimazole from plasma with organic solvents seems to be rather variable. Some authors claim that they have achieved about 70% recovery [4] when extracting into chloroform as organic solvent whereas others have achieved 54% recovery [3] using the same solvent. Extraction of methimazole from plasma with chloroform or ethyl acetate during the course of this work showed a variation in the extraction yield ranging from below 50%

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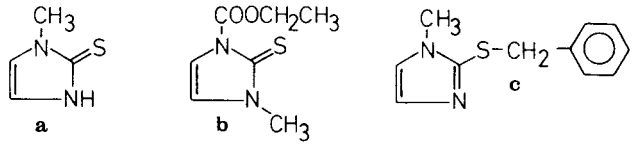


Fig. 1. Structural formulae of (a) methimazole; (b) carbimazole; (c) S-benzylated methimazole.

to about 70%. The plasma samples used for these extractions were collected from different persons on different occasions. These results indicate that it is unsatisfactory to rely on methods depending on this type of extraction when different plasmas are used.

In the method described in this paper, methimazole is extracted as an ion-pair into the organic phase where it is benzylated. The S-benzyl (or pentafluorobenzyl) derivative of methimazole (Fig. 1) is quantified on a gas chromatographic—mass spectrometric (GC—MS) system equipped with a glass capillary column. A deuterium-labelled analogue, 1-trideuteromethylimidazole-2-thiol, is used as internal standard (IS).

MATERIALS AND METHODS

Instrumental

The measurements were carried out in a Finnigan 4000 gas chromatograph—mass spectrometer equipped with a multiple ion monitoring device (Finnigan, Sunnyvale, Calif., U.S.A.). The injector of the gas chromatograph was of the Grob capillary type and operated at 200° in a splitless mode. The injector was equipped with valves which were programmed to vent the injector 60 sec after injection. The glass capillary column used was a 20-m UCON HB 5100 (Jaeggi, Trogen, Switzerland). A pressure of 20—25 kPa of helium was applied on the column which was directly interfaced to the ion source. The electron energy was 70 eV. The temperature of the GC oven was programmed to rise from 140 to 180° at a rate of 10°/min. The samples could be injected with 7—8 min intervals. For the injection, solid sample syringes (SGE, North Melbourne, Australia) were used. They were cleaned in a Hamilton syringe cleaner between the injections. A Jeol FX-100 NMR spectrometer was used for obtaining the NMR spectra.

Internal standard

Preparation of 1-trideuteromethylimidazole-2-thiol: Acetalylthiocarbimide [5] (5.45 g), prepared from aminoacetal, was mixed with trideuteromethyl amine HCl (2.86 g) and 5 ml ethanol in a screw-capped tube. The mixture was cooled in iced water and sodium hydroxide (1.64 g) was added. The tube was then slowly shaken until it reached room temperature (22°), where it was allowed to stand for a couple of hours. The sodium chloride that had formed was filtered off and the solvent evaporated. The residue was hydrolysed by refluxing it with 20 ml 30% sulphuric acid for 30 min. After cooling, it was neutralised with 4 *M* sodium hydoxide solution and extracted with ethyl acetate (4 × 50 ml). Evaporation of the solvent gave a crude residue which was purified on a silica column eluted with chloroform containing 4% ethanol.

The amount of pure 1-trideuteromethylimidazole-2-thiol isolated was about 800 mg.

Extraction of methimazole and formation of derivatives

To 1 ml of plasma in a screw-capped tube were added 100 μl water containing internal standard (concentration 200 ng/ml) and 50 μl 0.1 *M* tetrabutylammonium solution followed by 1 ml 0.5 *M* carbonate buffer (pH 10). The tetrabutylammonium solution was prepared by adding an equimolar amount of 4 *M* sodium hydroxide to tetrabutylammonium hydrogen sulphate and then adding water until the desired concentration was reached. The sample mixture was extracted with 5 ml dichloromethane containing 2.5% (v/v) of benzyl chloride (or pentafluorobenzyl bromide when determining plasma concentrations below 20 ng/ml). The extraction was performed in a water-bath at 50° for 20 min. After cooling to room temperature the tube was centrifuged (500 *g*) and the organic phase was then transferred to a new tube and evaporated to dryness with a stream of nitrogen. The residue was dissolved in 20 μl of ethyl acetate and 1—2 μl was evaporated on the needle of the solid sample syringe.

RESULTS

Extraction and alkylation

Methimazole is extracted from plasma as an ion-pair with the tetrabutylammonium ion into dichloromethane. Since methimazole is converted to a more lipophilic compound in the organic phase through reaction with benzyl chloride (or pentafluorobenzyl bromide), it is efficiently extracted from the aqueous phase. Possible variations in the conditions are compensated for by the use of the labelled methimazole as internal standard.

The extraction and alkylation step is carried out at a temperature of 50°. The yield of S-benzyl methimazole decreased considerably when the extractive alkylation was performed at room temperature but this could be circumvented to some extent by an increase of the concentration of benzyl chloride in the organic phase. A comparison of the yields is shown in Table I, where the yield in the reaction at 50° has been set to 100. The reaction at 50° is completed within 20 min. The effect of elevated temperature has been reported previously

TABLE I

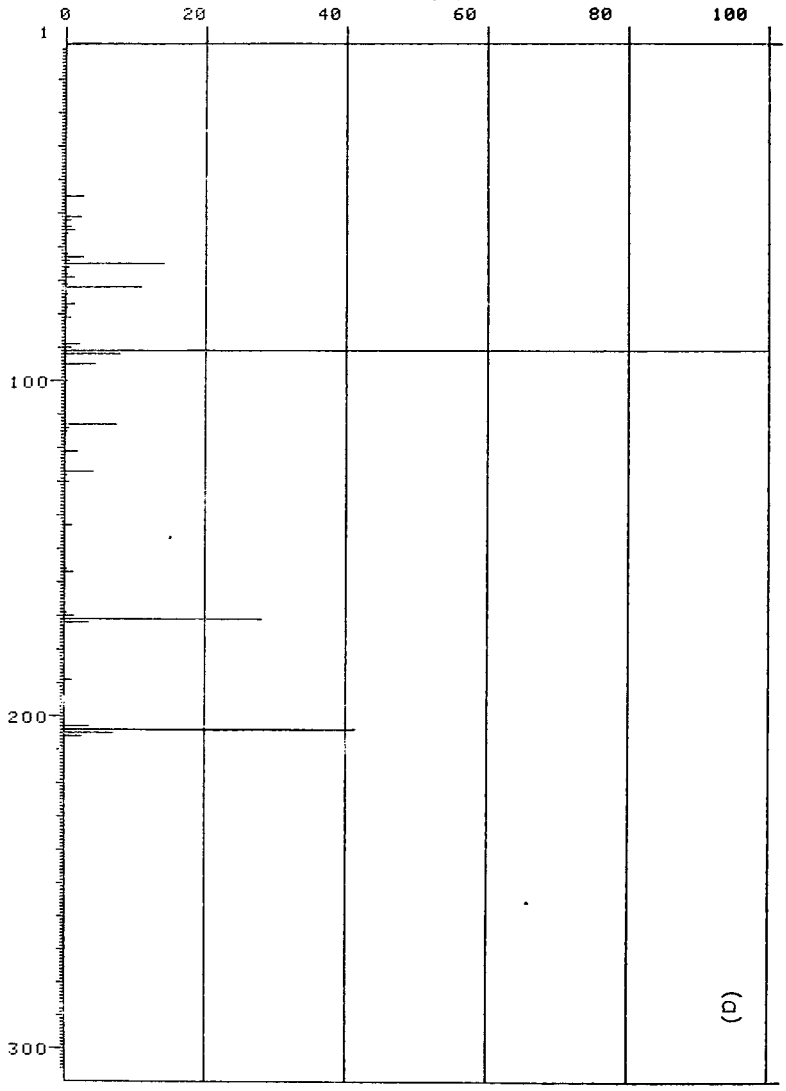
EXTRACTIVE BENZYLATION OF METHIMAZOLE IN CARBONATE BUFFER (pH 10)—DICHLOROMETHANE SYSTEM

The reaction time was 1 h.

Benzyl chloride concentration (%)	Temperature (°C)	Yield (%)
2.5	50	100
2.5	22	32
5	22	60
10	22	75

RUN IDENTIFICATION BENSYLMETIMAZOL
SPECTRA FILE NAME QQQ
FILE POSITION 24
BACKGROUND 10
MAXIMUM INTENSITY 11129 % OF TOTAL ION 33.0
OUTPUT MASS RANGE 1 TO 492
SCAN SPEED 5

MASS	91	204	171	65	72	92	113	205	95	127
INTENSITY	10000	5261	2831	1430	1105	824	774	735	456	444



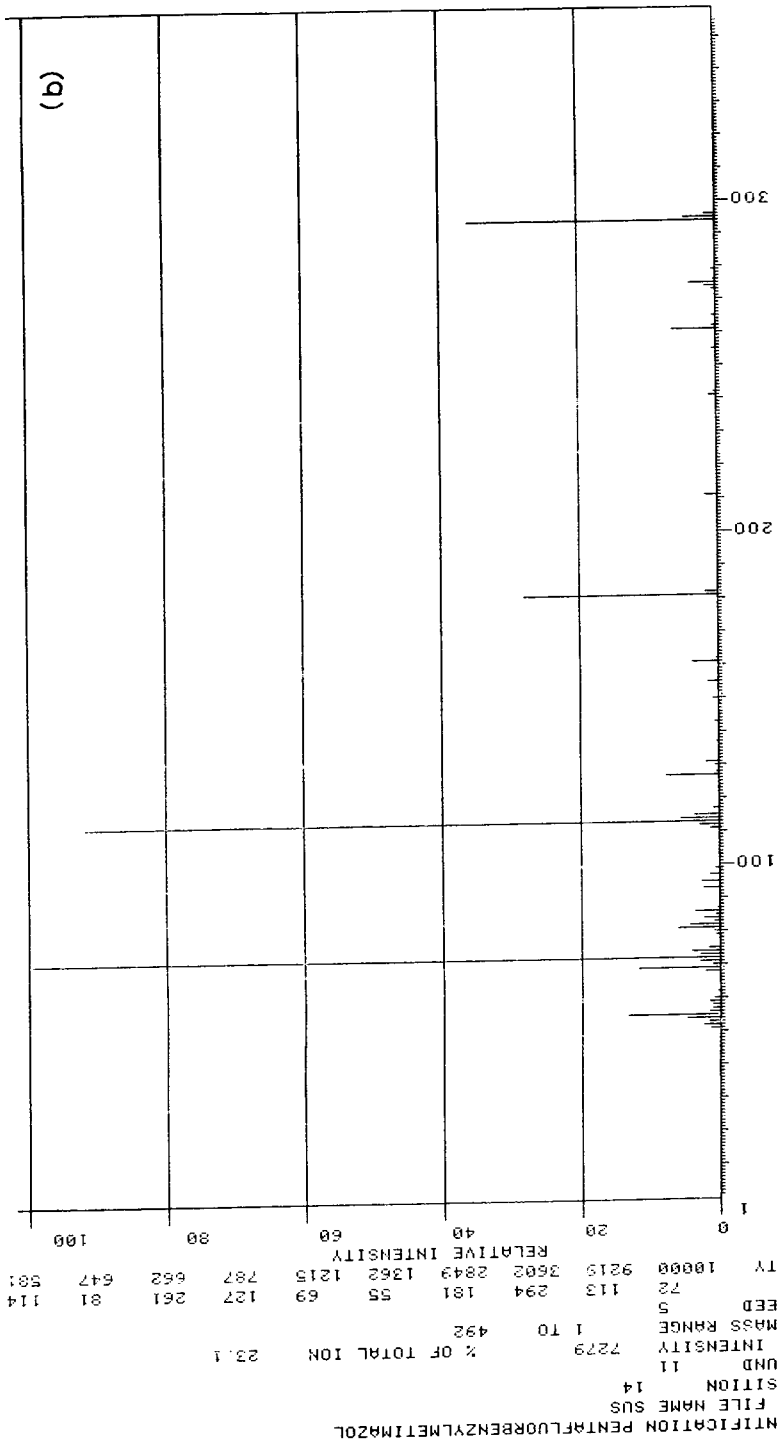


Fig. 2. Mass spectra of (a) S-benzylated methimazole and (b) S-pentafluorobenzylated methimazole.

in a report [6] which contains a survey of compounds that yield derivatives when subjected to extractive alkylation. In a series of extractions, the pH of the aqueous phase was increased from 7 to 12 and an improved extraction could be demonstrated up to but not above pH 10. The alkylation with pentafluorobenzyl bromide resulted in increased sensitivity because a higher molecular weight moves the molecular ion into a range with a lower background. Benzyl chloride is used as alkylating agent for samples where the concentration is likely to be above 20 ng/ml because it is cheaper and readily available. The structure of the benzyl derivatives was indicated by ^{13}C-NMR spectra. The thiocarbonyl carbon situated at 159.9 ppm relative to TMS disappeared and a new peak appeared at 138.9 ppm corresponding to =C(H)—S.

Gas chromatography

The UCON HB 5100 glass capillary column was used to achieve good peak symmetry when chromatographing the methimazole derivatives and the low background produced by this column was also beneficial to the sensitivity of the system. The benzyl and pentafluorobenzyl derivatives eluted after 4.2 and 6.2 min respectively. The mass spectrometer was focused on the molecular ions of the two types of methimazole/IS derivatives which had *m/e* values of 204/207 and 294/297 respectively. Fig. 2a and b shows mass spectra of benzyl and pentafluorobenzyl methimazole. Figs. 3 and 4 illustrate chromatograms recorded when the molecular ions of the different methimazole derivatives were monitored.

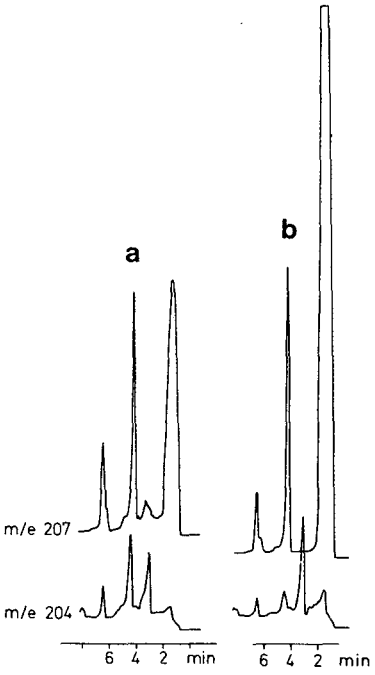


Fig. 3. (a) Chromatogram of plasma containing 20 ng/ml of methimazole obtained after benzylation; benzyl methimazole corresponds to the peak eluting after 4.2 min. (b) Chromatogram of blank plasma sample, internal standard, *m/e* = 207.

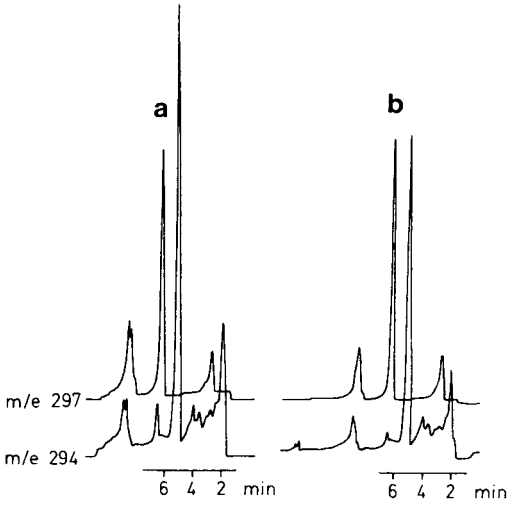


Fig. 4. (a) Chromatogram of plasma sample containing 5 ng/ml of methimazole obtained after pentafluorobenzylation; pentafluorobenzyl methimazole corresponds to the peak eluting after 6.2 min. (b) Chromatogram of blank plasma sample, internal standard, *m/e* = 297.

In the chromatograms of the blank samples a background peak can be observed which interferes with the methimazole derivatives. In the case when benzyl derivatives were used this background peak corresponds to about 7 ng/ml of methimazole in a plasma sample. The use of the pentafluorobenzyl derivative gave a background peak corresponding to about 1 ng/ml. The background peak originates from the plasma samples since no interfering peak could be detected when a sample of pure water was analysed according to the method. Due to the blank peak the detection limits of the method have been set to 15 and 2 ng/ml of methimazole for the benzyl and pentafluorobenzyl derivatives respectively. Blank plasma samples have been analysed both from healthy volunteers and from thyreotoxic patients. In these samples no significant variation in the interfering background peaks could be detected.

Calibration graph

The two calibration graphs in Fig. 5a and b were prepared by adding different amounts of methimazole to plasma samples and analysing them according to the described method. In Fig. 5a, the ratio of the peak heights resulting from the molecular ions 204 and 207 of the benzyl derivatives were plotted versus the concentration of methimazole. The amounts of methimazole that had been added to the plasma samples ranged from 15 to 150 ng/ml. In Fig. 5b, a similar plot is shown using the molecular ions 294 and 297 obtained from the pentafluorobenzyl derivatives. In this case the amounts that had been added to the plasma samples ranged from 2—50 ng/ml. The calibration graphs were both linear in the range investigated. The two graphs were constructed in the lowest range possible for each derivative. The precision of the method was determined by adding 50 ng/ml of methimazole to plasma samples, which were analysed according to the method. The precision was 2.5 (n = 10) and 1.5% (n = 10) for the benzyl and pentafluorobenzyl derivatives

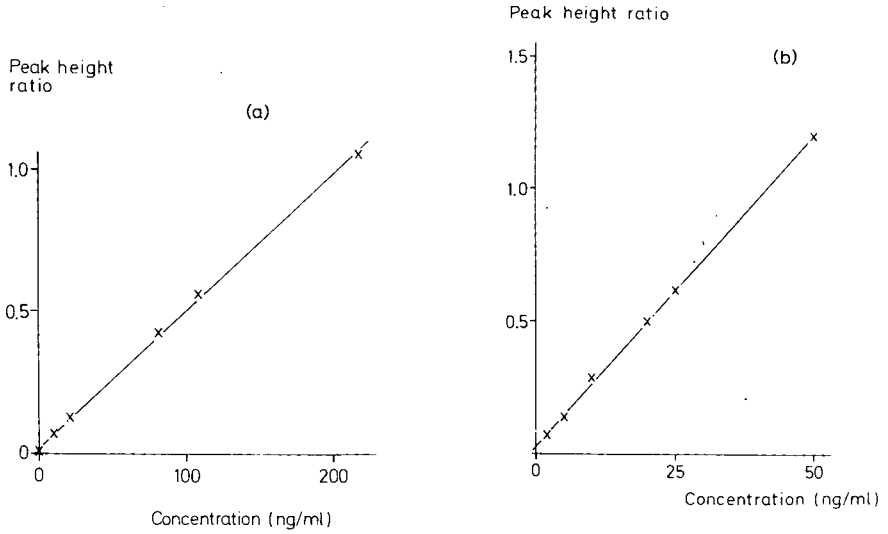


Fig. 5. Typical standard curves constructed by (a) plotting the 204/207 peak height ratios versus concentration of methimazole and (b) plotting the 294/297 peak height ratios versus concentration of methimazole.

respectively. The precision at the 5 ng/ml level of methimazole determined as pentafluorobenzyl derivatives was found to be about 6% (n = 9).

ACKNOWLEDGEMENTS

The authors are grateful to Mr. G. Everitt for measuring the NMR spectra and to Mr. S. Eriksson for drawing the figures.

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Biomedical Applications

CHROMBIO. 504

MICROASSAY FOR PRIMIDONE AND ITS METABOLITES PHENYLETHYLMALONDIAMIDE, PHENOBARBITAL AND *p*-HYDROXYPHENOBARBITAL IN HUMAN SERUM, SALIVA, BREAST MILK AND TISSUES BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY USING SELECTED ION MONITORING

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(Received August 31st, 1979)

SUMMARY

A method for the quantitative determination of primidone and its metabolites phenobarbital, phenylethylmalondiamide (PEMA) and hydroxyphenobarbital (free and conjugated) in serum, urine, saliva, breast milk and tissue has been developed. Following the addition of the methyl analogues of primidone, phenobarbital and PEMA as internal standards and of saturated ammonium sulphate, the samples (5—100 μl) were extracted twice with ethyl acetate—benzene (20:80). The extracts were divided into two equal portions; one portion was ethylated by Greeley's method for the analysis of primidone, phenobarbital and hydroxyphenobarbital, while the other was trimethylsilylated for the analysis of primidone and PEMA. A gas chromatographic—mass spectrometric system was used for the analysis of the derivatized extracts. Linear calibration curves were obtained in the concentration range studied (between 100 ng/ml and 30 μg/ml). The recoveries of the drugs were between 80 and 93%. The relative standard deviations were between 3.2 and 5.9% (100-μl serum samples containing 1 μg/ml of the drugs). The lower detection limits were found to be between 1.4 and 3.7 ng/ml using serum samples of 100 μl.

