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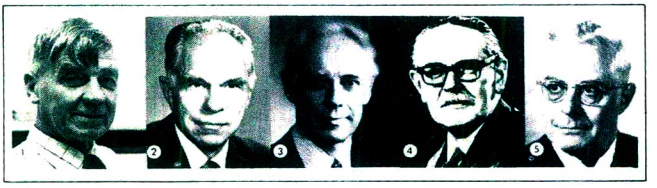
75 Years of Chromatography

A Historical Dialogue

L. S. ETTRE *and* A. ZLATKIS (Editors).

Journal of Chromatography Library - Volume 17

On the occasion of the 75th anniversary of the invention of chromatography, this book compiles the personal stories of 59 pioneers of the various chromatographic techniques (including five Nobel Prize laureates). In their contributions to this volume, these pioneers review the events which influenced them to enter the field; explain the background of their inventions; summarize their activities and results during their professional lives; and discuss their interactions with other scientists and other disciplines.



This book is more than a nostalgic recollection of the past for those who have been in chromatography for some time. It also provides, for the younger generation of chromatographers, a unique record of how present-day knowledge was accumulated.
The final chapter is devoted to "Those who are no longer with us".

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Biomedical Applications

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ISOLATION, IDENTIFICATION AND QUANTITATION OF URINARY ORGANIC ACIDS

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(First received April 25th, 1978; revised manuscript received October 11th, 1978)

SUMMARY

An application of the HISLIB program for the comparison of gas chromatographic—mass spectrometric profiles of urinary organic acids isolated by extraction and ion-exchange methods is described. Ion-exchange methods are clearly superior to solvent extraction in terms of the variety of compounds isolated. However, the former method has practical difficulties which make solvent extraction more attractive for rapid analyses. For the compounds isolated by both methods, the precision of analysis is similar, with standard deviations of relative concentration in the range 10—30% for most compounds.

INTRODUCTION

Experience has shown that only a few inborn errors of metabolism can be diagnosed by clinical criteria alone, and that detection generally depends upon screening patients with suspicious symptoms for a wide range of metabolites. The application of gas chromatography (GC) and combined gas chromatography—mass spectrometry (GC—MS) for the profiling of classes of metabolites has led to the identification of several new disorders [1]. The large numbers of components often seen in such mixtures have led to the application of new techniques such as capillary gas chromatography [2, 3] and advanced computer methods [4—7].

The literature on the use of GC—MS procedures in the analysis of organic acids has been replete with controversy over the choice of isolation method. Over the last several years we have routinely isolated organic acids from urine by acidification followed by solvent extraction. A recent publication [8] directed our attention to the purported superiority of anion-exchange isolation methods. Preliminary experiments with this method were not encouraging [5],

and we found the method considerably more time-consuming. Furthermore, there has been some controversy about the best methods for ion exchange isolation of organic acids [9, 10]. It was the purpose of this study to utilize the data processing techniques described recently [5] to compare critically the two isolation methods. During the course of this investigation we examined a third method for isolation of organic acids and have reported that method in a preliminary communication [11].

EXPERIMENTAL

We have previously described the chromatographs, mass spectrometer and data analysis equipment which are routinely used for our analyses [5]. Standard samples of organic acids were obtained from a variety of commercial sources including Sigma (St. Louis, Mo., U.S.A.), Aldrich (Milwaukee, Wisc., U.S.A.) and Eastman Organic (Rochester, N.Y., U.S.A.). Further samples were obtained from research groups in the Stanford University Department of Chemistry. All solvents were of the highest purity available commercially.

Urine extraction methods

Urine from a 30-year-old male was used to test the different isolation methods. A first morning sample was collected and kept frozen until use. The sample had a creatinine level of 224 mg/dl.

A. Manual extraction. To 3 ml of freshly thawed urine is added the internal standard, 3-chlorophenylacetic acid (0.212 mg), followed by hydroxylamine hydrochloride (30 mg). The pH is adjusted to 12 with 2 *N* NaOH. The sample is heated at 60° for 30 min to form the oximes of keto acids. After cooling, the pH is adjusted to 1 with concentrated HCl, and the acids are extracted with three 6-ml portions of diethyl ether—ethyl acetate (1:1). The combined organic extracts are dried (Na_2SO_4) and evaporated to dryness. The extract is then transferred to a vial with ethyl acetate—methanol (1:1). The solvent is removed with a stream of dry nitrogen, and the sample is sealed with a PTFE-lined cap.

B. Ion exchange. The ion-exchange method is based on the method of Thompson and Markey [8] with some minor variations [12]. Thus, urine (3 ml) and 3-chlorophenylacetic acid (0.212 mg) are treated with barium hydroxide solution (0.1 *M*, 3.0 ml), stirred and centrifuged for 15 sec. The supernatant is removed, treated with hydroxylamine hydrochloride (30 mg) and heated at 60° for 30 min. The cooled solution is adjusted to pH 7—8 with dilute HCl and applied to a short column (5.0 cm × 1.0 cm) of Sephadex A-25. The anion exchange resin was prepared as previously described [8]. Neutral and basic components are eluted with 50 ml distilled water and the acids are eluted with 40 ml of pyridinium acetate buffer (1.5 *M*). The extract is dried by lyophilization at 10—25 mm Hg (normally for 10—15 h) and transferred to a vial with ethyl acetate—methanol as above. We have occasionally found this extract to be highly hygroscopic, and in such cases a further drying period is necessary.

Preparation of aldonic acids.

Certain of the aldonic acids and lactones, which are important components of the ion exchange extract, were prepared by bromine oxidation of the corre-

sponding aldose [13]. The sugar (1 mg) was dissolved in water and treated with bromine (2 drops) and saturated $NaHCO_3$ solution (2 drops). After 30 min at room temperature the solution was evaporated and derivatized. This procedure yielded a mixture of the free aldonic acid and the corresponding 1,4- and 1,5-lactones.

Derivatization and GC

The extract from A, B, or a standard acid sample is derivatized with bis(trimethylsilyl)trifluoroacetamide (100 μl) at 60° for 30 min. The derivatized sample (about 1 μl) and a mixture of hydrocarbon standards (C12, C18, C24, about 1 μg each) for determination of relative retention indices [5] are coinjected onto the GC column at 70—80°. As previously described we use columns of 10% OV-17 on 100—120 mesh Gas-Chrom Q [5]. Starting temperatures are chosen to allow for quantitation of lactic acid. After a wait of 4 min, temperature programming is begun at 4°/min, and the chromatographic effluent is allowed to enter the mass spectrometer. A total of 600 spectra are recorded to a final temperature of 280°.

METHODS

Data analysis

The procedures for analyzing the raw data from our GC—MS computer system are basically the sequence of programs, CLEANUP, TIMSEK, SEARCH and HISLIB, described previously [5]. The following is a brief description of the function of each program:

(i) CLEANUP. The functions of the CLEANUP program include: (1) detection of spectra of components in the raw GC—MS data; (2) resolution of overlapping components; (3) removal of background from column or septum bleed. The current program differs slightly from the one described previously [14] in the following ways: (1) the chemist can specify criteria for component detection; (2) provision is made for mass spectrometric scans of the mass range in either direction and for non-zero dead time between scans; (3) improved multiplet resolution. The output of CLEANUP is a set of representative mass spectra for detected components.

(ii) TIMSEK. The TIMSEK program calculates relative retention indices (RRI's) and relative concentrations of each component detected by CLEANUP. As previously described [5] this requires a hydrocarbon calibration curve for each GC column and each starting temperature, and use of internal standards for quantitation.

(iii) SEARCH. Our mass spectral library search program attempts identification of mixture components by comparison of the unknown mass spectrum with a library of known mass spectra. The acid library began with the TMS components (about 1300) of the Markey library [15]. From this we selected 400 spectra of the acids and neutrals likely to occur in urine extracts. We have added about 70 new spectra to the library from commercially available standards and from literature spectra. A total of 350 of the library spectra have RRI values associated with them. As many of the library spectra are duplicates, there are at present 220 independent RRI values in the library. The SEARCH

program considers only those library components within a retention index window of 20 RRI units. We have also begun to save the spectra of unknown urinary components in a separate library. These spectra are searched during the analysis of new data giving an indication of how often the unknowns are seen.

(iv) HISLIB. The HISLIB program accumulates GC—MS profiles (each profile resulting from the operation of the preceding three programs), while compiling statistics on the occurrence of components (based on both RRI and spectral matching) and their relative concentrations [5]. This program was utilized to compile the data on reproducibility of various methods of extraction of urinary organic acids.

Compilation and use of RRIs

Our procedures for analysis of GC—MS data and comparison of GC—MS profiles do not require the identification of the structure of each component. However, meaningful chemical and biochemical interpretation of results obviously depends on the structural identity of compounds. The HISLIB procedure does require RRIs [16] in conjunction with mass spectra in order to perform comparison of profiles [5]. In addition, the similarity of mass spectra of certain polytrimethylsilyl compounds [17] makes structural identification difficult on the basis of MS data alone. The utility of RRIs in assignment of structure in such cases has been demonstrated by several workers [4, 5, 18]. Because of these considerations we have compiled an extensive list of RRIs of known compounds in order to facilitate component identification and interpretation of results.

At present our list of the RRI values for organic acid TMS derivatives (available from the authors) contains 215 entries. The majority of these are based on authentic samples of the compounds. However, some of the values were obtained from urine components and identified by comparison with library spectra [15, 19—25]. Other values have been adapted from the literature [26—29]. Petersson [27] reports retention data on three liquid phases for over 150 organic acids as TMS derivatives. This listing is based on the determination of retention times and RRI values under isothermal conditions with only one retention time standard present. We had independently determined the RRI values of over 50 of the compounds listed by Petersson. A comparison of our data can be seen in Fig. 1. Interestingly, the two sets of results coincide at an RRI value slightly above 1800, where Petersson's single retention time standard, glucitol-hexa-TMS, elutes. We calculated the least-squares best fit line to the data and used the equation to correct Petersson's RRI values for our use. The differences between our values and the corrected Petersson values for these 50 compounds represent the likely errors we will encounter in using corrected Petersson values with our identification programs. Among the 50 compounds we shared, the corrected values were within 20 RRI units for all but two (oxalic acid and erythrono-1,4-lactone). The standard deviation of the differences between the corrected Petersson RRIs and ours was 7.4 RRI units, while the average absolute difference was 5.5.

The listing of Butts [26] contains retention indices for over 200 biological compounds as TMS derivatives on both OV-1 and OV-17 stationary phases,

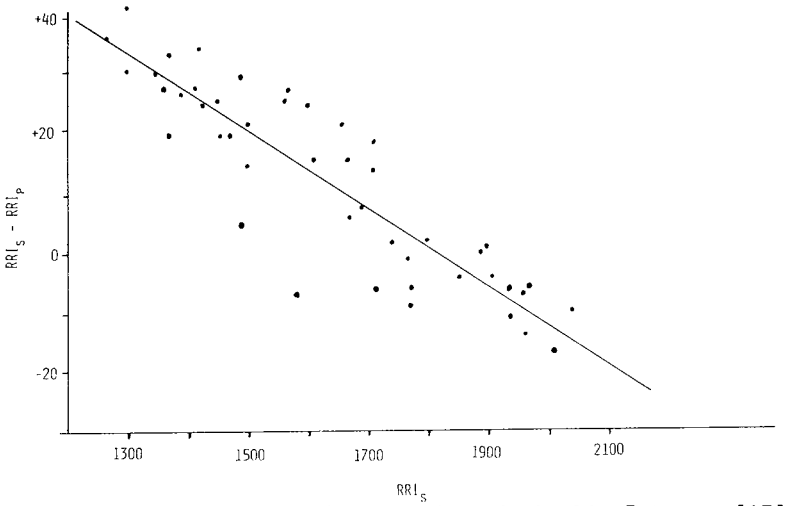

Fig. 1. Comparison between the RRI values obtained by Petersson [27] and our work for selected organic acid TMS derivatives, plotted as the difference between the values against our value. Least squares fit, $RRI_s = 0.938\ RRI_p + 112$.

measured by co-chromatography with a mixture of alkanes and reported as methylene units. Methylene units differ from our RRI values by a factor of 100. A comparison of 50 of the values reported by Butts with those obtained in our laboratory indicate an average difference of 3.0 RRI units with a standard deviation of 7.6. Thus there are no large systematic differences between the two data sets. The average absolute difference between the two sets of RRIs is 6.4 units, which is not much greater than the measured standard deviation of our own values over a long period (see below). Two of the Butts values are significantly different from ours. He reports the retention index of indoleacetic acid as 2093 while we measured 2187 for the di-TMS derivative of indoleacetic acid (Mamer et al. [30] mention the formation of both mono- and di-TMS derivatives of indoleacetic acid). N-Acetylphenylalanine, the mono-TMS derivative of which we recorded at 2051, was reported by Butts at 1933 (this may be a di-TMS derivative).

Over the last 18 months we have used many different GC columns including different batches of OV-17. Changes in operating conditions are taken into account by periodically obtaining a new calibration curve for each column. The standard deviations for the RRI values of 18 common organic acids determined during this period were between 1.8 and 8.4 with an average of 4.1. These standard deviations are based on 25 separate analyses of each component. The two most variable components are lactic (σ, 6.8) and hippuric (σ, 8.4) acids. The former is the first detected component in most runs and is the most sensitive to small changes in GC starting temperature [5]. The latter component often dominates acid profiles and is commonly present in sufficient quantities to overload the GC column or the mass spectrometer, leading to peak broadening and poor RRI reproducibility.

Isolation, identification and profile comparison

We carried out five replicate analyses with the isolation methods discussed in the Experimental section. Aliquots of the same urine were used in all analyses. Each organic acid fraction so obtained was analyzed by GC—MS and the set of computer programs described above. Structural assignments were made on the basis of comparison of RRI values and mass spectra. The set of five GC—MS profiles for a given isolation method was collected in an historical library using HISLIB. The resulting library contains data on the relative concentrations (and precision thereof) for all components detected, whether or not structural identity has been established.

RESULTS AND DISCUSSION

Extraction

The results for the isolation of organic acids by solvent extraction (Table I and Fig. 2, top) indicate satisfactory quantitation of the small aliphatic molecules (e.g. lactic, glycolic) and the aromatic acids (e.g. the hydroxyphenyl-

TABLE I

QUANTITATIVE ANALYSIS OF ORGANIC ACIDS ISOLATED FROM URINE BY TWO METHODS

Quantitative results are expressed in relative concentration units with the internal standard, 3-chlorophenylacetic acid, concentration set at 100. The initial concentration of the standard was 71 μg/ml urine. The relative concentration values have not been corrected for differential extraction or detection coefficients. The coefficient of variation (C.V.) represents five separate analyses.

	Acid*	RRI**	Extraction		Ion exchange	
			Rel. concn.	C.V.	Rel. concn.	C.V.
1	Lactic	1094	78	14	50	9
2		1108	—	—	21	19
3	Glycolic	1132	119	14	100	13
4	Phenol	1133	—	—	34	26
5		1177	—	—	18	28
6	3-Hydroxyisobutyric	1205	51	18	34	11
7	Pyruvic	1222	41	15	26	6
8	Cresol	1239	18	23	91	14
9	3-Hydroxyisovaleric	1243	18	3	—	—
10	?-Hydroxypyridine	1263	—	—	29	12
11		1299	—	—	56	65
12	Phosphate	1353	—	—	346	20
13	Urea	1366	491	35	—	—
14	Glyceric/4-deoxyerythronic	1368	—	—	87	40
15	4-Deoxythreonic/catechol	1383	—	—	170	7
16		1397	43	61	45	27
17	3-Deoxytetronic	1449	36	18	110	21
18	2-Deoxytetronic	1465	84	12	250	11

TABLE I (*continued*)

	Acid*	RRI**	Extraction		Ion exchange	
			Rel. concn.	C.V.	Rel. concn.	C.V.
19		1547	33	30	99	11
20	Erythronic	1557	—	—	914	20
21	4-Hydroxycyclohexane-carboxylic	1565	70	11	44	19
22	Threonic	1587	—	—	222	17
23	Adipic	1602	24	18	—	—
24		1648	—	—	18	7
25	2-Deoxyerythropentonic	1663	—	—	165	22
26	5-Hydroxymethylfuroic	1689	305	16	354	6
27	1,4-Arabinolactone	1710	—	—	61	36
28	3-Hydroxyphenylacetic	1733	82	10	116	25
29	1,4-Xylonolactone	1744	—	—	37	20
30	4-Hydroxyphenylacetic	1765	186	6	257	16
31		1839	—	—	1004	34
32	3-Hydroxyphenylpropionic	1846	32	27	—	—
33	Aconitic	1854	65	34	—	—
34	Citric	1886	174	45	—	—
35	Furoylglycine	1891	72	36	48	17
36	3-(3-Hydroxyphenyl) hydracrylic	1935	684	15	1675	12
37	1,4 + 1,5-Gluconolactone	1949	—	—	77	28
38		1966	—	—	84	13
39	Hippuric-di-TMS	1989	650	83	—	—
40		2007	—	—	701	26
41	3,4-Dihydroxyphenyl propionic	2046	—	—	14	15
42	Hippuric-mono-TMS	2111	1788	41	2900	20
43	3-Hydroxyhippuric	2379	841	24	—	—

*An empty space indicates an unknown or mixture of unknowns.

**Relative retention index of the peak.

acetics, 5-hydroxyfuroic acid). Tricarboxylic acids (e.g. aconitic, citric) are isolated but the reproducibility is poor. Hippuric acid tends to give a mixture of the mono- and di-TMS derivatives after solvent extraction, apparently due to variable dryness of the samples prior to derivatization. During the course of this work we developed an improved solvent extraction technique based on the adsorption of urine onto a solid-phase cellulose resin and elution of the hydrophobic acids and neutrals with organic solvents [11]. This technique is qualitatively comparable to the manual extraction method in terms of the type of compound isolated but yields better results in terms of recovery and precision.

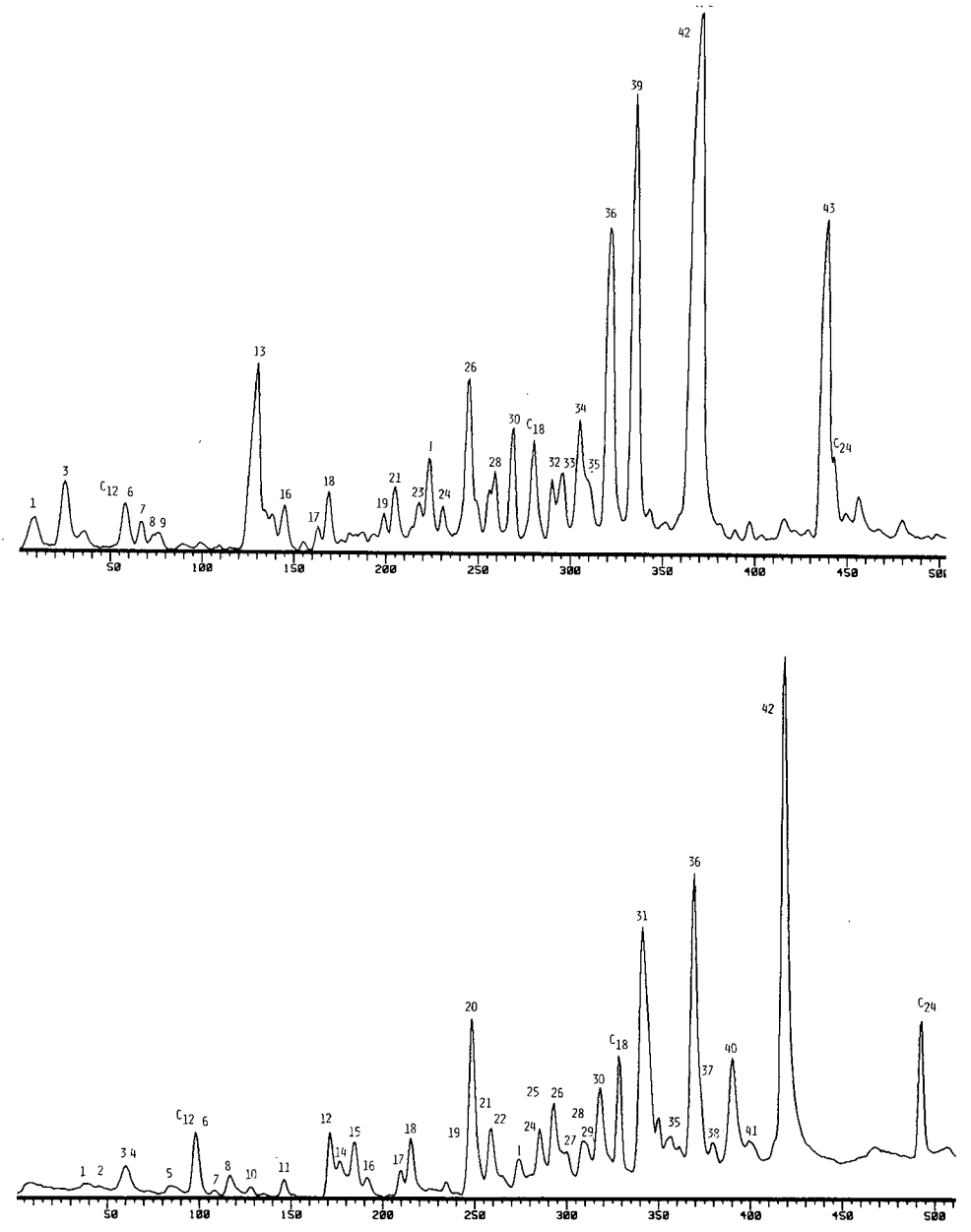

Fig. 2. Top: total ion current trace of the TMS derivatives isolated by manual extraction. Numbers refer to components in Table I. I is the internal standard, 3-chlorophenylacetic acid. Bottom: total ion current trace of the TMS derivatives isolated by ion exchange.

Ion exchange

In our preliminary work with the anion exchange method [5] we had difficulty in obtaining reproducible results for the isolation of organic acids using the procedure of Thompson and Markey [8]. Based on a suggestion of Gates et al. [12], the volume of eluting solvent used in the procedure was increased from 18 ml to 40 ml. With this change the ion exchange method yields reproducible isolation of the majority of the organic acids (Table I and Fig. 2, bottom). This is the only method that isolates carbohydrate derived acids such as glyceric acid. Ion exchange also achieves satisfactory reproducibility for the aliphatic and aromatic acids. Poor results are obtained for the tricarboxylic acids such as citric as these are co-precipitated by the initial barium treatment. A further complication of the ion exchange method is the tendency for aldonic acids to lactonize partially during isolation. For example, the ion-exchange-isolated acids from a child with galactosemia contained a major component identified as galatono-1,4-lactone, but significant quantities of galactono-1,5-lactone and galactonic acid were also observed [31]. Other low level components are not detected in the ion exchange fraction, being obscured beneath the dominant polar acids. We have experienced occassional difficulty in obtaining the ion-exchange-isolated acid fraction in a sufficiently dry state for derivatization. Furthermore, the overnight lyophilization period required to remove 40 ml of water makes this method by far the most time consuming of the methods which we have investigated.

CONCLUSIONS

We have found the use of relative retention indices to be of immense value in the computer assisted identification of organic acids. When measured carefully with co-injected hydrocarbon standards, the RRI values on a particular GC stationary phase are reproducible both with changing conditions in one laboratory and among different laboratories. Furthermore, values determined under less stringent conditions [27] can sometimes be utilized. With the increasing interest in metabolic profiling of body fluids, there is a continuing need for lists of normal components and data repositories [32]. The regular reporting of RRI values of biochemical compounds on OV-17 and other stationary phases along with their mass spectra will be of service to researchers whether established in this field or just entering it.

One of the more critical needs in the evaluation of the diagnostic uses of metabolic profiling is the definition of normal metabolites and their excretion levels [33]. Before the question of normal values can be meaningfully dealt with, methods of isolation and identification must be evaluated as to reproducibility and suite of compounds quantitated. At present none of the available methods is capable of quantitating all organic acids. The ion-exchange method, though clearly more comprehensive than solvent extraction, is limited by the time involved in its application. At present the isolation method of choice will depend on the class of metabolites of interest. For studies with aromatic acids (e.g. catecholamine metabolism), fatty acids or tricarboxylic acid cycle intermediates we recommend solvent extraction techniques [11]. The ion exchange method will continue to be necessary for polar acids. Possible

improvements in the ion exchange method which we hope to pursue are a resolution of the phosphate—citrate problem and a way to avoid the occurrence of both free acid and lactone forms of the aldonic acids. The literature contains some possible solutions to the latter problem, but no application to a complex biological extract [34, 35].

A recent publication from the Michigan State group [12] has addressed several questions on the utility of the data processing techniques which are described in this and previous reports from our laboratory [5, 14]. We have expressed our disagreement with several points raised in the paper of Gates et al. in a separate letter [36], and at this time wish to emphasize an important aspect of our techniques. Our forward library search methods do not require the accumulation of a large data base prior to the analysis of a new compound class as does the MSSMET program [12]. We have successfully applied the methods described here to amino acids, sugars and methyl esters from body fluids [31, 37] and to the analysis of polynuclear hydrocarbons from carbon blacks [38]. Thus we can detect and quantitate (relative concentrations, with absolute values requiring response factors) unknown or previously undetected compounds in these mixtures *in addition* to those observed previously.

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ANALYSIS OF FATTY ACIDS FROM HUMAN LIPIDS BY GAS CHROMATOGRAPHY

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SUMMARY

A rapid, quantitative method is described for the analysis of fatty acids from human lipids, namely serum lipids and lipids from adipose tissue biopsies. The method includes extraction of serum lipids with chloroform—methanol, hydrolysis with tetramethylammonium hydroxide, methylation with methyl iodide and N,N-dimethylformamide and gas chromatographic analysis on a Supelcoport SP-2330 column. Fat biopsies are analysed without extraction. Optimal hydrolysis conditions have been investigated.

INTRODUCTION

Many procedures have been described for the quantitative analysis of fatty acids [1]. All these procedures are time-consuming with regard to extraction, hydrolysis and esterification for gas chromatography (GC). Because of the interest at our hospital in fatty acids of adipose tissue from diabetic children and of serum lipids from children with cystic fibrosis and also from adults, as a part of a population screening programme, we searched for more rapid procedures. Greeley [2] described a new method for alkylation of acidic substances for GC and West [3] modified this method to prepare methyl esters from glycerides, alkyl paint resins and ester plasticizers. We tried this method on our samples and modified it to obtain rapid hydrolysis and methylation of the lipids under mild conditions. Combined with a simple extraction procedure and an appropriate GC procedure, an efficient analysis of fatty acids was achieved for clinical chemical purposes.

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MATERIALS AND METHODS

Chemicals

All chemicals were of analytical grade and were purchased from Merck (Darmstadt, G.F.R.) except the following chemicals: fatty acids and methyl esters of the fatty acids were from Applied Science Labs., (State College, Pa., U.S.A.), tetramethylammonium hydroxide, 24% in methanol, was from Fluka (Buchs, Switzerland), trilinolein was from ICN Pharmaceuticals (Plainview, N.Y., U.S.A.), and 10% SP-2330 on Chromosorb W AW (100—120 mesh) was from Supelco (Bellefonte, Pa., U.S.A.). High-performance thin-layer chromatography plates, silica gel 60, 10 × 20 cm, were from Merck.

Procedures

Standard concentrations of fatty acids and their methyl esters were made by dissolving 15—20 mg of each lipid in 100 ml of chloroform. The following fatty acids and their methyl esters were used: myristic acid (C_{14}), pentadecanoic acid (C_{15}), palmitic acid ($C_{16:0}$), palmitoleic acid ($C_{16:1}$), stearic acid ($C_{18:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$) and arachidonic acid ($C_{20:4}$). Pentadecanoic acid, 130 mg dissolved in 100 ml of chloroform, was used as an internal standard.

To 0.5 ml serum, 0.2 ml of internal standard solution was added in a reagent tube. For the extraction of serum lipids and the free fatty acids, 1.5 ml of water, acidified with concentrated HCl to pH 2, and 5 ml of chloroform—methanol (7:3, v/v) were added to the mixture of serum and internal standard. The tubes were shaken twice for about 30 sec on a vortex mixer. After centrifugation for 5 min at 700 *g*, the chloroform layer was removed and the extraction was repeated with 3 ml of chloroform—methanol. Both the chloroform layers were combined. The chloroform extract was evaporated under a stream of nitrogen at 40° to complete dryness.

For the saponification of the lipids 0.1 ml of tetramethylammonium hydroxide solution and 0.1 ml of methanol were added. The tubes were tightly capped and were allowed to stand for 10 min at 70° in a heating block. After cooling to room temperature the methyl esters were prepared by adding 1 ml of N,N-dimethylformamide and 0.1 ml of methyl iodide. The tubes were shaken and the precipitate was removed by centrifugation. The supernatant was ready for injection into the gas chromatograph. Fat biopsies were weighed and then saponified and methylated as described above.

The gas chromatograph used was a Packard-Becker Model 428 equipped with flame ionization detectors. A 5-μl aliquot of the sample was injected on a 2-m glass column (2 mm I.D.) packed with 10% SP-2330 on Chromosorb W AW, 100—120 mesh. The carrier gas was nitrogen, flow-rate 20 ml/min. The injection port temperature was 220° and the detector temperature 250°. The oven temperature was programmed to run for 5 min isothermally at 130°, followed by a temperature rise of 5°/min to 220°.

Methyl ester standards in chloroform of all the acids were injected before and after each series of sample analyses. When differences of less than 5% in the peak heights of all the fatty acids, relative to the internal standard (I.S.), were observed between the first and the last standard run, the first standard run was

used for calculating the amounts of the individual fatty acids (FA) in terms of unmethylated acids. Calculations were made according to the equation

$$\mu g \text{ FA per sample} = \frac{\text{peak height FA} \times \mu g \text{ I.S.}}{\text{peak height I.S.}} \times \text{factor}$$

where factor (standard) is

$$\frac{\mu g \text{ FA} \times \text{peak height I.S.}}{\text{peak height FA} \times \mu g \text{ I.S.}}$$

Thin-layer chromatography was performed according to the method of Van Gent [4] using high-performance silica-gel plates with consecutive development in chloroform and hexane—chloroform (3:1). The slides were sprayed with the charring agent, 10% sulphuric acid in acetone—water (1:1).

NMR spectra were made on a Brucker spectrometer Model HX-360 (360 MHz). Further details are given in the figures. Mass spectra were made on a Varian gas chromatograph—mass spectrometer combination Model MAT 112, on a 2-m column (1.2 mm I.D.) packed with 10% SP-2330 on Chromosorb W AW, 100—120 mesh. The carrier gas was helium, flow-rate 9 ml/min. The oven temperature was 190°, separator temperature 250°, injection port temperature 250° and the source temperature 200°. The ionization energy was 70 eV and the scan speed 200 a.m.u./sec.

Methylation with diazomethane as a reagent was performed according to the procedure described by Schenk and Gellerman [5].

RESULTS

The methylation procedure was checked by methylating a fatty-acid mixture of known composition in chloroform (see Table I). Of this mixture 1 ml was evaporated under a stream of nitrogen at 40°. To the dry fatty acids, all the saponification—methylation reagents were added as described above, omitting heating for saponification. The 5 μl of the sample were injected, followed by 5 μl of the standard mixture of methyl esters of the fatty acids. To see whether the methylation was complete the amount of each acid, including pentadecanoic acid, was calculated, considering pentadecanoic acid not as an internal standard. The methylation was also compared to the procedure using diazomethane as reagent. As can be seen in Table I, the methylation is almost complete for all fatty acids, and gave results comparable with the diazomethane procedure.

The original method of West [3] described the saponification at 100°. Because loss of the polyunsaturated fatty acids is possible at this temperature, we performed the same procedure with the fatty acid mixture at different saponification temperatures to see whether temperature effects were present. The results are given in Table II. Recoveries of both polyunsaturated acids $C_{18:2}$ and $C_{20:4}$ are temperature dependent. Performing the procedure with linoleic acid at high saponification temperature resulted in the appearance of two new peaks in the gas chromatograms immediately after the partly disappeared linoleic acid peak. Mass spectrometric analysis of these peaks revealed that they were due to compounds with the structure and molecular weight of methyl lino-

TABLE I

METHYLATION OF A STANDARD MIXTURE OF FATTY ACIDS WITH THE REPORTED PROCEDURE AND WITH DIAZOMETHANE AS METHYLATING REAGENT

Figures are the mean recovery values for 24 estimates (duplicate injections of three samples on four different days), expressed in μg per sample and as percentages of the original standard values. Recoveries were calculated by dividing average peak heights of the methylated free fatty-acid standard by those of the average peak heights of methyl ester standard.

	C_{14}	C_{15}	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$
Standard mixture								
Amount present (μg)	160	199	161	195	168	161	163	169
Reported procedure								
Amount recovered (μg)	161	193	176	203	167	164	164	154
Percentage	101	97	109	104	99	102	101	91
Diazomethane								
Amount recovered (μg)	150	179	166	196	161	158	165	160
Percentage	94	90	103	101	96	98	101	95

TABLE II

EFFECT OF DIFFERENT SAPONIFICATION TEMPERATURES ON THE FATTY-ACID STANDARD MIXTURE

Figures are the mean values for 6 estimates, expressed in μg per sample and as percentages (in parentheses) of the original standard values.

Temperature (°C)	C_{14}	C_{15}	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$
60	161 (101)	193 (97)	176 (109)	202 (104)	165 (98)	163 (101)	166 (102)	157 (93)
70	165 (103)	197 (99)	177 (110)	204 (105)	164 (98)	163 (101)	166 (102)	149 (88)
80	165 (103)	195 (98)	175 (109)	202 (104)	161 (96)	162 (100)	160 (98)	120 (71)
90	159 (99)	191 (96)	175 (109)	202 (104)	167 (99)	165 (103)	152 (93)	44 (26)
100	163 (102)	195 (98)	177 (110)	204 (105)	169 (100)	168 (105)	70 (43)	4 (2)

leate (Fig. 1). NMR spectral analysis showed that these compounds were the result of migration of the double bonds to conjugated positions during the saponification step (Fig. 2). The double bonds are probably located in the 9—10-*cis*, 11—12-*trans* and 10—11-*trans*, 12—13-*cis* positions instead of the 9—10-*cis*, 12—13-*cis* positions, as in linoleic acid. We expect that a similar process occurs during saponification of arachidonic acid, at an even faster rate (Table II). Losses by oxidative processes during the analytical steps were not observed because the sum of the amounts of the $C_{18:2}$ acids was the same as the original amount of linoleic acid. To prevent the migration of double bonds, saponification at lower temperatures, where losses of linoleic acid and arachidonic acid are minimal, is necessary. To test the saponification at lower temperatures, trilinolein was used. Trilinolein was dissolved in chloroform (19.9 mg/100 ml) with pentadecanoic acid as an internal standard (15.3 mg/100 ml). Of this solution, 0.5, 1, 2 and 3 ml were evaporated under a stream of nitrogen at 40°. Saponification was carried out as described above at 70° for 10 min. Under these conditions the saponification is almost complete with no losses of linoleic acid (Table III).

To determine whether hydrolysis of serum lipids is complete under these circumstances, aliquots of pooled serum were analyzed, using different tempera-

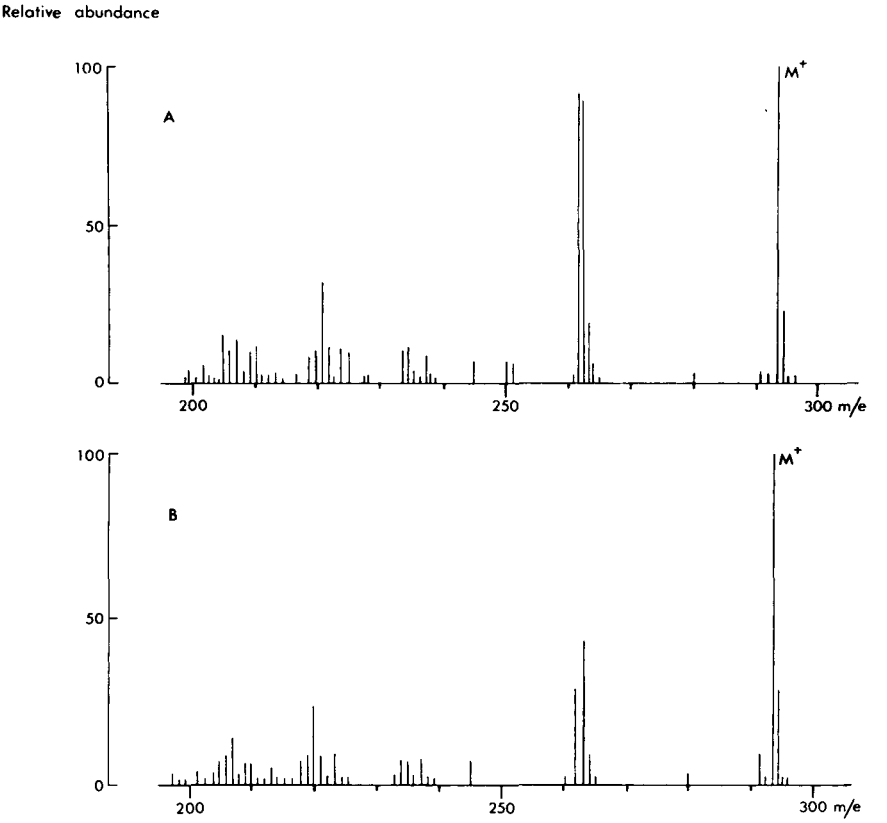

Fig. 1. Parts of the mass spectra of: (A) methyl linoleate and (B) the formed compounds.

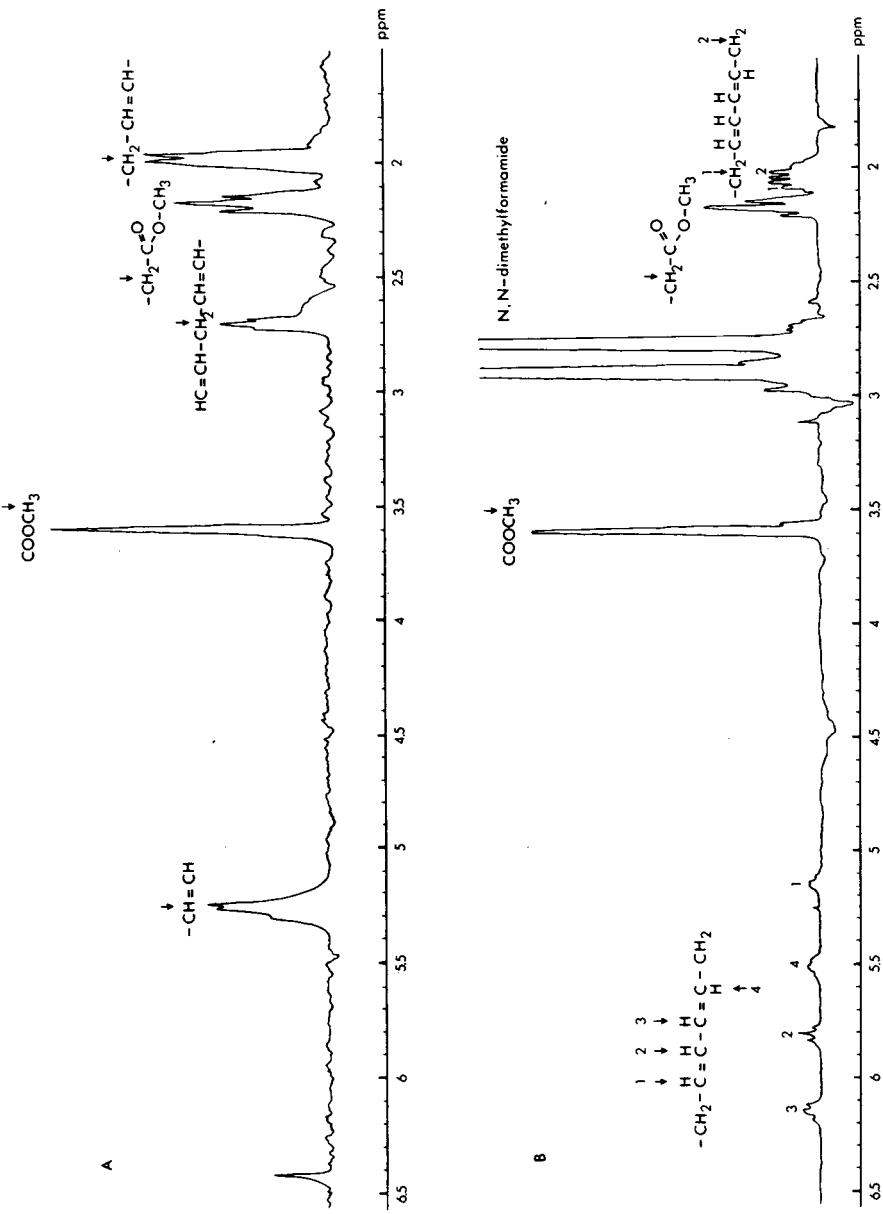

Fig. 2. NMR spectra of: (A) methyl linoleate and (B) the formed compounds. Solvents: (A) tetrachloromethane and (B) N,N-dimethylformamide. Internal standard, trimethylsilane; sweep width, 3000 Hz; temperature, 25°; number of scans, 100.

tures and incubation times during the saponification step. The results are shown in Table IV. Silica-gel thin-layer chromatography [4] revealed that saponification of the sample at 100° was complete after 30 min because no fatty-acid esters were visible in the chromatogram (Fig. 3).

Moreover, the presence of cholesterol in the serum samples is clearly shown, as should be expected. However, its presence does not interfere in the GC analysis. This was proved by injecting a solution of free cholesterol. After 1 h,

TABLE III

SAPONIFICATION OF TRILINOLEIN FOR 10 MIN AT 70°

Figures are the mean recoveries of duplicate injections of duplicate samples expressed in μg linoleic acid per sample and in percentages of the amount present.

Sample (ml)	Linoleic acid (μg per sample)		
	Present	Found	Recovery (%)
0.5	95	93	98
1.0	190	193	102
2.0	380	383	101
3.0	570	576	101

TABLE IV

EFFECT OF TEMPERATURE AND TIME ON THE SAPONIFICATION OF SERUM LIPIDS

Pooled serum (0.5 ml) was used according to the procedure described in the text, including internal standard. Figures are the mean values of duplicate injections of duplicate samples expressed as percentages of the highest yield found.

Time (min)	Temperature (°C)	C_{14} (%)	$C_{16:0}$ (%)	$C_{16:1}$ (%)	$C_{18:0}$ (%)	$C_{18:1}$ (%)	$C_{18:2}$ (%)	$C_{20:4}$ (%)
10	70	89	85	83	89	83	87	87
10	80	87	88	83	90	87	91	60
10	90	89	89	84	92	89	100	43
10	100	95	92	92	95	93	18	0
30	70	89	88	83	89	86	92	78
60	70	94	89	93	91	88	98	77
90	70	100	90	99	93	90	100	54
30	60	84	90	80	92	87	85	77
60	60	88	89	86	94	89	93	100
90	60	92	91	93	97	93	96	98
30	100	100	100	100	100	100	4	0

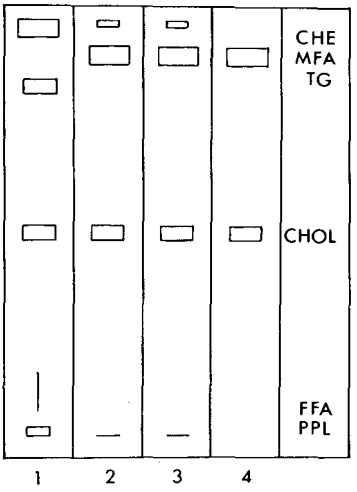

Fig. 3. Thin-layer chromatography on silica gel of a serum lipid extract in chloroform (1), and of serum extracts saponified for 10 min at 70° (2), for 90 min at 60° (3) and for 30 min at 100° (4). CHE = cholesterol esters; MFA = methyl fatty acids; TG = triglycerides; CHOL = cholesterol; FFA = free fatty acids; PPL = phospholipids.

TABLE V

EXTRACTION RECOVERY OF FATTY ACIDS AND TRINOLEIN ADDED TO SERUM

All figures are the mean values of duplicate injections of duplicate samples, expressed in μg per sample and as percentages of the values of the pure standards.

	C_{14}	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$
Fatty-acid standard mixture	160	179	215	171	171	172	155
Serum	8	357	48	71	329	271	53
Serum with added fatty-acid standard mixture	168	541	267	241	509	475	217
Amount recovered	160	184	219	170	180	204	164
Recovery percentage	100	103	102	99	105	119	106
Trinolein standard	—	—	—	—	—	191	—
Serum with added trinolein standard	8	364	48	73	335	470	56
Amount recovered	—	—	—	—	—	199	—
Recovery percentage	—	—	—	—	—	104	—

keeping the oven at 220°, no peak was observed. Most likely cholesterol has a very large retention time at this temperature or is completely retained on the column. The samples saponified at 70° and 60° still contained traces of cholesterol esters and phospholipids. Triglycerides were not seen. As can be seen in Table IV, no ideal conditions, in which complete hydrolysis and no loss of polyunsaturated acids occur together, can be found. However, results for 10 min at 70° and 90 min at 60° are quite satisfactory.

The extraction procedure was checked by adding fatty acids and trilinolein to serum and determining the recovery. Dried aliquots (1 ml) of both a fatty-acid standard mixture and trilinolein were determined without the extraction step and also after dissolving them in 0.5 ml serum, followed by extraction. Recoveries are shown in Table V.

The reproducibility of the method was tested by analysing pooled serum ten times according to the above procedure (Table VI).

The fatty-acid composition of the pooled sera of 40 healthy laboratory technicians (aged 18—55 years) together with some values from the literature are listed in Table VII.

Some examples of the usefulness of the method for clinical purposes are given in Tables VIII and IX. Table VIII shows the composition of the serum

TABLE VI

REPRODUCIBILITY OF TEN ANALYSES OF THE SAME SERUM

	C_{14}	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$
Mean (mg/l)	22.4	661.7	53.1	197.1	641.6	740.3	131.5
Standard deviation	1.36	22.95	3.61	12.29	31.78	47.52	20.88
Coeff. of variation (%)	6.0	3.5	6.8	6.2	5.0	6.4	15.9

TABLE VII

FATTY-ACID COMPOSITION OF SERUM LIPIDS OF THE POOLED SERA OF 40 LABORATORY TECHNICIANS COMPARED WITH SOME VALUES FROM THE LITERATURE

Figures represent percentages of the total amount of the fatty acids considered.

	C_{14}	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$	Others
This method	1.2	27.8	2.3	8.1	22.7	32.9	5.0	—
Ref. 6 (calculated)	1.5	29.5	7.0	8.0	29.3	18.6	6.0	—
Ref. 7	1.5	23.5	5.3	9.5	29.3	27.0	—	3.9
Ref. 8	1.6	26.7	4.4	9.0	25.9	27.8	3.9	—

TABLE VIII

FATTY-ACID COMPOSITION OF SERA OF HEALTHY CHILDREN COMPARED WITH CHILDREN SUFFERING FROM CYSTIC FIBROSIS

Figures are expressed as percentages of the total amount of the fatty acids considered.

	C_{14}	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$
Normal (age 0.5—12 years, n = 21)							
%	1.9	29.5	3.9	9.8	26.2	25.2	4.6
S.D.	0.88	3.92	1.04	2.14	3.85	5.17	1.26
Cystic fibrosis (age 1—16 years, n = 22)							
%	1.9	30.5	7.1	9.0	31.7	17.6	2.7
S.D.	0.85	3.10	2.15	2.26	5.51	6.99	1.70

TABLE IX

FATTY-ACID COMPOSITION OF FAT BIOPSIES OF DIABETIC CHILDREN COMPARED WITH VALUES FROM THE LITERATURE

Figures are expressed as percentages of the total amount of the fatty acids considered.

	C_{14}	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$	Others
Diabetic children (n=23)								
%	2.8	20.9	6.7	4.0	46.7	15.8	2.3	—
S.D.	0.77	2.09	1.40	1.24	2.58	3.31	0.82	—
Ref. 11								
%	2.7	24.0	5.0	8.4	46.9	10.2	—	2.5

fatty acids of children with cystic fibrosis compared with healthy children. Our results are in agreement with the results of others [9, 10], i.e., higher values for the monounsaturated and lower values for the polyunsaturated acids. The mean values of the fatty acid composition of fat biopsies compared with literature values are given in Table IX.

DISCUSSION

The method presented in this paper has many advantages in comparison with other procedures. The extraction is simple because no further extractions to purify the samples from non-lipid material are necessary. Saponification for 10 min at 70° and the subsequent methylation are very simple and rapid and

occur under mild circumstances without oxidation or double-bond migration in the polyunsaturated acids. However, the saponification can not be totally complete without loss of polyunsaturated acids, but the same probably holds true for other saponification procedures. For practical reasons we have taken a saponification procedure of 10 min at 70°, which will give about 85% saponification, although about 92% saponification can be achieved with the conditions 90 min at 60°.

Reproducibility is good and the results of determinations of the fatty-acid composition of 40 laboratory technicians agree fairly well with values found in the literature. Any differences could be due to dietary circumstances. Furthermore, the method is very well suited to the determination of the fatty-acid composition of fat biopsies.

In a laboratory with good automated equipment for GC, about 50 samples can be analysed daily by one technician. This method should also be suitable for the assay of other fatty acids and for the analysis of other types of sample.

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CORTICOSTEROID ANALYSIS IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive, specific, and reproducible high-performance liquid chromatographic assay for the simultaneous determination of prednisone, prednisolone and cortisol in biological fluids was developed with dexamethasone as the internal standard. Samples are extracted with methylene chloride, washed with sodium hydroxide and then water, and chromatographed on a microparticulate silica gel column with UV detection at 254 nm. Sensitivity was greater than 15 ng for all four steroids. Specificity was supported by use of dual wavelength UV detection and/or radioimmunoassay. The assay has been applied in pharmacokinetic studies and a typical plasma concentration—time profile for the three steroids is presented for one subject who received 50 mg of prednisone.

INTRODUCTION

Bioavailability and pharmacokinetic studies of synthetic glucocorticoids require efficient, sensitive, specific and reproducible analytical techniques. Most studies have employed radioimmunoassay (RIA) techniques to assess the bioavailability of prednisone or prednisolone formulations [1—3]. The RIA technique for these drugs, however, suffers from poor specificity [4] and poor reproducibility [1]. Only recently have high-performance liquid chromatography (HPLC) techniques been developed for glucocorticoid determinations in plasma [5—8]. However, these methods are either not efficient or not specific.

This report describes an HPLC method for the simultaneous assay of prednisone, dexamethasone, cortisol and prednisolone in plasma, urine, or saliva.

EXPERIMENTAL

Materials

The HPLC system consisted of a continuous flow, constant volume Model 6000A solvent delivery system with a U6K universal loop injector and a Model 440 UV absorbance detector with dual wavelength capability (Waters Assoc., Milford, Mass., U.S.A.). Wavelenghts of 254 and 280 nm were used. A 0.25 m × 4.6 mm I.D. stainless-steel column packed with 5—6 μm Zorbax™ SIL particles (DuPont, Wilmington, Del., U.S.A.) was attached to a 6 × 70 mm stainless-steel Whatman precolumn packed with HC Pellosil® (Whatman, Clifton, N.J., U.S.A.). Solvents for the mobile phase were purchased from Burdick & Jackson Labs., Muskegon, Mich., U.S.A. Analytical standards of prednisone, prednisolone, cortisol and dexamethasone were purchased from Sigma, St. Louis, Mo., U.S.A. Beclomethasone, 17α,20β,21-trihydroxy-1,4-pregnadiene-3,11-dione and 17α,20α,21-trihydroxy-1,4-pregnadiene-3,11-dione were gifts from Schering, Kenilworth, N.J., U.S.A. Methylprednisolone was a gift from Upjohn, Kalamazoo, Mich., U.S.A. and 6β-hydroxycortisol was a gift from Lederle Labs. (American Cyanamide), Pearl River, N.Y., U.S.A.

Prednisolone-6,7-^{3}H(N) with a specific activity of 45.4 Ci/mmole and cortisol-1,2-^{3}H(N) with a specific activity of 125 mCi/mg were obtained from New England Nuclear (Boston, Mass., U.S.A.). Pharmaceutical grade decolorizing carbon was obtained from the Amend Drug and Chemical Co., Irvington, N.J., U.S.A.

Preparation of standards. Four grams of decolorizing carbon were added to 100 ml of human plasma and stirred for 2 h at room temperature. Charcoal was then removed from the plasma by centrifugation overnight at 24,000 *g*. To this cortisol-stripped plasma, standards of dexamethasone, prednisone, prednisolone and cortisol in acetonitrile—methanol (1:1) were added to provide concentrations of 50—500 ng/ml.

Extraction procedure. Samples of saliva, plasma, or urine (1 ml) were added to 10 ml of methylene chloride. The internal standard, dexamethasone (125 ng) was then added and the glass culture tubes were shaken for 20 min. The tubes were centrifuged and the aqueous layer and creamy interface aspirated. The organic phase was then washed with 1 ml of 0.1 *N* sodium hydroxide and subsequently with 1 ml of water. After aspirating the aqueous phase, 1 g of anhydrous sodium sulfate was added to dry the organic phase. The latter was evaporated at 45° under a nitrogen gas stream.

Chromatography. The residue was reconstituted with approximately 200 μl of mobile phase for injection. The mobile phase was methanol—methylene chloride (3:97) at a column flow-rate of 2 ml/min. Concentrations of the individual steroids in plasma, urine, or saliva were determined from the slope of the plot of the peak height ratio of steroid: dexamethasone against standard concentrations of steroids.

Extraction recoveries. Trace quantities of tritiated cortisol or prednisolone were added to serum containing 50 or 500 ng/ml concentrations of dexamethasone, prednisone, prednisolone, and cortisol to determine the extraction

efficiency of the steroids. Four replicates of each concentration were extracted together with four samples of charcoal-stripped plasma. The washed organic phase was evaporated to dryness in a liquid scintillation counting vial and 10 ml of Aquasol® (New England Nuclear) were added. Samples were counted in a Packard Tri-Carb Model 3255 liquid scintillation counter (Packard, Downers Grove, Ill., U.S.A.) with a counting error of less than 1%. The sample channels ratio method of quench correction was employed to determine counting efficiency. The extraction efficiency was then calculated from dpm's obtained from samples and dpm's obtained from vials containing spike concentrations of ^{3}H-prednisolone or ^{3}H-cortisol.

RESULTS

A chromatogram of an extract of cortisol-stripped plasma spiked with prednisone, dexamethasone, cortisol and prednisolone is shown in Fig. 1. The

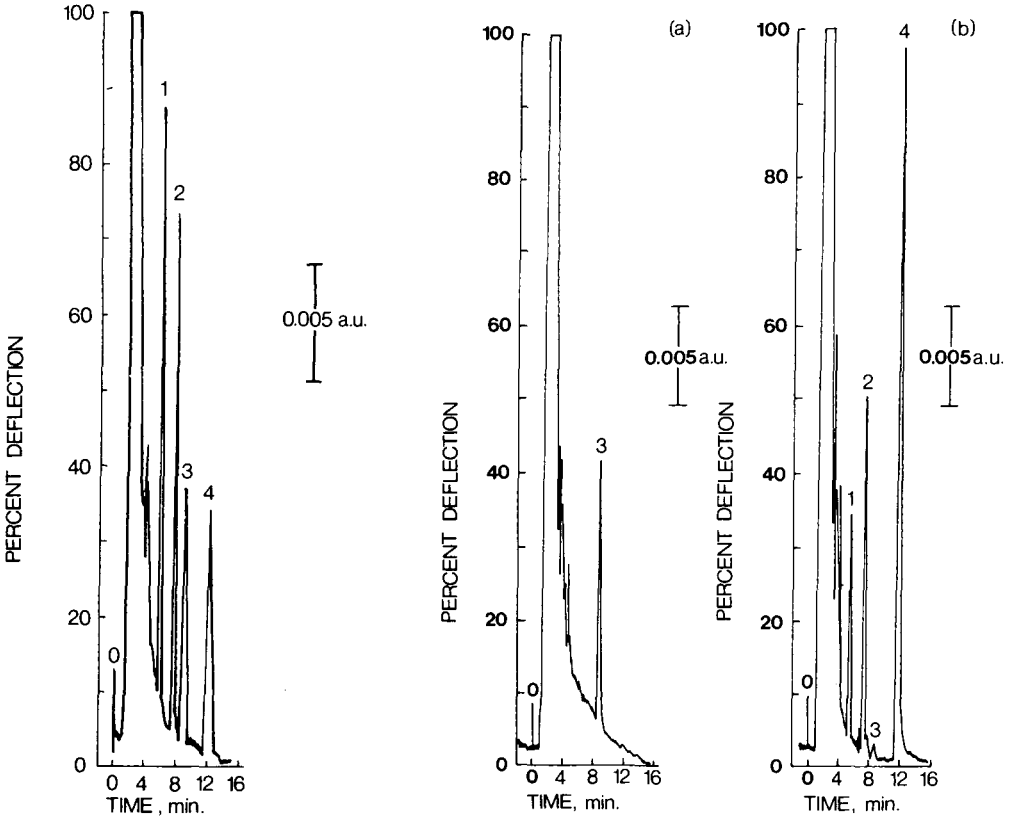


Fig. 1. Chromatogram of an extract of 1.0 ml of human plasma spiked with 100 ng of: (1) prednisone; (3) cortisol; (4) prednisolone. Dexamethasone, 125 ng (2) is the internal standard. The symbol (0) designates the injection point.

Fig. 2. Chromatograms of extracts of plasma: (a) taken prior to a dose of prednisone; (b) taken 3 h after a single oral dose of 50 mg prednisone. See Fig. 1 for peak assignments.

chromatogram illustrates the response to steroid concentrations of 100 ng per ml plasma and to 125 ng of dexamethasone, which was used as the internal standard. In Fig. 2a, the chromatogram of a plasma sample taken before the administration of a 50-mg tablet of prednisone is shown without the internal standard. Fig. 2b illustrates the response to steroid concentrations 3 h after the dose of prednisone, and represents concentrations of 56 ng/ml for prednisone, 10 ng/ml for cortisol, and 426 ng/ml for prednisolone.

Steroid concentrations in urine and saliva were also determined with this method after an oral dose of 50 mg of prednisone. Chromatograms of saliva and urine concentrations of steroids are shown in Fig. 3. In Fig. 3b, a chromatogram of 1.0 ml of urine taken from a 0—3 h urinary collection interval is monitored at wavelengths of 254 and 280 nm. A constant ratio of peak height of a steroid at the two wavelengths served as an added measure of specificity of the assay. The 280 to 254 nm ratios for the four steroids were: prednisone, 0.07; dexamethasone, 0.08; cortisol, 0.03; prednisolone, 0.09. While monitoring at dual wavelengths improves assay specificity, the 254 nm wavelength yields nearly optimum absorbance of several corticosteroids as demonstrated in Fig. 4. This wavelength is common, essentially, to all HPLC instruments which contain a mercury lamp and increases the general applicability of the assay technique.

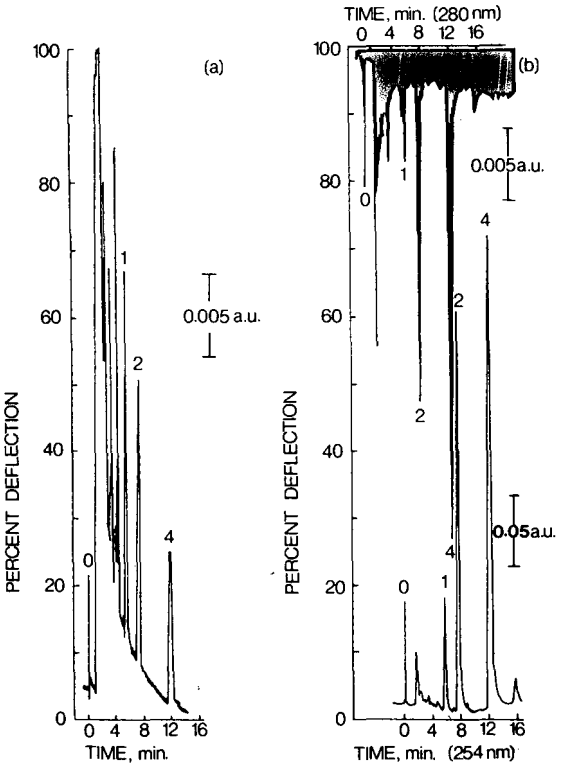


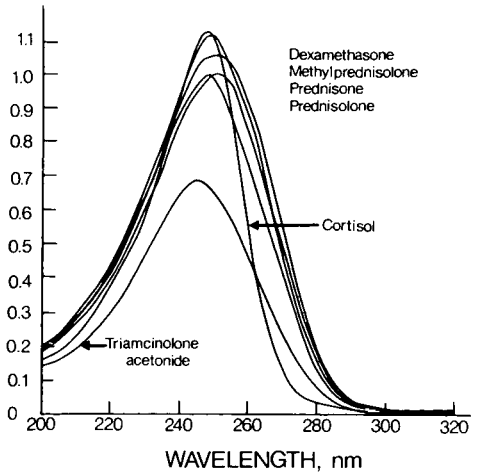
Fig. 3. Chromatograms of: (a) an extract of 3.0 ml of saliva collected 3 h after a 50-mg oral dose of prednisone; (b) an extract of human urine taken after a 50-mg oral dose of prednisone and monitored at wavelengths of 254 and 280 nm. See Fig. 1 for peak assignments.

The recovery of cortisol and prednisolone from plasma was approximately 83% (Table I) and was independent of concentration. The sensitivity limit of the assay for prednisone, cortisol and prednisolone was about 5 ng/ml when a signal-to-noise ratio of 2.5 or greater was used as a criterion for a significant response.

The response of the HPLC system was linear over the 0 to 500 ng/ml steroid concentration range, and as demonstrated in Fig. 5, a greater response was seen with prednisone than with cortisol or prednisolone.

The precision of the assay was determined with successive samplings of pooled patient plasma. Sixteen successive samplings gave coefficients of variation of 12, 5, and 8% for prednisone, prednisolone, and cortisol, respectively, as demonstrated in Fig. 6.

The specificity of the assay was determined by comparing retention times of standards to those of samples, by comparing the peak height ratio of a



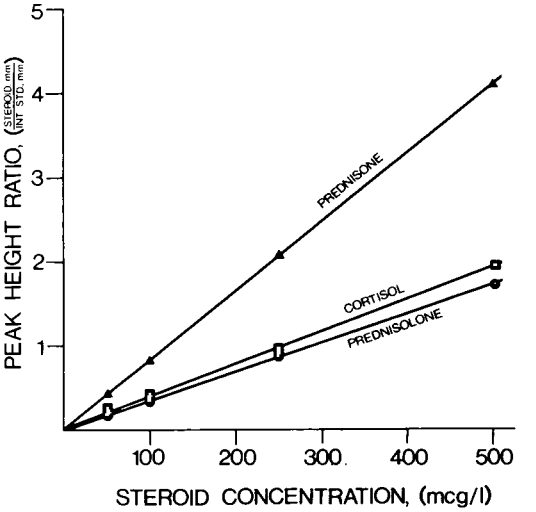


Fig. 4. UV absorbance spectra of several corticosteroids at concentrations of 25 μg/ml in acetonitrile—ethanol (1:1).

Fig. 5. Calibration curve for the determination of prednisone, prednisolone and cortisol in a 1.0-ml plasma extract.

TABLE I

EXTRACTION EFFICIENCY OF ^{3}H-PREDNISOLONE OR ^{3}H-CORTISOL FROM PLASMA CONTAINING DEXAMETHASONE, CORTISOL, PREDNISONE AND PREDNISOLONE

Steroid concentration (ng/ml)	Recovery (%)	(%)
	^{3}H-Prednisolone	^{3}H-Cortisol
50	83.0 ± 2.68*	82.8 ± 2.6
500	83.9 ± 2.6	81.4 ± 3.7

*Mean ± S.D. of 4 determinations.

steroid at two wavelengths (254 and 280 nm) to the ratio of the standards, and also by correlating the observed concentrations of a steroid assayed by HPLC with concentrations determined by an RIA technique. For the latter, concentrations of prednisolone following a single intravenous dose of 40 mg of prednisolone sodium succinate were determined by this HPLC method and are compared with concentrations determined by RIA in Fig. 7. Nearly perfect

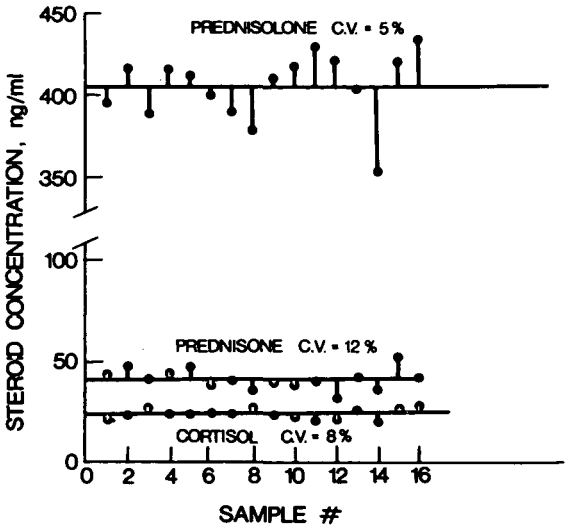


Fig. 6. Reproducibility of the HPLC assay method determined by 16 successive assays. The mean (horizontal bar) and coefficient of variation (C.V.) are as indicated.

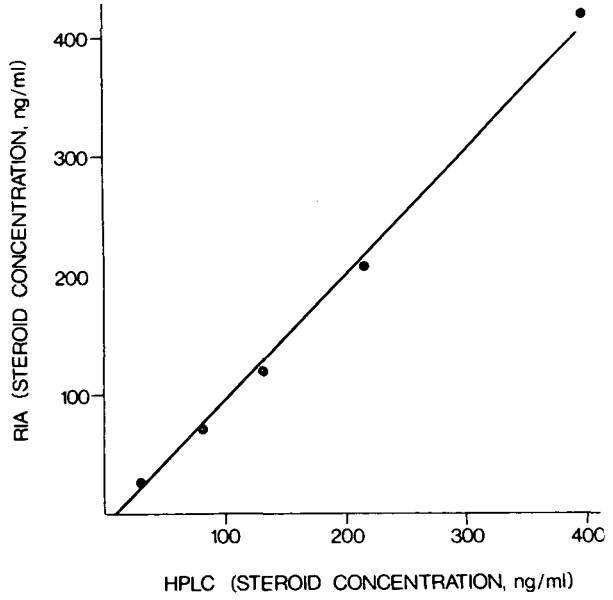


Fig. 7. Correlation between plasma prednisolone concentrations measured by HPLC and RIA ($r = 0.985$).

TABLE II

RETENTION TIMES OF SELECTED GLUCOCORTICOIDS AND THEIR METABOLITES

Steroid	Retention time (min)
Cortisone	4.2
Triamcinolone acetonide	4.3
Prednisone	5.4
Beclomethasone	6.5
Dexamethasone	7.1
Cortisol	8.4
Methylprednisolone	10.5
Prednisolone	11.6
17α,20α,21-Trihydroxy-1,4-pregnadiene-3,11-dione	15.5
17α,20β,21-Trihydroxy-1,4-pregnadiene-3,11-dione	18.0
6-β-Hydroxycortisol	18.4

agreement is seen. An additional check for specificity involved examination of other glucocorticoids and/or metabolites of prednisone. These materials had retention times as listed in Table II. The assay method clearly separates this series of closely related compounds. Many other conjugated metabolites of these steroids probably do not warrant concern as the organic extraction technique precludes the lipid-insoluble biotransformation products.

DISCUSSION

The simultaneous determination of prednisone, cortisol and prednisolone by this HPLC method proves to be efficient, precise, sensitive and selective. The procedure facilitates the characterization of prednisone and its pharmacologically active metabolite, prednisolone. Further, it allows examination of the effect these steroids have on circulating cortisol concentrations. The data in Fig. 8 were obtained in a healthy male volunteer who received a 50-mg prednisone dose in a bioavailability trial. These and other pharmacokinetic data confirm the expected disposition characteristics of these corticosteroids and the effect on cortisol secretion. The pharmacokinetics of prednisone and prednisolone will be reported more fully in subsequent reports.

Recent pharmacokinetic and bioavailability studies of prednisone have employed RIA techniques. However, poor precision with such RIA methods, as suggested by a coefficient of variation of 12–25% over the therapeutic concentration range [1], and a significant cross-reactivity with endogenous steroids or metabolites [4] complicate these results. In addition, endogenous interferences confound the assay performance to such an extent that standards must be constituted in each patient's zero hour plasma sample [1].

HPLC has been used only recently for steroid analysis in plasma [5–8], but such methods are not as efficient, specific or as versatile as the proposed procedure. Our method has proved specific and reliable. Over 2000 samples

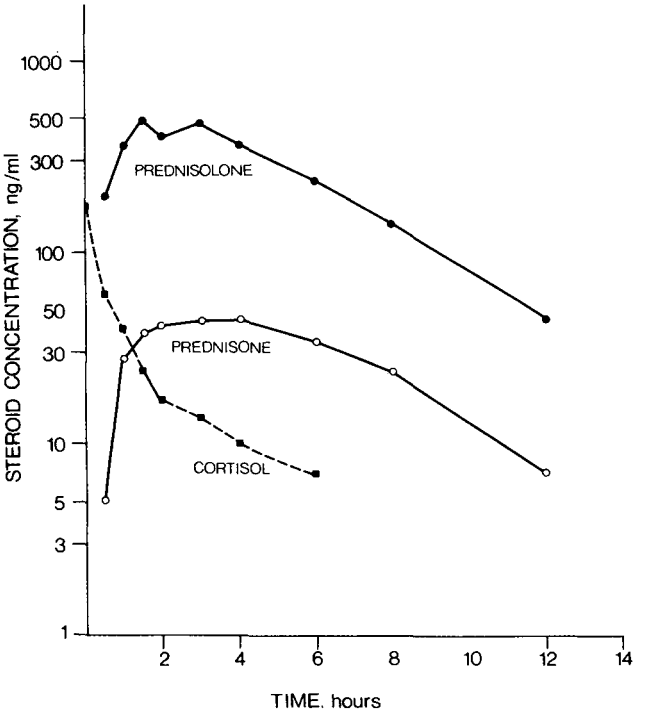


Fig. 8. Steroid plasma concentration—time profile of a human volunteer given a 50-mg oral dose of prednisone.

have been analyzed by this method in studies of the disposition kinetics of prednisone, cortisol, dexamethasone, prednisolone and methylprednisolone.

ACKNOWLEDGEMENTS

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Biomedical Applications

CHROMBIO. 275

DETERMINATION OF FREE, TOTAL, AND ESTERIFIED CHOLESTEROL BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for measuring free, total, and esterified cholesterol in blood serum in which reversed-phase liquid chromatography is used and the eluate is monitored at 200 nm. The sample for total cholesterol is prepared according to the Abell-Kendall procedure, and for free cholesterol an extract of serum—isopropanol (1: 5, v/v) is used. The column is a μBondapak C_{18}, 10 μm, and the mobile phase for total cholesterol is isopropanol—acetonitrile (50:50, v/v); for free cholesterol, it is isopropanol—acetonitrile—water (60:30:10). An approximation of the free cholesterol, triglycerides, and individual cholesteryl esters is obtained from single chromatograms of isopropanol extracts of serum if the first mobile phase is used. In a comparison study with the Abell-Kendall method for total cholesterol, the correlation is excellent and the precision is acceptable.

INTRODUCTION

Measuring serum cholesterol is one of the most frequently performed assays in the clinical laboratory, for which a wide variety of methods [1] are available and used. The classical method is a colorimetric assay based on the photometric measurement of the color formed when cholesterol reacts with a Lewis acid [2—4]. However, because of the hazards associated with using the strong acid medium in which the color is formed and the increasing emphasis on measuring both the free and esterified cholesterol, alternate methodologies have been and are being developed. Most of the newer methods are based on enzymatic hydrolysis and oxidation [5—7] or on chromatographic analysis [8—15]. The enzymatic reactions are followed by colorimetric [6] or electrochemical analysis [7], and the chromatography may be either gas—liquid [8—12] or thin-layer [13—15].