These methods have been applied to the study of the placental transfer and neonatal disposition of primidone and its metabolites in the human.

INTRODUCTION

Pharmacokinetic studies of placental transfer and neonatal metabolism (for recent reviews cf. ref. 1), particularly if low levels of metabolites are to be determined, require extremely sensitive methods of analysis, since only small amounts of tissue and body fluids are available. The methods presently available for pharmacokinetic studies of primidone are either directed only to the

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analysis of the parent drug for the purpose of anticonvulsant monitoring [2—12], or are too insensitive and require relatively large amounts of sample [13—17].

We have therefore developed methods for the assay of primidone and its metabolites (see Fig. 1) phenobarbital, phenylethylmalondiamide (PEMA) and hydroxyphenobarbital (free and conjugated) in small volumes (5—100 μl) of human serum, saliva, urine, breast milk and fetal tissues. Analysis was performed by a gas chromatograph—mass spectrometer—computer system (GC—MS—computer) operated in the selected ion monitoring mode (mass fragmentography). The sensitivity and selectivity of the GC—MS methods enabled us to use very simple and rapid sample handling procedures.

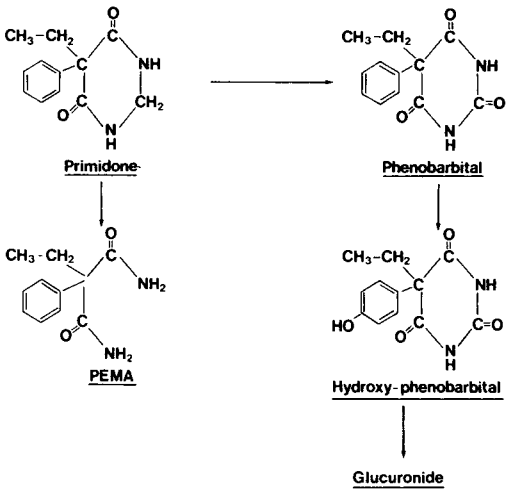

Fig. 1. Metabolic scheme of primidone.

MATERIALS AND METHODS

Chemicals and reagents

The internal standards (i.s.) used were: i.s. 1, 4-methylprimidone, for primidone; i.s. 2, 5-ethyl-5-(*p*-tolyl)barbituric acid, for phenobarbital, and i.s. 3, 2-ethyl-2-(*p*-tolyl)malonic acid diamide for PEMA, all obtained from EGA-Chemie, Steinheim, G.F.R.

These compounds were dissolved in methanol (1 mg/ml) and the methanolic solutions were added to benzene—ethyl acetate (both "Nanograde" from Promochem, Wesel, G.F.R.) (80:20, v/v) to a final concentration of 300 ng/ml each. Pyridine ("getrocknet"), N,N-dimethylacetamide and ethyl iodide were obtained from Merck (Darmstadt, G.F.R.), β-glucuronidase/aryl sulfatase from Boehringer (Mannheim, G.F.R.). Regisil RC-2, bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane from Regis (Hedinger, Stuttgart, G.F.R.), and tetramethylammonium hydroxide (20% in methanol) from EGA-Chemie.

Hydrolysis of conjugated metabolites

To 5—100 μl serum or urine were added an equal volume of 1 *N* sodium acetate buffer (pH 5.0) and 10 μl (per 100 μl sample volume) of β-glucuronidase/aryl sulfatase (5 U/ml, Boehringer). This mixture was slowly agitated at 37° for 16—20 h and then processed as described below.

Extraction procedure

A sample (5—100 μl) of serum, urine, saliva, breast milk or tissue homogenate was pipetted into a disposable 1.5-ml Eppendorf reaction vessel, diluted to 100 μl and 100 μl of saturated ammonium sulfate and 1 ml of benzene—ethyl acetate (80:20, v/v) containing the internal standards were added. The tube was shaken for 15 min and then centrifuged for 2 min in a 5012 Eppendorf centrifuge. One 400-μl portion of the supernatant organic phase was transferred to another 1.5-ml reaction vessel, and a further 400-μl portion to a 1.5-ml glass serum vial. The extraction was repeated using 1 ml of solvent mixture without internal standards. The combined extracts were evaporated at 40° under a stream of nitrogen.

The samples in the conical Eppendorf reaction vessels were ethylated according to the procedure of Greeley [18] by adding 40 μl dimethylacetamide, 5 μl 20% tetramethylammonium hydroxide and, after vortexing, 10 μl ethyl iodide. The samples were centrifuged for 2 min and 2 μl of the clear supernatant were injected into the GC—MS system. The samples in the glass vials were trimethylsilylated with a mixture of 20 μl pyridine and 30 μl Regisil RC-2 at 60° for 1 h. A 2-μl aliquot of the clear solution was injected into the GC—MS system.

GC—MS—computer analysis

A Perkin-Elmer F-22 gas chromatograph was coupled via a Watson-Biemann separator to a Varian MAT CH-7A mass spectrometer. A 2-m glass column (6 mm O.D. and 2.5 mm I.D.) packed with 3% OV-17 on 120—140 mesh Gas-Chrom Q was used (Applied Science Labs., Serva, Heidelberg, G.F.R.). The ethylated samples were injected at 220°, and the trimethylsilylated samples at 190°. After an initial period of 1 min the column temperature was raised 30° at a rate of 10°/min. During this period the derivatives to be measured eluted from the GC column. The temperature was then raised quickly (20°/min) to 270° to elute the accompanying substances. During this time, the selected ion records were plotted and the peak height ratios calculated. After the GC column had cooled down to the initial temperature, the next sample was injected. Six to eight samples were analyzed in this way within one hour.

The mass spectrometer was controlled by the SS-100 Varian data system and operated in the selected ion monitoring mode. The following ions were selected for the ethylated samples: *m/e* 246 (primidone), 260 (phenobarbital and i.s. 1 for primidone), 274 (i.s. 2 for phenobarbital and hydroxyphenobarbital) and 275 (hydroxyphenobarbital). For the trimethylsilylated samples: *m/e* 232 (primidone), 235 (PEMA), 246 (i.s. 1 for primidone), and 249 (i.s. 3 for PEMA). The results were displayed on a Textronix 4010 oscilloscope and plotted on a Textronix hardcopy unit.

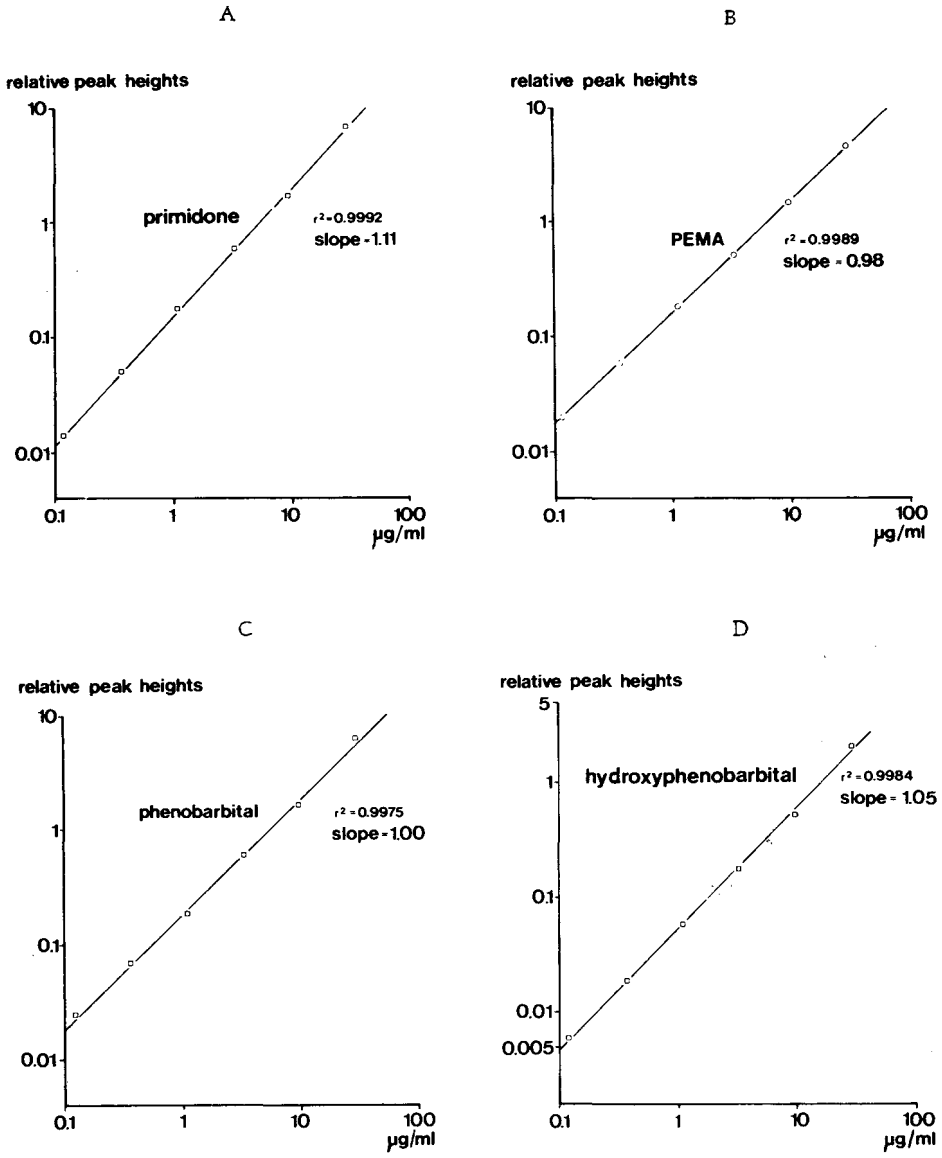

Fig. 2. Double logarithmic plots of the peak heights of the selected ions from derivatives of primidone and its metabolites (relative to the corresponding internal standards used; see Experimental) vs. amounts present in 100-µl serum samples. (A) *m/e* 232, the M−130 of the bis-trimethylsilylated primidone derivative; (B) *m/e* 235, the M−115 of the bis-trimethylsilylated PEMA derivative; (C) *m/e* 260, the M−28 ion of the diethylated phenobarbital derivative; and (D) *m/e* 275, the M−57 ion of the triethylated hydroxyphenobarbital derivative. The calibration graph for the ethylated primidone, monitoring the ion *m/e* 246 (M−28) of the diethylated derivative, is not shown.

Quantitation

Standard calibration graphs were obtained by the analysis of 100-μl portions of drug-free human serum, to which were added known amounts of primidone, phenobarbital, hydroxyphenobarbital and PEMA (Fig. 2). These samples were processed as described above. The stored samples were kept frozen at −30° and the concentrations of primidone and phenobarbital present were periodically checked by comparative analysis of antiepileptic drug calibrator serum standards (Emit-aed., Merck).

RESULTS AND DISCUSSION

We have evaluated a number of solvents for the extraction of the drugs, for example chloroform, ethyl acetate, benzene, methylene chloride and some of their mixtures. A mixture of benzene—ethyl acetate (80:20) and saturated ammonium sulfate for "salting out" [19,20] led to good yields (Table I). Furthermore, this mixture is less dense than water which simplified the transfer of the organic phase. Most importantly, low baselines and clean selected ion records (mass fragmentograms) were obtained even after conjugated substances had been released by glucuronidase/arylsulfatase.

TABLE I

RECOVERIES, RELATIVE STANDARD DEVIATIONS AND LOWER DETECTION LIMITS

Compound	Derivatization*	Recovery of isolation procedure (%)	Lower detection limit** (ng/ml)	Relative standard deviation*** (%)
Primidone	Ethyl.	89	2.5	3.9
	TMS		2.2	5.9
Phenobarbital	Ethyl.	89	1.4	3.2
p-Hydroxyphenobarbital	Ethyl.	80	3.7	4.1
PEMA	TMS	93	1.7	3.9

*Ethyl., ethylation according to Greeley [18]; TMS, trimethylsilylation.
**Signal-to-noise ratio = 2 using 100-μl serum samples.
***Twelve samples (100 μl serum) containing 1 μg/ml of each compound were analyzed as described.

Primidone, phenobarbital and hydroxyphenobarbital were ethylated, PEMA and primidone were trimethylsilylated prior to analysis by GC—MS. Similar results were obtained for primidone with either method. The derivatization reactions were performed by simply adding the reagents. After an appropriate reaction time the mixture could be directly injected into the GC—MS system. The derivatives were found to be stable: similar results were obtained by analyzing the same samples on two consecutive days.

The mass spectra generated by electron impact are shown in Fig. 3. All intense ions above *m/e* 200 were tried but those indicated in the legend of Fig. 2 were selected in the final method by the criteria of favourable baselines and

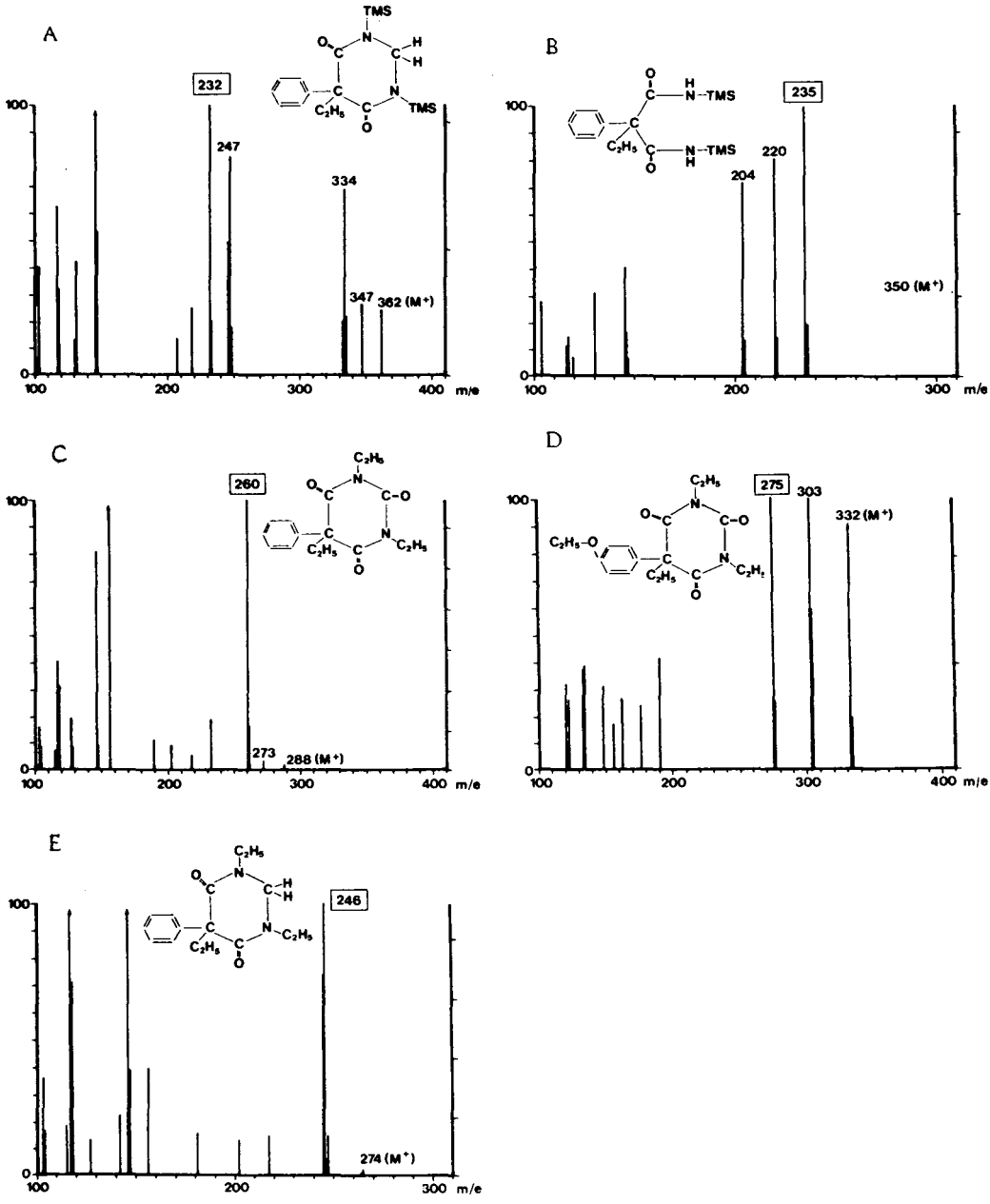

Fig. 3. Electron impact mass spectra of the trimethylsilylated derivatives of primidone (A) and PEMA (B) as well as the ethylated derivatives of phenobarbital (C), hydroxyphenobarbital (D), and primidone (E).

lower detection limits. The ions selected for the internal standards were 14 mass units higher than those of the corresponding drugs.

The reproducibility of the method was evaluated by the analysis of 12

100-μl serum samples which contained 1 μg/ml of the substances studied. The relative standard deviations are presented in Table I. The lower detection limits were found to be in the low ng/ml range using 100-μl sample volumes (Table I). Linear dependence of the peak height ratios vs. amounts of drug and metabolites added was found throughout the calibration range studied (between 100 ng/ml and 30 μg/ml corresponding to 10 ng per sample and 3 μg per sample, the square of the correlation coefficient r^2 exceeded 0.99 (Fig. 2).

The methods developed for the assay of primidone and its metabolites were well suited for the analysis of small samples of saliva (Fig. 4), serum (Fig. 5), urine, breast milk and tissue homogenates. Owing to the high sensitivity of the

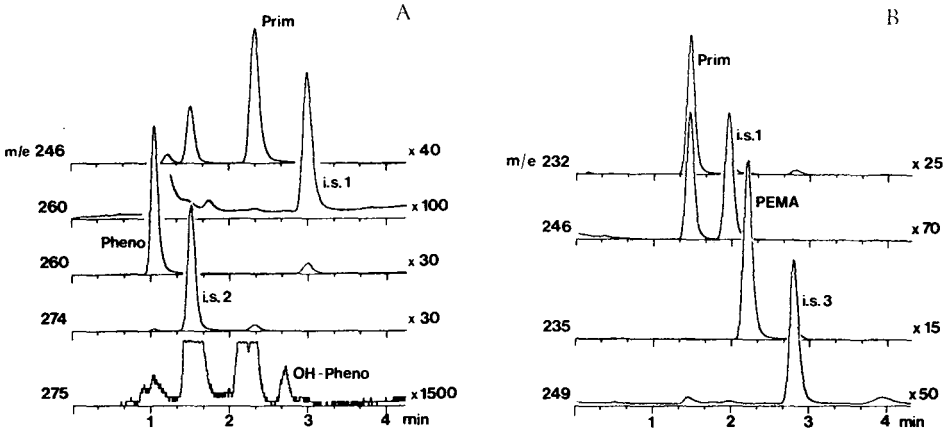

Fig. 4. Selected ion records of a 100-μl saliva sample of an epileptic woman treated with primidone (daily dose: 1125 mg). (A) Ethylated portion of the sample extract and (B) trimethylsilylated portion of the sample extract. Concentrations found: primidone (Prim), 4.9 μg/ml; phenobarbital (Pheno), 8.6 μg/ml; PEMA, 8.0 μg/ml; hydroxyphenobarbital (OH-Pheno), 50 ng/ml. For the description of the internal standards (i.s. 1—3) see Experimental.

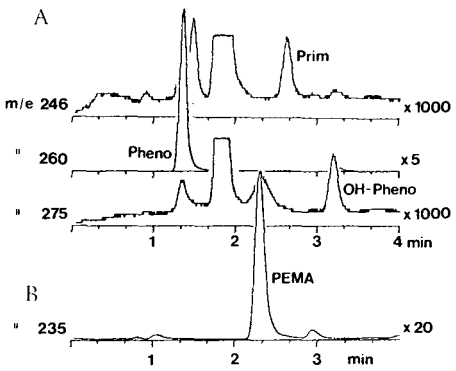

Fig. 5. Selected ion records of a 50-μl serum sample from a human neonate two days after birth. Last maternal dose: 500 mg primidone 4 h before delivery. (A) Ethylated portion of serum extract and (B) trimethylsilylated portion of serum extract. Concentrations found: primidone (Prim), 0.14 μg/ml; phenobarbital (Pheno), 22.4 μg/ml; PEMA, 8.5 μg/ml; hydroxyphenobarbital (OH-Pheno), 0.25 μg/ml.

method, low levels of metabolites such as hydroxyphenobarbital could be determined (Figs. 4 and 5). The highly specific and sensitive GC—MS method allowed us to use simple and rapid sample handling procedures (see Experimental).

We have applied these methods to the study of the placental transfer of primidone and its metabolites during early pregnancy and at term. Analysis of human fetal tissues obtained following interruption of pregnancy during the first trimester indicated that the compounds analyzed were present in comparable amounts in fetal tissues and maternal serum [21]. At term, the levels of primidone and metabolites in cord blood samples also approached those present in the corresponding blood samples of the epileptic mothers who had been treated by primidone for seizure control [22]. Thus, all compounds studied readily passed the placental membranes, both during early pregnancy and at term. Interestingly, the conjugated *p*-hydroxyphenobarbital was already present in neonatal blood at birth. The concentration ratio of total hydroxyphenobarbital (free and conjugated) to unconjugated hydroxyphenobarbital in the neonatal blood was found to be approximately 2.

The biological half-lives of primidone during the first five postnatal days of four neonates studied were found to vary widely between 6 and 60 h [22]. This range overlapped with that of normal adults (6—12 h) [23,24]. The concentrations of PEMA decreased only slightly during the first five postnatal days, while those of phenobarbital even showed small but significant increases. Apparently PEMA and phenobarbital were continuously formed by neonatal metabolism of primidone. Since one or both of these metabolites may be biologically active, the determination of their levels in the neonate is of great importance.

The analysis of saliva samples from epileptic patients treated with primidone confirmed earlier reports on the usefulness of monitoring the concentrations of primidone and phenobarbital present in this fluid [25—27]. In addition, we have also measured the saliva concentrations of PEMA; the saliva:plasma concentration ratios found indicated very low plasma protein binding for this metabolite [28]. Also hydroxyphenobarbital was found in saliva in low concentrations (Fig. 4). Monitoring of metabolites in saliva may be useful for the study of drug metabolism in man [28].

ACKNOWLEDGEMENTS

This work was supported by a grant from the Deutsche Forschungsgemeinschaft to the Sonderforschungsbereich 29. The preparation of the manuscript by Ms. E. Gottschalk and N. Nau is gratefully acknowledged. We are also indebted to Prof. J. Langman and Dr. C. Liddiard for their helpful editorial advice.

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GAS CHROMATOGRAPHIC DETERMINATION OF BROMO AND FLUORO DERIVATIVES OF BENZODIAZEPINE IN HUMAN BODY FLUIDS

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(First received August 2nd, 1979; revised manuscript received November 28th, 1979)

SUMMARY

A rapid, sensitive and accurate method for the determination of bromazepam and flunitrazepam in plasma and urine using gas chromatography has been developed. Bromazepam was extracted with diethyl ether and flunitrazepam with hexane at pH 7. A nitrogen detector was used to determine bromazepam and an electron-capture detector was used for flunitrazepam.

INTRODUCTION

Bromazepam, a bromo derivative of benzodiazepine, is an anti-anxiety and hypnotic drug administered in therapeutic doses of 3—18 mg daily.

Flunitrazepam, a fluoro derivative of benzodiazepine, is an anaesthetic drug given in the dose range of 0.01—0.03 mg/kg. Such low therapeutic doses are connected with the strong action of these drugs and result in low concentrations in plasma and in urine. Literature reports describe the determination of these compounds by differential polarographic [1], spectrofluorodensitometric [2] and gas chromatographic [3, 4] methods. In the latter case benzophenones, formed as a result of hydrolysis of the mentioned compounds, were determined. Columns with different packings were used with an electron-capture detector (ECD). The hydrolysis of these compounds significantly prolongs the determination time.

Vree et al. [5] determined flunitrazepam quantitatively by high-performance liquid chromatography. Kaniewska [6], studying bromo and fluoro derivatives of benzodiazepine by gas chromatography, elaborated a sensitive identification and determination method for these compounds omitting the hydrolysis process.

Differential polarographic and spectrofluorodensitometric methods used for

determination of compounds in human body fluids are not sufficiently sensitive when therapeutic doses are administered.

It was the aim of this work to optimize the extraction conditions of bromazepam and flunitrazepam from human plasma and urine and then to determine them directly by gas chromatography [6]. The method was developed to facilitate pharmacokinetic investigations of the drugs.

EXPERIMENTAL

Materials and reagents

Bromazepam (Lexotan®) was obtained from Hoffmann-La Roche (Nutley, N.J., U.S.A.), the purity, determined by a titration method was 99.7%; flunitrazepam (Rohypnol®) was from Hoffman-La Roche, m.p. 167—168° and the purity determined by a titration method was 99.93%.

Apparatus

A Pye 104 model 84 gas chromatograph, a Perkin-Elmer Model 3903 gas chromatograph, and a Mechanika Precyzyjna centrifuge, type 317a were used.

Determination of bromazepam in human plasma

Determinations of bromazepam in human plasma were performed on a Perkin-Elmer Model 3903 chromatograph equipped with a nitrogen detector. A glass column (2 m × 0.3 cm I.D.), packed with 10% UCC-W-98 on Chromosorb W AW DMCS (80—100 mesh) was used. The column was conditioned at 240° for 4 h with no gas flow; then at 280° for 48 h. Operating temperatures were column 265°, injection port 275°, detector 275°. Nitrogen was used as the carrier gas at a flow-rate of 45 ml/min; the hydrogen flow-rate was 9.2 ml/min and the air flow-rate 150 ml/min. The sensitivity was set at 1 × 16. Diazepam was used as an internal standard in the quantitative analysis.

Standard solutions. Two stock solutions were made up: (1) 100 mg bromazepam in 100 ml ethanol and (2) 50 mg diazepam in 100 ml ethanol. From the stock solutions, four standard solutions were prepared containing 2.5, 5, 7 and 10 μg of bromazepam per 100 μl.

Preparation of calibration curves. From the standard solutions calibration curves were constructed omitting the extraction process (curve I) and after an extraction from human plasma (curve II). In the latter case to each of the four centrifugal tubes 1 ml of human plasma and 100 μl of the standard bromazepam solutions were added and stirred. The mixtures were extracted with 4 ml diethyl ether three times, shaking each time for 15 min. After centrifugation for 5 min at 4000 *g* the upper organic layer was removed using a pipette, transferred to a conical centrifuge tube and dried with anhydrous sodium sulphate. After filtration the diethyl ether was evaporated under vacuum below 30°. The residue was dissolved in 0.2 ml of the internal standard solution (10 μg/ml). To prevent solvent evaporation the tubes were cooled in iced water. An aliquot of 2 μl of the final solution was injected onto the chromatographic column.

The linearity of the detector was determined by the calibration curves. Calibration curves I and II expressed the ratio dependence of the bromazepam

peak of that of internal standard on the concentration of bromazepam. From the curves thus obtained the recovery of bromazepam from human plasma was determined.

The content of bromazepam may be determined in 1—4 ml of human plasma under the same extraction conditions as those for the preparation of calibration curve II.

The concentration of bromazepam was calculated from calibration curve II prepared separately for each series of determinations.

Determination of bromazepam in human urine

Preparation of calibration curves. To each of four centrifuge tubes 1 ml of urine was added and adjusted to pH 7.0 with borate buffer, pH 9.0. Then 100 μl of the ethanol standard solutions containing 2.5, 5, 7.5, and 10 μg of bromazepam were added. Extraction and determination processes were performed as described for the determination of bromazepam in plasma.

From the standard solutions calibration curve I was constructed directly, i.e. omitting the extraction process; calibration curve III was prepared after the extraction from urine (Fig. 1).

For determination of unknown aliquots of bromazepam 1—4 ml of urine was used and the extraction conditions were the same as those given for the standard curves. The concentration of bromazepam was determined from calibration curve III which was prepared separately for each series of determinations.

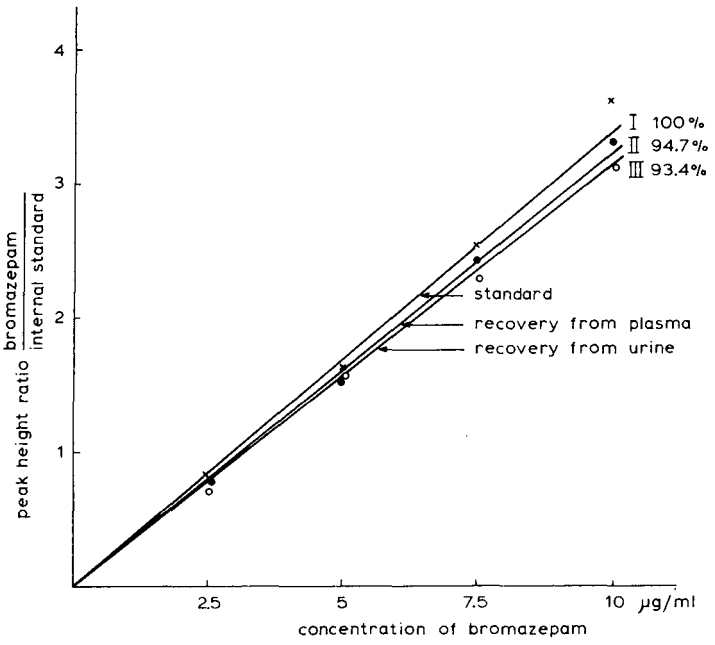

Fig. 1. Calibration curves of bromazepam: I, (x) omitting the extraction process; II (•) after extraction from plasma; III, (○) after extraction from urine.

Determination of flunitrazepam in human plasma

Determination of flunitrazepam in human plasma was performed on a Pye 104 gas chromatograph equipped with an ECD by the method developed previously [6]; a glass column (1.65 m × 0.4 cm I.D.) packed with 10% UCC-W-98 on Chromosorb W AW DMCS (80—100 mesh) was used. Operating temperatures were: Column 260°, injection port, 290°, and detector, 300°. Argon was used as the carrier gas at a flow-rate of 60 ml/min, the hydrogen flow-rate was 45 ml/min and the air flow-rate 300 ml/min. The sensitivity was set at 10×10^2. Diazepam was used as an internal standard in the quantitative analysis.

Standard solutions. Two stock solutions were made up: (1) 5 mg flunitrazepam in 100 ml ethanol and (2) 10 mg diazepam (internal standard) in 100 ml ethanol.

From the stock solutions three standard solutions were prepared containing 12.5, 25 and 50 ng of flunitrazepam per 100 µl.

The extraction process and determinations were as indicated for bromazepam except that hexane was used and the internal standard concentration amounted to 500 ng/ml.

From the standard solutions calibration curve I was construced directly, i.e. omitting the extraction process; calibration curve II was prepared after extraction from plasma.

The curves obtained were then used for calculation of the recovery and verification of the detector linearity in the concentration range 12.5—50 ng/ml (Fig. 2).

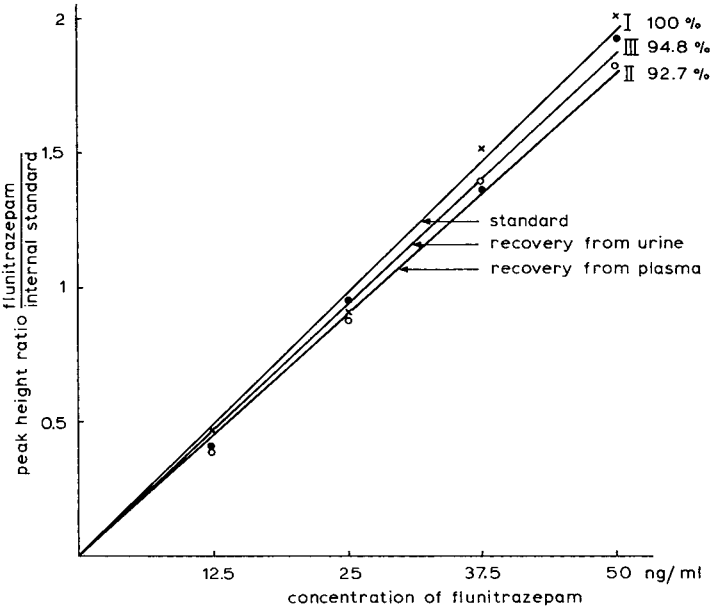

Fig. 2. Calibration curves of flunitrazepam: I, (x) omitting the extraction process; II, (○) after extraction from plasma; III (●) after extraction from urine.

For determination of unknown aliquots of flunitrazepam 1—4 ml of plasma was used and the extraction conditions were the same as those for the preparation of the calibration curve for nitrazepam.

The concentration of flunitrazepam was determined from calibration curve II prepared spearately for each determination series.

Determination of flunitrazepam in human urine

Preparation of calibration curves. To each of three centrifuge tubes 1 ml of urine was added and adjusted to pH 7.0 with borate buffer, pH 9.0. Then 100 μl of the standard solutions of flunitrazepam (12.5, 25, 50 ng) were added and extracted three times with 4 ml of hexane, shaking each time for 15 min.

After centrifugation (5 min at 4000 *g*) the upper organic layer was transferred to a conical centrifuge tube and dried with anhydrous sodium sulphate. After filtration the hexane was evaporated under vacuum at 40°. The residue was dissolved in 0.2 ml of internal standard solution (500 ng/ml).

Aliquots of 2 μl of each of the final solutions were injected on the chromato-

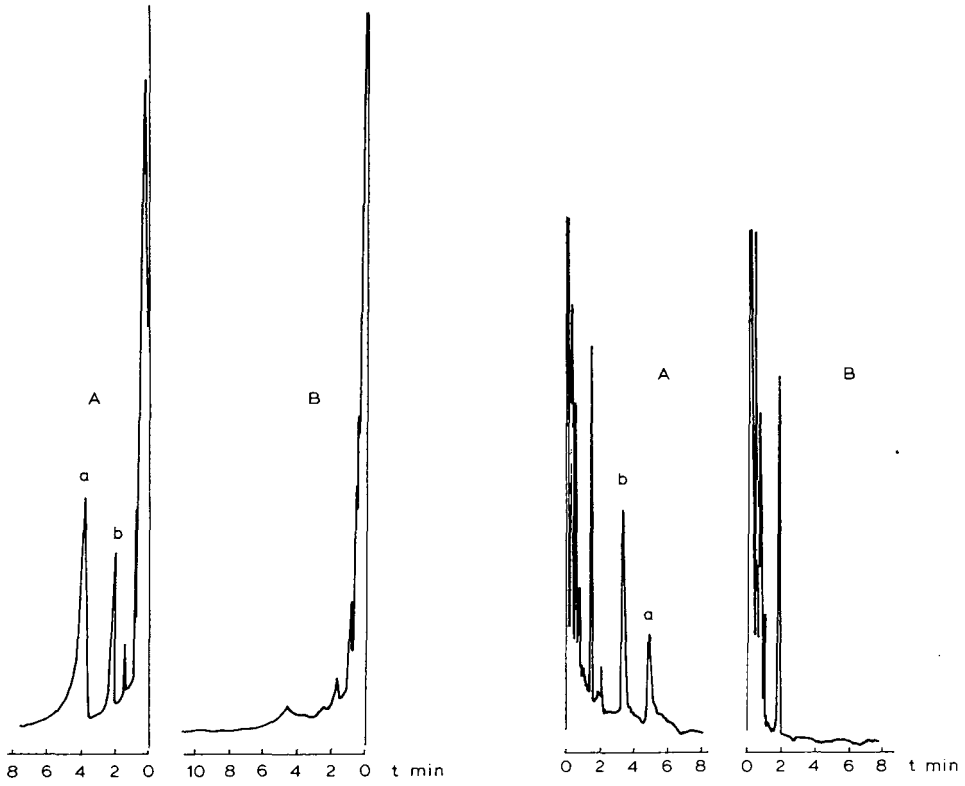

Fig. 3. Gas chromatograms of the extract from plasma containing bromazepam and diazepam (A) and of the extract from blank plasma (B). Peaks : (a) bromazepam, (b) diazepam (internal standard).

Fig. 4. Gas chromatograms of the extract from plasma containing flunitrazepam and diazepam (A) and of the extract from blank plasma (B). Peaks: (a) flunitrazepam, (b) diazepam (internal standard).

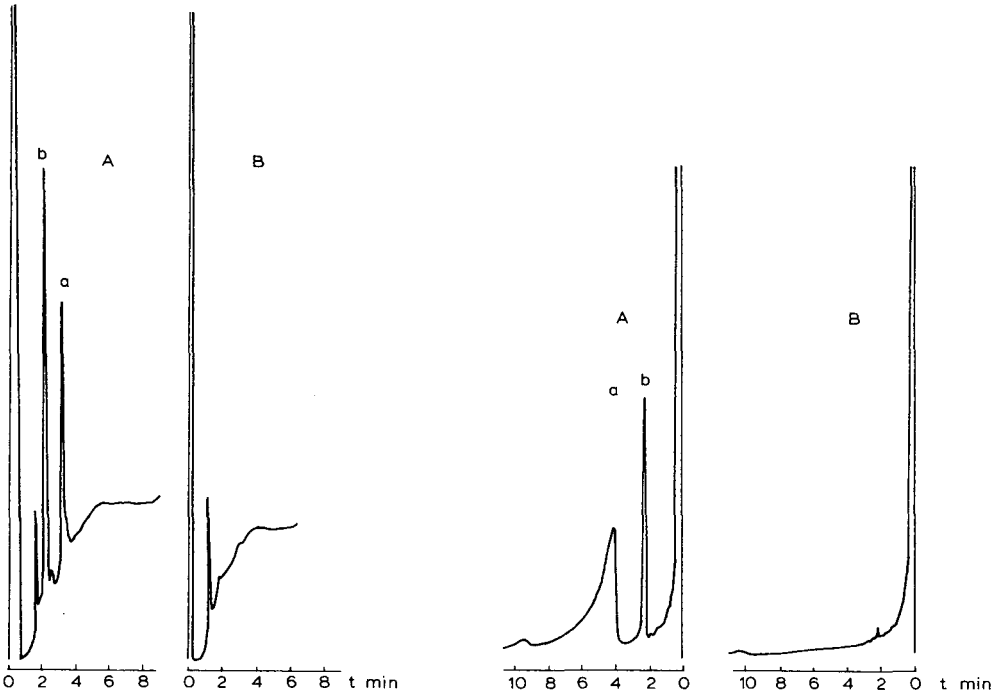

Fig. 5. Gas chromatograms of the extract from urine containing flunitrazepam and diazepam (A) and of the extract from blank urine (B). Peaks: (a) flunitrazepam, (b) diazepam (internal standard).

Fig. 6. Gas chromatograms of the extract from urine containing bromazepam and diazepam (A) and of the extract from blank urine (B). Peaks: (a) bromazepam, (b) diazepam (internal standard).

graphic column and the calibration curve was prepared. At the same time the calibration curve of flunitrazepam was constructed omitting the extraction process (Fig. 2).

For the determination of unknown concentrations of flunitrazepam 1—4 ml of urine was used and the extraction conditions were the same as those given for the standard curves. The concentration of flunitrazepam was determined from calibration curve III prepared separately for each series of determinations.

RESULTS AND DISCUSSION

The use of diethyl ether and hexane for the extraction of bromazepam and flunitrazepam from plasma and urine was convenient because of their relatively low specific weights. The organic layer accumulated at the top and was easy to separate. The extraction process was optimal at pH 7.0, thus requiring the urine to be adjusted with buffer to pH 7.0.

Fig. 3 shows chromatograms of plasma with added bromazepam and diazepam (internal standard), and blank plasma. Fig. 4 shows chromatograms of plasma with added flunitrazepam and diazepam, and blank plasma. Fig. 5 shows chromatograms of urine with added flunitrazepam and diazepam, and

blank urine. Fig. 6 shows chromatograms of urine with added bromazepam and diazepam, and blank urine.

For detecting bromazepam by gas chromatography a nitrogen detector was used because despite the fact that this compound contains a halogen in the molecule it gave poor peak shape (broad tailing peaks) and poor ECD response.

This allows the determination of bromazepam in plasma and urine in the concentration ranges 2.5—10 μg/ml where the recovery of bromazepam from plasma amounted to 94.7 ± 7.5% and from urine to 93.4 ± 8% (n = 10). The sensitivity limit of the method for this compound was 0.6 μg/ml.