Although liquid chromatography (LC) has been used in analyses of lipids in general [16], its specific application in cholesterol methodology has been limited because cholesterol and related compounds absorb very little UV radiation in the wavelength range in which most UV detectors used in LC operate. Consequently, LC has been used in assays in which column chromatography is followed by chemical analysis of the collected chromatographic fractions [17—19] or in which the eluate is monitored on-line with other types of detectors. These detectors include the refractive index detector [20, 21], the moving-wire flame-ionization detector [22—25], and a laser infrared detector [26]. However, there are problems associated with these instruments, including slow analysis time, and lack of sensitivity.

With recent advances in LC-detector technology, high-performance spectrophotometers with low-volume flow cells are now commercially available that allow chromatographic eluates to be monitored at wavelengths as low as 200 nm. By coupling a high-performance reversed-phase chromatographic column and such a detector and by eluting with the appropriate solvents, we are now able to monitor photometrically at 200 nm and to measure cholesterol as it is eluted from the chromatographic column. In this paper, we describe the development and evaluation of an LC procedure to determine free and total cholesterol in blood serum and to calculate esterified cholesterol as the difference between them. This technique may also perhaps be used to measure individual cholesteryl esters.

MATERIALS AND METHODS

*Reagents**

Standard Reference Material (SRM 911a) cholesterol was purchased from the National Bureau of Standards (NBS) (Office of Standard Reference Materials, Washington, D.C. 20234). The cholesteryl esters were purchased from Sigma St. Louis, Mo., U.S.A.; the glycerides from P-L Biochemicals, (Milwaukee, Wisc., U.S.A.). Spectrophotometric grade isopropyl alcohol (IPA) and acetonitrile (CH_3CN) were obtained from Burdick & Jackson (Muskegon, Mich., U.S.A.). Water was deionized, distilled in a glass apparatus, and filtered through a membrane with pores of 0.22 μm.

Preparation of standard solutions

A stock solution of cholesterol standard (10.00 g/l; 25.86 mmole/l) was prepared by dissolving 100.0 mg of the NBS cholesterol in 10 ml of isopropanol (IPA). The solution was then dispensed in 1-ml aliquots into small (1.7 ml) glass vials with Teflon™-lined caps and stored at −13°. Working standards were prepared from an aliquot of the stock solution which was thawed and diluted as necessary to cover the desired range of cholesterol concentrations. Stock solutions of each of the standards containing cholesteryl esters were prepared by dissolving a given quantity of ester in a specified volume of IPA: cholesteryl arachidonate, 5 mg/5 ml (1.00 g/l; 1.485 mmole/l); cholesteryl

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*Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or by the U.S. Department of Health, Education, and Welfare.

linoleate, 5 mg/5 ml (1.00 g/l; 1.541 mmole/l); cholestery oleate, 5 mg/10 ml (0.50 g/l, 0.768 mmole/l); and cholesteryl palmitate, 5 mg/10 ml (0.50 g/l; 0.800 mmole/l). A working solution of standards of cholesterol and the esters was prepared by combining the required volume of each of the stock solutions and diluting to 875 μl with IPA so that this final volume contained the following concentrations: cholesterol, 0.12 g/l (0.310 mmole/l); cholesteryl arachidonate, 0.07 g/l (0.103 mmole/l); cholesteryl linoleate, 0.30 g/l (0.462 mmole/l); cholesteryl oleate, 0.10 g/l (0.154 mmole/l); and cholesteryl palmitate, 0.04 g/l (0.080 mmole/l). A 50-μl aliquot of this solution was then injected onto the column for a chromatographic analysis of the standards.

Samples

Frozen serum from individual patients and from serum pools was obtained from the Metabolic Biochemistry Branch of the Clinical Chemistry Division at the Centre for Disease Control (CDC). The pooled samples have been used in standardization programs and cover a wide range of cholesterol concentrations. Fresh samples of serum were obtained from patients visiting the CDC Public Health Service Clinic.

Sample and standard preparation

For the total cholesterol determination, serum was saponified and extracted according to the Abell-Kendall (AK) method [4]. Five ml of alcoholic KOH (6 ml of 33% KOH in H_2O, diluted to 100 ml with absolute alcohol) was added, with a Cornwall syringe, to 0.5 ml of serum in a 20 × 150 mm glass test tube equipped with a Teflon™-lined cap. The tubes were incubated at 45° for 60 min and then allowed to cool to room temperature. After the addition of 5 ml of water to each tube, it was cooled to room temperature, 10 ml of hexane were added to each, and the tubes were shaken mechanically for 10 min. A 4-ml aliquot of the hexane layer was pipetted into a 16 × 130 mm glass test tube and evaporated in a 45° oven at a reduced pressure of 20—28 p.s.i. The residue was then dissolved in 800 μl IPA and the extract used for sampling for injection onto the column of the chromatograph. In the comparison studies, a 4-ml aliquot of the hexane extract was also evaporated for analysis by the AK method.

For the free cholesterol determination, 100 μl of serum was vortexed with 500 μl of IPA for 2 min, centrifuged, and the supernatant removed. This supernatant was used for sampling for the chromatographic analysis of free cholesterol and to obtain a profile of the individual cholesteryl esters and other lipids.

The standards for determining total cholesterol were prepared by adding 5 ml of alcoholic KOH to 0.5 ml of each of the standards, which contained 1.00, 2.00, 3.00 and 4.00 g cholesterol per l (respectively, 2.60, 5.17, 7.76 and 10.34 mmole cholesterol per l), and then incubating, extracting, evaporating, and dissolving the residue as described above. IPA dilutions of the stock standard containing cholesterol were used in measurements of free cholesterol.

Chromatographic system

The system used for the chromatographic analyses included a Varian Model

4200 liquid chromatograph (Varian, Walnut Creek, Calif., U.S.A.) equipped with a Waters Model U6K injector (Waters Assoc., Milford, Mass., U.S.A.); a Waters μBondapak C_{18}, 10 μm, reversed-phase column (30 cm × 4 mm I.D.) preceded by a precolumn (5 cm × 2 mm I.D., packed with Waters C_{18}/Porasil B, 37—75 μm); a Varian Vari-Chrom variable wavelength detector; and a Varian Model A-25 recorder. A Varian Model CDS 101 integrator was used to record the peak area electronically from the detector signal.

Different mobile phases were used for the chromatographic measurement of total and free cholesterol. To measure total cholesterol, an isopropanol—acetonitrile (50:50, v/v) mobile phase (Solvent A) was used and isopropanol—acetonitrile—water (60:30:10, v/v) mobile phase (Solvent B) for nonesterified (free) cholesterol. Solvent A was used also to obtain a profile of cholesterol, triglycerides, and cholesteryl esters.

Procedures

Chromatography. To measure total and free cholesterol, respectively, aliquots of either the AK (30 μl) or IPA (50 μl) extracts were injected onto the chromatographic column. Cholesterol was eluted at a flow-rate of 1 ml/min and a pressure of either 600—1000 p.s.i. (Solvent A) or 1500 p.s.i. (Solvent B), and dectected by monitoring the column eluate at 200 nm. The cholesterol was quantitated by comparing the peak areas (expressed as counts and printed by the integrator) of the eluted cholesterol of the sample with those of the cholesterol standards. The standards and the samples were analyzed during the same half-day.

Calibration. Aliquots of either the AK (30 μl) or IPA (50 μl) extracts of the cholesterol working standards were chromatographically analyzed with Solvent A (Solvent B for free cholesterol determination), and a peak area measured for each of the cholesterol standards. A response factor was obtained from either the 3.00 g/l (7.76 mmole/l) standard or from the reciprocal of the slope of the standard curve.

Total cholesterol. A 30-μl aliquot of each of the AK extracts was chromatographically analyzed with Solvent A, and the resultant cholesterol peak area was multiplied by the appropriate factor to obtain the total cholesterol content of each sample. Although either Solvent A or B can be used as the eluting solvent, Solvent A is preferable because the analysis can be done more quickly with it.

Comparison of HPLC method with the AK method. Several of the serum pools were assayed for their total cholesterol content by the HPLC method and a manual version of the AK method (MAK). An AK extract was prepared for each sample and analyzed in duplicate with both methods.

Free cholesterol. A 50-μl aliquot of the IPA extract of unsaponified serum was analyzed chromatographically with Solvent B, and the cholesterol peak area was multiplied by the appropriate response factor to obtain the free cholesterol content of each sample. Between chromatographic runs, 1 ml of IPA was injected to remove other lipids, and the column was equilibrated with Solvent B for at least 10 min.

Esterified cholesterol. To determine the esterified cholesterol content, both the total and free cholesterol content of each sample were chromatographically

measured, and the esterified portion was calculated as the difference between the two. An approximation of the free and specific ester content of a serum sample can be obtained by comparing the chromatogram of a single extract of the serum with Solvent A and that of the standard containing cholesterol and cholesteryl esters. We call such a chromatogram a lipid profile.

Lipid profiles. Fifty μl of the IPA extract of (unsaponified) serum was chromatographically analyzed with Solvent A. Fifty μl of the standard containing cholesterol and cholesteryl esters was analyzed on the same day. The eluates from several analyses of serum were collected in fractions corresponding to the peaks recorded on the chart. These fractions were analyzed for cholesterol (Liebermann-Burchard reaction) and triglycerides (periodic acid oxidation followed by diacetyl acetone and ammonia) with the Technicon AutoAnalyzer II (AAII) [27] and with a direct-probe mass spectrometer.

RESULTS AND DISCUSSION

Fig. 1 illustrates a series of typical chromatograms obtained when the standard solutions and sera were analyzed by high-performance liquid chromatography (HPLC) for their total cholesterol content. Chromatograms A and B are replicates obtained from the 3.00 g/l (7.76 mmole/l) cholesterol standard, and peaks C—F represent four samples of serum which contained 2.65, 3.91, 4.00, and 1.53 g/l (6.86, 10.12, 10.35, and 3.96 mmole/l, respectively) of total cholesterol.

As demonstrated in Fig. 1, approximately 8 min are required per chromatographic run, with cholesterol being cleanly resolved and eluted in approximately 6 min.

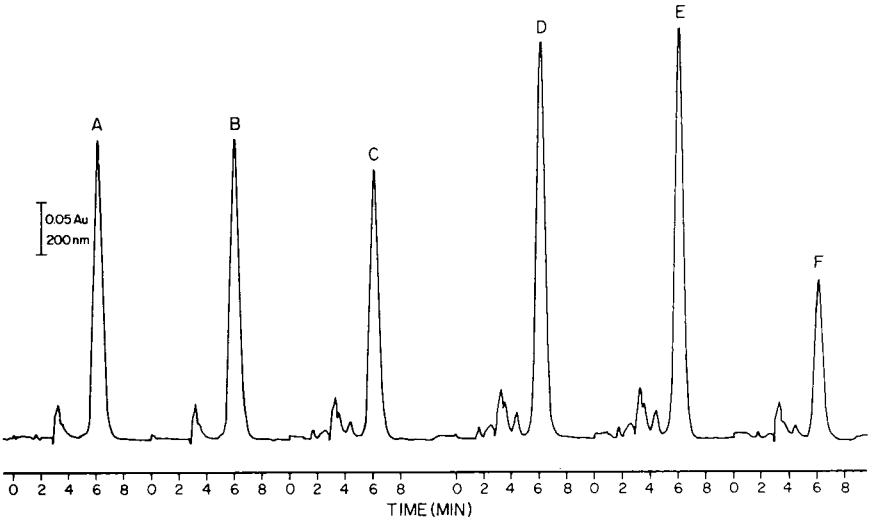

Fig. 1. Separation and measurement of total serum cholesterol by HPLC. Chromatograms A and B represent 3.0 g/l (7.76 mmole/l) cholesterol standards. Chromatograms C, D, E, and F represent samples of serum that contain 2.65, 3.91, 4.00, and 1.53 g/l (6.8, 10.12, 10.35, and 3.96 mmole/l) of total cholesterol, respectively. Chromatographic conditions: mobile phase: IPA—CH_3CN (50:50, v/v); pressure, 500 p.s.i.; flow-rate, 1 ml/min; 0.5 a.u.f.s.; band width, 8 nm; wavelength, 200 nm; column, μBondapak C_{18}; injection volume, 30 μl.

When peak areas expressed as integrator counts are plotted against the quantity of cholesterol injected or as the equivalent cholesterol concentration expressed as mg/dl, the data indicate that the method is linear over a concentration range of 0.25—5.00 g/l (0.65—12.93 mmole/l) of cholesterol. The lower limit of detection with the method is 0.25 μg of cholesterol at a detector sensitivity of 0.1 a.u.f.s. This is equivalent to a serum cholesterol concentration of 0.03 g/l (0.008 mmole/l).

The reproducibility of the HPLC method for measuring total cholesterol is shown in Tables I and II in which are summarized the precision data obtained

TABLE I

REPRODUCIBILITY OF CHROMATOGRAPHIC DETERMINATION OF TOTAL SERUM CHOLESTEROL: CHOLESTEROL STANDARDS WITH AND WITHOUT SAPONIFICATION AND EXTRACTION

	Cholesterol concentration (mg/dl)	Precision			
		Within-day		Day-to-day	
		n	C.V. (%)	*n*	C.V. (%)
Standards in IPA	100	5	1.58		
	200	5	0.65	15*	1.24
	300	5	0.55		
	400	5	0.45		
Standard processed by saponification and extraction	300	8	1.62	50**	2.02

*Three days of five replicate analyses each.
**Ten days of five replicate analyses each.

TABLE II

COMPARISON OF ANALYTICAL RESULTS OBTAINED BY ANALYZING SERUM POOLS FOR TOTAL CHOLESTEROL CONTENT BY BOTH HPLC AND MANUAL AK METHODS

n = number of days × 2 (duplicate samples were run each day).

Pool number	*n*	Analytical Results			
		HPLC		Manual AK	
		Cholesterol concentration (mg/dl)	Precision (C.V.%)	Cholesterol concentration (mg/dl)	Precision (C.V.%)
1	20	163	2.94	158	1.90
2	20	250	2.52	250	1.48
3	20	295	1.68	297	2.38
4	12	135	2.72	132	1.63
5	12	185	2.49	181	1.37
6	12	239	2.70	236	2.26
7	12	282	2.35	284	1.04
8	12	331	2.70	336	1.15
Mean		235	2.51	234	1.65

for standard cholesterol solutions and serum pools, respectively. In Table I, data are included for cholesterol standards analyzed directly with HPLC and for standards that had been processed prior to HPLC by the AK saponification and extraction procedures. With this data, we estimated not only the imprecision resulting from the chromatography, but also the combined error resulting from sample processing plus chromatography. As shown in Table I, the within-day imprecision of the chromatography step is inversely related to cholesterol concentration and ranges from 0.45% (expressed as coefficient of variation, C.V.) at a cholesterol concentrationof 4.00 g/l (10.35 mmole/l) to 1.58% at the 1.00 g/l (2.59 mmole/l) level. Day-to-day imprecision of the chromatography step is slightly higher than within-day variation (e.g., 1.24% vs. 0.65% at the 2.00 g/l (5.17 mmole/l) level. As expected, there were slight increases in analytical variation when the standards were processed by the saponification and extraction procedures prior to HPLC analysis. Table II indicates that the day-to-day variation of the AK method (mean C.V. = 1.65%) was slightly less than that of the HPLC method (mean C.V. = 2.51%).

For comparison purposes, five pooled serum samples were assayed for their total cholesterol content with both the HPLC and AK methods. As demonstrated in Fig. 2, the results obtained from the HPLC and the AK methods correlate (r = 0.998) and compare well. The data in Table II also indicate good correlation (r = 0.9997). Table III shows the results of HPLC determination of the free and total cholesterol in 20 samples of individual human sera. The

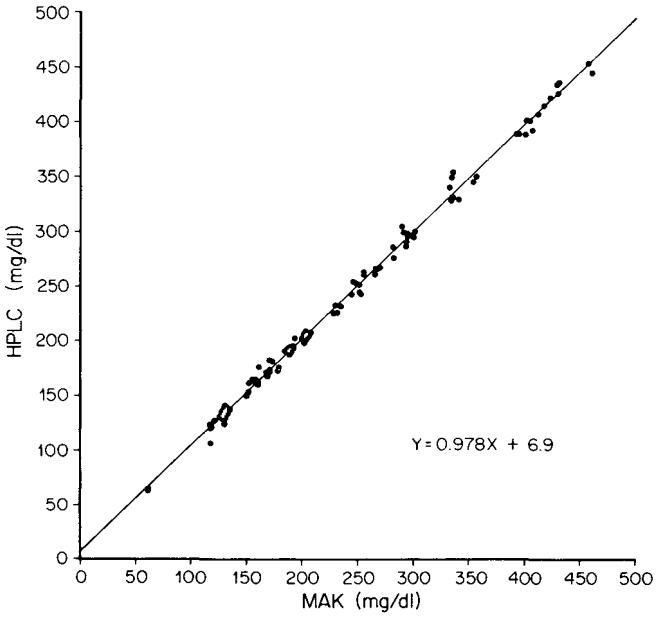

Fig. 2. Correlation between analytical values obtained for HPLC and AK analyses of human serum samples for their total cholesterol content. The 102 points represent duplicate analyses of equal aliquots of 51 samples of serum. Correlation coefficient = 0.998; standard error of estimate = 5.96.

TABLE III

RELATIVE PERCENTAGE OF FREE AND ESTERIFIED CHOLESTEROL IN HUMAN SERA DETERMINED BY HPLC

Sample No.	Cholesterol content (mg/dl)		Free (%)	Esterified (%)
	Total*	Free**		
1	299	61	20.4	79.6
2	299	56	18.7	81.3
3	230	54	23.5	76.5
4	314	65	20.7	79.3
5	279	56	20.1	79.9
6	131	27	20.6	79.4
7	205	37	18.0	82.0
8	202	62	30.6	69.4
9	217	64	29.5	70.5
10	237	58	24.5	75.5
11	355	96	27.0	73.0
12	217	59	27.2	72.8
13	230	58	25.2	74.8
14	158	36	22.8	77.2
15	192	45	23.4	76.6
16	194	48	24.7	75.3
17	242	56	23.1	76.9
18	297	78	26.3	73.7
19	301	84	27.9	72.1
20	242	66	27.9	72.1
Mean	242	58	24.1	75.9
Range	131—355	27—96	18—31	69—82

*Mobile Phase A.
**Mobile Phase B.

esterified cholesterol was calculated as the difference between the two. The percentage of free cholesterol relative to the total amount of cholesterol ranged from 20—31%, which is a range comparable to that of 17—39% reported in previous studies [13, 28].

Several compounds interfere in various cholesterol analyses [1]. For example, bilirubin interferes in the Liebermann-Burchard method [2, 3] if it is not removed before colorimetric analysis is performed, and it interferes also in the enzymatic method [1]. When we used the HPLC method, we found bilirubin did not interfere because it was removed in the AK extraction for the total cholesterol, and it was eluted from the column in the void fraction (3 min) before the cholesterol was eluted (6 min) in the free cholesterol determination. We found that vitamins A and D also did not interfere because both were eluted before cholesterol.

Steroids with a structure similar to cholesterol which are also potential interferences include: 5-cholestan-3β-ol (cholestanol), 5-cholest-7-ene-3β-ol (Δ7-cholestenol) and cholesta-5,7-diene-3β-ol (7-dehydrocholesterol). Cholestanol does not interfere in the HPLC method since it lacks the double bond present in cholesterol and thus would not absorb and be detected at 200 nm. In Solvent A, the latter two steroids co-elute with cholesterol and thus could inter-

fere in the measurement of total cholesterol. However, with this technique and appropriate solvents the steroids can be resolved.

Fig. 3 is representative of a series of chromatograms obtained from the HPLC analysis of unsaponified serum with Solvent A. Chromatogram I was obtained from the standard solution of cholesterol and cholesteryl esters, and chromatograms II and III were obtained from two serum samples. Approximately 20 min were required per chromatographic run with the compounds eluting in the following order: cholesterol (B), cholesteryl arachidonate (E), cholesteryl linoleate (F), cholesteryl oleate (G), and cholesteryl palmitate (H). There was a very small peak after the palmitate which co-chromatographed with cholesteryl stearate. Triglycerides in the eluates corresponded to peaks C and D and contributed also to peak E (cf. Fig. 4). Cholesterol was not resolved well with Solvent A if triglycerides were present in relatively high concentration, but Solvent B (which did resolve cholesterol and was used when free cholesterol was measured) did not produce satisfactory chromatograms of the cholesteryl esters.

Fig. 4 shows a chromatogram of an analysis similar to those in Fig. 3 but with bar graphs above the chromatogram which illustrate the percentage of glyceride in each fraction relative to the total amount of glycerides and the percentage of cholesterol in each fraction relative to the total amount of cholesterol. The known compounds that co-chromatographed with the fractions are as in Fig. 3. Direct-probe mass spectrometry of fractions collected during the chromatography of serum showed the molecular ion for cholesterol

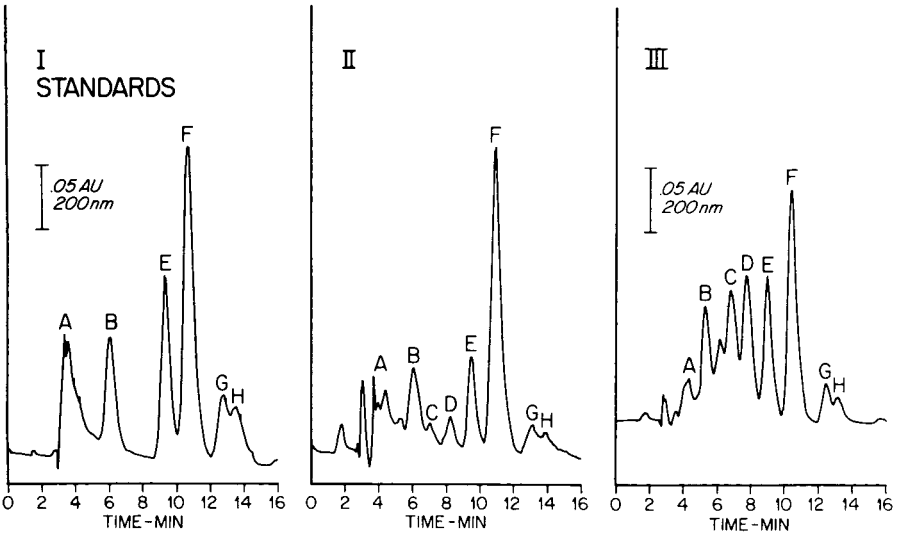

Fig. 3. Separation of cholesterol, triglycerides, and cholesteryl esters by HPLC. Chromatogram I represents a standard solution containing known quantities of the following materials in the 50-μl injection: cholesterol, 6 μg (B); cholesteryl arachidonate, 3.5 μg (E); cholesteryl linoleate, 15 μg (F); cholesteryl oleate, 5 μg (G); and cholesteryl palmitate, 2.5 μg (H). Chromatograms II and III represent 50 μl of 1:5 serum—IPA extracts. Peaks C and D represent unidentified glycerides and peak E represents unidentified glycerides and cholesteryl arachidonate. Chromatographic conditions are the same as those listed in Fig. 1.

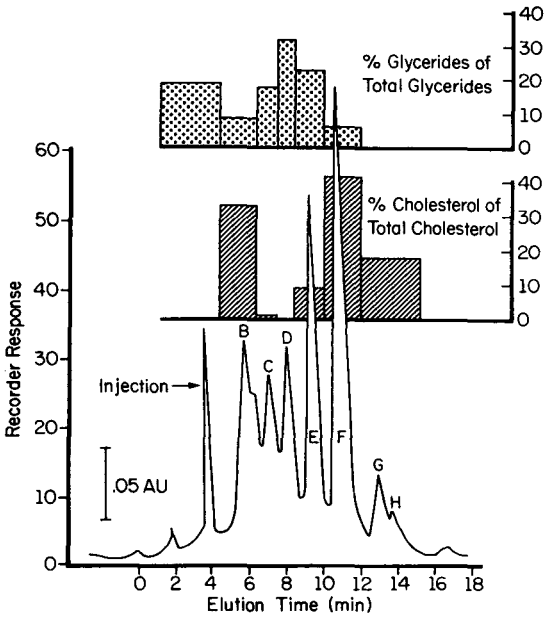

Fig. 4. Comparison of the chromatogram of 50 μl of a 1:5, serum—IPA extract with the analysis of the fractions of the eluate for triglycerides and cholesterol. The peaks are cholesterol (B); glycerides (C,D); glycerides and cholesteryl arachidonate (E); cholesteryl linoleate (F); cholesteryl oleate (G); and cholesteryl palmitate (H). Bar graphs above the chromatogram indicate the percentage of glycerides in the corresponding peak relative to the total amount of glycerides in all the peaks and the percentage of cholesterol in the corresponding peak relative to the total amount of the cholesterol in all the peaks. The bar widths represent the fraction collected during the elution of the underlying peaks. Chromatographic conditions are the same as in Figs. 1 and 3.

in fraction (peak) B, and that the cholesteryl esters in fractions (peaks) F, G, and H were, respectively, cholesteryl linoleate, cholesteryl oleate, and cholesteryl palmitate. Specific glycerides were not identified in peaks C, D, and E, but triolein and 1,2-dipalmitoylolein, which are eluted about the same time as the components of fraction E, do not co-chromatograph with any of the fractions collected from the chromatographic analysis of human serum. The approximately 20% of the total glycerides which (top bar graph, Fig. 4) is eluted before cholesterol is presumably a phospholipid-containing fraction, because we did not use the Zeolite-Lloyd reagent [27] in our glyceride analyses. Monoglycerides and diglycerides were also eluted before cholesterol.

In order to obtain a semiquantitative lipid profile of a serum, we compared the peak areas in a chromatogram (as II and III, Fig. 3) with the areas in a chromatogram of a standard (as I, Fig. 3). Cholesteryl oleate and palmitate concentrations thus determined ranged from 0.09 to 0.31 g/l (mean 0.25) and from 0.04 to 0.11 g/l (mean 0.09), respectively. A similar quantitation for cholesteryl linoleate indicated a range of 0.51—1.86 g/l (mean 1.35), probably heightened by the approximately 8% of the triglycerides present in this fraction (Fig. 4). The observed values for these cholesteryl esters were comparable to

those reported for the distribution of fatty acids in cholesteryl esters from human sera [28—30]. The values for free cholesterol similarly determined (Fig. 3) ranged from 0.26 to 0.96 g/l (mean 0.6 g/l) and were similar to those obtained with the HPLC method (Solvent B) for free cholesterol except that sera with high levels of triglycerides had erroneously high values for free cholesterol because the triglycerides were resolved poorly with Solvent A.

The HPLC method provides analytical results which compare favorably with those obtained with the widely used AK method and yields information about free, total, and esterified cholesterol from one easily prepared extract. Concentrated acid reagents are not used with HPLC and the metabolite is not destroyed and can be recovered for further study. An additional advantage is that with even as little as 50 μl of serum it is possible to measure total, free, and esterified cholesterol.

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CHROMBIO. 268

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF BIOGENIC AMINES IN BIOLOGICAL MATERIALS AS *o*-PHTHALALDEHYDE DERIVATIVES*

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SUMMARY

A remarkably sensitive, simple and selective reversed-phase high-performance liquid chromatographic (HPLC) method has been developed, allowing, for the first time, the direct measurement of histamine, norepinephrine, octopamine, normetanephrine, dopamine, serotonin and tyramine in a single sample of plasma (2 ml), tissue (0.2 g), or urine. The biogenic amines were modified by pre-column derivatization with *o*-phthalaldehyde which stabilizes the molecules, aids in extraction, and improves HPLC detection at the nanogram level. To minimize losses during the sampling procedure a careful collection procedure was designed.

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We developed a simple sample cleanup in which the samples were thawed, neutralized with KOH, immediately derivatized, extracted into ethyl acetate (EtOAc) and then chromatographed by HPLC. The derivatives were stable in EtOAc for more then 24 h. Interfering amino acids were removed from the EtOAc by partitioning twice with Na_2HPO_4 buffer (pH 10.0). Complete separation was achieved in ca. 60—90 min on a μBondapak phenyl column using a stepwise gradient of acetonitrile and/or methanol—phosphate buffer (pH 5.1). A variable wavelength fluorometer with a 5-μl flow-cell was used (excitation 340 nm; emission 480 nm). Linearity ranged from 200 pg to 50 ng onto the column. Precision (R.S.D.) for retention times was 1% and for derivatization and injection 2.5%. Recoveries of the seven biogenic amines from plasma spiked with 25 ng/ml averaged 70%, with a relative standard deviation of 6%. Separation studies were also done using a μBondapak C_{18} column. The effects of various eluents are presented. Gas—liquid chromatography was also investigated but lacked the sensitivity achieved by HPLC. The HPLC method is used routinely for the determination of biogenic amines in plasma from pigs with malignant hyperthermia and thermally stressed bovine. Significant differences in levels of biogenic amines were noted between stressed and non-stressed animals. Data on rat brain tissue samples were compared with the trihydroxyindole method and canine heart tissue was analyzed for ventricular norepinephrine and dopamine. Application of the method to urine from normal persons and a patient with a brain tumor has been demonstrated.

INTRODUCTION

In addition to being neurotransmitters, biogenic amines function as hormones which influence and modify the secretion of a variety of other hormones, hypothalamic releasing and inhibiting factors, pituitary tropic hormones, and adrenal steroids. The abnormal secretion and/or metabolism of biogenic amines are associated with many diseases such as pheochromocytoma [1], neuroblastoma [2], schizophrenia [3], malignant hyperthermia [4], and possibly essential hypertension [5—7].

To gain more insight into the role biogenic amines play in hormonal control, and in the etiology and pathogenesis of disease, more advanced, simple, sensitive and quantitative methods are needed for bioscience research. Analytical methods are available for the analysis of biogenic amines such as the trihydroxyindole and ethylenediamine fluorescence methods [8—10], gas—liquid chromatography (GLC) [11, 12], and gas chromatography—mass spectrometry [13, 14]. These methods have in general proved inadequate for the determination of biogenic amines in the plasma due to high background levels, instability of derivatives at low concentrations, and lack of accuracy and precision. More sensitive methods include the double-isotope derivative [15] and radioenzymatic assays [16], but reproducibility, time, cost, and the small number of biogenic amines that are quantitated make these methods difficult to work with.

Initially, GLC was investigated in our laboratory because it appeared to have the potential for good separation, specificity, and sensitivity for the analysis of biogenic amines. This approach to sample analysis showed some success but ultimately was discontinued. It became evident that this approach could not easily meet the subnanogram sensitivity, efficient sample cleanup, and derivative stability; all requirements of a routine biogenic amine analysis. In addition, the cleanup procedure was not selective enough. The biogenic amines are very water soluble and in the attempt to extract them from an

aqueous sample considerable interfering material was also extracted. Finally, the use of an electron capture detector (ECD) was complicated by the fact that the ECD and the derivatizing agent (trifluoroacetic anhydride, TFAA) are incompatible and the TFAA must be vented. With all of the TFAA removed from the derivatives they tended to decompose.

At this point the development of a GLC method was discontinued and high-performance liquid chromatography (HPLC) was initiated. The prediction that a good HPLC procedure would circumvent all of the decomposition problems and also solve the extraction dilemma was to be proved correct.

The application of HPLC to the analysis of biogenic amines is a recent development. Molnár and Horváth [17] demonstrated the separation of catecholamines and their metabolites on a reversed-phase column (octadecylsilica) with aqueous, isocratic elution, and studied the effects of pH, temperature, and ionic strength on catecholamine retention time. An alternative approach, reported by Kissinger et al. [18] combines HPLC with electrochemical detection for analysis of urine and tissue but is not well-suited for plasma.

Fluorescence detection coupled with HPLC separation offered the needed specificity and sensitivity to analyze biogenic amines quantitatively at the nanogram level. To enhance the natural fluorescence of biogenic amines, derivatization reagents have been employed. The two most commonly used are fluorescamine (fluram; 4-phenylspiro[furan-2(3H), 1′-phthalan]-3,3′-dione), which reacts with primary amines [19], and *o*-phthalaldehyde (OPT; OPA; Fluoropa), which together with 2-mercapthoethanol forms a strongly fluorescent adduct (Fig. 1) that is twenty times stronger than the native compound [20, 21]. OPT has been used in HPLC for post-column [22] but not pre-column derivatization of biogenic amines (Fig. 1).

We introduced the concept of pre-column derivatization of biogenic amines with OPT to improve the separation, sensitivity, and quantitation by HPLC, and to simplify the sample cleanup. Together with reversed-phase partition chromatography and fluorometric detection we have developed for the first time a rapid, highly sensitive, and simple analytical method for measuring histamine, norepinephrine, octopamine, normetanephrine, dopamine, sero-

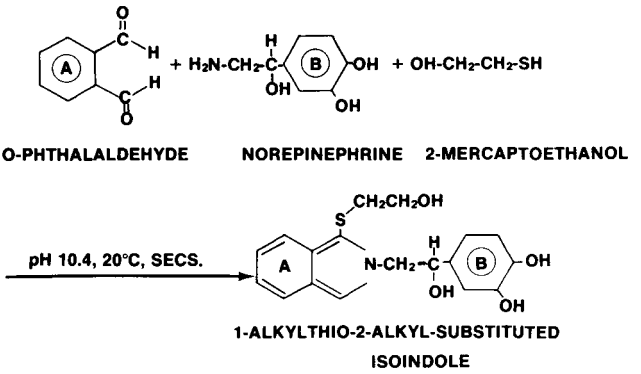


Fig. 1. Formation of isoindole fluorescent adduct from OPT and 2-mercaptoethanol [21].

tonin, and tyramine, at the nanogram level. We are using the method routinely for the chromatographic analysis of biogenic amines in plasma, tissue and urine.

EXPERIMENTAL

Apparatus

A Model 6000A Solvent Delivery System and U6K Universal Injector (Waters Assoc., Milford, Mass., U.S.A.) were used with the HPLC system. The fluorescence detector was a Spectrofluoro Monitor Model FS970 with a 5-μl flow-cell and selectable monochromatic excitation wavelength (Schoeffel, Westwood, N.J., U.S.A.). The recorder was a Fisher Recordall Model 5000 (Houston Instruments, Austin, Tex., U.S.A.). The HPLC columns used were μBondapak phenyl/Porasil and μBondapak C_{18}, 300 × 4 mm I.D. (Waters Assoc.) with a Co:Pell ODS pre-column (Whatman, Clifton, N.J., U.S.A.).

The temperature of the HPLC column was controlled by a constant-temperature circulating water bath Model FJ (Haake, Saddle Brook, N.J., U.S.A.) connected to an aluminum column jacket. The jacket was composed of two aluminum blocks (24 × 7 × 2.2 cm) precisely grooved to accommodate two columns and a thermometer, when bolted together. Each block had two holes (6.0 mm) drilled completely through the block, lengthwise, and fitted with Swagelok fittings and copper tubing to allow the controlled-temperature water to circulate along four sides of the columns before recycling through the bath. The aluminum column jacket blocks were specially designed and made in the University of Missouri (Columbia) Science Instrument Shop.

Peak areas, retention times, relative weight response values, and concentrations based on an internal standard were calculated by a Hewlett-Packard 3352B Laboratory Data System (Hewlett-Packard, Avondale, Pa., U.S.A.).

The samples were prepared for HPLC analysis using all-glass 12-ml PTFE faced screw-cap, round-bottom culture tubes, and graduated centrifuge tubes (KIMAX; Kimble Glass Works, Toledo, Ohio, U.S.A.).

An IEC Clinical Centrifuge Model CL (Damon/IEC Division, Needham Hts., Ma., U.S.A.), rotary shaker Model S-500 (Kraft, Mineola, N.Y.,U.S.A.), and a Corning Model 12 research pH-meter (Corning, Corning, N.Y.,U.S.A.) were used.

A special reagent-grade, Nanopure water system utilizing deionization and reverse osmosis produced all the water necessary to prepare aqueous solutions, eluents and to rinse all glassware.

Chemicals

All reagents used were of highest purity available (A.C.S. certified grade). Monobasic sodium phosphate (A.C.S. certified grade; Fisher Scientific, Fairlawn, N.J., U.S.A.), methanol, glass-distilled (Burdick & Jackson, Muskegon, Mich., U.S.A.), and acetonitrile (HPLC grade; Fisher Scientific), were used to prepare buffers.

Boric acid, 2-mercaptoethanol, and potassium hydroxide (A.C.S. certified

grade; Fisher Scientific, and OPT (Sigma, St. Louis, Mo., U.S.A.) were used in preparing the derivatization reagent.

Sodium metabisulfite, disodium ethylenediaminetetraacetate (EDTA), dibasic sodium phosphate, sodium chloride, perchloric acid (A.C.S. certified and reagent grade; Fisher Scientific), and ethyl acetate (spectrophotometric grade; Aldrich, Milwaukee, Wisc., U.S.A.) were used in the preparation of the samples.

Calibration standards

Histamine, norepinephrine, and octopamine, were purchased from Sigma, dopamine and tyramine from Aldrich and normetanephrine and serotonin from Calbiochem (San Diego, Calif., U.S.A.) and stored in methanol at 4° for daily use.

Buffer preparation

Two stock solutions of NaH_2PO_4 were prepared (25 and 50 mmole/l) in acetonitrile and/or methanol and stored at 5°. Buffers for daily use were then prepared by checking the pH and adjusting if necessary with a few drops of 1 *N* sodium hydroxide or 1 *N* hydrochloric acid. The buffer was then filtered through a 0.22-μm membrane filter and degassed.

Preparation of o-phthalaldehyde reagent

The reagent was prepared by dissolving 0.50 g of boric acid in 19 ml of water using a 50 ml beaker, adjusting the pH to 10.40 ± 0.02 with a 45 g/100 ml KOH solution, and then transferring the solution into a dark glass bottle with a PTFE-lined screw cap. Separately, 17.5 mg of OPT were dissolved in 200 μl of methanol (glass-distilled) using a 5 ml beaker. The OPT solution was added to the borate solution along with 40 μl of fresh 2-mercaptoethanol (A.C.S. reagent grade), and stored under nitrogen at 5°. The reagent was stable for seven working days.

Sample collection and storage

To minimize losses a careful sample collection was necessary. Only acid-cleaned glassware, which was exhaustively rinsed, and PTFE equipment was used in handling the samples.

Plasma. The whole blood was rapidly collected from in-dwelling catheters using a sterile 15-ml syringe which had been rinsed on the inside with sterile saline—heparin solution (100 I.U./ml). The collected blood was then emptied immediately into an ice water chilled 25-ml screw-cap glass centrifuge tube (Scientific Products, St. Louis, Mo., U.S.A.) which contained 1 ml of freshly made EDTA—metabisulfite solution (20 mg/ml EDTA, 10 mg/ml sodium metabisulfite). The tube was capped with a PTFE-lined screw-cap and inverted gently several times to mix the blood and reagent, then brought to a cold room (4°), and immediately spun for 10 min at 4000 *g* . The plasma was removed from the packed cells and measured at the same time with a 10 ml all-glass syringe with a Kel-F ever-hub and 3-inch PTFE tube (Scientific Products). Working in the cold room, the volume of plasma was recorded and made 0.4 mole/l with concentrated perchloric acid and mixed with a vortex

mixer. The tube was allowed to stand in the cold for 15 min to complete perchlorate precipitation of protein. The sample was then centrifuged at 20,000 *g* at 4° for 20 min, the supernatant was removed and immediately frozen and stored at −70° in the dark.

Tissue. Up to 1 g of tissue was homogenized in 5 ml of 0.4 mole/l perchloric acid in a glass tissue grinder submerged in an ice bath. The homogenate was transferred to a 25-ml screw-cap glass centrifuge tube and spun at 30,000 *g* at 4° for 15 min. The supernatant was removed and immediately frozen for storage at −70° in the dark.

Urine. The sample was collected in the presence of sodium metabisulfite (0.5 mg/ml urine) made 0.4 mole/l with concentrated perchloric acid and immediately frozen to −70° in the dark. Prior to analysis it was centrifuged at 5000 *g* for 15 min.

Cleanup of samples for HPLC analysis

The plasma samples were thawed in flowing water at room temperature, mixed thoroughly, and a 2-ml aliquot was pipetted into a 12-ml conical culture tube. The deproteinated sample was then adjusted to a pH of 7.0 ± 0.2 with 0.5 mole/l potassium hydroxide, and immediately derivatized with 400 µl (350 µg) of OPT at pH 10.40 ± 0.02. Two grams of sodium chloride were added to break any emulsion formed during the double extraction with 2 ml of ethyl acetate and to aid in partitioning the derivatives into the EtOAc. The sample was shaken for 1 min during each extraction and spun at 3400 *g* to separate the phases. After extraction the ethyl acetate was partitioned twice with 2 ml of 50 mmole/l dibasic sodium phosphate buffer (pH 10.0 ± 0.1) shaken for 1 min, and spun at 3400 *g*. The final ethyl acetate volume was reduced to 100 µl under a sweep of ultrapure, oxygen-free, dry nitrogen gas and stored in the cold room (4°) until analysis.

Reversed-phase HPLC separation and quantitation of biogenic amines

The ethyl acetate extract containing the derivatized biogenic amines was brought to room temperature, and 10—50 µl were injected and chromatographed with a NaH_2PO_4 buffer (pH 5.10), containing either methanol or acetonitrile for the first elution step and methanol for the second elution step. The chromatography was performed on a 300 × 4 mm I.D. µBondapak phenyl column with a flow-rate of 1.5 ml/min. The derivatives were quantitated by their fluorescence intensity at 340 nm (excitation) and at 480 nm (emission).

The areas under the peaks were integrated and the amount (ng/ml) of each biogenic amine (BA) was calculated based on an internal standard (IS) as follows:

$$\text{Amount of BA (ng/ml)} = \left[\frac{\text{area}_{BA}}{\text{area}_{IS}}\right]_{\text{sample}} \times \left[\frac{1}{RWR_{BA/IS}}\right] \times \left[\frac{ng_{IS}}{ml}\right]_{\text{sample}}$$

$$\text{where } RWR_{BA/IS} = \left[\frac{\text{area}_{BA}}{ng/ml_{BA}} \times \frac{ng/ml_{IS}}{\text{area}_{IS}}\right]_{\text{standard}}$$

The relative weight response (RWR) values for each of the biogenic amines were determined by at least ten independent analyses of calibration standards of the biogenic amines; thereafter, the RWR was determined daily. In the above expression for $RWR_{BA/IS}$, the concentration units are ng/ml (or μg/l).

Peak identification

The biogenic amines were identified on the basis of retention time by comparison with standards and also by co-chromatography of standards in different solvent systems. The biogenic amines were well resolved on the μBondapak phenyl columns, thus the chromatograms are relatively simple and identification was certain. In addition, the identity of norepinephrine and dopamine was confirmed by correlating the values from the trihydroxyindole determination and OPT—HPLC analyses of the same rat brain samples. For further confirmation of chromatographic peaks in plasma, urine and tissue samples, and the identification of unknown peaks, work is in progress to utilize high-resolution mass spectrometry. HPLC fractions are being collected, concentrated and analyzed by this technique.

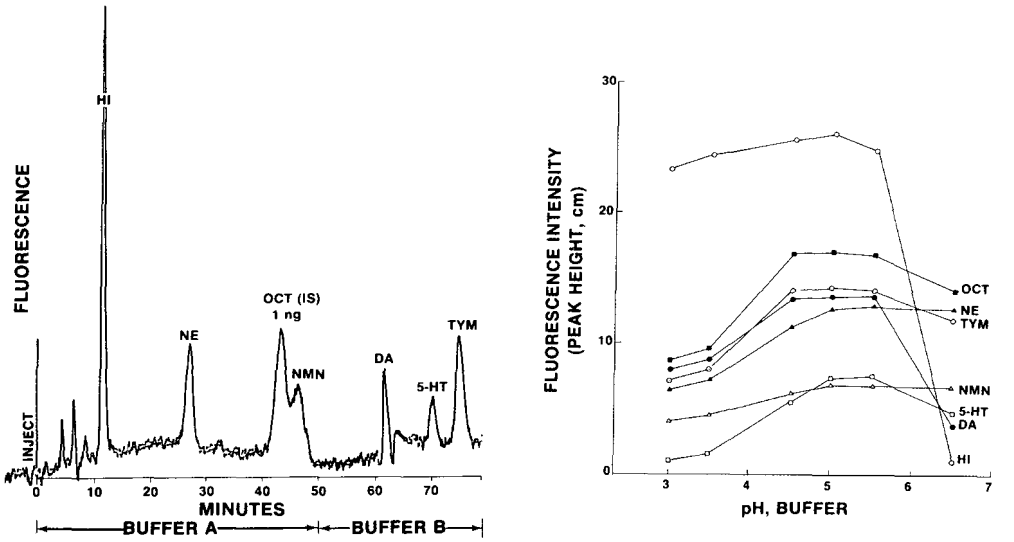
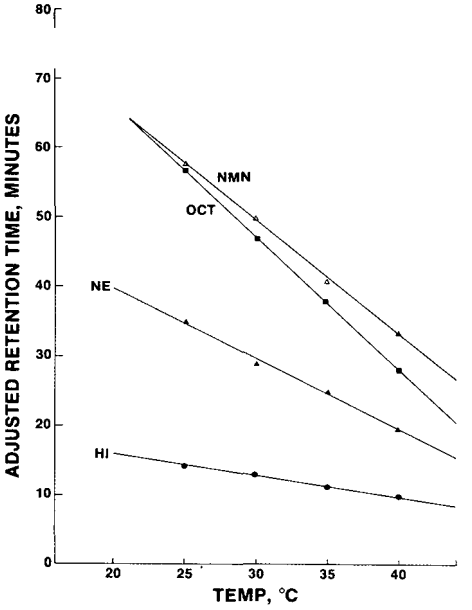


Fig. 2. Reversed-phase HPLC separation of seven biogenic amine standards. Sample: 1.0 μl standards, 500 pg each; column: μBondapak phenyl (300 × 4 mm); buffer: 0.05 mole/l NaH_2PO_4 (pH 5.10) with 320 ml of methanol per l for first elution step (A) and 450 ml of methanol per l for second elution step (B); flow-rate: 1.5 ml/min; detector: Schoeffel FS970, 0.10 μA full-scale, excitation 340 nm, emission 480 nm; temperature: 30°. The internal standard (IS) is octopamine. Peaks: HI, histamine; NE, norepinephrine; OCT, octopamine; NMN, normetanephrine; DA, dopamine; 5-HT, serotonin; TYM, tyramine.

Fig. 3. Effect of eluent pH on fluorescence intensity. This figure demonstrates that maximum fluorescence intensity is obtained at pH 5.0. All other conditions and abbreviations as in Fig. 2.



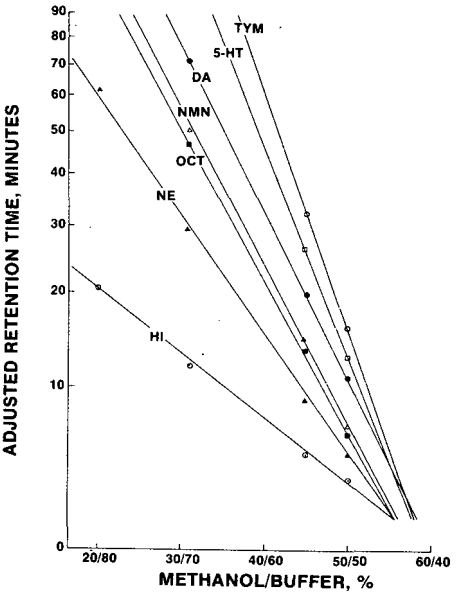


Fig. 4. Effect of column temperature on adjusted retention time. Sample: 10 μl standards, 10 ng each; buffer: 0.05 mole/l NaH_2PO_4 (pH 5.10) with 320 ml of methanol added per liter. All other conditions and abbreviations as in Fig. 2.

Fig. 5. Effect of eluent methanol concentration on adjusted retention time. Abbreviations and all conditions, except methanol concentration of buffers, are the same as in Fig. 2.

TABLE I

RELATIVE WEIGHT RESPONSE OF BIOGENIC AMINES IN HPLC ANALYSIS

Eluent is 50 mmole/l NaH_2PO_4 buffer (pH 5.10) containing 32% methanol for the first elution step and 45% methanol for the second elution step.

Biogenic amine	Retention time (min)*	RWR**
Histamine	13.21	1.676
Norepinephrine	30.41	0.904
Normetanephrine	51.76	0.859
Dopamine	69.33	0.749
Serotonin	77.46	0.437
Tyramine	82.97	1.058
Octopamine (IS)	48.13	1.000

*Uncorrected for void volume (3.0 ml). Pumping rate, 1.5 ml/min.
**RWR compared to IS, octopamine = 1.000. Each value is an average of 10 independent analyses with a relative standard deviation of 2%.

RESULTS AND DISCUSSION

Reversed-phase HPLC analysis of biogenic amines

Optimization of chromatography. Fig. 2 shows the two-step isocratic separation of a standard solution containing 500 pg each of seven biogenic amines in less than 80 min using a μBondapak phenyl column. An internal standard, octopamine, was used to facilitate accurate quantitation of the biogenic amines in plasma. Other internal standards are being investigated for urine because octopamine is indigenous to the sample matrix. The eluent used for each separation is outlined in the legend for the chromatograms. A 300 × 4 mm I.D. μBondapak phenyl column with a 70 × 2 mm I.D. Co:Pell ODS pre-column (Whatman) hand packed with octadecyl-silica particles was used, and a flow-rate of 1.5 ml/min was maintained. The conditions of eluent pH, column temperature, and eluent methanol concentration on separation and fluorescence intensity had been systematically studied to find the optimal conditions (Figs. 3—5).

Minimum detection limit. The high efficiency of the HPLC separation combined with the enhanced sensitivity of fluorescence detection allows an extremely low detection of about 100 pg for all seven of the biogenic amines of a standard mixture, shown in Fig. 2.

Retention times and RWR

The retention times and RWR, compared to the internal standard octopamine, for six biogenic amine derivatives are presented in Table I. The relative weight response, $RWR_{BA/IS}$ values are given for comparative purposes and must be determined in each laboratory. The RWR values represent averages of ten independent analyses with an R.S.D. of less than 2%. The RWR values remained constant over a six-month period.

Stability of OPT derivatives

Fig. 6 demonstrates the stability of the OPT derivative in the ethyl acetate extract (stored at 4°) over time. The good stability of the adduct enables one to prepare samples in advance and to use an auto-injection system for chromatographic analyses.

Precision of HPLC analysis

Biogenic amine standards added to plasma were analyzed with good precision at concentrations comparable to those in samples of tissue and biological fluids (Table II). Repeated injections of 5—10 ng each of the seven biogenic amine derivatives gave an average R.S.D. of less than 3%. The retention time was not affected by the sample matrix, thus excellent precision of retention times for five biogenic amines was obtained in routine analysis over a four-day period for eight different plasma samples (Table III). The R.S.D. ranged from 0.9 to 2.8%. Table IV demonstrates the precision obtained from eight independent analyses of a pooled plasma sample on four different days for an average R.S.D. of 4.3%.

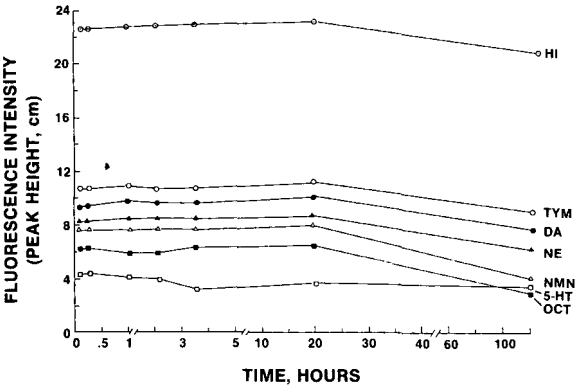


Fig. 6. Stability of biogenic amine OPT derivatives in ethyl acetate at 4°. Abbreviations and all conditions as in Fig. 2.

TABLE II

PRECISION OF RECOVERY FOR BIOGENIC AMINES BY THE EXTERNAL STANDARD METHOD

Each value is the mean of eight or more analyses.

Biogenic amine	Biogenic amines added (ng/ml)*		* Average R.S.D. (%)
	25.0	50.0	
Histamine	54.3**	56.4**	
R.S.D. (%)	5.2	4.0	4.6
Norepinephrine	67.0	69.5	
R.S.D. (%)	5.3	4.1	4.7
Normetanephrine	77.8	75.0	
R.S.D.(%)	3.7	3.2	3.5
Dopamine	75.3	78.0	
R.S.D. (%)	8.6	8.5	8.6
5-Hydroxytryptamine	76.0	74.1	
R.S.D. (%)	7.4	6.2	6.8
Tyramine	68.5	69.0	
R.S.D. (%)	8.6	7.4	8.0

*The standards were added to bovine plasma prior to sample cleanup and HPLC analysis.
**Percentage recovery of biogenic amines added prior to sample cleanup.

Linearity

All seven biogenic amines responded linearly at concentrations ranging from 200 pg to 15 ng injected onto the column (Fig. 7), thus providing a more than adequate range for the analysis of biogenic amine levels found in biological materials.

TABLE III

PRECISION OF RETENTION TIMES OF BIOGENIC AMINE ANALYSIS IN BOVINE PLASMA

Eluent was 32% methanol in 50 mmole/l NaH_2PO_4 buffer for the first elution step, then 45% methanol in 50 mmole/l NaH_2PO_4 buffer (pH 5.1).

	Retention time (min)					
	HI**	NE	OCT (IS)	DA	5-HT	TYM
$\bar{x}$	13.5	30.5	48.7	70.2	81.6	86.2
σ	0.34	0.85	0.58	1.0	0.73	0.86
R.S.D. (%)	2.5	2.8	1.2	1.5	0.9	1.0

*$\bar{x}$ is the average of eight independent analyses.
**Abbreviations of the amines: HI, histamine; NE, norepinephrine; OCT, octopamine; DA, dopamine; 5-HT, 5-hydroxytryptamine; TYM, tyramine.

TABLE IV

PRECISION OF HPLC ANALYSIS FOR BIOGENIC AMINES IN BOVINE PLASMA

Each value is the average of eight independent runs on four different days from a pooled bovine plasma sample.

Biogenic amines	Mean ± σ (ng/ml)	R.S.D. (%)
Histamine	28.5 ± 0.94	3.3
Norepinephrine	2.4 ± 0.08	3.3
Dopamine	1.2 ± 0.05	4.2
Serotonin	14.5 ± 0.90	6.2
Tyramine*	28.5 ± 1.31	4.6

*An unknown peak co-eluted with tyramine and both were integrated together.

Recovery of biogenic amines added to pooled bovine plasma

The recoveries of biogenic amines added to eight 2-ml pooled bovine plasma samples prepared on four different days are shown in Table V. Recoveries were computed by comparing the spiked value to eight 2-ml samples of pooled bovine plasma to which no biogenic amines were added. The difference was calculated as the recovery. Each of the seven standard biogenic amines was added at a level of 25 ng per ml of plasma. The recoveries ranged from 55 to 78% with an R.S.D. of ca. 6%.

Chromatograms of a standard mixture of seven biogenic amines, of a plasma sample, and of an identical plasma sample to which biogenic amine standards were added before analysis, are shown in Fig. 2, 8, and 9, respectively.

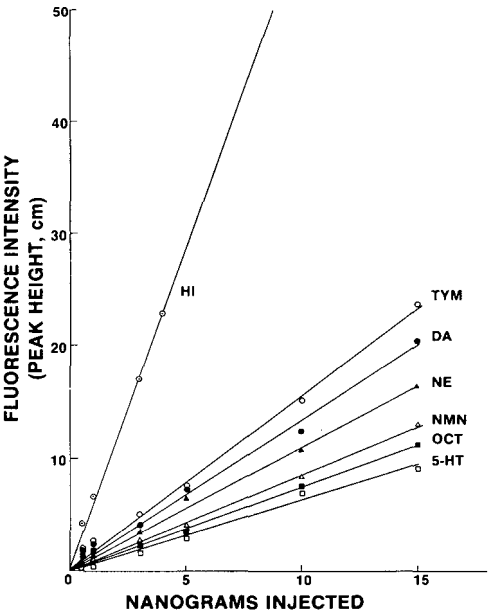


Fig. 7. Linearity of detection for seven biogenic amine standards. Sample: 1.0—15 μl standards, 0.2—15 ng each. All other conditions and abbreviations as in Fig. 2.

TABLE V

ANALYTICAL RECOVERY OF BIOGENIC AMINES ADDED TO POOLED BOVINE PLASMA

Each value is the average of eight independent runs on four different days.

Biogenic amine	Amine (ng/ml)			Average recovery (%)
	Plasma	Plasma + spike*	Spike found	
Histamine	30.0	55.0	13.6	54.4
Norepinephrine	2.0	27.0	16.8	67.2
Normetanephrine	<0.2	25.0	19.4**	77.6**
Octopamine	<0.2	25.0	15.8	63.2
Dopamine	1.0	26.0	18.8	75.2
Serotonin	15.0	40.0	19.0	76.0
Tyramine	30.0***	55.0***	17.0	68.0

*Spike added at a level of 25 ng/ml.
**Average recovery = (19.4 ng found)/(25 ng added) × 100 = 77.6%.
***An unknown peak eluted with tyramine and they were integrated together.

Analysis of biogenic amines in plasma, tissue and urine

In addition, the previously described method has been applied to the routine analysis of plasma samples from control pigs and pigs afflicted with malignant hyperthermia [23]. Chromatograms illustrating the difference in biogenic

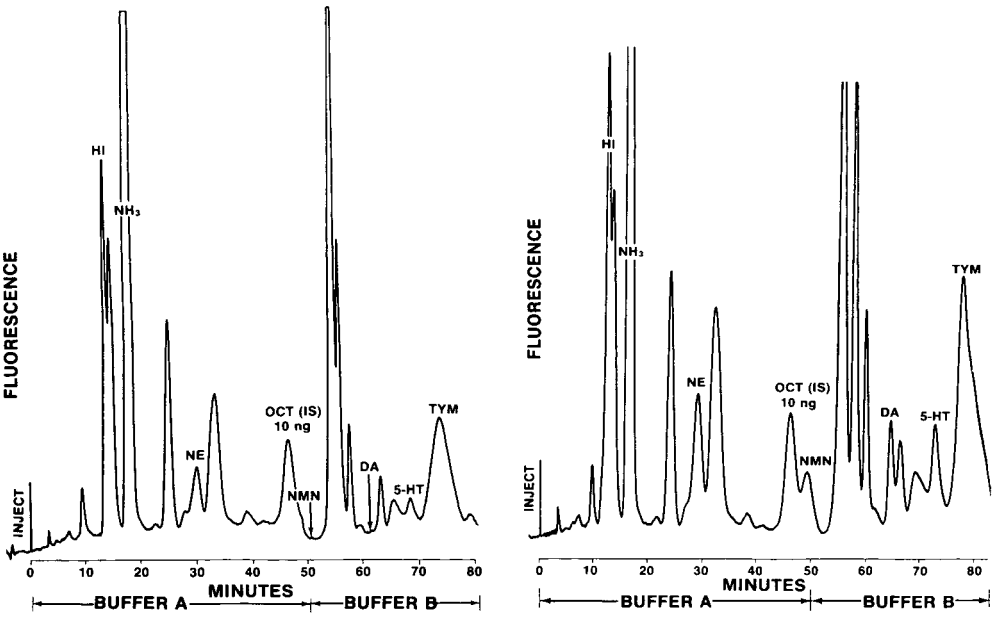


Fig. 8. Reversed-phase HPLC separation of biogenic amines in bovine plasma. Sample: 10 μl, equivalent to 0.2 ml plasma. All other conditions and abbreviations as in Fig. 2.

Fig. 9. Reversed-phase HPLC separation of biogenic amines in bovine plasma with six biogenic amines added. Mixture of biogenic amines shown in Fig. 2 added to bovine plasma shown in Fig. 8 to obtain sample for analysis shown in this figure. Sample: 10 μl, equivalent to 0.2 ml plasma with 5 ng of each biogenic amine. All other conditions and abbreviations as in Fig. 2.

amine levels between control and malignant hyperthermic pigs are shown in Figs. 10 and 11.

Furthermore, the HPLC method has also been applied to quantitating biogenic amines in rat brain tissue (Fig. 12), dog ventricle (Fig. 13), human control urine (Fig. 14), and urine from a patient with a brain tumor (Fig. 15). Note the large difference in the peak heights of the urine sample from the patient with a brain tumor (Fig. 15) and of the control (Fig. 14). Both samples were from 24-h total collections ranging from 1100—1200 ml of urine.

A comparison of the OPT—HPLC method with the trihydroxyindole procedure

Samples of five whole rat brains were analyzed for norepinephrine and dopamine by both the trihydroxyindole and OPT—HPLC procedures. The mean value ± S.E. obtained for the trihydroxyindole procedure was 0.44 ± 0.06 μg/g (range, 0.33—0.60 μg/g) for norepinephrine and 0.89 ± 0.01 μg/g (range 0.86—0.92 μg/g) for dopamine. The mean value ± S.E. determined by the HPLC method was 0.6 ± 0.09 μg/g (range 0.54—0.93 μg/g) for norepinephrine, and 1.02 ± 0.12 μg/g (range 0.67—1.2 μg/g) for dopamine. When the values for norepinephrine and dopamine obtained by both methods were analyzed by linear-regression statistics the slope of the linear least-squares line was 1.02,

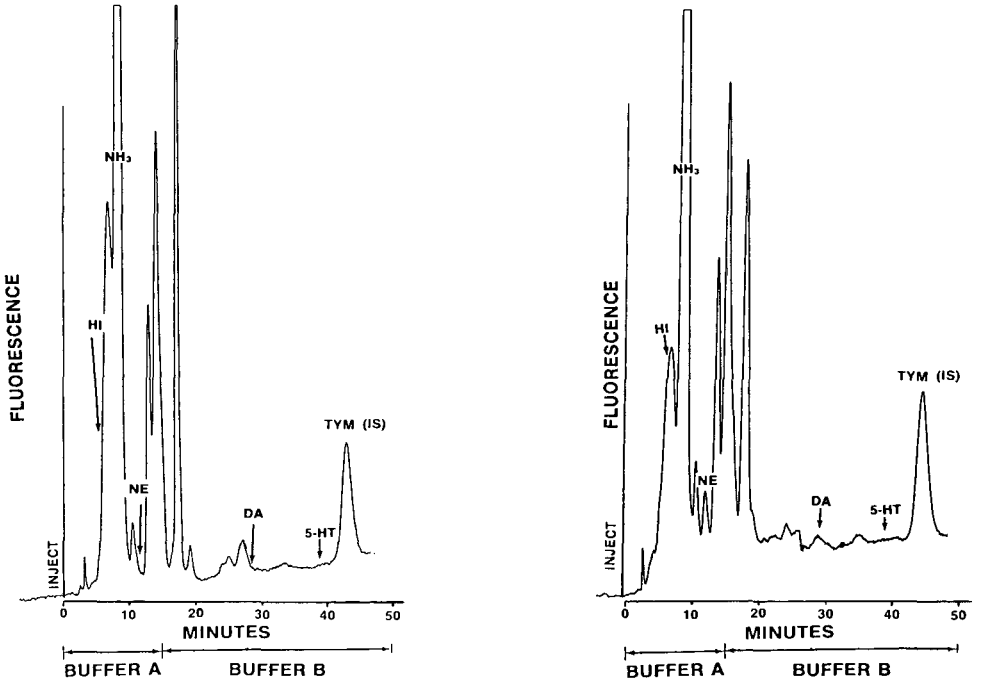


Fig. 10. Reversed-phase HPLC separation of biogenic amines in plasma from a control pig under halothane anesthesia. Sample: 10 μl, equivalent to 0.20 ml plasma; column: μBondapak phenyl (300 × 4 mm); buffer: 0.025 mole/l NaH_2PO_4 (pH 5.10), with 250 ml acetonitrile added per liter for first elution step (A) and 450 ml of methanol added per liter for second elution step (B); flow-rate: 1.5 ml/min; detector: Schoeffel FS970, 0.10 μA full-scale, excitation 340 nm, emission 480 nm; temperature: 26°. IS is tyramine because no indigenous tyramine was detected in the pig plasma. Abbreviations as in Fig. 2.

Fig. 11. Reversed-phase HPLC separation of biogenic amines in plasma from a malignant hyperthermic pig under halothane anesthesia. Sample: 10 μl, equivalent to 0.20 ml plasma. All other conditions as in Fig. 10. Abbreviations as in Fig. 2.

the intercept was 0.15, and the coefficient of correlation was 0.7. The low correlation may be due to the poor precision and recovery from the cation-exchange resin cleanup prior to the trihydroxyindole analysis.

Eluent composition and separation of biogenic amines

Prior to our separation studies on the μBondapak phenyl column, extensive research was done to separate the biogenic amines by isocratic elution using a μBondapak C_{18} column.

The effect of the organic solvent in the eluent mixture on the separation is demonstrated in Figs. 16 and 17. We found that under optimal temperature conditions using only a methanol and phosphate buffer mixture, an adequate separation appeared possible (Fig. 16). Norepinephrine seemed to be identified correctly by co-injection and dopamine co-eluted with a larger peak. By adding N,N-dimethylformamide (5%, v/v) to the eluent (methanol—phosphate

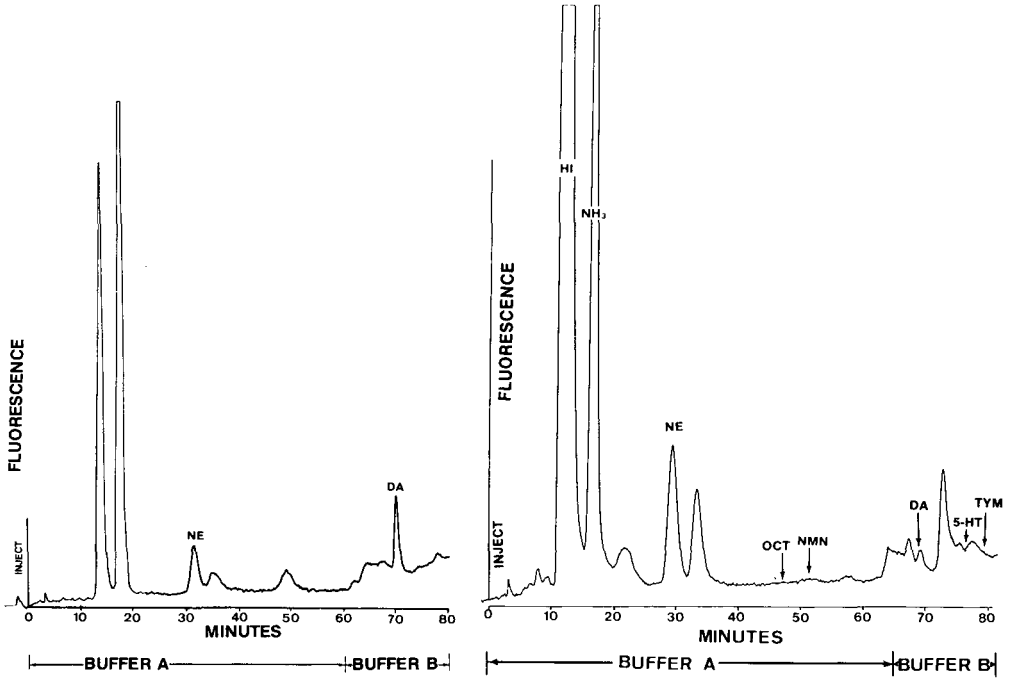


Fig. 12. Reversed-phase HPLC separation of biogenic amines in rat brain tissue after Bio-Rex 70 cleanup. Sample: 10 μl, equivalent to 12.5 mg whole brain. Samples were quantitated by external standard. The first elution step (A) consisted of 300 ml of methanol per liter. All other conditions and abbreviations as in Fig. 2.

Fig. 13. Reversed-phase HPLC separation of biogenic amines in dog heart tissue. Sample: 10 μl, equivalent to 15.1 mg heart. All other conditions as in Fig. 12. Abbreviations as in Fig. 2.

buffer) it was found, based on co-injection of a norepinephrine standard, that the previously symmetrical norepinephrine peak was not homogeneous (Fig. 17). Similarly, an improvement of the selectivity for norepinephrine was observed when a mixture of 40% (v/v) methanol 10% (v/v) acetonitrile in 0.05 *M* phosphate was used as the eluent.

Since norepinephrine, normetanephrine, dopamine and 5-hydroxytryptamine were not retained sufficiently on the C_{18} column for a good separation from interfering primary amines, we investigated the more selective and polar reversed-phase μBondapak phenyl column. This column proved to be far superior in efficiently and selectively separating the biogenic amines in various biological materials, after studying the effects of eluent composition and temperature.

Our work has shown that the following molecules are removed by the cleanup or do not chromatograph under the present experimental conditions: polyamines, N-acetylserotonin, melatonin, amino acids, *p*-methoxybenzylamine, 5-methoxytryptamine, 3-methoxytyramine, 6-hydroxydopamine, DOPA, phenylethylamine and 3-methoxy-DOPA.

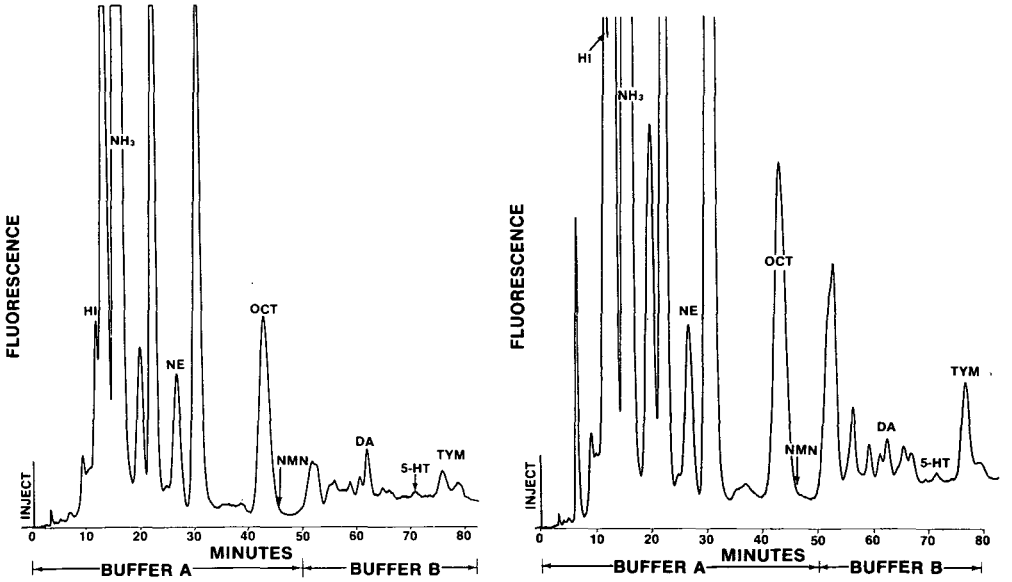


Fig. 14. Reversed-phase HPLC separation of biogenic amines in control urine. Sample: 15 μl, equivalent to 15 μl urine. All other conditions and abbreviations as in Fig. 2 except that no IS was used.

Fig. 15. Reversed-phase HPLC separation of biogenic amines in urine of brain tumor patient. Sample: 15 μl, equivalent to 15 μl urine. All other conditions and abbreviations as in Fig. 2 except that no IS was used.

Experiments are underway to collect the major unknowns that are chromatographed and identify them by high-resolution mass spectrometry. These unknowns change in response to experimental treatments and disease and thus may indicate important biochemical relationships.

CONCLUSIONS

The reversed-phase partition mode of HPLC with fluorescence detection and pre-column derivatization with OPT for separation provides a rapid, highly sensitive, simple and quantitative method for the simultaneous analysis of many biogenic amines at the subnanogram level.

The pre-column derivatization with OPT proved very advantageous by stabilizing the labile molecules, aiding in their extraction with organic solvent and allowing for sensitive HPLC fluorescence detection at the subnanogram level. The classical aluminum oxide or weak cation-exchange resin cleanup methods can also be used (Fig. 12) if only two to three biogenic amines need quantitating and if the recoveries are adequate at physiological levels.