Statistical evaluation for the extraction of bromazepam from plasma is as follows: $\bar{x}$ = 96.65%, s = 3.36, confidence interval 96.65% ± 3.52 for p = 0.95, n = 7, C.V. = 3.24%. For extraction from urine $\bar{x}$ = 95.33%, s = 4.23, confidence interval 95.33% ± 3.25 for p = 0.95%, n = 9, C.V. = 4.44%.

The use of the ECD which is sensitive to halogens allowed the determination of flunitrazepam in plasma and urine in the concentration range 12.5—50 ng/ml. The sensitivity limit of the method for this compound was 3 ng/ml in plasma and urine. In the given concentration ranges of flunitrazepam in plasma the average recovery was 92.7 ± 8% (n = 15) and 94.8 ± 6.5% (n = 6) in urine.

Statistical data for the extraction of flunitrazepam from plasma are as follows: $\bar{x}$ = 97.25%, s = 4.47, confidence interval 97.25% ± 5.11 for p = 0.95, n = 7, C.V. = 4.59%. For the extraction from urine $\bar{x}$ = 98.61%, s = 3.69, confidence interval 98.6% ± 4.24 for p = 0.95, n = 6, C.V. = 3.81%.

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Note

High-performance liquid chromatographic determination of hippuric acid in human urine

Preliminary results for normal urine levels

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(First received September 4th, 1979; revised manuscript received November 28th, 1979)

Hippuric acid (HA) is the glycine conjugate of benzoic acid. It is the normal excretory metabolite of benzoic acid and is a common constituent of human urine. After exposure to toluene, which is oxidized in the body to benzoic acid, drastically increased levels of HA are found and the determination of HA in urine is thus important for estimating the degree of exposure [1]. In the Lesch-Nyhan syndrome [2] and in liver dysfunction [3], Ha is decreased or absent; it has been found in large quantities in hypertense females [3].

Routine determination of HA is normally hampered by the low sensivity and poor specificity of known methods. Photometric methods [4, 5] are unspecific and salicyluric acid (a metabolite of salicylic acid) and *m*- or *p*-methylhippuric acid (metabolites of *m*- or *p*-xylene) also react.

The isotachophoretic method [6] is complex and gas chromatography [7, 8] is time consuming and laborious (derivatization with diazomethane). Thin-layer chromatography [9] is simple, but only for screening, and is lengthy. A high-performance liquid chromatographic (HPLC) procedure for measurements of the urine concentration of HA has been published recently by Matsui et al. [10].

In a search for a more satisfactory and less difficult technique, HPLC was used with the expectation that it would offer decisive advantages over existing methods. This paper reports a liquid chromatographic method using a simplified procedure as compared to the above mentioned method of Matsui et al. [10] i.e. no extractions are performed. This technique is simple, rapid and specific. The present study is an application of this method to investigate the normal distribution of HA in human urine.

EXPERIMENTAL

Reagents

Hippuric acid, acetonitrile (Licrosolv^{D} grade) and interfering substances used were purchased from Merck (Darmstadt, G.F.R.).

Apparatus

A high-performance liquid chromatograph (LC3, Pye Unicam, Cambridge, Great Britain) was equipped with a variable-wavelength UV detector (Pye Unicam). The effluent stream was monitored at 250 nm.

A 250 mm × 4.6 mm I.D. column packed with C_{18}-bonded silica gel (particle size 10 μm: Partisil ODS-2; Whatman, Clifton, N.J., U.S.A.), a septumless syringe loading sample injector (20-μl injector, Rheodyne, Berkeley, Calif., U.S.A.) and a chart speed of 5 cm/min were employed.

Mobile phase

An amount of 500 ml of mobile phase was prepared fresh daily by thoroughly mixing 400 ml of 1% aqueous acetic acid with 100 ml of acetonitrile. The mobile phase was filtered through a 5-μm glass filter prior to use, degassed by a helium stream during the determination and pumped at a constant flow-rate of 1.35 ± 0.05 ml/min.

Urine samples

Normal 24-h urine specimens were obtained from laboratory personnel and from hospital patients with normal renal and hepatic functions. The samples were filtered (0.22 μm, Millipore, Bedford, Mass., U.S.A.) and stored at −24° if not analyzed immediately.

Calibration curves

A calibration curve was prepared daily by appropriate dilutions of a stock solution of HA (10 g/l in water) with 1 *N* acetic acid. The concentration range was 0.1—1.5 g/l (0.56—8.4 m*M*).

Urine determinations

Aliquots of 100 μl of 24-h urine specimens were diluted with 900 μl of 1 *N* acetic acid. Exactly 20 μl of this solution were then injected into the chromatograph. The peak height of HA was measured and the unknown concentration was calculated from the calibration curve.

RESULTS AND DISCUSSION

The separation of HA was satisfactory and retention time was 250 ± 10 sec (mean ± S.D.). A typical chromatogram is shown in Fig. 1. Endogenous and exogenous constituents such as creatinine, urea, oxalic acid, glycolic acid, glyoxylic acid, benzoic acid, salicylic acid, salicyluric acid, malonic acid and acetaminophen do not coelute with HA.

The influence of acidity on the mobile phase was studied. The effect of pH on linearity (Fig. 2) shows that the best results were obtained with 1% aqueous

acetic acid. Decreasing the pH of the diluent used for urinary determinations increases sensitivity. The optimal pH of 3.1 was obtained by diluting samples with 1 *N* acetic acid.

Good linearity was observed in the range of 0—1.5 g/l (0—8.4 m*M*) (r = 0.9999, four determinations for each point). An additional sample dilution was necessary at concentrations greater than 1.5 g/l.

The precision of this method was determined by first assaying 16 samples of pooled normal urine, followed by assaying samples of this pooled urine to

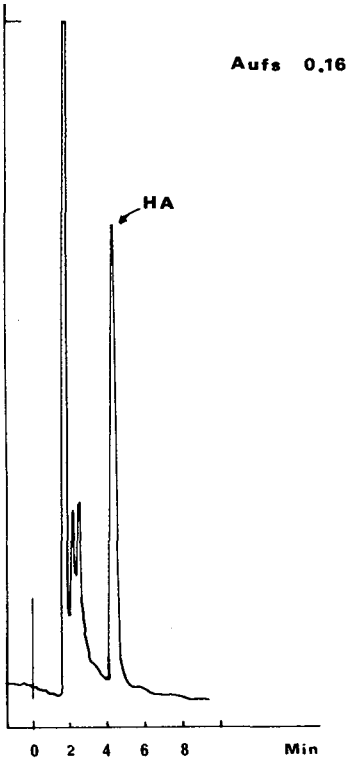

Fig. 1. HPLC separation of HA from normal human urine. Concentration of HA 0.82 g/l (4.56 m*M*).

TABLE I

PRECISION AND ACCURACY OF HA DETERMINATION

	Mean (n = 16) HA (mg/l)	Standard deviation (%)	Standard error (%)
Pooled normal urine	253	6.2	1.6
Pooled normal urine spiked with 50 mg/l HA	301	4.5	1.1
HA found	48 (96% recovery)		

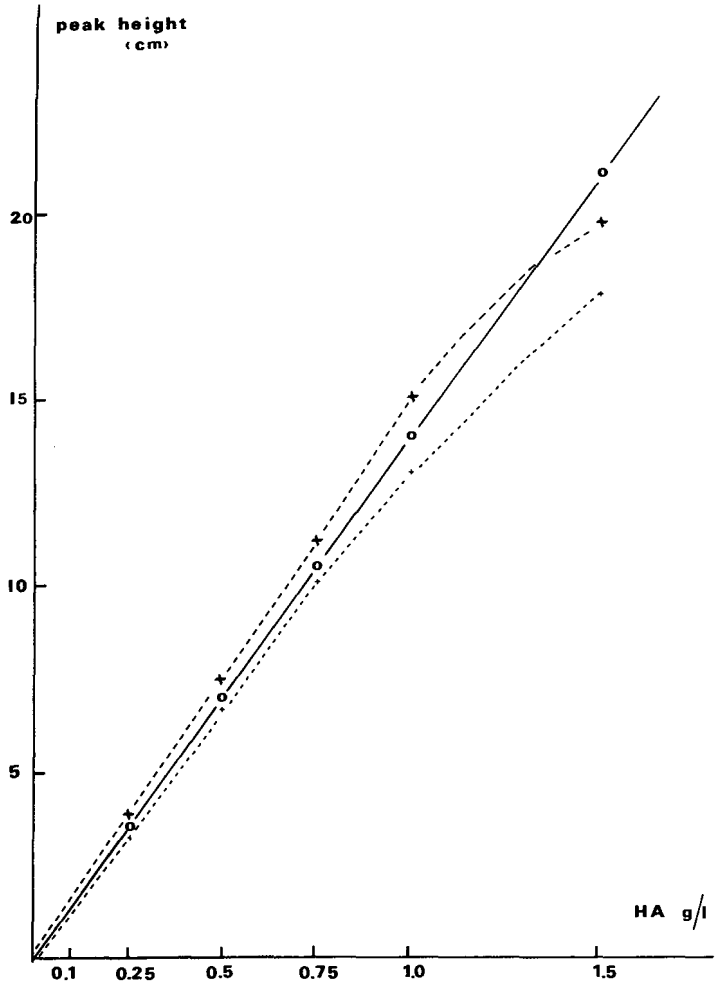

Fig. 2. Calibration curves for various concentrations of acetic acid in the mobile phase. (x——x) 0.1% acetic acid, (+· · · · +) 0.2% acetic acid and (O——O) 1% acetic acid ($y = 139.9\ X - 0.38$; $r = 0.9999$).

which 50 mg/l of HA (280 μM) were added. The accuracy was evaluated from recovery data (difference between mean values of pooled normal urine and urine containing 50 mg/l HA) (Table I).

Our HPLC method showed good day to day reproducibility. The results of the analysis of pooled normal urine and HA spiked urine (2 g/l, 11.2 mM) (3 replicate analyses of each on 30 consecutive days) are shown in Table II.

Biological results

Preliminary results for normal urine levels of HA were obtained. The reference range for hippuric acid content has been reported to be 2.55 ± 1.19 mmoles/24 h [9].

TABLE II

REPRODUCIBILITY OF HA DETERMINATION

	Mean HA found (mg/l)	Standard deviation (mg/l)	Standard deviation (%)
Normal urine (pooled)	273	15	5.5
HA-spiked urine (2 g/l)	2077	125	6.0

Our results obtained with a control group (no exposure to toluene and normal renal and hepatic functions) (n = 69) are in agreement with this value but we found evidence for a bimodal distribution of HA excretion in normal subjects. Fig. 3 shows the frequency distribution histogram for HA excretion. A bimodality is seen with a mean for one subpopulation at 1.07 ± 0.51 mmoles/24 h (66%) and a mean for the other subpopulation at 4.4 ± 1.1 mmoles/24 h (33%) (t-test: $p < 0.01$). Sex is not an interfering factor.

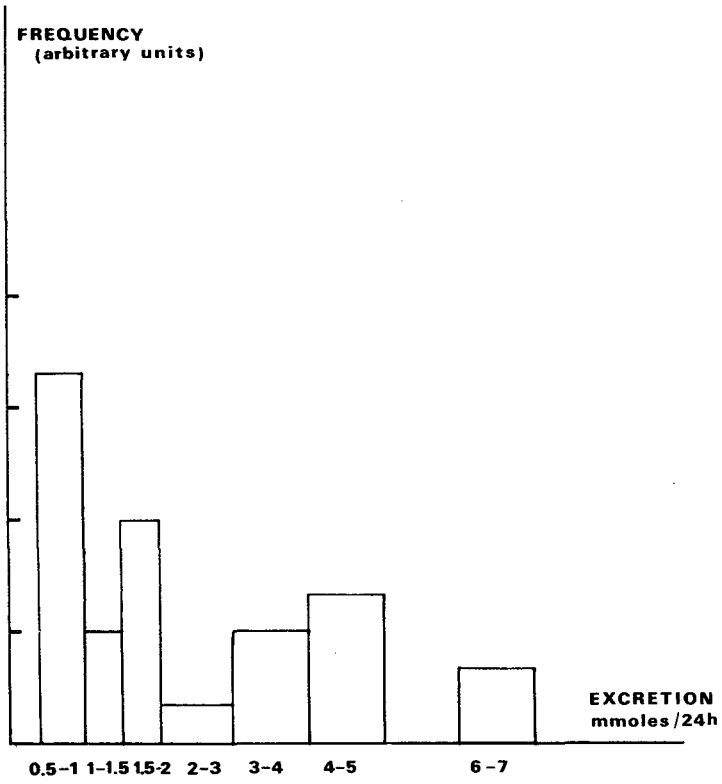

Fig. 3. Frequency distribution histogram for hippuric acid in normal human urine (n = 69). First subpopulation (66%: 1.07 ± 0.51 mmoles/24 h); other subpopulation (33%: 4.4 ± 1.1 mmoles/24 h).

CONCLUSION

The HPLC method described for the determination of hippuric acid in human urine is specific, accurate, reproducible, rapid and simple. It promises to be of great help in cases of toluene exposure as well as for biochemical studies. Further work is in progress [11].

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Note

Chromatographic separation and quantification of type I and type III collagens

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(Received October 9th, 1979)

Several genetic variants of the connective tissue protein collagen are known [1]. Types I, II, and III collagens are collectively designated as interstitial collagens and type IV collagen is designated as basement membrane collagen. Type I collagen is the predominant type, and is found in tendon and bone, while cartilage collagen is termed type II. Types I and III collagens are found together in several tissues (skin, intestine, liver, lung, aorta, and uterus). The functional significance of collagen polymorphism is not known. However, changes in the relative distribution in tissues of the different collagen types with age [2], and in several diseases [3] have been documented.

Type III collagen molecule contains about 30% more hydroxyproline than type I collagen molecule [3]. Therefore, total collagen determination for tissues containing both types I and III collagens cannot be made accurately using hydroxyproline values unless the relative proportion of the two collagens in the specimen is known [3]. Separation procedures [2, 4] involving differential salt precipitation of pepsin-solubilized collagens, followed by molecular sieve and/or ion-exchange chromatography, result in very pure fractions of collagen types suitable for structural studies. However, because of the low recoveries of collagen, which in certain tissues may be as low as 20% [2], the above methods are not suitable for quantitative investigations involving analysis of multiple tissue samples of limited availability. Recently we reported a simple procedure for the separation of types I and III collagens by differential renaturation [5]. About 85% of the pepsin-solubilized hydroxyproline was recovered as collagens by this procedure. The present paper describes the chromatographic separation and quantification of types I and III collagens with 96—100% recoveries.

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EXPERIMENTAL

Pepsin solubilization of collagens

Skin (1 g) from a 1-week-old rat was finely minced with scissors, extracted overnight with chloroform—methanol (2:1, v/v) and then with methanol for 6 h at 4°. The defatted tissue was suspended (10 mg/ml) in 0.5 *M* acetic acid. Crystalline pepsin (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) was added at a concentration of 1 mg/ml [4] and the mixture was stirred for 24 h at 8°. The pepsin digest was centrifuged at 30,000 *g* for 1 h at 4°. The skin residue was redigested overnight with additional pepsin and centrifuged as before. The supernatants were pooled and dialyzed overnight against 4 l of 0.02 *M* Na_2HPO_4 and the resultant collagen precipitate was separated by centrifugation at 30,000 *g* for 1 h at 4°.

Denaturation of pepsin-solubilized collagens. The collagen precipitate was suspended (2—10 mg/ml) in 2 *M* guanidine·HCl containing 0.05 *M* Tris·HCl (pH 7.5), and was denatured by heating to 45° for 30 min. The resultant solution was centrifuged at 30,000 *g* for 40 min at 22° and the supernatant containing the denatured collagens was obtained after filtration through glass wool.

Chromatographic separation of types I and III collagens. Two glass columns (1.6 cm diameter) were connected via a flow adapter and packed with Sepharose CL-6B (Pharmacia Fine Chemicals, Piscataway, N.J., U.S.A.) to provide a total bed length of 210 cm. A 5-ml aliquot of the denatured collagen solution was applied to the molecular sieve column which was equilibrated and eluted with 2 *M* guanidine·HCL containing 0.05 *M* Tris·HCL (pH 7.5), at 22°, with a flow-rate of 20 ml/h. The absorbance of the eluent at 230 nm was continuously monitored with a Fracto-Scan ultraviolet monitor (Buchler Instrument, Nuclear Chicago, Fort Lee, N.J., U.S.A.) and fractions of 3 ml were collected automatically. The ultraviolet-absorbing fractions for each peak were pooled, dialyzed against deionized water until free of salt, and then lyophilized.

Determination of relative purity of collagen fractions

Polyacrylamide gel electrophoresis. Polyacrylamide gel electrophoresis of the collagen samples was carried out in 5% cylindrical gels (85 × 7 mm), containing 0.3% N,N′-methylene-bisacrylamide [5]. Gel and electrode buffers were 0.1 *M* Tris—borate (pH 8.5) containing 0.1% sodium dodecyl sulfate (SDS). The gels were pre-electrophoresed at 2 mA/gel, 50 V, for 60 min. Collagen samples (25—100 μg) were incubated at 60° for 5 min with gel buffer containing 1% SDS and 1 *M* urea. Aliquots of the samples were also reduced with dithiothreitol (DTT) and applied to the gels. After electrophoresis at 2 mA/gel, 50 V, for 190 min, the gels were stained with Coomassie Blue R-250 [6]. All gels after removal of excess stain were scanned at 550 nm in a Gilford recording spectrophotometer equipped with a linear gel transport.

Proline:hydroxyproline ratios. Aliquots of the collagen samples were hydrolyzed in constant-boiling HCl and assayed for proline [7] and hydroxyproline [8]. The ratio of proline to hydroxyproline was used as an additional index of the identity and relative purity of the separated types I and III collagens [5, 9].

Quantification of types I and III collagens

From absorbance at 230 nm. The relative quantities of pepsin-solubilized types I and III collagens were determined by tracing the Sepharose CL-6B column elution patterns on paper, cutting out the traced outlines, and weighing the appropriate individual pieces of paper [9].

From hydroxyproline. The relative amounts of type I and type III collagens were also calculated assuming types I and III contain 13.7 and 17.9% of hydroxyproline, respectively [5, 9].

RESULTS AND DISCUSSION

Solubilization of collagen

The amount of collagen solubilized by digestion with pepsin varies with the type of tissue, age, and pathological state of the donor. We have been able to solubilize 92—95% of the total collagen expressed as hydroxyproline from young rat dermis (Fig. 1) and fetal human skin [5] after two digestions with pepsin.

Removal of pepsin

A small amount of pepsin always coprecipitated with collagen when pepsin digests of tissues were dialyzed against 0.02 *M* Na_2HPO_4 [5] (Fig. 2a). Heat denaturation in 2 *M* guanidine·HCl removed the contaminant pepsin as precipitate. Other denaturing agents such as $CaCl_2$ or urea which are used for solubilizing collagen were found to be unsuitable because they also solubilized denatured pepsin (ChandraRajan, unpublished results).

Chromatographic separation of collagen types I and III

Chromatography of the denatured collagens on a calibrated Sepharose CL-6B column (Fig. 3) separated type III collagen (γ component, mol. wt. 285,000) from type I collagen (α chains, mol. wt. 95,000). The use of a longer column (210 cm) of the cross-linked agarose was necessary to obtain separations comparable to those obtained on a shorter (115 cm) column of agarose [9]. However, because of the greater stability of the cross-linked agarose in 2 *M* guanidine·HCl, and the increased flow-rate, the separation time remained essentially the same.

Characterization of the separated collagen types I and III

The identity and relative purity of each of the two chromatographic fractions were confirmed from their respective proline:hydroxyproline ratios (Fig. 1), by SDS—polyacrylamide gel electrophoresis (Fig. 2), and by cyanogen bromide peptide analysis (not shown) according to the method of Benya et al. [10].

Quantification of types I and III collagens

Estimation of the relative proportions of type I and type III collagens in 1-week-old rat dermis from absorbance at 230 nm or hydroxyproline content of the separated fractions gave essentially the same results (71% type I and 29% type III, Fig. 1). Because types I and III collagens contain different amounts of hydroxyproline [1], appropriate factors [5, 9] must be used for calculating the

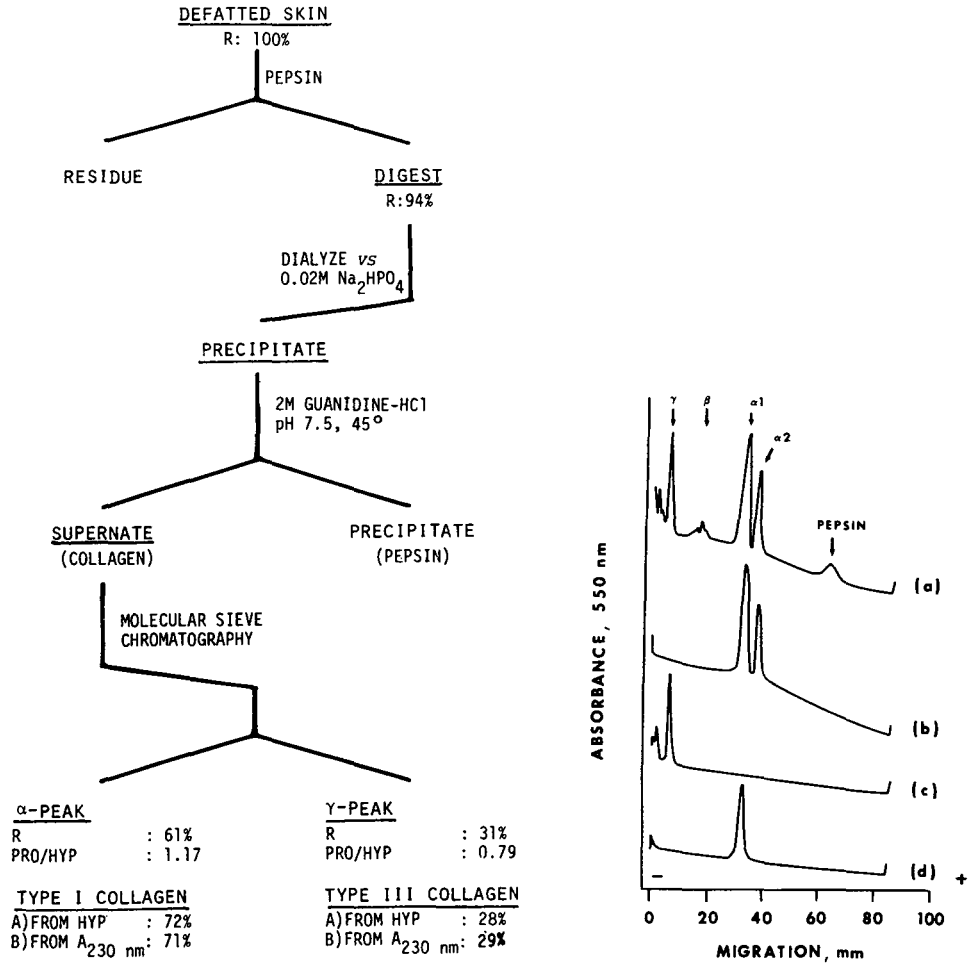

Fig. 1. Outline of the procedure for the separation and determination of relative proportions of types I and III collagens in rat dermis. R indicates recovery of collagen from defatted skin expressed as hydroxyproline. Molar ratio of proline to hydroxyproline was calculated as an index of the identity and relative purity of the collagen types. Relative amounts of types I and III collagens were determined from: (A) hydroxyproline, assuming types I and III contain 13.7 and 17.9% of hydroxyproline, respectively; and (B) from A_{230nm} of α (type I) and γ (type III) peaks on Sepharose CL-6B column (Fig. 3).

Fig. 2. SDS—polyacrylamide gel electrophoresis of pepsin-solubilized collagens. (a) Collagens precipitated by dialysis against 0.02 *M* Na_2HPO_4; (b) type I collagen (α peak); (c) type III collagen (γ peak) obtained after molecular sieve chromatography (Fig. 3); (d) same as (c) but reduced with dithiothreitol. The gels were stained for protein with Coomassie Blue and scanned at 550 nm.

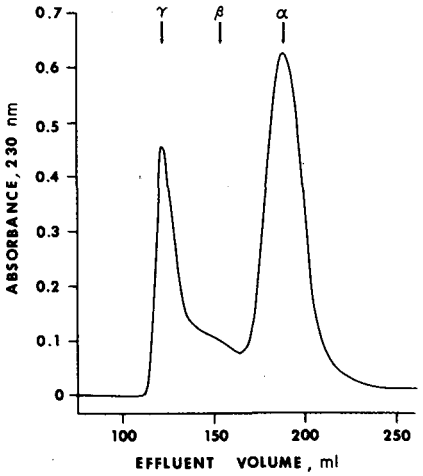

Fig. 3. Separation of type I (α) and type III (γ) collagens by molecular sieve chromatography on a Sepharose CL-6B column (210 × 1.6 cm). The sample was eluted with 2 *M* guanidine·HCl—0.05 *M* Tris·HCl (pH 7.5) at room temperature. The absorbance at 230 nm of the eluent was monitored continuously and 3-ml fractions were collected.

amounts of collagen from hydroxyproline values. Otherwise, type III collagen which contains more hydroxyproline would be overestimated. A recently reported method [11] for the quantitative assay of types I and III collagens from proline plus hydroxyproline total radioactivities would be subject to similar error because the amount of proline plus hydroxyproline for type I is 662 residues per molecule while it is 711 residues for type III collagen [1].

For the ultraviolet absorbance to be a reliable index of the amount of collagen, it was found necessary to defat the tissues prior to digestion with pepsin. Because ultraviolet-absorbing lipids elute from molecular sieve columns approximately in the region of the γ component (type III collagen), failure to remove lipids from samples would also result in overestimation of type III collagen. The success of separation and estimation of types I and III collagens using the method described by Adam et al. [12] depended on the preliminary removal of ultraviolet-absorbing proteoglycans during several steps of fractional precipitation and/or DEAE-cellulose column chromatography. No interference from proteoglycans was observed while using the present procedure for the estimation of collagen types I and III.

Recoveries of collagen

Ninety-two per cent of the defatted skin collagen (96—100% of the pepsin-solubilized collagen) expressed as hydroxyproline was recovered after molecular sieve chromatography (Fig. 1). Multiple precipitation steps to remove pepsin from pepsin-solubilized collagens usually resulted in low (20%) recoveries of collagen [2], while a single denaturation step has been shown by us [5] to remove the contaminant pepsin without loss in collagen. Therefore, the major advantage of the present procedure is the high recoveries without a reduction in relative purity of the types I and III collagen fractions.

ACKNOWLEDGEMENTS

We thank Mr. James Murtagh for his excellent technical assistance. This research was supported by grants AG-00361 and HL-16206 from the National Institutes of Health.

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Note

Separation and characterization of bilirubin conjugates by high-performance liquid chromatography

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(First received August 22nd, 1979; revised manuscript received December 4th, 1979)

Before excretion into bile, bilirubin is conjugated in the liver. Under physiological conditions, little unconjugated bilirubin appears in bile [1, 2]. Azopigment analysis has revealed that in man and rat, bilirubin mono- and diglucuronide are the main conjugates [3, 4]. Dog bile contains a mixture of glucuronide, glucoside and xyloside, mono-, di- and mixed conjugates [5]. These bilirubin conjugates react with diazo reagents, e.g. diazonium salts of ethyl anthranilate and *p*-iodoaniline, to form azodipyrrole pigments [6]. Azopigment analysis has led to the recognition that, besides bilirubin diglucuronide, other bilirubin conjugates exist [3—5]. A disadvantage of azopigment analysis is that through cleavage in dipyrryl derivatives, structural information about the composition of tetrapyrrole pigments is lost. Therefore methods are needed to separate the tetrapyrroles. Tetrapyrrole bile pigments can be separated directly by thin-layer chromatography (TLC) [3, 5, 7].

However, bile pigments are oxygen and light sensitive, and during work-up by TLC, considerable losses occur [4]. This paper describes a new method for the direct separation of bile pigments by means of high-performance liquid chromatography (HPLC). HPLC is particularly suited for bile pigment analysis because it is a rapid and simple technique and can be carried out under exclusion of light and oxygen.

MATERIALS AND METHODS

Chemicals

Tetrabutylammonium hydrogen sulphate was obtained from Fluka (Buchs, Switzerland). Acetonitrile came from Merck (Darmstadt, G.F.R.).

HPLC

A high-performance liquid chromatograph, Spectra Physics (Santa Clara, Calif., U.S.A.) Model 3500B, with a 20-μl injection loop was used, connected to a Spectra Physics variable-wavelength monitor (Model 770), set at 450 nm. A LiChrosorb 5 RP-18 column (150 mm × 4.6 mm I.D., particle size 5 μm; Chrompack, Middelburg, The Netherlands) was used.

Acetonitrile—0.01 *M* Tris—HCl buffer (40:60, v/v), containing 9.0 m*M* tetrabutylammonium hydrogen sulphate, was adjusted to pH 8.0 with 4 *M* sodium hydroxide and was used as the mobile phase. The flow-rate was 0.7 ml/min.

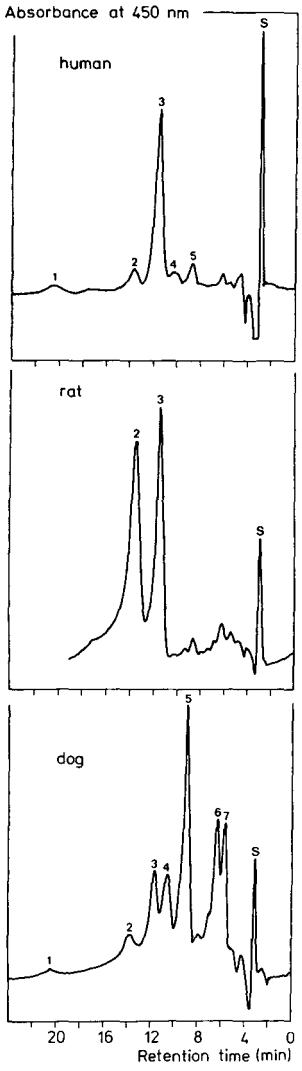

Fig. 1. HPLC analysis of human, rat and dog bile. Bile was diluted with citrate—phosphate buffer and injected directly into the HPLC column. S = solvent front, for peaks 1—7 see Table I.

Sample preparation

Rat bile was collected from fluothane anesthetized Wistar rats provided with bile duct cannulas. Dog bile was aspirated from the gall bladder of Labradors and Beagles. Human bile was obtained from a cholecystectomized patient with a bile duct drain. To prevent isomerisation [8], human bile and dog bile were diluted with 0.1 *M* citrate—0.2 *M* disodium hydrogen phosphate buffer (pH 4.0). Rat bile was collected in the dark, in ice, in tubes containing citrate—phosphate buffer. Diluted bile samples were injected directly into the HPLC column.

The preparation of reference material was carried out in the following way. After extraction from bile with chloroform, the bilirubin conjugates were separated using TLC [5]. The yellow pigments were eluted in methanol and injected into the HPLC column. Samples of the methanol eluates were diazotized with ethyl anthranilate as diazo reagent [6]. The azopigments were analysed by means of TLC [6].

RESULTS

Fig. 1 shows the HPLC analysis of human, rat and dog bile. Dog bile shows seven main peaks, enumerated 1—7. Peak 3 is the main pigment in human bile, peaks 2 and 3 in rat bile and peak 5 in dog bile. For identification of the

TABLE I

PEAK IDENTIFICATION

The tetrapyrrole pigments of human, rat and dog bile extracts were separated using TLC [5]. The R_F values of the pigments are shown in the table. The pigments were analyzed as follows. Samples of the separated pigments were submitted to azopigment analysis. According to the nomenclature proposed by Heirweigh et al. [5], α_0 is unconjugated azopigment; δ, azodipyrryl glucuronide; α_2, azodipyrryl xyloside; and α_3, azodipyrryl glucoside. In case two different azopigments were found, their ratio is shown within brackets. In addition, samples of the separated pigments were injected directly into the HPLC column. The retention times are shown. The pigment numbers correspond to the peak numbers in Fig. 1 and follow from comparison of the retention times.

Pigment	HPLC retention time (min)	TLC R_F value	Azopigments	Bilirubin conjugate
1	21	0.95	α_0	α_0—α_0, unconjugated bilirubin
2	13.5	0.58	α_0, δ (1:1)	α_0—δ, bilirubin monoglucuronide
3	11.5	0.38	δ	δ—δ, bilirubin diglucuronide
4	10.5	—	—	unknown
5	9.0	0.45	α_3, δ (1:1)	α_3—δ, bilirubin monoglucoside monoglucuronide
6	6.5	0.68	α_2, α_3 (1:1)	α_2—α_3 bilirubin monoxylosi-de monoglucoside
7	5.5	0.63	α_3	α_3—α_3, bilirubin diglucoside

peaks, bile was extracted in chloroform and the bile pigments were separated by means of TLC [5]. The separated pigments were analyzed by HPLC and diazotized as to study their azo-pigment composition.

Table I summarizes the results. Peak 2 is bilirubin monoglucuronide; peak 3, diglucuronide; peak 5, bilirubin monoglucoside monoglucuronide; peak 6, bilirubin monoxyloside monoglucoside; and peak 7, bilirubin diglucoside. Peak 4 could not be identified. This pigment is lost during extraction of bile with chloroform or during TLC.

DISCUSSION

HPLC is an excellent technique for the separation of tetrapyrrole bile pigments. With acetonitrile—water (70:30, v/v) as eluent, Lim [9] separated conjugated from unconjugated bilirubin, using a reversed-phase column. For the separation of mono- and diglucuronides Lim [9] used a μBondapak carbohydrate column. In the present paper a method is described for the separation of unconjugated bilirubin and several conjugates, in a single run on a reversed-phase column. Tetrabutylammonium hydrogen sulphate acts as a counter-ion. Without this compound all peaks run with the solvent front. At concentrations lower than 9.0 m*M*, the method can be used to separate the bilirubin IIIα, IXα, and XIIIα isomers (unpublished observations).

Gordon et al. [3] using a TLC technique, found that human bile contains 86% bilirubin diglucuronide, 7% bilirubin monoglucoside monoglucuronide diester, 4% bilirubin monoglucuronide and 3% bilirubin. According to the same authors, the main pigments in dog bile are bilirubin diglucuronide and bilirubin monoglucoside monoglucuronide diester [3]. Dog bile also contains bilirubin monoglucuronide, diglucoside, bilirubin and traces of xylose-containing conjugates [3]. Noir [4], also using a TLC method, found that rat bile contains mainly bilirubin mono- and diglucuronides. Our results are in agreement with these findings.

There is a current interest in the importance of unconjugated bilirubin in human bile for the formation of gall stones [10]. The HPLC method described in this paper seems to be well suited to study this problem.

ACKNOWLEDGEMENTS

We thank Dr. A. Huijbregts and Dr. J.H.M. van Tongeren for their helpful suggestions in this study. The expertise of Mr. P. Spaan, who did the animal surgery, is gratefully acknowledged.

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Note

Accurate and sensitive analysis of ethyl anthranilate azopigments from bile by reversed-phase high-performance liquid chromatography

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(First received June 29th, 1979; revised manuscript received November 9th, 1979)

Van Roy and Heirwegh [1] developed a very elegant thin-layer chromatographic (TLC) method by which it was elucidated that the conjugates of bilirubin are composed of glucoside and xyloside in addition to glucuronide [2]. This finding reflects the considerable progress being made in research techniques in the field of conjugated bilirubin structure. However, Bonnett and Stewart pointed out that photodecomposed substances of bilirubin were incompletely resolved on TLC [3]. In order to overcome these difficulties, we introduced the new high-performance liquid chromatographic (HPLC) method in 1973 and reported our results at the Second International Symposium on Bilirubin Metabolism in the Newborn, held in Jerusalem in 1974 [4]. Lately, Billing has pointed out that the most promising approach would appear to be that of HPLC [5]. We concluded that HPLC is a useful method for separation of azopigments, conjugated bilirubin [6] or geometric isomers of bilirubin in biological fluids, and have published a preliminary report of these results [7, 8]. The present paper describes the conditions for the complete separation of azopigments of dog bile by HPLC.

MATERIALS AND METHODS

High-performance liquid chromatography

A Shimadzu LC-2 liquid chromatograph with an SPD-1 detector and Chromatopac C-R1A was used for all HPLC procedures. The column used was a Shimadzu PCH-05/S2504 (25 cm × 4.6 mm) packed with a reversed-phase packing material based on silica gel (5-μm particles). Separations were achieved at room temperature. The variable-wavelength detector was set at 530 nm. The mobile phase was a mixture of acetonitrile, distilled water and sodium acetate.

Separation was best achieved by using a linear gradient of acetonitrile [20% to 60% (v/v) in 80 min] in 0.1 *M* acetate buffer (pH 4.0). Peak assignments were conducted using two methods; one was the TLC method described below, the other by comparison with the retention time of reference samples on HPLC. The ethyl anthranilate azopigments derived from bilirubin conjugates which were obtained from incubating bilirubin with UDP-glucuronic acid (or either UDP-glucose or UDP-xylose) and adult human liver homogenate, yielding bilirubin glucuronides (or either glucosides or xylosides), respectively, were used as a reference substance. Fourier transform proton NMR spectrometry was also performed for the identification of azodipyrrole (*exo*-vinyl and *endo*-vinyl isomer).

Formation of ethyl anthranilate azo derivatives

A 1% (v/v) suspension of ethyl anthranilate in 0.15 *M* HCl was prepared, and 0.6 ml was diluted with 9.4 ml of water. To this was added 0.6 ml of $NaNO_2$ solution (5 mg/ml) and, 5 min later, 0.2 ml of ammonium sulphamate solution (10 mg/ml). The diazo reaction was carried out by mixing 1 vol. of bile with 1 vol. of citrate—phosphate buffer (pH 6.0), 0.5 vol. of formamide—ethanol (1:1, v/v) and 1 vol. of the diazo reagent. After 45 min at 0°, 1 vol. of ascorbic acid solution (10 mg/ml) in glycine—HCl buffer (pH 2.0) was added to destroy the excess diazo reagent and to lower the pH for extraction. The azopigments were extracted without delay by vigorous shaking with 2 vols. of pentan-2-one [9]. The organic phase was dried at room temperature in a rotary evaporator connected to an oil vacuum pump. The residue was dissolved in the primary eluent of HPLC before analysis.

Analysis of azopigments by TLC

Portions of the extracted azopigments with pentan-2-one were applied to precoated silica gel plates (DC-kieselgel, E. Merck, Darmstadt, G.F.R.) and developed at room temperature in the dark. Separation was best obtained by developing the plates first with chloroform—methanol (17:3, v/v) for 3 cm, followed by chloroform (containing 0.6—1% ethanol) for 18 cm, and then chloroform—methanol—water (65:25:3, v/v) for 15 cm [10]. The azopigments were eluted from the thin-layer plates with ethanol.

Pigments and reagents

Bilirubin (Merck) and ethyl anthranilate (Kodak, London, Great Britain) were used without further purification. Pentan-2-one (Tokyo Kasei, Tokyo, Japan) was distilled before use. UDP-glucuronic acid, UDP-glucose and UDP-xylose were purchased from Sigma (St. Louis, Mo, U.S.A.). Acetonitrile was of analytical grade.