The use of a two-step gradient elution, with the μBondapak phenyl column decreased analysis time since the more strongly retained compounds eluted earlier than by isocratic elution. In addition, the more polar phenyl column efficiently separated the biogenic amines from the sample matrix, while the

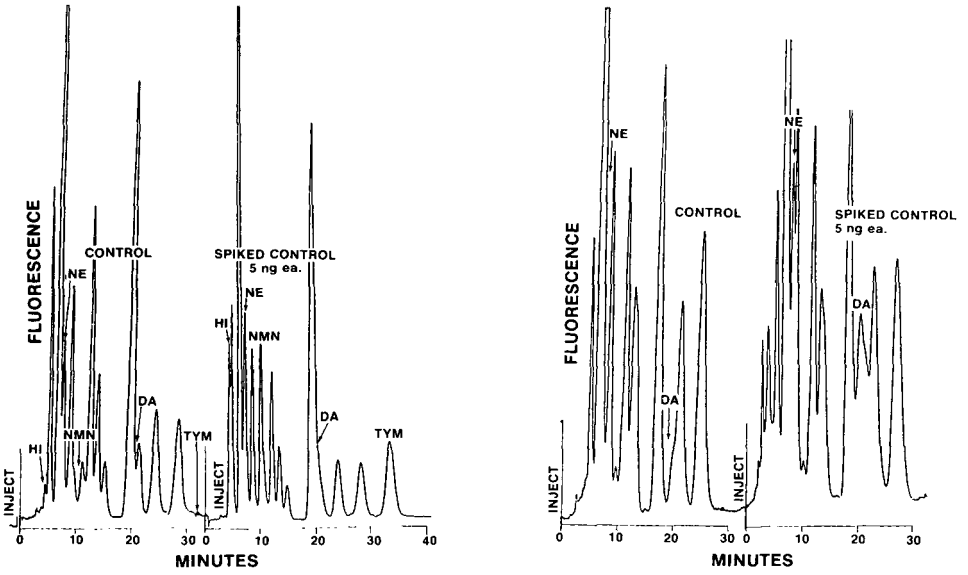


Fig. 16. Effect of eluent composition on separation of biogenic amines in plasma. Sample: 5 μl, equivalent to 0.05 ml plasma; column: μBondapak C_{18} (300 x 4 mm); eluent: 0.05 mole/l NaH_2PO_4 (pH 5.10) with 480 ml of methanol per l; flow-rate: 1.5 ml/min; temperature: 35°. Abbreviations as in Fig. 2.

Fig. 17. Effect of eluent composition on separation of biogenic amines in plasma. All conditions as in Fig. 16 except eluent, made with 450 ml of methanol and 50 ml of DMF per l; and temperature: 25°. Abbreviations as in Fig. 2.

less-selective C_{18} column did not. Even complex mixtures of organic solvents and buffers did not satisfactorily improve the separation on the C_{18} column.

This HPLC chromatographic method is now used routinely in our laboratory. Instead of quantitating only two or three biogenic amines in a single sample we are able to measure seven biogenic amines in one analysis. In addition, eight to ten well-separated yet unidentified primary amines appear in the chromatograms, which could have physiological significance. The method provides a powerful research and clinical tool for studying various diseased states in both man and animals.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF DOPAMINE SULFOCONJUGATES IN URINE AFTER L-DOPA ADMINISTRATION*

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SUMMARY

A procedure was developed for the separation and determination of dopamine-3-O-sulfate (DM-3-S) and dopamine-4-O-sulfate (DM-4-S) in the urine of subjects administered L-DOPA. The method consists of sample preparation using cation- and anion-exchange resins followed by determination of the sulfates by high-performance liquid chromatography. The addition recoveries were 96 ± 2.9% (S.D.) for DM-3-S and 93 ± 3.0% (S.D.) for DM-4-S. Twenty samples could be measured per day. When every 2-h urine specimen from normal subjects was analysed after L-DOPA administration (0.5 g), the maximum excretion of each sulfate was observed in the second 2-h specimen. For the first 6 h 7.5 ± 1.5% (S.D.) of the administered L-DOPA was excreted as DM-O-sulfates. During this time, the ratio of DM-4-S to the DM-O-sulfates was 11.7 ± 0.58% (S.D.).

INTRODUCTION

From the results of acid and enzyme hydrolysis, the main conjugate of dopamine (DM) in urine had been thought to be O-sulfate [1—5]. Jenner and Rose [6] first identified the conjugates as DM-3-O-sulfate (DM-3-S) and DM-4-O-sulfate (DM-4-S), which were excreted in the urine of Parkinsonian patients with the intake of high doses of L-DOPA, an immediate precursor of DM. They also reported that a rat brain preparation sulfated DM to DM-3-S and DM-4-S in the ratio 1.7:1, whereas rat liver supernatant produced mainly DM-3-S, suggesting the importance of separate determination of these conjugates [7]. The method they used for the estimation of the sulfates in urine was time-consuming and tedious. Recently, Bronaugh et al. [8] developed a method to

*Part of the work was presented at the 97th Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, 1977.

estimate the conjugates by high-performance liquid chromatography (HPLC). However, the sample preparation procedure they used required more than 12 h and the separation of DM-3-S from DM-4-S by HPLC seemed incomplete even with a 2-m column of Zipax SAX (DuPont), and the sensitivity of the detection was low.

In this paper we report on a simpler, more rapid and sensitive method for the measurement of DM-3-S and DM-4-S in urine. The method consists of isolating the conjugates from urine by passing the urine successively through small columns of cation and anion exchange resin, and the quantitation of the conjugates by using silica gel HPLC to separate them. The method was applied to the estimation of the conjugates in the urine of normal subjects who were administered 0.5 g of L-DOPA orally.

EXPERIMENTAL

Materials

Dowex 50W-X8 (200—400 mesh; Dow Chem., Midland, Mich., U.S.A.) and Dowex 1-X8 (200—400 mesh) were used. DM-3-S and DM-4-S were synthesized according to the method of Jenner and Rose [7]. Wakogel LC-5K (spherical silica gel for HPLC, 5 μm, Lot No. LDM4300) was the gift from Wako (Osaka, Japan). All the other chemicals were of reagent grade.

Administration of L-DOPA and collection of urine

Subjects were 9 normal men (from 22 to 35 years; the mean age was 25.8 years). The subjects were obliged to fast after the last evening meal until 1 p.m. The first morning urine was discarded. Then some water was taken, and at 9 a.m. the first urine (0-h urine) was collected and 0.5 g of L-DOPA (Larodopa: Hoffman-La Roche) was administered orally. 2, 4 and 6 h later, every 2-h urine was collected and designated 0—2 h, 2—4 and 4—6 urine, respectively. To each sample were added 50 mg of ascorbic acid and 25 mg of EDTA-2Na to preserve catecholamines. The urines were stored at −20°.

Sample preparation for HPLC

The urine sample was thawed and centrifuged at 1000 *g* for 5 min. One ml of the supernatant was applied to a Dowex 50W (H+) column (6.4 cm × 1.0 cm I.D.) in a cold room at 6°, and the column was washed with 4 ml of chilled water. The effluent and the washings were discarded. Then a column (1.1 cm × 0.5 cm I.D.) of Dowex 1 (Cl^-) was connected to the bottom of the Dowex 50W column. A 25-ml volume of chilled water was added to the top of the Dowex 50W column to elute the DM-O-sulfates from both the columns. The eluate from the Dowex 1 column was rotary evaporated under a reduced pressure by a vacuum pump at 30°. The resultant residue was redissolved in 100 μl of water and transferred to a conical tube (Eppendorf Reactionsgefaesse 3810, 4 cm × 0.9 cm I.D.). The tube was centrifuged at 1300 *g* for 3 min. Five μl of the supernatant were analysed by HPLC.

HPLC of the conjugates

Wakogel LC-5K, spherical silica gel, was packed in a 33 cm × 2 mm I.D.

glass tube (Kyowa Seimitsu, Tokyo, Japan) by suspending the gel in water. Water was circulated through a jacket surrounding the column by a water-bath circulator (Model BT-35, Yamato Scientific) to maintain a temperature of 30°. A mobile phase, acetonitrile—3% aqueous ammonia (pH 10.0 by HCl) (85:15, v/v), was pumped through the column at a rate of 0.31 ml/min by a high-speed pump (Mitsumi Scientific Model SF-0396-57). Samples were applied via the injection port at the top of the column while the flow was stopped. The effluent from the column was monitored by a spectrophotometric detector (Shimadzu Model SPD-1, or Hitachi Model 634-0513) for absorbance at 277 nm, recorded with an Ohkura desk-top recorder.

RESULTS

The mobile phase for HPLC was chosen on the basis of a preliminary experiment for the separation of catecholamine-O-sulfates (3- and 4-O-monosulfates of DM, norepinephrine and epinephrine) on a silica gel thin layer [9]. The resolution of the peaks varied with the injection volume, and a volume of 5 μl was found to be sufficient for general use (Fig. 1). The peak height in absorbance was proportional to the amount of the conjugate from 50 ng (Fig. 2) to 5 μg.

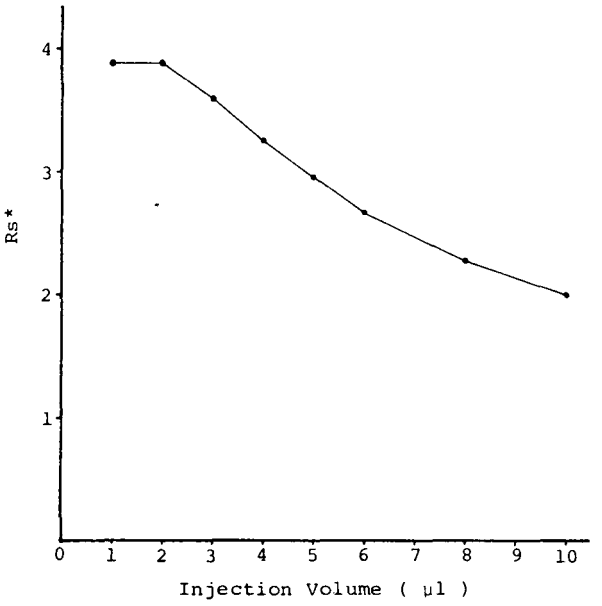


Fig. 1. Effect of injection volume on the resolution of dopamine-O-sulfates in HPLC. *Resolution value (Rs) was represented by the equation:

$$Rs = \frac{\text{interval between two dopamine-O-sulfate peaks}}{\text{mean band width of two dopamine-O-sulfate peaks}}$$

Conditions: column, Wakogel LC-5K (33 cm × 2 mm I.D.); eluent, acetonitrile—3% aqueous ammonia (pH 10.0 by HCl) (85:15, v/v); flow-rate, 0.31 ml/min; temperature, 30°.

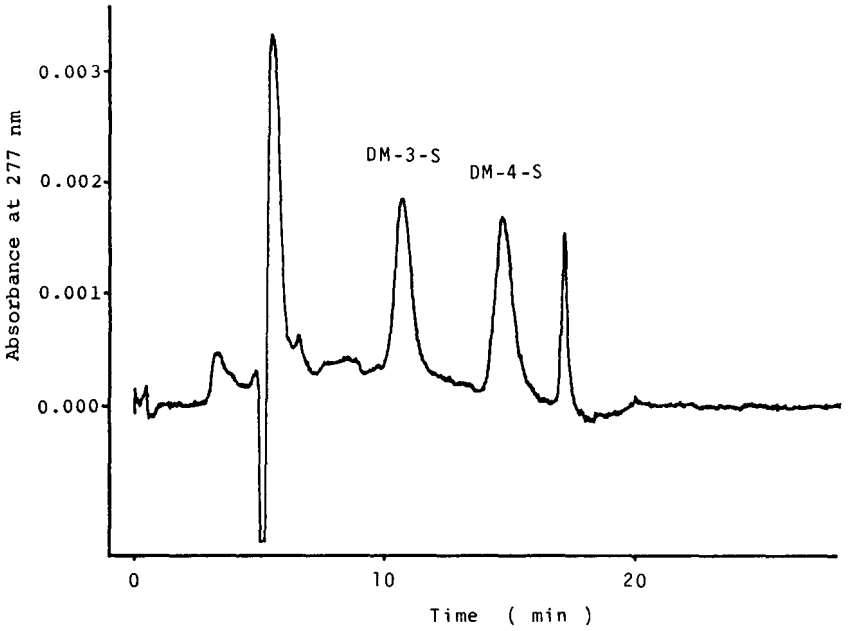


Fig. 2. High-performance liquid chromatogram of standard dopamine-O-sulfates. Five μl containing 50 ng each of dopamine-3-O-sulfate (DM-3-S) and dopamine-4-O-sulfate (DM-4-S) were injected and the eluate from the column was monitored by a Shimadzu spectrophotometric detector Model SPD-1. Other conditions are the same as in Fig. 1. The peaks other than those of DM-3-S and DM-4-S observed in the chromatogram were caused by the water used as the solvent for the standard dopamine-O-sulfate solutions.

Other constituents of urine, together with metabolites of L-DOPA, DM and dihydroxyphenylacetic acid (DOPAC), interfered when the sample urine was analysed directly. A Dowex 50W (H^+) column and a Dowex 1 (Cl^-) column removed these constituents through the sample preparation procedure. In order to protect the sulfates from hydrolysis on the column of Dowex 50W, a strong cation-exchange resin, chilled water was used for the elution.

Recoveries through the whole procedure of DM-3-S (25 or 50 μg) and DM-4-S (5 or 10 μg) added to 1 ml of urine were 96 ± 2.9% (S.D.) and 93 ± 3.0% (S.D.), respectively; these values were used for correction purposes.

The chromatogram obtained from the 2—4 h urine of a subject after the intake of 0.5 g of L-DOPA is depicted in Fig. 3. The accuracy of the amount of DM-3-S or DM-4-S estimated by HPLC was confirmed by the coincidence of the amounts in the DM-O-sulfate fractions on HPLC determined by two methods: (i) rechromatography of sulfate fractions, and (ii) fluorometric determination [10, 11] of liberated DM after acid hydrolysis of the O-sulfates (Fig. 4).

DM-3-S was detected in the 0—2 h, 2—4 h and 4—6 h urine; the largest amount was observed in 2—4 h urine in all cases, as shown in Fig. 5. Almost the same patterns were obtained for DM-4-S in a smaller amount (Fig. 6).

During 6 h, 6.6 ± 1.3% (S.D.) of orally administered L-DOPA (0.5 g) was excreted as DM-3-S in urine of normal subjects tested, while 0.9 ± 0.2% (S.D.) was excreted as DM-4-S. The ratio of DM-4-S to total DM-O-sulfates excreted in urine was 12.4 ± 0.56% (S.D.) for 0—2 h urine, 11.4 ± 0.69% (S.D.) for 2—4 h urine and 11.4 ± 0.63% (S.D.) for 4—6 h urine (the mean value during 6 h was 11.7 ± 0.58%).

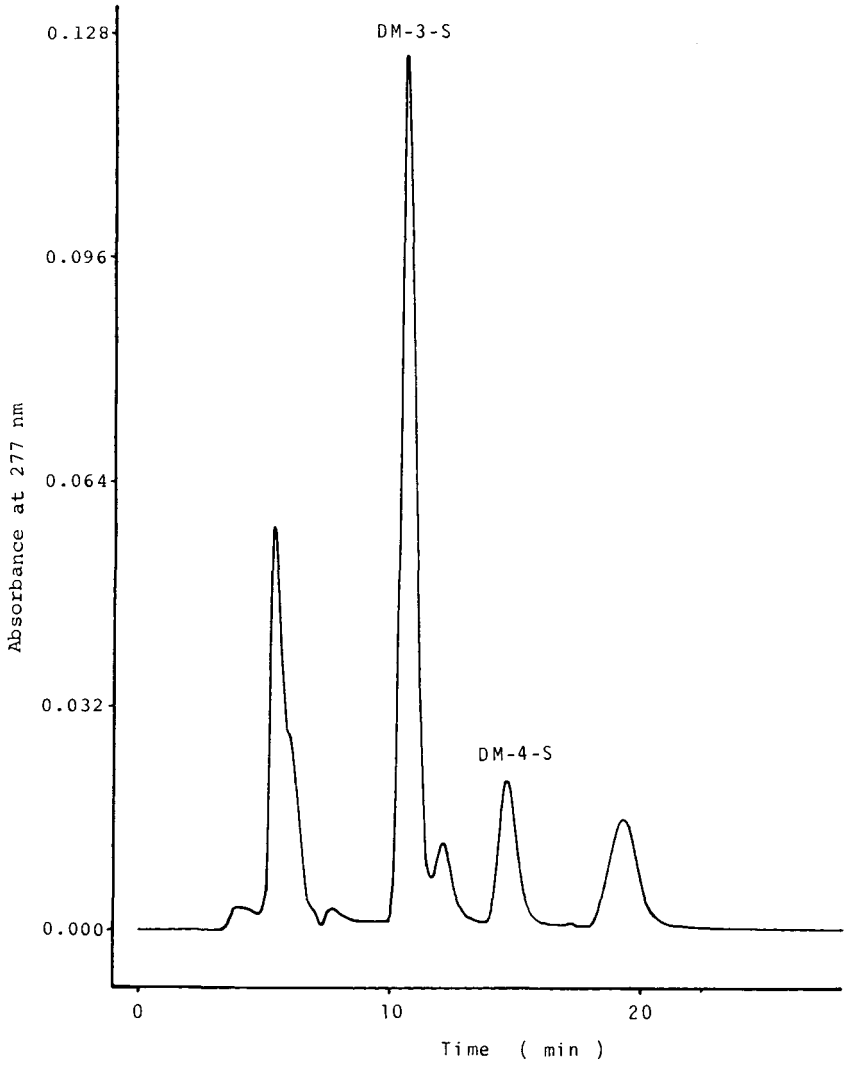
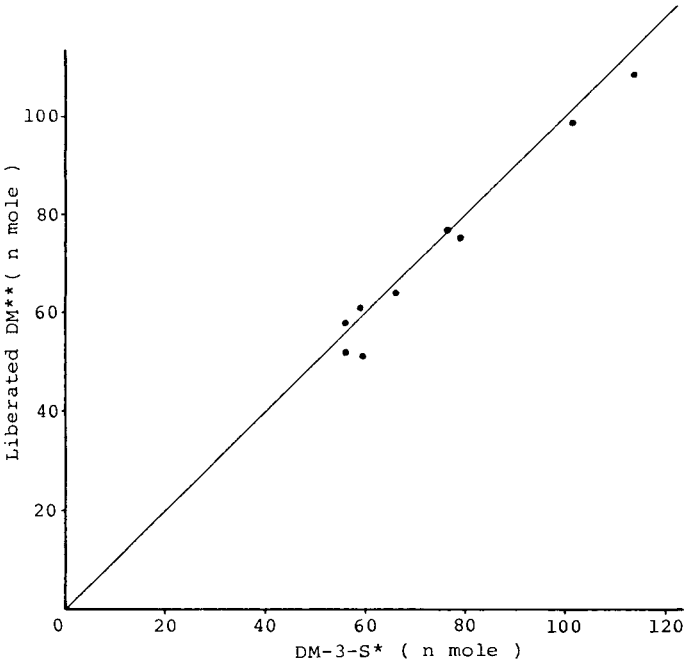


Fig. 3. High-performance liquid chromatogram of a urine sample. Injection: 5 μl of the sample preparation from the 2—4-h urine of a subject after administration of 0.5 g of L-DOPA. The conditions for HPLC were the same as in Fig. 2.



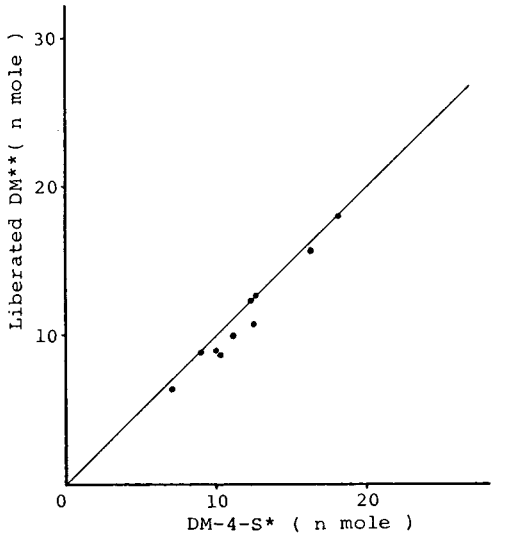


Fig. 4. Correlation between the amounts in the dopamine-O-sulfate fractions in HPLC determined by two methods. The fractions corresponding to each DM-O-sulfate in HPLC were collected after injection of 5 μl of several urine samples chosen at random. The collection was duplicated if necessary. Each fraction was evaporated and dissolved in 100 μl of water. *The amount of DM-O-sulfate in this solution was determined by the present HPLC method. **An aliquot of the solution was added to 1 ml of 0.1 *N* hydrochloric acid containing ascorbic acid (0.1 mg/ml) and EDTA·2Na (0.05 mg/ml), heated at 100° for 30 min, and the liberated dopamine was determined fluorometrically [10, 11]. The results were corrected for the efficiency of the hydrolysis procedure obtained by using standard dopamine-O-sulfate. The lines drawn in the figures represent the theoretical correlation.

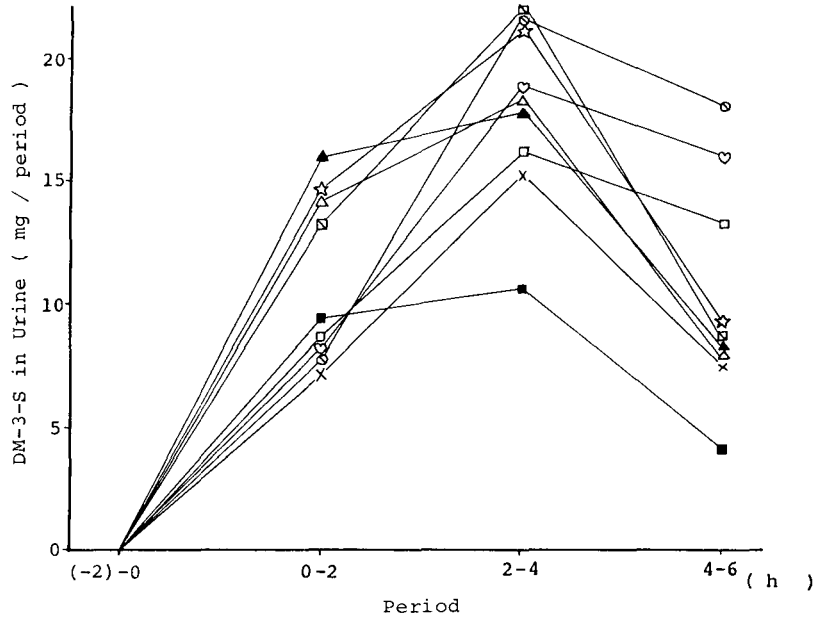


Fig. 5. Excretion of dopamine-3-O-sulfate after administration of 0.5 g of L-DOPA. The various symbols represent the individuals from whom samples were obtained.

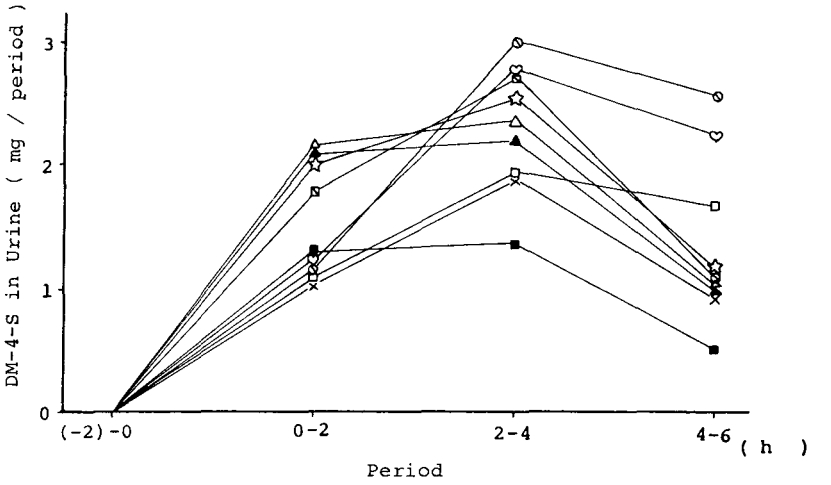


Fig. 6. Excretion of dopamine-4-O-sulfate after administration of 0.5 g of L-DOPA. Symbols are the same as in Fig. 5.

DISCUSSION

To determine urinary DM-O-sulfates in patients administered L-DOPA, Jenner and Rose [6] added ^{35}S-labeled DM-O-sulfates to 50 ml of urine sample for the correction of recoveries, concentrated the sample by lyophilisation, and separated the sulfates by 60-h elution on a Dowex 1 (CH_3COO^-) column (35 cm × 1.4 cm I.D.). They lyophilised 100 ml of each fraction of the sulfates, hydrolysed with acid and measured liberated DM by the trihydroxyindole

method. The method of Bronaugh et al. [8] is also tedious and time-consuming, as described in our introduction.

In the present work, high resolution and sensitive detection of the two DM-O-sulfates was achieved by silica-gel HPLC. Impurities of the peaks from urine were proved to be easily removed by sample clean-up on small columns. Consequently, only 1 ml of urine and 3.5 h were required for the whole procedure. The columns for sample preparation were easily made and regenerated, and when ten pairs of these columns were simultaneously used, as many as twenty samples were analysed per day.

So far as it has been investigated, the present method is simple, rapid, sensitive, accurate and reproducible and therefore suitable for the routine assay of DM-3-S and DM-4-S in urine of subjects administered L-DOPA.

In the present work the time course of urinary excretion of the conjugates after L-DOPA administration was first investigated, and DM-3-S was found to be the major sulfate of DM even in normal subjects. The higher ratio of DM-4-S to total DM-O-sulfates excreted in 0—2 h urine than in 2—4 h urine found for all subjects might be caused by the difference in DM-O-sulfate-producing organs [7]. The ratio of DM-4-S to total DM-O-sulfates during the 6 h following administration of L-DOPA was 11.7 ± 0.58% (S.D.), demonstrating the small range of variation in normal subjects. The ratios of 24-h urine of Parkinsonian patients reported by other workers were quite different from each other (16.3% by Jenner and Rose [6] and 4.9% by Bronaugh et al. [8]). It would be of interest to study the time course of urinary excretion of these DM-O-sulfates from Parkinsonian patients using the present method.

ACKNOWLEDGEMENTS

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Biomedical Applications

CHROMBIO. 231

RAPID SEPARATION OF DNA CONSTITUENTS, BASES, NUCLEOSIDES AND NUCLEOTIDES, UNDER THE SAME CHROMATOGRAPHIC CONDITIONS USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH A REVERSED-PHASE COLUMN

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SUMMARY

Four components of three sets of DNA constituents, bases, deoxyribonucleosides and deoxyribonucleoside 5'-monophosphate, were sufficiently resolved under one set of chromatographic conditions using high-performance liquid chromatography with a reversed-phase column (Zorbax ODS) and the solvent 0.4 *M* $NH_4H_2PO_4$ (pH 3.5). The effect of pH and salt concentration in the solvent on the retention of these compounds in the column was thoroughly investigated. Proportionality of peak height to the content, and reproducibility and recovery of the four bases were satisfactory under appropriate conditions and as little as 1 μg of DNA could be analysed for its base composition by this method.

INTRODUCTION

A high-performance liquid chromatographic (HPLC) procedure was developed for analysis of nucleic acid bases, nucleosides and nucleotides [1]. In most cases, a cation-exchanger resin is employed for the separation of bases or nucleosides, and an anion exchanger is used for nucleotides. It is desirable to separate these nucleic acid constituents using a simple chromatographic system with a single column and a single solvent. Quantitative analysis of both nucleotides and nucleosides in a mixture of enzymatically degradated products of DNA is a necessary step for studying the mode of action of deoxyribonuclease. Simultaneous separation of components of these two sets of DNA constituents, if possible, would be especially useful for the determination of nucleoside composition, terminal nucleoside and chain length of DNA fragments produced by the action of deoxyribonuclease.

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Ho and Gilham [2] and Asteriadis et al. [3] reported a simultaneous separation of nucleotides and nucleosides with a polystyrene anion-exchange column using a solvent containing ethanol. But their procedure is rather tedious and time-consuming. Reversed-phase HPLC on a column of a quaternary ammonium derivative on polychlorotrifluoroethylene beads (RPC-5) was also used to separate mononucleotides and nucleosides in the sequence analysis of tRNAs [4,5], but the separation of nucleosides was not satisfactory even under the best conditions for nucleotides.

In order to simplify the routine work and to reduce the necessary time for analysis of these DNA constituents, we established an ideal chromatographic system with one reversed-phase column consisting of a porous silica containing a covalently bonded octadecyl hydrocarbon (Zorbax ODS) and one solvent.

MATERIALS AND METHODS

Standard samples

All nucleic bases, deoxyribonucleosides and deoxyribonucleoside 5'-monophosphate used in this study were purchased from Sigma (St. Louis, Mo., U.S.A.). These were all dissolved in 0.1 *N* HCl. The following millimolar extinction coefficients were used to check the true concentration of the standard solution of bases for the quantitative determination [6]: adenine, 13.2 at 262.5 nm; cytosine, 10.0 at 276 nm; guanine, 11.4 at 248 nm and thymine, 7.9 at 264.5 nm. Radioactive bases, [2-^{14}C]thymine, [8-^{14}C]adenine, [8-^{3}H]-guanine sulphate and [2-^{14}C]cytosine sulphate, were obtained from The Radiochemical Centre (Amersham, Great Britain) and purified once by chromatography as described in this article.

Chromatographic procedure

A Shimadzu—DuPont Model LC-1 high-performance liquid chromatograph equipped with a prepacked stainless-steel column of Zorbax ODS (25 cm × 2.1 mm I.D.) was used throughout this study. Routinely, the column was run at a pressure of 140 kg/cm^2 and a temperature of 40°. The flow-rate of solvent through the column was maintained at 0.6 ml/min under such conditions. The solvent of a certain concentration of ammonium phosphate was prepared by dissolving $NH_4H_2PO_4$ (analytical grade, Wako, Osaka, Japan) in water at the desired concentration and the pH was adjusted by the addition of concentrated phosphoric acid. The sample was applied to the column with the aid of a microsyringe (Hamilton, 801NR) and a septum injection valve interrupting the flow of the eluent momentarily. The effluent was monitored at 260 nm with a Shimadzu model SPD-1 spectrophotometric detector.

Digestion of DNA

DNA from the sperm of the Pacific salmon (*Oncorhynchus keta*) was extracted by treatment with sodium dodecyl sulphate—phenol [7] and purified by successive treatment with ribonuclease A (Boehringer, Mannheim, G.F.R.) [8] and Pronase (Calbiochem, Los Angeles, Calif., U.S.A.) [9]. The DNA was hydrolysed with formic acid in a sealed tube (3 mm I.D.) according to the method of Bendich [10].

Radioactivity measurement

An aliquot (0.5—1.0 ml) of the column eluant with 0.2 ml of distilled water was mixed with 6 ml of PCS^{TM} (Amersham—Searle, Arlington Heights, Ill., U.S.A.) in a counting vial. Radioactivity was measured in a Searle Analytical Model Mark II liquid scintillation counter and the quenching was corrected by the sample-channel ratio method.

RESULTS

Analytical conditions

Fig. 1 illustrates a typical separation of authentic samples of DNA bases, deoxyribonucleosides and deoxyribonucleoside 5'-monophosphate on the Zorbax ODS column with a single solvent system of 0.4 *M* ammonium phosphate (pH 3.5). Four components in three sets of DNA constituents were adequately resolved by this chromatographic system.

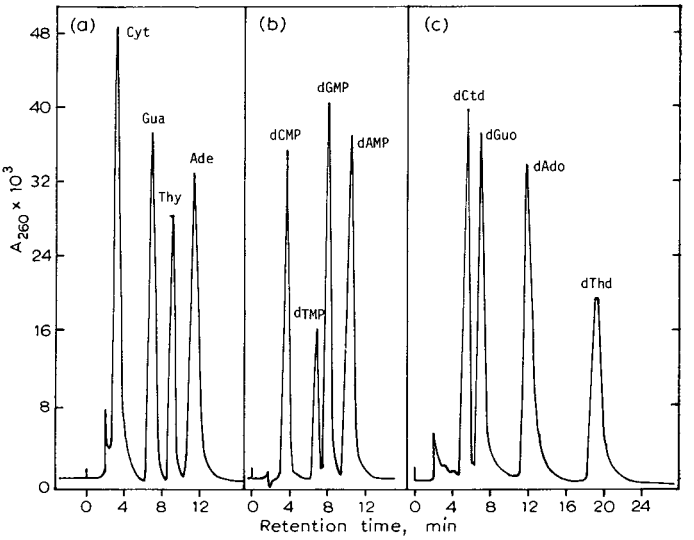


Fig. 1. Separation of: (a) DNA bases, (b) deoxyribonucleoside 5'-monophosphates, and (c) 2'-deoxyribonucleosides, on a Zorbax ODS column (25 cm × 2.1 mm I.D.) with 0.4 *M* ammonium phosphate (pH 3.5) as the solvent, under the conditions described in Methods. Of each compound, 1.0 nmole was applied to the column.

Fig. 2a shows the effect of the solvent pH (0.2 *M* ammonium phosphate) on the elution of DNA bases. The elution position of thymine and cytosine was not changed within the pH range of 2.5—4.0, whereas the retention of guanine and adenine was considerably affected by the pH. Adenine particularly had a higher retention time as the pH increased. Fig. 2b and c indicate the effect of the pH on the resolution of nucleosides and nucleotides. The elution of dAdo*

*Abbreviations and symbols: Ade=adenine; Thy=thymine; Gua=guanine; Cyt=cytosine; dAdo=2'-deoxyadenosine; dThd=thymidine; dGuo=2'-deoxyguanosine; dCyd=2'-deoxycytidine; dAMP=2'-deoxyadenosine 5'-monophosphate; dTMP=thymidine 5'-monophosphate; dGMP=2'-deoxyguanosine 5'-monophosphate; dCMP=2'-deoxy 5'-monophosphate.

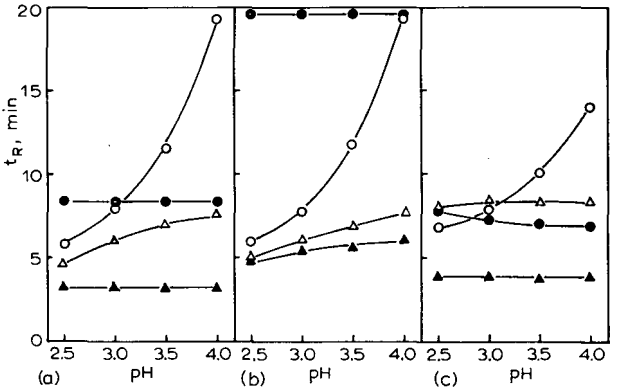


Fig. 2. Effect of pH of solvent (0.2 *M* $NH_4H_2PO_4$) on the separation of: (a) bases, (b) deoxyribonucleosides and (c) deoxyribonucleoside monophosphate. In (a): ○, Ade; ●, Thy; △, Gua; ▲, Cyt. In (b): ○, dAdo; ●, dThd; △, dGuo; ▲, dCyd. In (c): ○, dAMP; ●, dTMP; △, dGMP; ▲, dCMP.

and dAMP from the column was increasingly delayed as the pH increased. Other nucleosides and nucleotides were maintained at almost the same elution position within the pH range examined.

Fig. 3 depicts the effect of concentrations of ammonium phosphate in the solvent (pH 3.5). The resolution of four bases and four nucleosides was not satisfactory when the solvent of low salt concentration was used. As the concentration of ammonium phosphate was increased, Ade and dAdo were eluted more rapidly from the column, but dThd, dAMP, dCMP and dTMP were eluted more slowly. The retention of the other compounds was not affected by the change of the salt concentration in the solvent.

Increase of the pressure applied to the column caused a rapid separation of these materials without a significant change of resolution, within the range 130—150 kg/cm^2. Increasing the column temperature decreased the retention of all the bases on the column with a good separation.

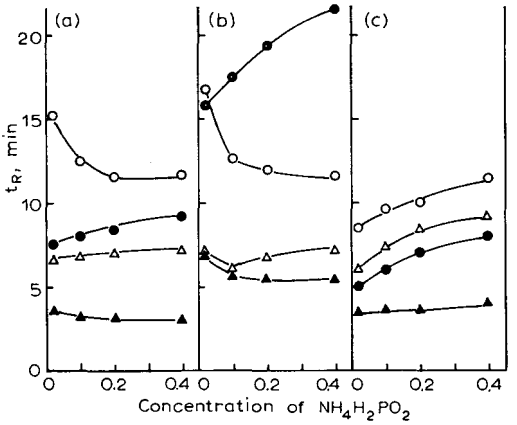


Fig. 3. Effect of $NH_4H_2PO_4$ concentration in solvent (pH 3.5) on the separation of: (a) bases, (b) deoxyribonucleosides and (c) deoxyribonucleoside monophosphate. The symbols are as in Fig. 2.

Reproducibility

The reproducibility of retention was examined by repeated analysis of the standard mixture of the four authentic bases of DNA under the same conditions. The peak positions of each base was fairly reproducible, as seen in Table I, except for Ade, the retention time of which was gradually reduced as the amount applied to the column was increased. Graphs of peak height vs. concentration were found to be linear for each base over the concentration range of interest, as shown in Fig. 4. Since measurement of peak width at half height was difficult, the peak height was chosen for quantitative calculation.

The recovery rate of each base after chromatography was measured by the use of radioactive bases. Table II indicates that the recoveries of bases were

TABLE I

REPRODUCIBILITY OF RETENTION TIME (t_R)

Results represent the mean ± S.D. of five experiments. C.V.= Coefficient of variation.

Base	nMoles	t_R (min)	C.V. (%)
Cyt	0.2	3.20 ± 0.038	1.2
	2.0	3.24 ± 0.048	1.5
	10.0	3.20 ± 0.052	1.6
Gua	0.2	7.00 ± 0.060	0.9
	2.0	7.16 ± 0.080	1.1
	10.0	6.88 ± 0.080	1.2
Thy	0.2	9.04 ± 0.080	0.9
	2.0	9.28 ± 0.088	1.0
	10.0	9.12 ± 0.12	1.1
Ade	0.2	12.04 ± 0.056	0.5
	2.0	11.80 ± 0.092	0.8
	10.0	10.72 ± 0.19	1.8

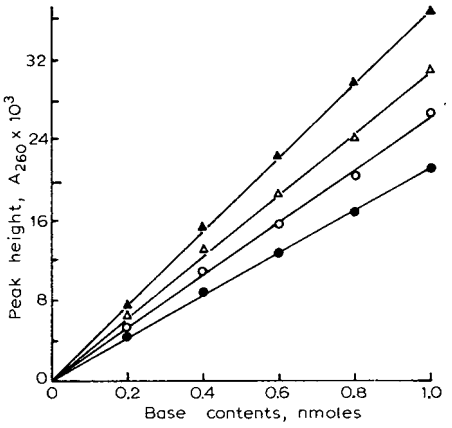
TABLE II

RECOVERY OF BASES

Results represent the mean disintegrations per minute ± S.D. of five experiments. C.V.=coefficient of variation.

Base	Amount applied (dpm)	Amount recovered (dpm)	Recovery (%)	C.V. (%)
[^{14}C]Cytosine	5567 ± 84	5286 ± 44	95.6 ± 0.81	0.85
[^{3}H]Guanine	16519 ± 480	14719 ± 1112	89.2 ± 6.7	7.5
[^{14}C]Thymine	9146 ± 127	7806 ± 164	85.4 ± 1.8	2.1
[^{14}C]Adenine	6807 ± 116	6432 ± 341	90.0 ± 4.5	5.4

between 85 and 95%. The radiochromatogram of each base in Fig. 5 shows that trace amounts of Thy, Gua, and Ade appeared near the position of Cyt and that Ade was eluted with slight tailing.



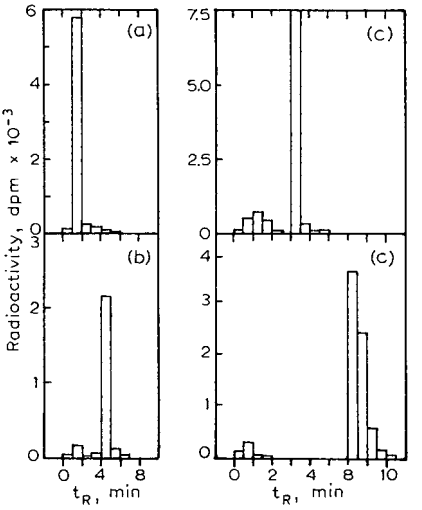


Fig. 4. The relationship between peak height and content of bases. The symbols are the same as in Fig. 2.

Fig. 5. Examination of elution profiles of bases by radiochromatography. (a) [^{14}C]Cytosine, (b) [^{3}H]guanine, (c) [^{14}C]thymine and (d) [^{14}C]adenine were eluted respectively by HPLC and fractions of the eluant at 1-min intervals were collected and the radioactivity measured in a liquid scintillation counter.

TABLE III

ANALYSIS OF BASE COMPOSITION OF SALMON SPERM DNA

Data represented by the mean ± S.D. of five or more determinations. Numerals in parentheses indicate the coefficient of variation (%).

Amount of DNA examined (μg)	Base composition (%)			
	Cyt	Gua	Thy	Ade
1	20.4 ± 0.65 (3.1)	21.4 ± 0.82 (1.3)	29.9 ± 1.68 (5.6)	28.3 ± 1.86 (6.5)
5	20.6 ± 0.34 (1.6)	20.9 ± 0.12 (0.5)	29.7 ± 0.00 (0.0)	28.9 ± 0.46 (1.5)
20	21.4 ± 0.07 (0.3)	21.0 ± 0.04 (0.1)	30.0 ± 0.04 (0.1)	27.5 ± 0.10 (0.3)

TABLE IV

COMPARISON OF ANALYTICAL RESULTS OF BASE COMPOSITION OF SALMON SPERM DNA

Results are expressed as percentage base composition.

Chromatography	Cyt	Gua	Thy	Ade
Zorbax ODS column	21.4	21.0	30.0	27.5
Cation-exchange column*	21.0	20.8	30.3	28.0
Paper partition**	20.0	21.5	30.4	27.6
Paper partition***	20.4	20.8	29.1	29.7

*From Ref. 12

**From Ref. 13

***From Ref. 14

Base composition of salmon sperm DNA

The base composition of salmon sperm DNA was analysed by this method after formic acid digestion in a small-bore glass tube. The result obtained from different amounts of the DNA showed that as little as 1 μg of DNA could be analysed for its base composition using this method (Table III).

DISCUSSION

Four components in each group of three sets of DNA constituents were readily separated in a simple chromatographic system consisting of one reversed-phase column and a single solvent using a high-performance liquid chromatograph. The Zorbax ODS column proved to be suitable for these purposes. We attempted to separate eight components of nucleotides and nucleosides simultaneously by this system. But the present results revealed that the separation of dGuo from dTMP and dAdo from dAMP were not satisfactory. An improved resolution would be expected by a system with a longer column or employing gradient elution.

The chromatographic behavior of Ade, dAdo and dAMP was somewhat different from that of the others. The retention time of these three compounds was greatly increased as the pH of the solvent increased. Of these, dAMP was found to have a somewhat shorter retention time than the other two. The retention of only Ade and dAdo was reduced as the ammonium phosphate concentration increased, the retention of dAMP and other compounds not being affected or being slightly increased. Another peculiarity of Ade in chromatographic elution was that its retention decreased as the amount applied to the column increased. We could not explain the reason for these phenomena. Singhal and Cohn [11] observed a similar behaviour of adenosine on cation-exclusion chromatography on an anion-exchange column (Aminex A-25); that is, adenosine appears nearer the front as the pH of solvent (0.01 *M* ammonium

formate) is lowered. They concluded that this is due to its positive charge and its purine nature.

The elution profiles of bases traced by radioactivity indicated that small amounts of Gua, Thy and Ade were inclined to spill near the position of Cyt and that Ade eluted with tailing. These would be reasons for the somewhat lower recoveries of these compounds. Moreover, that this method would inevitably result in a slightly higher content of Cyt in DNA than the other methods may not be excluded.

According to this method at least 1 μg of DNA can be analysed for its base composition as shown in Table III. Our previously published results for the base composition of salmon sperm DNA are presented together with the data obtained by the present study in Table IV. The data in the second row of the table were obtained from DNA of the same origin as used in the present study, but the DNA used by Chargaff et al. [14] was from sperm of Atlantic salmon (*Salmo salar*), a different species from our source, and that of Darlington and Randall [13] was of commercial origin, the source of which is unknown. Although the methods and the respective origins of DNA are different, the results are in good accord with each other.

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CHROMBIO. 282

ION-EXCHANGE CHROMATOGRAPHIC SEPARATION AND FLUOROMETRIC DETECTION OF GUANIDINO COMPOUNDS IN PHYSIOLOGIC FLUIDS

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SUMMARY

A high-performance liquid chromatographic procedure has been developed for the separation and fluorometric detection of guanidino compounds in physiologic fluids. All guanidino compounds were separated on a 17 × 0.46 cm cation-exchange column using a stepwise pH gradient. The chromatographic system was designed to enable the use of the specific reagent 9,10-phenanthrenequinone as a means of monitoring the guanidino compounds of physiologic fluids. This new analytical method is so sensitive that it enables the analysis at the picomole level. Our automatic guanidino-compound analyzer was successfully applied to the quantitative determination of all guanidino compounds in physiologic fluids from normal controls and uremic patients.

INTRODUCTION

Guanidino compounds such as methylguanidine [1—5] and guanidino succinic acid [3, 6—8] have been demonstrated to be elevated significantly in the sera of uremic patients compared with normal controls. The analysis of these guanidino compounds has been carried out using the colorimetric method after separation with ion-exchange [5, 9—13], paper [14—17] and thin-layer chromatography [18] and electrophoresis [19]. These methods require a large amount of time and effort, and have the limitation that only some guanidino compounds can be analyzed. Guanidino compounds have also been determined by gas—liquid chromatography [20—26]; however, this method involves a complex preparation procedure for derivatizing the volatile compounds before injection into the gas-chromatographic column. It has recently been reported that the automatic quantitative analysis of the various guanidino compounds in plasma has been performed with a modified automatic amino-acid analyzer [27—30]. However, this analytical method is still time-consuming and the colorimetric determination used lacks sensitivity.

The purpose of this study was to develop a rapid and sensitive method for the quantitative analysis of all guanidino compounds in physiologic fluids. A high-performance cation-exchange chromatographic procedure has been developed for separating all guanidino compounds in physiologic fluids, and detecting these compounds fluorometrically by utilizing the fluorophor that is formed when guanidino compounds react with 9,10-phenanthrenequinone. 9,10-Phenanthrenequinone has been reported to form a fluorescent product with mono-substituted guanidines in alkaline media [31]. The reagent has been used for manual fluorometric assay of arginine, arginine peptides and mono-substituted guanidines and it has been demonstrated to be more sensitive than the Sakaguchi reaction.

EXPERIMENTAL

Chemicals

The standard samples of guanidinosuccinic acid, guanidinoacetic acid, guanidinopropionic acid, guanidinobutyric acid, 4-guanidinobenzoic acid, arginine, creatine, creatinine, guanidine hydrochloride and methylguanidine hydrochloride were obtained from Sigma (St. Louis, Mo., U.S.A.). α-Amino-β-guanidinopropionic acid hydrochloride was purchased from Pierce (Rockford, Ill., U.S.A.). 9,10-Phenanthrenequinone was obtained from Tokyo Chemicals (Tokyo, Japan). N,N-Dimethylformamide was purchased from Nakarai Chemicals (Kyoto, Japan).

Eluent and reagent solutions

The separation conditions for guanidino compounds were studied using buffer solutions in the pH range 3—11, and alkali solutions, which will be described later. The buffer solutions established as eluents are listed in Table I. The buffers were prepared in 4-l batches to ensure reproducibility during the analyses. The pH of each eluent was adjusted with either concentrated HCl or 1.0 *N* NaOH. 9,10-Phenanthrenequinone is unstable in alkaline solution and insoluble in water, so it is necessary to prepare it in an organic solvent such as methanol or dimethylformamide. A 2.5 m*M* solution of 9,10-phenanthrenequinone was prepared by dissolving the reagent in dimethylformamide. The

TABLE I

COMPOSITION OF BUFFERS

Reagent	Buffers			
	First	Second	Third	Fourth
pH	3.35	4.90	8.10	
Na^+ concentration (*N*)	0.20	0.20	0.20	0.50
Sodium citrate $2H_2O$ (g/l)	19.60	19.60	19.60	0
Hydrochloric acid (36%) (ml/l)	12.4	6.3	0	0
Boric acid (g/l)	0	0	0.50	0
Sodium hydroxide (g/l)	0	0	0	20.0
Pentachlorophenol (ml/l)	0.2	0.2	0.2	0

reagent solution was stored in a refrigerator at 5° to prevent degradation of the reagent. A 2.0 *N* NaOH solution was prepared in 4-l batches to ensure accuracy during the analyses.

Chromatographic system

Studies on the reaction of guanidino compounds with 9,10-phenanthrenequinone were first carried out by a manual procedure to establish optimal conditions for reaction time, alkaline concentration, concentration of reagent and other variables. These findings were used in the design of the automated 9,10-phenanthrenequinone detection system. Fig. 1 is a schematic diagram of the automatic guanidino-compound analyzer which was constructed. Three minipumps served to pump the eluent, the alkaline solution and 9,10-phenanthrenequinone solution through the system. An 8-port motor valve was inserted between the four buffer chambers and the eluent pump. The eluent selecting valve was controlled by the electric programmer of the analyzer through a relay, and allowed any one of the eluents from the buffer chambers

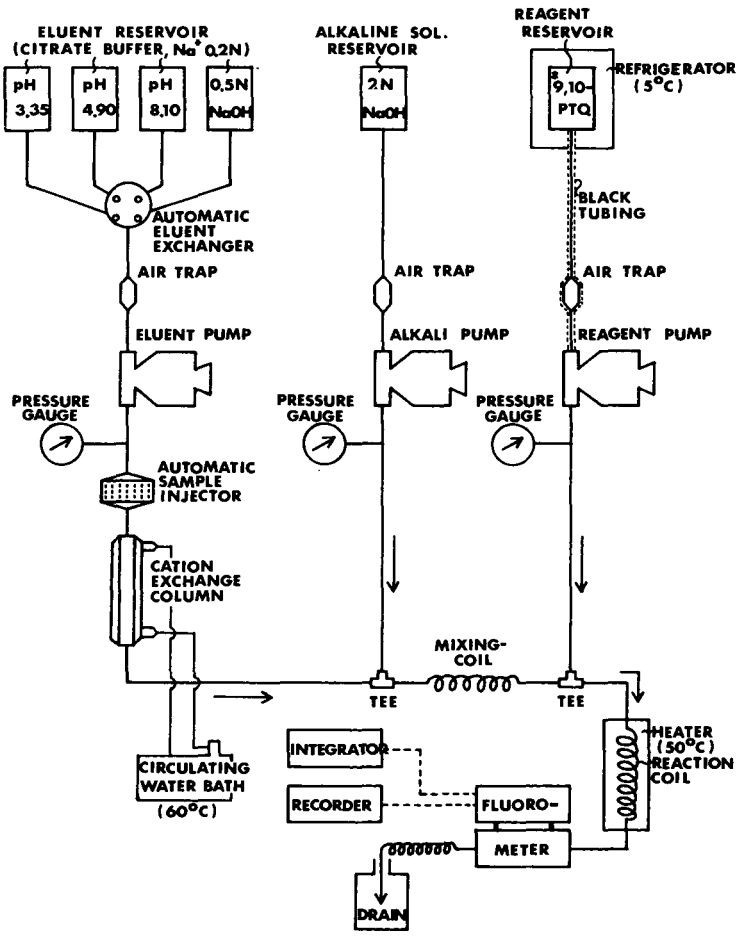

Fig. 1. Schematic diagram of automatic guanidino-compound analyzer.

to be supplied to the eluent pump. The eluent was pumped through an automated sample injection valve supplied with a 100-μl sample loop. A jacketed, stainless-steel column, 17 × 0.46 cm I.D., was used in this investigation. The column was slurry packed with CK-10-S cation-exchange resin with a mean particle size of 11.5 μm (Mitsubishi Kasei Co., Tokyo, Japan). The PTFE tubing connecting the reagent reservoir to the reagent pump was covered with black tubing to prevent exposure of the reagent to light. The column was operated at 60° using a Haacke constant-temperature circulator. The column effluent was first mixed with a stream of 2 *N* NaOH by means of a mixing coil. The alkalinated stream was then fed to another tee-junction where the 9,10-phenanthrenequinone reagent stream was introduced. The mixture flowed through a reaction coil, 800 × 0.05 cm I.D. where it was heated to 50°. At a total flow-rate of 48 ml/h the reaction coil provided approximately 2 min for dwell time.

An FP-100 fluorescence photometer (Japan Spectroscopic Co., Tokyo, Japan) was used to detect the fluorescence components eluting from the column. The excitation lamp was a high-pressure mercury lamp which emits strong 365 nm light. Visible light emitted from the light source was blocked by a primary filter; visible light above 460 nm emitted from the flow-cell passed through a secondary filter and impinged on the photosensitive elements of a photocell. The chromatogram was recorded with a strip chart recorder (Rikadenki, Tokyo, Japan). The chromatographic peak areas were determined with a SIC Model 500A digital integrator (Scientific Instruments, Tokyo, Japan) with baseline display which was connected between the fluoromonitor and the recorder.

Operation of the chromatograph for analysis

The chromatographic operation is controlled automatically and requires a minimum of operator effort. Table II describes the sequence of events for the high-performance liquid chromatographic (HPLC) analysis.

The guanidino compounds were separated on a cation-exchange resin using a stepwise pH gradient. The first buffer was pumped through the column for 11 min after the introduction of the sample, the second buffer for 32 min, the third buffer for 18 min and the fourth buffer for 33 min. Seven samples of deproteinized physiologic fluids were loaded in 100-μl sampling loops of the injection valve and automatically introduced into the analytical column at an appropriate time. The column was operated at a flow-rate of 24 ml/h and a column inlet pressure of 30—50 kg/cm^2. An alkalinating reagent was pumped into the mixing coil at a flow-rate of 12 ml/h. The 9,10-phenanthrenequinone reagent solution was pumped into the reaction coil at a flow-rate of 12 ml/h. As mentioned previously, the reaction coil was heated up to 50°, the optimum temperature for forming the fluorescent 9,10-phenanthrenequinone derivatives of the guanidino compounds for fluorescence measurement (see later).

Preparation of physiologic fluid samples

Serum and cerebrospinal fluid samples from normal controls and from uremic patients were used for the analysis of guanidino compounds. A 1-ml aliquot from each serum sample was centrifuged at 170 *g* for 2 h in a CF-

TABLE II

SEQUENCE OF EVENTS IN OPERATION OF AUTOMATIC GUANIDINO-COMPOUND ANALYZER

Time (min)	Events
	The main switch is turned on.
	Seven samples are loaded in the sample loops.
	The eluent pump, the alkaline pump and the reagent pump are started.
	The first eluent is pumped through the column.
000	The automatic programmer is started.
001	The repeat program is turned on. The first eluent continues.
002	The sample is introduced into the column. The integrator is started.
013	The eluent-selecting valve is switched to the second eluent.
045	The eluent-selecting valve is switched to the third eluent.
063	The eluent-selecting valve is switched to the fourth eluent.
100	The eluent-selecting valve is switched to the first eluent.
110	The integrator prints out the retention times, peak heights, peak areas and so on. The first eluent continues.
140	The programmer is automatically recycled, or the repeat programmer is turned off.
142	The alkaline flow-line is washed with water and the reagent flow-line washed with dimethylformamide.
200	The main power is turned off.

25 Centriflo® membrane [32] (Amicon, Lexington, Mass., U.S.A.), yielding approximately 0.5 ml of ultrafiltrate. A 0.1-ml aliquot of the deproteinized ultrafiltrate was applied to the column of the chromatographic system. The cerebrospinal fluid sample was used directly for analysis without any deproteinization treatment.

RESULTS

Effect of pH on the capacity factor of guanidino compounds

The effect of the pH of a 0.2 *N* sodium citrate buffer on the capacity factors of different guanidino compounds is shown in Fig. 2. For the guanidino compounds that have their pK_a values within the studied pH range, a significant drop of retention is expected with increasing pH as these solutes become less cationic. The capacity factors of guanidinosuccinic acid and guanidinoacetic acid decreased markedly around pH 3.0—3.5 and were constant above pH 5.0. The values of k for guanidinopropionic acid, creatinine and guanidinobutyric acid showed a steep decrease around pH 5.0—6.0 and were constant above pH 7.0. For guanidino amino acids such as α-amino-β-guanidinopropionic acid and arginine, a significant decrease in k was observed at a higher pH of 8.0 and 10.0, respectively. Methylguanidine and guanidine are strongly basic, and so are strongly adsorbed by the column and not eluted with the buffers studied. With a strong alkaline solution, such as 0.4 *N* or stronger NaOH, they are eluted in a reasonable time.

From these data, it was determined that a stepwise pH gradient should be adopted for the separation of a series of these guanidino compounds in a single run. Furthermore, the pH values of the buffer eluents for the gradient elution were determined using these data.

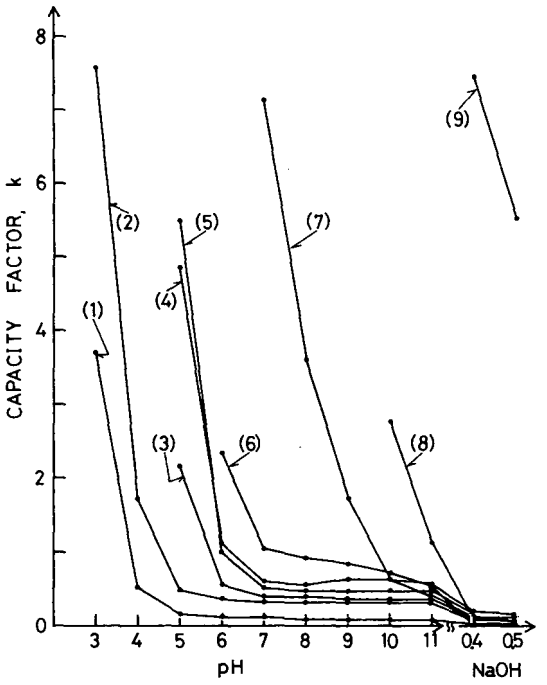

Fig. 2. Dependence of capacity factor k upon pH and alkaline concentration of eluent. (1) GSA; (2) GAA; (3) GPA; (4) CRN; (5) GBA; (6) 4-GBA; (7) α-A-β-GPA; (8) ARG; (9) MG.

Fluorescence properties of 9,10-phenanthrenequinone derivatives of guanidino compounds

9,10-Phenanthrenequinone derivatives of guanidino compounds show a fluorescence maximum around 495—525 nm and an excitation maximum around 370—386 nm. The effect of temperature on the reaction of guanidino compounds with 9,10-phenanthrenequinone was studied in the range 20—90° using a chromatographic system. Fig. 3 shows a plot of the fluorescence intensity as a function of reaction-coil temperature after passing through the coil for 2 min. The degree of fluorescence conversion of all guanidino compounds increased with increase in reaction-coil temperature up to a maximum, and then decreased at higher temperatures with increase in reaction temperature. This decrease is assumed to result from a partial degradation of the reagent or products at higher temperatures. The optimum temperature for fluorescence conversion of guanidinoacetic acid and methylguanidine is 50°, whereas that for α-amino-β-guanidinopropionic acid and guanidinosuccinic acid is 60° and for creatinine, 4-guanidinobenzoic acid, arginine and guanidinobutyric acid 70°

From these data it appeared that a temperature of 50—60° should be chosen for coil operation as a compromise between speed of fluorescence conversion, sensitivity and stability of the fluorescent derivatives formed. A temperature of 50° was tentatively chosen for coil operation for the rest of the analysis.

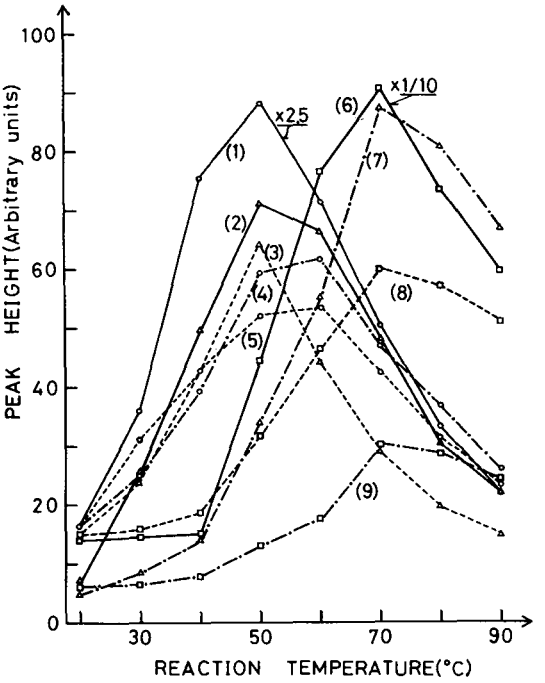

Fig. 3. Effect of reaction temperature on fluorescence conversion of guanidino compounds. (1) GAA; (2) MG; (3) α-A-β-GPA; (4) GPA; (5) GSA; (6) CRN; (7) 4-GBA; (8) ARG; (9) GBA.

Chromatographic separation and quantitative response

Fig. 4 shows a typical separation of a standard mixture of guanidino compounds, each at a concentration of 2.5 nmole/100 μl (creatinine 25 nmole/100 μl). Guanidinosuccinic acid (GSA), guanidinoacetic acid (GAA), guanidinopropionic acid (CPA), creatinine (CRN), guanidinobutyric acid (GBA), 4-guanidinobenzoic acid (4-GBA), α-amino-β-guanidinopropionic acid (α-A-β-GPA); arginine (ARG), guanidine (G) and methylguanidine (MG) were all completely resolved, except for guanidine—methylguanidine pairs. For such a chromatogram as guanidine—methylguanidine pairs, which have an R_S value of about 0.9 and a peakheight ratio (MG:G) of 23:1, the guanidine area will be underestimated [33]; this is confirmed in Table III. Therefore, quantitative analysis of guanidine was performed by using peak heights in the rest of the analysis for physiologic fluid samples. A more sensitive and rapid HPLC method has been developed for the quantitative analysis of guanidine in physiological fluids; this has been presented in a separate paper [34]. Creatine, which was eluted between the peaks of guanidinosuccinic acid and guanidinoacetic acid, could not be detected in this analytical system. The entire analysis can be completed in less than 100 min under the conditions described above. The retention times, peak heights and peak areas of equimolar quantities of the guanidino compounds are presented in Table III. The reproducibility of the

TABLE III

RETENTION TIMES, PEAK HEIGHTS AND PEAK AREAS OF A STANDARD MIXTURE OF GUANIDINO COMPOUNDS

Numerals shown in the Table are the mean values (n = 20) obtained with a digital integrator.

Compound	Retention time (min)	Peak height	Peak area
GSA	16.29	385	197086
GAA	25.02	1778	633102
GPA	37.94	632	331372
CRN	48.16	339	214222
GBA	56.82	130	48939
4-GBA	61.66	122	51527
α-A-β-GPA	65.95	1011	477322
ARG	72.76	227	103036
G	91.36	32	7636
MG	94.76	903	581403

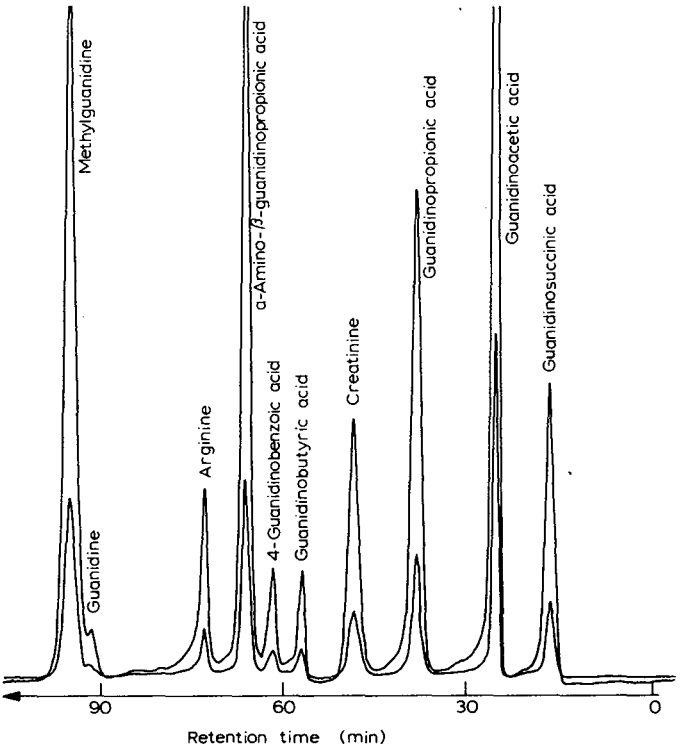

Fig. 4. Chromatogram of a standard mixture of guanidino compounds, each at a concentration of 2.5 nmoles/100 μl (creatinine 25 nmoles/100 μl).

analytical system was evaluated with twenty chromatograms obtained from the standard samples. Retention times of all guanidino compounds showed a relative standard deviation of $\leqslant 1.5\%$. The standard deviations for peak heights and peak areas were within 4.0% and 5.4%, respectively, for all guanidino compounds.

The fluorescence response was linear for all guanidino compounds up to at least 2.5 nmoles. As examples, guanidinosuccinic acid, guanidinoacetic acid, guanidinopropionic acid and methylguanidine are shown in Fig. 5, plotted for amounts of 0.025—2.50 nmoles. The amount of each guanidino compound in an aliquot of physiologic fluid taken for the analysis was generally less than 2 nmoles, except for arginine and creatinine. For arginine and creatinine, the linear range was tested up to 25 nmoles and 100 nmoles, respectively. The linearities of these compounds were obtained using peak areas. The minimum detectable quantity for each guanidino compound is listed in Table IV. The limit of detection was determined by the peak height at twice the noise level.

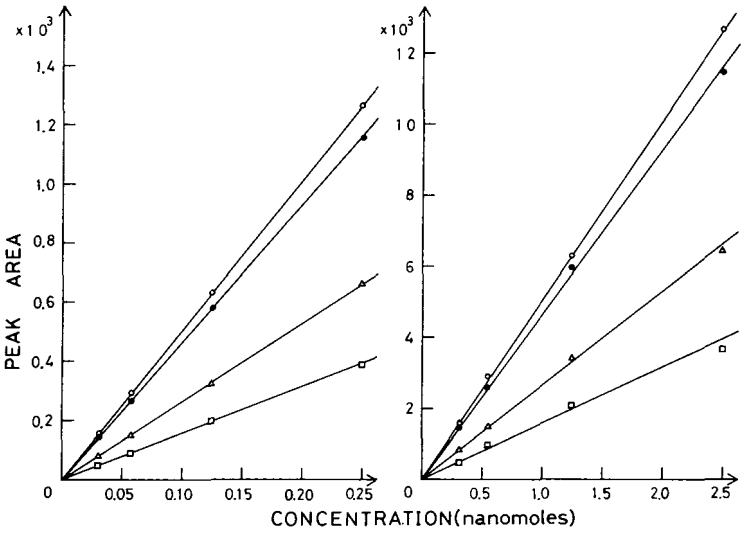

Fig. 5. Calibration curves for some guanidino compounds. GAA (○); MG (●); GPA (△); GSA (□).

TABLE IV

MINIMUM DETECTABLE AMOUNT AND CONCENTRATION OF GUANIDINO COMPOUNDS IN PHYSIOLOGIC FLUIDS

	GSA	GAA	GPA	CRN	GBA	α-A-β-GPA	ARG	G	MG
pmoles	6.5	1.4	3.9	73.0	19.0	2.5	11.0	78.0	2.7
μg/dl	0.11	0.19	0.51	8.26	2.76	0.37	1.92	4.61	0.20

Analysis of physiologic fluid samples

A typical example of a serum sample from a uremic patient is shown in Fig. 6. The labeled peaks were identified from the retention times compared to those of standard mixtures. Recently we obtained an authentic sample of taurocyamine and could identify the first peak (unlabeled) as taurocyamine. A significant peak, which is unidentified, is observed between the peaks of taurocyamine and guanidinosuccinic acid. Smaller unknown peaks also appear at 28 min, 32 min and 63 min. The amounts of the labeled peaks were estimated to be 1.96 nmoles for GSA, 0.38 nmoles for GAA, 0.26 nmoles for GPA, 63.6 nmoles for CRN, 0.41 nmoles for GBA, 0.27 nmoles for α-A-β-GPA, 0.675 nmoles for ARG, 0.31 nmoles for G and 0.68 nmoles for MG. When calculated in terms of serum levels, they represent a concentration of 343.4 μg/dl for GSA, 44.5 μg/dl for GAA, 34.1 μg/dl for GPA, 7.20 mg/dl for CRN, 59.5 μg/dl for GBA, 39.5 μg/dl for α-A-β-GPA, 1.18 mg/dl for ARG, 18.5 μg/dl for G and 49.7 μg/dl for MG.

Fig. 7 represents a typical example of a serum sample from a person in normal health. The first peak (unlabeled) was identified as taurocyamine by comparison of its retention time. A smaller unknown peak at 28 min, which is present in uremic serum, was also observed in normal serum. The amounts of

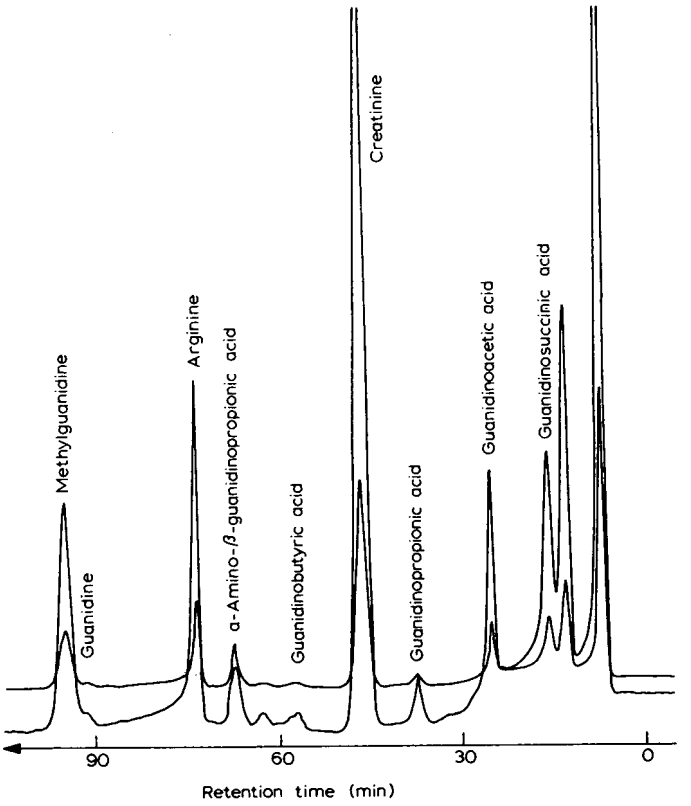

Fig. 6. Analysis of a serum sample from a uremic patient.

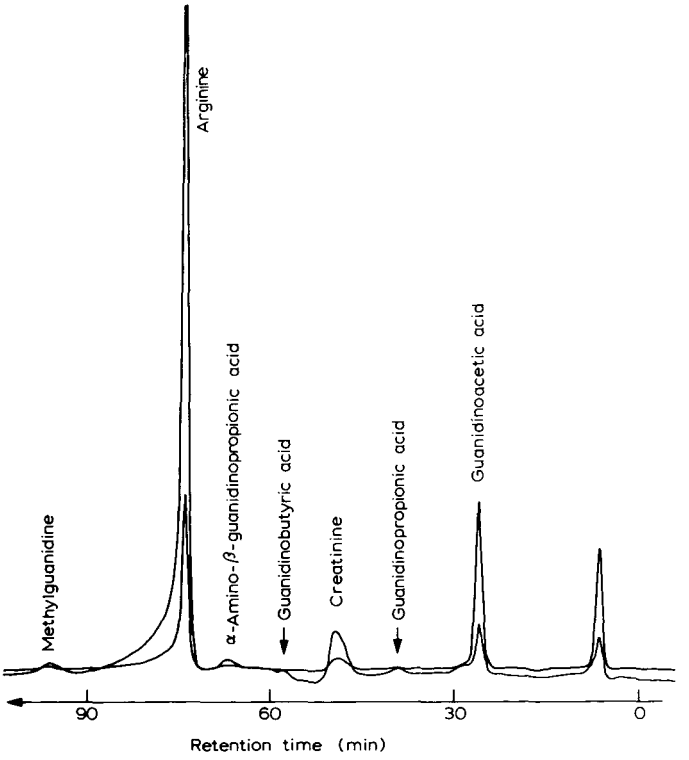

Fig. 7. Analysis of a serum sample from a normal control.

the labeled peaks were determined to be 0.303 mmoles for GAA, 0.035 nmoles for GPA, 6.17 nmoles for CRN, 0.078 nmoles for GBA, 0.030 nmoles for α-A-β-GPA, 9.46 nmoles for ARG and 0.01 nmoles for MG. In terms of serum concentration there are 35.5 μg/dl for GAA, 4.59 μg/dl for GPA, 0.698 mg/dl for CRN, 11.3 μg/dl for GBA, 4.38 μg/dl for α-A-β-GBA, 1.65 mg/dl for ARG and 0.73 μg/dl for MG.

Fig. 8 shows a chromatogram of a cerebrospinal fluid sample from a uremic patient. In the analysis of cerebrospinal fluid, the baseline was irregular because deproteinization treatment of the sample was omitted; when the filtration membrane was used the baseline became stabilized. The first peak at 5.09 min was identified as taurocyamine. An unidentified peak at 10.55 min, which also appeared in the uremic serum sample, was also observed with a significant peak. The quantities of the labeled peaks were estimated as 78.30 μg/dl for GSA, 2.01 μg/dl for GAA, 6.87 mg/dl for CRN, 3.79 mg/dl for ARG and 19.25 μg/dl for MG.

DISCUSSION

All of the analytical techniques used in the past have been directed toward determination of some specific guanidino compounds [1—8] such as guanidinosuccinic acid [3, 6—8] and methylguanidine [1—5]. The determination of

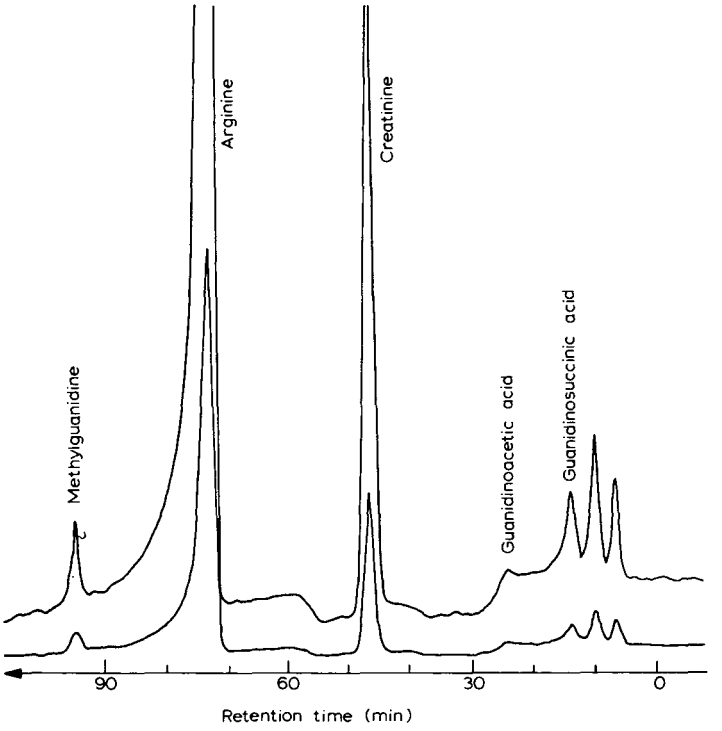

Fig. 8. Analysis of a cerebrospinal fluid sample from a uremic patient.

these guanidino compounds was mainly carried out with cation-exchange column chromatography followed by manual assay using the Sakaguchi reaction. Such methods require a large amount of time and effort, and specialized analysts. The recently described analytical method using a modified automatic amino-acid analyzer [27—30] enabled the separation of most of the guanidino compounds in biologic fluids. The entire analysis requires 4 h or more, which is still time-consuming. In this method, automatic quantitative determination was performed with biacetyl-α-naphthol, a specific reagent for guanidino compounds. The color development time required was 16—20 min [29], which made it difficult to shorten the analysis time. We have developed a high-performance cation-exchange chromatographic method for the analysis of guanidino compounds using 9,10-phenanthrenequinone reagent. Experiments were carried out to determine the optimum separation conditions for shortening the analysis time. A series of guanidino compounds were efficiently separated using a stepwise pH gradient in which a considerably stronger alkaline solution is used for the elution of stronger basic compounds. Furthermore, our efforts were focused on methods for reducing the volume of the reaction system and the fluorescence conversion time to a minimum. The present study has shown that fluorescence conversion is achieved in 2 min when the reaction is carried out at temperature above 50°. Shortening the reaction time made it

possible to give a baseline separation and to complete the analysis in less than 100 min.

The conventional analytical method based on the colorimetric assay requires a large amount of sample (5—20 ml) and concentration of the samples [4]. A recent analytical method using an amino-acid analyzer also requires concentration techniques such as lyophilization prior to introduction into the chromatographic column [29]. Nevertheless, some of the guanidino compounds containing methylguanidine were not quantitatively determined, even for the uremic serum [29, 30], because of the low sensitivity of the colorimetric method used. The limiting factor in sensitivity for the conventional analytical method for guanidino compounds is the reagent used for detection. 9,10-Phenanthrenequinone has been reported to form a highly fluorescent product with mono-substituted guanidines and has been demonstrated to be more sensitive than the Sakaguchi reaction for the detection of arginine [31]. We adopted the 9,10-phenanthrenequinone reagent for the determination of the guanidino compounds eluted from the chromatographic column. The fluorometric detection method used in our analytical system is found to be two orders of magnitude more sensitive than the conventional colorimetric detection method. Therefore, no such concentration treatment as lyophilization or evaporation is required for our analytical method. As little as 0.3 ml of physiologic fluid sample is sufficient for the analysis, since our analytical method requires a net volume of only 0.1 ml of sample. In the analysis of serum, as little as 1 ml of serum is deproteinized with the filtration membrane and then approximately 0.3 ml of the filtrate is loaded onto the analytical system. With our analytical method, all of the physiologic fluid guanidino compounds were quantitatively determined with ease in serum and cerebrospinal fluid samples from normal subjects as well as from uremic patients. The sensitivity of the method was sufficiently high for routine clinical laboratory use. Furthermore, the sensitivity of the method has proved advantageous where only samples of limited volume are available, for example cerebrospinal fluid or sera of small animals. An instrument based on the present report is now available from Japan Spectroscopic Co.

A large number of serum and cerebrospinal fluid samples from normal subjects and uremic patients are currently being analyzed and the results obtained with our analytical method will be the subject of a later report.

ACKNOWLEDGEMENTS

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ANALYTICAL ISOTACHOPHORESIS OF UREMIC BLOOD SAMPLES

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SUMMARY

Uremic blood samples were analyzed for ionogenic substances using analytical isotachophoresis. Multicomponent separations proved that the uremic state shows significant differences from the normal state, especially with regard to anionic low-molecular-weight substances. As a quantitative parameter the ratio of anionic higher-molecular-weight substances to anionic low-molecular-weight substances is proposed: the HL ratio. Separation patterns and HL ratios were studied during nine weeks for one patient on chronic hemodialysis. The patient showed a low HL ratio due to excess of low-molecular-weight substances. Separation patterns before and after hemodialysis showed clear differences and the HL ratio increased. The method of analysis is neither time- nor sample-consuming and sample preparation is not needed. Experimental procedures are easily standardized and results are reliable.

INTRODUCTION

Despite the fact that in recent years considerable progress has been made in hemodialysis techniques, patients still suffer from substantial disabilities. For a long time symptoms of uremic intoxication were attributed to excess of low-molecular-weight substances [1]. The possible importance of middle-molecular-weight substances was established when Babb et al. [2] suggested the middle molecule hypothesis.

Obviously the uremic state is in many respects different from the normal state, not least of which is the biochemical aspect [3]. The clinical state of patients suffering from renal failure can be improved by dialysis of the blood, thus removing the toxic products of metabolism that would normally be excreted by the kidneys. However, solutes removed by the artificial "kidney" may differ from those eliminated by a normally functioning kidney. Moreover some are eliminated in excess and others are poorly removed. As a result, concentrations of many metabolites in the body fluids of uremic patients are different from those of normal subjects.

The search for uremic toxins has been highly specific, assessing the possible toxicity of single known substances. Due to the complex and nonspecific nature of uremic toxins and their interrelations, such studies are rather elaborate and difficult. In recent years, however, some research groups have followed a screening approach to the problem [4, 5], in which chemical multicomponent analyses of biological fluids, derived from uremic patients, play an important part. The obvious drawback of such an approach is the extraordinary complexity of such fluids.

Analytical chemistry has been successful in the development of techniques that permit separation, identification, quantification and even isolation of many metabolites in biological fluids. For the qualitative and quantitative evaluation of ionic solutes several analytical techniques are available and each has its own disadvantages and limitations. Isotachophoresis [6] is a rather new analytical technique that seems to be compatible with the basic requirements for screening approach procedures: multicomponent information, rapid completion, reliable and inexpensive. Moreover the flexibility of the technique offers a vast spectrum of possibilities for detailed studies. Hence isotachophoresis can provide useful information on the occurrence of ionic solutes in uremic biological fluids.

EXPERIMENTAL

All isotachophoretic experiments were performed in the equipment developed by Everaerts and coworkers [6—8]. The separation capillary was 20 cm × 0.2 mm I.D. Samples were introduced by means of a microliter syringe. The electrical driving current was stabilized at 20 μA. Voltages varied between 1 and 16 kV. Separation times were less than 20 min. Separated zones were detected by means of conductimetric and UV (254 nm) detection systems.

All chemicals used were of analytical grade or additionally purified by conventional methods. Water was of double distilled quality. Exact specifications of the electrolyte systems are given in Tables I and II. In both systems anionic solutes can be analyzed. The operational system of Table I has a large difference between the mobilities of the leading and the terminating ionic species. Due to this large mobility gap the system is particularly suitable for screening approach procedures. As a more differentiating system the operational system of Table II was chosen.

Blood samples were obtained from a 61-year-old female patient with polycystic renal disease, on intermittent hemodialysis since August 6th, 1973.

TABLE I

OPERATIONAL SYSTEM FOR THE DETERMINATION OF THE HL RATIO

	Electrolyte	
	Leading	Terminating
Anion	Chloride	HEPES*
Concentration	0.01 *M*	0.01 *M*
Counterion	Histidine	TRIS**
pH	6.02	8.50
Additive	0.1% HEC***	
Solvent	Water	
Current density	0.064 A/cm^2	
Temperature (°C)	Ambient air, 21°	

*HEPES = N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Sigma, St. Louis, Mo., U.S.A.) recristallized from water—ethanol.
**TRIS = Tris(hydroxymethyl)aminomethane (Merck, Darmstadt, G.F.R.).
***HEC = hydroxyethylcellulose (Cat. No. 5568 Polysciences, Warrington, Pa., U.S.A.); a 1% stock solution was purified by ion exchange (Ionenaustauscher V; Merck).

TABLE II

OPERATIONAL SYSTEM FOR ANIONIC SEPARATION AT LOW pH

	Electrolyte	
	Leading	Terminating
Anion	Chloride	Caproate
Concentration	0.01 *M*	0.01 *M*
Counterion	β-Alanine	Na^+
pH	3.75	6.20
Additive	0.1% HEC*	
Solvent	Water	
Current density	0.064 A/cm^2	
Temperature (°C)	Ambient air, 21°	

*HEC = hydroxyethylcellulose.

Dialysis was performed with a poly-acrylonitrile membrane three times per week for 4 h in a RP6 open system [9]. The dialysate composition is given in Table III. During a period of 2½ months, 1.5-ml blood samples were taken in EDTA just before and immediately after hemodialysis. After centrifugation, samples were stored at −20°. Before analysis, samples were diluted eleven fold with double-distilled water. Reference samples were obtained from several healthy subjects and analyzed under the same experimental conditions.

RESULTS

In applying isotachophoresis as a screening approach much information must be obtained in a relatively short analysis time. We therefore limited the time of

TABLE III

COMPOSITION OF THE DIALYSATE

Sodium	Na^+	134.5 mequiv./l
Potassium	K^+	1.0 mequiv./l
Acetate	CH_3COO^-	45.0 mequiv./l
Chloride	Cl^-	95.5 mequiv./l
Magnesium	Mg^{++}	1.0 mequiv./l
Calcium	Ca^{++}	4.0 mequiv./l

analysis to 20 min at moderate current densities. Concerning the multicomponent information, a methodological choice has to be made between anionic and cationic separations. Preliminary experiments showed that anionic separations give more information, so we confined our investigations to the anodic separation mode. Fig. 1 gives a representative wide range result of a predialysis uremic blood sample, analyzed in the operational system of Table I. From the linear conductivity tracing (Fig. 1 LIN), it can be seen that there are many anionic solutes present within this sample, as indicated by the stepwise change of the conductivity signal.

Qualitative information, for identification purposes, is obtained in isotachophoresis from the conductance of a zone relative to that of the leading zone [6, 8]. The reciprocal conductance axis, at the left hand side of Fig. 1, has been calibrated with respect to this. It should be emphasized that within a

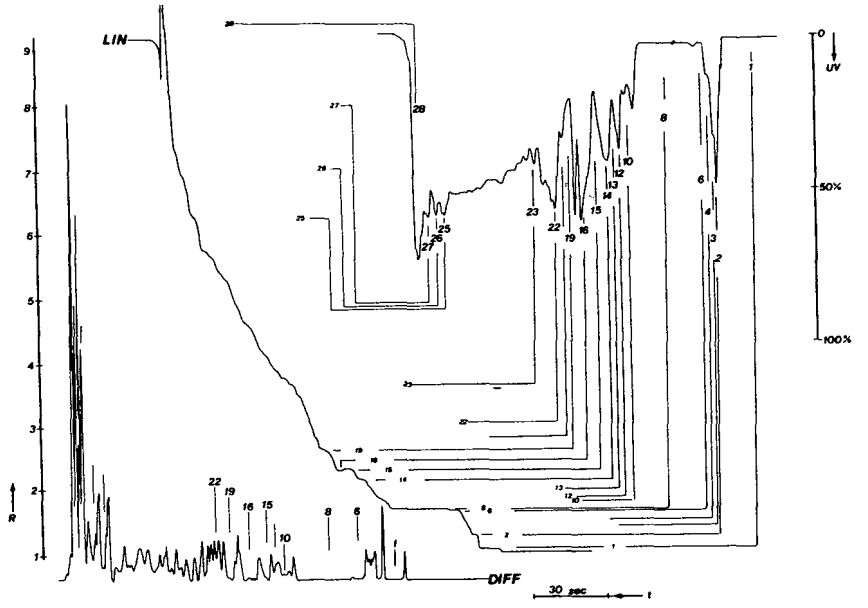

Fig. 1. Uremic plasma sample analyzed in the operational system of Table I. Sample: elevenfold diluted plasma from J.B. (3 μl). 0 = Chloride, 1 = sulphate, 5 = acetate, 6 = lactate, 8 = phosphate, 14 = hippurate, 16 = urate. R = resistance; t = time; UV = UV absorption 254 nm. For further explanation see text.

given operational system relative stepheights are species specific. Hence component number 8 (phosphate) will always migrate at a relative stepheight of $R = 1.70$, whatever the nature of the sample.

Additional information is obtained when the sample zones are migrating through the UV detection system. Each zone will have or not have a specific UV absorption at 254 nm. Since in isotachophoresis the steady-state configuration is no longer a function of time, the UV tracing (Fig. 1 UV) can be superimposed upon the conductivity tracing. In this way, zones that contain UV absorbing solutes are easily localized. For a number of zones the relationship between the UV and the conductivity tracing is indicated by numbered lines. Some solutes are given in the legend of Fig. 1. The advantage of the dual detection system is obvious from the fact that a non-UV-absorbing solute like sulphate, cannot be located by the UV detector. Its presence is nevertheless clearly indicated by the conductometric detection system (Fig. 1, LIN No. 1). Due to the large mobility gap and the large number of solutes that are present, several mixed zones, stable with respect to time, can be expected. The numbering of the zones therefore is based upon the characteristics of the UV profile.

In isotachophoresis the length of a zone is proportional to the amount of the ionic species in the sample. In order to facilitate the measurement of zone lengths the linear conductivity signal has been differentiated. From the differential tracing (Fig. 1, DIFF) distances between zone boundaries can be measured. Sample concentrations are calculated from the measured zone length by methods of calibration or using an internal standard [6, 8].

Several uremic patients have been screened within this operational system and they all show similar profiles, though minor individual differences occur. In order to assess the difference between the normal and the uremic state, blood samples of normal subjects were analyzed. Representative UV separation profiles of four normal subjects are given in Fig. 2. Differences with the uremic state are obvious, especially in the low-molecular-weight region. This region comprises the zones 1 up to 22 and has a relative conductance $G_R < 3$. Fig. 3 shows a blank run of the operational system of Table I and analyses of samples derived from a uremic patient before and after hemodialysis. From Fig. 3a it can be seen that there is only a small impurity present originating from the electrolyte system. Though the separation profiles of Figs. 3b and 3c show a large similarity, it can be concluded from the decrease in UV absorption that the sample after hemodialysis, Fig. 3c, contains fewer UV absorbing solutes. Moreover in Fig. 3c, acetate, originating from the dialysate is clearly visible. In the legend of Fig. 1 some other solutes are given.

Since the excess of lower-molecular-weight substances is a characteristic difference between the normal and the uremic state it is advantageous to describe this difference as a single quantitative parameter. The ratio of anionic higher-molecular-weight substances to anionic low-molecular-weight substances seems appropriate, in which the HL ratio is given by the quotient of two zone-lengths:

$$HL = (\Delta l_{28-22}) : (\Delta l_{22-2})$$

Most anionic low-molecular-weight substances, mol. wt. < 400, have low dis-

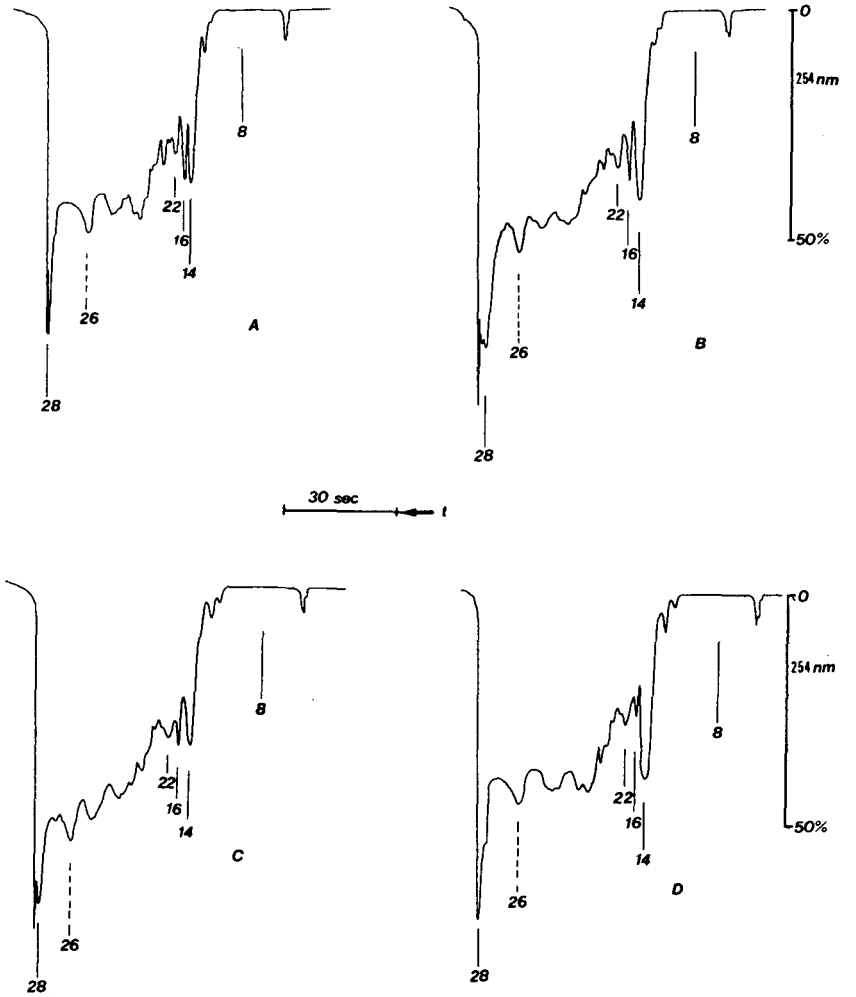

Fig. 2. Normal plasma samples analyzed in the operational system of Table I. Samples: eleven-fold diluted plasma (3 μl). A: female, B: male, C: female, D: male. UV (254 nm) tracings; t = time.

sociation constants, $pK_a < 6$, and migrate within the operational system of Table I as rather mobile species. They will generally migrate at a low relative conductance, i.e. $G_R < 3$. Higher-molecular-weight substances like peptides have higher dissociation constants and a low ionic mobility and for that reason migrate in the less mobile part, $G_R < 3$. Most amino acids have fairly high pK_a values and will not migrate isotachophoretically within this operational system. It should be noticed that even relatively high-molecular-weight substances, like heparin, can migrate as rather mobile species, provided they have a large net electrical charge. As a criterion for finding the ratio we chose zone No. 22, since this zone seems characteristic for all separations done up until now. In order to study the relevancy of this ratio a patient was screened during several weeks of hemodialysis. Samples were analyzed according to the operational

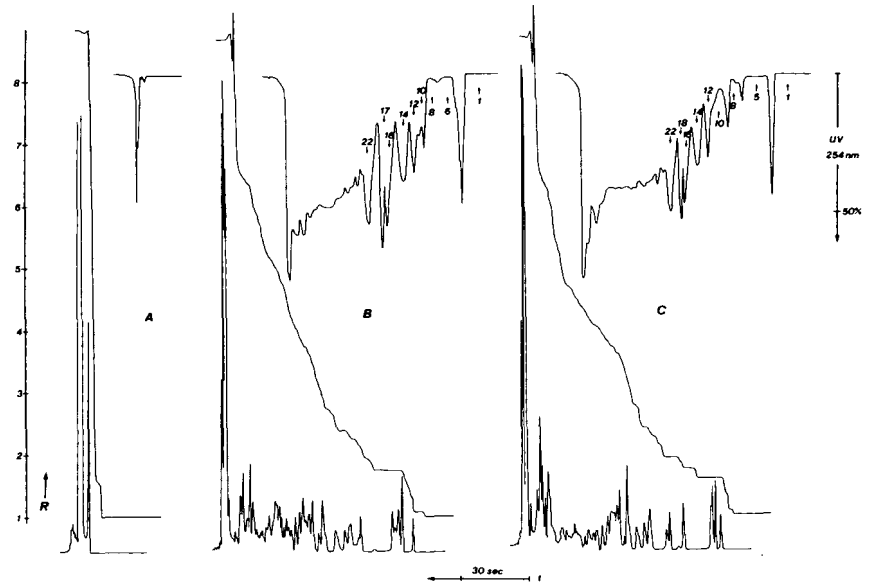

Fig. 3. Uremic plasma samples analyzed in the operational system of Table I. Sample: eleven-fold diluted plasma from T.M. (3 μl). A: Blank run, B: predialysis, C: postdialysis.

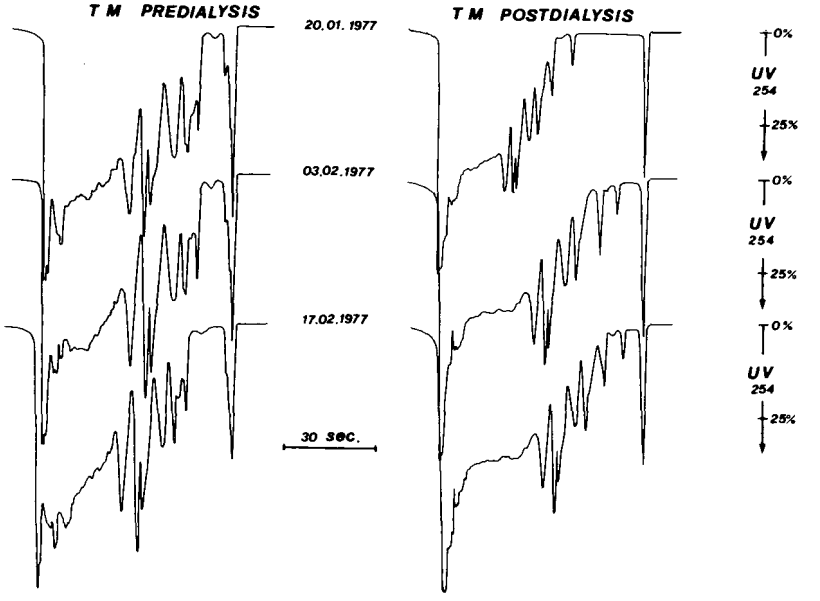

Fig. 4. The longitudinal behaviour of sample from patient T.M. Operational system: Table I. Sample: eleven-fold diluted plasma (3 μl).

system of Table I. Some representative results are given in Fig. 4. Comparison of these results with the UV tracings from Figs. 1 and 3 indicates the strong similarity of the separation patterns, especially in the lower-molecular-weight region. Moreover the strong resemblance of the separation profiles for different data suggests a fairly constant response from the patient to intermittent

hemodialysis. In Table IV the measured HL ratios have been summarized. Normal values are significantly higher than unity, whereas predialysis values are smaller than unity due to the excess of anionic low-molecular-weight substances. Post-dialysis values, corrected for acetate, are slightly larger than unity.

TABLE IV

HL RATIOS: NORMAL-, PRE- AND POSTDIALYSIS VALUES

										Mean	C.V. (%)
Normal	1.10	1.25	1.22	1.17							
T.M. pre	0.86	0.91	0.91	0.79	0.86	0.84	0.77	0.79	0.72	0.83	8
T.M. post	0.93	0.79	0.81	0.46	0.84	0.80	1.00	0.77	0.77	0.80	20
T.M. post*	1.19	1.15	0.94	0.90	1.10	1.03	1.21	0.98	1.00	1.06	10

*Corrected for acetate content.

With the operational system of Table II blood samples were analyzed for the content of weakly acidic and acidic anionic solutes of low molecular weight. Fig. 5a shows a representative result of a normal serum in which phosphate and lactate can be determined. Fig. 5 b and c shows the same samples as Fig. 3 but now analyzed within the operational system of Table II. The differentiating capabilities of this system are better compared to those of Table I, but the mobility gap is considerably smaller. For the determination of concentrations this system is more appropriate. From a calibration run the concentrations of three constituents could be determined. Results are given in Table V.

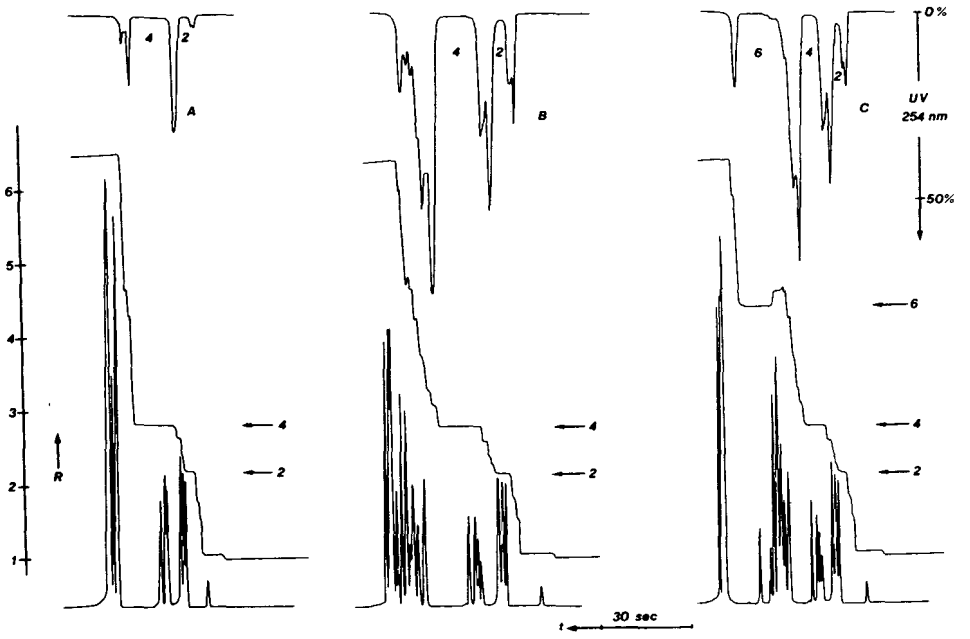

Fig. 5. Normal and uremic samples analyzed in the operational system of Table II. Samples: eleven-fold diluted (3 μl). A: Normal, B: T.M. predialysis, C: T.M. postdialysis. 2 = Phosphate; 4 = lactate; 6 = acetate.

TABLE V

DETERMINED CONCENTRATIONS (m*M*)

	Normal	T.M. predialysis	T.M. postdialysis
Phosphate	0.9	1.8	1.1
Lactate	3.9	4.8	2.5
Acetate	Trace	Trace	4.4

DISCUSSION

Isotachophoresis provides multicomponent information of uremic blood samples in a short analysis time. Due to the minimal sample preparation and the rigidly standardized experimental conditions results have good reproducibility and a high reliability.

For the screening approach of anionic solutes in uremic blood samples we used a wide range electrolyte system, for which the total time of analysis is less than 20 min. The fairly high dissociation constant of the terminating ion N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid, $pK_a = 7.55$, guarantees that almost all acidic and weakly acidic solutes are being analyzed. The majority will migrate isotachophoretically as rather mobile anions: Fig. 1, $G_R < 3$. Moreover various peptides and peptide-like substances will be analyzed without rigid limitation to molecular weight: Fig. 1, $G_R > 3$. The resolving capabilities of this electrolyte system will be relatively poor for the less mobile part of the mobility gap. In the mobile part, $G_R < 3$, acidic and weakly acidic organic and inorganic solutes are separated with satisfactory differentiation.

Bultitude and Newham [5] have reported a method for comparing plasma samples from patients with chronic uremia before and after dialysis, using combined gas chromatography—mass spectrometry. They showed that the concentrations of several low-molecular-weight substances increase in uremia. Comparison of the normal sera separations of Fig. 2 with the uremic sample separations, Figs. 1, 3 and 4, confirm these findings. After dialysis Bultitude found that concentrations became approximately the same as from plasma of healthy subjects. Considering the low-molecular-weight organic acids we must conclude that, though individual concentrations are lower after hemodialysis, they are still considerably higher than normal values. This means that the concentration level of several of these substances will be in the millimolar range. Identification and quantification of more solutes is the subject of present investigations.

During hemodialysis only a slight change in the higher-molecular-weight region has occurred. This region seems of particular interest since the growth of evidence showing the importance of substances of middle molecular weight: mol. wt. 400—5000 [2, 4, 10, 11]. Most analytical information about these substances has been obtained with gel chromatography [4, 10, 11]. Although little is known of the exact nature of these substances, undoubtedly some will be analyzed within the wide range system of Table I. Gel chromatographic studies have proved the removal of middle molecules by adequate hemodialysis

[4, 10]. Our isotachophoretic experiments suggest only a small change in the higher-molecular-weight region. It should be emphasized however, that the separation mechanism of isotachophoresis is quite different from that of gel chromatography. Hence the higher-molecular-weight substances, as analyzed by isotachophoresis, will be different from those separated by gel chromatography.

Considering the HL ratio it can be concluded that the uremic state is characterized by a relatively low ratio, due to excess of organic acids. From Table V it follows that the ratio after hemodialysis has not changed significantly, due to the high acetate concentration originating from the dialysate. Moreover variations in the acetate content are responsible for the high value of the coefficient of variation. Since acetate is assumed to be metabolized rather quickly [12], it seems appropriate to correct the HL ratio for the acetate content. The increase of the ratio after hemodialysis then suggests a slight correction of the uremic state. This correction is also demonstrated by the more differentiated separations in the operational system of Table II. Within this operational system the metabolically important constituent acetate [13] can be easily determined. Further work on the determination of HL ratios in different hemodialysis strategies, identification and quantification of uremic solutes is the subject of current investigations.

We conclude that isotachophoresis is compatible with most of the demands for screening approach procedures. Particularly in the field of ionic solutes, isotachophoresis proves to be a useful analytical tool.

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RELIABLE ROUTINE METHOD FOR THE DETERMINATION OF PLASMA AMITRIPTYLINE AND NORTRIPTYLINE BY GAS CHROMATOGRAPHY

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SUMMARY

A gas chromatographic method has been developed for the determination of amitriptyline and nortriptyline in plasma. OV-17 is used in a 1 m long packed column, with a flame ionization detector and an electronic integrator. Five internal standards are added. The base-specific extraction procedure and the method of calibrating the chromatograph are described in detail. The accuracy, precision and reliability of the method are demonstrated by the results of nearly 700 determinations of each drug, at concentrations ranging from 5 to 400 ng/ml in the plasma. An interlaboratory comparison with a double radioactive isotope derivative assay for nortriptyline has also shown satisfactory agreement.

INTRODUCTION

The routine determination of amitriptyline and its demethylated metabolite nortriptyline in plasma remains difficult although numerous methods have been described in the last two years. Plasma levels in patients treated with amitriptyline may reach only 20 ng/ml, and in pharmacokinetic studies using single doses, concentrations of a few ng/ml need to be measured. The tendency of tricyclic antidepressants to adsorb onto surfaces complicates extraction procedures at these low concentrations, and contamination with interfering materials occurs easily. Routine reliability is particularly difficult to achieve.

Gas chromatography (GC) with mass spectrometry (MS) [1,2] seems to offer the best available solution of the main problems. Garland [2] used deuterated amitriptyline and nortriptyline as internal standards and showed, as expected, that the ratios of labelled to unlabelled compounds were unchanged by extraction and chromatographic procedures. Thus the deuterated compounds acted as ideal internal standards and controlled for losses in chromatography as well as in extraction. The chemical ionization mass spectrometer was highly

sensitive and provided considerable (though not absolute) specificity of detection. Coefficients of variation were under 4% for samples containing only about 6 ng of nortriptyline and 9 ng of amitriptyline. Garland found that absolute recoveries of both drugs varied, and at the lower concentrations the percentage recoveries were smaller and more variable. He attributed these erratic losses in part to adsorption and stressed the consequent importance of using isotopic internal standards.

Unfortunately many investigators find the cost of MS equipment prohibitive.

Internal standards labelled with ^{14}C have been used for double radioactive isotope methods, in which tricyclic and related antidepressants are determined by acetylation with tritiated acetic anhydride. However, only the secondary amines such as maprotiline [3], nortriptyline [4] and desmethylclomipramine [5] can be directly acetylated. Clomipramine has been acetylated after chemical demethylation [5], but the procedure is laborious and sensitivity is lost. Furthermore, the radioactive acetylation methods lack specificity: at best, thin-layer chromatography is used to separate the desired compound from other substances that will acetylate, such as natural components of plasma, other drugs and drug metabolites. Carnis et al. [5] found that even after a base-specific extraction followed by thin-layer chromatography, the acetylation of plasma components limited the accuracy of the method.

Radioimmunoassay [6,7] has recently been introduced for amitriptyline and nortriptyline. The available antisera do not distinguish between these two drugs, so that both are estimated together [6], or alternatively they may be separated [7] before assay by a differential extraction comparable with the procedure needed for chromatographic methods.

All other published methods for determining amitriptyline with acceptable sensitivity and specificity have been chromatographic, using internal standards chemically different from the drugs estimated. Most authors have used GC with electron capture detectors [8—10], flame ionization detectors [11—14], or nitrogen detectors [15—23]. Some recent methods [24—26] employ high-performance liquid chromatography, but so far this technique does not seem to offer appreciable advantage: the sensitivity is no greater and the problems of quantitative extraction are unchanged. These limitations also apply to high-performance thin-layer chromatography [27], an interesting new technique in which the time taken for chromatography is much reduced.

This paper describes a method for estimating amitriptyline (AT) and nortriptyline (NT) in plasma by GC. The method is conventional, using only minimum equipment including flame ionization detectors. The detailed procedure has been very fully investigated and developed to provide a high degree of reliability. The present version has been in routine use for ten months and the results of nearly 700 determinations of each drug are reported here.

The compounds are extracted from alkaline plasma into heptane and thence into HCl, which is made alkaline and re-extracted with 50 μl heptane. A 10-μl aliquot of this extract is injected into the chromatograph without derivative formation. We follow Cooper et al. [20] in avoiding the evaporation of any solvent, and Jorgensen [15] in adding a low-molecular-weight amine (in our case diethylamine) which prevents adsorption of the tricyclic bases to glass.

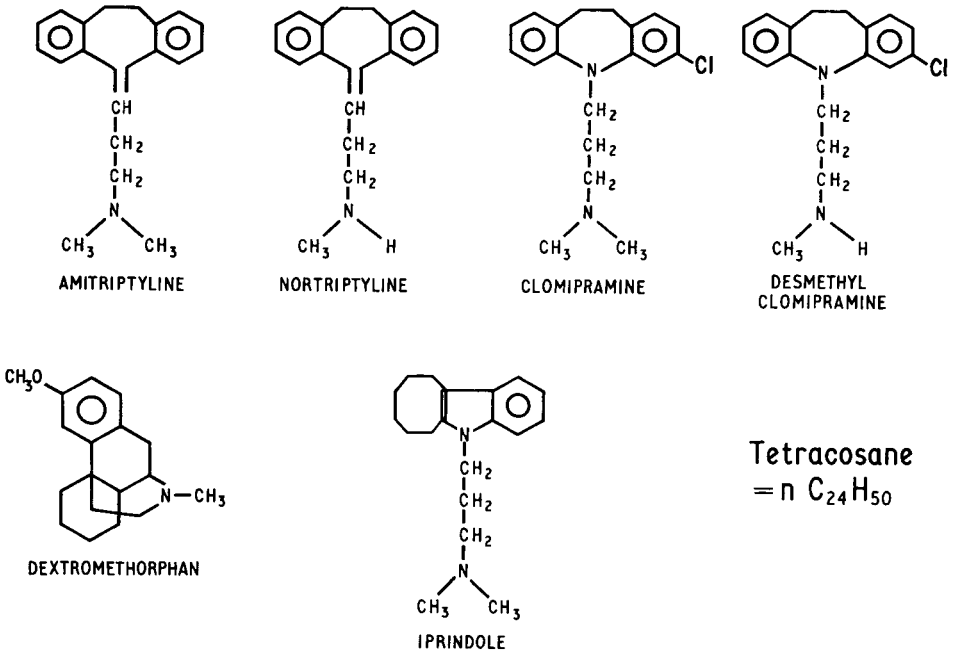

Fig. 1. Molecular structures of AT, NT, internal standards and tetracosane.

Four internal standards are used simultaneously: dextromethorphan and clomipramine are added as hydrochlorides in aqueous solution to the plasma sample; iprindole and desmethylclomipramine are added as bases in heptane, to the first extraction step. A fifth compound, tetracosane, is included in the 50 μl of solvent used in the final extraction, to allow calculation of the over-all yield in the processing of each sample. Fig. 1 shows the molecular structures of these compounds, AT, NT and tetracosane. Dextromethorphan is normally chosen as the primary internal standard for calculating AT and NT concentrations, but the additional internal standards have been found helpful both in developing the method and in routine work.

The chromatogram usually shows all four basic standards in approximately the proportions in which they were added. However, on some occasions the proportions differ, giving warning that the estimation must be carefully considered. Patients' plasma often contains other basic drugs, prescribed or otherwise. These may interfere on the chromatogram with internal standards, and it is always possible that the patient has taken a drug that is used as an internal standard. Furthermore, in routine work peaks from accidental contamination at the ng level are not unknown. In all these instances the presence of several internal standards in fixed ratios usually allows the estimation to be rescued; the procedure insures against errors that might otherwise pass undetected.

MATERIALS AND METHODS

Reagents

n-Heptane (99.5%, international pesticide specification), *n*-pentane (99%)

and diethylamine (analytical reagent grade) were obtained from BDH (Poole, Great Britain) and all were distilled before use.

Compounds

Tetracosane was obtained from Aldrich (Milwaukee, Wisc., U.S.A.). Drug compounds were kindly donated by the following firms: amitriptyline hydrochloride by Merck, Sharpe & Dohme (Hoddesdon, Great Britain), nortriptyline hydrochloride by E.R. Squibb and Sons (Twickenham, Great Britain), dextromethorphan hydrobromide by Parke Davis (Pontypool, Great Britain), iprindole hydrochloride by Wyeth Labs. (Maidenhead, Great Britain), clomipramine and desmethyl clomipramine hydrochlorides by Geigy Pharmaceuticals (Macclesfield, Great Britain).

Preparation of standard solutions

Aqueous salt solutions. An amount of each drug equivalent to 50 mg base is weighed, and dissolved in 0.01 *M* HCl to give 200 ml stock solution. Dilutions are prepared as follows:

(i) Internal standard mixture, containing dextromethorphan (1 μg/ml) and clomipramine (2 μg/ml) in 0.01 *M* HCl.

(ii) Amitriptyline (1 μg/ml) and nortriptyline (1 μg/ml) in 0.01 *M* HCl. This solution is added to drug-free plasma to give standard samples.

The aqueous solutions are stored at 4° and all except iprindole remain stable for several months. Iprindole is best kept as a base (see below).

Base solutions in heptane. A standard solution of the basic form of each drug is prepared from 25 ml of the stock salt solution, by extraction with 25 ml heptane and 500 μl diethylamine in a glass-stoppered tube for 45 min on a rotary tumbler. Each solution contains 0.25 mg base per ml, in heptane containing about 0.5% of diethylamine.

Tetracosane is weighed, and dissolved in heptane containing 0.5% (v/v) of diethylamine. The stock solution contains 100 μg/ml and is stable at room temperature for many months. A working solution containing 5 μg/ml is prepared by dilution with the same solvent.

Mixtures of bases are prepared in this solvent, for calibrating the chromatograph. Ten microlitres of each contain 50 ng tetracosane, 75 ng dextromethorphan, 100 ng iprindole and amounts of AT, NT, clomipramine and desmethylclomipramine ranging from 1 to 150 ng.

An internal standard base mixture is prepared, containing iprindole (0.75 μg/ml) and desmethylclomipramine (1 μg/ml) in the same solvent.

All the base solutions are stable at room temperature for several months.

Glassware

The extraction of each plasma sample requires three glass-stoppered tubes: a 35-ml centrifuge tube, a 15-ml test-tube and a 10-ml conical centrifuge tube. After use, each tube is rinsed with ethanol and then distilled water. The conical tubes are then soaked in chromic acid overnight, rinsed with distilled water, soaked in 2 *M* NaOH for at least 1 h and rinsed again in distilled water. All tubes are finally soaked overnight in Decon 90 detergent (Decon Labs., Brighton, Great Britain), rinsed with distilled water and dried in an oven.

The 15-ml tubes and conical tubes, with their stoppers, are silanised

by standing for 30 min in 2.5% dimethyldichlorosilane in toluene, rinsed in toluene, soaked in methanol for 10 min, rinsed twice with methanol and dried. These tubes are rinsed with pentane immediately before use.

Extraction of plasma

Into a 35-ml tube is pipetted 1.0 ml of a solution prepared by diluting 50 ml 4 *M* NaOH and 20 ml diethylamine to 200 ml with water. The tube is stoppered and rotated in the hand until the whole surface has been wetted with alkali.

To the plasma sample (1—10 ml) in a polystyrene tube are added 500 μl of the aqueous internal standard solution of dextromethorphan and clomipramine. The sample is mixed and then washed quantitatively into the 35 ml tube with water. One millilitre of the internal standard base mixture containing iprindole and desmethylclomipramine is added, and 10 ml heptane. Water is then run in until the tube is almost full. It is stoppered and mixed at 30 rpm on a rotary tumbler for 30 min. The dilution of the plasma and the gentle tumbling action prevent emulsification.

After centrifuging (without stopper) for 15 min at 2000 *g*, the heptane layer is transferred to a 15 ml tube, using a Pasteur pipette that has been rinsed first with diethylamine and then with heptane. (Between successive samples the pipette is rinsed with heptane only.) A 2-ml volume of 0.1 *M* HCl is added, the tube tumbled at 30 rpm for 10 min and then centrifuged briefly with the stopper in place. Most of the heptane is removed by aspiration.

Into a conical tube are pipetted 300 μl of a solution prepared by diluting 50 ml of 4 *M* NaOH and 30 ml diethylamine to 200 ml with water. The whole surface is wetted with this solution as before. The acid layer from the 15-ml tube is transferred to this conical tube, using a Pasteur pipette rinsed in 0.1 *M* HCl before use and between samples. Care is taken to avoid transferring any heptane layer.

A 50-μl volume of the working tetracosane solution is then added and the tube vibrated on a Whirlimixer for 10 sec. After centrifuging briefly with the stopper in place, most of the lower layer is removed with a Pasteur pipette previously rinsed in diethylamine and then in pentane. Between one sample and the next the pipette is simply wiped carefully on paper tissue. The tube is centrifuged again and all the remaining aqueous layer removed, using a drawn-out capillary pipette rinsed in the same way as the previous one. Ten microlitres of the resulting extract are injected into the chromatograph. This is usually done the same day, but the extracts can be kept at room temperature until the following day, without change.

Gas chromatography

The Pye-Unicam GCV chromatograph is fitted with dual columns, flame ionization detectors and amplifiers, and a Philips PM8221 two-pen chart recorder. Pye-Unicam DP88 integrators are used to measure retention times and peak areas.

Columns are silanised glass, 1 m × 4 mm I.D., packed with 10% OV-17 on Gas-Chrom Q 80—100 mesh. It is important to leave space above the packing at the inlet end, so that the injection needle does not enter the packing material.

(If the needle regularly penetrates into the packing, the resolution of the column deteriorates rapidly.) The packing is supplied by Jones Chromatography (Llanbradach, Great Britain). Columns are conditioned at 270° for about 45 h with low carrier flow.

The carrier gas is nitrogen, flow-rate 75 ml/min. Hydrogen and air flow-rates are 50 ml/min and 300 ml/min, respectively. Working temperatures are: column oven 230°, injection oven 230°, detector oven 270°.

All injections are of 10 μl volume, from syringes manufactured by Scientific Glass Engineering (Melbourne, Australia).

RESULTS AND DISCUSSION

Gas chromatography

The standard mixed base solutions are used daily to check the chromatographic system. Injection of large amounts (one hundred to several hundred ng) of the various compounds gives peak area ratios proportional to the relative amounts present. However, when smaller amounts of AT and NT are injected their peak areas are lower than would be expected from simple proportion. Each day, the base solution containing 150 ng of AT and NT per 10 μl is first injected several times. Peak area ratios are calculated, and seldom differ by more than 2% from the mean values shown in Table I. Mixtures containing smaller amounts of AT and NT are then injected and, each time, the peak areas of the internal standards are used to calculate the expected areas of AT and NT by simple proportion. The actual areas are subtracted from expected areas to find the losses of AT and NT in chromatography. These losses are small but significant; they differ from day to day and between columns, but are usually between 0.5 and 1 ng of AT and between 1 and 2 ng of NT. When mixtures containing different amounts (1—30 ng) of AT and NT were compared, it was found that the loss of each compound in chromatography was approximately independent of the quantity injected. Routinely, 7.5 ng of AT and NT are injected several times for the determination of area losses.

Plasma extracts are then injected and the area of each AT and NT peak is corrected by adding the loss already estimated. Corrected areas, together with

TABLE I

RELATIVE RETENTION TIMES AND PEAK AREAS

Compound	Relative retention time (AT = 1)	Peak area ratio for equal weights of compounds (AT = 1)
Tetracosane	0.68	1.05
Dextromethorphan	0.84(5)	0.87
Amitriptyline	1.00	1.00
Nortriptyline	1.16(5)	0.96
Iprindole	1.53	0.87
Clomipramine	2.00	0.82
Desmethylclomipramine	2.40	0.76

the area ratios determined at 150 ng, are used to calculate AT and NT concentrations in the plasma sample by simple proportion. It is thus assumed that the relative amounts of the various compounds in the extract are the same as those in the original plasma sample after addition of internal standards.

The ratios of the peak areas of the different internal standards in each extract are routinely calculated, and the ratio of dextromethorphan to tetracosane is used to estimate the over-all yield of the extraction process, which normally averages between 70 and 75%. Thus 10 μl of plasma extract contains the amounts of AT and NT in approximately one-seventh of the plasma sample, together with about 50 ng tetracosane, 75 ng dextromethorphan, 110 ng iprindole, 150 ng clomipramine and 150 ng desmethylclomipramine. A loss of 1 ng AT or NT in chromatography corresponds to about 1.5 ng/ml in a 5-ml plasma sample.

Fig. 2 shows a chromatogram from 5 ml of blank plasma carried through the usual procedure, but without the addition of internal standards or tetracosane. Fig. 3 shows a chromatogram from 4.8 ml of the plasma of a subject who had taken a single dose of 100 mg amitriptyline 4 h previously. The calculated drug concentrations were AT 71 ng/ml and NT 13 ng/ml. The retention time of AT

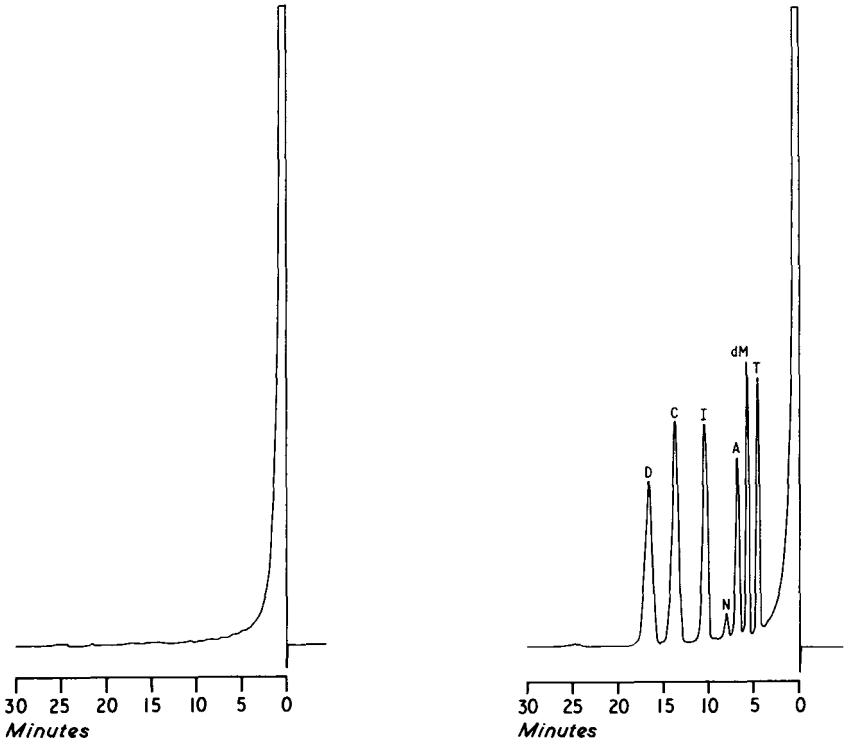

Fig. 2. Chromatogram from 5 ml blank plasma carried through the extraction procedure. Attenuation the same as for Fig. 3.

Fig. 3. Chromatogram from 4.8 ml of plasma from a subject who had taken a single dose of 100 mg amitriptyline 4 h previously. Calculated drug concentrations were AT 71 ng/ml, and NT 13 ng/ml. Peaks: T, tetracosane; dM, dextromethorphan; A, amitriptyline; N, nortriptyline; I, iprindole; C, clomipramine; D, desmethylclomipramine.

was 6.9 min; it differs between columns but is usually between 6 and 7 min. The retention times of the other compounds relative to AT are shown in Table I.

A column packing material containing a high proportion (10%) of liquid phase has been adopted, because all packings with lower percentages that were tried gave greater losses of AT and especially of NT. Some batches of the current packing obtained from various other suppliers were also found to be inadequate, but with good batches sufficient resolution is obtained in 1-m columns, which give about 2000 theoretical plates with these compounds.

Assay results

Each batch of 12 samples processed usually contains two of drug-free plasma. One is a blank, and known amounts of AT and NT are added to the other. Table II shows the results of 120 such determinations, most of which were carried out on different days. Usually, equal amounts of AT and NT were present.

The two drugs showed no interaction in the estimation. In 5 ml of plasma, 20 ng/ml of added AT gave the same result when 20 ng/ml of NT were also added as when no NT was present. Similarly, the results with 20 ng NT per ml were unaffected by the presence or absence of 200 ng AT per ml.

Neither the amount of plasma present nor the anticoagulant used affected the assay. Ten millilitres of plasma, either citrated from the blood bank or heparin-treated from volunteers, 1 ml plasma of either type, and water were compared. Firstly 1000 ng of AT and NT were added to each sample and then

TABLE II

RESULTS OF DETERMINATIONS ON 5-ml PLASMA SAMPLES WITH ADDED DRUGS

Number of estimations = 20 for each concentration.

Drug	Amount added (ng/ml)	Amount found (ng/ml)		Coefficient of variation (%)
		Mean	S.D.	
Amitriptyline				
	5	5.2	0.47	9.1
	10	10.0	0.70	7.0
	20	19.1	0.69	3.6
	40	39.4	1.25	3.2
	100	99.9	2.70	2.7
	400	415	11.2	2.7
Nortriptyline				
	5	5.7	0.60	10.7
	10	10.6	0.96	9.0
	20	19.5	0.87	4.5
	40	39.2	1.08	2.8
	100	98.0	2.65	2.7
	400	414	10.8	2.6

the experiment was repeated with 75 ng per sample. No differences were found at either drug level.

For the determinations summarized in Table II, AT and NT concentrations were calculated as explained above, assuming that the extraction yield of each drug, at all concentrations from 5 to 400 ng/ml, was identical with the yield from 100 ng dextromethorphan per ml in the same sample. The close agreement in Table II between the amounts of drug added and the mean amounts estimated shows that the assumption is justified and that attempts to calibrate the extraction process, by using the results of extracted standard samples to calculate the results for unknown samples, would be unlikely to improve accuracy. This is satisfactory. If extraction yields vary with concentration or differ between different compounds they are likely to be inherently unstable, making any corrections unreliable. Our efforts to improve the extraction procedure in order to eliminate such variations appear to have been successful, within the limits shown in Table II.

Table II shows that when the AT concentration is 40 ng/ml or greater, coefficients of variation are effectively constant at about 3%. At lower levels, coefficients of variation rise: the standard deviation falls to about 0.7 ng/ml (3.5 ng per sample) but thereafter remains constant. Variations in the losses in chromatography are probably responsible for much of this residual standard deviation. NT gives figures very similar to those for AT: standard deviations at low concentrations are marginally higher, as are the losses in chromatography. The minimum amount of either drug that can be detected with certainty is about 15 or 20 ng per sample.

Treatment of blood samples

Blood is collected in heparin-coated polystyrene tubes and usually centrifuged soon afterwards. The plasma is transferred to fresh polystyrene tubes and kept frozen until analysed. However, when blood samples are taken in the evening they are left at room temperature until they are centrifuged next morning, and some samples of plasma are received by post, taking up to seven days to arrive.

Patients' blood samples were used for the following six experiments:

(1) Ten blood samples left at room temperature for 24 h before centrifuging were found to give the same plasma concentrations of AT and NT as duplicate blood samples centrifuged immediately after collection.

(2) Three plasma samples left at room temperature for ten days gave the same AT and NT concentrations as duplicate plasma samples frozen immediately, and analysed with the others ten days later.

(3) Eight plasma samples frozen and left five to seven months in the freezer before analysis showed little or no loss of AT and NT, compared with duplicate samples analysed at the beginning. The nominal mean losses were 6% AT and 4% NT, which were probably within the errors of the estimation over this time interval.

(4) On storage in the freezer, plasma samples develop precipitates. These precipitates were shown not to adsorb appreciable amounts of AT or NT from the plasma. Two samples analysed complete with precipitate gave the same results as duplicate samples of clear supernatant after centrifuging. Analysis of

TABLE III

PRECISION OF AMITRIPTYLINE ESTIMATIONS FROM DUPLICATE ANALYSES OF PLASMA FROM PATIENTS AND NORMAL VOLUNTEERS

Range (ng/ml)	0—10	10—20	20—30	30—50	50—70	70—100	100—150	over 150
Mean	6.3	14.8	25	40	59	83	117	168
Number of pairs	28	27	13	42	60	44	33	5
S.D.	0.54	0.58	1.4	2.1	2.5	4.3	5.5	8.8
Coefficient of variation (%)	8.5	4.0	5.6	5.3	4.2	5.2	4.7	5.2

TABLE IV

PRECISION OF NORTRIPTYLINE ESTIMATIONS FROM DUPLICATE ANALYSES OF PLASMA FROM PATIENTS AND NORMAL VOLUNTEERS

Range (ng/ml)	0—10	10—20	20—30	30—50	50—70	70—100	100—150	150—200	over 200
Mean	6.2	14.3	24	40	59	86	123	168	251
Number of pairs	35	49	15	22	38	41	33	16	18
S.D.	0.74	0.65	1.55	3.1	2.5	4.3	4.8	7.7	10.4
Coefficient of variation (%)	11.9	4.5	6.4	7.6	4.2	5.0	3.9	4.6	4.1

the precipitates from 24 ml of old stored plasma showed only traces of AT and NT.

(5) Adsorption of AT and NT from plasma on to glass pipettes or polystyrene tubes was shown to be negligible: two samples of clear centrifuged plasma were each sucked up and down in five ordinary 2-ml glass pipettes in series. Two further samples were each poured into five polystyrene tubes, from one to the other in series. No loss of AT or NT could be detected. The binding of the drugs to plasma proteins probably prevents adsorption on to other surfaces.

(6) In view of the problems experienced with storing dilute aqueous solutions of AT and NT in polystyrene tubes in the freezer (see later), plasma was tested for the effect of freezing and thawing in these tubes. Plasma was poured into a clean polystyrene tube and frozen. Next day, it was thawed, poured into a fresh tube and refrozen. This process was carried out five times altogether, on each of three samples. There was no change in AT or NT concentrations, compared with duplicate samples frozen once only and kept frozen.

Duplicate determinations on plasma samples

The precision of the method was confirmed by analysis of the results of duplicate determinations, using the method of Snedecor [28]. Plasma samples were either from psychiatric patients being treated with AT or NT, or from normal subjects who had ingested single doses of AT. The volume of plasma used in each estimation ranged from 3 to 6.5 ml, with a mean of about 4 ml. Duplicates were always estimated on different days. The results are given in Tables III and IV. (One pair of duplicates with grossly discrepant values for both drugs has been omitted.)

The coefficients of variation are satisfactory for most purposes but are slightly larger at high concentrations than those of Table II. These duplicate determinations at the higher concentrations (patients in steady state) were carried out over a period of ten months, during which period improvements were being made to the procedures. The estimations in Table II, however, and those in the low-concentration groups of Tables III and IV (single-dose studies), were carried out within the last three months. These improvements were probably responsible for the better precision obtained.

Additional peaks on the chromatogram

Normal plasma contributes no peaks (Fig. 2). In order to avoid contamination during extraction, extreme cleanliness of the glassware, and particularly of the final conical tube, is essential. The present extraction method replaced an earlier technique that included the evaporation of 5 ml pentane to dryness. Not only did recoveries of the compounds become more reproducible, but background peaks were largely eliminated.

Metabolites of AT and NT. Peaks with retention times of 1.89 and 2.20 relative to AT are usually found in small amounts in plasma extracts from patients treated with AT, but never in extracts from drug-free plasma with or without added drugs. Patients treated with NT show only the later peak. These compounds are probably 10-hydroxy-AT and 10-hydroxy-NT, respectively, well-known metabolites of AT and NT [29]. At a column temperature of 212° their relative retentions were found to be 1.98 and 2.41, respectively,

in good agreement with the values of 1.96 and 2.38 reported by Hucker and Stauffer [13] for the authentic compounds on OV-17 at this temperature.

These peaks are not resolved from clomipramine and desmethylclomipramine, respectively. However, if the amounts present are large enough to add significantly to the measured peak areas of these internal standards, the metabolites can be clearly seen as shoulders on the leading edges of the respective standard peaks. In such samples, the value of clomipramine and/or desmethylclomipramine as internal standards is of course reduced.

Contaminants. Two extraneous peaks still often appear on the chromatograms, at retention times of 2.12 and 3.6 relative to AT. These substances probably enter during the last stage of the extraction and all efforts to exclude them completely have failed. The later peak merely increases the necessary time interval between injections, but the earlier one forms a shoulder on the rear edge of the clomipramine peak. As with 10-hydroxy AT, it can be seen if there is enough present to add appreciably to the integrated clomipramine area.

If any heptane layer is accidentally transferred into the conical tube, it contributes a peak from plasma constituents, at a retention time of 10 relative to AT. This peak is likely to intrude upon the chromatograms of subsequent samples.

Other drugs

The relative retention times of 29 basic drugs and some of their metabolites are given in Table V. Retention time depends broadly on molecular weight and polarity. However, small, highly polar amines that might give peaks in the region of interest will be excluded by the extraction process. Usually, metabolites are more polar than the parent drug and hence have longer retention times; the demethylation of tertiary amines and the hydroxylation of hydrocarbon structures both have this effect. N-Oxidation of tertiary amines can shorten retention time [15] but the oxide is not basic and so cannot appear in the plasma extract. It seems possible, however, that where a drug is an ester, metabolites formed after hydrolysis might have shorter retention times than the parent compound.

The information in Table V suggests that large numbers of benzodiazepines and phenothiazines can be eliminated as sources of interference with dextromethorphan, AT or NT. Medazepam, the least polar benzodiazepine that we have encountered, overlaps with iprindole on the chromatogram. The introduction of a carbonyl group, to give diazepam, considerably increases retention time as expected. All the other benzodiazepines familiar to us, including oxazepam, lorazepam, flurazepam and clonazepam, are more polar and/or larger than diazepam and are therefore extremely unlikely to interfere. Similarly, the phenothiazines promethazine, trimeprazine and promazine, appear in the iprindole region of the chromatogram. The introduction of a chlorine atom, giving chlorpromazine, increases retention time as expected. Thioridazine, with an extra ring on the basic side-chain, comes very much later. All the phenothiazines that we have encountered, including fluphenazine, trifluoperazine, perphenazine and levomepromazine, are larger and/or more polar than chlorpromazine.

Haloperidol is also large and polar and was excluded by Bailey and Jatlow

TABLE V

RELATIVE RETENTION TIMES OF TWENTY-NINE BASIC DRUGS

Drug	Relative retention time (AT=1)	Drug	Relative retention time (AT=1)
Lidocaine*	0.36	Promethazine	1.45
Orphenadrine*(1)	0.41	Trimeprazine	1.48
Diphenyl pyraline	0.75	Medazepam	1.48
Bromodiphenhydramine	0.85	Iprindole	1.53
Procyclidine*(2)	0.88	Benztropine	1.55
Dextropropoxyphene*(3)	0.88	Maprotiline	1.71
Propranolol	0.92	Promazine*(6)	1.76
Butriptyline	0.93	Dothiepin	2.06
Trimipramine*(4)	1.04	Antazoline	2.14
Imipramine	1.11	Chlorpromazine*	2.83
Atropine	1.14	Diazepam*	3.16
Mianserin*(5)	1.20	Nitrazepam	11.7
Doxepin	1.22	Chlordiazepoxide	15.3
Desipramine	1.32	Thioridazine*	21.9
Protriptyline	1.35		

*These drugs have been identified in extracts prepared as usual from patients' plasma. Metabolite peaks have also been found, with retention times relative to AT, as follows: (1) 0.44; (2) 1.00 and 1.33; (3) 0.81 and 4.00; (4) 1.27; (5) 1.57; (6) 2.18.

[19], who chromatographed 47 basic drugs on OV-17 at 235°. Of these, only atropine, scopolamine, doxepin and imipramine were reported to interfere with AT or NT peaks, although no retention times were given. The remaining 43 non-interfering drugs include 36 that do not appear in Tables I or V of the present paper.

Four further drugs that have retention times within 10% of those of AT or NT are shown in Table V. All but propranolol are themselves antidepressants and are therefore usually avoidable in combination with AT or NT. No drugs have yet been found whose retention times are so close to those of AT or NT that their presence would pass undetected. However, it is necessary to remain aware of the possibility of interference and to test any suspect basic drug that a patient is known to be taking. Pharmaceutical preparations can readily be treated with 0.1 *M* HCl and the supernatant solution made alkaline and extracted with heptane for injection into the chromatograph.

Interference by metabolites, particularly of drugs with retention times shorter than that of AT, remains more difficult to eliminate. Such metabolites can only be sought in extracts of the plasma of subjects who are taking the drug in question without AT or NT. We have found that a metabolite of procyclidine gives a very small peak coincident with that of AT; furthermore, the peak of procyclidine itself is close to that of AT and often very large.

Comparison with double radioactive isotope assay of nortriptyline

Twenty-four frozen plasma samples whose duplicates had been analysed for NT by the double isotope derivative dilution assay [4] were sent by air from Melbourne, Australia. They were estimated by a slightly earlier version of our GLC method, and the subsequent comparison of results has been reported elsewhere [30]. Agreement was satisfactory: the mean ratio between the two results for each sample was 0.99, and the joint coefficient of variation was 8.6%.

Development of the method

The hydrocarbon tetracosane, with its ideal chromatographic behaviour, has been found useful in overcoming problems encountered in chromatography, as well as for checking the over-all yield of the extraction process.

The four basic internal standards were chosen by trial and error as lipophilic bases with conveniently spaced retention times. Several compounds more closely related to AT and NT were tried and abandoned: protriptyline on account of the very active adsorption of its base on chromatography columns and on glassware; and all the tricyclic drugs in Table V from butriptyline to desipramine inclusive, because of inadequate separation from AT or NT. We used clomipramine as the primary standard until we discovered the presence of hydroxy-AT in many plasma extracts. This peak interferes with clomipramine and is also close to maprotiline. Desmethylclomipramine is more variable in its extraction yield than are the other compounds used, and suffers interference from hydroxy-NT. Iprindole has the slight disadvantage that it is used in heptane solution, being unstable on storage in dilute acid. Dextromethorphan remains the best internal standard that we have found, in spite of the difference in chemical structure between it and the tricyclic drugs.

We find it useful to add the four internal standards in two separate solutions, to guard against pipetting errors, which would cause a characteristic pattern of peak area ratios.

AT and NT bases were found to adsorb very markedly from solution in pure heptane, particularly on to the glass barrel and metal needle of the injection syringe. Much more NT than AT was adsorbed. The addition of 0.5% diethylamine prevented the effect, and in this mixed solvent all six bases used can be diluted, pipetted, etc., without significant losses. In the extraction, the 35-ml tube does not adsorb appreciable amounts of the compounds although it is not silanised. The chief purpose of silanising the conical tube is to improve the shape of the interface when the last of the aqueous layer is being removed.

On the recommendation of Carnis et al. [5] for clomipramine and desmethylclomipramine, 0.01 *M* HCl was adopted as the aqueous solvent for all the compounds. However, these solutions have not been found easy to handle. On pouring them into a series of tubes, from one to the next, adsorptive losses have been encountered on polystyrene, on glass and to a lesser extent on silanised glass. It seems possible that ion pair formation in the acid solution may be involved. Of the six drugs used here, clomipramine and desmethylclomipramine are the most strongly adsorbed. Unlike the bases, in which the adsorption on glass and on poor column packings is much stronger for the secondary amine than for the related tertiary amine, the adsorption of the salts

is approximately equal for secondary and tertiary, but depends markedly upon the tricyclic nucleus. Silanising the 15-ml tube used in the extraction is essential to minimise salt adsorption. In preparing and diluting the standard solutions in 0.01 *M* HCl, the glassware is silanised, small vessels are avoided and pipettes are thoroughly pre-rinsed with solution to saturate their surfaces.

Extraction of the bases from the heptane layer into 2 ml of 0.1 *M* HCl appears to be complete. In three experiments, compounds were sought in the discarded heptane layer:

(1) The heptane was evaporated to dryness and the residue taken up in solvent and injected into the chromatograph. Pentane was also tried instead of heptane in this experiment.

(2) The heptane was re-extracted with a further 2 ml of 0.1 *M* HCl which was processed and chromatographed as usual.

(3) Using very high drug concentrations, 10 μl of the discarded heptane layer was injected directly.

None of the six drugs was found in any experiment, although in each case it was shown that a few percent of the original amount of any of them would have been detected.

It was also found that neither the yield nor the peak area ratios were altered when an ordinary HCl layer was re-extracted successively with two extra 10-ml volumes of heptane, before proceeding with further treatment. Thus any ion pair formation in the acid solution does not result in significant losses of the compounds in the heptane phase.

The dilute aqueous solutions to be added to plasma were at one time divided out into 10-ml polystyrene tubes and frozen. They were then thawed daily as required. However, in about 20% of these tubes large erratic losses were encountered, particularly of dextromethorphan. Solutions have since been stored successfully in flasks at 4°.

CONCLUSIONS

The precision and accuracy of this method have been tested over a long period, and with nearly 700 estimations, as shown in Tables II—IV. The sensitivity could probably be improved by using the Perkin-Elmer nitrogen detector [20], albeit at considerable cost. However, as the internal standards must remain chemically different from the drugs estimated, it is not clear whether in routine practice the processes of extraction and chromatography could be made reproducible enough to take advantage of any large increase in sensitivity.

The present method has been used successfully for pharmacokinetic studies both in patients undergoing treatment with amitriptyline or nortriptyline and in normal subjects after single doses of amitriptyline. Because the results are not affected by the volume of plasma, large samples can be used if available, to increase precision at low concentrations.

We believe that the method could readily be adopted by other laboratories without further developmental work.

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CHLORPHENIRAMINE

I. RAPID QUANTITATIVE ANALYSIS OF CHLORPHENIRAMINE IN PLASMA, SALIVA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method was developed for the rapid quantitative analysis of chlorpheniramine in plasma, saliva and urine using high-performance liquid chromatography. A diethyl ether or hexane extract of the alkalinized biological samples was extracted with dilute acid which was chromatographed on a reversed-phase column using mixtures of acetonitrile and ammonium phosphate buffer as the mobile phase. Ultraviolet absorption at 254 nm was monitored for the detection and brompheniramine was employed as the internal standard for the quantitation. The effects of buffer, pH, and acetonitrile concentration in the mobile phase on the chromatographic separation were investigated. A mobile phase 20% acetonitrile in 0.0075 *M* phosphate buffer at a flow-rate of 2 ml/min was used for the assays of plasma and saliva samples. A similar mobile phase was used for urine samples. The drug and internal standard were eluted at retention volumes of less than 17 ml. The method can also be used to quantify two metabolites, didesmethyl- and desmethylchlorpheniramine, in the urine. The method can accurately measure chlorpheniramine levels down to 2 ng/ml in plasma or saliva using 1 ml of sample, and should be adequate for biopharmaceutical and pharmacokinetic studies. Various precautions for using the assay are discussed.

INTRODUCTION

Chlorpheniramine maleate, a potent antihistamine with little side-effect of somnolence, is incorporated in a variety of pharmaceutical preparations for the symptomatic alleviation of the common cold and various allergic diseases [1]. However, despite its widespread use, surprisingly little has been reported

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about its pharmacokinetic profiles in man. Most of the reported studies were confined to the estimation of renal excretion of the drug [2—4] or the drug and its metabolites [5—7]. These studies showed a large variation in the amount of the unchanged drug excreted in urine, as pointed out by Kamm et al. [7].

In some earlier reports, spectrophotometric methods were used to assay chlorpheniramine in urine for urinary excretion studies [2, 3]. More specific gas—liquid chromatographic (GLC) methods for the assay of chlorpheniramine in biological fluids have since appeared in the literature [4—12]. Beckett and Wilkinson [4], using a GLC method for the assay of drug in urine samples, showed that the renal excretion of chlorpheniramine in man was dependent on the urinary pH and flow-rate. The same general assay procedure was employed in the subsequent studies on the renal excretion of chlorpheniramine and its metabolites in man by Kabasakalian and coworkers [5, 6]. Kamm et al. [7] studied the metabolism of chlorpheniramine in rats and dogs using another GLC method. These investigators showed that didesmethyl- and desmethylchlorpheniramine were the major metabolites of chlorpheniramine [7]. Albert and Windheuser [8] used ion-pair column extraction to separate chlorpheniramine and its metabolites from the biological samples in their GLC assays. All the above-mentioned GLC assays, as well as the methods of Kazyak and Knoblock [9], and Street [10], were developed only for the quantitation of drug and/or metabolites in urine.