RESULTS AND DISCUSSION

The azopigments were obtained from dog bile treated with diazotized ethyl anthranilate [9]. This was followed by confirmation of the separation of azodipyrrole, its xyloside, glucoside and glucuronide at bands α_0, α_2, α_3 and δ (Fig. 1), respectively, by TLC, as demonstrated by Heirwegh et al. [2]. Then, using

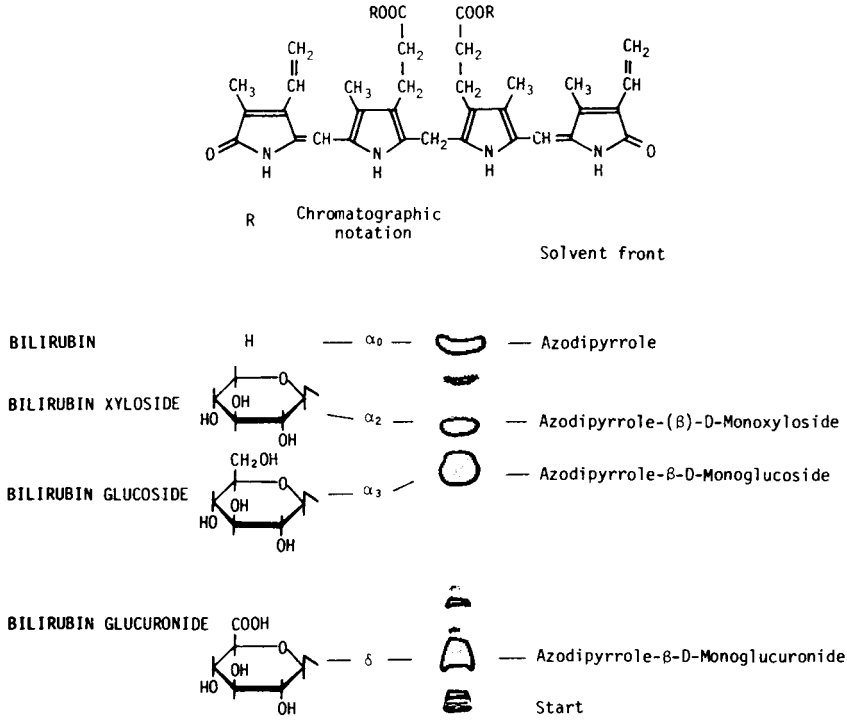

Fig. 1. Schematic diagram of TLC of ethyl anthranilate azopigments (α_0, α_2, α_3 and δ) derived from diazo coupling of bilirubin and its conjugates in dog bile.

the HPLC method, it was observed that each band (α_0, α_2, α_3 and δ) had a pair of peaks (Fig. 2). Moreover, by analyzing each α_0 peak using Fourier transform proton NMR spectrometry [11], it was confirmed that the former peak was *endo*-vinyl isomer and the latter *exo*-vinyl isomer (Fig. 3). Accordingly, it was demonstrated that the mixture of the *endo*- and *exo*-vinyl isomers of azopigment which gave one band on the TLC plate obtained by several developments could be clearly separated by only one HPLC run, and that the *exo*-vinyl peak was usually smaller than that of *endo*-vinyl and easily degraded despite careful attention to light, oxygen and related factors.

The calibration curve of peak area, obtained from the electronically integrated peak area calculated by Chromatopac C-R1A, versus amount of bilirubin loaded, is linear up to 1–3 μg of bilirubin. If the concentration is above this range, the value can be precisely estimated either by decreasing the amount of sample injected into the chromatograph or by diluting the sample with the primary eluent.

ACKNOWLEDGEMENTS

We thank Dr. K.P.M. Heirwegh for valuable advice and comments on this work. This research was supported by Research Grant No. 487097 from the Ministry of Education of Japan. We thank Professors J. Ogawa and Y. Wada for encouragement.

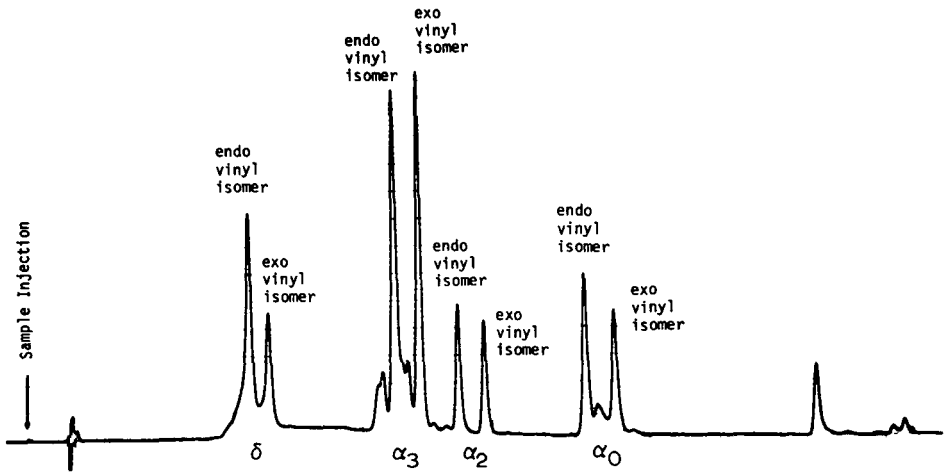

Fig. 2. Separation of ethyl anthranilate azopigments (α_0, α_2, α_3 and δ) derived from dog bile. Conditions: column, Shimadzu PCH-05/S2504; mobile phase, a linear gradient of acetonitrile (20% to 60% in 80 min); flow-rate, 1 ml/min; detection, 530 nm, 0.02 a.u.f.s.

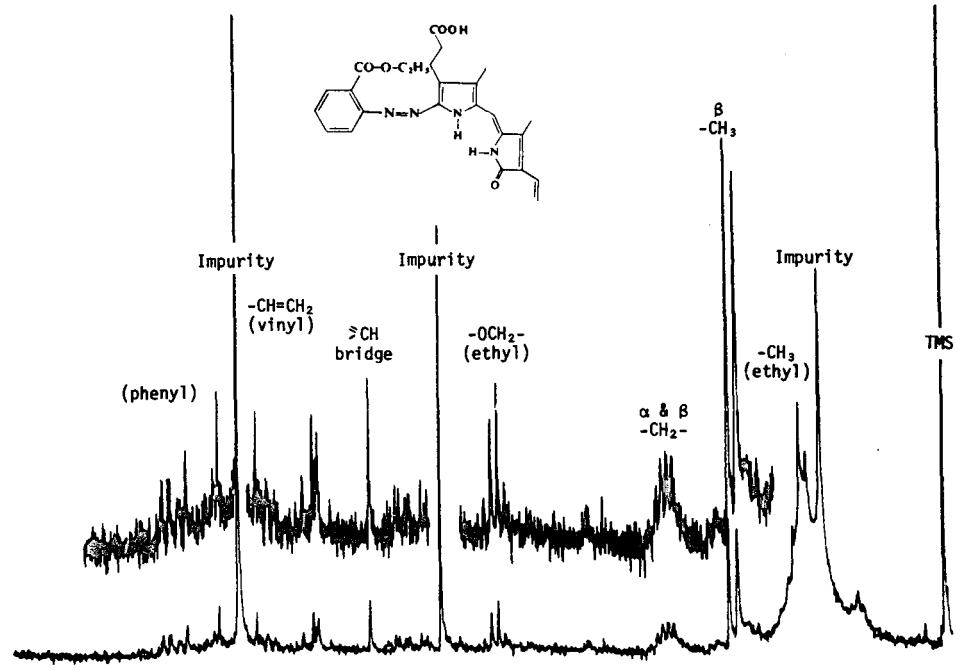

Fig. 3. Fourier transform proton NMR spectrum of *exo*-vinyl isomer of azodipyrrole (α_0) run on a JMN-PFT-100 type NMR (JEOL) instrument with tetramethylsilane as an internal standard.

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Note

Thin-layer chromatographic method for the quantitative analysis of L-tryptophan in human plasma

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(First received July 31st, 1979; revised manuscript received November 7th, 1979)

Considerable interest in monitoring plasma L-tryptophan levels during treatment with anti-inflammatory drugs has been awakened by Aylward and co-workers [1—3].

During an investigation into the possible interaction between aspirin and diclofenac (an anti-inflammatory drug marketed by Ciba-Geigy, Basle, Switzerland), the plasma levels of salicylic acid, diclofenac and L-tryptophan were monitored. In addition, several other parameters in the plasma had to be monitored to satisfy the protocol requirements of this clinical trial. These requirements made it necessary to develop simple analytical methods by which salicylic acid and L-tryptophan could be quantitatively measured with precision and accuracy, employing the smallest possible volume of plasma in order to avoid drawing excessive amounts of blood.

Although numerous methods exist for the determination of L-tryptophan [4—14], none of them were found to be entirely suitable, mainly from the point of view of sample requirement or because the method was deemed too cumbersome and time-consuming. Previous experience with thin-layer chromatographic (TLC) methods involving measurement of in situ fluorescence of the spots on the TLC plate [15, 16] prompted an investigation into the possibility of using such a method for the determination of L-tryptophan. This paper describes a TLC method by means of which L-tryptophan can be determined in plasma. Only 50 μl of plasma are required if the same aliquot is used to determine salicylic acid in the sample as well.

EXPERIMENTAL

Reagents

All reagents used were of guaranteed reagent grade (Merck, Darmstadt, G.F.R.) and were used without further purification. L-Tryptophan was obtained from Merck, and also from Sigma (St. Louis, Mo., U.S.A.), and was used as received.

Apparatus

A Perkin-Elmer MPF3 spectrofluorimeter equipped with a TLC scanning attachment was used to measure the fluorescence of the spots on the TLC plates using the following conditions: light source, xenon lamp; excitation wavelength, 287 nm; emission wavelength, 335 nm; excitation slit width, amplifier sensitivity and sample adjustment were set so as to obtain approximately 80% full-scale deflection on the recorder when the strongest spot in the chromatogram was being scanned.

The other apparatus used consisted of a Shandon S/P Chromatank, Hamilton dosing syringes (10 μl and 50 μl), 5-μl disposable glass capillary micropipettes calibrated at 1 μl intervals (Clay Adams Division of Becton Dickenson & Co., Parsipany, N.Y., U.S.A.), and a Desaga Autospotter.

Stock solutions

A single stock solution containing 1.00 mg of L-tryptopan per millilitre of solution was prepared by making 50 mg of L-tryptophan up to 50 ml in 0.1 *N* NaOH. This solution was kept in a refrigerator at 4°.

Plasma standards

Fresh human plasma (10 ml) was shaken with 100 mg of activated charcoal for 5 min and then filtered under pressure through a Celite bed (1 cm high) in a disposable plastic syringe. This procedure was shown to remove tryptophan quantitatively from the plasma. Four standard solutions of L-tryptophan were prepared by adding, with a Hamilton syringe, 5, 10, 15 and 20 μl of the stock solution in each instance to 1 ml of the charcoal-treated plasma contained in plastic-capped sample tubes. Corrections were applied to compensate for the small volume changes. These standard solutions were kept frozen at —20° when not in use.

Preparation of plasma samples for chromatography

A 50-μl volume of plasma (standard or unknown) was measured accurately with the dosing syringe into a small plastic-stoppered, disposable, conical centrifuge tube. To the plasma were added 100 μl of methanol in order to precipitate the proteins partially. After thorough mixing on a vortex mixer, the stoppered tube was centrifuged for 1 min to produce a clear supernatant.

Spotting the plates

A 3-μl aliquot of the clear supernatant was spotted on cellulose TLC plates without fluorescence indicator (Merck) in three applications of approximately 1 μl at a time, while drying with a hairdrier between applications. In this fash-

ion sixteen spots with unknown (X) and standard (S) alternating in duplicate were applied to a single 10 cm × 20 cm plate ($S_1X_1S_2X_2S_3X_3S_4X_4$). Alternatively, 6 μl were spotted using the Desaga Autospotter.

Chromatography

The mobile phase used was *n*-butanol—acetone—glacial acetic acid—water (35:35:7:23). Ascending development was carried out in an unsaturated tank up to a height of 6.5 cm; the elution time was ca. 20 min. The plates were dried briefly with a hairdrier and then allowed to stand open to a clean atmosphere at room temperature for 15 min before being scanned. (Drying in an oven at 100° was found to have a deleterious effect on the baseline of the densitograms.) The R_F value of L-tryptophan was found to be 0.52.

Each spot was then scanned in the TLC scanning attachment of the MPF_3 spectrofluorimeter in the direction of the solvent flow. Standard curves were constructed by plotting peak height versus plasma concentration of the known standards. An equation for the best straight line fit was obtained by linear regression analysis and this equation used to calculate the plasma concentrations of unknowns by substitution of the peak heights of the unknowns into the equation.

RESULTS AND DISCUSSION

Fig. 1 represents part of a densitogram showing the peaks obtained for standard plasma containing the indicated amount of L-tryptophan, plasma samples obtained from two different persons, as well as a blank plasma after charcoal treatment. The second peak at R_F 0.29 (indicated by an asterisk in the densitogram) was obtained in all normal plasma samples investigated. Generally, a good linear relationship existed between the concentration of L-tryptophan (5—20 μg/ml) and peak height so that an equation of the best straight line fit could be found by linear regression analysis ($r^2 > 0.98$ normally)

A summary of the recoveries of L-tryptophan added to charcoal-treated plasma is presented in Table I.

To ascertain whether it would be feasible to use aqueous standards of L-tryptophan instead of standards made up in plasma, standards of L-tryptophan were prepared in water in exactly the same way as described for the plasma. These were then treated in the same way as the plasma standards and spotted on the same plates alternating with the plasma standards. The results obtained are illustrated in Fig. 2 in which were plotted the peak heights of plasma standards versus peak heights corresponding to aqueous standards for adjacent spots on the chromatogram. A good linear correlation between the peak heights for the two types of standards was apparent but the slope indicated that L-tryptophan would be underestimated by a factor of 1.06 if aqueous standards were used. This factor was shown to be constant over a period of one year, suggesting that aqueous standards could be used provided a correction factor were applied.

The effect of scanning the spots across the direction of the solvent flow was investigated. The results are given in Table I. An example of a chromato-

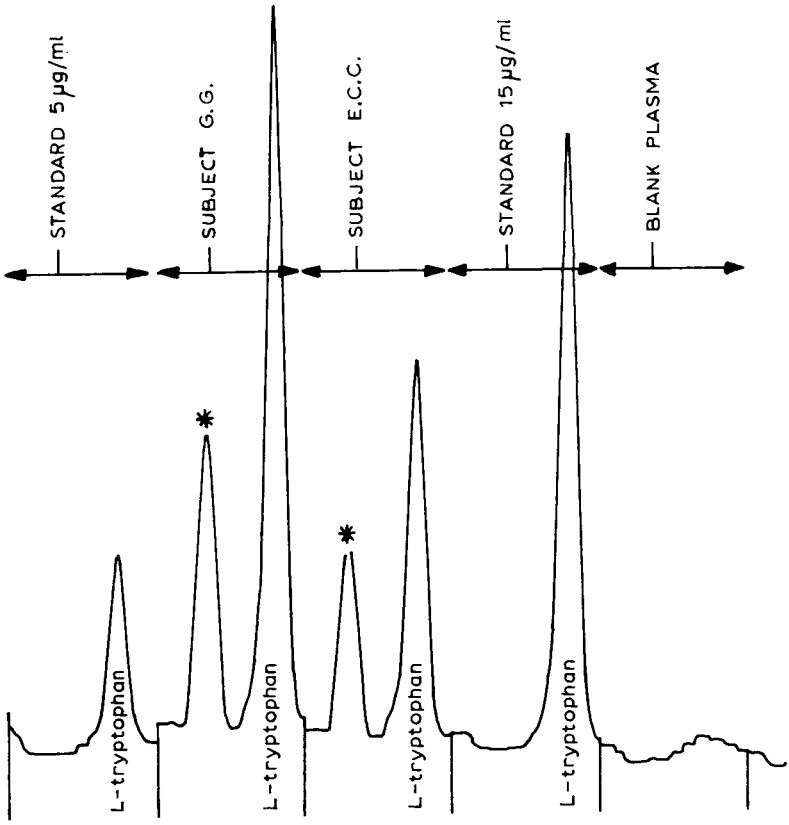

Fig. 1. Densitogram of L-tryptophan in plasma of two volunteers and in plasma standards. Asterisk (*) indicates peak (R_F 0.29) due to endogenous substance present in all normal plasma samples.

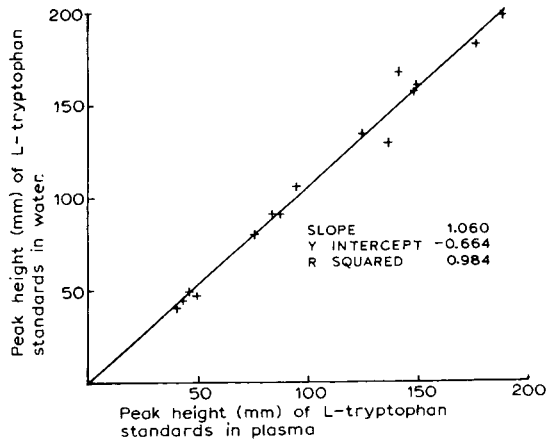

Fig. 2. Comparison of L-tryptophan standards in plasma with L-tryptophan standards in water.

TABLE I

RECOVERY OF L-TRYPTOPHAN FROM HUMAN PLASMA

Method*	L-Tryptophan added (μg)	Mean recovery (μg)	C.V. (%)	*N*
a	8.00	8.18	5.50	4
	11.00	10.74	3.45	4
	14.00	13.97	7.59	4
	17.00	17.32	6.18	4
b	8.00	7.85	7.39	4
	11.00	10.45	10.43	4
	14.00	13.53	3.62	4
	17.00	17.59	2.96	4
c	5.00	4.39	9.11	4
	12.50	12.45	8.59	4
	17.50	16.52	8.60	4
	20.00	19.02	2.72	4

*(a) Spots scanned in direction of solvent flow; (b) spots scanned across direction of solvent flow; (c) spots applied with Desaga Autospotter.

gram scanned across the direction of solvent flow is presented in Fig. 3. From the results obtained it can be seen that the chromatograms can be scanned across the direction of solvent flow, with considerable saving of time, without significant difference in accuracy or precision.

For comparison with the manual spotting method, spiked plasma samples were treated as before but 6 μl of the supernatant were spotted using the Desaga Autospotter. A summary of the recoveries of L-tryptophan in this case is presented in Table I. It is evident that lower accuracy and precision were attained using the Autospotter.

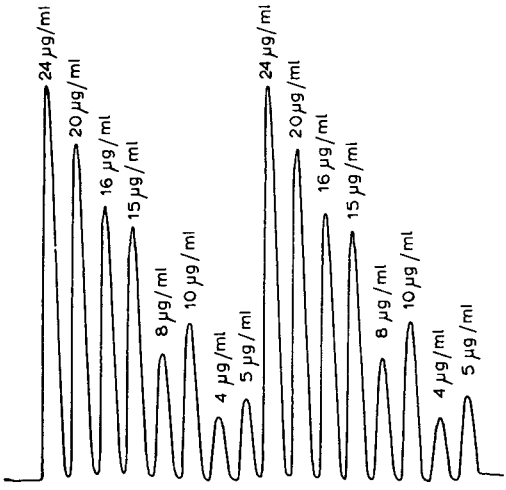

Fig. 3. Densitogram of spiked plasma samples scanned across the direction of solvent flow.

The optimum excitation and emission wavelenghts were found to be quite critical. In contrast with assays of many other compounds performed by in situ fluorescence measurement on thin-layer plates, cut-off filters for the emitted light could not be used in this case.

No interference with the determination was observed with the following compounds tested: mephenamic acid, flufenamic acid, niflumic acid, thiaprofenic acid, nalidixic acid, flubiprofen, fenoprofen, ketoprofen, ibuprofen, indoprofen, fenbufen, alclofenac, diclofenac, oxyphenbutazone, phenylbutazone, naproxen, probenecid, sulindac, indomethacin, penicillin G, furosemide, piretinide, all at a concentration of 20 μg/ml; paracetamol at 50 μg/ml; and salicylic acid at a concentration of 300 μg/ml. Likewise, no interference from metabolites of salicylic acid was experienced.

L-Tryptophan plasma levels (mean 12.33 μg/ml, range 7.15—19.94 μg/ml, S.D. 2.59) determined in 20 normal volunteers taking no medication are within the range reported by other workers [1, 10—14].

This method was used to determine L-tryptophan in a large number of plasma samples obtained from volunteers participating in a clinical trial involving the ingestion of aspirin. Determination of salicylic acid in the plasma samples was carried out by TLC on silica gel plates using the same supernatant aliquot described above. The small amount of sample required for the assay (the determination can be scaled down to 5 μl of plasma) makes the method ideally suitable for the study of L-tryptophan in plasma or other biological fluids where only small volumes of plasma are available.

Two recent publications [17, 18] on the determination of L-tryptophan in plasma came to our attention while this manuscript was being prepared.

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Journal of Chromatography, 182 (1980) 116—120
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Note

Improved gas—liquid chromatographic method for the simultaneous determination of phenobarbital, phenytoin, carbamazepine and primidone in biological fluids

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(First received September 6th, 1979; revised manuscript received October 31st, 1979)

Among the techniques which have contributed to the present widespread determination of antiepileptic drugs, gas—liquid chromatography after flash-heater alkylation remains largely used by reason of its selectivity, sensitivity and reliability [1].

However, the analytical performances of flash-heater derivatization methods for phenobarbital (PB) and carbamazepine (CBZ) are questionable, since these two drugs decompose in the alkylating medium at high temperature. This problem can be overcome by using either an automatic injector [2—4], or internal standards structurally related to the drugs to be assayed. Such an appropriate internal standard is now often used for PB but, until now, was not available for CBZ.

Another problem encountered with CBZ determinations is the difficulty in achieving an efficient and specific extraction into the alkylating reagent. Interfering biological constituents can be eliminated by time-consuming clean-up procedures [2, 3, 5]. More conveniently, specificity can be improved by use of a nitrogen-selective detector [4, 6].

The method proposed here for the simultaneous determination of PB, phenytoin (DPH), CBZ and primidone (PM) in biological samples is an improvement on other published flash-heater methylation methods [4, 6, 7]. The two above-mentioned problems have been solved by the combination of appropriate internal standards (in particular, a new internal standard for CBZ), a modified rapid extraction procedure, and the use of a thermionic detector.

MATERIALS AND METHODS

Reagents and standards

Reagent grade dichloromethane, methanol and *n*-hexane were obtained from Merck (Darmstadt, G.F.R.). CBZ and 10-methoxycarbamazepine (MCBZ) were kindly supplied by Ciba-Geigy (Basel, Switzerland) and PM by Imperial Chemical Industries (Macclesfield, Great Britain). 5-Ethyl-5-*p*-tolylbarbituric acid (TEB), 4-methylprimidone (MPM) and 5-(*p*-methylphenyl)-5-hydantoin (TPH) were from Aldrich-Europe (Beerse, Belgium). PB and DPH meet the specifications of the United States Pharmacopeia (USP XVIII). MethElute® (trimethylphenylammonium hydroxide 0.2 mol/l methanol) was from Pierce Eurochemie (Rotterdam, The Netherlands).

The buffer used was 0.3 mol/l KH_2PO_4/Na_2HPO_4 (pH 6.7).

The extraction solvent was dichloromethane containing 4.5 mg of TEB, 7.5 mg of TPH, 11.0 mg of MCBZ and 5.0 mg of MPM per liter, as internal standards.

MethElute—methanol—water (2:2:1, v/v) was used as methylating solution. This solution was prepared daily.

Calibration serum. One volume of drug-free serum pool mixed with 0.025 vol. of a stock solution of drugs. This stock solution was prepared by dissolving PB, DPH, CBZ and PM in KOH (10 mol/l)—methanol—water (0.4:50:50, v/v). The following serum concentrations are obtained: 100 (PB), 60 (DPH), 30 (CBZ) and 60 (PM) μmol/l. The calibration serum, divided into 1-ml aliquots, was found to be stable for at least six months when stored at −30°.

Apparatus and operating conditions

An Intersmat IGC 120 DFL gas chromatograph equipped with a thermionic detector (alkali source: electrically heated rubidium glass bead) (Chelles les Coudreaux, France) was used. The glass column was 6 ft. × 0.4 in. O.D., packed with a mixture of equal volumes of 3% OV-1 and 3% OV-17 on 100—120 mesh Gas-Chrom Q. Separate packings were obtained from Applied Science Labs. (State College, Pa., U.S.A.). The injector temperature was 280°, the oven temperature 215° and the detector temperature 250°. The flow-rate of nitrogen (carrier gas) was 25 ml/min, of air 120 ml/min, and of hydrogen 1.2 ml/min. The attenuation was 4×10^{-9} A/mV.

Procedure

Into a 10-ml glass centrifuge tube, pipette 200 μl of sample (serum, plasma, saliva or cerebrospinal fluid) or of calibration serum, add 200 μl of buffer and 650 μl of extraction solvent. Shake on a Vortex-type mixer for 30 sec and centrifuge. With an Eppendorf pipette, transfer 500 μl of the organic layer into a stoppered conical glass centrifuge tube. Evaporate to dryness at 40° under a stream of nitrogen. Add 600 μl of *n*-hexane and 10 μl of methylating solution to the residue. Shake on a Vortex-type mixer for 30 sec and centrifuge for 2 min at 2000 *g*. Inject 1 μl of the lower layer into the chromatograph.

Quantitation

The following peak height ratios are calculated for each sample: PB/TEB,

DPH/TPH, CBZ/MCBZ and PM/MPM.

The concentration of each drug is calculated by comparing the ratio obtained for the unknown sample with that obtained for the calibration serum.

RESULTS AND DISCUSSION

A chromatogram of a calibration serum extract is shown in Fig. 1A. Retention times of the drugs and internal standards are: 106 (PB), 146 (TEB), 179 (CBZ), 204 (PM), 285 (MPM), 334 (MCBZ), 406 (DPH) and 570 (TPH) sec. A mixture of equal volumes of OV-1 and OV-17 has been selected for its excellent ability to separate these eight compounds without need of temperature programming.

Calibration curves are linear up to at least 350 (PB), 200 (DPH), 85 (CBZ) and 120 (PM) μmol/l of serum.

Owing to the short procedure time (about 30 min for an overall analysis of one sample), one analyst can easily process 30 samples per day.

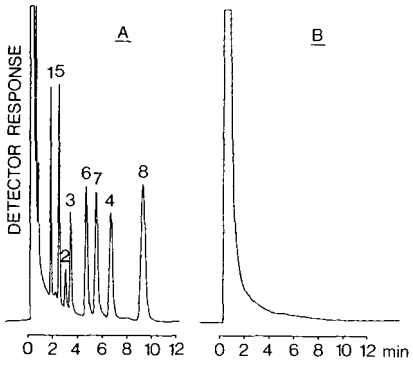

Fig. 1. (A) Gas chromatogram of a calibration serum containing PB (1), CBZ (2), PM (3) and DPH (4). Internal standards are TEB (5), MPM (6), MCBZ (7) and TPH (8). (B) Gas chromatogram of a drug-free serum extracted without internal standards.

Extraction procedure

To achieve a good recovery of CBZ into the methylating reagent, the procedures already published include evaporation of the organic extract and reconstitution of the residue with a methylating reagent [2—5, 7—9] or with methanol [6]. However, these procedures do not offer the advantages of the alkaline *extraction*, wherein a smaller volume of methylating reagent can be used, thus ensuring a greater sensitivity. For this reason, we first reconstitute the dried residue with *n*-hexane, and then add the methylating solution.

The composition of the methylating solution has been selected in order to achieve a good compromise between (a) complete derivatization of DPH, (b) complete breakdown of CBZ into iminostilbene, and (c) minimal methylation of iminostilbene [3]. DPH, CBZ and their respective internal standards are recorded each as a single peak.

Precision

Repeatability and reproducibility have been estimated from duplicates of patients' sera analysed under routine conditions. The results (coefficients of variation, C.V., %) are given in Table I, together with the limits of detection.

For the quantitation of each drug, we have selected the most appropriate internal standard, i.e. the respective *p*-methyl derivatives of PB, DPH and PM, and the 10-methoxy derivative of CBZ. This last substance was first introduced by Schwertner et al. [10] for the determination of CBZ as its N-pentafluorobenzamide. Table II demonstrates the importance of a good selection of the internal standards to ensure the greatest precision. For example, repeatability of the CBZ determination is 4.2% when calculated with MCBZ as internal standard, whereas the C.V. ranges from 9.6 to 16.9% when calculated by reference to the three other internal standards. Dudley [11] has also emphasized the usefulness of multiple internal standards to improve the precision of flash-heater alkylation methods.

TABLE I

PRECISION AND DETECTION LIMITS

	PB	DPH	CBZ	PM
Within-day C.V. (%)*	3.6 ($2n$=64)	3.7 ($2n$=62)	4.2 ($2n$=72)	3.4 ($2n$=58)
Between-day C.V. (%)*	3.2 ($2n$=86)	5.3 ($2n$=76)	4.5 ($2n$=62)	3.0 ($2n$=60)
Detection limit (μmol/l)	0.2	0.5	2.0	0.5

*Coefficient of variation on duplicates.

TABLE II

WITHIN-DAY COEFFICIENTS OF VARIATION CALCULATED ON PATIENTS' SERA ANALYSED IN DUPLICATE

For each drug, the precision has been calculated by reference to each of the four internal standards.

Internal standard	Drug			
	PB ($2n$=64)	DPH ($2n$=62)	CBZ ($2n$=72)	PM ($2n$=58)
TEB	3.6	11.5	16.9	11.8
TPH	10.4	3.7	11.8	6.3
MCBZ	14.7	13.5	4.2	10.8
MPM	11.7	7.7	9.6	3.4

Specificity and accuracy

The possible interference of several drugs and drug metabolites has been tested. Valproic acid, trimethadione, paramethadione, phenacemide, phenylethylmalonamide, ethylphenacemide, ethosuximide, methsuximide, phensuximide, ethotoin, mephenytoin and 5-ethyl-5-phenylhydantoin elute before PB and do not interfere. No peak is recorded within 30 min for sulthiame. 10,11-Epoxycarbamazepine is decomposed to several compounds which give no mea-

surable peaks. Mephobarbital yields the same N,N-dimethyl derivative as PB. Caffeine appears on chromatograms of some patients' sera. This substance has a retention time of 126 sec and does not interfere.

No peaks are recorded on chromatograms of drug-free sera extracted without internal standards (Fig. 1B). Serum constituents are recorded when a flame ionization detector is used instead of a thermionic detector [3, 7–9]. One of these observed peaks is not resolved from TEB, and thus interferes with the determination of PB. Some authors avoid this problem by tedious clean-up of the organic extract [2, 3]. We have chosen the alternative of using a thermionic detector. Being highly specific for substances containing nitrogen or phosphorus [12], this detector can be used in conjunction with our rapid extraction procedure.

Our participation in the St. Bartholomew's Hospital Quality Control Scheme [13] has enabled us to check the reliability of our method. At the present time, we have analysed eleven interlaboratory quality-control samples. None of our results fell outside the mean ± 1 S.D. limits of the accepted values reported by the participating laboratories.

ACKNOWLEDGEMENT

We gratefully acknowledge Mrs. J. Genin-Ramakers for her excellent technical assistance.

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Note

Determination of caffeine in serum by straight-phase high-performance liquid chromatography

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(First received July 3rd, 1979; revised manuscript received November 6th, 1979)

Caffeine (1,3,7-trimethylxanthine) and analogous dimethylxanthine derivatives such as theophylline (1,3-dimethylxanthine) and theobromine (3,7-dimethylxanthine), are stimulators of the central nervous system. Caffeine is the most potent stimulator of the central nervous system, especially of the medullary respiratory centre [1]. This property makes caffeine the most useful drug of the three commonly available methylxanthine derivatives in the prevention of apnea in premature infants. Aranda et al. [2] showed the efficacy of treating apnea in low-birth-weight infants with caffeine.

Caffeine is mainly metabolised in the liver, undergoing demethylation and oxidation [3]. The most important metabolite is paraxanthine (1,7-dimethylxanthine), and its physiological activity should not be disregarded. Also the formation of theobromine and theophylline has been described [4]. The oxidised and demethylated metabolites 1-methyluric acid, 1-methylxanthine and 1,3-methyluric acid appear in the urine. Sometimes the biotransformation of the dimethylxanthine derivatives is paradoxical. Bory et al. [5] and Boutroy et al. [6] described the treatment of premature infants suffering from apnea with theophylline, which was biotransformed to caffeine. So even in the use of theophylline one should be aware of the possibility that caffeine might be present.

Unchanged caffeine is excreted in the urine in negligible amounts. The analysis of urinary products has no therapeutic value. The biological half-life ($t_{1/2}$) of caffeine in adults is approximately 3.5 h. The $t_{1/2}$ in premature infants is very much prolonged up to 97 h and the values are spread over a wide range: 40.7—230 h [2]. The reason for this is the incomplete development of the liver functions in the preterm infant. As a consequence of these enormous differ-

ences in rate of metabolism, serum concentrations need to be monitored to optimise the therapy and to avoid toxic or subtherapeutic levels.

In analogy to theophylline, caffeine has no significant efficacy below a serum concentration of 5 mg/l. The optimal level is taken to be 10—15 mg/l. Within this range there is a significant decrease in the incidence and severity of apnea [2]. The serum concentration should not exceed 20 mg/l because of risk of toxicity. Below a level of 20 mg/l no toxic symptoms have been described. In man the fatal oral dose of caffeine is estimated to be about 10 g. Untoward reactions, however, may be observed following the ingestion of 1 g or more.

Several methods for the determination of caffeine in biological fluids have been described [4, 7—10]. When caffeine is used for the indication apnea in premature infants, the serum determination should require very small amounts of serum and be accurate and rapid. We have modified the straight-phase high-performance liquid chromatographic (HPLC) method for the determination of carbamazepine described by Westenberg and De Zeeuw [11].

MATERIALS AND METHODS

Chemicals and reagents

Dichloromethane, p.a. grade, and tetrahydrofuran "rein" were from Merck (Darmstadt, G.F.R.); caffeine was obtained from A.C.F. (Maarssen, The Netherlands), and carbamazepine was from Ciba-Geigy (Basle, Switzerland). Water was double-distilled from glass columns.

Standard solutions

Standard solutions of caffeine were prepared in dichloromethane, water and spiked serum, in the concentration range 5—25 mg/l. The internal standard solution was carbamazepine, 0.5 mg/l in dichloromethane.

Apparatus

A Siemens Model S100 high-performance liquid chromatograph with a Valco UH Pa7 valve with 0.05-ml sample loop was used connected to a UV detector (Zeiss PM_2 DLC) which was operated at 272 nm. The column was stainless steel (10.0 cm × 4.6 mm I.D.) packed with silica gel (Partisil 5, Chrompack, Middelburg, The Netherlands; particle size 5 μm). The eluent was 20% tetrahydrofuran in dichloromethane, the flow-rate 1.5 ml/min (pressure drop 80 bar).

A Finnigan 3200 mass spectrometer connected to a Finnigan 9500 gas chromatograph was operated as follows: column, 3.8% SE-30 on Chromosorb W AW DMCS 80—100 mesh; column oven temperature, 240°; mass spectrometry ionizing voltage, 70 eV; electromultiplier voltage, 1600 V.

Assay

Transfer 0.2 ml of a serum sample into a 10-ml test-tube, and add 5.0 ml of internal standard solution. Rotate for 1 min on a Vortex mixer and centrifuge at 2000 *g* for 5 min. Transfer 4 ml of the organic layer into a test-tube and evaporate to dryness by heating the tube in a water-bath of ca. 37°. Add 0.250 ml of dichloromethane and inject 0.020—0.050 ml into the chromatograph.

RESULTS

The standard solutions of caffeine in dichloromethane, water and spiked serum were determined as described in the assay procedure. Peak height ratios were calculated and plotted against the known concentrations. The slopes and correlation coefficients were calculated using a least-squares procedure. There is a good linear correlation between peak height ratios and serum levels in the range of the calibration curve (correlation coefficient = 0.9997). The standard error of the determination at 12 mg/l (n = 12) is 1.5%. There is no significant difference between the extraction of caffeine from serum and water. With these results in mind, we use an aqueous solution for the caffeine standard for routine determinations since blank serum might be contaminated with caffeine.

The detection limit of the method is 4 ng per injection with a signal-to-noise ratio of 4:1. Thus, with the described procedure it is possible to detect caffeine accurately using 0.1 ml of serum. Fig. 1 shows a chromatogram of the serum from a patient treated with caffeine in a single dose of 5 mg/kg. The peaks numbered 3 and 4 were identified by gas chromatography—mass spectrom-

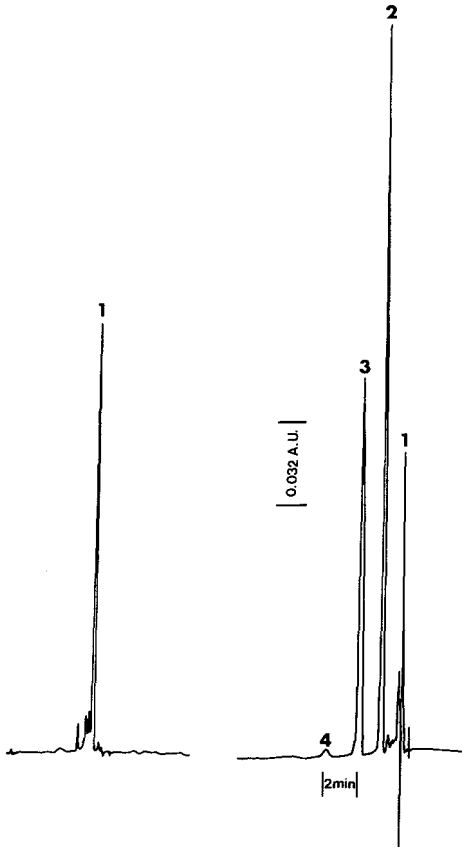

Fig. 1. Chromatogram of blank serum (left) and of a patient's serum sample (right). Peaks: 1, solvent; 2, internal standard (carbamazepine); 3, caffeine; 4, paraxanthine. Peak 3 represents 560 ng of caffeine.

etry, peak 4 being first flash-ethylated using a modified method of Brochman-Hansen and Oke [12] to obtain a better peak shape.

The three possible dimethylxanthine derivatives have the same retention time. Whenever one of these is present in significant amounts its identity can be determined using an other eluent [4] or mass spectrometry.

CONCLUSION

The described method is suitable for the routine determination of therapeutic serum levels of caffeine as well as for toxicological purposes.

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Journal of Chromatography, 182 (1980) 125—129
Biomedical Applications

CHROMBIO. 489

Note

Determination of two new metabolites of 1-hexylcarbamoyl-5-fluorouracil in biomedical specimens by high-performance liquid chromatography

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(Received August 13th, 1979)

A recently discovered anticancer agent, 1-hexylcarbamoyl-5-fluorouracil (HCFU) [1], has more favorable therapeutic ratios than its parent compound, 5-fluorouracil (FU), by oral administration [2]. A study of the metabolic fate of HCFU showed that the major metabolites in the mammalian body were FU, 1-(carboxypentylcarbamoyl)-5-fluorouracil (CPEFU), and 1-(carboxypropylcarbamoyl)-5-fluorouracil (CPRFU) [3]. The structural formulae of these compounds are shown in Fig. 1. We have reported the high-performance liquid chromatographic (HPLC) determination of HCFU and its three metabolites in biomedical specimens [4].

In the analysis of sera of patients administered HCFU, we noted in the chromatograms a small, but well-defined peak, which does not arise from endogenous serum components. It is concluded that the peaks can be attributed to two hitherto unknown metabolites, 1-(5′-oxohexylcarbamoyl)-5-fluorouracil (OHCFU) and 1-(5′-hydroxyhexylcarbamoyl)-5-fluorouracil (HHCFU). The present paper is concerned with the HPLC analysis of the two new metabolites of HCFU.

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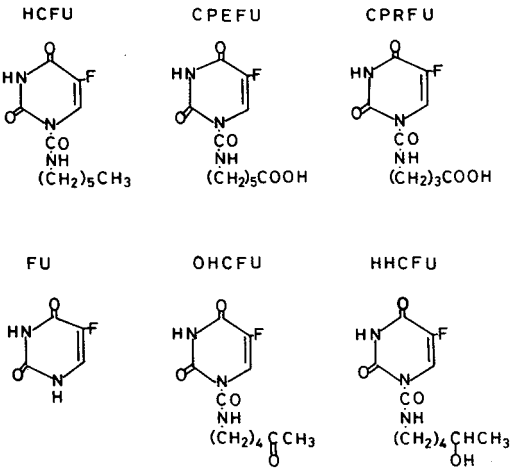

Fig. 1. Structures of HCFU and its metabolites.

EXPERIMENTAL

Materials

OHCFU, HHCFU, CPEFU and CPRFU were synthesized in the Laboratory of Mitsui Pharmaceutical (Tokyo, Japan), and are the generous gift from that company. The methods of synthesis and analytical data will be reported in the near future. Chemicals and the solvents for HPLC were of certified grade and products of Wako (Osaka, Japan).

HPLC instrumentation

A Waters Assoc. liquid chromatograph equipped with a Model 6000 solvent delivery system, a Model U6K universal injector, and a Model 440 UV detector, operated at 254 nm, and a μBondapak C_{18}/Porasil (particle size, 8—10 μm; 300 mm × 3.9 mm I.D.) column were used. The flow-rate of the mobile phase was 1 ml/min, at a pressure of about 1800 p.s.i.

Method

Standard solutions were prepared in analytical grade methanol so as to contain OHCFU and HHCFU at 0.5—10.0 ng/μl, and CPEFU and CPRFU at 1.0—25 ng/μl. Samples were injected into the chromatograph in a volume of exactly 1.0 μl using a 10-μl Hamilton syringe.

Human serum was collected from patients administered HCFU. HCFU-free biomedical samples were obtained either from a healthy adult man or from patients who had not been administered HCFU.