Townley et al. [11] reported a GLC method that can measure nanogram quantities of chlorpheniramine in plasma. However, their procedure is quite complicated, involving six replicates of extractions between plasma and solvent and between solvent and aqueous acid or base, and requires up to 5 ml of plasma for each assay [11]. More recently, another GLC method was reported for the assay of plasma chlorpheniramine in a study to investigate relative bioavailability among dosage forms [12]. This method requires 1 ml of plasma to detect 100 ng of drug. It is quite doubtful that such sensitivity is suitable for the measurement of chlorpheniramine concentration in plasma after normal dosing. Lange et al. [13] reported another method for the assay of chlorpheniramine in plasma. These authors used thin-layer chromatography (TLC) to separate the drug from the constituents of plasma supernate, extracted the drug from the adsorbant on the developed TLC plates, and measured the fluorescence generated after reaction with rose bengal [13]. This method is obviously too elaborate and lengthy.

The purpose of this paper is to describe a simple and sensitive high-performance liquid chromatographic (HPLC) method for the quantitation of chlorpheniramine in plasma, saliva and urine after a single dose. In addition, a simpler procedure is also developed for the simultaneous determination of chlorpheniramine and its two N-demethylated metabolites in urine.

EXPERIMENTAL

Reagents

Chlorpheniramine maleate, brompheniramine maleate, desmethylchlorpheniramine and didesmethylchlorpheniramine were all generously supplied by Schering (Bloomfield, N.J., U.S.A.). Brompheniramine was used as an in-

ternal standard. Glass-distilled acetonitrile was obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). Most of the other chemicals were of reagent grade.

Source of biological samples

Samples of plasma, saliva and urine were collected at various time intervals for one or two days after single doses of chlorpheniramine maleate administered to healthy subjects either orally or intravenously. Blood samples were collected in heparinized tubes by venipuncture and centrifuged to separate the plasma. Saliva samples were collected in glass vials by mastication and transferred to small culture tubes for centrifugation to remove any particulate debris. The volume and pH of urine from each voidance were measured and aliquots of about 10 ml were preserved. All the biological samples were stored frozen in screw-capped culture tubes until analysis. Blank samples of plasma, saliva and urine were obtained from each subject before drug administration and processed likewise to serve as controls.

HPLC instrumentation

An HPLC solvent delivery pump (Model M-6000A), a sample injector (Model U6K), a 30 cm × 3.9 mm I.D. column prepacked with 10 μm porous silica particles chemically bonded with a monomolecular layer of octadecylsilane (μBondapak C_{18}), and a UV detector with an 8-μl flow-cell and 254 nm filter (Model 440), all from Waters Assoc. (Milford, Mass., U.S.A.), were used in this study.

Selection of mobile phase

Aqueous solutions of chlorpheniramine and the internal standard were initially chromatographed to select a suitable mobile phase. The binary mixtures consisted of various proportions of acetonitrile—water, acetonitrile—phosphoric acid (0.05%, pH 2.5), and acetonitrile—phosphate buffer which were tested as mobile phases in the initial studies. The phosphate buffer was prepared by acidifying ammonium phosphate (monobasic, 0.05 *M*) with phosphoric acid to a pH of about 2.5. All aqueous components of the mixtures were passed through a 0.45 μm HA filter (Millipore, Bedford, Mass., U.S.A.) prior to mixing with acetonitrile. From the chromatograms so obtained, the chromatographic parameters of capacity (k'), selectivity (α), and resolution (R) were calculated. The mobile phase that gave desirable retention and resolution was then tried with any necessary adjustment for the chromatography of biological samples.

Sample preparations

Plasma and saliva samples. Plasma samples in 1-ml aliquots in 13 × 100 mm screw-capped disposable culture tubes were supplemented with 100 μl of brompheniramine maleate (1000 ng/ml) in 0.5% phosphoric acid, basified with 300 μl of 5% KOH to a pH of about 11.5, and extracted with 3 ml of diethyl ether by shaking for 3 min followed by centrifugation at ca. 900 *g* for 2 min. These sample tubes were allowed to stand in a shallow bath of dry-ice—alcohol mixture such that only the lower aqueous layers were allowed to submerge for

freezing. The diethyl ether layers were poured into another set of disposable tubes containing 100 μl of 0.5% phosphoric acid. These mixtures were shaken, centrifuged, and the lower layer frozen as before. The diethyl ether layers were poured out and discarded. The aqueous layers were kept at room temperature in a vortex-evaporator (Buchler Instruments, Fort Lee, N.J., U.S.A.) at reduced pressure for 10—15 min to remove traces of diethyl ether. Most of the remaining aqueous solutions were taken up in a syringe for chromatography. Saliva samples were prepared in the same manner.

Urine samples. Aliquots of 500 μl of urine samples were transferred to 13 × 100 mm screw-capped disposable culture tubes. These tubes were supplemented with 100 μl of aqueous stock solution of brompheniramine maleate, 10 μg/ml, basified with 100 μl of 10% potassium hydroxide, and extracted with 3 ml of hexane (the screw-cap was lined with a piece of aluminum foil to prevent possible leaching of chemicals from the cap and also adsorption of the drugs and metabolites onto the cap) by vortexing for 1 min. After centrifugation at ca. 900 *g* for 3 min, approximately 2 ml of the hexane extract were transferred to another disposable tube containing 200 μl of 0.5% phosphoric acid. After vortexing and centrifugation aliquots of 50 μl of the aqueous acid extracts were injected into the column through the injector for chromatography.

Chromatographic separation and quantitation

For plasma and saliva samples the mobile phase used was acetonitrile—phosphate (0.075 *M* $NH_4H_2PO_4$ in 0.16% H_3PO_4) solution (20:80, v/v). For urine samples the phosphate solution was made up of 0.05 *M* $NH_4H_2PO_4$ in 0.11% H_3PO_4. The flow-rate was set at 2 ml/min. The effluent from the column was monitored by UV absorption at 254 nm with a 0.002 a.u.f.s. sensitivity setting. The chart speed of the recorder was set at 10, 15, or 20 cm/h. Peak height ratios were used for the quantitation based on the calibration curves established on the same day. The calibration curves were prepared from the results of assays on biological fluids supplemented with known quantities of the drug, metabolites and the internal standard. All the chromatographic separations were carried out at ambient temperatures.

RESULTS AND DISCUSSION

Octadecylsilane-bonded microparticulate silica is one of the most commonly used column packings in reversed-phase HPLC. In this type of chromatography, not only is the column efficiency extremely high but also the chromatographic behavior is usually quite predictable on the basis of pH of the eluent and the pK_a and partition coefficient of the eluate [14]. When the pure chlorpheniramine and internal standard were chromatographed on a reversed-phase column using nonacidified mobile phase, they were strongly retained. For example, their retention volumes were greater than 90 ml using an eluent of 40% acetonitrile in water. When the mobile phase was acidified, these compounds were eluted in much smaller retention volumes. This is probably due to the basic property of the compound. Furthermore, with the acidic mobile phase (pH 2.5) the peaks obtained were more symmetrical than the

peaks obtained with a higher pH of the mobile phase.

Chromatograms with differing degrees of resolution were found from the mobile phases with different acetonitrile composition (ranging from 20 to 70%) and dilute phosphoric acid. The chromatographic parameters, k', α, and R, calculated from these chromatograms, are summarized in Table I. Both the α and R values increased as the acetonitrile content was reduced. However, the k' values were found to decrease initially with the reduction in acetonitrile content in the mobile phase system containing more than 40% of acetonitrile. The k' values then gradually increased as the acetonitrile content was further reduced. The above biphasic phenomenon can probably be rationalized by the opposing effect of acidity (amount of phosphoric acid) and water content in the mobile phase. The higher the acidity of the mobile phase, the higher the solubility of chlorpheniramine in the mobile phase and hence the lower the capacity factor. The opposite is true for the water content. The above results also show that a better resolution of chromatograms could be obtained with a higher ratio of the aqueous phase at the expense of longer elution time. With 20% acetonitrile mobile phase, the internal standard had a retention volume of about 24 ml.

Surprisingly, the incorporation of the inorganic salt, ammonium phosphate, in the eluent was found to reduce the retention volumes and improve the separation. The chromatographic behavior of chlorpheniramine and the internal standard was studied using mobile phases containing ammonium phosphate (0.05 *M*, monobasic) with acetonitrile in the useful range of 20—25%. The

TABLE I

EFFECTS OF COMPOSITION OF THE MOBILE PHASE ON CHROMATOGRAPHIC PARAMETERS OF CHLORPHENIRAMINE AND BROMPHENIRAMINE

Capacity factor subscripts 1 and 2 for chlorpheniramine and brompheniramine, respectively. Resolution calculated as quotient of difference of retention times divided by average of peak widths, and not obtainable with acetonitrile 40% or higher in phosphoric acid.

Mobile phase	Capacity factor		Selectivity factor	Resolution
	k'_1	k'_2	(α)	(R)
Acetonitrile—phosphoric acid*				
70:30	2.98	3.09	1.04	—
50:50	2.56	2.71	1.06	—
40:60	2.10	2.25	1.07	—
30:70	2.52	2.95	1.17	0.58
25:75	2.82	3.73	1.32	0.74
20:80	3.46	4.71	1.36	0.85
Acetonitrile—phosphate buffer**				
25:75	1.66	2.15	1.30	0.70
22:78	2.79	3.67	1.31	1.10
20:80	2.80	3.87	1.38	1.12

*0.05% Phosphoric acid.

**Ammonium phosphate (monobasic) 0.05 *M* with the pH adjusted to 2.5 by addition of phosphoric acid.

results of this study are also summarized in Table I. All the parameters, k', α, and R, were found to increase with reduction of the proportions of the organic solvent in this range. When the ammonium phosphate concentration was increased to 0.075 M, there was a slight improvement in the chromatography. Thus, the mobile phases consisting of 20—25% acetonitrile and acidified phosphate buffer (0.05 or 0.075 M) appears to give the best results as regards resolution and elution time. In our study on plasma, saliva and urine samples, a mobile phase containing 20% acetonitrile was chosen due to a better separation of chlorpheniramine peak from the interfering peaks of endogenous substances.

Typical chromatograms from plasma and urine samples of normal adult subjects after receiving chlorpheniramine maleate, together with those from their blank samples, are shown in Figs. 1 and 2. A typical plasma level profile is shown in Fig. 3. Chromatograms from saliva samples are similar to the plasma samples. No interferences with peaks of the drug and internal standard

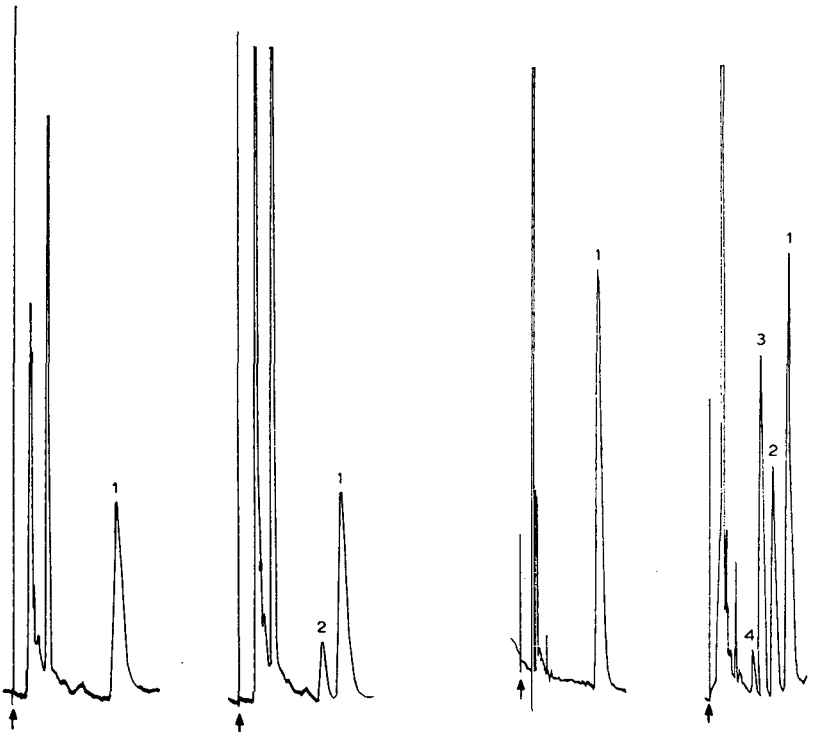

Fig. 1. Chromatograms from a control plasma sample (left) and a plasma sample (right) collected 4 h after an oral dose of 10 mg chlorpheniramine maleate solution to a normal subject. Peaks: 1, brompheniramine; 2, chlorpheniramine (15.4 ng/ml). The arrows mark the point of injection.

Fig. 2. Chromatograms from a control urine sample (left) and a urine sample (right) collected between 24 and 36 h after an oral dose of two 4-mg chlorpheniramine maleate tablets to a normal subject. Peaks: 1, brompheniramine; 2, chlorpheniramine (394 ng/ml); 3, desmethylchlorpheniramine (568 ng/ml); 4, didesmethylchlorpheniramine (62 ng/ml). The arrows mark the point of injection.

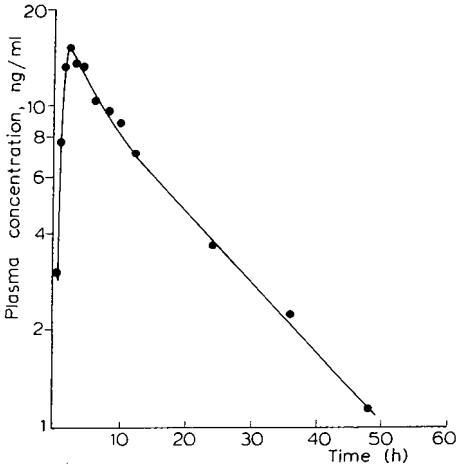

Fig. 3. Chlorpheniramine plasma concentration profile following an oral dose of 10 mg chlorpheniramine maleate in aqueous solution to a normal subject.

were found in most plasma, saliva and urine samples obtained from numerous normal subjects participating in the oral absorption study. For the plasma and saliva samples the retention times for the drug and internal standard were 6.6 and 7.8 min, respectively. For the urine samples the retention times for didesmethylchlorpheniramine, desmethylchlorpheniramine, chlorpheniramine and brompheniramine were 4.4, 5.24, 6.6, and 8 min, respectively. Chlorpheniramine and its metabolites were found in all urine samples collected up to 48 h after an oral dose of 8 mg of chlorpheniramine maleate. No metabolites could be detected in plasma and saliva by the present method.

Within the concentration range of 3.52—25.16 ng/ml (equivalent to 5—40 ng/ml of maleate salt) for both plasma and saliva samples, the drug—internal standard peak height ratios were found to be linear. This is illustrated in the response factor data for plasma samples as shown in Table II. The coefficient of variation of the response factors studied in the same day is usually between 5 and 8% for plasma samples. For the same plasma sample supplemented to yield 3.52 ng/ml of chlorpheniramine the coefficient of variation of the response factors obtained on 10 days over a three-week period is 10.5%. Excellent reproducibility of plasma assay was demonstrated with two HPLC units from our laboratory and four HPLC units from ArnarStone Labs. (Mount Prospect, Ill., U.S.A.) involving several thousands of sample analyses. The over-all extraction recoveries of the drug and internal standard from plasma and saliva samples were about 85%.

For urine samples the detector responses were found to be linear for both the drug and its two metabolites in the range 100—1500 ng/ml. The results are summarized in Table III. In one replicate study (n = 5) on a urine sample collected between 24 and 34 h after an 8 mg oral dose in an adult, the coefficients of variation for didesmethylchlorpheniramine (43.5 ng/ml), desmethylchlorpheniramine (335 ng/ml) and chlorpheniramine (205.6 ng/ml) were 6.62, 9.11 and 4.24%, respectively. The extraction efficiencies for urine samples

TABLE II

TYPICAL RESPONSE FACTORS OF CHLORPHENIRAMINE AT VARIOUS CONCENTRATIONS IN PLASMA

Chlorpheniramine concentration (ng/ml)		Response factor* (ml/ng)
3.5		0.0156
7		0.0167
14		0.0167
21		0.0181
28		0.0174
	Mean ± S.D.	0.0169 ± 0.00096
	C.V. (%)	5.68%

*Drug–internal standard peak height ratio divided by the drug concentration.

TABLE III

RESPONSE FACTORS OF CHLORPHENIRAMINE AND ITS TWO METABOLITES AT VARIOUS CONCENTRATIONS IN URINE

Response factor calculated as drug or metabolite–internal standard peak height ratio × 10^3, divided by the concentration of drug or metabolite based on the average of two determinations.

Spiked concentration (ng/ml)	Chlorpheniramine (ml/ng)	Didesmethyl chlorpheniramine (ml/ng)	Desmethyl chlorpheniramine (ml/ng)
100	1.364	1.799	1.504
250	1.291	1.734	1.321
500	1.281	1.589	1.316
1000	1.309	1.744	1.355
1500	1.426	1.701	1.402
Mean ± S.D.	1.334 ± 0.061	1.713 ± 0.078	1.380 ± 0.078
C.V. (%)	4.54	4.55	5.62

were found to be 66, 76, 98 and 84% for didesmethylchlorpheniramine, desmethylchlorpheniramine, chlorpheniramine and brompheniramine, respectively.

Although the UV absorption maximum of chlorpheniramine in the mobile phase was found to be at about 263 nm, the detection at 254 nm used in this study did give satisfactory sensitivity. The excellent signal-to-noise ratio using the 254 nm fixed wavelength detector (mercury lamp) allowed use of a 0.002 a.u.f.s. sensitivity setting for plasma, saliva and urine samples. A higher sensitivity can also be obtained by using 2 or 3 ml of plasma or saliva samples.

A slow chart speed of 10 cm/h was employed in most studies which would not only reduce the cost of assay but also make the peak height measurement easier and more consistent. The accuracy of the measurement of peak heights, especially for those smaller than 2 cm, appears to be enhanced greatly by the

use of a micrometer (Vernier Caliper from Fisher Scientific, Chicago, Ill., U.S.A.) as opposed to the conventional ruler.

Somewhat different extraction procedures and chromatographic conditions are required for plasma/saliva samples and for urine samples. These modifications were made to accommodate the large differences in the concentrations of chlorpheniramine in these samples and to avoid interferences by the normal constituents of these biological fluids. In general, the concentrations of chlorpheniramine in urine were found to be as much as 50—100 times higher than in plasma and saliva. The flow-rate of the mobile phase had an effect on the peak height obtainable at a given concentration of drug. A flow-rate of 2 ml/min appeared to give peaks about 5—10% higher with slightly slower or faster flow ratios. This flow-rate was used for all samples studied. The extraction and chromatographic procedures for the plasma/saliva samples are also suitable for urine samples. The procedure outlined for the urine samples, however, is simpler and faster, and yet with sufficient sensitivity for the concentrations of the drug and its two metabolites normally encountered in urine.

There was found to be no interference in the HPLC assay reported by the presence of the following commonly used drugs in the same sample: aspirin, salicylic acid, acetaminophen, phenacetin, theophylline, phenobarbital, dilantin, procainamide, propranolol, and ephedrine. This specificity study was carried out on biological samples supplemented with these drugs. No in vivo study of concomitant administration of these drugs with chlorpheniramine was made. Biological samples taken from subjects who took pseudoephedrine, atropine, scopolamine, hysocycamine, and phenylpropanolamine showed no interference with the assay.

In the assay development a significant fraction of chlorpheniramine from its aqueous solutions at low concentrations (5—100 ng/ml) was found to be adsorbed by various types of glassware. This problem was overcome by preparing stock solutions in dilute (0.05%) phosphoric acid solution. It is of interest to note that the source of disposable glass culture tubes used in the extraction could markedly affect the reproducibility and efficiency of the extraction. Those from Fisher Scientific (Cat. No. 14-959-25A) were found to be superior to the Kimble brand (Cat. No. 73750; Vineland, N.J., U.S.A.). A much better recovery of chlorpheniramine was obtained using 0.5% phosphoric acid rather than dilute sulfuric acid with a similar pH for the last extraction step in the plasma assay.

In plasma and saliva assays a freezing technique using the dry-ice—alcohol mixture was employed twice for phase separation. It is important to note that either over-freezing or under-freezing may result in poor quantitation.

Leaching of chemicals from some syringes used for the collection of blood samples may seriously interfere with the assay. In the present study the 6-ml Monoject sterile disposable syringe from Sherwood (Deland, Fla., U.S.A.) was found to leach out various interfering chemicals into blood or water. Syringes from Becton—Dickinson (Rutherford, N.J., U.S.A.) were found to be acceptable.

An unknown impurity from the ultraviolet-grade hexane from the Burdick & Jackson Labs. was found to interfere with the urine assay as it had the same retention time as the internal standard. The pesticide-grade hexane from Fis-

her Scientific contained an impurity with a retention time of 20.8 min. Therefore, caution should be taken when using this hexane for extraction in the urine analysis. For example, it is recommended that a second injection can be started after the appearance of the internal standard peak from the first injection and the third injection should await the appearance of this interfering peak from the first injection.

In summary, simple, rapid, and sensitive high-performance liquid chromatographic methods were developed for the determination of chlorpheniramine in plasma and saliva, and also for the simultaneous determination of chlorpheniramine and its two N-demethylated metabolites in urine. These methods should be useful in biopharmaceutical and pharmacokinetic studies in humans after single or multiple doses. The results of our preliminary pharmacokinetic study in humans will be reported elsewhere.

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Note

Specific and sensitive method for the determination of C_6—C_{10} dicarboxylic acids in serum and urine by selected ion monitoring

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The C_6—C_{10} dicarboxylic acids, adipic, suberic, and sebacic acids, have been shown in recent years to be of biological interest. Substantial amounts of adipic and suberic acids have been found in urine from patients with ketosis [1, 2], and from a patient with carnitine deficiency [3]. In the metabolic disturbances glutaric aciduria, type II [4], and dicarboxylic acidurias [5] large urinary excretions of all three acids have been detected. Small amounts have been measured in urine from patients with glycogen storage disease [6] and in normal newborn infants [7, 8].

The gas chromatographic (GC) methods employed so far in the dicarboxylic acid analyses lack both specificity and sensitivity for quantitative work in serum and in urinary concentrations below 10—20 μg/mg creatinine [7, 8].

We now report a reliable selected ion monitoring (SIM) method for the quantitation of adipic, suberic and sebacic acids in serum and urine. The method has been used for the determination of dicarboxylic acids in serum from ten normal children, aged 5—13 years and in the urine from ten normal children, aged 2.5—10 years and ten normal neonates, aged 1—4 days.

MATERIALS AND METHODS

Chemicals

Adipic acid (1,6-hexanedioic acid), suberic acid (1,8-octanedioic acid), and sebacic acid (1,10-decanedioic acid) were purchased from Koch-Light (Colnbrook, Great Britain). Diethylglutaric acid (1,5-(3,3-diethyl)-pentanedioic acid; DEGA) was obtained from EGA Chemie (Albuch, G.F.R.). The stationary phase for GC, Dexsil 300, was obtained from Analabs (North Haven, Conn.,

U.S.A.) and column support, Chromosorb W HP from Koch-Light. The silylation mixture bis-(trimethylsilyl)-trifluoroacetamide (BSTFA)—trimethylchlorosilane (TMCS) (100:1) was purchased from Pierce (Rockford, Ill., U.S.A.).

Clinical material

Urine was collected from ten full-term neonates (six males and four females), aged 1—4 days. The first morning micturition was collected from ten normal children (five males and five females), aged 2.5—9 years old. Serum was obtained from ten hospitalised children without any clinical or biological signs of a metabolic disorder. All specimens were kept at −20° until analysis.

Analytical equipment

The measurements were performed by means of an AEI MS-30 double focusing mass spectrometer equipped with a Pye Unicam 104 gas chromatograph, a 6 channel selected ion monitoring unit and a 6 channel oscillographic ultra violet recorder. The analytical conditions were as follows: The GC column was a glass coil (7 ft. × 1/4 in. I.D.) packed with 3% Dexsil 300 on Chromosorb W HP. The column temperature was programmed from 200° at 15°/min and the helium carrier flow-rate was 40 ml/min. Injection port temperature was 280°. The interface between the gas chromatograph and mass spectrometer consisted of a glass transfer line and a membrane separator at 250°. The temperature of the electron impact ion source was 220°. Ionizing and accelerating potentials were 70 eV and 4 kV, respectively.

Analytical procedure

Urine samples containing 2 mg of creatinine were mixed with water and 200 μl of a solution of 400 mg DEGA/l (internal standard) to a final volume of 10 ml. The solution was saturated with NaCl and pH was adjusted to 1 with 5 *M* HCl solution prior to four consecutive extractions with 40 ml diethyl ether. The organic phases were dried (Na_2SO_4) and the solvent was evaporated in a stream of dry nitrogen. Silylation of the remainder was performed with 400 μl BSTFA—1% TMCS at 60° for 1 h before 4 μl of the mixture were injected into the SIM system. The silylated dicarboxylic acids remained stable for at least one month when kept at −20°.

Serum samples (2 ml) were mixed with 4 ml ethanol. The precipitated protein was removed by centrifugation, then the ethanol was evaporated in a stream of nitrogen. The resulting aqueous solution of the serum dicarboxylic acids was then diluted to 10 ml with water and the extraction and silylation procedures were performed exactly as described above for the urine samples, except that the remainder was silylated with 50 μl BSTFA—TMCS mixture. The fragment ions used in the quantitative analysis of the dicarboxylic acids were in all cases $M^+ - 15$; *m/e* 275 for adipic acid, 303 for suberic acid, 317 for DEGA, and 331 for sebacic acid. The heights of the respective SIM-peaks were used as a measure of the amounts of compounds. The ratios of *m/e* 275, 303 and 331 to *m/e* 317 were calculated and compared with the standard curves (Fig. 1).

As the detector response ratios were very dependent on the conditions of the mass spectrometer, i.e. tuning and source condition, the slope of the standard

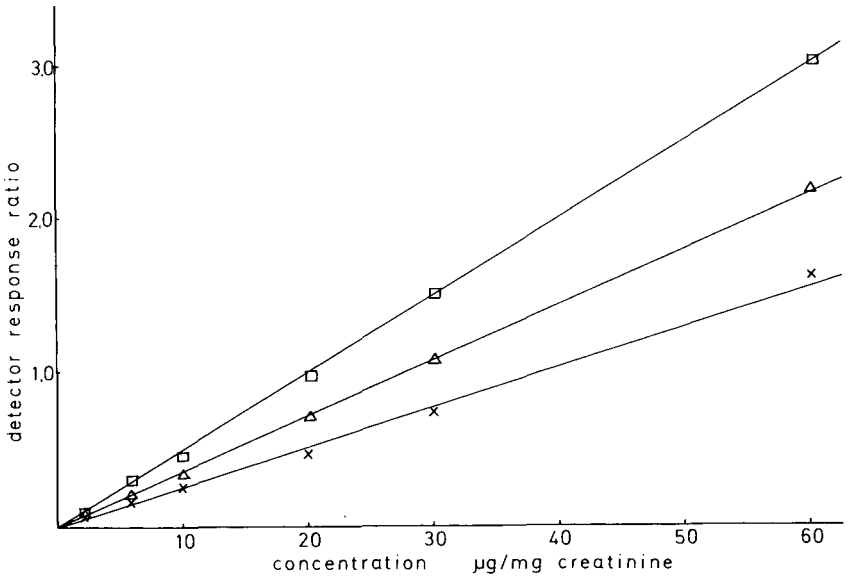

Fig. 1. Standard curves for adipic acid (x), suberic acid (△), and sebacic acid (□) in normal urine. Increase in detector response ratios (peak height of SIM peaks 275 (x), 303 (△) or 331 (□) divided by the peak height of 317) versus dicarboxylic acids added to normal urine.

curve for each acid was determined before each series of 10—20 samples. Standard solutions for urine and serum of 0 and 60 µg/mg creatinine and 0 and 50 µg/100 ml, respectively, were used in this determination of the slope. Standard solutions were prepared by enrichment of normal serum and urine samples with adipic, suberic and sebacic acids.

RESULTS AND DISCUSSION

Fig. 2A shows the SIM profiles of the ion fragments *m/e* 275, 303 and 331 from a silylated extract of a mixture of serum from a 12 year old child. The concentrations of adipic, suberic, and sebacic acids in this mixture were 4, 4 and 3 µg/100 ml, respectively. This result illustrates the high sensitivity and specificity of the present method. The specificity, expressed as the ability to differentiate between compounds with the same nominal mass, but with different atomic compositions, is about 1000 ppm, the resolution of the mass spectrometer. The interference of other compounds with the SIM peaks in the 30 investigated serum and urine samples was negligible. This is a considerable improvement compared to the GC technique with flame ionisation detector [8], where the lack of specificity resulted in a limit of precise detection for adipic acid of about 10 µg/mg creatinine. The extraction efficiency, measured as the fraction of dicarboxylic acids extracted from pure water compared to freeze drying of the same solution, was for all the dicarboxylic acids including the internal standard, above 70% at a concentration of 6 and 30 µg/2 ml solution.

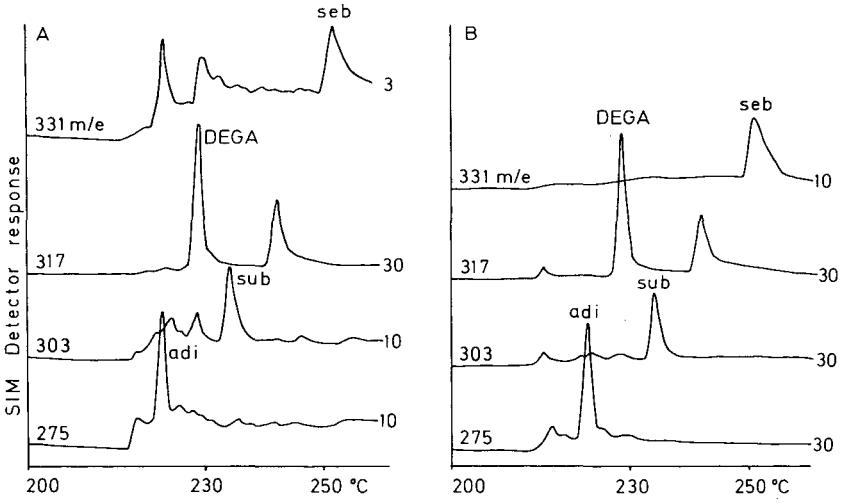

Fig. 2. Mass fragmentograms of: A, silylated extract from normal serum; B, silylated extract from normal serum enriched by 10 μg dicarboxylic acids per 100 ml serum. Peaks: adi = adipic acid; sub = suberic acid; DEGA = diethylglutaric acid (internal standard); seb = sebacic acid. The mass fragment ions are shown to the left on each trace and the relative attenuations to the right (GC conditions, see text).

Because of small variations in this extraction efficiency and also because of variation in the detector responses, it is essential for the SIM measurements to use an internal standard with a chemical structure similar to that of the compounds of investigation.

The results of the analysis of the variation between series of urines are shown in Table I. They are expressed as the standard deviation of the determinations of 10 aliquots from the same enriched urine, extracted and analysed on 10 different days. The variation coefficient of 10—15% is of the same order of magnitude as in the conventional GC method [8]. The variation within a series, i.e. the standard deviation of 10 consecutive determinations of 10 different extracts of the same urine, was 3—8% (results not shown).

The coefficient of variation for the serum analysis was 4—15%, measured at concentrations 10 and 50 μg/100 ml serum (results not shown).

The reliability of the present SIM method is shown by the results of the analysis of urine from neonates and children (Table II), and serum from children (Table III).

TABLE I

VARIATION BETWEEN SERIES OF DICARBOXYLIC ACID DETERMINATIONS

The figures represent the detector response ratios. Number of measurements = 10.

	Adipic acid		Suberic acid		Sebacic acid	
Concentration (μg/mg creatinine)	9	32	8	30	6	29
Mean ratio	0.23	0.83	0.29	1.08	0.33	1.48
Standard deviation	0.03	0.10	0.02	0.10	0.03	0.20
Variation coefficient	13%	12%	7%	9%	9%	14%

The finding of adipic, suberic, and sebacic acids in the serum and urine of all the investigated children indicates very strongly that the ω-oxidation pathway from long-chain monocarboxylic acids [9] is operative under normal physiological conditions and not only in pathological states [1—6]. The larger excretion of the dicarboxylic acids in the urine of the neonates than in that of the children (Table II) most probably reflects the high lipolytic activity and increased catabolism of fatty acids in the neonates [10].

TABLE II

EXCRETION OF ADIPIC, SUBERIC, AND SEBACIC ACIDS IN THE URINE OF NEONATES AND CHILDREN (μg/mg CREATININE)

	Adipic acid		Suberic acid		Sebacic acid	
	Range	Median	Range	Median	Range	Median
Neonates (n = 10) aged 1—4 days	2—62	15	3—19	6	0.2—12	6
Children (n = 10) aged 2.5—9 years	2—15	5	1— 9	2	0.6— 7	3

TABLE III

CONCENTRATION OF ADIPIC, SUBERIC AND SEBACIC ACIDS IN SERUM FROM CHILDREN

Concentrations in μg/100 ml; n = 10 children.

	Adipic acid		Suberic acid		Sebacic acid	
	Range	Median	Range	Median	Range	Median
Children aged 5—13 years	2—5	3	2—5	3	1—3	2

ACKNOWLEDGEMENT

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Note

Bromide measurement in serum and urine by an improved gas chromatographic method

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Bromide ion is an end product [1] of halothane metabolism following halothane anaesthesia in man. During an investigation of halothane metabolism we required a rapid and convenient method, suitable for routine use, to measure bromide ion in serum and urine in the 10—100 μg Br^-/ml range. Various techniques have been reviewed [2]. Most involve lengthy procedures and are either not sensitive enough [3, 4] for our requirements, or use specialised equipment [5, 6]. Gas chromatographic (GC) methods appeared to offer the best approach. Various reagents have been tested [7—9] but again we required a simpler and more sensitive procedure. A further potentially useful GC method for serum bromide has been reported [2, 10]. This method utilised the principle of oxidation of bromide to bromine followed by coupling the bromine to an unsaturated compound and assaying the bromo derivative by GC. In this paper we present an improved GC method based on the above work [2, 10] but having a tenfold higher sensitivity. Our method has been applied to the routine measurement of bromide at the 10—100 μg/ml level in both serum and urine from subjects undergoing halothane anaesthesia, and a report of the detailed clinical findings is in preparation.

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EXPERIMENTAL

Reagents

Sodium bromide, trichloroacetic acid (TCA), activated charcoal (powder, acid washed, for decolourising purposes), *n*-hexane, bromine and sulphuric acid were purchased from BDH, Poole, Great Britain; sodium hypochlorite (15% available chlorine), petroleum spirit (b.p. below 40°) and 2,4-dimethylphenol were from Koch-Light, Colnbrook, Great Britain; 2-cyclohexen-1-ol was from Aldrich, Milwaukee, Wisc., U.S.A. Standard sodium bromide solution was 1.29 mg/ml (1.0 mg/ml with respect to bromide ion) in water for use in standard curves and reproducibility tests. Sulphuric acid (2 *N*), TCA (30% in water) and 2,4-dimethylphenol (DMP; 200 mg in 20 ml petroleum spirit) were freshly prepared for each batch of analyses. Purity of the solvents and of DMP was checked using the GC procedure below and purification, where necessary, was achieved by alumina chromatography, or redistillation, or both.

Internal standard, 2,3-dibromocyclohexanol (DBCH)

The standard was prepared [11] by addition of a slight excess of bromine in CCl_4 to a solution of 2-cyclohexen-1-ol in CCl_4. After removal of excess bromine and concentration of the sample, the product (m.p. 56°) was recrystallised from CCl_4 at - 10° and characterised by gas chromatography—mass spectrometry (GC—MS). [M^+ group *m/e* 260, 258, 256 (1:2:1); M—Br at 179, 177; M—(Br+OH) at 160, 162; M—(Br_2 +H) at 97, corresponding to $C_6H_9(OH)Br_2$. (GC on a 1.5m × 4 mm glass column packed with 3% OV-1. at 150°, with He as carrier gas, 40 ml/min, a retention time of 4.0 min and MS electron impact 25 eV, 300 μA.)]

Between 30 and 60 mg of DBCH was dissolved in 1 ml of ethanol. The concentration of DBCH solution was adjusted so that 10 μl of stock added to the sample before extraction gave approximately the same peak area in the GC analysis as that of the bromo product formed from 40 μg/ml bromide (Fig. 1).

Samples

Serum (10 ml) and urine (20-ml portions from pooled 24-h samples) were collected from patients undergoing orthopaedic surgery under halothane anaesthesia. Pre-operative control sera and further control sera and urines from healthy laboratory staff were also collected. Samples were stored at - 20°.

Extraction procedure

Samples were thawed and each mixed thoroughly before portions were taken for assay.

Serum. To 1.0 ml of serum in a glass centrifuge tube add 2.0 ml 30% TCA solution and mix on a vortex mixer. Add 50 mg of charcoal, mix thoroughly and leave for 5 min. Centrifuge down the denatured protein and charcoal (bench centrifuge, e.g. MSE Minor, speed 7; 200 *g* for 2—3 min). Filter the supernatant through a glass wool plug packed into a pasteur pipette and transfer 1.0 ml of filtrate to a capped vial (2.0 ml size). Add 10 μl of internal standard DBCH and 30 μl of sodium hypochlorite, mix well, then add 0.3 ml of DMP solution. Close the vial securely and extract by shaking vigorously for 1

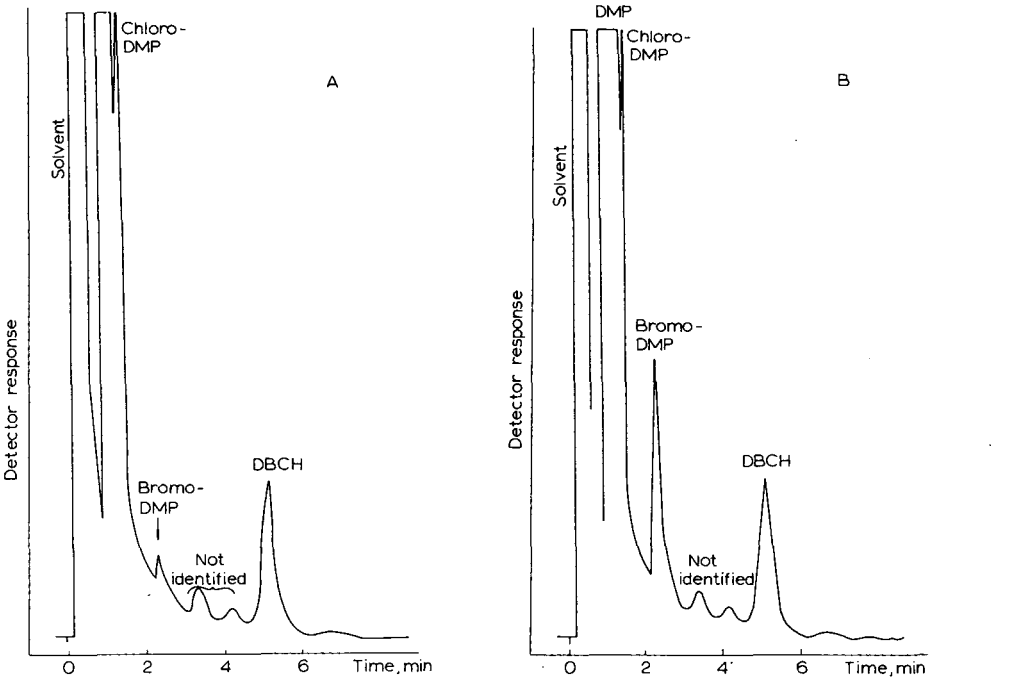

Fig. 1. GC traces from the analysis of: (A) pre-operative serum control; (B) 6 days post-halothane anaesthesia showing a level of bromo-DMP equivalent to 29 μg Br^-/ml serum. Phase OV-17 at 145°, bromo-DMP is measured derivative, DBCH is internal standard.

min. After separation of the solvent layers (by brief centrifugation if necessary), transfer the upper petroleum phase, containing the bromo-DMP product, to a small vial (800-μl Pye-Unicam Autojector sample holders are suitable). The solvent is evaporated off under a stream of dry nitrogen and the samples are stored, if this is necessary, without solvent at - 10°. Hexane (20 μl) is added and the samples warmed to room temperature for 10 min before the GC analysis.

Urine. Add 0.15 ml of 2 *N* H_2SO_4 to 1.5 ml of urine in a glass centrifuge tube. Mix well, and add 75 mg of charcoal. Stand 6 min with occasional shaking. Centrifugation and subsequent steps are the same as for serum above except that only 20 μl of sodium hypochlorite is used.

Controls, standard curves and other tests. Pre-operative sera or pooled blank serum and urine were used for controls and standard curves. Standard curves, multiple replicate tests and yield tests were performed by adding the appropriate volume of standard NaBr (1 μg/μl Br^- equivalent) at the beginning of the procedure or to the 1.0-ml aliquot taken after filtration.

Gas chromatography and assay procedure

The bromo-DMP product was assayed, using a Pye 104 chromatograph fitted with a flame ionization detector (FID) on a 2.1 m × 2 mm I.D. glass column packed with 3% OV-17 on Diatomite CQ, 100–120 mesh. Conditions were: column temperature, 145°; injector, 200°; carrier gas, nitrogen at a flow-rate of 15 ml/min; sample volume, 2 μl. Retention times: bromo-DMP, 2.1 min;

DBCH, 5.0 min. The GC column was purged for 15 min at 230° after 20 to 25 samples had been run.

Peak areas were measured by electronic integration with baseline correction, and the bromo-DMP values adjusted to constant internal standard recovery. A linear regression programme was used for the standard curves and the sample values read directly from this using a programmable calculator.

RESULTS AND DISCUSSION

GC traces from the analysis of pre-operative serum control (Fig. 1A) and six days post-halothane anaesthesia (Fig. 1B) are shown for patient PL. The bromo-DMP level in Fig. 1B is equivalent to 29 μg Br^-/ml. Small amounts (usually $<5\mu$g/ml) of bromide were found in all control sera. Identification of the peaks marked was made from standards and confirmed by GC—MS (conditions as given for DBCH). The phase OV-17 was preferred to Poly A-103 [10] because bromo-DMP and the internal standard both elute in clear areas; also a rapid analysis time of 6 min is possible. Any effect on the bromo-DMP measurement caused by the proximity of the large DMP plus chloro-DMP peak is corrected by an angular baseline correction applied by the integrator. The chloro-DMP arises by reaction of DMP with chlorine released from the sodium hypochlorite.

Table I summarizes the tests on recovery, precision and the results from applying the method to serum and urine analysis. Linearity of the standard curves was obtained over the range 15—150 μg/ml, but values became inaccurate under 10 μg/ml (see below). Few samples had Br^- values exceeding 100 μg/ml, so the routine standard curves to 100 μg/ml were extrapolated for values above 100 μg/ml. Recovery and reproducibility were considered to be adequate for our investigations because of the fairly large changes of bromide concentration observed in the experimental samples.

Some results showing these changes in serum bromide concentration are given in Table II. The overall recovery (73%) of bromide from serum was

TABLE I

SUMMARY OF EXPERIMENTAL FINDINGS

Parameter	Serum	Urine
Range of samples tested (μg Br^-/ml)	<10—123	<10—65
Mean of sample values (μg Br^-/ml)	32	25
Number of samples assayed	56	50
Variation limits (%) in replicate analyses (10 samples each at 20 μg Br^-/ml and 40 μg Br^-/ml)	± 3.9	± 5.8
Overall recovery of bromide (%)	73	57
Working range (μg/ml)	10—150	

TABLE II

SOME OBSERVED CHANGES IN SERUM BROMIDE LEVELS (μg/ml) AFTER HALOTHANE ANAESTHESIA

Patient	Pre-operative (control)	Post-operative		
		Day 2	Day 6	Day 9
MC	<0.5	21	123	97
RK	<0.5	12	42.2	8.7
AP	<0.5	28	27	9
PL	<0.5	4	29	37
SC	<0.5	5	15	<0.5

similar to that found previously [10], indicating that in the 10—1,000 μg/ml range, a constant proportion of the bromide is not recovered. Recovery from urine was lower (57%) but the proportion remained constant. The effect possibly contributes to the lower accuracy for urine measurements, for below about 15 μg/ml the quantitative recovery of bromide falls away markedly. This may be due to a generally poor recovery, or high adsorption of bromide to protein or charcoal at these low levels. Calculated results therefore become inaccurate below this level, and 10 μg/ml is essentially the lower limit of accuracy (but not necessarily detection) of the method.

The internal standard is in effect an extraction standard. Initial experiments showed that variation between replicate analyses was attributable to the derivative formation and extraction stage. A standard was found which was soluble in both water and petroleum spirit. Trials showed that the efficiency of formation and recovery of bromo-DMP was paralleled by the amount of DBCH extracted into the petroleum phase. Thus DBCH is a reliable internal standard for use in quantitative measurements. When the "trial" serum procedure (modified from ref. 10) was applied to urine no bromo-DMP was formed. This was presumably due to interference by unsaturated compounds in urine; this interference noted previously [12], provided the basis for development of the coupling method for serum.

The problem of how to remove these unsaturated compounds was solved simply by the addition of activated charcoal. An enhancement of the yield was found also on the addition of charcoal to the serum samples; therefore, the charcoal step was included in both procedures. One drawback to the use of charcoal is that the internal standard cannot be added until after the removal of charcoal.

GC methods [13] for analysis of μg/ml levels of inorganic anions are often unsatisfactory for biological samples. The available GC methods for bromide [2, 10] have lower detection limits of about 100 μg Br^-/ml. By using charcoal adsorbent and an effective internal standard, we have increased the sensitivity to a lower limit of 10 μg Br^-/ml. This gives a method which is rapid enough to be suitable for routine measurements of normal post-operative bromide levels after normal anaesthetic doses of halothane. These bromide levels (10—

100 μg/ml) previously could only be measured by activation analysis or X-ray fluorescence.

The system described, therefore, provides a method for the measurement of bromide in serum and urine. We anticipate that the method could be applied also to bromide determinations in other fluids, both biological and chemical.

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Note

Method for the fluorimetric estimation of dopamine

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Fluorimetric determination of dopamine has been performed by the trihydroxyindole method [1, 2] and the ethylenediamine condensation method [3, 4] after extraction of catechol compounds from biological extract by alumina adsorption. The ethylenediamine condensation method was automated and coupled with a high-performance liquid chromatograph [5, 6], and fluorescent derivatives of dopamine and norepinephrine have been separated by high-performance liquid chromatography (HPLC) on silica gel [7] or a synthetic gel [8]. By these methods, dopamine in both tissue extract and urine could be successfully estimated, but they were not sufficiently sensitive for the determination of picogram amounts of dopamine present in human plasma.

On the other hand, one of us [9] has reported that catecholamines could be estimated fluorimetrically by oxidizing them with hexacyanoferrate(III) in the presence of ethylenediamine in an alkaline borate buffer at 75°. Oxidation of dopamine dissolved in a borate buffer (pH 9.0) containing a primary amine or amino acid with hexacyanoferrate(III) at 80° yielded also fluorescent product(s), and among the compounds tested, *p*-aminobenzoic acid gave the best result. When the method was automated and used to measure dopamine eluted from a column of Amberlite IRC-50 with a mixed buffer of pH 6.3, 0.1 ng of dopamine could be detected at a signal-to-noise ratio of 10, and by this method, dopamine in human plasma could be estimated.

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EXPERIMENTAL

Reagents

Epinephrine hydrogen tartrate was purchased from Nakarai Pharmaceuticals (Kyoto, Japan), norepinephrine hydrogen tartrate, deoxyepinephrine hydrochloride, dopamine hydrochloride and *p*-aminobenzoic acid were purchased from Yashima Pharmaceuticals (Osaka, Japan). Other chemicals were of reagent grade. Stock solutions of catecholamine base were prepared in 0.01 *M* HCl.

Apparatus

A constant flow pump (Jasco, Model TRI ROTAR) was used to pump buffer through a chromatographic column. Peristaltic pumps (Atto, Model SJ-1211 H and L) were used to pump air and reagents into a gas-segmented-flow reaction detector. A spectrofluorimeter (Jasco, Model FP-550) equipped with a flow cell (square cross section, 4 × 4 × 20 mm internal dimension) was used to measure fluorescence. Samples were injected into the column by a motor-driven injector (Kyowa Seimitsu, Auto-Injector Model KUH-6000).

Preparation of Amberlite CG-50 column

Amberlite CG-50 (type 2) was graded according to size, washed and buffered as described previously [10]. The buffered resin was poured into a tube with phosphate buffer of pH 6.5 (0.4 *M*) and allowed to settle under gravity to a height of 12 cm (the tube was 20 × 0.4 cm I.D. with a 10-ml reservoir); the column was washed with 2 ml of water before use.

Preparation of Amberlite IRC-50 column

Amberlite IRC-50 (45—55 μm in sodium ion form) was prepared and washed as described previously [11], and suspension of the washed resin (sodium ion form) was buffered at pH 6.3 with a succinic acid solution (0.5 *M*) and then washed with an eluent. The eluent is a mixed buffer of pH 6.3 containing 0.35 *M* boric acid, 0.12 *M* succinic acid and 0.002 *M* disodium ethylenediamine tetraacetate. The washed resin was suspended in an equal volume of the eluent and the suspension was poured into a chromatographic tube (15 × 0.8 cm I.D.), and allowed to settle under gravity. Then the tube was fitted with a column adjuster and the eluent was pumped through the column at the rate of 0.8 ml/min for several hours at 42°. The height of the resin column was 12 cm.

Preparation of catecholamine fraction from human plasma

Heparinized blood was drained into a chilled tube containing 5 m*M* reduced glutathione and centrifuged immediately in the cold at 1500 *g* for 15 min. The plasma was divided into 1.0 ml portions and stored at −20°. A frozen plasma sample (1.0 ml) was mixed with 1.0 ml of 0.1 *M* HCl and thawed. Then it was deproteinized with 1.0 ml of 2.0 *M* perchloric acid and centrifuged in the cold at 1500 *g* for 15 min. The supernatant was transferred to a 20 ml beaker, the precipitated protein was mixed with 2 ml of 0.5 *M* perchloric acid and centrifuged again. The supernatant was combined, chilled in an ice bath, and after 0.5 ml each of 5% (w/v) disodium ethylenediamine tetraacetate and

0.5% (w/v) ascorbic acid solution were added, was neutralized to pH 6.2 with a 1 *M* potassium carbonate. The supernatant was added to a column of Amberlite CG-50, the precipitate of potassium perchlorate was washed with 2 ml of cold 0.05% (w/v) disodium ethylenediamine tetraacetate (pH 6.2) and the washing was also added to the column of Amberlite GC-50. The column was washed with 4 ml of deionized water and then with 1.0 ml of 2/3 *M* boric acid solution, then another 2.0 ml of the boric acid solution were used to elute catecholamines from the column. The eluate was collected in a test-tube containing 0.07 ml of a solution of 1 *M* HCl containing sodium dihydrogen phosphate (0.5 *M*) and disodium ethylenediamine tetraacetate (0.005 *M*). The eluate in the test-tube was adjusted to pH 6.3 with 1 *M* HCl containing sodium dihydrogen phosphate (0.5 *M*) and disodium ethylenediamine tetraacetate (0.005 *M*) and diluted to 4.0 ml with a succinate buffer of pH 6.3 (0.08 *M*), and stored in a refrigerator.

Chromatographic separation of samples

A 1-ml volume of a solution of the amines in the eluent to be used for chromatography, or 1.0 ml of catecholamine fraction prepared as described above was injected into the column of Amberlite IRC-50. Then, elution was carried out with the eluent at a flow-rate of 0.7 ml/min.

Fluorimetric determination of dopamine

A gas-segmented-flow reaction detector was assembled from commercial parts and pyrex coils. Pyrex coils were made by winding 4-mm pyrex tubing around a brass tube of 14 mm O.D. As shown in Fig. 1, eluate from the column was fed to the detector and segmented by air, mixed with 1% (w/v) *p*-aminobenzoic acid solution in 0.1 *M* disodium hydrogen phosphate (mixing coil, 7 turns), 0.8 *M* sodium hydroxide containing 0.1% (w/v) Brij-35 (mixing coil, 7 turns), 0.3% (w/v) hexacyanoferrate (III) (mixing coil, 7 turns), and

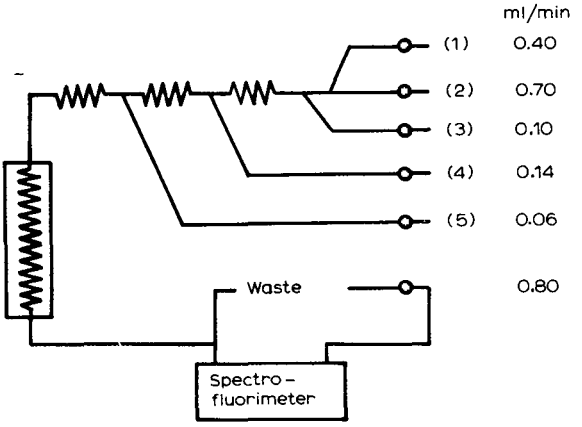


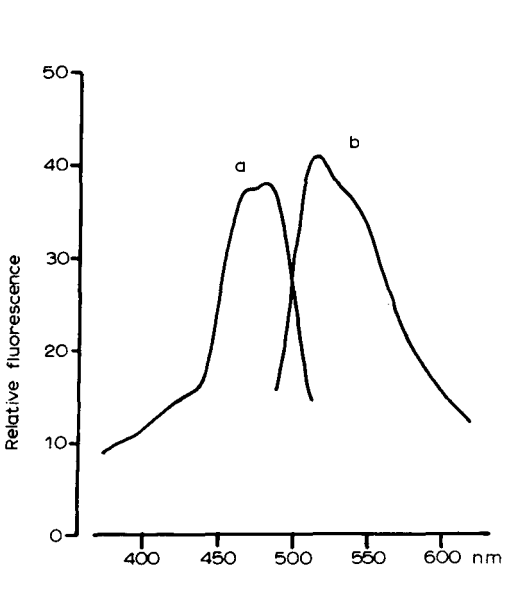
Fig. 1. Schematic diagram of the gas-segmented-flow reaction detector. pH of the waste was 9.0. (1) air; (2) eluate; (3) 1% (w/v) solution of *p*-aminobenzoic acid in 0.1 *M* disodium hydrogen phosphate; (4) 0.8 *M* NaOH—0.1% (w/v) Brij-35, (5) 0.3% (w/v) hexacyanoferrate(III).

heated at 80° (heating coil, 30 turns). Bubbles were removed from the stream and the fluorescence was measured at 520 nm, with excitation at 465 nm. The slit width of both excitation and emission was 20 nm.

RESULTS AND DISCUSSION

p-Aminobenzoic acid was used for the formation of fluorescent product(s) from dopamine in an alkaline reaction medium containing hexacyanoferrate (III) as an oxidizing agent. The method was automated as shown in Fig. 1. Eluate from a column was mixed with the reagents and heated at 80° and the times for mixing and heating were 6 and 5.5 min, respectively. Oxidation was necessary for the formation of fluorescent product(s) from dopamine, and the optimum pH for the reaction was 9.0. Oxidation of dopamine with hexacyanoferrate(III) in the presence of *p*-aminobenzoic acid under alkaline conditions was preferred to oxidation at neutral pH prior to the addition of *p*-aminobenzoic acid and sodium hydroxide solution since the latter reaction sequence has a lower yield of fluorescent product(s). Reduction of hexacyanoferrate(III) was not necessary, because hexacyanoferrate(III) in the reaction mixture absorbed less than 5% of the excitation light.

A flow cell with a square cross section was used since it gave lower background fluorescence than a flow cell with round cross section. Excitation and



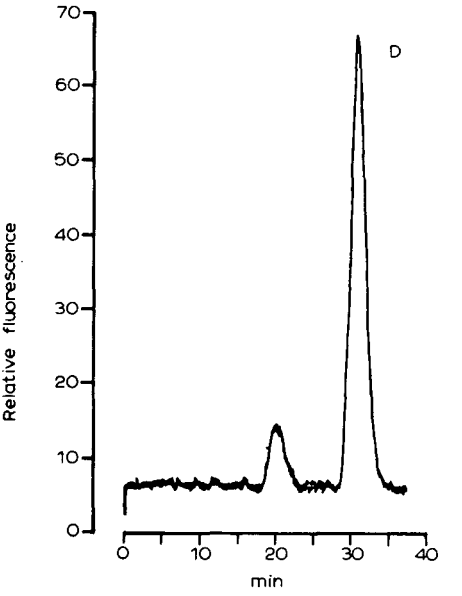


Fig. 2. Fluorescence spectra (uncorrected) of the reaction product(s) of dopamine. A solution of dopamine in the eluent (6 μg/ml) was fed to the detector. Slit width of excitation and emission was 5 nm and 10 nm respectively. (a) = excitation spectrum, emission wavelength was 520 nm; (b) = emission spectrum, excitation wavelength was 465 nm.

Fig. 3. Elution and fluorimetric estimation of dopamine. One nanogram of dopamine was separated on the Amberlite IRC-50 column and estimated using the detector described in Fig. 1. Peak D = dopamine. The first peak = impurity present in sample solution.

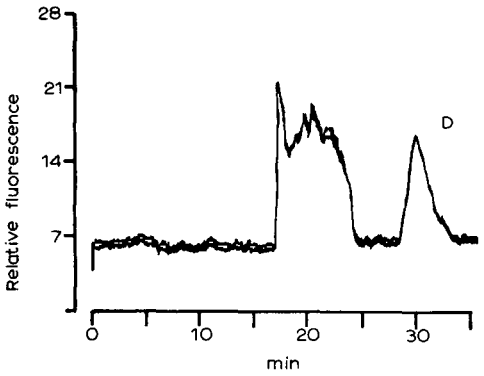


Fig. 4. Elution and fluorimetric estimation of dopamine from human plasma. Retention time of peak D corresponded to that of dopamine.

emission spectra of the fluorescent product(s) from dopamine are shown in Fig. 2. Epinephrine and norepinephrine yielded virtually no products which fluoresce following excitation at 465 nm, but deoxyepinephrine could be measured at a sensitivity of ca. one sixth and dopa at ca. the same sensitivity as that of dopamine. A shorter column than that reported previously, was used in this experiment [9], because it gives a shorter analysis time and higher sensitivity, and deoxyepinephrine, which was not completely separated from dopamine, will not interfere with the estimation of dopamine in human plasma or in urine, since it is not usually found in these samples.

The elution pattern of dopamine was readily reproducible and 1.0 ng of dopamine could be measured at a signal-to-noise ratio of 100 (Fig. 3). A linear relationship between the peak height and the amount of dopamine added to the column was obtained over the range of 0.05—100 ng. As shown in Fig. 4, a peak with a retention time corresponding to that of dopamine was clearly separated from the peaks of impurities present in a catecholamine fraction from human plasma. When 1.0 ng of dopamine was added to plasma samples before deproteinization, the recovery was 87 ± 1.5% (S.E.; 5 determinations). Dopamine content of human plasma taken from 26 individuals (morning; at rest and before breakfast) ranged from 0.60 ng to 0.09 ng, and the mean value was 0.23 ng ± 0.17 (S.D.) per ml.

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Note

Analysis of urinary 4-hydroxy-3-methoxyphenylethylene glycol as vanillyl alcohol by high-performance liquid chromatography with amperometric detection

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4-Hydroxy-3-methoxyphenylethylene glycol (HMPG) is a major metabolite of norepinephrine [1] that is found in urine and cerebrospinal fluid (CSF). It has been determined that 25—50% of urinary HMPG is derived from brain norepinephrine [2—4].

Urinary HMPG exists as the non-conjugated, free molecule (HMPG-free), as the conjugate of sulfuric acid (HMPG-SO_4) and as the β-conjugate of glucuronic acid (HMPG-Glu). In human urine, HMPG exists mainly in the conjugated form [5—7]. The sulfate conjugate has been reported to be derived mainly from the metabolism of brain norepinephrine [2, 4, 8], while the β-glucuronide conjugate has been reported to be derived mainly from the metabolism of systemic norepinephrine [4, 7, 8]. The origin of HMPG-free remains uncertain although HMPG exists in CSF predominantly as the non-conjugated molecule [9].

Hydrolysis of conjugated HMPG, either enzymatically or under acidic or basic catalysis, affords the non-conjugated molecule. Acid catalyzed hydrolysis of conjugated HMPG is not recommended because HMPG has been reported to decompose under the conditions of the reaction [10]. Enzymatic hydrolysis of conjugated HMPG in urine can be selectively performed through the use of the appropriate enzymes [3, 5, 8]: Aryl sulfatase type VI (EC 3.1.6. 1) liberates HMPG from HMPG-SO_4 [11]; β-gluconidase type V-A liberates HMPG from HMPG-Glu while β-glucuronidase type H-1 (contains aryl sulfatase activity) liberates HMPG from HMPG-SO_4 and HMPG-Glu. The sum of HMPG-free, HMPG-SO_4 and HMPG-Glu equals HMPG-total. It is HMPG-total that has been analyzed and reported in most clinical investigations.

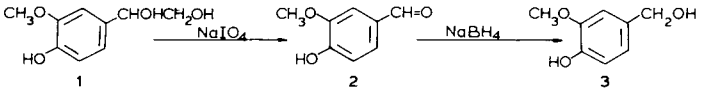
We were interested in ascertaining the differences in the excretion of HMPG-free, HMPG-SO_4, HMPG-Glu and HMPG-total by schizophrenic patients and mentally normal control subjects. It was hoped that one of these forms of

HMPG might provide a clue to brain norepinephrine metabolism in schizophrenia.

The quantitative analysis of HMPG in body fluids has been performed using gas—liquid chromatography (GLC) with flame ionization detection [12], electron capture detection [4, 8, 10, 13, 14] and gas chromatography—mass spectroscopy (GC—MS) [7, 15—17]. GC methods for the determination of HMPG in body fluids have the necessary sensitivity to detect HMPG-free (approximately 0.20 ng/μl in urine) and may have the necessary selectivity to detect HMPG-free. No matter what type of detector one employs in the GC analysis, HMPG must be derivatized to be volatile. The commonly used derivatives of HMPG are unstable and are not specific for HMPG. Other compounds present in the urine extract could become gas chromatographically active when subjected to the derivatization process and may interfere with the HMPG analysis. The derivatization process leaves a gas chromatographically active residue which may also interfere with the analysis.

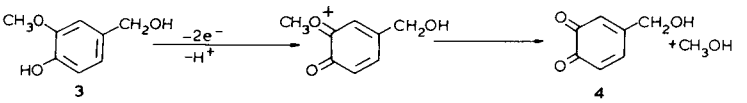
The quantitative analysis of HMPG in body fluids by reversed-phase high-performance liquid chromatography (HPLC) with amperometric detection was investigated because of the high selectivity afforded by both the μBondapak C_{18} HPLC column [18] and the oxidizing electrochemical detector [19—22] and the high sensitivity of the oxidizing electrochemical detector [19, 20] towards easily oxidizable organic functional groups such as phenols [22]. Reversed-phase HPLC columns and the amperometric detector permit the use of aqueous buffer as the mobile phase. This is ideal for the analysis of biological compounds isolated from body fluids. The pH of the buffer affects the mobility of the compounds on the reversed-phase column [23] thus affecting the selectivity of the system. The compounds being analyzed do not require derivatization but must be electrochemically oxidizable. Compounds that are electrochemically inactive cannot be detected. The oxidation potential of the detector can be varied so that functional groups with low oxidation potentials can be detected while functional groups with higher oxidation potentials are not detected. This increases both the selectivity and the sensitivity of the detector.

Utilizing the procedure of Felice and Kissinger [24], HMPG (1) was oxidized by periodate to vanillin (2) and the vanillin thus formed was reduced by sodium borohydride to vanillyl alcohol (3) via the procedure of Schwedt et al. [25].



1 2 3

At the carbon paste electrode of the detector, vanillyl alcohol (3) was converted into an *o*-quinone (4) via a two electron process [26].



3 4

MATERIALS AND METHODS

Materials

Glass distilled ethyl acetate, methanol and toluene were obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.) and were used without further purification. Water was glass distilled from an alkaline permanganate solution. All chemicals were reagent grade. Aryl sulfatase type VI (EC 3.1.6.1), lot No. 127C-6820; β-glucuronidase type V-A (from *E. coli*), lot No. 107C-6810 and β-glucuronidase type H-1 (from *Helix pomatis*), lot No. 67C-0393 were obtained from Sigma (St. Louis, Mo., U.S.A.) and were used without further purification.

Solution

The internal standard, isovanillyl alcohol (Aldrich, Milwaukee, Wisc., U.S.A.), was used as a 500 ng/μl aqueous solution. A standard 58.6 ng/μl aqueous HMPG solution (free acid) was used.

Apparatus

The analyses were performed on an LC-50 liquid chromatograph (Bioanalytical Systems) with an oxidizing electrochemical detector, employing a carbon paste electrode and an Ag/AgCl reference electrode. A μBondapak C_{18} (Waters Assoc., Milford, Mass., U.S.A.) HPLC column (30 cm × 3.9 mm I.D.) containing octadecyl silane chemically bonded to 10 μm Porasil (10%, w/w) was used for the separation.

Analysis of HMPG-free, HMPG-SO_4, HMPG-Glu and HMPG-total from urine [24, 25]

Samples were prepared in duplicate as follows:

Sample 1: 3.00 ml of standard urine (24 h pool utilized for all analyses).

Sample 2: 3.00 ml of standard urine plus 25 μl of a 58.3 ng/μl HMPG solution.

Sample 3: 3.00 ml of standard urine plus 50 μl of a 58.3 ng/μl HMPG solution.

Sample 4: 3.00 ml of standard urine plus 100 μl of a 58.3 ng/μl HMPG solution.

Sample 5: 3.00 ml of unknown urine.

Sample 6: 3.00 ml of unknown urine plus 150 μl of aryl sulfatase type VI (EC 3.1.6.1).

Sample 7: 3.00 ml of unknown urine plus 30 mg of β-glucuronidase type V-A plus 15 μl $CHCl_3$.

Sample 8: 3.00 ml of unknown urine plus 30 mg of β-glucuronidase type H-1.

To each sample was added 0.5 ml of a 2% EDTA solution. Each sample was further modified as follows:

Samples 1—5: Added 1.0 ml of 0.45 *M* acetate buffer, pH 6.8 and the pH of the sample was adjusted to 6.5.

Sample 6: Added 1.0 ml of 0.45 *M* acetate buffer, pH 7.0 and the pH of the sample was adjusted to 7.1 (at pH 7.0, sulfatase had no β-glucuronidase activity).

Sample 7: Added 1.0 ml of 0.45 *M* acetate buffer, pH 6.5 and the pH of the samples was adjusted to 6.8.

Sample 8: Added 1.0 ml of 0.45 *M* acetate buffer, pH 5.0 and the pH of the sample was adjusted to 5.2.

All samples were kept in a constant temperature bath for 18—22 h at 37°. After incubation, the pH of each sample was adjusted to 6.5. Each sample was extracted 3 times with 10 ml of ethyl acetate. The ethyl acetate pool from each sample was washed with 1.0 ml of 1.0 *M* $NaHCO_3$ to remove carboxylic acid contaminants. The ethyl acetate layer was taken to dryness at 37° under a stream of nitrogen.

The residue from each sample was redissolved in 1.5 ml of 1.0 *M* K_2CO_3. To each solution was added 0.1 ml of a 3% $NaIO_4$ solution. The samples were kept for 10 min at 39°. Excess $NaIO_4$ was decomposed by the addition of 0.1 ml of a 10% $Na_2S_2O_5$ solution to each sample. The pH of each sample was adjusted to 6.5 by the addition of 0.3 ml of 5.0 *M* acetic acid and 0.6 ml of 1.0 *M* acetate buffer, pH 6.3 [24]. Each sample was extracted 3 times with 5 ml of toluene [24]. To the pooled toluene from each sample was added 10 μl of a 500 ng/μl isovanillyl alcohol solution (internal standard). The pooled toluene was extracted 3 times with 1 ml of 1.0 *M* K_2CO_3. $NaBH_4$ (100 mg) was added to the pooled K_2CO_3 solution from each sample and allowed to react for 20 min at ambient temperature. Excess $NaBH_4$ was quenched and the pH readjusted to 8.6 by the addition of 0.7 ml of 5.0 *M* acetic acid to each sample. Each sample was extracted 3 times with 5 ml of ethyl acetate. The pooled organic layer from each sample was taken to dryness at 39° under a stream of nitrogen. The residue from each sample was redissolved in 1.0 ml of glass distilled water.

HPLC analysis. 20 μl of each solution was placed on to a μBondapak C_{18} column via a loop injector. The mobile phase, 0.5 *M* acetate buffer, pH 4.7 containing 15% methanol, was pumped through the column at a flow-rate of 1.5—1.7 ml/min. The electrochemical detector was set at + 0.85 V vs. an Ag/AgCl reference electrode. The sensitivity of the detector was set at 10 nA/V with the recorder set at 1.0 V full scale deflection and a chart speed of 0.5 cm/min. The average retention time for vanillyl alcohol was 5 min while the average retention time for isovanillyl alcohol was 7 min.

Calculations. A standard curve using the method of standard additions was prepared from the analytical results of samples 1—4 by plotting the vanillyl alcohol/isovanillyl alcohol peak height ratio versus the [HMPG] of the standard urine. Linear regression analysis was employed to afford the best linear graph. The equation for the standard curve was $y = 0.37x + 0.072$. The slope of the linear regression line was used to determine the HMPG in unknown urine samples. The average of each duplicate analysis was recorded.

Sample 5 afforded the HMPG-free of the unknown sample.

Sample 6 afforded the HMPG-free + HMPG-SO_4 of the unknown urine sample.

Sample 7 afforded the HMPG-free + HMPG-Glu of the unknown urine sample.

Sample 8 afforded the HMPG-total of the unknown urine sample.

Subtracting HMPG-free from the HMPG in samples 6 and 7 afforded HMPG-SO_4 and HMPG-Glu respectively.

Using this procedure, urinary HMPG concentrations of 50 pg/μl could be measured with a range of linearity from 30 pg/μl to 5.00 ng/μl.

RESULTS AND DISCUSSION

Attempts to analyze HMPG directly from the ethyl acetate extract of urine failed because of our inability to separate the HMPG peak from much larger interfering peaks. Conversion of the extracted HMPG to vanillin (2) using periodate oxidation according to the method of Felice and Kissinger [24] yielded a much cleaner chromatogram. However, we still could not completely resolve the vanillin peak from an interfering peak in the chromatogram. Felice and Kissinger [24] analyzed urinary vanilmandelic acid (VMA) as vanillin and reported no interfering peaks. VMA exists in urine in much higher concentrations than does HMPG-free. In their analysis, Felice and Kissinger utilized a detector sensitivity of 50 nA/V while we used a detector sensitivity of 10 nA/V. At our higher sensitivity, any small interfering peak unnoticed by Felice and Kissinger in their analysis would be very large in our HMPG analysis and would greatly interfere with vanillin derived from HMPG.

Oxidation of HMPG (1) to vanillin (2) by periodate followed by the reduction of the vanillin to vanillyl alcohol (3) by sodium borohydride was found to be specific for HMPG. No peaks other than those due to vanillyl alcohol and isovanillyl alcohol (internal standard) were present in the chromatogram (Fig. 1).