To 1.0 ml serum, 0.1 ml of 1 *N* HCl and 8 ml of ethyl acetate were added and the sample was extracted with vigorous shaking. The organic layer was separated by centrifugation and evaporated to dryness using a water-bath at 30° and a water-pump vacuum. The residue was dissolved in 50—200 μl of methanol for the HPLC analysis.

RESULTS AND DISCUSSION

Fig. 2 is a chromatogram of an extract of serum obtained from a patient administrered HCFU. Water—tetrahydrofuran (65:35) was used as the mobile phase. CPRFU and CPEFU were detected under these conditions [4]. On the chromatogram, an unassigned peak is seen between the peaks of CPRFU and CPEFU. The corresponding peak was absent in HCFU-free sera. The peak was likely to be due to a new metabolite.

The typical metabolic pathways in mammals for an alkyl group in a foreign compound are ω-, ω—1-, and β-oxidation. CPEFU is an ω-oxidation product of HCFU, and CPRFU is a β-oxidation product of CPEFU. An ω—1-oxidation product has not been found among the metabolites of HCFU.

The unassigned peak was suspected to be related to an ω—1-oxidation product. The probable ω—1-oxidation products are OHCFU and HHCFU.

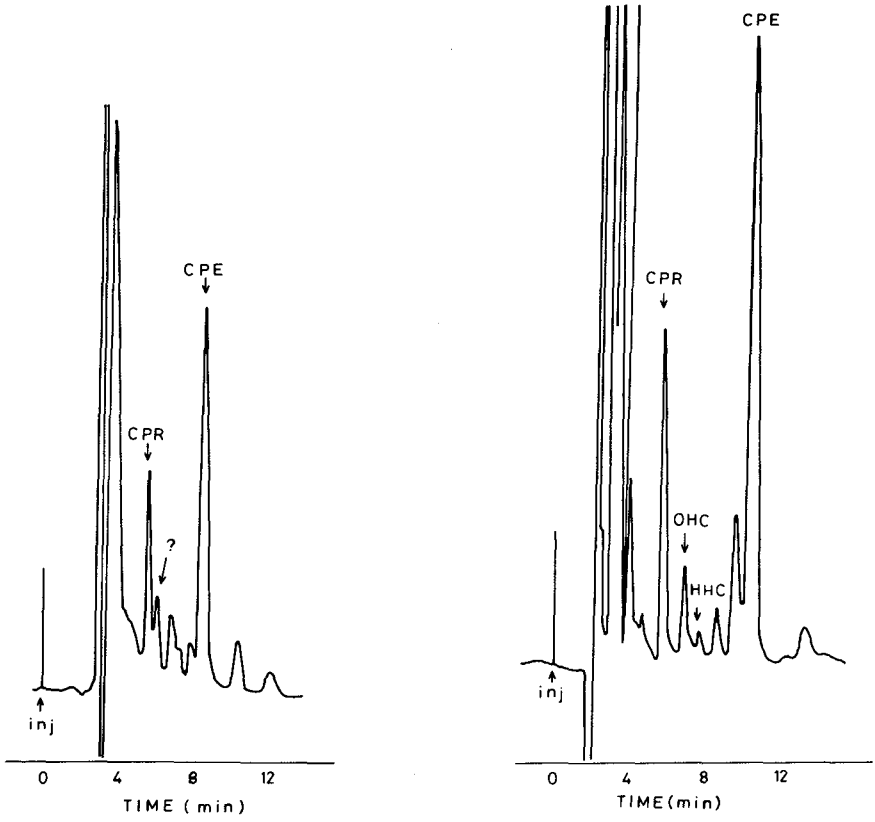

Fig. 2. Chromatogram of an extract of serum collected 3 h after the oral administration of 200 mg of HCFU to an adult man with cancer. The mobile phase is water—tetrahydrofuran (65:35).

Fig. 3. Chromatogram of an extract of serum collected 0.5 h after the oral administration of 200 mg of HCFU to an adult man with cancer. The mobile phase is water—tetrahydrofuran (75:25).

Therefore, OHCFU and HHCFU were synthesized. A methanolic solution of OHCFU, HHCFU, CPRFU and CPEFU was submitted to HPLC. With water—tetrahydrofuran (65:35) as the solvent system, the peaks of OHCFU and HHCFU partially overlapped, and had the same retention time as the unassigned peak of the serum. With water—tetrahydrofuran (75:25) as the solvent system, OHCFU and HHCFU were well separated from each other and from CPRFU and CPEFU. With the latter solvent system, four well-separated peaks with retention times of 5.85, 6.45, 6.75, and 10.15 min were obtained from the serum (Fig. 3). The peaks with retention times of 5.85 and 10.15 are attributed to CPRFU and CPEFU, respectively. Addition of OHCFU to the serum enhanced the peak at 6.45 min, whereas addition of HHCFU intensified the peak at 6.75 min. These results indicate that the peaks with retention times of 6.45 and 6.75 min in the chromatograms of the serum can be attributed to OHCFU and HHCFU, respectively.

Standard methanolic solutions of CPRFU, CPEFU, OHCFU and HHCFU were diluted with methanol to give the desired concentration. An aliquot of each solution was injected into the liquid chromatograph. Standard curves obtained by plotting the peak heights against the amounts of the substances injected were linear in the range 0.5—25 ng; 0.5 ng of each metabolite can be

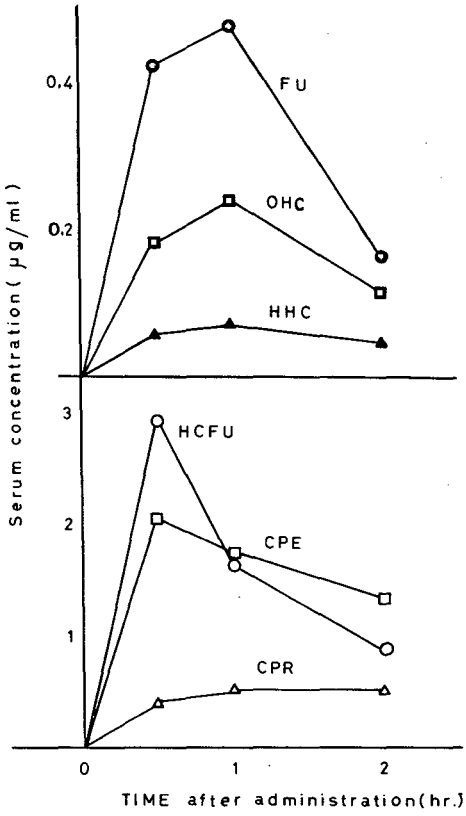

Fig. 4. Concentrations of HCFU and its metabolites in the serum of an adult man with cancer administered 200 mg of HCFU orally.

estimated using the available detector. In the determination of the substances in solutions containing 10 ng, standard deviations were 0.57, 0.55, 0.58, and 0.54 ng for OHCFU, HHCFU, CPRFU, and CPEFU, respectively. Appropriate amounts of these substances were added to HCFU-free serum and these spiked standards were carried through the procedure. Plots of the peak heights against the amounts of spiked metabolites gave straight lines, and comparison of the slopes with those obtained with the standard methanolic solutions afforded the recoveries, which were 101.2 ± 7.7% and 100.0 ± 5.3% for OHCFU and HHCFU, respectively. The recoveries of CPRFU and CPEFU were the same as those reported using the water—tetrahydrofuran (65:35) solvent system [4].

The present method permits the accurate determination of OHCFU, HHCFU, CPRFU and CPEFU in biological specimens in concentrations as low as 50 ng/ml, and is suited for monitoring the drug in therapeutic doses (600 mg/day). Fig. 4 is an example of the time—concentration curves in the serum of a patient administered 200 mg of HCFU orally. OHCFU and HHCFU seem to be minor metabolites and were not detected in the urine of patients administered HCFU.

These pharmacokinetic measurements of HCFU and its metabolites in tumor-bearing patients are continuing. The correlation of therapeutic effects to concentration in body fluids and tissues is under investigation.

ACKNOWLEDGEMENT

This work was supported by a Grant-in-Aid for Cancer Research from the Ministry of Health and Welfare, for which we are grateful.

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Journal of Chromatography, 182 (1980) 130—135
Biomedical Applications

CHROMBIO. 484

Note

Rapid high-performance liquid chromatographic method for the determination of dapsone and monoacetyldapsone in biological fluids

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(First received July 9th, 1979; revised manuscript received October 29th, 1979)

Dapsone (DDS, diaminodiphenylsulfone) is an important drug in the treatment of leprosy (Fig. 1). In humans it is mainly metabolised to monoacetyldapsone (MADDS). The determination of the MADDS:DDS ratio has been found to be

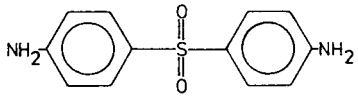

Fig. 1. Chemical structure of dapsone.

useful for the characterisation of the acetylator phenotype [1]. For this purpose, for therapy control and for biopharmaceutical studies, a rapid and reliable determination of DDS and MADDS in biological fluids is needed.

Until the nineteen-seventies blood concentrations were measured with spectrophotometric methods. A well-known colorimetric method for aromatic amines was published in 1939 by Bratton and Marshall [2]. This method is frequently used for the determination of sulfones and many minor modifications of it have been described [3, 4]. Fluorimetric methods have been described since 1968 [5, 6]. They require prior extraction with, for example,

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ethyl acetate or dichloroethane. These methods are seldom specific and are more erratic than modern chromatographic methods. Thin-layer and paper chromatography gave the first impetus in this field [7]. A gas chromatographic method with electron-capture detection has been described by Burchfield and co-workers [8, 9]. The method is laborious since derivatisation appeared to be necessary. Much easier and more quick to perform are the high-performance liquid chromatographic (HPLC) methods. Ion-exchange HPLC methods have been described by Murray and co-workers [10, 11], Orzech et al. [12] and Ribi et al. [13]. Reversed-phase methods have been reported by Mannan et al. [14] and recently by Carr et al. [1]. UV absorption and fluorimetry were used for the detection.

All HPLC methods were preceded by an extraction procedure. In this paper we present a rapid, non-extractive absolute method as a modification of the method of Carr et al. Proteins are removed by precipitation with perchloric acid. The supernatant is neutralised by potassium carbonate. Excess perchloric acid is thus precipitated as potassium perchlorate.

MATERIALS AND METHODS

A 0.5-ml serum sample was pipetted into a tube containing 50 μl of 70% perchloric acid and mixed for 30 sec on a whirl-mixer. Then 50 μl of a saturated potassium carbonate solution and 400 μl of a mixture containing 5% acetic acid, 65% acetonitrile and finally aqueous solutions of standards were added and mixed. The water:acetonitrile ratio of the sample equalled that in the mobile phase. The potassium perchlorate was precipitated by centrifuging for 10 min. An aliquot (100 μl) of the supernatant was injected onto the column with a syringe.

Analyses were performed using a Waters Assoc. (Milford, Mass., U.S.A.) Model M-6000 A pump and Model 440 absorbance detector. A reversed-phase system was used, consisting of a μBondapak C_{18} column (30 cm × 4 mm I.D.) with a particle size of 10 μm (Waters Assoc.). The mobile phase solvent system, acetonitrile—1.5% (v/v) acetic acid (26:74), was delivered at a rate of 2 ml/min at room temperature. Absorbance was monitored at 280 nm. The detector was operated at a sensitivity of 0.05 a.u.f.s. Peak heights were used for quantitation.

The method was compared with a non-extractive internal standard method. As an internal standard monopropionyldapsone (MPD) was used as recommended by Carr et al. [1]. MPD was synthesised, without using dapsone as an intermediate, by reacting 4′-amino-4-nitrodiphenylsulfone with propionylchloride in pyridine and hydrogenation. Before use, purity and identity were checked by HPLC, NMR and infrared spectroscopy. No measurable amounts of dapsone were present. MPD was dissolved in water and added to the serum. Then acetic acid and acetonitrile were added. The MPD concentration in the sample was then 2 μg/ml. Apart from the internal standard the procedure was the same as that described above.

The method was also compared with the extractive, internal standard method described by Carr et al. [1].

Calibration curves

Normal plasma and serum were spiked with known amounts by mixing concentrated standard solutions with serum (5:95) of DDS and MADDS over the range 0.2—5 μg/ml and determined by the three methods described above. To define the standard curves for HPLC measurements, the peak heights, or the ratios of the DDS or MADDS peak heights to the heights of the internal standard peaks, were plotted against the DDS or MADDS concentrations. Regression coefficients and *y*-intercepts could be calculated by linear regression (least-squares method).

RESULTS

A representative chromatogram of spiked serum is shown in Fig. 2. The retention times for DDS, MADDS and MPD were 4.8—4.9, 5.6—5.7 and 9.4—9.5 min, respectively. Results of the calibration curves are given in Table I.

The within-run variation of the non-extractive absolute method could be determined by repetitive injection of samples of 1 and 3 μg/ml and is presented as the variation coefficient in Table II. The between-run variation was determined by injecting, on 30 consecutive days, spiked standard serum samples of 1, 3 and 4 μg/ml (freshly prepared daily). The results are presented in Table III. The within-run variation makes up the error of the method, the

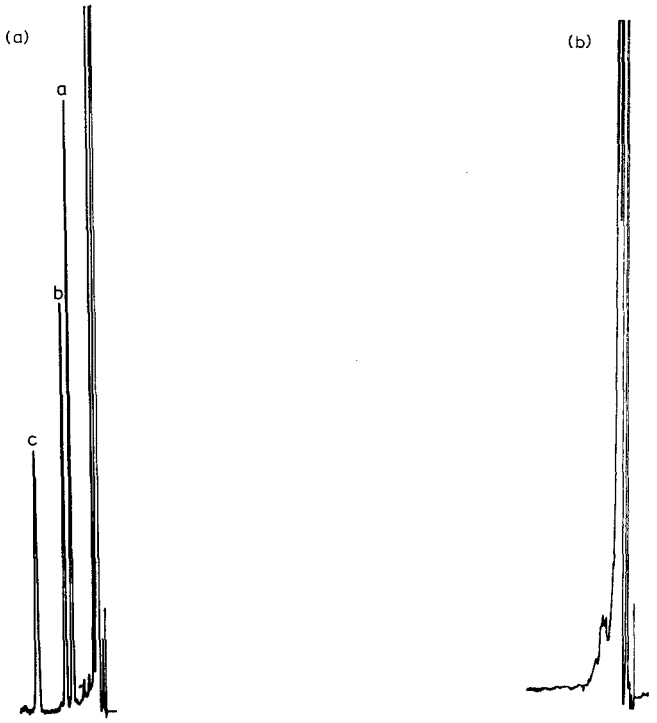

Fig. 2. (a) A representative HPLC chromatogram of human serum containing: a, dapsone (DDS), 4 μg/ml; b, monoacetyldapsone (MADDS), 4 μg/ml; c, internal standard (MPD), 2 μg/ml. (b) A representative blank sample.

TABLE I

COMPARISON OF THE METHODS BY REGRESSION COEFFICIENTS AND *y*-INTERCEPTS

The results represent an average of *m* serum standard curves, obtained from serum or plasma from different volunteers. Each curve was calculated from *n* points, representing different concentrations.

Method*	Regression coefficient (mean ± var. coeff.)	*y*-Intercept (mean ± var. coeff.)	Correlation coefficient (mean ± S.D.)		
DDS:					
n.e. abs.	35.4 (± 3.1%)	7.0 (± 21.4%)	0.999 ± 0.000	n = 5	
				m= 4	$P<0.01$
	35.3	7.3	0.998	n =20	
				m= 1	$P<0.01$
n.e. + i.s.	0.559 (±3.6%)	0.121 (± 22.3%)	0.998 ± 0.001	n = 5	
				m= 4	$P<0.01$
	0.573	0.055	0.976	n =20	
				m= 1	$P<0.01$
e + i.s.	0.501 (± 7.4%)	0.425 (± 33.4%)	0.995 ± 0.004	n = 6	
				m= 4	$P<0.01$
	0.517	0.396	0.983	n =24	
				m= 1	$P<0.01$
MADDS:					
n.e. abs.	26.5 (± 5.3%)	0.82 (± 89.0%)	0.999 ± 0.000	n = 4	
				m= 4	$P<0.01$
	26.3	0.8	0.996	n =16	
				m= 1	$P<0.01$
n.e. + i.s.	0.425 (± 1.7%)	0.019 (± 94.7%)	0.999 ± 0.001	n = 4	
				m= 4	$P<0.01$
	0.423	0.215	0.999	n =16	
				m= 1	$P<0.01$
e. + i.s.	0.391 (± 3.8%)	—0.000	0.997 ± 0.003	n = 4	
				m= 6	$P<0.01$
	0.396	—0.001	0.997	n =25	
				m= 1	$P<0.01$

*n.e. abs. = non-extractive absolute method; n.e. + i.s. = non-extractive method with internal standard; e. + i.s. extractive method with internal standard.

TABLE II

COMPARISON OF THE METHODS BY THE WITHIN-RUN VARIATION

n = 10. Abbreviations as in Table I.

Conc. (μg/ml)	n.e. abs.		n.e. + i.s.		e. + i.s.	
	DDS	MADDS	DDS	MADDS	DDS	MADDS
1	4.8%	8.8%	3.5%	7.3%	17.3%	2.2%
3	3.4%	5.8%	3.4%	5.7%	3.7%	2.7%

TABLE III

BETWEEN-RUN VARIATION OF THE NON-EXTRACTIVE ABSOLUTE METHOD

(μg/ml)	DDS	MADDS	*n*
1	12.0%	11.3%	61
3	7.8%	10.2%	26
4	10.6%	8.4%	25

between-run variation makes up both the error of the method and the "spike error".

The recovery of the extraction procedure could be calculated from a plot of the absolute method against an extractive method with external standard, being the regression coefficient. The recovery with diethyl ether was essentially complete (r.c. = 1.03). The standard deviation of the regression line was S_r = 0.36 and the standard deviation of the regression coefficient was S_a = 0.12. It can be calculated that at a level of $P \leqslant 0.05$ the recovery of 100% was significantly different from recovery of 85% and lower.

DISCUSSION

This HPLC method is easy and quick to perform; it involves no extraction and can be successfully performed with 0.5 ml of serum, or even smaller samples.

We could demonstrate that the results of both DDS and MADDS determinations with the three methods are well-fitted by a straight line ($P<0.01$) over the range 0.2—5 μg/ml. The extraction method with internal standard has been published by Carr et al. [1]. The authors did not mention a *y*-intercept. We found a large *y*-intercept in all of the calibration curves using this method, so we cannot confirm their results. The mean *y*-intercept was significantly different from zero ($P<0.01$) and significantly different (larger) from the mean *y*-intercept as calculated for the non-extractive method with internal standard ($P<0.01$). Thus the advantages of the non-extractive DDS determination methods are a smaller *y*-intercept, a better precision and time gained by deleting the extraction procedure.

There is no difference in the precision and *y*-intercept between the non-extractive methods with or without internal standard as compared by the variation coefficients and *y*-intercept versus regression coefficient ratio.

The variation coefficient of the regression coefficient makes up the error of the method and the "spike error". The within-run variation of the non-extractive DDS determination methods was smaller than that of the extraction method (Table II). The difference between the non-extractive internal standard method and the absolute method was not significant. Advantages of the absolute method are the performance time (a simpler calculation), and the fact that no interference can occur with an internal standard peak.

The MADDS regression lines are quite accurate. The *y*-intercepts are not significantly different from zero ($P<0.01$). The precision of the three methods is good and they are not significantly different from each other.

Thus, from comparing the non-extractive methods and the extractive method, it can be concluded that the non-extractive methods are to be preferred for the determination of DDS in terms of precision, accuracy, chance of interfering peaks and performance time. For the determination of MADDS this can only be said with respect to the chance of interfering peaks and the performance time. The gain in time for twenty samples was about 3 h.

ACKNOWLEDGEMENTS

The authors are grateful to Dr. T. Eggelte for preparing monopropionyldapsone, to Mr. A.M.A. Prins for his technical assistance, and to Mrs. B.J.M. Eckmann for her help in typing the manuscript.

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2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 H. C. S. Wood and R. Wrigglesworth, in S. Coffey (Editor), *Rodd's Chemistry of Carbon Compounds, Vol. IV, Heterocyclic Compounds*, Part B, Elsevier, Amsterdam, Oxford, New York, 2nd ed., 1977, Ch. 11, p. 201.
4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

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ELECTROPHORESIS

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