When a known amount of HMPG, 1.00 μg to 6.50 μg, was added to 3.00 ml aliquots of water and carried through the procedure, a straight line, $y=0.91x$

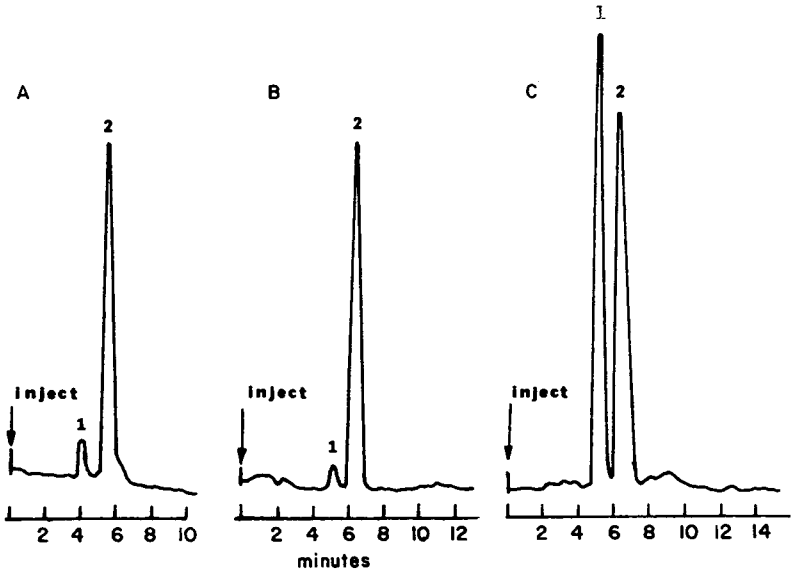


Fig. 1. Chromatogram of HMPG detected as vanillyl alcohol by HPLC with amperometric detection. Peaks: 1, vanillyl alcohol; 2, isovanillyl alcohol. (A) HMPG (0.33 ng/μl) from water; (B) HMPG-free (0.18 ng/μl) from urine; (C) HMPG-total (2.93 ng/μl) from urine. The mobile phase is 0.5 *M* sodium acetate, pH 4.7 containing 15% (v/v) methanol. The flow-rate is 1.5 ml/min. The detector was set at 10 nA/V at an oxidation potential of + 0.85 V vs. an Ag/AgCl reference electrode.

- 0.03 (correlation coefficient 0.991) resulted. When a known amount of HMPG, 1.00 μg to 6.00 μg was added to 3.00-ml aliquots of standard urine (a 24-h urine pool) and carried through the procedure, a straight line, $y=0.37x + 0.072$ (correlation coefficient 0.983) resulted. It is more difficult to extract HMPG from urine than from water. Once the HMPG was removed from urine, all of the extractions were 91—97% efficient.

Because of the reactivity of *o*-methoxyphenols with periodate [27, 28] some loss of HMPG will occur during the oxidation of HMPG to vanillin. Allowing the reaction to proceed for a specific time span, 10 min at 37° gives the same percentage loss per sample as shown by the linearity of the results obtained from the addition of HMPG to water and urine. Isovanillyl alcohol, the internal standard, reacted completely with periodate during the 10 min oxidation reaction. Thus necessitating the addition of isovanillyl alcohol to each sample after the periodate oxidation. The procedure gave reproducible results.

The extent of enzymatic hydrolysis of conjugated HMPG varies with the urine sample. In some urine samples, endogenous inhibitors of the hydrolytic enzymes are present [7]. In other instances, the use of an excess of the enzyme will cause inhibition [7]. The sum of the experimentally determined HMPG-free + HMPG-SO_4 + HMPG-Glu should be 100 ± 20% of the experimentally determined HMPG-total. A lower limit of 80% was judged as being acceptable. This idealized condition was difficult to meet because of the variability of the

TABLE I

HMPG OBTAINED FROM THE HPLC-AMPEROMETRIC DETECTION ANALYSIS OF HUMAN URINE

Sample volumes (ml)	HMPG-free		HMPG-SO_4*		HMPG-Glu*		HMPG-total		Percent of HMPG-free + HMPG-conj as HMPG-total
	(ng/μl)	(mg)	(ng/μl)	(mg)	(ng/μl)	(mg)	(ng/μl)	(mg)	
24-h samples									
1258	0.13	0.16	0.48	0.60	1.21	1.52	2.14	2.69	85
1557	0.30	0.47	0.65	1.01	0.91	1.42	2.31	3.60	81
Individual samples									
264	0.16	0.04	1.34	0.35	1.72	0.45	3.83	1.01	84
113	0.23	0.03	1.51	0.17	1.59	0.18	3.84	0.43	87
130	0.15	0.02	1.16	0.15	1.42	0.18	3.05	0.40	90
280	0.14	0.02	0.44	0.12	0.76	0.21	1.68	0.47	80
340	0.12	0.04	0.31	0.11	0.31	0.11	0.75	0.26	99
240	0.13	0.03	0.54	0.13	1.39	0.33	2.36	0.57	86
388	0.13	0.05	0.76	0.30	0.72	0.28	1.98	0.77	82
82	0.29	0.02	1.02	0.08	1.45	0.12	2.96	0.24	93
124	0.31	0.04	0.72	0.09	1.35	0.17	2.02	0.25	118
100	0.47	0.05	1.92	0.19	2.32	0.23	4.09	0.41	115
96	0.31	0.03	0.57	0.06	0.94	0.90	2.31	0.22	82

*HMPG-free is not included in these values.

hydrolytic efficiency of the enzymes from urine sample to urine sample. In every determination, the same amount of enzyme was used per urine sample. This variability was not a result of a change in potency of the enzyme from lot to lot because only the same lot number was used for each analysis.

From the 24-h urine samples and the individual (non-24 h) urine samples that have been analyzed and which fall within the 100 ± 20% range, it can be seen that the HMPG-SO_4 is less than or equal to the HMPG-Glu (Table I). This is in contrast to the results of Bond and Howlett [8], who reported HMPG-Glu to be less than HMPG-SO_4 in human urine. Our results are comparable to those of Murray et al. [7] and Joseph et al. [4], who reported HMPG-SO_4 to be less than or equal to HMPG-Glu in 24-h human urine samples.

The normal 24-h urinary HMPG values obtained by this method are: HMPG-free (0.16—0.47 mg); HMPG-SO_4 (0.60—1.01 mg); HMPG-Glu (1.42—1.52 mg); HMPG-total (2.69—3.60 mg).

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Note

Simultaneous separation of β-lipotrophin, adrenocorticotropic hormone, endorphins and enkephalins by high-performance liquid chromatography

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The object of this study has been to develop and evaluate methods of high-performance liquid chromatography (HPLC) for the separation of polypeptide hormones. Tissues active in the synthesis of such hormones often produce and/or contain a spectrum of related materials which may be precursors and fragments. This is especially apparent in their ectopic production by tumours when the normal balance of synthesis, intracellular processing, secretion and degradation may be altered. Our aim has been, therefore, to ascertain whether HPLC can achieve their rapid separation, identification and measurement in a manner analogous to that which we have already described for steroid hormones [1].

Several recent papers have shown that peptides can be separated using reversed-phase partition HPLC systems [2—6]. A number of hormones, including somatostatin [2], oxytocin [3], melanotrophin (α-MSH) and angiotensin [4], releasing factors [5], insulin [6] and some endorphins [7], have been individually chromatographed under a variety of different HPLC conditions. Systematic examinations of the capability of single optimised systems for separating simultaneously a wide range of related hormones are, however, few at present. As part of such a study we present here an HPLC system capable of rapidly separating at least 13 members of the adrenocorticotropic hormone (ACTH)—lipotrophin series, ranging in size from β-lipotrophin (91 residues) to enkephalin (5 residues). This chromatographic system does not destroy the bio- and immunoactivity of ACTH.

MATERIALS

Chemicals

Synthetic human adrenocorticotrophin ($ACTH_{1-39}$) and [D-Ser^1, Lys^{17,18}]-ACTH-(1—18)-amide (Ciba 41'795-Ba) were a gift from Dr. W. Rittel (Ciba-Geigy, Basle, Switzerland) and corticotrophin-(1—24)-tetracosapeptide (Synacthen, $ACTH_{1-24}$) was obtained from Ciba, Horsham, Great Britain. Corticotrophin-like intermediate lobe peptide (CLIP, synthetic human sequence $ACTH_{18-39}$), and β-lipotrophin (LPH) extracted from human pituitaries, were gifts from Dr. P.J. Lowry (St. Bartholomew's Hospital, London, Great Britain). Chromatographically purified porcine pituitary $ACTH_{1-39}$ (150 U/mg) was obtained from Sigma (Poole, Great Britain) and all other peptides from Bachem (Torrance, Calif., U.S.A.). A full list of polypeptides tested is given in Table I.

Acetonitrile (HPLC S grade) was obtained from Rathburn Chemicals (Walkerburn, Great Britain) and other reagents for chromatography were AnalaR (BDH, Poole, Great Britain) dissolved in double glass-distilled water.

Apparatus

Chromatography was carried out using a DuPont Model 830 chromatograph with 838 programmable gradient module, coupled in series to a DuPont Model 837 variable wavelength spectrophotometer and a Schoeffel Model 970 fluorescence detector. Samples were injected via a Rheodyne Model 7120 septumless valve fitted with a 175-μl injector loop on to stainless-steel columns (100 × 5 mm I.D.) slurry-packed with Hypersil-ODS (5 μm) according to the manufacturer's instructions (Shandon Southern, Runcorn, Great Britain). Eluted polypeptides were collected for subsequent bio- and radioimmunoassay into polyethylene minitubes, using an LKB 2112 Redirac fraction collector.

TABLE I

LIST OF POLYPEPTIDES TESTED

+ = Strong fluorescence. — = Weak or no fluorescence.

Peptide	Trivial name	Amino-acid residues	Fluorescence (275 nm)
$ACTH_{1-39}$	Adrenocorticotrophin	39	+
$ACTH_{1-24}$	Synacthen	24	+
*$ACTH_{1-18}$	41'795-Ba	18	+
$ACTH_{1-13}$	α-MSH	13	+
$ACTH_{4-10}$	Memory peptide	7	—
$ACTH_{18-39}$	CLIP	22	—
$ACTH_{34-39}$	—	6	—
β-LPH	β-Lipotrophin	91	+
$\beta\text{-LPH}_{39-45}$	—	7	—
$\beta\text{-LPH}_{61-65}$	Met-enkephalin	5	—
*$\beta\text{-LPH}_{61-65}$	Leu-enkephalin	5	—
$\beta\text{-LPH}_{61-76}$	α-Endorphin	16	—
$\beta\text{-LPH}_{61-91}$	β-Endorphin	31	—

*Analogues of naturally occurring peptide hormone sequences.

METHODS

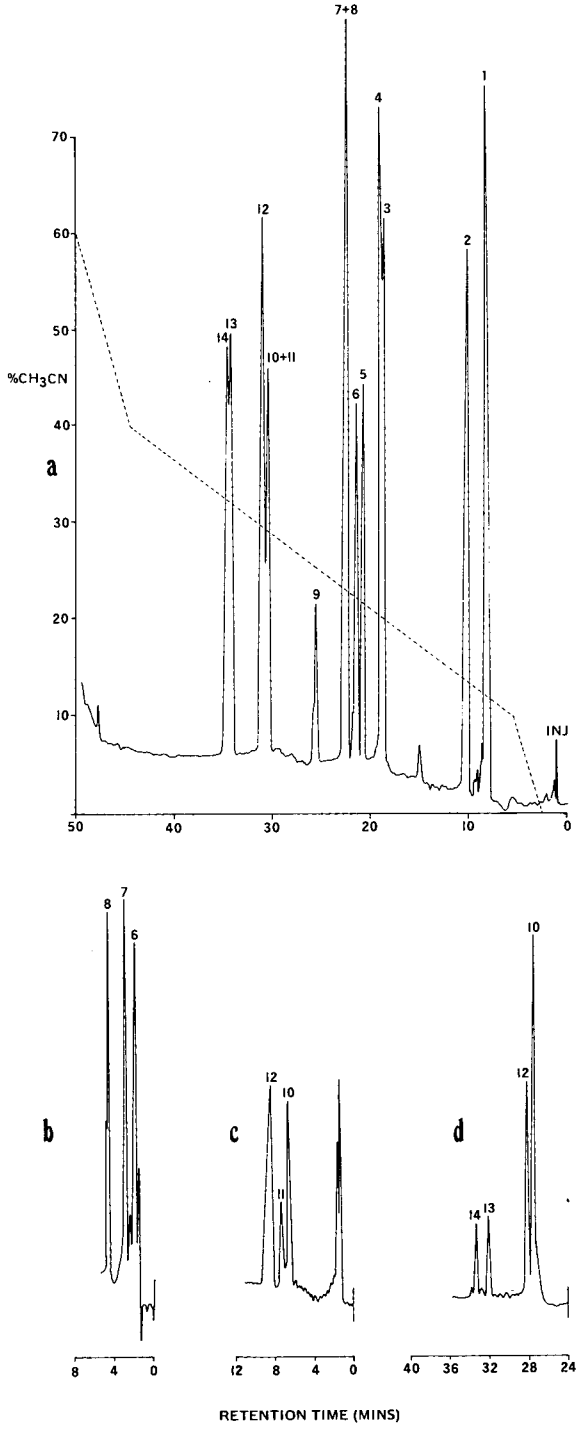
For chromatography, polypeptide standards were dissolved in the primary solvent (0.1 *M* sodium dihydrogen phosphate adjusted to pH 2.1 with orthophosphoric acid). Between 250 ng and 10 μg of each peptide was injected onto the columns, in 10—20 μl primary solvent, together with 250 ng of L-tryptophan as an internal standard. A loading period of 2.5 min pumping primary solvent only was employed to concentrate the polypeptides on the head of the column. They were then sequentially eluted over 50 min using a binary gradient with acetonitrile as the secondary solvent (Fig. 1a). The final concentration of organic modifier reached was 60% (v/v). Chromatography was carried out at ambient temperatures with an initial flow-rate of 1.0 ml/min (625 p.s.i.). These conditions, which are based on one of the systems used by Molnár and Horváth [4] were empirically optimised for the separation of compounds in Table I. The eluted polypeptides were detected by UV absorbance at 225 nm, and by fluorescence (see Table I) at an activation wavelength of 275 nm (emission filter 370 nm). Column efficiencies determined by isocratic peptide elution were 10,000—20,000 theoretical plates per metre.

RESULTS AND DISCUSSION

Complete or partial separation of 11 of the 13 polypeptides tested was achieved in a single run of 50 min at the ambient temperature using a linear gradient of acetonitrile in acid phosphate buffer with Hypersil-ODS (Fig. 1a). The system will effectively separate the endorphins, enkephalins, $ACTH_{1-39}$ and a number of related peptide fragments, and partially separate β-endorphin from β-lipotrophin. Compounds which failed to separate completely on this system could be resolved by a simple modification of the chromatographic conditions. α-Endorphin and leu-enkephalin could be separated by isocratic chromatography with 20% acetonitrile, CLIP and $ACTH_{1-39}$ at 24%, and separation of β-endorphin from β-lipotrophin was achieved by raising the temperature to 45° (see Fig. 1, b—d). Thus a potential for the complete separation of all polypeptides tested was apparent.

No deterioration of peak shape or peak broadening occurred with late-eluting polypeptides such as $ACTH_{1-39}$ and β-endorphin. Injection of the individual polypeptides under isocratic conditions showed that the chromatography of each occurred only over a very narrow range (3—4%) of organic modifier concentration with no retention or irreversible binding above or below these limits. This feature of the chromatography of the polypeptide hormones is probably responsible for the lack of peak broadening and also for the very high precision with which individual retention times could be reproduced. Thus, repeated injections of $ACTH_{1-24}$ with a mean retention time of 21.5 min gave a coefficient of variation of 0.57% (n = 7), i.e. ± 8 sec. This feature is of considerable importance for predictable chromatography of complex biological polypeptide-containing samples.

Retention times were minimally influenced (< 10%) by different flow-rates (0.5—1.5 ml/min) or different temperatures (15—70°) although the latter could be used to enhance certain separations, as shown above. Nevertheless, to avoid


7+8
1
4
3
2
12
13
14
10+11
5
6
9
INJ
%CH3CN
70
60
50
40
30
20
10
a
50
40
30
20
10
0
8
7
6
b
8
4
0
12
10
11
c
12
8
4
0
d
10
12
14
13
40
36
32
28
24
RETENTION TIME (MINS)

possible decomposition of unstable polypeptides we opted for routine chromatography at ambient temperature. The presence of both acid (pH 2.1) and salt (0.1 *M* NaH_2PO_4) in the primary solvent was essential for efficient reproducible chromatography. Omission of either resulted in marked deterioration of peak shape and loss of resolution of late-eluting compounds, and omission of both in the irreversible binding of many polypeptides to the ODS column packing.

It is evident from the behaviour of ACTH and lipotrophin-related hormones that separation and retention orders are not dictated by molecular weight with compounds of similar size eluted both very early (β-LPH_{39-45} at 8.5 min) and very late ($ACTH_{34-39}$ at 31 min). Furthermore, a very small change in the sequence of a large polypeptide could alter its retention time significantly, particularly if a hydrophobic amino-acid residue was involved. Thus porcine $ACTH_{1-39}$ was completely separated from human $ACTH_{1-39}$ (Fig. 2) although it differs from the latter only in the substitution of leucine for serine at position 31 [8].

Detection limits for individual peptides varied according to their size and amino-acid composition. A tryptophan-containing hormone such as $ACTH_{1-24}$ could be detected at levels down to 15 ng per peak by fluorescence while the corresponding minimum value for UV detection (see Methods) was 120 ng, with a linear response up to at least 100 μg. The conditions of fluorescence measurement used in this study were optimised for the detection of endogenous tryptophan present in some polypeptide hormones. The molar fluorescence ratio of tryptophan—tyrosine was 30:1, with the latter, and other amino-acid residues therefore not contributing significantly to polypeptide detection by this method. Table I indicates those fluorescent hormones which contain tryptophan (one residue per molecule in each case), and for which detection limits were 5—15-fold lower than by using the UV absorption contributed by the peptide bonds. These limits, while low in comparison with conventional methods of chromatography, are still high with respect to hormone levels in vivo.

A capability for retaining bio- and immunoactivity of eluted polypeptides, as well as the capacity to resolve a series of closely related materials in biological extracts, is a prerequisite for a biologically useful HPLC system. To test whether HPLC meets these criteria, we have used commercially available "chromatographically purified" porcine pituitary ACTH known to contain several components on polyacrylamide gel electrophoresis. Fig. 2 illustrates results

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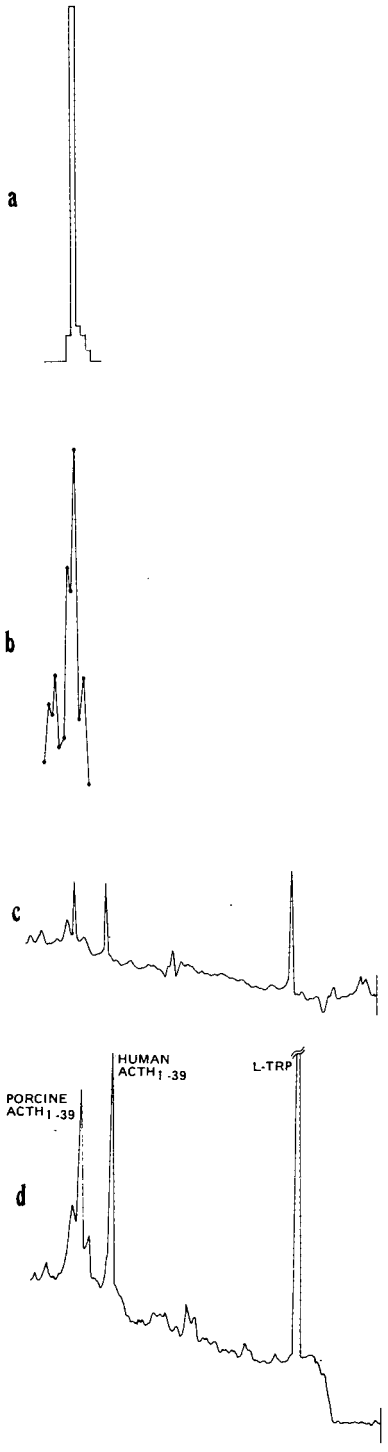
Fig. 1. Separation of polypeptide hormones by HPLC on Hypersil-ODS. (a) Chromatogram of 250 ng—10 μg of polypeptide standards in 0.1 *M* NaH_2PO_4—H_3PO_4 buffer (pH 2.1) eluted with an acetonitrile gradient (dotted line) at ambient temperature. Detection by UV absorbance (225 nm, 0.16 a.u.f.s.). INJ = injection artifact; 1 = β-LPH_{39-45}; 2 = L-tryptophan; 3 = $ACTH_{1-18}$; 4 = met-enkephalin; 5 = $ACTH_{4-10}$; 6 = $ACTH_{1-24}$; 7 = α-endorphin; 8 = leu-enkephalin; 9 = α-MSH; 10 = $hACTH_{1-39}$; 11 = CLIP; 12 = $ACTH_{34-39}$; 13 = β-endorphin; 14 = β-lipotrophin. See Table I for abbreviations. (b) Chromatogram of $ACTH_{1-24}$ (6), α-endorphin (7) and leu-enkephalin (8) eluted isocratically with 20% acetonitrile in acid-phosphate buffer at ambient temperature. (c) Chromatogram of $hACTH_{1-39}$ (10), CLIP (11) and $ACTH_{34-39}$ (12) eluted isocratically with 24% acetonitrile in acid-phosphate buffer at ambient temperature. (d) Chromatogram of $hACTH_{1-39}$ (10), $ACTH_{34-39}$ (12) β-endorphin (13) and β-lipotrophin (14) with gradient elution at 45°, other conditions as in (a).


a
b
c
HUMAN
ACTH 1-39
L-TRP
PORCINE
ACTH 1-39
d

obtained with this preparation. First, several UV-absorbing and fluorescent peaks are resolved. These compounds can be individually re-chromatographed with identical retention times indicating that they are not degraded by chromatography and also suggesting that they are individual compounds, not conformational isomers. Second, the eluted peaks have been successfully radioimmunoassayed for ACTH after evaporation of the organic solvent, neutralisation of the acid and dilution of buffer 1:4 to reduce its ionic strength, giving a detection limit of 200 pg per peak. Last, the eluted peaks have been successfully bioassayed for ACTH, using an isolated adrenal cell suspension [9] and a fluorescence assay for corticosteroid [10]. Only one peak showed significant bioactivity with over 90% of the activity recovered therein, in contrast to the multiple peaks revealed by radioimmunoassay with an N-terminal-directed antibody (Fig. 2). Thus the HPLC system described here conforms to these criteria for biological use.

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Fig. 2. HPLC of porcine pituitary ACTH preparation (25 μg) with L-tryptophan and synthetic human $ACTH_{1-39}$ as internal standards. Conditions of chromatography as Fig. 1a. Chromatograms show (a) bioactive and (b) immunoreactive materials eluted in 30 sec fractions, compared with (c) UV absorbance (225 nm, 0.16 a.u.f.s.) and (d) fluorescence profiles (275 nm activation, 0.2 μA) obtained from this preparation. Retention times of standards were identical to those given in Fig. 1a and that of the bioactive porcine pituitary ACTH peak was 33.25 min.

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Note

Simultaneous determination of retinol and α-tocopherol in human serum by high-performance liquid chromatography

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All-*trans*-retinol is the predominant form of vitamin A in serum (>95%), whereas *d*-α-tocopherol represents 88% of the total vitamin E pool of serum (2% exists in the *d*-β and 10% in the *d*-γ form) [1—3]. The sparing action of vitamin E on the expenditure of vitamin A has been suggested [4—6]. In view of this hypothesis the combined analysis of these two vitamins might prove to be clinically useful.

The simultaneous determination of vitamin A and E in serum has only rarely been performed. Two procedures [7, 8] using fluorimetry have been reported. Both methods are based on the difference in fluorimetric properties of the two molecules.

Chromatographic separation and determination of these two vitamins using thin-layer chromatography (TLC) [9], gel chromatography [10, 11] and high-performance liquid chromatography (HPLC) [12] have been described. However, these methods have not been applied to biological samples. Separate HPLC assays for vitamin A [13] and E [14] in serum were recently reported by us.

We now propose an HPLC method for the simultaneous determination of retinol and α-tocopherol in serum (or plasma).

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EXPERIMENTAL

Materials

Methanol, ethanol, *n*-hexane were all analytical-grade reagents from E. Merck (Darmstadt, G.F.R.) and used without further purification.

All-*trans*-retinol was of crystalline purity from Sigma (St. Louis, Mo., U.S.A.); *d*-α- and *d*-β-tocopherol were purchased from Eastman-Kodak (Rochester, N.Y., U.S.A.) and *d*-γ-tocopherol was obtained from Supelco (Bellefonte, Pa., U.S.A.).

The internal standard *dl*-tocol was purchased from Koch-Light (Colnbrook, Great Britain).

High-performance liquid chromatography

We used a Hewlett–Packard 1084A liquid chromatograph equipped with a Pye Unicam LC3 variable-wavelength detector.

The chromatographic support RSIL C_{18}, a 10-μm heavily loaded octadecyl bonded phase (18% organic material) and the column tubing (25 cm × 4.6 mm I.D., Lichroma SS) were obtained from RSL (St. Martens-Latem, Belgium). The column was packed by the slurry technique under following conditions: slurry liquid, tetrachloromethane (analytical grade, E. Merck); slurry concentration, 8% (w/v); pump, Varian 8500; packing pressure, 210 bar; pressurising liquid, methanol. At optimum flow-rate (0.25 ml/min), a column efficiency of 11,000 plates (h = 2.3) was obtained for α-tocopherol.

Elution was performed by methanol at a flow-rate of 2 ml/min (pressure = 96 bar). The oven temperature was set at 40° and the column effluent was monitored at 292 nm.

Sample preparation

In a PTFE-capped centrifuge tube (100 × 10 mm), 10 μl of a tocol internal standard solution (0.1 μg/μl) in ethanol and 100 μl of pure ethanol were added to a serum (or plasma) sample of 100 μl.

After mixing, the sample was extracted with 1 ml of *n*-hexane by interrupted mixing on a Vortex-type mixer for 4 min. After centrifugation (10 min, 1500 *g*) the organic layer was evaporated at 40° in a gentle stream of nitrogen. The residue was dissolved in 50 μl of methanol and injected on top of the RP-18 column.

Quantification

Known amounts of vitamins A and E covering the range of 125–1000 μg/l and 5–25 mg/l respectively, were added to samples of a serum pool. Calibration curves were made by plotting peak height ratios ($h_{\text{retinol}}/h_{\text{i.s.}}$; $h_{\alpha\text{-tocopherol}}/h_{\text{i.s.}}$) against vitamin A and E concentrations.

RESULTS

Fig. 1 shows a typical chromatogram obtained from a serum extract to which a known amount of tocol as internal standard had been added. Retention characteristics are given in Table I. The β- and γ-tocopherol isomers elute

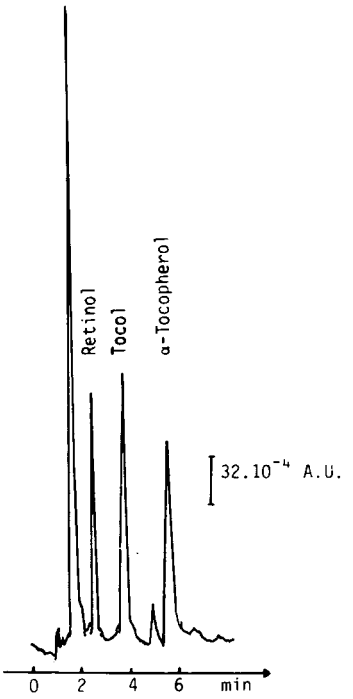

Fig. 1. High-performance liquid chromatogram of a serum extract. Column: 25 cm × 4.6 mm I.D., packed with 10-μm RSIL C_{18}. Eluent: methanol. Flow-rate: 2.0 ml/min. Oven temperature: 40°. Detection: 292 nm.

TABLE I

CAPACITY RATIOS (k') OF CHROMATOGRAPHIC PEAKS

Compound	k'
All-*trans*-retinol	1.06
Tocol (internal standard)	2.10
β- and α-Tocopherol*	3.01
α-Tocopherol	3.54

*Composite peak.

as one peak. The total elution time of the two vitamins and the internal standard takes less than 6 min.

A linear relationship between peak height ratios (peak height of retinol or α-tocopherol:peak height of tocol) and retinol or α-tocopherol concentrations was found. These calibration curves ($y = 0.0012\ x + 0.9398$, $r = 0.9991$ and $y = 0.0671\ x + 0.6748$, $r = 0.9994$, respectively) are used after subtraction of the intercepts, which represent the endogenous levels of the vitamins in the serum pool. Concentrations in unknown serum samples are then easily determined after calculation of the peak height ratios.

The mean extraction recoveries from spiked serum, as given in Table II, were 96.8 and 95.5% for retinol and α-tocopherol, respectively.

In serum the lower detection limit for retinol was estimated at 60 μg/l, whereas a value of 0.6 mg/l was found for α-tocopherol. A within-day precision (coefficient of variation, C.V.) of 2.47% and 1.62% was obtained for retinol and for α-tocopherol, respectively, analyzing 10 samples of a normal serum pool ($\bar{x}$ = 674 μg/l, S.D. = 16.6 μg/l for retinol; $\bar{x}$ = 11.27 mg/l, S.D. = 0.18 mg/l for α-tocopherol). The day-to-day precision (C.V.) as measured over a period of 20 days was 4.9% for retinol and 3.2% for α-tocopherol, at the respective concentration levels of 635 μg/l and 10.8 mg/l. For these experiments, serum samples were stored at —18° under a nitrogen atmosphere.

TABLE II

EXTRACTION RECOVERY

Compound	$\bar{x}$ (%)	S.D. (%)	C.V. (%)	n	Range (mg/l)
Retinol	96.8	3.0	3.1	4	0.25—1.00
α-Tocopherol	95.5	3.7	3.9	5	5 —25

Peaks obtained from a serum extract are identified on the basis of retention times. The specificity of the method was also checked by determining the $\lambda_{max.}$ of the peaks eluting at the retention times of retinol, α, β, γ-tocopherol and tocol. Therefore, aliquots of an extract of a 1-ml serum sample were run at different wavelength settings and peak heights of compounds of interest were plotted vs. wavelength. The $\lambda_{max.}$ we found ($\lambda_{max.}$ (retinol) = 330 nm, $\lambda_{max.}$ (tocopherols) = 295 nm) agreed with values reported in the literature [15, 16]. In addition, for the tocopherols the specificity of the procedure was further verified by gas—liquid chromatography and gas chromatography—mass spectrometry [14].

As a test of the applicability of the method, serum from a series of 25 human donors was analyzed for retinol and α-tocopherol. Data are given in Table III.

DISCUSSION

This simultaneous determination of vitamins A and E in serum by HPLC, provides high specificity due to a chromatography column with a high separation capacity. The addition of a known amount of tocol as an internal standard before the extraction step, compensates for possible losses, caused by evaporation or spilling, and improves the precision of the method. Tocol was chosen as an internal standard on the basis of its retention characteristics (it elutes between the two vitamins assayed), commercial availability and structural analogy to the component with the lowest extinction coefficient i.e. α-tocopherol. The major absorption band of α-tocopherol and tocol is relatively

TABLE III

SERUM RETINOL AND α-TOCOPHEROL CONCENTRATIONS FOR SOME ADULT HUMANS

Age (years)	Sex	Retinol (μg/l)	α-Tocopherol (mg/l)
69	F	625	11.4
72	F	835	9.5
64	M	1000	7.9
32	F	505	5.0
50	M	980	7.3
15	M	635	6.2
65	F	745	6.0
62	F	775	9.4
70	M	710	6.4
77	F	790	9.4
31	F	565	7.4
45	M	610	8.2
51	F	760	15.3
39	M	775	9.8
83	F	495	10.6
54	M	270	7.7
77	M	715	18.5
67	M	230	8.8
57	M	830	19.8
56	M	570	11.3
68	F	565	10.5
56	M	270	5.5
65	M	370	6.2
75	M	342	10.5
71	F	403	6.6

sharp and occurs at 292 nm (ϵ = 3500 and 3900 $l \cdot mol^{-1} \cdot cm^{-1}$, respectively). This compound has practically no residual absorbance at 325 nm, the $\lambda_{max.}$ of retinol (ϵ = 52600 $l \cdot mol^{-1} \cdot cm^{-1}$). However, retinol still absorbs significantly at 292 nm, the $\lambda_{max.}$ of α-tocopherol. We, therefore, monitored the column effluent at 292 nm, the wavelength of the component with the weakest absorbance.

The method proposed is sensitive as only 100 μl of serum (or plasma) are needed. The overall procedure is simple and the total analysis time is relatively short (less than 30 min including sample preparation, extraction and chromatography). Compared to the fluorimetric method of Hansen and Warwick [8], our method proved to be more precise: the C.V. reported for the fluorimetric technique was 7% against 3% for our method. The specificity of our retinol determination should also be better, because it is well-documented that other (fluorescent) co-extracted compounds interfere with the fluorimetric method. To overcome this problem, Thompson et al. [7] introduced a correction formula which compensates specifically for the presence of phytofluene. However, because fluorescence must be measured at two emission and three excita-

tion wavelengths, their technique appears more complicated. On the other hand the HPLC method uses only one wavelength (292 nm), improving the reliability of the measurement, and no tedious calculations have to be made.

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Note

New principle for the separation of plasma lipoprotein lipids without ultracentrifugation

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The diagnosis and therapeutic management of disorders in lipid metabolism requires detailed yet simple analytical procedures for the determination of plasma lipid and lipoprotein (LP) lipid concentrations [1—6].

Lipid extraction from serum samples followed by chemical lipid determination is time-consuming and relatively large sample volumes are needed. In a recent report [7], however, the direct application of 0.5-μl plasma samples to high-performance thin-layer plates prior to lipid separation by thin-layer chromatography (TLC) was shown to be feasible. The serum proteins were denatured by methanol.

The separation of plasma LP fractions by ultracentrifugation followed by lipid analysis using chemical methods is also widely used. Although accepted as the reference method for LP fractionation, this procedure is restricted to clinics and institutes specialized in this technique. In addition, large sample volumes are required. Therefore, a procedure has been developed for the quantitative enzymatic determination of cholesterol in LP fractions separated by electrophoresis on agarose gel [8—10]. The agarose samples containing the LP fractions of 15 μl capillary plasma were dissolved in HCl and, after neutralization, a commercial test combination was used for the enzymatic determination of cholesterol. No influence of the HCl on cholesterol was observed.

These principles (applying serum or plasma directly to commercial TLC plates, and dissolving agarose samples containing LP lipids in HCl) have now been applied to a combined electrophoresis and TLC procedure for the separation of plasma lipids and LP lipids on a microscale and without ultracentrifugation.

MATERIALS AND METHODS

Chemicals

All chemicals were of analytical grade. TLC plates were pre-coated with silica gel 60 without fluorescent indicator (20 × 20 cm; Merck, Darmstadt, G.F.R.). [1α, 2α-^{3}H]Cholesterol (>30,000 mCi/mmole) and Insta-Gel liquid scintillation agent were supplied by Amersham/Buchler, Braunschweig, G.F.R.

Electrophoresis

The LP fractions of 20 μl of freshly prepared blood serum or plasma were separated on agarose gel and made visible by opalescence as previously described [8].

Direct application of the agarose samples containing the LP fractions to TLC plates

The opalescent LP fractions were cut with a scalpel. After evaporation of adherent moisture, the samples were transferred to the TLC plate. Moisture in the agarose gel was evaporated in vacuo for a few minutes. Caution was required to ensure that the agarose samples did not lift from the silica gel layer. This was guaranteed as long as a white circle was visible around the agarose sample on the silica gel layer. When the white circle was clearly visible, the vacuum was released very carefully. The agarose samples appeared as a transparent film. Immediately, a few microlitres of concentrated HCl were deposited on each sample, thereby destroying the agarose structure. The HCl was evaporated under a stream of cold air from a hair dryer.

Direct application of the serum or plasma samples to the TLC plates

A few microlitres of concentrated HCl were deposited on the TLC plate prior to and after the application of 20 μl of sample. The HCl was evaporated under a stream of cold air.

Separation and detection of lipids

Lipids were separated and detected as described previously [7].

Radiochemical examination of the quality of the HCl procedure

[^{3}H]Cholesterol (20 μCi) was dissolved in benzene and applied to a piece of filter paper [11]. After evaporation of the solvent, the piece of paper was added to 3 ml of freshly prepared serum or plasma. Incubation was carried out for 1 h at 37°. The samples labelled with [^{3}H]cholesterol were analysed by electrophoresis and TLC as described above. The lipid spots were scraped from the TLC plates and transferred to liquid scintillation counting vials containing 10 ml Insta-Gel. For measuring radioactivity of the total serum, 20-μl samples were mixed with 10 ml Insta-Gel. Radioactivity was counted using a liquid scintillation spectrophotometer (Packard, Downers Grove, Ill., U.S.A.) [12].

RESULTS AND DISCUSSION

Principle of the procedure and its radiochemical examination

In a recent study, serum samples could be applied directly to TLC plates prior to the separation of the lipids. The serum proteins were denatured by methanol [7]. In the present study, the same end was attained by the addition of concentrated HCl to the serum sample after its direct application to the TLC plate. In Fig. 1, the lipid patterns of a serum sample and of the corresponding lipid extract are shown. No marked differences are apparent between the samples (Fig. 1A). From this lipid extract, free and esterified cholesterol and triacylglycerols were isolated by preparative TLC. Following exposure of these lipids to the HCl procedure on the TLC plate, re-chromatography did not show changes due to HCl (Fig. 1B).

Fig. 2 demonstrates the lipid patterns of a sample with known lipid concentrations (Precilip) and of a serum of a subject with its corresponding LP fractions. The separation of the neutral lipids was satisfactory. Semiquantitation of the lipids by visual examination compared to the known lipid content of Precilip is possible.

For radiochemical examination of the procedure, serum was incubated with [^{3}H]cholesterol for 1 h at 37°. Of the ^{3}H-label, 50—60% was taken up by the serum LPs and distributed between the LP fractions, as demonstrated in

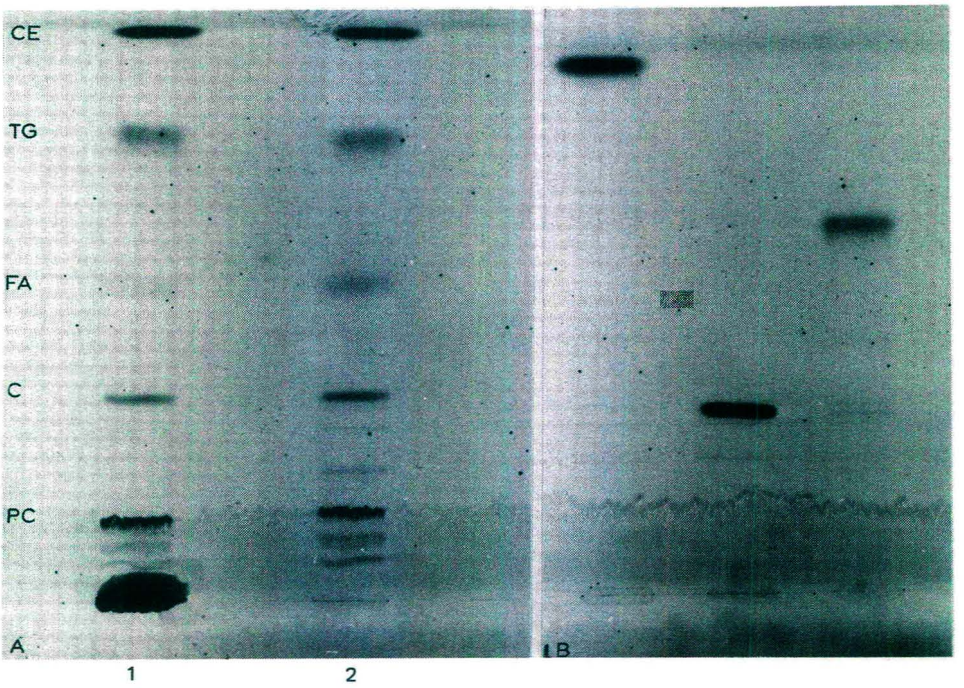


Fig. 1. Denaturation of serum lipids with HCl on the TLC plates. Twenty microlitres of serum (position 1) and the corresponding amount of lipid extract (position 2) were applied to the TLC plate and the HCl procedure was carried out (A). From this lipid extract, cholesterol esters (CE), free cholesterol (C) and triacylglycerols (TG) were prepared by preparative TLC and exposed to HCl on the plate (B). FA, fatty acids; PC, phosphatidyl choline.

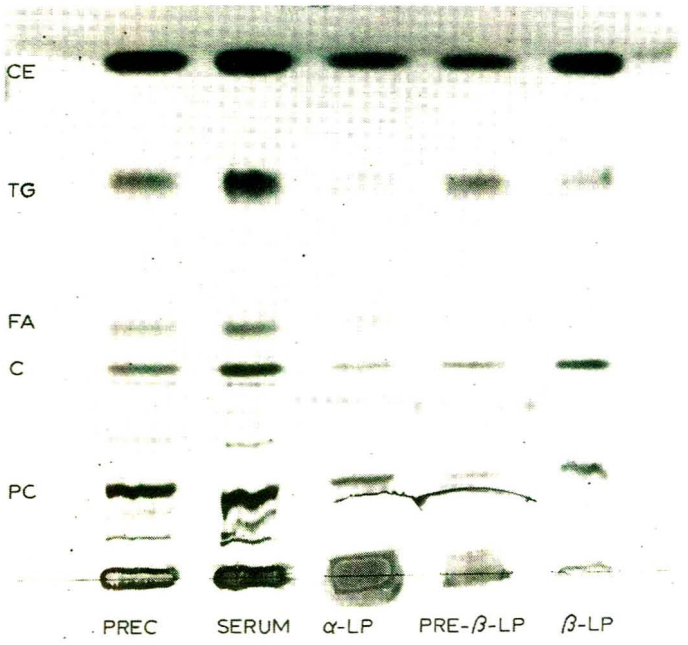


Fig. 2. Direct application of agarose samples containing LPs to the TLC plate. The LP fractions were obtained by electrophoresis on agarose gel and applied to the TLC plate followed by the HCl procedure. After TLC the distribution of the serum lipids among the LP fractions can be examined. Semiquantitative evaluation is possible by comparison with a sample (Precilip = PREC) containing known lipid concentrations (CE = 2.16 mmole/l, C = 0.86 mmole/l, and TG = 0.63 mmole/l). Abbreviations as in Fig. 1.

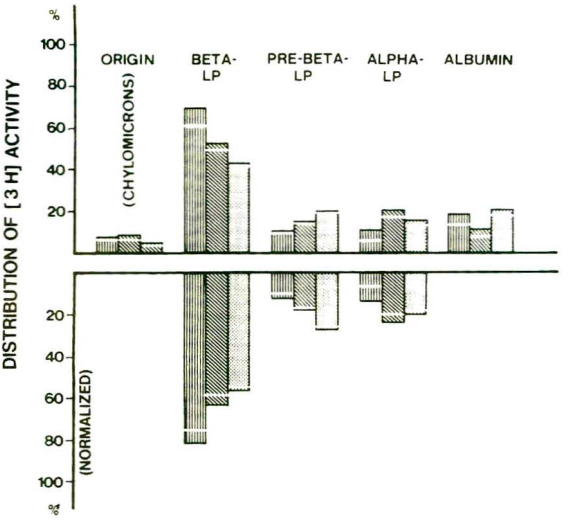


Fig. 3. Labelling of serum LPs. Serum was incubated with [^{3}H]cholesterol for 1 h at 37° prior to electrophoresis. The fractions were cut and radioactivity measured. Two samples from each of three subjects were studied. Normalized scale: distribution of radioactivity among LP fractions alone.

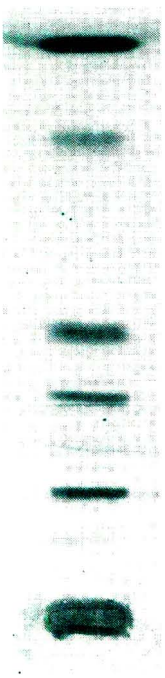
Fig. 3. Traces of label were found at the origin of the electropherogram. The remainder was preferentially taken up by the β-LPs and to a much lesser extent by the other fractions. ^{3}H-Label found in albumin was probably due to α-LPs included in this fraction.

TLC and counting of ^{3}H-activity in the different areas of the TLC layer showed that approximately 85—95% of the [^{3}H]cholesterol was recovered as free cholesterol with high precision (coefficient of variation, C.V. = 2—3%), and 10% as esterified cholesterol (C.V. <15%) (Table I). Only trace amounts of radioactivity were found in the other areas and at the origin of the chromatoplates. It was postulated from these results that negligible amounts of free and esterified cholesterol remain at the origin during TLC. Elution of the origin spots followed by re-chromatography showed that practically no cholesterol or other neutral lipids had remained there during the TLC procedure.

TABLE I

RADIOCHEMICAL EXAMINATION OF THE COMBINED ELECTROPHORESIS AND TLC PROCEDURE

The serum LPs labelled with [^{3}H]cholesterol were separated by electrophoresis. The LP fractions were applied directly to the TLC plates and, after HCl treatment of the samples, TLC was carried out. The cholesterol esters (CE), free cholesterol (C) and the areas marked by brackets were scraped from the plate and radioactivity was counted. Data are expressed as a percentage of total radioactivity; coefficients of variation (C.V., %) are given in parentheses. Values are averages of 10 samples ± S.D.



	Serum	β-LP	pre-β-LP	α-LP
CE	6.9 ± 0.7 (10.1)	2.4 ±0.2 (8.3)	3.2 ± 0.5 (15.6)	12.2 ± 1.6 (13.1)
	1.2/0.9	1.2/0.9	1.7/2.5	1.4/1.0
C	91.6 ± 1.6 (1.7)	95.9 ± 1.4 (1.5)	92.9 ± 2.1 (2.3)	85.6 ± 2.6 (3.0)
	1.3 ± 0.1 (7.7)	1.5 ± 0.2 (13)	1.4 ± 0.2 (14)	1.3 ± 0.2 (11)

Application to the evaluation of esterifying activity in LP fractions of ten healthy subjects

The procedure described above was extended to the analysis of the serum of 10 healthy (control) subjects. Fig. 4 demonstrates that the serum LP fractions exchanged their cholesterol with [^{3}H]cholesterol during the incubation. Up to 10% of this [^{3}H]cholesterol was esterified and mainly localized in the α-LPs (Fig. 4A). The distribution of free [^{3}H]cholesterol among the LP fractions represents to a certain extent the total cholesterol moieties in these fractions (Fig. 4B). The [^{3}H]cholesterol esters, however, were present in equal amounts in the α- and β-LPs independent of the very different amounts of cholesterol in these fractions. This result is consistent with the observation that plasma esterifying activity due to lecithin:cholesterol acyl transferase (LCAT) is mainly localized in the α-LPs [13–18], and that the β- and pre-β-LPs participate in this process, perhaps by exchanging the newly synthesized cholesterol esters with the α-LPs [19].

Typical LP patterns in control and hyperlipidemic subjects

The procedure was applied to the separation of the plasma lipids and LP lipids of healthy and hyperlipidemic subjects. Fig. 5 presents the lipid patterns

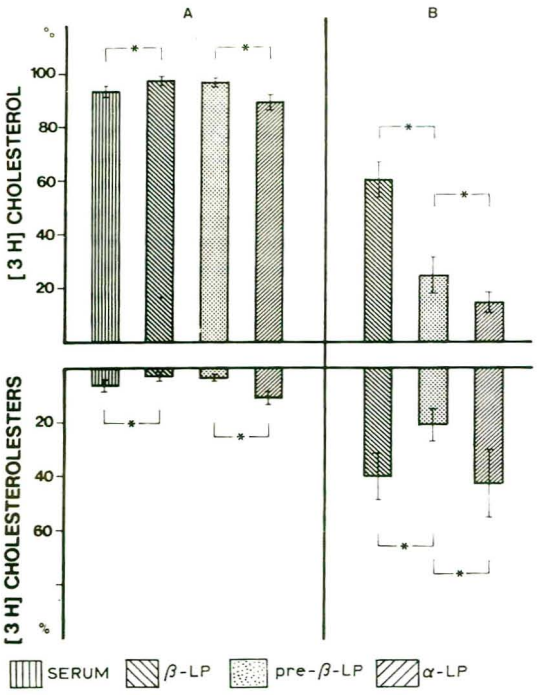


Fig. 4. Cholesterol esterifying activity in ten healthy subjects. Serum of ten healthy subjects was incubated with [^{3}H]cholesterol for 1 h at 37°, after which the combined electrophoresis and TLC procedure was carried out. Radioactivity was counted in the free and esterified cholesterol. A: Proportion of free and esterified [^{3}H]cholesterol in each sample. B: Distribution of free [^{3}H]cholesterol and [^{3}H]cholesterol esters among the three LP fractions. Asterisks (*) indicate significant differences.

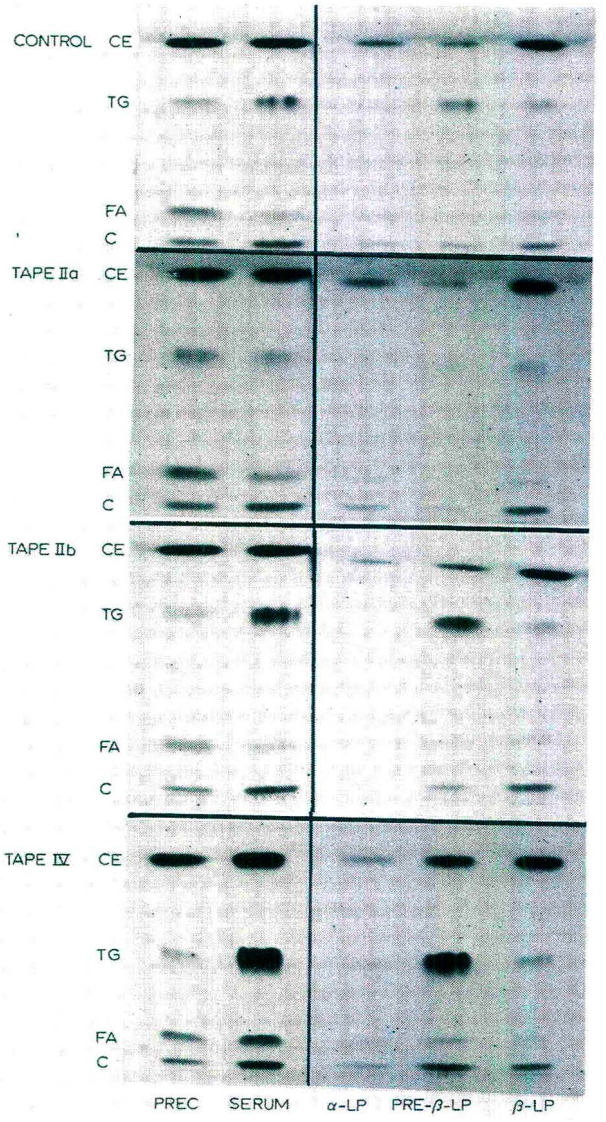


Fig. 5. Typical lipid patterns. The neutral lipids of a known sample (Precilip = PREC) are compared with those in serum and the corresponding LPs of four subjects. Results on one healthy (control) subject and on patients with three types of hyperlipidemia are shown. The lower parts of the chromatograms have been deleted. Abbreviations as in Fig. 1.

of a healthy (control) subject, and of Type IIa, Type IIb and Type IV hyperlipidemic patients. The contents of the plasma lipids and their distribution among the LP fractions are compared to a sample with known amounts of lipid. Visual examination of the chromatoplates gives information on the qualitative and semiquantitative lipid composition of the LP fractions (the quantitation of these lipids is in progress). The distribution of the serum cholesterol among the LP fractions is consistent with data previously published [8]. This distribution is quite similar for normal and Type II subjects, regard-

less of the total cholesterol level. The pre-β-LPs of Type IV hyperlipedemic patients, however, carry larger amounts of cholesterol. In all types of serum, the triacylglycerols are carried predominantly by the pre-β-LPs.

CONCLUSIONS

Serum LP fractions obtained by electrophoresis on agarose gel were applied directly to the TLC plate and HCl used to dissolve the agarose structure prior to TLC of the lipids. The quality of the procedure was examined by radiochemical analysis of [^{3}H]cholesterol-labeled LP fractions.

The method was applied to the examination of the esterifying activity of LP fractions from healthy subjects. Furthermore, typical lipid patterns of serum and serum LP fractions were demonstrated. The visual examination of the chromatoplates gave satisfactory results. The quantitation of the lipids is in preparation.

This new micro-procedure can serve for further studies in phenotyping hyperlipidemias, in therapeutic management of the different dyslipoproteinemias, and in identifying subjects with a high risk of atherogenesis.

ACKNOWLEDGEMENTS

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Note

Direct thin-layer chromatography—fluorimetric quantification of pharmacological plasma concentrations of an antiarrhythmic steroid (Org-6001)

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(Received May 18th, 1978)

Org-6001 (3α-amino-5α-androstan-2β-ol-17-one hydrochloride) is an aminosteroid possessing antiarrhythmic properties without hormonal activity. It has been shown that Org-6001 is effective in the treatment of ventricular tachycardia induced by digitalis [1], and when administered either intravenously or orally is effective in the treatment of experimental arrhythmias induced by coronary occlusion [2]. For future clinical evaluation of this substance a thin-layer chromatography (TLC) method has been developed, which permits specific determination of Org-6001 in plasma to a lower limit of 10 ng/ml with a variability at this concentration of 14%. The fluorescence was developed in situ on thin-layer chromatograms by treatment of the TLC-plate with a solution of perchloric acid using a previously described method for amitriptyline/nortriptyline analysis [3], with a minor modification [4].

MATERIALS AND METHODS

Apparatus

A Zeiss spectralphotometer with TLC scanning equipment KM 3 (Carl Zeiss, Oberkochen, Württemberg, G.F.R.), linked to a Servogor Sb RE 646 recorder

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(Goerz Electro, Vienna, Austria), was used with the following settings: Monochromator 345 nm; fluorescence filter cutting at 430 nm; voltage selector 2; scanning speed 120 mm/min.

Chemicals

Sodium hydroxide (analytical grade) was obtained from Elektrokemiska (Bohus, Sweden) and 96% ethanol from DDSF (Copenhagen, Denmark). All other chemicals were analytical grade from E. Merck (Darmstadt, G.F.R.). Org-6001 was from Organon (Oss, The Netherlands) and the filterpaper was from Frisenette (Ebeltoft, Denmark).

Thin-layer chromatography

TLC was performed on 20 × 20 cm pre-coated silica gel 60 thin-layer plates with a layer thickness of 0.25 mm and without fluorescent indicator, from E. Merck. Duplicate samples were always run on separate TLC plates, with 12 samples spotted on each plate, 4 of which were extracts from spiked plasma samples. The chromatography tank was lined with Whatman No. 3 chromatography paper and the solvent was chloroform—methanol—diethylether—25% ammonia (30:15:5:0.25) or 1-butanol—chloroform—25% ammonia (35:15:2.5). Org-6001 has R_F values of 0.22 and 0.50 respectively in these solvent mixtures. The chromatography was conducted with the exclusion of light, then the plates were dried at 50° for 30 min.

Procedures

One ml of plasma was pipetted into a 10-ml PTFE stoppered centrifuge tube, mixed with 0.5 ml glycine—sodium hydroxide buffer, pH 9.9, and extracted twice with 7 ml of chloroform for 15 min. Centrifugation for 5 min at 1500 *g* separated the two phases. The two organic phases were washed twice, each with 3 ml 0.1 *N* NaOH. Five-ml samples from each of the organic phases were then combined and transferred to a conical centrifuge tube and evaporated to dryness under a stream of nitrogen at 40°. The residue was dissolved in 50 μl chloroform and the extracted material was spotted under a stream of nitrogen onto the TLC plate using a 10-μl constriction pipette. An additional 20-μl portion of chloroform was added to the tubes, shaken and then spotted onto the plate. After chromatography, the spots of Org-6001 were oxidized in situ to produce fluorescence as follows: The TLC plates were dipped in a mixture of ethanol—water—perchloric acid, 135 : 135 : 12 (freshly prepared) for 7 sec and any excess of reagent was removed by pressing the wet layer against filter paper No. 617. The TLC plates were then immediately placed in an oven at 90° for 35 min. To ensure uniform heating, the TLC plates were placed on copper plates, 3 mm thick and after heat treatment were left at room temperature for 15 min before scanning, to ensure full development of the spots.

Calculations

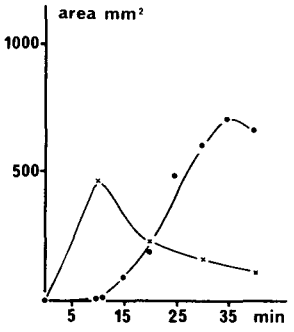
The amount of Org-6001 was calculated by comparison of the peak areas of the samples and standards. Standards with known amounts of Org-6001 were prepared from Org-6001-free plasma spiked with 10 μl methanol per ml of

plasma containing Org-6001. All calculations were corrected to give the free base of Org-6001.

RESULTS AND DISCUSSION

Sample preparation

Extraction of Org-6001 was found to be optimal at pH 9.9. When either the pH was increased or the two washings with 0.1 *N* NaOH were omitted, interference with an unknown substance from the plasma became apparent. Recovery at pH 9.9, by extraction of spiked plasma samples containing 112 and 224 ng/ml of Org-6001, was 90%. Furthermore, it was shown that the recovery by extraction of plasma samples spiked with methanolic Org-6001 solution was constant for an equilibration time between 5 and 240 min. An internal standard was not used because it would have required further clean-up steps. The fluorescence reaction is time and temperature dependent as shown in Fig. 1. Heating for 35 min at 90° was chosen and Fig. 2 shows the development of fluorescence after this heat treatment. The reproducibility of the development of fluorescence, tested with different amounts of pure substance, is seen in Table I.



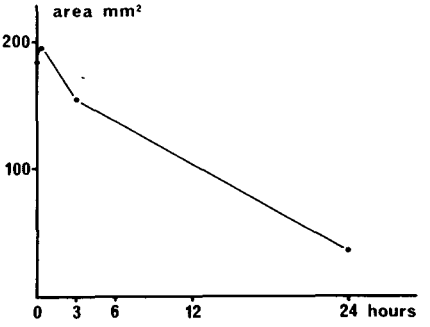


Fig. 1. Effect of heating time on measured areas (•, 90° ; x, 105°) scanning immediately after heating.

Fig. 2. Measured peak area as a function of time after heating for 35 min at 90°.

TABLE I

REPRODUCIBILITY OF THE DIRECT QUANTITATIVE ANALYSIS CARRIED OUT WITH PURE SUBSTANCE

Values from 12 measurements

Amounts applied per spot (ng) of Org-6001	Coefficient of variation (%)
112	5.3
7	11
3.5	19

Specificity, sensitivity and reproducibility

Plasma from subjects who had not ingested Org-6001 showed no interfering peaks when analyzed. An insignificant peak, with the same R_F value as Org-6001, could sometimes be seen if the aqueous phase had not been carefully aspirated. A typical scan is shown in Fig. 3. No interference was found between Org-6001 and other steroids, i.e. aldosterone, cortisol, testosterone, digoxin, and oestrogens. The lower limit for reliable quantitation of Org-6001 using 1 ml of plasma is 10 ng/ml. Reproducibility studies were performed on plasma samples spiked with Org-6001, and are outlined in Table II.

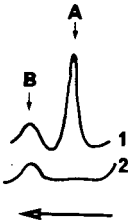


Fig. 3. Chromatogram scan showing 45 ng Org-6001 extracted from 1 ml plasma (1), and 1 ml plasma without Org-6001 extracted in the same manner (2). A is Org-6001 and B is an unknown substance from the plasma sample.

TABLE II

REPRODUCIBILITY OF THE ANALYSIS WHEN SPIKED PLASMA SAMPLES WERE ANALYSED ON DIFFERENT DAYS

Amount (ng) of Org-6001 in plasma sample	Coefficient of variation (%)	*n*
9	14.2	5
22	14.8	6
45	7.4	5
90	4.3	5
112	7.0	7

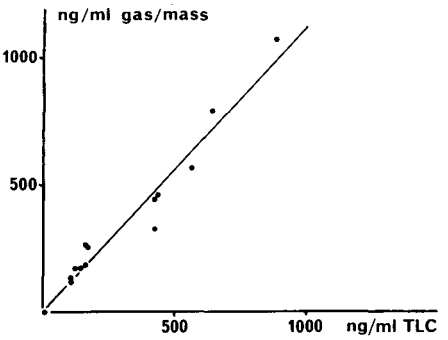


Fig. 4. Comparison of a GC—MS method (see text) with the present TLC method. Linear regression showed that the slope of the line is 1.1 and r^2 = 0.95.

Plasma samples from two patients actually treated with Org-6001 were measured both with gas chromatography—mass spectrometry (GC—MS)* and with the present TLC method. The two methods correlate well, as shown in Fig. 4. Fig. 5 shows the plasma concentrations of Org-6001 in two patients following intravenous administration of 350 mg. The dose was given in three parts (100 mg, 100 mg, 150 mg) at two-minute intervals. A rough estimate of the plasma half-life (Fig. 5) in cases A and B suggests a half-life of 10 h and 22 h, respectively. The relatively long half-life suggests that the plasma concentrations should be followed for a long period in future studies.

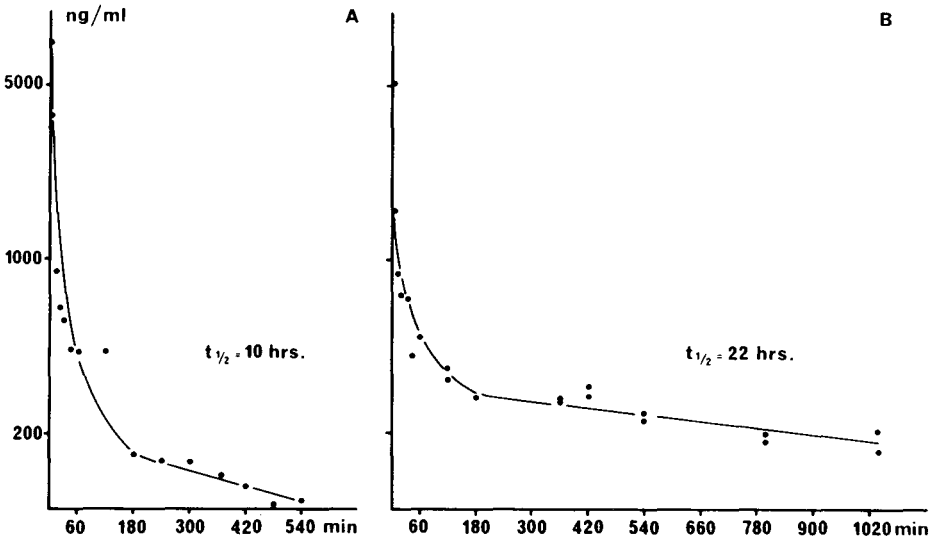


Fig. 5. Plasma concentrations of Org-6001 in two patients following intravenous administration of 350 mg Org-6001.

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Brock and Michelsen, Copenhagen are thanked for their kindness in placing a Zeiss TLC scanner in our laboratory. Johs. Christiansen is thanked for his advice and for helpful discussion of the analytical procedure.

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*The GC—MS procedure can be outlined as follows: extraction, several clean-up steps, derivatization with tertiary butyl-dimethylchlorosilane—imidazole in dimethyl formamide, using [9,11,16-$^2H_3$]—Org-6001 as internal standard (Organon), GC and finally measurement of the peak heights at *m/e* 390 and *m/e* 393.

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Note

Analysis of γ-amino-β-hydroxy butyric acid (GABOB) by chromatography and electrophoresis

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γ-Amino-β-hydroxy butyric acid (GABOB) has been found in the brains of mice, rabbits, cattle, and humans [1], but it has not been found in normal urine and plasma. In Japan, however, GABOB has been tested as a drug for the treatment of convulsions and mental retardation.

Urine from a patient with mental retardation was examined for inborn errors of metabolism. Sodium buffer column chromatography showed a large unknown peak corresponding to tyrosine, but the tyrosine metabolites were normal. Lithium buffer was added to the urine and the unknown peak appeared, like homocystine between phenylalanine and β-alanine. By using paper chromatography and electrophoresis however, tyrosine and homocystine were eliminated as possibilities. Then it was disclosed that the patient had been given GABOB and by adding pure GABOB to the urine, the unknown peak increased correspondingly for all four methods of chromatography.

MATERIALS AND METHODS

Urine and blood samples were obtained from a 21 year old man with anophthalamus and mental retardation. He was given 1300 mg of GABOB (Ono Pharmaceuticals, Tokyo, Japan) daily. The day before the examination, fruit and vegetables were limited, and fasting urine and blood samples were collected early in the morning.

Procedure

For the determination of the urinary amino acid, the urine was deproteinized by adding 5 mg sulfosalicylic acid per ml of urine. The blood was collected in a heparinized centrifuge tube and shaken several times. The heparinized blood was immediately centrifuged (1620 *g*, 5 min) and the plasma was carefully removed from the upper layer. 120 mg dry sulfosalicylic acid were added to 4 ml of plasma, vigorously shaken and then centrifuged (1620 *g*, 5 min) [2].

Two dimensional paper chromatography

Whatman No. 1 paper, 20 × 20 cm, was used in Shandon equipment for ascending chromatography at 23°. Run 1: the solvent was pyridine—water—acetone—ammonia (45:30:20:5). Run 2: the solvent was isopropanol—formic acid—water (75:12.5:12.5). Both runs were 16 h.

Spray reagents: 3 g ninhydrin solution in 950 ml isopropanol and 50 ml collidin.

Hydrolysis

A volume (2 ml) of urine was hydrolyzed with an equal volume of 6 *N* HCl for 24 h at 110°. The hyrolyzates were dried in an evaporator at 55° and dissolved in 0.01 *N* HCl.

High-voltage paper electrophoresis was carried out with Pherograph-Original (Frankfurt, G.F.R.) using Paper No. 214, 35 × 40 cm from Macherey Nagel (Düren, G.F.R.). The buffer was pH 2 (1.5 *M* formic acid—2 *M* acetic acid, 1:1) and the electrophoresis was run at 100 mA and 2000—2500 V for 1.5 h. The paper was dried at 50°, and then used at right angles with isopropanol—acetic acid—water (8:1:1) as the solvent, for ascending chromatography at 23° for 21 h.

Liquid column chromatography

The amino acids were examined with the Technicon and Hitachi Model KLA-5 amino acid auto-analyzers. The technicon analyzer was equipped with a column (140 × 0.6 cm I.D.) filled with Chromobeads B resin. The gradient buffer solution contained lithium instead of sodium, according to Perry et al. [3]. The column was operated at 35° until glutamine had been eluted, then the temperature was raised to 70°. The Technicon integrator—calculator was used to quantitate all the amino acid peaks. Norleucine was used as internal standard. The concentration of the ninhydrin positive substances is expressed in μmole/l of plasma and μmole/mg creatinine in urine. The Hitachi analyzer had a column (25 × 0.9 cm I.D.) filled with Hitachi custom ion-exchange resin

No. 2615, and with 0.38 *N* sodium citrate buffer and was kept at 60° for basic amino acids. For acid and neutral amino acids, a column (55 × 0.9 cm I.D.) filled with Hitachi custom ion-exchange resin No. 2614 and with 0.2 *N* sodium citrate buffer, was used between 32° to 60°.

RESULTS

Two dimensional paper chromatography (urine)

Fig. 1 illustrates the positions of ninhydrin positive substances in two dimensional paper chromatography. Fig. 1(1) shows one unknown strongly ninhydrin positive spot near the alanine in the urine from the patient receiving GABOB. Fig. 1(2) shows the same ninhydrin positive spot to be present, two days after oral administration of GABOB was withdrawn. Fig. 1(3) shows the typical amino acid pattern of normal urine. Fig. 1(4) shows the spot of 10 μg pure GABOB added to the sample used in Fig. 1(3). GABOB shows up in the same position as the unkown compound in Fig. 1(1).

After hydrolysis, the ninhydrin positive GABOB did not disappear.

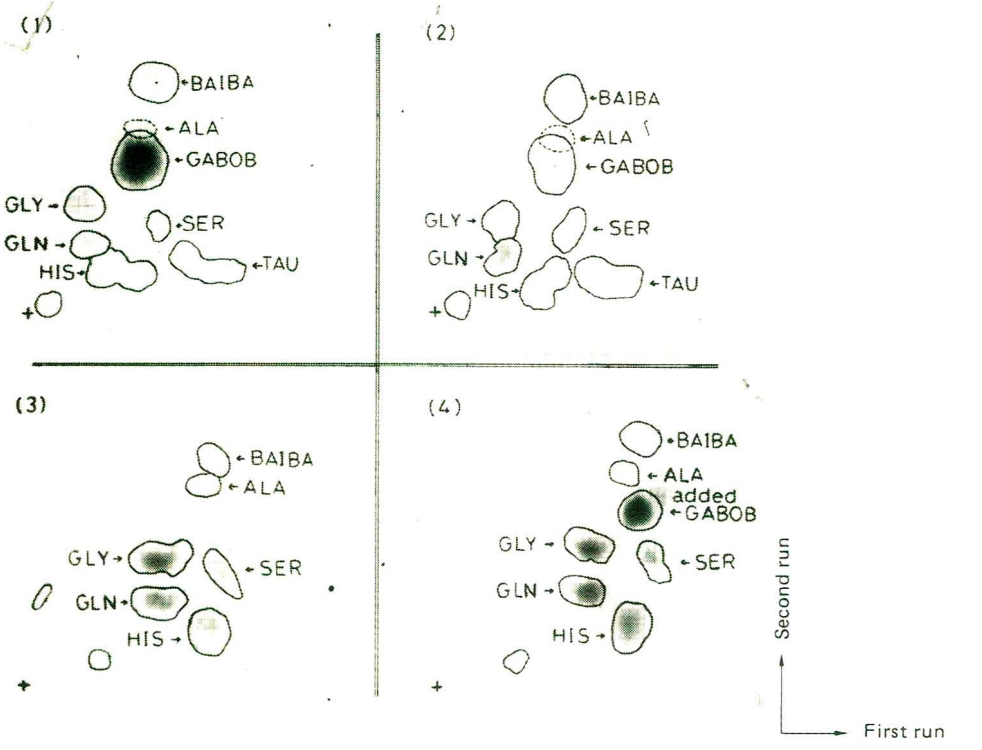

Fig. 1. Two dimensional paper chromatography of amino acids. Solvent pair: pyridine—water—acetone—ammonia (45:30:20:5) and isopropanol—formic acid—water (7.5:12.5:12.5). Detection reagent is ninhydrin. Chromatograms: (1) and (2) = urine samples from a patient receiving GABOB; (3) = normal urine sample; (4) = normal urine sample with added GABOB.

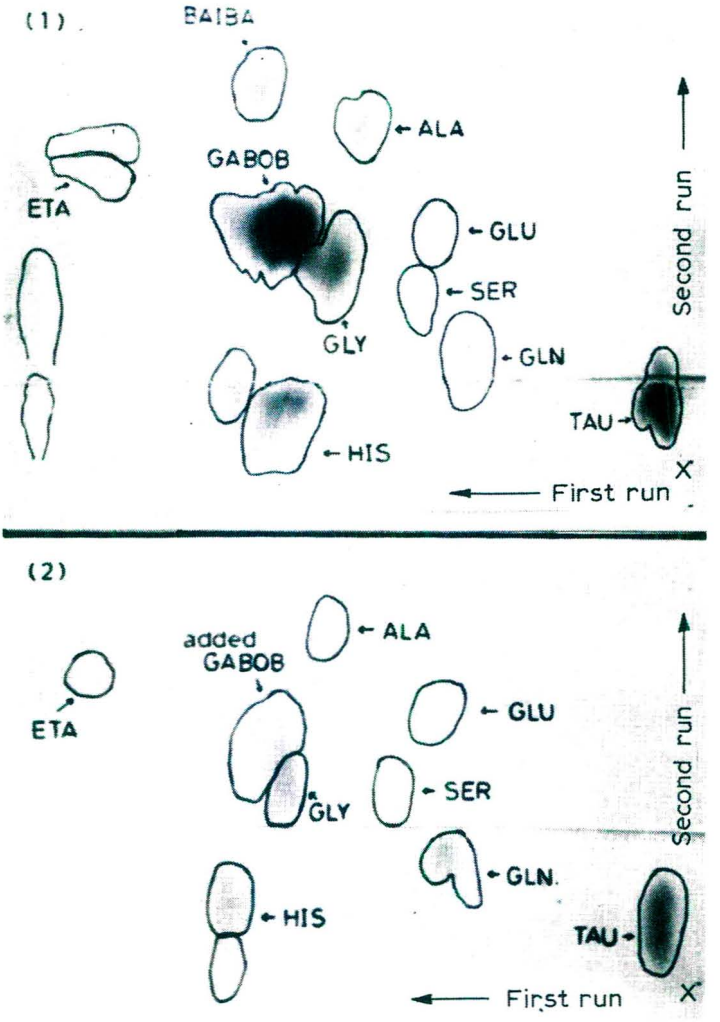

Fig. 2. Amino acids in a two dimensional separation using high voltage electrophoresis in the first run and isopropanol—acetic acid—water (8:1:1) for the second run. Chromatograms: (1) = urine sample from a patient receiving GABOB; (2) = normal urine sample with added GABOB.

High voltage paper electrophoresis (urine)

Fig. 2 shows the position of ninhydrin positive substances in the two-dimensional separation, using electrophoresis in the first dimension and isopropanol—acetic acid—water for the second dimension. Fig. 2(1) shows one strongly ninhydrin positive spot near the glycine in the urine from the patient receiving GABOB. Fig. 2(2) shows a similar ninhydrin positive spot in the same place after adding 20 μg of GABOB to the normal urine.

Even if the chromatogram was heated for 2—3 min in an oven at 105°, the colour did not change.

Liquid column chromatography with detection by amino acid auto-analyzer

(a) Lithium buffer method. Fig. 3 shows the chromatograms of ninhydrin positive substances from the Technicon amino acid auto-analyzer. Fig. 3(1) shows the ninhydrin positive GABOB peak 42 min after phenylalanine was added to the urine. Fig. 3(2) shows the peak from plasma in the same position; both samples were from one patient receiving GABOB. Two days after the withdrawal of oral administration of GABOB, the GABOB peak was still present in the urine, but not in the plasma.

(b) Sodium buffer method. Fig. 4 shows the chromatograms of ninhydrin positive substances from the Hitachi Model KLA-5 amino acid auto-analyzer. Fig. 4(1) shows the ninhydrin positive GABOB peak including leucine between isoleucine and tyrosine. One day after the withdrawal of oral administration of GABOB, the GABOB peak was not found in the plasma, as shown Fig. 4(2).

DISCUSSION

γ-Aminobutyric acid (GABA) has been shown to be converted to GABOB by beta oxidation and also to glutamic acid by transamination between GABOB and α-ketoglutaric acid [4]. Takada et al. [5] found two GABOB metabolites in the urine of rats receiving ^{14}C-GABOB. One was the γ-acetoamino-β-hydroxybutyric acid (N-acetyl GABOB) and the other was an unknown metabolite in low concentration.

Quantitative determination of amino acid in physiological fluid with the automatic amino acid analyzer is being used as a routine procedure for detecting inborn errors of metabolism [6, 7]. It is, however, very dangerous to diagnose metabolic diseases using only one method of amino acid analysis. Our case was originally diagnosed as tyrosinosis using the Hitachi auto-analyzer, because the GABOB peak appeared at the expected place for tyrosine. When using the Technicon analyzer, GABOB appeared close to homocystine. Paper chromatography and high voltage electrophoresis, however, ruled out these amino acids and identified GABOB.

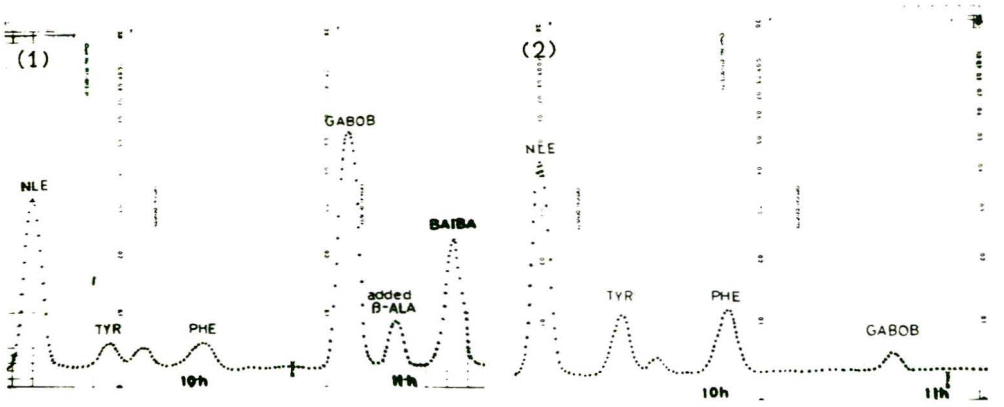

Fig. 3. Chromatograms from Technicon amino acid auto-analyzer using lithium buffer solution: (1) = urine sample from a patient receiving GABOB with added β-alanine; (2) = plasma sample from the same patient receiving GABOB.

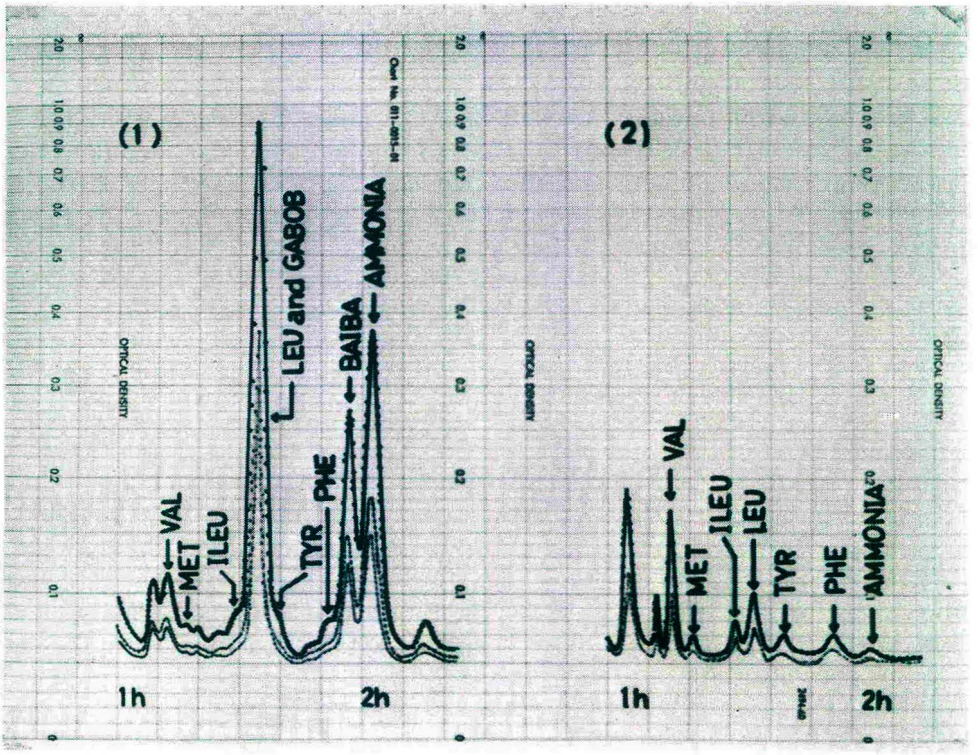

Fig. 4. Chromatograms from Hitachi Model KLA-5 amino acid analyzer using sodium buffer solution: (1) = urine sample from the patient receiving GABOB; (2) = plasma sample 1 day after withdrawal of GABOB.

ACKNOWLEDGEMENTS

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Note

Mass fragmentographic determination of 4-amino-3-*p*-chlorophenylbutyric acid (baclofen) in cerebrospinal fluid and serum

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Baclofen (4-amino-3-*p*-chlorophenylbutyric acid) is a γ-amino-butyric acid (GABA) analogue which, unlike the natural amino acid, is capable of passing the blood-brain barrier. Baclofen is generally assumed to be a GABA agonist but the compound has also been suggested to antagonize the effects of substance P [1].

Clinically baclofen is used in the treatment of multiple sclerosis and other spastic conditions [2]. Recently baclofen was suggested as being beneficial also in the treatment of schizophrenia, preferentially in combination with conventional neuroleptic drugs [3]. Other authors, however, have not been able to confirm this claim [4—7].

For the achievement of an optimal pharmacotherapy with baclofen, an analysis of the relationships between therapeutic effects and drug concentration in the body fluids may be valuable. A gas chromatographic method for the determination of baclofen using electron capture detection has been described [8]. However, for the determination of baclofen concentrations in cerebrospinal fluid (CSF) the latter method is insufficient [9]. Therefore a more specific and sensitive method with high precision is required.

In the present paper we describe a mass fragmentographic method for the determination of baclofen. The method has been applied in a preliminary analysis of drug levels in serum and CSF of baclofen treated patients. The analytical steps are shown in Fig. 1.

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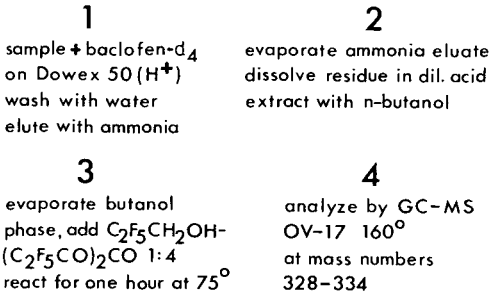

Fig. 1. Scheme of the analytical procedure.

EXPERIMENTAL

Materials

Baclofen-d_4 (2.5 mg) [10] was dissolved in 100 ml of water. An aliquot of this solution was diluted 10 times and 100 μl (250 ng of the compound) were added to all samples as internal standard. Authentic baclofen (Hässle-Ciba-Geigy, Mölndal, Sweden) was dissolved in water. A stock solution containing 1 mg in 100 ml was used. After two ten-fold dilutions the solution was used for the preparation of calibration curves. Standard solutions for CSF and serum contained 0—100 ng and 0—200 ng of baclofen respectively.

Dowex 50W-X4 (200—400 mesh) in the protonated form was treated with an excess of aqueous ammonia, washed with water to neutrality, regenerated with an excess of 4 *M* HCl and finally washed with water to neutral pH. This treatment removed impurities that interfered with the analysis. The pentafluoropropionic anhydride was purchased from Produktkontroll, Stockholm, Sweden, and 2,2,3,3,3-pentafluoropropanol was obtained from Columbia Organic Chemicals, S.C., U.S.A. Other chemicals were of analytical grade and were obtained from standard sources.

Sample preparation

Procedure. The deuterated standard (250 ng) was added to 1 ml of CSF or serum and the samples were mixed (Fig. 1). Columns of Dowex 50 (15 × 5 mm I.D.) were prepared in disposable pipettes plugged with a small amount of glass wool. The volume of the column above the resin corresponded to approximately 2 ml. The columns were washed with two portions of water after which the samples were allowed to flow through with gentle air pressure when required. The effluent and three washings with 2 ml of water were discarded. Baclofen was eluted with 2 ml of 10% ammonia, which was collected in small conical test tubes. The samples were blown to dryness in a stream of nitrogen gas with gentle heat from a hair drier. After evaporation, the samples were redissolved in 1 ml of 10^{-4} *M* HCl and extracted with 2 ml of *n*-butanol. The butanol phases were transferred to conical test tubes and evaporated as above. When the residue was completely dry, 100 μl of a 4:1 mixture of pentafluoropropionic anhydride and pentafluoropropanol was added. The test tubes were closed by glass stoppers and kept at 75° for 1 h. After removal of excess reagent in a desiccator at reduced pressure 25 μl of ethyl acetate containing 2%

pentafluoropropionic anhydride was added. The samples were analysed by gas chromatography—mass spectrometry (GC—MS) as described below.

GC—MS conditions

The analyses were performed on a Finnigan 3200 gas chromatograph—mass spectrometer system equipped with a vacuum diverter and operated in the electron-impact mode. An OV-17 column (1.5 m × 2 mm I.D.) was used for the GC separation. The column temperature was 160—170° with a helium gas flow of about 20 ml per min. The electron energy was set at 50 eV. Other spectrometer parameters were as reported elsewhere [11]. The mass numbers routinely monitored were 328 and 334, which have a relative intensity in the mass spectra of about 40% and correspond to the loss of pentafluoropropionamide from the molecule [10]. There is no loss of label in that process. The base peak in the region 170—500 is 273 but it retains no label. The molecular peak is not discernible.

Peak heights were determined manually and the ratios between non-labelled and labelled compounds were determined. The data from the standard solutions were used for the calculation of the equation of the calibration line. Unknown samples were then interpolated according to this equation using a small desk calculator.

Sampling of CSF and serum

Serum and CSF were collected from psychotic patients by venipuncture and lumbar puncture. Samples were taken before the morning dose after the patients had been treated for 2 and 4 weeks with baclofen (Lioresal®, Hässle-Ciba-Geigy; 10 mg, 3 times daily). Blood and CSF were centrifuged and all samples were frozen to −20° within 1 h.

Serum was also collected from a healthy, male volunteer who received 20 mg baclofen in a single oral dose. The compound was given at 8 a.m. with the subject fasting. Venous blood samples were removed as indicated in Table I.

RESULTS AND DISCUSSION

From preliminary experiments, using the more easily available baclofen-d_2 as internal standard, it was found that the mass number 332 (which must be used due to the ^{37}Cl present in non-labelled baclofen) was unsuitable. Compounds present in serum and CSF interfered at this mass number and could not be separated chromatographically without excessive prolongation of the retention time.

Therefore we decided to use a d_4-standard which was found to be more satisfactory. The calibration curves invariably show good linearity with a correlation coefficient usually higher than 0.99. When baclofen was added to samples of plasma and CSF a satisfactory recovery and experimental error was obtained (Table II). Preliminary investigations on serum and CSF sampled from patients treated with baclofen indicate that the levels are of the magnitude added to the samples shown in this table. Analysis of CSF and serum from untreated patients gave blank values of ± 0.5 ng/ml and ± 2 ng/ml, respectively.

Typical mass fragmentograms from serum and CSF of baclofen treated sub-

TABLE I

AMOUNTS OF BACLOFEN FOUND IN SERUM FROM A HEALTHY SUBJECT RECEIVING A SINGLE ORAL DOSE OF BACLOFEN 20 mg

The deviation from mean is the difference between the mean and either of the determined values.

Time from start of experiment (h)	Baclofen (ng/ml)	Deviation from mean (ng)
0	-1.9	0.4
1	3.1×10^2	0.2×10^2
2	260	2
3	197	3
4	150	2
6	92	4
8	68	2
12	36.2	0.6
24	7.7	0.1

TABLE II

STANDARD DEVIATION AND RECOVERY OF ASSAY OF BACLOFEN ADDED TO PLASMA AND CSF

Sample (n=5)	Amount added (ng/ml)	Found (ng/ml)		Found as % of added
		Mean	S.D.	
CSF	6.7	6.7	0.7	100
	13.3	13.2	0.9	99
Plasma	33.5	32.5	2.1	97
	100	96.9	3.2	97

jects are shown in Fig. 2. It can be seen from the mass fragmentograms that some interfering compounds are present both in serum and CSF. For serum, it was necessary to include a waiting period of a few minutes in order to elute a slowly moving component from the column. During that period the flow was diverted to the separator pump by the use of the vacuum diverter. A part of this peak can be seen in Fig. 2. The waiting period, however, did not cause an undue prolongation of the analysis time.

To determine the applicability and precision of the procedure for determination of baclofen levels in serum, the compound was given in a single dose to a healthy volunteer. Blood samples were removed as indicated in Table I and

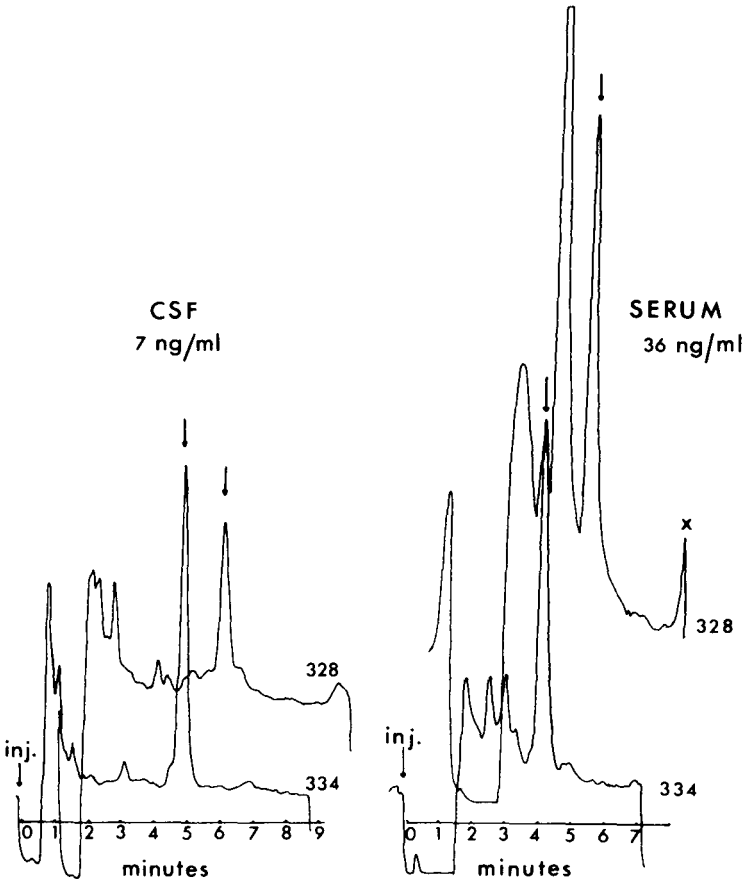

Fig. 2. Typical mass fragmentograms of CSF and serum extracts from subjects receiving baclofen. Arrow indicates retention time of baclofen. x is a slowly moving compound possibly interfering with the analysis.

serum was analysed in duplicates. The mean percentage deviation was 2.4 ± 1.6% and the correlation between the two sets of analyses was 0.995. The fact that there was a negative amount of compound at zero time may be due to a compound giving a small contribution at mass number 334, thus making the ratio 328:334 smaller than for the pure standard.

Being a 4-aminocarboxylic acid, there is a possibility of baclofen cyclizing to give the corresponding lactam. However, solutions of baclofen in water, kept at 4° for three months, did not show any change in their concentration. Therefore lactamization should be very slow, at least in water solutions at pH 7.

In summary, the method described in this paper for the determination of baclofen in serum and in CSF showed a high reproducibility and sensitivity. In CSF baclofen could be determined in concentrations down to about 5 ng/ml. In preliminary experiments the drug concentration in CSF from baclofen treated patients has been shown to be above this concentration. As compared to the previously described GC method [8] the present method has an approximately 4-fold increase in sensitivity.

ACKNOWLEDGEMENTS

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Note

Quantitative analysis of naphthoxylactic acid, a major metabolite of propranolol in plasma in man

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To be able to understand fully the multiplicity of actions produced by the beta-adrenergic receptor blocking drug propanolol in man it is important to understand its metabolism and disposition. Thus, as more than 98% of the drug is metabolized following oral administration in man [1, 2], it is possible that metabolites may contribute to its actions. It has already been shown that 4-hydroxypropranolol, equipotent with propranolol as a beta-blocker [3], can be present in plasma in similar concentrations to its parent compound in patients chronically treated with propranolol [4].

Although the major metabolite of propranolol in human urine has been indicated to be naphthoxylactic acid (NLA) [5—7], no information is available on its plasma concentrations, in particular after chronic propranolol therapy.

The present study describes a sensitive and specific technique for NLA determinations in plasma by electron capture gas chromatography (GC) following methylation of the carboxyl group with diazomethane and acylation of the hydroxyl group with heptafluorobutyric anhydride. The method was applied to quantitative determinations in the plasma of patients chronically treated with propranolol.

EXPERIMENTAL

Standards and reagents

Naphthoxylactic acid (NLA) was kindly supplied by I.C.I. (Macclesfield, Great Britain).

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Trimethylamine was obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.) and was dissolved in nanograde benzene. Nanograde benzene was obtained from Mallinckrodt (St. Louis, Mo., U.S.A.). Diazomethane was prepared from N-methyl-N-nitroso-*p*-toluene-sulfonamide in diethyl ether using a Diazald® kit from Aldrich (Milwaukee, Wisc., U.S.A.). Heptafluorobutyric anhydride was obtained from Pierce (Rockford, Ill., U.S.A.) in 1-ml ampules and was stored in airtight PTFE-capped 3-ml vials (Reacti-Vials, Pierce).

Aqueous reagents, including pH 6.0 phosphate buffer, 0.5 *M*, and hydrochloric acid, 1 *M*, were prepared from glass-distilled water and stored in glass bottles with PTFE-lined caps.

Synthesis

The ethyl ester of NLA, used as the internal standard, was prepared by extractive alkylation. To 50 mg of NLA ($2 \cdot 10^{-4}$ mole) in a 15-ml centrifuge tube were added 0.75 ml of 10% tetrabutylammonium hydroxide in water ($3 \cdot 10^{-4}$ mole), 30 μl ethyl iodide ($3 \cdot 10^{-4}$ mole) and 4 ml of methylene chloride. The mixture was shaken vigorously for 1 h and was then centrifuged. The top, aqueous, layer was removed and the methylene chloride was evaporated at 50° under a stream of nitrogen. To the residue was added 5 ml of benzene. After washing with 2 ml of pH 6.0, 0.5 *M*, phosphate buffer the benzene was evaporated to dryness. A white powder was obtained, melting point 49—51°. The ethylation was quantitative as confirmed by gas chromatography—mass spectrometry (GC—MS).

Glassware

All glassware was cleaned in chromic acid. Conical and round-bottomed glass centrifuge tubes were silanized as previously described [8].

Instruments

GC. A Varian Model 1440 gas chromatograph equipped with a ^{63}Ni electron capture detector was used. The column (180 cm × 2 mm I.D.) was made of pyrex glass and packed with 80—100 mesh Chromosorb W coated with 5% OV-17. The column was conditioned at 250° for 48 h before use. Operating conditions were: column temperature 185°, injector temperature 265°, detector temperature 270°, and carrier gas (nitrogen) flow-rate 30 ml/min.

GC—MS. The combination instrument was an LKB 9000S, operated at an accelerating voltage of 3.5 kV, ionization voltage of 20 eV, and a trap current of 60 μA. A pyrex column (120 cm × 2 mm I.D.) with 4% OV-17 at 190° was employed. The carrier gas (helium) flow-rate was 20 ml/min.

Patients

Blood samples were obtained 2 h after the morning dose from seven hypertensive patients chronically treated with propranolol, 40 mg every 6 h, and were studied in a clinical research unit [9]. The blood samples were collected in heparinized tubes and immediately centrifuged at 6000 *g* for 15 min in a refrigerated centrifuge and the clear plasma layer removed. The plasma samples were either analyzed immediately or stored at —70° in an ultra-cold freezer.

Extraction and derivatization

A 1.00-ml plasma sample was acidified to ≈ pH 2 with 0.2 ml of 1 *N* hydrochloric acid in a 15-ml round-bottomed centrifuge tube. After the addition of the internal standard, 400 ng, the sample was extracted on a reciprocating shaker for 10 min. The organic phase was transferred to a conical centrifuge tube and evaporated to dryness in a water bath at 50—60° under a gentle stream of nitrogen.

Methanol, 100 μl, and the diethyl ether solution of diazomethane, 200 μl, were added and allowed to react for 10 min at room temperature. The excess reagent was evaporated as described above. A 400-μl volume of nanograde benzene was then added, together with 25 μl of 1 *M* trimethylamine in benzene and 50 μl of heptafluorobutyric anhydride. The tubes were capped and heated at 50° for 10 min. The reaction mixture was then washed with 1 ml of pH 6.0 phosphate buffer, 0.5 *M*, by vigorous shaking for 30 sec. After centrifugation, 0.5—4 μl of the benzene layer was injected.

Standard curves

Varying quantities of synthetic NLA (100—1000 ng) and 400 ng of the internal standard were added to 1.0-ml samples of control human plasma and carried through the analytical procedure. Peak area ratio measurements times the concentration of the internal standard were plotted against the concentration of NLA. Synthetic NLA was also extracted from 1.00-ml samples of distilled water as well as derivatized without extraction to obtain an index of recovery.

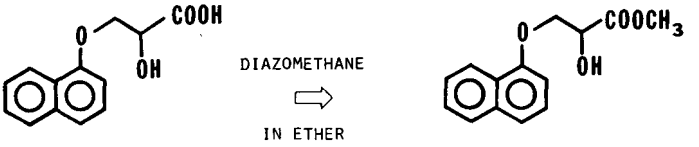
Propranolol was determined by a specific GC—MS assay [10] recently modified to include stable-isotope-labeled propranolol as the internal standard [11].

RESULTS AND DISCUSSION

The two-step derivatization procedure employed for NLA is depicted in Fig. 1. Both reactions proceed rapidly and quantitatively to yield the methyl,heptafluorobutyryl derivative, the structure of which was confirmed by GC—MS, Fig. 2A. A similar derivatization technique has been applied to acidic catecholamine metabolites [12]. The acylation step in the present study followed a procedure previously shown to lead to rapid acylation of a variety of compounds [13, 14], including propranolol [8] and several of its basic and neutral metabolites [10, 15]. Excess reagents were removed by a pH 6.0 buffer wash similar to the method of previous studies [8, 10, 14]. This procedure resulted in a minimum of interferences on the electron capture detector. The derivative was stable for more than 48 h in contact with the buffer.

While the methylation step was necessary in order to obtain good peak symmetry, the acylation of the hydroxyl group with heptafluorobutyric anhydride provided a unique tool to enhance the electron capture sensitivity of NLA specifically as compared with interfering endogenous carboxylic acids not containing a hydroxyl group. The minimum detectable quantity of the methyl, heptafluorobutyryl derivative of NLA was about 2 pg, which is similar to other heptafluorobutyryl esters [13].

STEP 1:



STEP 2:

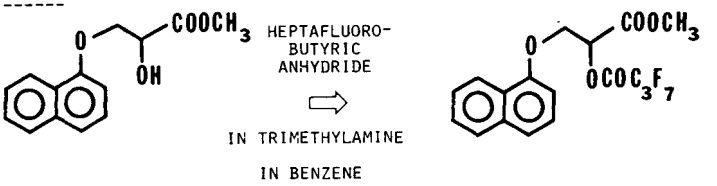


Fig. 1. Derivatization procedure for NLA.

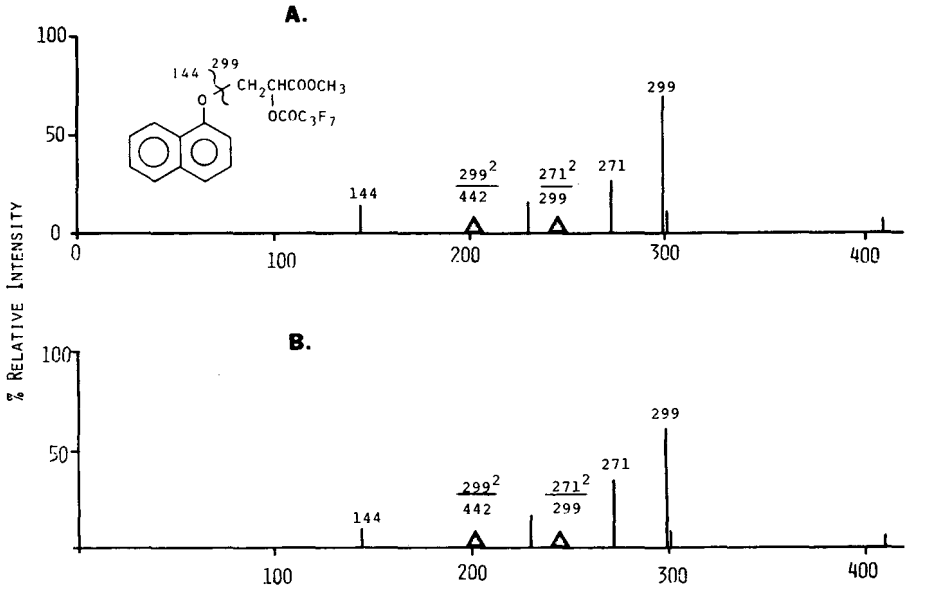


Fig. 2. Mass spectra of the methyl,heptafluorobutyryl derivative of NLA. (A) Synthetic NLA; (B) propranolol metabolite in plasma.

The chromatogram of a control plasma extract is shown in Fig. 3A, demonstrating no interference at the retention time of NLA and only a minor interference at the retention time of the internal standard, the ethyl ester of NLA. Fig. 3B shows the chromatogram of an extract of plasma from a patient chronically treated with propranolol, 160 mg/day, to which the internal standard, 400 ng/ml, had been added. The high NLA concentration (656

TABLE I

PLASMA LEVELS OF NLA AND PROPRANOLOL IN SEVEN PATIENTS ON CHRONIC PROPRANOLOL THERAPY, 160 mg PER DAY

All samples were collected at 2 h after the morning dose, i.e. at the time for peak plasma propranolol [9].

Patient	Propranolol* (ng/ml)	NLA (ng/ml)	NLA Mean ± S.D.	Ratio NLA : Propranolol
I	46	660 632 676	656 ±22	14.5
II	63	536 500 496	510 ±22	8.1
III	53	917 960 920	932 ±24	17.6
IV	51	365 320 365	350 ±26	6.9
V	45	540 545 521	535 ±13	11.9
VI	38	405 400 365	390 ±22	10.3
VII	100	460 510 450	473 ±32	4.7

*Determined as part of a separate study (see ref. 9).

ng/ml) present in this patient at this moderate propranolol dose indicates that the method developed is quite adequate for determinations of this propranolol metabolite in man. At these concentration levels the minor interference with the internal standard seen in Fig. 3A becomes insignificant. The high NLA concentration present also made it possible to obtain a complete mass spectrum of this metabolite in the plasma of this patient, Fig. 2B. This spectrum is identical to the mass spectrum of the synthetic material, Fig. 2A.

The standard curve for NLA in plasma was linear for the concentration range studied (100—1000 ng/ml) and went through the origin (slope 1.04; correlation coefficient >0.99). Extraction from water as well as derivatization without prior extraction gave the same slope, indicating no adverse effect due to the extraction. When the internal standard was added to the NLA plasma samples after the extraction but prior to the derivatization, a 20% lower value was obtained, indicating that the recovery of NLA in the extraction step was about 80%. The minimum detectable plasma concentration of NLA was about 2 ng/ml.

Table I summarizes the plasma concentrations of NLA measured in seven patients chronically treated with propranolol, 160 mg/day. Triplicate samples were measured for each patient. The NLA concentrations are expressed as the mean ± S.D. of these determinations. The table also gives the propranolol concentrations as a comparison. The specificity of the method is emphasized by the fact that no interferences were observed from several other drugs concomitantly administered to these patients. These drugs were clofibrate, digoxin, chlordiazepoxide, nitroglycerin, isosorbide dinitrate, tolazamide and warfarin.

The NLA concentrations in the seven patients ranged from 350 to 930 ng/ml, with a reproducibility for the individual patients of between 2.4 and 7.5%. These concentrations exceeded the propranolol concentrations measured in the same patients by five to eighteen times. There was no apparent relationship between the NLA and propranolol concentrations observed except for a similar, only 2.6-fold, between-patient variation in these concentrations, which supports the view [9] of only small between-patient variations in propranolol pharmacokinetics.

Although no pharmacological activity has been described for NLA, the high concentrations of this propranolol metabolite observed in plasma warrant further studies of both its pharmacokinetics and possible biologic activity.

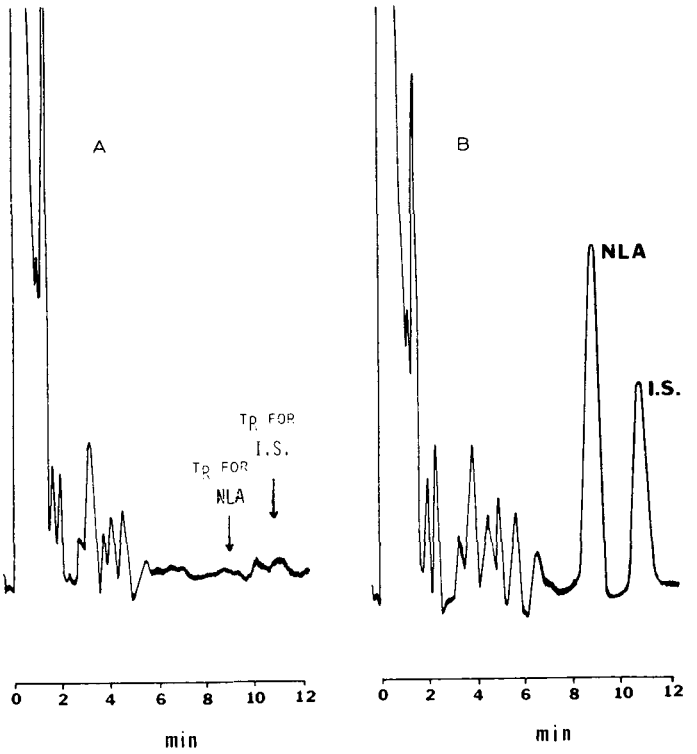


Fig. 3. Gas chromatograms of derivatized plasma extracts using electron capture detection. (A) Control plasma; (B) plasma from patient chronically treated with propranolol, 160 mg/day. Internal standard = ethyl ester of NLA, 400 ng/ml. NLA calculated = 656 ng/ml.

Furthermore, as lactic acid metabolites have been indicated to represent an important metabolic pathway for other beta-blockers [16—18], such studies extended to beta-blocking drugs in general may be important.

ACKNOWLEDGEMENTS

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Note

Use of formic acid in carrier gas: a rapid method to quantitate dipropylacetate in plasma by gas—liquid chromatography

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A rapid and sensitive method to quantitate drugs in biological fluids is most helpful in pharmacokinetic studies or in drug therapy, particularly when biological drug levels vary with rapid elimination and/or with individual variations, as is noticed with dipropylacetate (DPA) [1].

Dipropylacetic acid (valproic acid) is a branched chain fatty acid whose sodium salt is recognized to be effective in the treatment of petit mal epilepsy and, in association with other drugs in cases of grand mal epilepsy. As far as we know the methods already described for DPA determination require microdiffusion [2] or solvent extraction, often dessication and sometimes derivatisation [3—7]. Moreover, most of these methods need large samples and therefore are time consuming and not suitable for pediatric requirements or for pharmacokinetic studies stretching over 48 h.

In the direct procedure described below, no pre-chromatographic manipulations are necessary. This method is sensitive enough for quantitation to be carried out on small plasma samples. The biological sample is directly injected into a gas chromatograph, DPA is displaced from its plasmatic salts by a formic acid gas current saturating the carrier gas, and detected in approx. 10 min up to a minimum level of 2.5 ng.

MATERIALS AND METHODS

Reagents

Sodium dipropylacetate was used in the form of the commercial 20% solution (Depakine, Labaz, 33440 Ambares, France). Octanoic acid was purchased

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from Prolabo (Paris, France) and formic acid came from Baker (Deventer, The Netherlands) and chromatographic reagents from Varian Aerograph (Walnut Creek, Calif., U.S.A.). All volumes were measured with Hamilton syringes.

Gas chromatography (GC)

An Intersmat IGC 120 DFL bi-column gas chromatograph equipped with a flame ionisation detector (FID) was used without modification.

Both FID and injector were maintained at 160°. The injector was equipped with a glass pre-column, 100 × 2 mm I.D. (Insert tube Intersmat), carefully acid washed, rinsed out, then heated overnight at 160° in the injection port before use. This pre-column was used for up to ca. 20 injections with protein-containing samples (100 μl injected).

The metal column (2 m × 2 mm I.D.) was coated with 5% Carbowax 20 M on 80—100 mesh, Chromosorb P and was operated at 150° after a conditioning of 48 h at 160° under a stream of nitrogen, then one night at 100° without the carrier gas (ref. 8 slightly modified). The glass wool occluding the column was treated with phosphoric acid (85%) to avoid adsorption of DPA fractions on the column head.

Just before gas entry, the nitrogen flow (45 ml/min) was saturated with acid by flowing, without bubbling, over 1 ml of pure formic acid contained in a 5-ml screw cap vial equipped with an O-ring (for more details see [9]). The use of pure anhydrous formic acid was necessary to avoid the appearance of interfering peaks due to volatile compounds eventually present in the acid, and also the premature wear of the chromatograph. In such conditions formic acid vapors were able to displace DPA from its plasmatic salts.

Procedure

A 2.5-μl aliquot of internal standard (octanoic acid 12.6 mmole/l in aqueous alkaline solution, stored at —20° in a 1-ml vial until utilization) was added to 100 μl of heparinized plasma, possibly obtained from blood drawn from the finger tip, and 5 μl of the resulting solution were injected into the chromatograph. The volume of heparinized plasma is only limited by the injection volume and can be reduced to 20 μl.

Between injections of biological samples, 10 μl of 50% (v/v) formic acid were injected into the pre-column (5 μl to pre-column head, half a syringe needle and 5 μl to pre-column end), and the oven heated to 160° for 10 min in order to clean the pre-column of residual compounds eventually adsorbed on proteins denatured by injector heat.

RESULTS AND DISCUSSION

A typical chromatogram of DPA in patient's plasma is shown in Fig. 1. DPA and the internal standard were well separated and no interfering endogenous compounds were noticed. The DPA calibration graph indicates a linear relationship between added DPA in normal plasma and detector response, from 2.5 μg/ml to 170 μg/ml (minimum detectable amount 0.5 μg/ml; Fig. 2). This concentration range allows a wide margin for DPA therapeutic concentrations, (from 40 to 120 μg/ml). It seems suitable for drug concentration

TABLE I

PERCENTAGE RECOVERY OF ADDED DPA IN PLASMA

Internal standard: same concentrations in aqueous and plasma samples. Each value is the mean of three determinations and is measured at least one hour after plasma addition to allow proteic fixation of DPA and of internal standard.

Concentration of added DPA in water and plasma	$\frac{\text{Absolute DPA peak height (plasma)}}{\text{Absolute DPA peak height (water)}} \times 100(\%)$	$\frac{\text{DPA relative to internal standard peak height (plasma)}}{\text{DPA relative to internal standard peak height (water)}} \times 100(\%)$
43 $\mu g\ ml^{-1}$	92	108*
172 $\mu g\ ml^{-1}$	80	105*
344 $\mu g\ ml^{-1}$	83	100

*An increase in percentage recovery seems due to a lower recovery of octanoic acid in plasma (recovery of octanoic acid is 80% for ten determinations in water and plasma at internal standard concentrations).

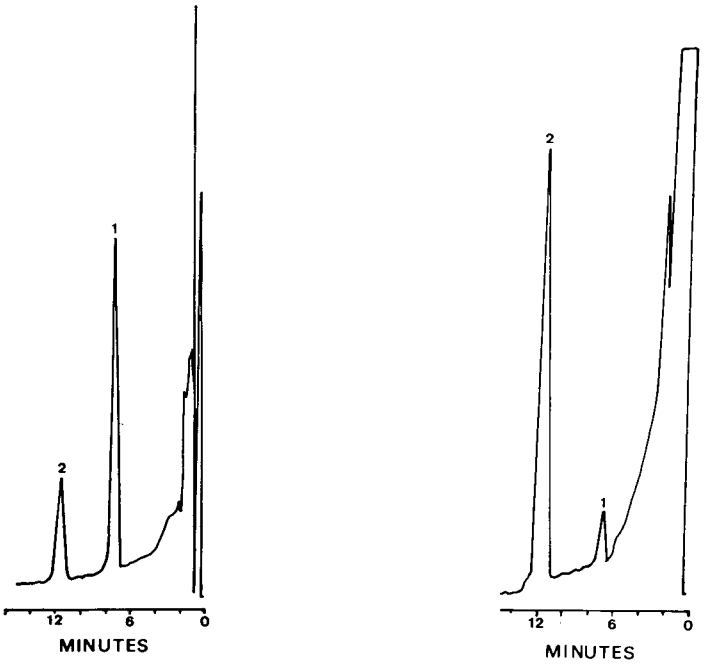

Fig. 1. Gas chromatogram for children's plasma one hour after ingestion of DPA 200 mg (Depakine). (1) DPA, 65 $\mu g \cdot ml^{-1}$; (2) internal standard octanoic acid, 47 $\mu g \cdot ml^{-1}$. For gas chromatographic details see text (5-μl injected).

Fig. 2. Gas chromatogram for plasma with added DPA near minimum detectable amount (5-μl injected). (1) DPA, 2.5 $\mu g \cdot ml^{-1}$; (2) internal standard, octanoic acid, 47 $\mu g \cdot ml^{-1}$. For gas chromatographic details see text.

studies in saliva or spinal fluid or for pharmacokinetic studies for long periods after drug administration [5].

The reproducibility of the method has been studied with plasma samples corresponding to the lowest therapeutic level (40 μg/ml). For the first ten injections, the standard deviation was found to be equal to 3.5% and for the following ten equal to 6%. This seems due to a slight adsorption of plasma DPA on protein residues in the pre-column. However, this reproducibility fell within acceptable limits for clinical purposes.

Choice of technical conditions

Since the original publication of James and Martin, many authors have noticed a column adsorption of free volatile acids analysed by GC. Adsorption generally gives rise to tailing peaks, irregular shaped peaks and ghosting. This phenomenon seems avoidable by heating the column without the carrier gas and by using formic acid to saturate the carrier gas. Poorly detectable by flame ionization, formic acid is more polar than DPA, thus strongly bound to adsorption sites, and so it prevents tailing peaks and ghosting. Moreover, formic acid is able to displace DPA from its plasmatic salts, rendering all previous sample treatments useless. In spite of the use of an acid carrier gas, no premature wear of the chromatograph could be noticed after several months of constant use

(certainly because of the pure anhydrous formic acid used). Utilization of such formic acid (the 5-ml vial was filled with 1 ml twice a week) allows the quantitation of DPA on very small blood samples.

A residual adsorption of plasmatic DPA by denaturated proteins in the pre-column was not completely prevented, as is shown by the percentage recovery studied in Table I. The percentage recovery of added plasma DPA compared to an aqueous DPA solution has been tested on three DPA concentrations, including the lowest therapeutic level. Taking into account the residual adsorption of DPA, it was obvious that the use of an internal standard was necessary, so, one chemically related to DPA was chosen. This residual adsorption justifies the injection of 10 μl of formic acid between two samples; by sweeping up waste from the pre-column, it allows repeated injections of biological samples. The pre-column precents column pollution by proteins.

The Carbowax phase was compared with the neopentylglycolsuccinate phase (NPGS) 10% on Chromosorb G. Carbowax 20 M was chosen since it gives a better resolution under the conditions used in spite of a longer retention time. Remesy and Demigne [8] had already shown the possibility of using Carbowax 20 M to assay free volatile acids, and they pointed out that eddy peaks are noticed over 130° due to the thermal decomposition of this phase. Such a phenomenon was not observed despite the use of a temperature of 150°. This could be due to the low coating that had been chosen in order to allow a rapid elution (absolute retention time for DPA is 7 min), i.e. mild conditions which avoid thermal decomposition yielding eddy peaks and premature wear of the column.

The use of formic acid in the carrier gas prevents most of the problems already described in volatile acid analysis by GC. It allows a simple procedure for DPA determination in plasma by direct injection of the biological sample and so makes this method particularly adaptable for pediatric requirements.

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Note

Sensitive gas—liquid chromatographic assay of underivatized 5-fluorouracil in plasma

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Several gas-chromatographic methods have been reported for the analysis of 5-fluorouracil (5-FU) in biological fluids [1—4]. These methods have in common that 5-FU is determined as a methyl or silyl [1, 2, 4] derivative. In our opinion gas—liquid chromatographic (GLC) analysis of underivatized 5-FU is possible, which might lead to a more direct analysis of the drug. In accordance with this view, already put into practice in the analysis of underivatized barbiturates [5, 6], we have developed a suitable GLC method for underivatized 5-FU.

MATERIALS AND METHODS

Apparatus

A Becker Model 420 gas chromatograph equipped with a Hewlett-Packard dual nitrogen—phosphorus flame ionization detector Model 18789 A was used. The radiochemical measurements were performed on a Packard Model 2450 Tri-carb® liquid scintillation spectrometer.

Solvents, standards and reagents

The solvents used were of analytical grade and obtained from Baker (Deventer, The Netherlands). Dimethyldichlorosilane was from Merck (Darmstadt, G.F.R.). Gas-Chrom Q, Versamid 900 and Carbowax 20M were obtained from Chrompack (Middelburg, The Netherlands). RBS-25 was from Hicol (Rotterdam, The Netherlands). 5-Fluorouracil was kindly supplied by Hoffmann-La Roche (Mijdrecht, The Netherlands). 5-Chlorouracil (5-CU) was purchased from Calbiochem (Los Angeles, Calif., U.S.A.) and 5-fluoro-[6-^{3}H]uracil (2.1 μCi/mmol) from the Radiochemical Centre in Amersham (Great Britain).

Instafluor was from Packard (Downers Grove, Ill., U.S.A.) and Triton X-100 from Koch-Light Labs. (Colnbrook, Great Britain).

Packing of the column

To 500 mg of Gas-Chrom Q (140—160 μm, carefully sieved and dried at 120°), 30 ml of a 10% (v/v) solution of dimethyldichlorosilane in toluene were added, and the mixture was refluxed for 1 h. The resilanized Gas-Chrom Q was filtered off, washed with toluene and refluxed in methanol. After filtration and washing with acetone the powder was dried at 120°. A solution of Versamid 900 (15 mg) in 15 ml chloroform—methanol (85:15) was added to the hot Gas-Chrom Q, and the solvent was removed using a Rotavap. A glass column (0.45 m × 0.8 mm I.D.) was cleaned with warm RBS 25 solution, water and acetone, and dried at 120°. The column was then silanized with dimethyldichlorosilane, cleaned by three methanol washings and dried at 120°. A 1% (w/v) solution of Carbowax 20M in methanol was sucked through the hot column. The air-dried column was packed with 3% Versamid 900 on Gas-Chrom Q.

Recovery experiments

For the radiochemical experiments a solution of 5-FU in methanol (4 ml, 100 ng/μl) containing 5-fluoro-[6-^{3}H]uracil (about 22 × 10^5 dpm per ml) was prepared. Several 20-μl samples of this solution were stored in the scintillation liquid in a counting vial as a reference. Aliquots of 10—60 μl were then pipetted into vials of glass, silanized glass and plastic, or Eppendorf tubes. After evaporation of the solvent the residue was dissolved in 200 μl methanol, the vial was heated at 50° and shaken by means of a Vortex mixer. Subsequently 20 μl of the methanolic solution were added to the scintillation liquid (1 ml water and 9 ml Instafluor Triton X-100, 2:1). A sample of 20 μl of pure methanol added to the scintillation liquid served as a background reference. The gas-chromatographic recovery experiments were carried out by the same procedure, omitting the addition of radioactively labelled 5-FU. Standard solutions of 5—FU (10—600 ng/μl) and 5-CU (200 ng/μl) in methanol were used; after evaporation 100 μl methanol were added to the residue prior to detection. The recoveries were calculated by comparison of the absolute peak heights obtained with those detected with the corresponding reference solutions.

Extraction method

To 0.2 ml of plasma in a plastic tube were added 100 or 200 ng of the internal standard, 5-CU. After two extractions with 3 ml of ethyl acetate the combined ethyl acetate layers were evaporated and the residue could be used in the GLC procedure. However, the lifetime of the column can be extended by further purification. Hence the ethyl acetate residue was dissolved in 1 ml of hexane, 0.5 ml of water was added and the mixture was shaken by means of a Vortex and centrifuged. The hexane layer was separated and the water layer again extracted with 1 ml of hexane. After evaporation of the remaining water layer the residue was suitable for further manipulation in the GLC procedure. For the construction of a calibration curve (5—200 ng) known amounts of

5-FU in the range 50—2000 ng were added to 0.2 ml of blank plasma, after which the extraction procedure described above was followed.

GLC procedure

The residue obtained by the extraction method was dissolved in 100 μl water. The injections were carried out with a solid-phase injection system (modified pyrolysis system Becker Model 767 [5]). The reference solution and sample solution (10 μl) were applied to the tip (0.5 mm O.D.) of a stainless-steel rod (2 mm O.D.) with a microsyringe. After evaporation of the solute, the rod was conducted through the sluice system and maintained in the upper part of the column for 10 sec. The retention times for 5-FU and 5-CU were approximately 1.4 and 3.5 min, respectively. The operating conditions were: carrier gas (helium) flow-rate 8.5 ml/min, additional scavenger gas (helium) up to 30 ml/min; hydrogen flow-rate 3.0 ml/min; air flow-rate 100 ml/min; inlet and detector temperature 300°; oven temperature 190°.

RESULTS AND DISCUSSION

Initial observations whereby 5-FU was found to adhere to glass prompted us to investigate this phenomenon in more detail. A number of recovery experiments were performed using vials of different material. Solutions of 5-FU were evaporated in glass and plastic vials. After redissolving the residue, both radiochemical and gas-chromatographic measurements indicated that considerable loss of 5-FU had occurred in the glass vials, whereas with the plastic vials almost quantitative recoveries were obtained. Some results of experiments on the adhesive properties of several materials are summarized in Fig. 1. Awareness of the adherence of 5-FU to non-deactivated glass is important in developing extraction procedures for this compound. Although we have no indication that 5-FU is adsorbed by glass from a solution, we have avoided the use of non-deactivated glass equipment during the analysis of 5-FU.

GLC of the polar compound 5-FU requires special conditions. In the analytical system the number of adsorption sites should be minimized. Hence all glass equipment used, including the glass column, was silanized with dimethyldichlorosilane in toluene. The glass surfaces were then relatively inert to 5-FU (see Fig. 1). The support material, Gas-Chrom Q, was carefully sieved, resilanized and loaded with the selected stationary phase Versamid 900 (3%, w/w). The use of a narrow-bore short column, also deactivated with Carbowax 20M and packed to only 30—50%, contributed to the elimination of active sites and permitted a rapid analysis. As an internal standard 5-CU was found to be highly suitable because of the structural similarity to 5-FU. The adhesive behaviour of 5-CU closely resembled that of 5-FU. In order to prevent solvent interference a solid-phase injection system was used, and to obtain a high degree of sensitivity nitrogen—phosphorus detection was used. In this way excellent results for the determination of underivatized 5-FU were obtained. In Fig. 2 three chromatograms illustrate the GLC method for 5-FU.

For the assay of 5-FU in the plasma of patients a series of samples of blank plasma, to which a known amount of 5-FU was added, has been analysed simultaneously according to the extraction and GLC procedure described. In

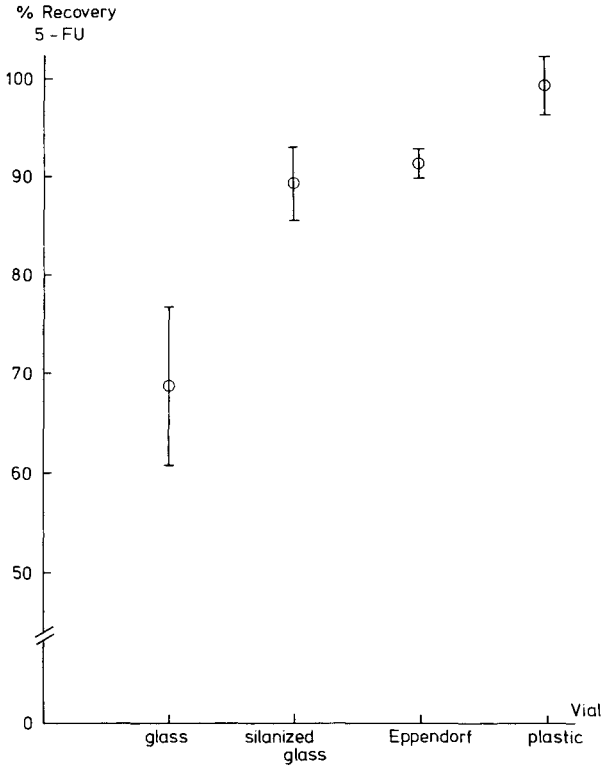

Fig. 1. Recovery of 5-fluorouracil from glass, silanized glass, Eppendorf tubes and plastic vials.

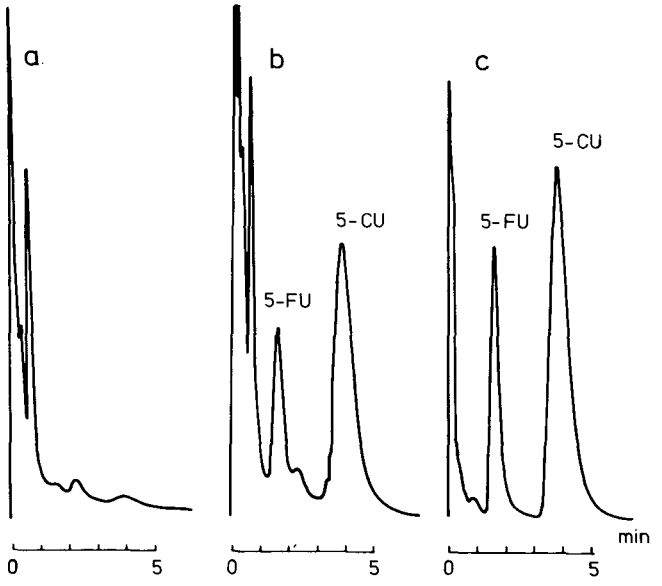

Fig. 2. GLC diagrams of: (a) blank plasma; (b) 50 ng of 5-FU and 200 ng of 5-CU from a plasma sample; (c) 50 ng of 5-FU and 200 ng of 5-CU in a standard solution in water.

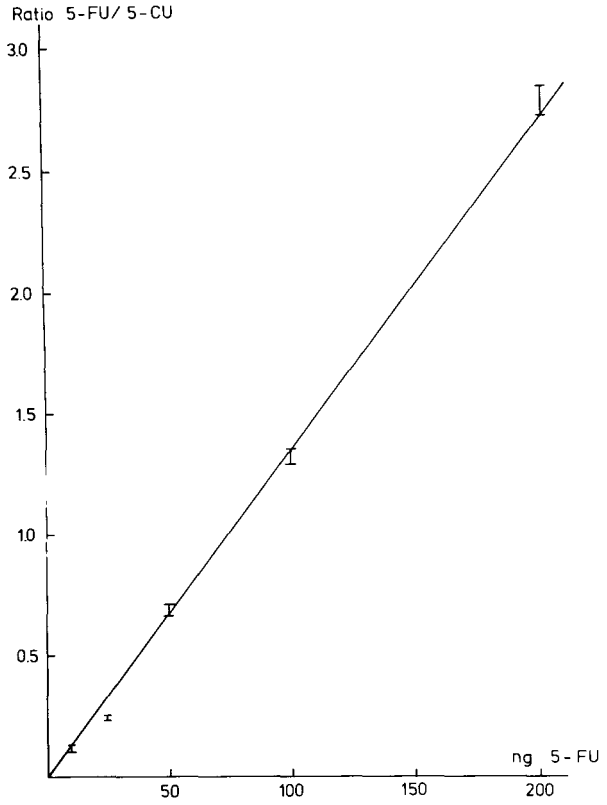

Fig. 3. Ratios of the peak heights of 5-FU and 5-CU measured according to the extraction method and the GLC procedure *vs.* the added amount of 5-FU.

Fig. 3 an example of a standard extraction line is presented. The recoveries for the extraction method were found to be in the range 50—80%.

The lower detection limit of 5-FU in patients' plasma according to the described extraction and GLC procedure is 50 ng/ml, whereas the limit per injection on the column is usually 1 ng per injection.

To demonstrate the practicability of the method, an example of a routine determination of 5-FU in plasma is shown in Fig. 4, which presents the 5-FU decay curve after intravenous and oral administration.

In conclusion, it can be stated that a suitable GLC analysis for underivatized 5-FU is now available, offering a more direct approach to the determination of 5-FU in only 0.2 ml plasma. The observed adherence of 5-FU to glass demands attention, as a considerable loss of 5-FU in extraction and GLC procedures may occur.

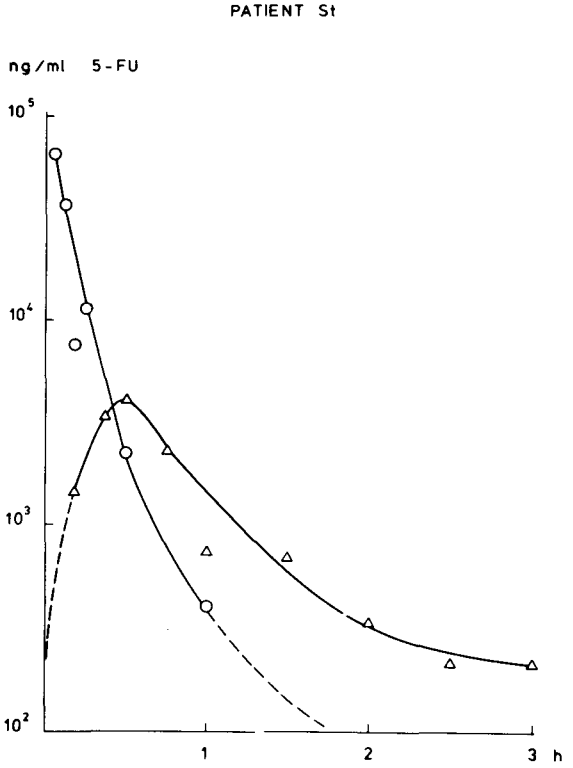

Fig. 4. Concentration of 5-FU in the plasma of a patient administered 500 mg of 5-FU orally (△) and by an intravenous (○) bolus injection.

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NOTE ADDED IN PROOF

As the described method was used routinely, it appeared that the estimation of the lower detection limit was too optimistic and is 5 ng per injection, or 250 ng/ml.

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Note

Assay of underivatized salicylamide in plasma, saliva and urine

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Several methods have been described for the determination of salicylamide (SAM) in plasma and urine. These comprise UV spectrophotometric [1—5], spectrofluorimetric [6] and gas—liquid chromatographic (GLC) methods [7, 8]. The UV spectrophotometric methods exhibit lack of sensitivity (detection limit about 10 μg SAM per ml sample). The spectrofluorimetric method is more sensitive (0.5 μg SAM per ml sample), but lacks specificity and blank values have to be subtracted [6]. The published GLC methods either require the formation of trimethylsilyl derivatives [7], or lack sensitivity [8].

We developed a rapid and sensitive method for the determination of underivatized SAM in human plasma and saliva and rat whole blood, using gas chromatography with nitrogen selective detection and solid injection system.

EXPERIMENTAL

Apparatus

A Hewlett-Packard 5750 gas chromatograph was used with an alkali flame ionization detector (nitrogen detector, HP 15151A, rubidium bromide crystal). Temperatures: injection port 280°; column 200°; detector 380°. A silanized borosilicate column (1.20 m × 0.8 mm I.D.), filled with 2% OV-225 + 1% OV-17 on Gas-Chrom Q, with a particle size of 200—220 μm, was used. Gas flow-rates: hydrogen 20—30 ml/min; air 180—200 ml/min; carrier (He) through the column 10 ml/min; auxiliary gas (He) was added to obtain a total carrier flow-rate of ± 60 ml/min. A solid injection system was used which has been described earlier for the determination of underivatized antiepileptic drugs [9] and underivatized nitrazepam and clonazepam [10].

Materials

The following materials were used: freshly distilled diethyl ether (Baker, Deventer, The Netherlands), kept over basic Al_2O_3 (Woelm) activity grade I; salicylamide (Reinst; Merck, Darmstadt, G.F.R.); N-methylhexobarbital (internal standard IS; synthesized by Drs. N.P.E. Vermeulen, by methylation of hexobarbital with diazomethane); ethanol (AR; Baker); glucuronidase and sulfatase (Limpet Acetone Powder, Patella Vulgata, crude, Type 1; Sigma, St. Louis, Mo., U.S.A.).

Extraction procedures

Extraction of SAM from human plasma and saliva and rat whole blood. To 1.0 ml plasma or saliva in a centrifuge tube, were added 25 μl ethanol containing 2.5 μg N-methylhexobarbital as internal standard and 1.0 ml acetate buffer (0.1 *M*, pH 5.0). After homogenization the mixture was extracted twice with 5 ml diethyl ether on a Cenco whirlmixer for 10 sec. The upper organic layer was removed each time with a pasteur pipette and transferred to a conical evaporation tube. The solvent was evaporated to dryness at 50—60° in a flow of dry nitrogen. The residue was dissolved in 0.1 ml of absolute ethanol and 2 μl of this solution were brought on the needle of the solid injection system. After evaporation of the solvent the needle was injected into the gas chromatograph. Blood samples (0.1 ml) of rats were analyzed in the same way, except that the extraction was carried out twice with 2 ml of diethyl ether.

Extraction of SAM from urine after hydrolysis. To 50 μl urine in a centrifuge tube of 10 ml, were added 2 ml acetate buffer (0.1 *M*, pH 4.9) containing 25 mg enzyme (9250 Fishman Units glucuronidase and 100 Enzyme Units sulfatase). After homogenization the mixture was incubated on a shaking waterbath for 3 h at 37°. After incubation, 25 μl ethanol were added containing 2.5 μg hexobarbital and the mixture was homogenized. Extraction was carried out in the same way as described for the extraction of SAM from plasma, except that 2 ml of diethyl ether were used.

Preparation of calibration curves

The concentrations of SAM in human plasma and saliva, whole blood (rat) and urine were calculated with the aid of calibration curves prepared by adding known amounts of SAM to 1.0 ml plasma or 50 μl urine. The samples were analyzed by the same procedures as described above and the ratios of the peak areas of SAM to internal standard were plotted against known concentrations of SAM. The same procedure was followed for estimation of the extraction-yield of SAM at various concentrations, except that now N-methylhexobarbital was used as an external standard. The ratios found were compared to the ratios of standard amounts of the drugs.

RESULTS AND DISCUSSION

Fig. 1 shows the gas chromatograms of the extracts from human plasma and saliva samples. There is no interference from endogenous substances according to the blank, the retention time is short and the detection limits are

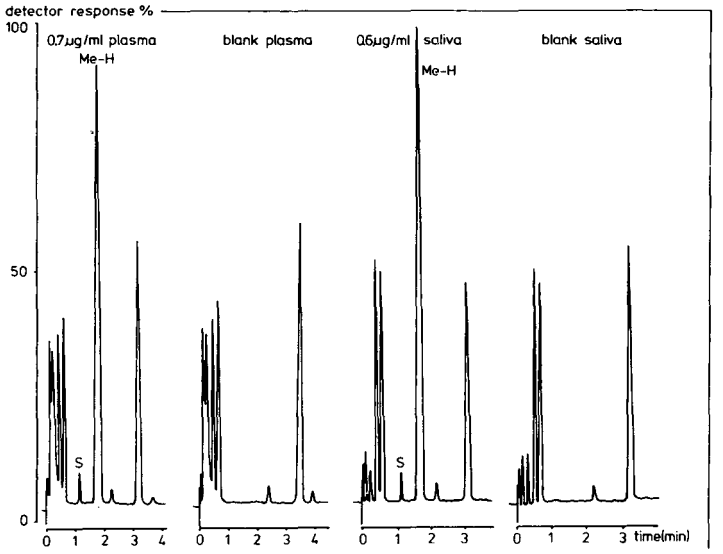

Fig. 1. Gas chromatograms of extracts of 1 ml plasma and saliva, obtained from a volunteer after 2½ and 1½ hours, respectively, and before (blank plasma and blank saliva) administration of 1500 mg salicylamide orally.
S = Salicylamide, Me-H = N-methylhexobarbital (IS, 2.5 µg/ml plasma or saliva). Salicylamide concentration: in plasma, 0.7 µg/ml; in saliva, 0.6 µg/ml.

about 0.25 µg SAM per ml plasma or saliva, or 0.25 µg SAM per 0.1 ml rat whole blood. Identification of the compounds eluting from the gas chromatograph was carried out by means of combined gas chromatography—mass spectrometry (LKB-2091 with PDP-11 computer system).

Metabolites [3] of SAM (SAM sulfate, SAM glucuronide, gentisamide and gentisamide glucuronide) do not interfere, primarily because these compounds are not co-extracted using the present extraction procedure. On the other hand the conjugates can be readily determined by the present assay, after hydrolysis (glucuronidase and sulfatase incubation) to the parent drug.

Linearity exists between detector response (expressed as the ratio of the peak area SAM:peak area N-methylhexobarbital) and the concentration of SAM from 1.0 to 25.0 µg/ml human plasma and saliva.

The mean of three plasma and saliva calibration curves obtained on different occasions showed, for each point in the concentration range from 1.0 to 25.0 µg SAM/ml, a standard deviation (S.D.) which was smaller than 5%. In spite of the short extraction time (10 sec) extraction yields of SAM from whole blood (rat) and human plasma and saliva are relatively high: 97%, 83% and 83%, respectively, and are constant over the concentration range 1–25 µg SAM per ml. Calibration curves for the extraction of SAM from urine (human and rat) after enzymatic hydrolysis were linear. In the concentration range of 1—5 µg SAM per 50 ml urine and 5—100 µg SAM per 50 µl urine different calibration curves were used, because of a bend in the calibration curve. The enzymatic hydrolysis was performed within 3 h instead of 16 h [3]. The calibration curve obtained following either hydrolysis time was repro-

ducible, with the difference that after hydrolysis for 3 h larger amounts of SAM were determined probably due to the fact that less was broken down in the shorter period. This was also noted by Levy and Matsuzawa [3], and was checked by adding known amounts of SAM to blank urine and performing the hydrolyses over 3 and 16 h, respectively. The results showed that the calibration curve after the 3-h hydrolysis was significantly higher than after 16 h. Further experiments showed that 25 mg enzyme was sufficient even for hydrolysis of large quantities of SAM-metabolites (equivalent to approximately 100 μg SAM per 50 μl urine) to SAM within 3 h.

Larger quantities of enzyme and longer hydrolysis times did not show any increase in SAM recovery. The recovery of SAM from urine (human and rat) after incubation with glucuronidase and sulfatase was 92% (S.D. $<$ 5.0%; n = 3) in the concentration range of 5–100 μg per 50 μl urine. In the urine assay of SAM, hexobarbital was used as IS because of an interfering peak when N-methylhexobarbital was used. Attempts were made to analyse gentisamide sulfate and gentisamide glucuronide in urine as gentisamide (a minor metabolite [3]), however, this compound decomposed during the enzymatic hydrolysis and could therefore not be accurately determined.

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Note

High-performance liquid chromatographic analysis of nalidixic acid in plasma after alkylation with methyl iodide

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Nalidixic acid (1-ethyl-1,4-dihydro-7-methyl-4-oxo-1,8-naphthyridine-3-carboxylic acid; NA) is used in the treatment of urinary-tract infections caused by Gram negative organisms other than Pseudomonas spp.

Most methods for its quantitative analysis are based on the fluorescence of NA [1, 2], but these methods have several disadvantages. They are non-specific, giving relatively high blank values and non-linear calibration curves and they need relatively large plasma volumes of 1—3 ml. A high-performance liquid chromatographic (HPLC) method using an ion-exchange column has been described [3], which needs only 1 ml of plasma, but in this method no internal standard is used. It is suggested that with this method the major metabolite, hydroxynalidixic acid (1-ethyl-1,4-dihydro-7-hydroxymethyl-4-oxo-1,8-naphthyridine-3-carboxylic acid; HNA) can also be determined, but this compound is not separated from the solvent peak in the system used.

In this paper we describe a method that can measure NA concentrations down to 0.6 μg/ml in a 100-μl plasma sample using a standard reversed-phase column. Because nalidixic acid gives very strongly tailing peaks in most chromatographic systems, the compound is methylated as described in the literature [4]. The calibration curve is linear up to at least 100 μg/ml and the intercept does not differ significantly from zero. Also the metabolite does not interfere with the assay. The method was used for determining plasma levels in patients treated with nalidixic acid.

EXPERIMENTAL

Apparatus

Chromatography was performed on a component system consisting of a Waters Assoc. (Milford, Mass., U.S.A.) Model 6000 A solvent delivery system, Model U 6 K injector and Model 440 absorbance detector operated at 313 nm. The two 30-cm columns used were μBondapak C_{18} (Waters Assoc.) and LiChrosorb 10 RP-18 (Chrompack, Middelburg, The Netherlands), both C_{18} bonded phases on 10-μm particles of silica gel. Peak areas were measured by means of a Spectra Physics SP 4000 data system.

Chemicals

The solvents toluene, acetone, dimethylformamide (DMF) and the catalyst caesium carbonate were purchased from Merck (Darmstadt, G.F.R.); methyl iodide and nalidixic acid were from Fluka (Buchs, Switerland).

The propylester of NA (PNA), which was used as an internal standard, was prepared by refluxing 400 mg NA for 24 h in 200 ml of acetone to which had been added 500 mg caesium carbonate and 25 ml propyl iodide (Fluka). After the solution had been evaporated to dryness by means of a rotary evaporator, 200 ml of chloroform (Merck) were added to the residue. The insoluble inorganic catalyst was removed by filtration of the solution through filter paper. The chloroform was evaporated and the product obtained was purified by recrystallisation from cyclohexane (Merck), yielding 225 mg of PNA (m.p. 108—112°). The mass spectrum of this compound showed the parent peak to be at *m/e* 274, in accordance with the empirical formula $C_{15}H_{18}N_2O_3$ (PNA); unreacted NA should give a peak at *m/e* 232, but this peak was not present.

The metabolite HNA was isolated from urine of a patient who was treated with NA, according to the method of McChesney et al. [1]. The identity of this product was confirmed by its melting point and mass-spectral analysis.

Procedures

Extraction. To 100 μl of plasma in a 7-ml glass centrifuge tube, 100 μl water and one drop of hydrochloric acid (2 *M*) were added. This solution was extracted with 2.00 ml of a stock solution of PNA in toluene (about 5 μg/ml). After mixing on a Vortex mixer for 1 min and subsequent centrifugation at 2500 *g* for 2 min, the toluene phase was transferred with a pasteur pipette into a 2-ml ampoule and evaporated to dryness under a stream of nitrogen at 80° in a metal heating block. Acetone (100 μl), caesium carbonate (about 5 mg) and methyl iodide (10 μl) were added, the ampoule was closed by melting after replacement of the air by nitrogen and shaken on a Vortex mixer for 5 sec. Esterification was completed after 15 min at 80°; the ampoule was opened at that time and evaporated to dryness under a stream of nitrogen at 50°. The residue was dissolved in 100 μl DMF; 20 μl of the clear solution was injected into the HPLC system.

Chromatography. The mobile phases used were 70% (v/v) methanol in water in combination with the LiChrosorb 10 RP-18 column and 63% (v/v) methanol in water for the μBondapak C_{18} column. The flow-rate was 1.5 ml/min in both cases. All chromatograms were obtained at ambient temperature.

RESULTS AND DISCUSSION

Toluene was used as extraction solvent as described by other authors [1, 2]; a very clear upper layer was obtained, which was easy to transfer. Because of the fact that underivatized NA gave very strongly tailing peaks on various stationary phases such as μBondapak C_{18}, Corasil Phenyl (Waters Assoc.) and μBondapak CN (Waters Assoc.) the methylester (MNA) was prepared and chromatographed.

For reasons of extraction, retention and detection, the propyl ester of NA was chosen as the internal standard. The ethyl ester is another potential internal standard but in this case there is a risk of the two peaks overlapping when the plate number of the column decreases.

Methyl iodide gave a peak very close to the MNA peak, so it was removed by evaporation. The residue was dissolved in DMF and not in the mobile phase or pure methanol, because by standing in the presence of caesium carbonate PNA was slowly transesterified to MNA by methanol; on column this phenomenon was not seen.

The stability of PNA during the derivatisation step was checked by heating 10 μg with methyl iodide and caesium carbonate in 100 μl acetone. Even after 18 h (72 × reaction time) at 80° no MNA could be detected.

Although the molar absorptivity of NA at 313 nm is lower than at 254 nm, the wavelength of 313 nm was chosen because at this wavelength no interfering plasma peaks are detected (Fig. 1).

The detection limit was about 10 ng MNA per injection. Calibration curves were made on a LiChrosorb 10 RP-18 column and a μBondapak C_{18} column; the concentration range of NA was 0.66—99.2 μg/ml. The straight lines were calculated by the method of least squares and were, respectively, $y = -0.0009\ (\pm 0.0063) + 0.0100\ (\pm 0.0001)x$ ($r^2 = 0.9972$, $n = 22$), and $y = -0.0056\ (\pm 0.0129) + 0.0091\ (\pm 0.0002)x$ ($r^2 = 0.9887$, $n = 20$), where y = peak area ratio MNA : PNA and x = concentration of NA in μg/ml.

The accuracy of the method is shown in Table I. Different amounts NA were added to blank plasma and analysed by a technician to whom the concentrations had not been revealed.

The day-to-day reproducibility of the calibration curve on the same column (μBondapak C_{18}), using the same stock solution of PNA in toluene, is shown in the two following equations for, respectively, days 1 and 4: $y = 0.0106\ (\pm 0.0111) + 0.0115\ (\pm 0.0002)x$ ($r^2 = 0.9985$, $n = 10$); and $y = 0.0067\ (\pm 0.0097) + 0.0113\ (\pm 0.0002)x$ ($r^2 = 0.9990$, $n = 8$). There are no significant differences, which makes it possible to use the calibration curve for several days. The intercepts of the calibration curves did not differ significantly from zero, so only a few samples of the same concentration are sufficient to construct a calibration curve, passing through the origin. The absolute recovery for the whole method was 84% for NA and 88% for PNA.

The metabolite HNA was found not to interfere with the determination of NA. When treated as described under procedures, HNA gave some small peaks before the MNA peak. Even concentrations of HNA twice as high as NA (respectively, 100 μg/ml and 49.6 μg/ml) did not interfere with the assay.

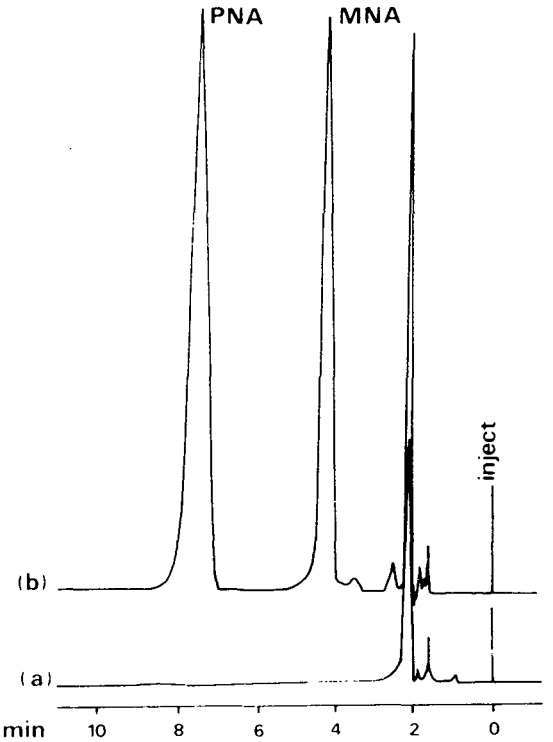

Fig. 1. Chromatograms obtained by the analysis of 100 μl plasma on a μBondapak C_{18} column (for conditions see text). (a) Plasma blank. (b) Plasma of a 15-year-old boy who was treated for 3 days, every 6 h with an infusion of 1 g NA over a period of 20 min. Plasma was taken just before the next period of infusion. The plasma level was calculated to be 52.1 μg/ml.

TABLE I

RECOVERY OF NA FROM PLASMA

NA (μg/ml)		Recovery (%)
Added	Found	
92.5	88.0	95.1
66.1	65.1	98.5
66.1	67.2	101.7
52.9	51.3	97.0
26.4	27.5	104.0
16.5	16.4	99.4
2.64	2.86	108.3
	Mean ± S.D.	100.6 ± 4.5

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Note

High-performance liquid chromatographic method for the simultaneous determination of lidocaine and its N-dealkylated metabolites in plasma

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Lidocaine (I) has been used for many years in coronary care units for the prevention and treatment of cardiac arrhythmias. Therapeutic and toxic responses to I appear to be related to its plasma concentration [1]. Therefore, it may be desirable that plasma levels of I be monitored in those patients who receive the drug; this would be particularly true if therapy is protracted, toxicity or failure of therapy is suspected, or if the patient has concurrent pathology which is known to alter the disposition of the drug [2]. However, the two dealkylated metabolites of I, namely monoethylglycinexylidide (II), and glycinexylidide (III), possess some pharmacological and toxicological activity [3, 4], and these compounds should therefore be included in the design of analytical methods which are intended to be used for plasma level monitoring as an aid to patient care [5]. Lidocaine and its metabolite (II) have been used as markers in pharmacokinetic studies [6, 7].

A large number of gas chromatographic and gas chromatographic—mass spectrometric methods have been described for the quantitation in plasma of I alone [8—14], and of I together with one (II) [15, 16], or both [17—19] of its dealkylated metabolites. A high-performance liquid chromatographic (HPLC) assay for I in plasma has been reported [20]. In that method I was extracted from the plasma, together with added procaine which served as an internal standard, by a charcoal adsorption technique. The chromatographic separation was subsequently carried out on an octadecyl reversed-phase column, and detection was by ultraviolet absorption. The lower limit of quantitation was 0.1 μg/ml using a 1-ml plasma sample. The method did not

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include quantitation of II and III, and the report of the method [20] did not state whether or not these metabolites of I were separated from the parent drug.

The purpose of the present paper is to describe a rapid, sensitive and specific HPLC method for the simultaneous quantitation of I, II, and III in a small volume of plasma. The method may be useful for routine clinical monitoring, or for pharmacokinetic studies in animals and humans.

EXPERIMENTAL

Reagents

Lidocaine (I), monoethylglycinexylidide (II), glycinexylidide (III), and ethylmethylglycinexylidide, all as their hydrochloride salts, were donated by Astra (Worcester, Mass., U.S.A.). Amounts and concentrations of these compounds are expressed as the salts, unless otherwise noted. Glass-distilled ethyl acetate and acetonitrile (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.), phosphoric acid, sulfuric acid (Fisher Scientific, Fair Lawn, N.J., U.S.A.), and sodium hydroxide (J.T. Baker, Phillipsburg, N.J., U.S.A.) were obtained commercially. A 0.006% solution of phosphoric acid in distilled water was prepared (final pH 2.8), and passed through a 0.45-μm membrane filter (Millipore, Bedford, Mass., U.S.A.) for subsequent use in the preparation of the HPLC mobile phase. An aqueous solution with a pH of 2.2 was prepared by dilution of sulfuric acid with distilled water.

HPLC system

The HPLC system consisted of a Model M-6000A pump, a Model U6K sample injector, a μBondapak alkyl phenyl reversed-phase column (30 cm × 3.9 mm I.D., 10-μm particle size) obtained from Waters Assoc., (Milford, Mass., U.S.A.), and a Model LC55 variable wavelength HPLC ultraviolet detector (Perkin-Elmer, Oakbrook, Ill., U.S.A.). The output from the detector was connected, via an attenuator, to a 25.4 cm recorder which had 1 and 10 mV calibrated positions (Linear Instruments, Irvine, Calif., U.S.A.).

The mobile phase for the chromatographic separation was prepared by mixing 30 parts of acetonitrile with 70 parts of 0.006% phosphoric acid solution. This mobile phase was pumped through the HPLC system at 2 ml/min, which resulted in a pump pressure of approximately 1800 p.s.i.g. The separation was carried out at ambient temperature (approximately 24°). The HPLC detector was operated at a wavelength of 200 nm, and the recorder chart speed was 0.33 cm/min.

Procedure

Preparation of plasma samples. To a 500-μl aliquot of plasma contained in a culture tube (13 × 100 mm) were added 2 μg of internal standard in an aqueous solution (ethylmethylglycinexylidide hydrochloride; 20 μg/ml), 100 μl of 1 *M* NaOH, and 3 ml of ethyl acetate. The tube was closed with a screw cap and vortexed for 1 min to promote mixing of the immiscible phases. After centrifugation (800 *g*; 2 min), most of the ethyl acetate was transferred by disposable pipet to a 12-ml tube which had a tapered base, and which con-

tained 0.1 ml of dilute sulfuric acid solution (pH 2.2). This tube was capped, and the contents were vortex-mixed for 1 min followed by brief centrifugation as described above. Approximately 20—30 μl of the lower aqueous phase were injected into the chromatograph.

Plasma assays were also performed using 100-μl aliquots of plasma together with 0.5 μg of internal standard (1 μg/ml aqueous solution) in the first extraction; all other conditions were as described above for the analysis of 500-μl aliquots of plasma.

Standard curves were prepared by spiking blank human plasma with I, II, and III, followed by extraction and chromatography as described above. The ratios of the peak heights of I, II, and III, to that of the internal standard, were plotted against the concentrations of I, II, and III, respectively.

For reproducibility studies one 5-ml aliquot of blank human plasma was spiked with I, II, and III at 0.5 μg/ml and another aliquot handled correspondingly at 2.0 μg/ml, with replicates (500 μl only) analyzed for I, II, and III at each concentration.

RESULTS AND DISCUSSION

Chromatograms resulting from the HPLC analysis of 500-μl aliquots of plasma which had been spiked with I, II, and III, together with a chromatogram of a similarly analyzed blank plasma sample, are shown in Fig. 1. The retention times of I, II, and III were 10.0, 7.4, and 5.9 min, respectively, and the internal standard had a retention time of 8.3 min. The drug, its dealkylated metabolites, and the internal standard were eluted as symmetrical peaks which were satisfactorily resolved from each other and from endogenous components in the plasma; no interfering peaks were observed when the blank plasma was analyzed. The total analysis time of a single plasma sample was approximately 18 min.

The standard curve for (I) in the concentration range from 0.1 to 10 μg/ml in plasma, using 500-μl aliquots of plasma, is shown in Fig. 2. Standard curves for (II) and (III) were prepared over the same concentration range but are not shown. Each standard curve was apparently linear at low concentrations but downward curvature occurred with increasing concentration. Such an effect could conceivably result from a non-linearity in the extraction steps during sample preparation, or in chromatographic separation. Injection into the HPLC of given amounts (0.025 to 0.3 μg) of I, II, and III in 20-μl aliquots of aqueous solution resulted in similarly curved standard curves when the peak height of each compound was plotted against amount injected. Since the injection volume was constant in this experiment, and no extractions were performed, the observed curvature of these standard curves may have resulted from a non-linearity of the distribution coefficients of the compounds during the HPLC separation. A close inspection of chromatograms (Fig. 1), indicates a small but obvious broadening of the peaks of I, II, and III, as the concentration of these compounds in plasma increased. The peak broadening, with resultant curvature of standard curves based on peak height ratio, was also observed when a different column, of the same type, was tested. The experimental data were well described by quadratic equations (Fig. 2) with r^2 values of 0.9968,

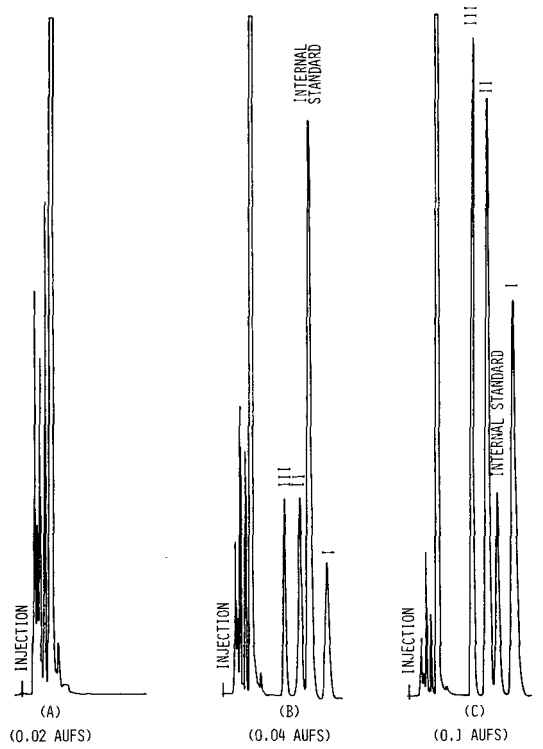

Fig. 1. Chromatograms resulting from the analysis of 500-μl aliquots of blank human plasma (A), and plasma which had been spiked with I, II, and III, each at a concentration of 1 μg/ml (B), and 10 μg/ml (C).

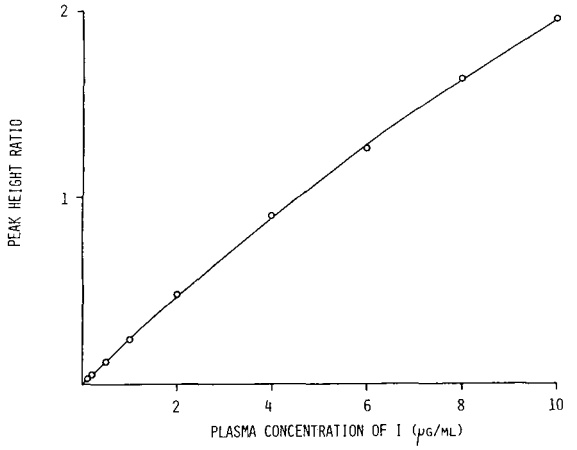

Fig. 2. Plot of the ratio of peak height of lidocaine (I) to that of the internal standard versus concentration of I in plasma, using 500-μl aliquots of plasma. $y = (-0.00428)x^2 + (0.2378)x + (0.00515)$, $r^2 = 0.9968$.

0.9998, and 0.9996 for the standard curves of I, II, and III, respectively. Linear calibration curves may result if peak areas are used to prepare standard curves.

Standard curves for I, II, and III using 100-μl aliquots of plasma were prepared over the concentration range from 0.5 to 10.0 μg/ml (Table I). These standard curves were apparently linear probably because smaller amounts of compounds were injected onto the HPLC column.

Based on a signal-to-noise ratio of 3:1, the lower limit of quantitation of I, II, and III using 500 μl of plasma was approximately 0.02 μg/ml; for 100 μl of plasma the corresponding value was 0.1 μg/ml. The reproducibility of the method at concentrations of 0.5 μg/ml and 2.0 μg/ml of each compound in plasma, using 500-μl aliquots, is summarized in Table II.

The extraction work-up procedures that are used to prepare plasma samples for HPLC analysis are relatively simple and rapid. The extraction of basified plasma with ethyl acetate followed by back extraction of the organic layer with a small volume of aqueous acid serves to provide a degree of sample clean-up. In addition, no evaporation steps are necessary because sufficient concentration of the samples can be obtained by using a small volume of acid for the back extraction step. The recoveries of I, II, and III were determined by comparing the chromatographic peak height of each compound from extracted 500-μl plasma aliquots with the corresponding peak height from an aqueous solution

TABLE I

STANDARD CURVES FOR I, II, AND III IN PLASMA USING 100-μl ALIQUOTS OF PLASMA FOR ANALYSIS

Results obtained are for single determination. Linear regression equations: I $y = 0.1778x - 0.00326$, $r^2 = 0.9989$; II $y = 0.2717x - 0.0178$, $r^2 = 0.9999$; III $y = 0.3100x - 0.0304$, $r^2 = 0.9996$. PHR = ratio of peak height of the compound to that of the internal standard; response factor = peak height ratio divided by the concentration of the compound in the sample.

Concentration in plasma (μg/ml)	I		II		III	
	PHR	Response factor	PHR	Response factor	PHR	Response factor
0.5	0.078	0.156	0.119	0.238	0.134	0.268
1.0	0.172	0.172	0.259	0.259	0.293	0.293
2.0	0.360	0.180	0.519	0.260	0.580	0.290
5.0	0.912	0.182	1.33	0.266	1.48	0.296
7.5	1.29	0.172	2.04	0.272	2.32	0.309
10.0	1.79	0.179	2.69	0.269	3.07	0.307

TABLE II

REPRODUCIBILITY DATA USING 500-μl ALIQUOTS OF PLASMA

PHR = peak height ratio; S.D. = standard deviation; C.V. = coefficient of variation.

Compound	0.5 μg/ml ($n = 7$)			2.0 μg/ml ($n = 8$)		
	Mean PHR	S.D.	C.V.	Mean PHR	S.D.	C.V.
I	0.117	0.0059	5.0	0.474	0.0085	1.8
II	0.174	0.012	6.9	0.688	0.012	1.7
III	0.185	0.014	7.6	0.744	0.029	3.9

of the compounds which had not been extracted; correction was made for volume changes. The mean (± S.D.) percentage recoveries (n = 5) at 6 μg/ml of each compound in plasma were 87.4 ± 2.4%, 86.4 ± 1.6%, and 62.3 ± 0.8% for I, II, and III, respectively.

Chromatograms resulting from the analysis of plasma samples collected from patients who were receiving lidocaine infusions are shown in Fig. 3. The initiation of lidocaine therapy had occurred 6, 30, and 24 h prior to collection of these plasma samples for patients A, B and C, respectively. Lidocaine infusion rates were 1—3 mg/min. The other medications being taken by these patients were procainamide, propranolol, potassium, diazepam, and morphine. The concentrations of I, II, and III in the plasma from these patients (Fig. 3) are of the same order as those previously reported in patients receiving lidocaine infusions [18].

Plasma samples collected from a number of patients who were receiving a variety of other drugs, but not lidocaine, were analyzed. The drugs known to have been taken by these patients included ampicillin, chlorthalidone, digoxin, furosemide, hydrocortisone, metaproterenol, methyldopa, penicillin, prednisone, terbutaline, and theophylline. No interfering peaks in the HPLC chromatograms were observed from the analysis of any of these plasma samples. Patients in clinical settings may concurrently receive lidocaine and another antiarrhythmic agent. Therefore, propranolol, 4-hydroxypropranolol,

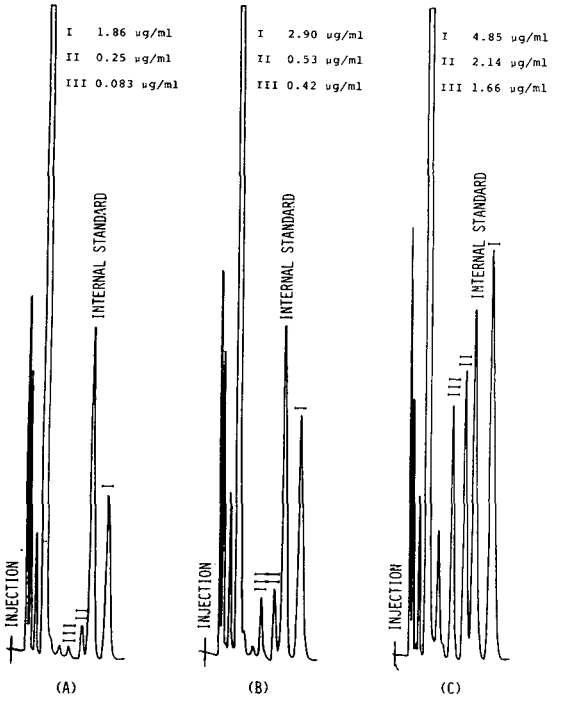

Fig. 3. Chromatograms resulting from the analysis of 500-μl aliquots of plasma samples collected from patients who were receiving lidocaine by infusion. The concentrations of I, II, and III (expressed as the base) in each sample are also shown. Detector sensitivity was 0.04 a.u.f.s.

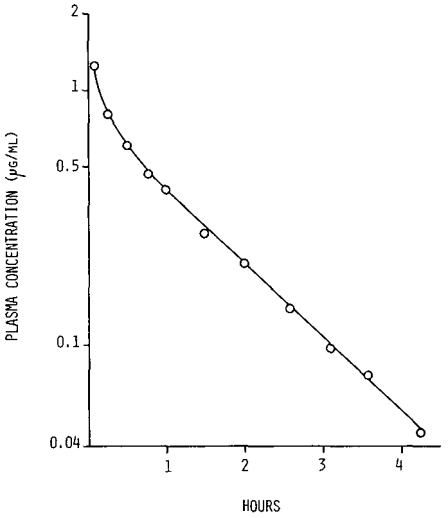

Fig. 4. Semilog plot of plasma concentration of lidocaine (I) versus time in a 4-kg albino rabbit which had been administered 8 mg of I during 30 sec by the intravenous route.

procainamide, N-acetylprocainamide, quinidine, and verapamil were tested for potential interference of the HPLC method by injecting stock solutions of these compounds into the chromatograph; however, no interference was observed.

The time course of plasma concentration of I in a rabbit following intravenous administration is shown in Fig. 4. In agreement with similar studies in man [21] plasma concentrations of II and III were too low to quantitate in this experiment which involved administration of a single dose of I. However, plasma concentrations of II and III do accumulate during chronic administration of I to cardiac patients (see Fig. 3), and probably contribute to the clinical responses elicited in such individuals [18].

The HPLC method reported here for simultaneous determination of lidocaine and its N-dealkylated metabolites may be useful for plasma level monitoring in patients, and for pharmacokinetic studies in animals and man.

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Note

Rapid assay for triamterene in plasma *

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A great deal of work has been done on the pharmacokinetics and pharmacodynamics of triamterene in animals [1—6]. Although Badinand et al. [7] have measured the serum concentrations of triamterene in humans and obtained profiles up to 5 h post administration in both normal volunteers and patients, no detailed pharmacokinetic studies in humans are available, possibly because of the lack of a rapid, sensitive and selective assay method for the drug in biological fluids.

This paper describes such an assay based on extraction of triamterene as the perchlorate ion pair from plasma and high-performance liquid chromatography (HPLC) of the extract coupled with fluorescence detection.

EXPERIMENTAL

Chemicals and Reagents

Triamterene (USP Reference Standard) was dissolved in water at the concentration of 100 μg/ml and diluted to 1 μg/ml in either blank plasma (for the standard curves) or in methyl isobutyl ketone (for the recovery experiments). Appropriate dilutions in the respective media were made to give the desired concentrations. Outdated plasma, obtained from the Canadian Red Cross, was used for blanks and calibration curves. Dyrenium tablets (50 mg triamterene, Smith, Kline & French, Montreal, Canada) were purchased locally.

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*Preliminary results of this study have been presented at the 9th Materials Research Symposium, National Bureau of Standards, Gaithersburg, Md., USA, 1978.

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The solvents used for chromatography were from Burdick & Jackson (Muskegon, Mich., U.S.A.). All other chemicals were of reagent grade, purchased locally. Perchloric acid, 3 *M*, was prepared with fluorescence-grade water. Methyl isobutyl ketone was saturated with 1 *M* perchloric acid before use.

HPLC

The chromatographic equipment consisted of a Waters Model 6000A pump and a U6K injector; the column, 250 × 3.2 mm I.D. stainless steel, was slurry packed with LiChrosorb Si 60, 5 μm (BDH, Toronto, Canada). The detector, Schoeffel LS990, was set at 335 nm excitation with a Schoeffel No. 7-54 filter (broad band pass centered at 320 nm) and the emission monitored using a sharp cut filter at 470 nm.

The chromatographic mobile phase consisted of dichloromethane—hexane—methanol—70% perchloric acid (57:35:8:0.1) at a flow-rate of 2 ml/min.

Thin-layer chromatography (TLC)

Concentrated effluents from HPLC, untreated urine or urine extracts were spotted on silica gel plates (Quanta/gram LQ6F, Quantum Ind., Fairfield, N.J., U.S.A.) and developed in ethyl acetate—methanol—25% aqueous ammonia (60:30:10) according to the method of Grebian et al. [5]. Triamterene and its metabolites were located visually by fluorescence under 360 nm light.

Treatment of the human volunteers

Two healthy male volunteers were given triamterene in the form of Dyrenium tablets. Subject 1 was administered one tablet (50 mg) after a normal light breakfast; subject 2 was fasted overnight, then administered four tablets (200 mg total). Venous blood was withdrawn into heparinized evacuated tubes (Vacutainers, Becton-Dickinson, Toronto, Canada) just prior to the dose, and at appropriate intervals thereafter (volunteer 1: 0.5, 1, 2, 3, 5 and 7.7 h; volunteer 2: 0.5, 1, 2, 3, 5, 8, 24 and 32.5 h). The blood samples were immediately centrifuged and the separated plasma kept at −18° until analysis. Urine was collected from subject 2 on the day of the experiment, giving a 7 h combined sample, which was kept at −18° until used.

Procedure

To 0.5 ml plasma or urine in a 10-ml round-bottomed glass tube fitted with a PTFE-lined screw cap, 0.25 ml 3 *M* perchloric acid was added. After mixing briefly on a vortex-type mixer, 1 ml methyl isobutyl ketone was added, and the mixture was shaken vigorously (Evapo-Mix, Buchler Instruments, Fort Lee, N.J., U.S.A.) for 5 min and centrifuged for 5 min at 3000 rpm using a table centrifuge (Model HN-S, International Equipment Co., Needham Heights, Mass., U.S.A.). An aliquot of exactly 50 μl of the supernatant was used for chromatography.

For the verification of the absence of interfering metabolites, plasma or urine extracts were chromatographed, and the eluted fractions corresponding to the peaks for triamterene and a possible metabolite collected separately. The fractions were washed with 0.1 *M* K_2HPO_4, the organic layers evaporated to dryness in a stream of nitrogen at 50° and redissolved in methyl isobutyl

ketone. Aliquots of these solutions, as well as that of the original extract and triamterene standard, were subjected to TLC as described above.

Quantitative analyses

The extraction efficiency was estimated by comparing the peak height obtained from spiked plasma with that from a standard solution of triamterene in methyl isobutyl ketone. The amount of the drug in plasma samples was estimated by comparing the peak height for the sample to a calibration curve, constructed daily, using spiked blank plasma.

RESULTS AND DISCUSSION

Extraction and chromatography

Triamterene was shown previously [8] to chromatograph on a silica gel column using a mobile phase of dichloromethane—hexane—methanol—perchloric acid. From its retention characteristics it was concluded that this drug, extracted from plasma as the perchlorate ion pair, should separate well from endogenous fluorescent materials. The extraction of the ion pair was expected to proceed with high efficiency in view of the three amino functions present in the molecule. Using the mobile phase described for quinidine, however, the retention time for triamterene was excessive (k' = 10.3). In order to reduce the time required for chromatography the methanol content of the mobile phase was increased from the original 5% to 8% at the expense of dichloromethane. Thus, with a mobile phase of dichloromethane—hexane—methanol—70% perchloric acid (57:35:8:0.1) the following k' values were obtained with authentic standards: triamterene, 4.6; 3-hydroxyquinidine, 4.2; quinidine, 2.7; 2'-quinidinone and dihydroquinidine, 2.4.

Chromatograms of plasma extracted and chromatographed as described above are shown in Fig. 1. While the maximum emission of triamterene is probably closer to 430 nm [5], better sensitivities were obtained at 470 nm, due to a quieter baseline. Blank plasma (Fig. 1A) does not appear to contain fluorescent material with retention characteristics similar to triamterene. The baseline noise is approximately 0.15 nA peak-to-peak. The inclusion of 10 ng drug per ml plasma (250 pg actually chromatographed) caused a peak of approximately 6 nA (Fig. 1B), which was well above the noise level.

For the recovery experiments blank plasma spiked with triamterene (100 ng/ml) was chosen. At this concentration the recovery was 88.6%. Since the calibration curve is a straight line passing through the origin (Table I), similar recoveries would be expected at all concentrations studied.

Fig. 1C and D represent traces obtained from plasma extracts from the two subjects 5 h after the 50 mg dose and 3 h after the 200 mg dose of the drug, respectively. Following the higher dose (Fig. 1D) a fluorescent peak appears with a k' value of 8.1. This substance has only been observed in the plasma and urine of subjects who have taken the drug.

Calibration curves

Preliminary experiments have shown the calibration curve to be linear to 1000 ng/ml with good coefficients of variation (C.V., 3.5% or less) between 100 and 1000 ng/ml. To verify the applicability of the method at lower con-

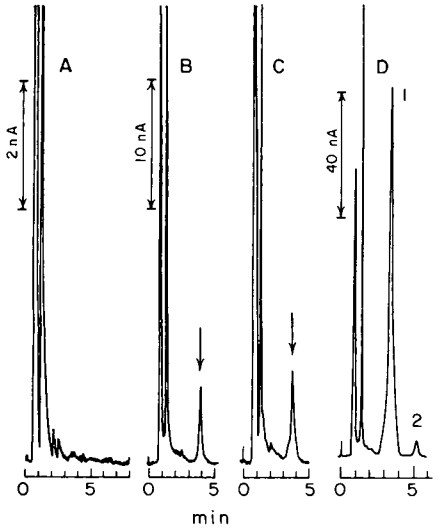

Fig. 1. HPLC of plasma extracts. A, blank plasma; B, blank plasma spiked with triamterene (10 ng/ml); C, plasma of volunteer 1, 5 h after an oral dose of 50 mg drug (estimated concentration 13.9 ng/ml); D, plasma of volunteer 2, 3 h after an oral dose of 200 mg drug (peak 1, triamterene, estimated concentration 350 ng/ml; peak 2, metabolite). Extraction and chromatography as described in the text.

TABLE I

CALIBRATION CURVE FOR TRIAMTERENE IN PLASMA

$n = 4$

Concn. (ng/ml plasma)	Peak height		Ratio peak height : concentration (nA/ng · ml^{-1})
	Mean (nA)	C.V. (%)	
2	0.81	9.26	0.405
4	1.54	6.15	0.385
6	2.29	2.75	0.382
8	3.06	5.73	0.383
10	3.98	3.16	0.398
20	7.39	3.04	0.370
30	11.85	0.84	0.395
40	15.00	1.22	0.375
50	19.15	4.85	0.383

centrations, nine concentrations from 2 to 50 ng/ml were prepared in blank plasma and assayed in quadruplicate (Table I).

Linearity was found to be good down to 2 ng/ml as shown by the steady values for the ratio of peak height to concentration; the coefficients of variation were good down to 4 ng/ml, and acceptable (<10%) at 2 ng/ml.

Interferences

As already mentioned, no endogenous interferences were found in blank plasma (Fig. 1A). However, to verify the identity and purity of the chroma-

tographic peaks, plasma from volunteer 2 (see below), collected between 1 and 3 h following the dose, was pooled, extracted and subjected to HPLC by the method described. Both the triamterene peak and the probable metabolite (peaks 1 and 2, respectively, Fig. 1D) were collected. Chromatography by TLC of these samples revealed a single spot for each peak, but the amounts obtained were judged to be too small to detect lesser contaminants. Since urine was shown by Lehman [4] to contain sizable amounts of the major metabolites of triamterene, the experiment was repeated using the 0—7 h urine of the same volunteer. The TLC of the urine extracts subjected to HPLC, as well as the original urine, urine extract and triamterene standard, gave the R_F values listed in Table II. Urine gave four spots: one unidentified, near the origin, and three

TABLE II

TLC OF TRIAMTERENE AND A METABOLITE EXTRACTED FROM URINE

Sample	R_F			
	Spot 1	Spot 2	Spot 3	Spot 4
Urine	0.09	0.51	0.66	0.77
Urine extract	0.06	—	0.63	0.75
Triamterene	—	—	—	0.77
LC peak 1	—	—	—	0.77
LC peak 2	—	—	0.63	—
Hydroxytriamterene sulphate*	—	0.40	—	—
Hydroxytriamterene *	—	—	0.52	—
Triamterene*	—	—	—	0.65

*Values published by Grebian et al. [5].

others having R_F values in the same order as those found by Grebian et al. [5] for hydroxytriamterene sulphate (spot 2), hydroxytriamterene (spot 3) and triamterene (spot 4). (The difference between the published R_F values and those obtained here may be due to differences in experimental conditions, for example the degree of activation of the TLC plates.) The organic extract gave three spots: the unidentified one near the origin, the presumed hydroxytriamterene and triamterene. Only the fastest-moving spot could be detected when peak 1 (Fig. 1D) from the HPLC was re-run on TLC, and this spot had an R_F value identical to that of authentic triamterene. The second peak from the HPLC also gave a single spot on TLC, with an R_F value similar to that reported for hydroxytriamterene by the above authors.

Quinidine, a commonly used fluorescent drug, did not interfere with the assay. One of its metabolites, 3-hydroxyquinidine, had retention characteristics similar to triamterene [8]. However, because of the less than optimal fluorescence at the wavelengths used and the relatively low amounts expected in plasma, there should be no significant interference from this substance. No other drugs were tested for interference.

Plasma concentrations in volunteers

The method was applied to the analysis of the drug in the plasma of two

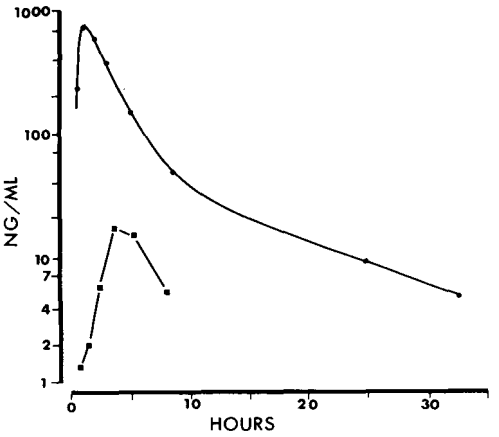

Fig. 2. Plasma concentration profiles of triamterene following a single oral dose of 50 mg (▪) and 200 mg (•) of triamterene to two different volunteers. Upper curve (volunteer 2): non-linear least-square regression fit of the experimental values to a two-compartment open model according to eqn. 2-207 of Wagner [9]. Estimated parameters: R^2, 0.997; K_a, 3.43 h^{-1}; α, 0.58 h^{-1}; β, 0.079 h^{-1}; A_1^*, 998 ng/ml; A_2^*, 61 ng/ml; A_3^*, −1059 ng/ml; lag time, 0.43 h. Assay as described in the text.

volunteers who had taken triamterene orally (Fig. 2). Plasma concentrations of volunteer 1, who was given one 50-mg tablet after a light breakfast, reached a peak of 16.2 ng/ml at 3 h, falling to 6.1 ng/ml at 7.7 h. In volunteer 2, who was given 200 mg of the drug (4 × 50-mg tablet) after overnight fasting, a peak plasma concentration of 706 ng/ml occurred at 1 h, followed by an apparent biphasic decay.

Badinand et al. [7] showed there was a great deal of individual variation in triamterene concentrations in human plasma following the administration of a single dose of 200 mg to fasted normal humans, with peak concentrations of 400—3000 ng/ml occurring at about 1 h. The second subject in our study was well within this range. However, subject 1 showed concentrations well below those expected from dose-independent kinetics. Whether this discrepancy was due to dose dependence, first pass metabolism, dietary interference or malabsorption cannot be ascertained without using a much larger number of volunteers. However, the rapidity and sensitivity of the method should make such studies feasible. Furthermore, the simplicity and selectivity of this method should also make it useful in clinical, toxicological and bioavailability studies.

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Note

Determination of serum chloramphenicol by high-performance liquid chromatography

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Chloramphenicol, D(—)-*threo*-2,2-dichloro-N-[β-hydroxy-α-(hydroxymethyl)-*p*-nitrophenethyl] acetamide is a broad spectrum antibiotic with bacteriostatic action. It is known to be highly toxic against the erythroid function of the bone marrow [1]. In premature infants, who lack an effective glucuronidation pathway, normal therapeutic doses can accumulate leading to fatal toxicity [2]. Despite this, it is the drug of choice in some situations, and paradoxically, its effectiveness against *H. influenzae* meningitis has lead to an increase in its use in infant patients who are, in turn, most susceptible to life-threatening toxicity from the drug. Under such circumstances it is clear that knowledge of the drug concentration in blood should permit optimum usage without fear of serious toxicity and that the availability of a rapid, accurate assay procedure would be an important adjunct to safe therapy.

Several techniques for the assay of chloramphenicol have been reported [3]. Of these, gas chromatographic procedures have been applied successfully to its analysis in serum [4, 5]. However, this approach requires prior derivatization to a silyl derivative and it is known that multiple peaks due to mono-, di- and trisilyl derivatives can ensue unless appropriate reagents and solvents are used [6]. High-performance liquid chromatography (HPLC) has been used to analyse chloramphenicol during its commercial production [7, 8], and recently has been applied to serum analysis [9]. This approach [9] is sensitive but requires a relatively large sample volume (500 μl) and a two-step extraction procedure. An internal standard was not included and accurate quantitation of unknowns must rest upon careful volume manipulations which can be tedious and error prone in the routine setting. It is noteworthy also that the

authors monitor the column effluent at 254 nm rather than 280 nm (λ_{max} chloramphenicol = 280 nm [10], and report the capacity factor, k', of 0 for thiamphenicol on a C_{18} reversed phase in a water methanol (70:30, v/v) system. We now report an HPLC assay specifically designed for routine analysis, which requires a single extraction step from a small volume of serum (50 μl) followed by reversed-phase chromatography. Thiamphenicol (D-*threo*-2,2-dichloro-N-[β-hydroxy-α-(hydroxymethyl)-*p*-(methylsulphonlyl) phenethyl] acetamide), a closely related antibiotic not used in clinical practice in North America serves as the internal standard.

EXPERIMENTAL

Apparatus

The liquid chromatogram used was an ALC Model 202, with Model 6000A pump, U6K injector and Model 440 absorbance detector (Waters Assoc., Milford, Mass., U.S.A.).

Chromatographic Conditions

Reversed-phase chromatography using two types of bonded phase was investigated and two equally appropriate systems arrived at.

System 1. A stainless-steel column (30 cm × 4 mm I.D.) packed with a stable reversed-phase stationary phase consisting of porous silica beads (mean diameter 10 μm) coated with a chemically bonded monolayer of octadecylsilane (μBondapack C_{18}, Waters Assoc.). The mobile phase is methanol—water (40:60, v/v) with a flow-rate of 1.7 ml/min and an operating pressure of 17.25 MPa (2500 p.s.i.).

System 2. A column of identical dimensions with a chemically bonded monolayer of cyanopropylsilane (μBondapack CN, Waters Assoc.). The mobile phase is methanol—water (20:80, v/v) with a flow-rate of 2.0 ml/min and an operating pressure of 17.25 MPa (2500 p.s.i.).

In both systems the operating temperature is ambient. The column effluent is monitored continuously at 280 nm with a full scale deflection of 0.05 A. A short methanol wash (20 ml at 1 ml/min) at the end of each analytical day removes strongly retained solutes from both phases.

Reagents

All chemicals are reagent grade. Chloramphenicol was donated by Parke Davis, Ontario, Canada. Thiamphenicol was purchased from Sigma (St. Louis, Mo., U.S.A.). Solvents are routinely filtered through 0.45-μm filters (Millipore Corp., Bedford, Mass., U.S.A.) prior to use in the liquid chromatograph.

Standards

Chloramphenicol (40 mg) is dissolved in methanol (10 ml). A 2-ml sample of this solution is diluted to 100 ml with plasma. This standard (80 mg/l) is serially diluted with plasma to prepare standards containing 60, 40, 20 and 10 mg/l, respectively. These preparations are divided into small aliquots (ca. 0.5 ml) and frozen (−20°). The internal standard, thiamphenicol (20 mg) is dissolved in ethyl acetate (1 l) and this solution serves as the extraction solvent

Extraction

Serum or plasma (50 μl) is added to a 50-ml glass tube fitted with a PTFE-lined screw cap. Ethyl acetate (5 ml), containing the internal standard is added, followed by sodium chloride (ca. 1 g). Extraction is for 10 min (Buchler Omnishaker), followed by centrifugation at 500 *g* for 2 min. A portion of the organic layer (ca. 4 ml) is transferred into a disposable tube and taken to dryness by warming under a stream of dry nitrogen. The residue is dissolved in methanol (ca. 40 μl) and 25 μl is injected into the chromatograph. This procedure is followed for patient and standard samples. Standard curves are constructed by plotting the peak height ratios of chloramphenicol to thiamphenicol against the chloramphenicol concentration in each standard. The level of chloramphenicol in an unknown sample is derived from this curve.

RESULTS AND DISCUSSION

Fig. 1 is a chromatogram of a 20 mg/l plasma standard on the C_{18} phase. In

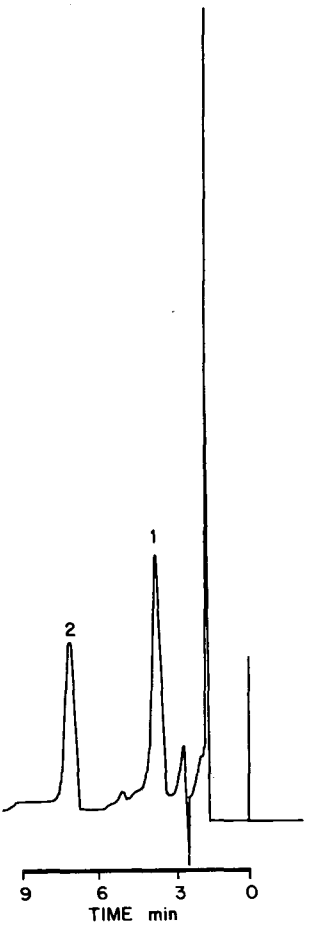

Fig. 1. Chromatogram of a plasma extract (20 mg/l) on the C_{18} phase. 1 = Thiamphenicol; 2 = chloramphenicol.

this case and on the —CN phase the chromatography is complete within 10 min with baseline separation between the two solutes. The k' values for chloramphenicol and thiamphenicol in the C_{18} system are 5.6 and 2.2 and in the —CN system 4.6 and 2.5, respectively (these data for thiamphenicol contrast with the $k' = 0$ reported by Wal et al. [9]. This illustrates the potentially wide variance in behaviour between bonded reversed-phase columns from different sources). The mechanisms of solute retention by —CN phases have not been investigated as fully as those for $-C_{18}$ alkyl phases. However, under the conditions employed herein, the $-C_2H_4CN$ function obeys "rule of thumb" reversed-phase behaviour and could be viewed as a short alkyl chain phase. In both systems the capacity factors of both solutes are reduced by increasing the methanol concentration of the solvent (Fig. 2) and it was by adjusting this component, together with flow-rate, that the optimum chromatographic conditions were achieved. Also the C_{18} phase exhibits much greater solute retention (Fig. 2) than the $-C_2H_4CN$ phase and this behaviour is in accordance with many observations in reversed-phase chromatography [11—14], which demon-

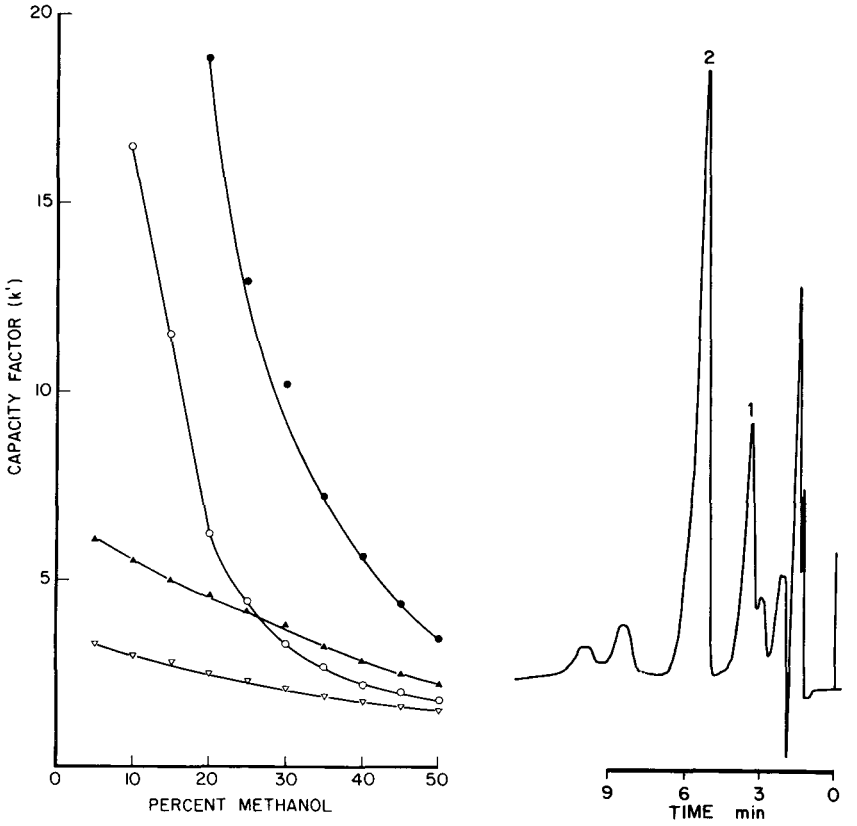

Fig. 2. Plot of k' vs. percent methanol. •, Chloramphenicol; ○, thiamphenicol, C_{18} phase; ▲, chloramphenicol; △, thiamphenicol, —CN phase.

Fig. 3. Chromatogram of patient plasma extract on the —CN phase. 1 = Thiamphenicol; 2 = chloramphenicol.

strate that k' values increase as the alkyl chain length and percentage carbon content of the bonded reversed phases increase.

Fig. 3 shows a chromatogram on the —CN phase from a patient on chloramphenicol therapy with found plasma levels of 76.0 ± 6.5 (1 S.D.) μg/ml (C_{18} approach) and 75.5 ± 5.7 (1 S.D.) μg/ml (—CN approach), this patient was an adult receiving large doses of chloramphenicol for disseminated intravascular coagulation caused by systemic bacterial infection. A level of about 15 μg/ml in plasma is considered effective against the majority of sensitive organisms, whereas anemia from the effect of the drug on bone marrow occurs regularly when levels of 25 μg/ml or higher are experienced [15]. Analysis of the standards and plasma blank by both approaches showed the relationship between the plasma concentration and peak height ratio of chloramphenicol to thiamphenicol to be linear between 0 and 80 μg/ml. Typical regression equations for the standard curves are $y = -0.23 + 0.37x$, $r = 0.9986$ (C_{18}) and $y = 0.05 + 0.34x$, $r = 0.9994$ (—CN) (y = peak height ratio drug:internal standard and x = chloramphenicol concentration). The limit of detection is 2 μg/ml for each method. A pool sample containing chloramphenicol (30 μg/ml) was processed to determine the accuracy and precision of the methods. The between batch variations are 8.6%, mean = 31.7 ± 2.7 (1 S.D.) (C_{18}, $n = 30$) and 7.5%, mean = 31.5 ± 2.4 (1 S.D.) (—CN, $n = 30$).

The above data demonstrate that each approach possesses the linearity, limits of detection, precision and accuracy acceptable for a routine assay aimed at monitoring blood levels of chloramphenicol. The sample work-up and fast analysis time allow regular analysis on a routine basis and moreover, the small sample requirement (50 μl), which can be met by the capillary sampling technique, obviates the need for venipuncture and enhances analysis of infant and neonatal patients who are particularly susceptible to harmful overdose from this drug.

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Note

Determination of indomethacin in plasma and urine by direct quantitative thin-layer chromatography

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Several methods for quantitative analysis of the antirheumatic drug indomethacin in biological fluids have been utilized in the past. These include radioactive [1—3], spectrophotometric [1] and spectrofluorometric [4] methods which, except for the isotope dilution technique of Duggan et al. [3] lack specificity due to interference from metabolites or salicylates. Recently, a specific gas chromatographic method has been published [5]. However, since indomethacin does not have an adequate vapour pressure to permit its direct gas—liquid chromatography (GLC) at temperatures below 300° the method involves a derivation step with diazomethane.

Indomethacin has been shown to undergo extensive biotransformation to 0-desmethylindomethacin, N-deschlorobenzoylindomethacin, 0-desmethyl-N-deschlorobenzoylindomethacin and their glucuronides in man and all laboratory animals so far examined [1, 3, 6]. All of these metabolites are present in significant amounts in plasma but are devoid of anti-inflammatory activity [7].

A method for separation of indomethacin, 0-desmethylindomethacin and N-deschlorobenzoylindomethacin using anion-exchange chromatography and spectrophotometry for detection of the individual compounds has been described [8] but the method lacks the sensitivity needed for clinical pharmacological studies.

The direct quantitative thin-layer chromatography (TLC) method described in this paper permits specific determination, without any derivation step, of

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indomethacin in plasma and urine at concentration levels far below the levels obtained from therapeutic doses of indomethacin.

MATERIALS AND METHODS

Apparatus

A Zeiss spectralphotometer with TLC scanning equipment KM 3 (Carl Zeiss, Oberkochen/Württemberg, G.F.R.) linked to a Servogor SB RE 646 recorder (Goerz Electro, Vienna, Austria) was used. The apparatus was equipped with two photomultipliers for simultaneous measurement of remission and transmission. The monochromator was set at 330 nm and the remission—transmission ratio used was 100:30. The scanning speed was 120 mm/min.

Chemicals

The following chemicals were used: 1,2-dichloroethane (Reinst), methanol (analytical grade), chloroform (analytical grade), citric acid (analytical grade) from E. Merck (Darmstadt, G.F.R.); sodium hydroxide (analytical grade) from Elektrokemiska (Bohus, Sweden); indomethacin from Dumex (Copenhagen, Denmark); metabolites of indomethacin from Alfred Benzon (Copenhagen, Denmark); filter paper Whatman No. 3 from A.G. Frisenette and Sons (Ebeltoft, Denmark).

Thin-layer chromatography

TLC was carried out using 20 × 20 cm pre-coated silica gel 60 thin-layer plates with a layer thickness of 0.25 mm and fluorescent indicator obtained from E. Merck. Twelve samples were spotted on each plate leaving 2 cm at the edges. Duplicate samples were always run on separate TLC plates and 4 of the 12 spots on each plate were extracts from spiked plasma samples used for preparing a standard curve. To ensure a reproducible spot size, the organic phase containing indomethacin was spotted, 10 μl at a time and the spot was completely dried under a stream of nitrogen before the next 10-μl portion was applied. The chromatography tank was lined with Whatman No. 3 chromatography paper. The mobile phase was chloroform—methanol (30:6). Indomethacin has an R_F value of 0.37 in this solvent. After chromatography the plates were dried in a ventilated oven at 50° for 15 min. The chromatography was conducted with the exclusion of light.

Procedures

One ml of plasma or urine was pipetted into each 10-ml PTFE-stoppered centrifuge tube and mixed with 1 ml citrate buffer (pH 5.0). A 7-ml aliquot of 1,2-dichloroethane was added and the tubes were shaken horizontally for 15 min at a rate of approximately 90 strokes per min. After centrifugation for 5 min at 1500 *g* the aqueous phase was removed by aspiration. A 3-ml volume of citrate buffer (pH 5.0) was added and the tubes were shaken for 5 min. The tubes were centrifuged and the aqueous phase was removed by aspiration. Five ml of the organic phase were then transferred to a conical centrifuge tube and evaporated to dryness under nitrogen, in a water bath at 40°. The residue was dissolved in 50 μl 1,2-dichloroethane using a Whirlimixer and the extracted material was spotted under nitrogen on the TLC plates using a 10-μl

constriction pipette. An additional 20-μl portion of 1,2-dichloroethane was added to each tube, shaken and spotted. After chromatography the TLC plates were scanned.

Calculations

The amount of indomethacin was calculated by comparison of the peak areas, calculated as peak height × width at half-height, for samples and standards. The standard curve fits a polynomium of the form $y = a_2x^2 + a_1x + a_0$, where y is the peak area and x is the concentration. The quadratic regression using the least-square method is made on a programmable calculator (Texas Instruments SR-52 or similar) and typically the correlation coefficients obtained are 0.99 or better.

RESULTS AND DISCUSSION

Precision, accuracy and sensitivity

The within-day as well as the day-to-day variations are shown in Table I.

When linear regression on added versus measured amount of indomethacin in the range 30—500 ng/ml plasma (72 samples) is made the correlation coefficient is 0.997 and the slope of the regression line is 0.97.

The recovery of indomethacin by extraction from plasma and urine with 1,2-dichloroethane has a maximum at pH 5.0. At this pH the recovery is 90%. It is important that the citrate buffer is freshly prepared every week.

The absorption spectrum of indomethacin when measured on a TLC plate in the remission mode, shows two maxima, one at 265 nm and one at 320 nm. However, to use the automatic baseline correction, inherent in the simultaneous measurement of remission and transmission, the lowest possible wavelength at which simultaneous measurement can be performed was chosen, i.e. 330 nm. Below this wavelength the absorption of transmitted light in the glass plate becomes too significant to allow simultaneous measurement. If the apparatus used is not equipped for simultaneous measurement it is advisable to measure in the remission mode at 265 nm.

No interference was found between indomethacin and the metabolites, O-desmethylindomethacin, N-deschlorobenzoylindomethacin, O-desmethyl-N-deschlorobenzoylindomethacin or the salicylates, in the analysis, because the R_F values of these compounds differed from that of indomethacin. Fig. 1 shows a typical scan of two samples extracted from plasma, one containing 250

TABLE I

WITHIN-DAY AND DAY-TO-DAY VARIATIONS AT DIFFERENT INDOMETHACIN CONCENTRATIONS

Concentration (ng/ml)	Within-day range	*n*	Day-to-day S.D. (%)	*n*
31	27— 31	4	12.2	22
63	60— 65	4	6.5	17
125	120—130	4	4.4	18
250	245—257	4	6.3	11
500	469—524	4	3.0	5

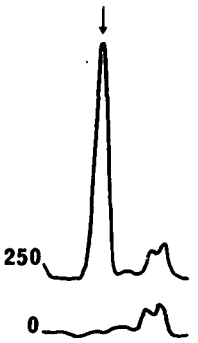

Fig. 1. Chromatogram scan showing 0 and 250 ng indomethacin, extracted from two 1-ml plasma samples.

ng and one no indomethacin. The lower limit for reliable quantitation of indomethacin in plasma and urine is 30 ng/ml using 1 ml samples (Table I). As no derivation step is involved the samples can be re-chromatographed in the same or in another solvent to yield a better separation, for example in case of interference from a new drug. From the following formula [9] $n = -1/\ln(1 - R_{F'})$, where n is the number of runs and $R_{F'}$ is the average R_F of the two solutes, the number of runs which yields maximum separation with a given solvent, can be calculated. In this case when the R_F value is 0.37, $n = 2$.

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Note

Fluorimetrische Bestimmung von Nifluminsäure aus Plasma durch direkte Auswertung von Dünnschichtchromatogrammen

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(Eingegangen am 17. Mai 1978; geänderte Fassung eingegangen am 9. August 1978)

Niflumínsäure [2-(*m*-Trifluormethyl-anilino)-nicotinsäure] ist ein in der Rheumatherapie häufig verwendetes Antirheumatikum und Antiphlogistikum. Es gibt jedoch bisher nur wenige in der Literatur beschriebene Verfahren zur quantitativen Analyse dieses Wirkstoffes in biologischem Material. 1968 und 1971 bestimmten Boisier et al. [1, 2] den Niflumínsäureplasmaspiegel spektralphotometrisch bei 286 nm. Das Verfahren erfordert jedoch eine Extraktion aus dem Plasma und beinhaltet keine Abtrennung der Substanz von evtl. bei gleicher Wellenlänge absorbierenden Stoffen (z.B. Metaboliten).

Die Messung radioaktiv markierter Niflumínsäure, 1973 von Lan et al. [3] durchgeführt, kann zwar auch zu Plasmaspiegelbestimmungen herangezogen werden, erfasst werden dabei jedoch gleichzeitig ausser der Niflumínsäure alle radioaktiv markierten Abbauprodukte.

Das von uns entwickelte fluorimetrische Verfahren besitzt gegenüber den genannten Methoden die Vorteile (1) einer niedrigen Nachweisgrenze ($\leqslant$ 10 ng pro Fleck); (2) einer chromatographischen Abtrennung der Substanz von Metaboliten und Plasmabestandteilen; und (3) eines geringen Arbeitsaufwandes und einer grossen Genauigkeit durch die direkte Bestimmung aus Plasma ohne vorherige Extraktion.

Bei dem von uns entwickelten Verfahren entsteht vermutlich aus der nicht fluoreszierenden Niflumínsäure durch Cyclisierung mit Formaldehyd, analog zu der von Dell und Kamp [4] beschriebenen Reaktion für Flufenaminsäure, ein fluoreszierendes Pyridooxazin-Derivat. Ein Zusatz von Ameisensäure als saurer Katalysator steigert die Fluoreszenzintensität erheblich.

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*Teilergebnisse der Dissertation A. Schumacher, in Vorbereitung.

**Anforderung von Sonderdrucken: Prof. Dr. Dr. Ernst Mutschler, Pharmakologisches Institut für Naturwissenschaftler der Johann Wolfgang Goethe-Universität, Robert-Mayer-Strasse 7—9, 6000 Frankfurt/Main, B.R.D.

METHODIK

Die zu chromatographierende Lösung wird wie folgt hergestellt: 1 Volumenteil Plasma wird mit 2 Volumenteilen Methanol versetzt und scharf abzentrifugiert, wobei das gefällte Eiweiss sedimentiert. Entsprechend der zu erwartenden Konzentration werden von der überstehenden Lösung 20—100 µl strichförmig mit dem Linomaten III (Camag) auf Kieselgel-60-Fertigplatten (Merck, Darmstadt, B.R.D.) 20 × 20 cm ohne Fluoreszenzindikator aufgetragen.

Bei einer Strichbreite von 10 mm pro Fleck und einem 25-mm-Abstand der äusseren Startlinien vom Plattenrand können acht Proben und drei Standards auf eine Platte aufgetragen werden.

Die Standards werden durch Zusatz von Nifluminsäure zu gepooltem Plasma hergestellt. Dazu werden 60.0 mg Nifluminsäure in 100.0 ml Aceton gelöst und 1.0 ml dieser Lösung nochmals auf 10.0 ml verdünnt, entsprechend einer Konzentration von 600.0 µg pro 10.0 ml Aceton. 5.0 ml dieser Lösung werden nun durch Einblasen von Stickstoff bis zur Trockne eingeengt, dann werden 50.0 ml gepooltes Plasma zugegeben. Dadurch ergibt sich eine Konzentration von 6.0 µg Nifluminsäure pro 1.0 ml Plasma. Nach Zugabe von 2 Volumenteilen Methanol und Zentrifugation wird der Überstand als Standardlösung verwendet.

Die Chromatographie erfolgt 10 Min nachdem der letzte Fleck aufgetragen ist, um ein gleichmässiges Trocknen der Proben auf der Platte zu erreichen. Mit dem Fliessmittel Chloroform—Methanol—Ameisensäure (95:7:7, v/v) bei einer Fliessstrecke von 12 cm wird die Substanz von ihren Metaboliten und Plasmabestandteilen abgetrennt.

Nach der Chromatographie wird die Platte für 15 Min bei 130° im Wärmeschrank getrocknet. Anschliessend erfolgt die Umsetzung mit Formaldehyd direkt auf der Dünnschicht-Platte während 30 Min bei 130° zu intensiv fluoreszierenden Flecken. Dabei steht die Platte in einer Dünnschicht-Kammer zusammen mit zwei Glasschiffchen, von denen das eine mit 2.5 g Paraformaldehyd und 0.5 ml konz. Schwefelsäure, das andere mit 1 ml Ameisensäure gefüllt ist.

Die direkte quantitative Messung, 30 Min nach Herausnahme der Platte aus der Kammer, erfolgt mit dem Chromatogramm-Spektralphotometer KM 3 der Firma Zeiss. Exzitation: Hg-Linie 265 nm der Hg-Mitteldrucklampe St 41, Spaltgrösse 1 × 8 mm. Emission: > 430 nm. Als Sperrfilter für die Exzitationsstrahlung verwendeten wir den Kantenfilter Fl 43. Die Intensität der Emissionsstrahlung wird durch einen Photoelektronenvervielfacher gemessen, die Aufzeichnung der Fluoreszenzintensitäts—Ortskurven erfolgt durch einen Perkin-Elmer-Recorder 56, Tischgeschwindigkeit 50 mm/Min, Schreibervorschub 120 mm/Min. Die Auswertung erforlgt über die Flächen unter den Fluoreszenzintensitäts-Ortskurven.

Nach der vorstehenden Methode wurden die Präzision und Richtigkeit des Verfahrens bestimmt. Da bei einer therapeutischen Tagesdosis von 3 × 250 mg Nifluminsäure-Blutspiegel van ca. 12 µg/ml erreicht werden, wurden zur Bestimmung von Präzision und Richtigkeit zu jeweils 1.0 ml Testplasma (1) 12.0 µg, (b) 6.0 µg, und (c) 1.0 µg Nifluminsäure zugesetzt. Bei den Untersuchungen auf Richtigkeit des Verfahrens verwendeten wir als Standards die jeweils gleichen Mengen Nifluminsäure, gelöst im Methanol—Wasser (2:1).

ERGEBNISSE UND DISKUSSION

Bei einer Konzentration von 12.0 µg Nifluminsäure pro ml Testplasma war die relative Standardabweichung 3.3%, bei 6.0 µg/ml 3.6%, und bei 1.0 µg/ml 7.7%.

Die Wiederfindungsraten bei der Bestimmung der Richtigkeit des Verfahrens lagen über 100%, was einerseits durch eine Volumenkontraktion des Plasma—Methanol-Gemisches und andererseits durch eine Volumenverminderung infolge des ausgefallenen Eiweisses erklärt wird. Wird die ursprüngliche, im Plasma vorhandene (durch Einwaage bekannte) Nifluminsäuremenge auf das Volumen des Plasma—Methanol-Gemisches nach der Zentrifugation berechnet, so liegt die Wiederfindungsrate etwa bei 100%. Geringe Schwankungen sind bei verändertem Eiweissgehalt des Plasmas zu erwarten. Deshalb erschien es uns am günstigsten, als Standard gepooltes Plasma zu verwenden und dies genauso wie die zu bestimmenden Proben zu behandeln. Dadurch wird der Fehler infolge der Volumenkontraktion ausgeschlossen und der Fehler durch schwankenden Eiweissgehalt so klein wie möglich gehalten.

Die Bestimmung der Linearität zwischen den Flächen unter den Fluoreszenzintensitäts—Ortskurven und den aufgetragenen Substanzmengen im Konzentrationsbereich von 10—200 ng/Fleck ergab einen linearen Regressionskoeffizienten von 0.998, gemittelt aus zwei Eichkurven. Die Eichkurven gehen durch den Nullpunkt. Zur Erstellung der Eichgeraden genügt daher jeweils ein einziger Kurvenpunkt, der zur Erhöhung der Genauigkeit aus drei Messwerten ermittelt wird.

Das Fluoreszenzmaximum des an das Sorbens gebundenen Umsetzungsproduktes der Nifluminsäure liegt, wie aus Fig. 1 hervorgeht, bei 450 nm.

Zur Überprüfung unserer Methode wurde einer Versuchsperson eine einmalige Dosis von 250 mg Nifluminsäure verabreicht und 5, 9 und 26 Stunden nach der Einnahme Blut entnommen. Die Proben wurden, Zusammen mit einem 6.0 µg Nifluminsäure pro 1.0 ml Plasma enthaltenden Standard, nach der beschriebenen Methode aufgearbeitet. Die Auswertung der Platten erfolgte 30 Min nach

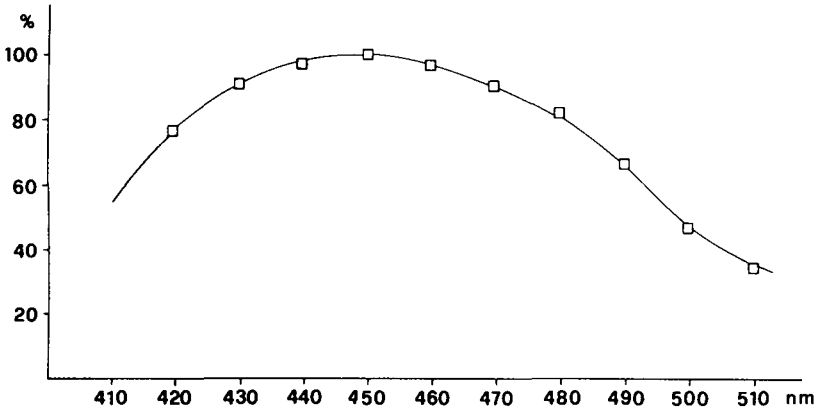

Fig. 1. Emissionsspektrum der mit Formaldehyd umgesetzten Nifluminsäure. Exzitation: Hg-Linie 265 nm.

Herausnahme aus der Kammer, da, wie auf Fig. 2 ersichtlich, nach anfänglich recht schneller Abnahme der Fluoreszenzintensität bis auf ein relativ stabiles Niveau, dann gut reproduzierbare Werte gefunden werden.

Bei längerer Applikation von Nifluminsäure ist mit dem Auftreten der von Cohen et al. [5] beschriebenen Hauptmetaboliten 5-Hydroxynifluminsäure und 4'-Hydroxynifluminsäure zu rechnen, da diese nach Lan et al. [3] Halbwertszeiten von 15 h aufweisen und daher nach mehrmaliger Gabe im üblichen 8-h-Rhythmus einen deutlich höheren Plasmaspiegel erreichen werden als nach einmaliger Gabe. Daher wurde ihr Verhalten bei unserer Bestimmungsmethode ebenfalls untersucht. Die Metaboliten wurden als Standards zu Testplasma zugesetzt und unter gleichen Bedingungen aufgearbeitet.

Nach erfolgter Methanolfällung wurden von dem Uberstand der 5-h-Probe 20 µl, der 9-h-Probe 40 µl und der 26-h-Probe ebenfalls 40 µl aufgetragen. Es ergaben sich Blutspiegel von 15.6 µg pro 1.0 ml nach 5 h und 5.0 µg pro 1.0 ml nach 9 h. Nach 26 h waren nur noch Spuren von Nifluminsäure vorhanden.

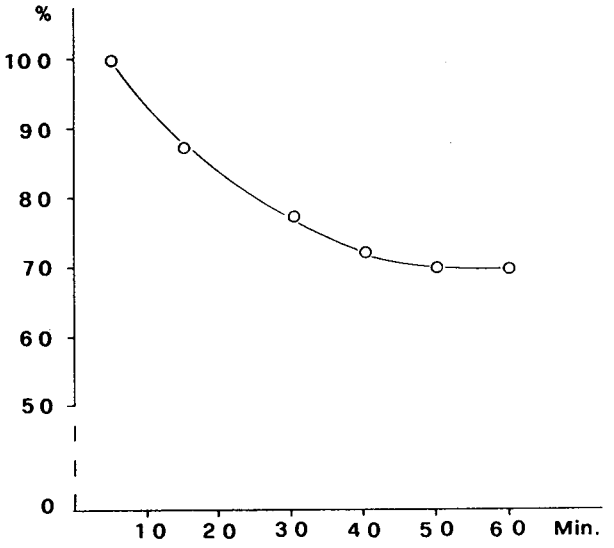

Fig. 2. Abnahme der Fluoreszenzintensität der mit Formaldehyd umgesetzten Nifluminsäure.

In Fig. 3 ist die Fluoreszenzintensitäts—Ortskurve des Chromatogramms des 9-h-Wertes dargestellt und als Vergleich dazu die Fluoreszenzintensitäts—Ortskurve eines Chromatogramms, bei dem 40 µl des Uberstandes einer Methanolfällung von 1.0 ml Plasma ohne Nifluminsäure aufgetrennt wurden. Der hR_F-Wert der Nifluminsäure beträgt 36. Die Hauptmetaboliten erreichen hR_F-Werte von 20 für 5-Hydroxynifluminsäure und 6 für 4'-Hydroxynifluminsäure.

4'-Hydroxynifluminsäure reagiert nach dem beschriebenen Verfahren nicht zu einem fluoreszierenden Produkt. Der hR_F-Wert wurde durch Fluoreszenzlöschung auf einer Kieselgel-60-Fertigplatte (Merck) 20 × 20 cm mit Fluoreszenzindikator ermittelt. Die 5-Hydroxynifluminsäure ergibt mit Formaldehyd

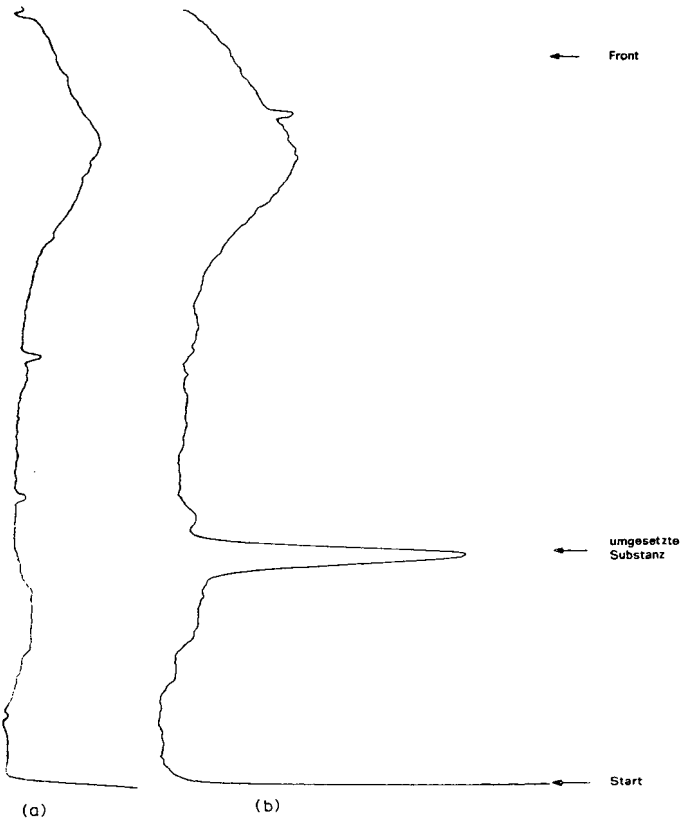

Fig. 3. Fluoreszenzintensitäts—Ortskurven der Chromatogramme eines Plasmas ohne Niflu-minsäure (a) und eines 5.0 μg pro 1.0 ml enthaltenden Plasmas (b). Das Nifluminsäure enthaltende Plasma wurde 9 h nach der Einnahme einer einmaligen Dosis von 250 mg Niflumin-säure gewonnen. Aufgetragen wurde je 40 μl eines Uberstandes von 1.0 ml Plasma und 2.0 ml Methanol. Die Fluoreszenzreaktion erfolgte nach der Chromatographie direkt auf der Platte mit Formaldehyd.

ein fluoreszierendes Produkt und kann daher gleichzeitig neben der Niflumin-säure quantitativ mitbestimmt werden. Bei einmaliger Gabe von 250 mg Niflu-minsäure ist jedoch nach 9 h, wie aus Fig. 3b ersichtlich, noch keine 5-Hydroxy-nifluminsäure im Plasma nachweisbar.

DANK

Wir danken der Dr. Robert Pfleger-Stiftung für eine Sachbeihilfe.

LITERATUR

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Book Review

Plasma proteins — Analytical and preparative techniques, by P.C. Allen, E.A. Hill and A.M. Stokes, Blackwell, Oxford, 1977, IX + 254 pp., price £ 11.75, ISBN 0-632-00279-4.

Occasionally it happens that one meets with a book that is a pleasure to read by the fire with a drink alongside. This need not necessarily be a novel and there are a few books in science that can be classified in this category. The present book is certainly one of these. Being written with a light pen it offers the reader up-to-date surveys of both analytical and preparative procedures that are carefully selected and classified. Objections can be raised against, for example, the arrangement of the analytical section: are immunoelectrophoretic methods not just part of electrophoretic techniques in general? Would it not be better to have electromigration techniques as one single chapter ? Analytical chromatographic techniques are not mentioned at all; the idea is, obviously, that the adequately skilled reader can easily derive them from the preparative ones. The preparative chromatographic procedures are described more than well so the demand is fulfilled. The practical descriptions of individual procedures are likely to be welcomed by all intending to use this volume as a laboratory handbook. There are a few errors or misleading formulations; the subtitle to Chapter 1 in the Contents may be one of the examples. Fig. 2.6 presents an excellent result of a polyacrylamide gel run, but it can hardly be called "densitometric analysis". It is not the aim to enumerate here these minor discrepancies. They are few and are unlikely to devalue the volume as a textbook. Blackwell publishers have supplied the reviewers with a leaflet justifying the retail price with respect to increasing costs of paper, printing, binding, etc. I am therefore going to withhold my comments on this point. Still, my personal feeling is that the book is a good buy for all workers in protein chemistry as most of the techniques described exceed the limitations of plasma proteins.

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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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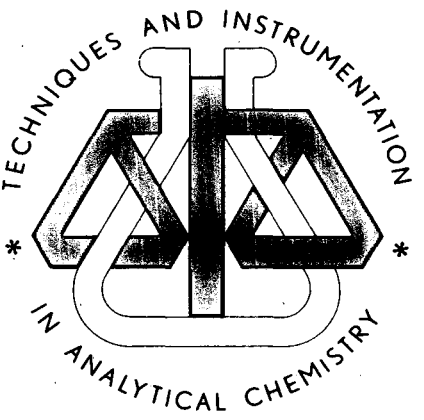

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