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Modern Liquid Chromatography of Macromolecules

by **B.G. BELENKII** and **L.Z. VILENCHIK,** Institute of Macromolecular Compounds, Academy of Sciences of the USSR, Leningrad, USSR

JOURNAL OF CHROMATOGRAPHY LIBRARY, 25

Researchers, technologists and students in the fields of physics, chemistry, biology and medicine will all find this book of interest. It considers the chromatography of high-molecular-weight compounds and its main theoretical and methodological features.

Recommendations are made for preparing high-performance chromatographic systems and for selecting the optimum conditions for their operation. Particular attention is paid to the problems of interpreting chromatographic data in order to obtain various molecular-weight and structural characteristics of the macromolecules investigated: AMW, MWD, indices of polymer branching, the compositional homogeneity of copolymers, the functionality of oligomers, etc. The authors also provide examples of various combinations of chromatographic and other methods that can be used to analyse complex polymer systems.

Written mainly for the reader who has some practical experience in chromatography, the book also contains much useful information for the beginner.

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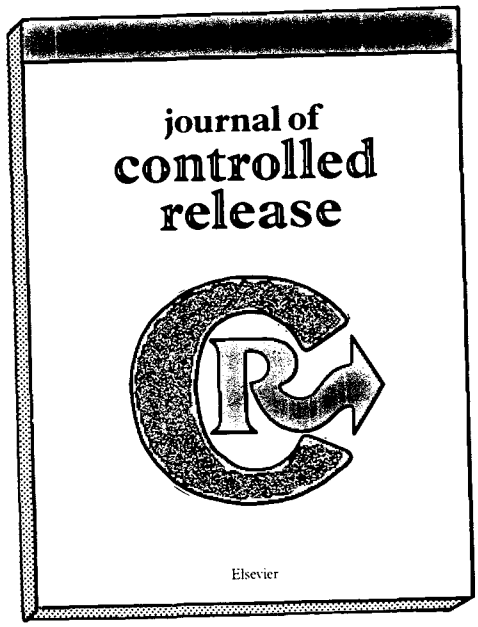

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RAPID METHOD FOR THE MEASUREMENT OF METHYLPREDNISOLONE AND ITS HEMISUCCINATE IN PLASMA AND URINE FOLLOWING "PULSE THERAPY" BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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(First received November 14th, 1984; revised manuscript received March 12th, 1985)

SUMMARY

A rapid method for the measurement of methylprednisolone and its 21-hemisuccinate ester in plasma and urine following high dose pulse therapy is described. The drugs were extracted using Extrelut® columns, eluted with ethyl acetate which was evaporated to dryness and the residue was reconstituted in chromatographic mobile phase. High-performance liquid chromatography was performed on a reversed-phase column using a mobile phase of acetonitrile—acetate buffer with detection at 251 nm. No interference from any drugs or endogenous compounds has been observed. The method has been used to analyse over 200 plasma and 150 urine samples from patients with rheumatoid disease or renal failure who have received high dose methylprednisolone hemisuccinate infusions.

INTRODUCTION

High doses of intravenous steroids have been useful in the treatment of several diseases. Solu-Medrone (Upjohn; methylprednisolone-21-hemisuccinate, MPHS) is a water-soluble ester which is rapidly hydrolysed to the active 21-hydroxycorticosteroid (MP) following parenteral administration [1] and has been used to treat glomerulonephritis, systemic lupus erythematosus, rheumatoid disease, shock and for the reversal of rejection episodes following organ transplantation. Intravenous infusions of MPHS lasting 15—20 min in doses of up to 2 g appear to be associated with a lower incidence of side-

*This work forms part of a thesis to be submitted to the University of Surrey for the degree of Ph.D.

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TABLE I

COMPARISON OF HPLC METHODS USED TO MEASURE MPHS AND/OR MP

Reference	MPHS	MP	Plasma	Urine	Dose	Sample volume (ml)	Acid preservative
7	No	Yes	Yes	No	MP 7 × 4 mg p.o.	2.5	No
10	Yes	Yes	Yes	No	MPHS 125 mg i.m.	0.5	No
8*	No	Yes	Yes	No	MP 20—40 mg p.o.	2.5	No
9	No	Yes	Yes	Yes	MPHS 12—20 mg/kg i.v.	1.0	No
11	Yes	Yes	Yes	No	MPHS 40 mg i.v.	"up to 1"	No
This paper	Yes	Yes	Yes	Yes	MPHS 0.5—2.0 g i.v.	0.5	Yes

*Modified from Garg et al. [7].

effects and complications than the more conventional, and smaller, oral maintenance doses [2].

The pharmacokinetics of such large intravenous doses of MPHS and MP are not well established. It is not always relevant to extrapolate from information on drugs obtained from low-dose to high-dose studies, especially in this case as MP is an analogue of prednisolone which has been shown to exhibit dose dependency [3]. Prednisolone is cleared more rapidly from serum when administered at higher doses. Sudden death following MPHS pulse therapy has been reported recently [4, 5]. This may be due to drug-induced arrhythmias. Monitoring MPHS and MP blood levels may be of use in investigating the aetiology of this complication.

Thin-layer chromatography has been used to measure corticosteroids although this technique lacks the required reproducibility. Radioimmunoassay is very sensitive, but the antibody (which is not available commercially) measures MPHS and MP equally [6]. This renders the method unsuitable for pharmacokinetic studies on the two drugs. High-performance liquid chromatography (HPLC) does possess the necessary selectivity and has been used to measure MP alone in serum [7—9] and urine [9], and MP and MPHS have been measured in serum following intramuscular injection [10] or intravenous infusion [11]. Assael et al. [9] are the only group to have examined blood and urine drug levels following high dose intravenous MPHS but just measured the hydrolysis product, MP using normal phase chromatography. In the present study both MPHS and MP were measured in blood and urine following high-dose MPHS infusion by the method described below.

Extraction	Chromatog. system (time, min)	Limit of detection (nmol/l)	Internal standard	Number of patients
Chloroform	Reversed (8)	80	MP acetate	1
Hexane Ethyl acetate	Reversed (7)	53 (both) 'estimated empirically'	17-OH-Progesterone	1
*	*	<27	*	5
Chloroform	Normal (11)	27	Dexamethasone	14
Methylene chloride	Normal (12)	27	Dexamethasone	1
Extrelut Ethyl acetate	Reversed (5)	MP-267 MPHS-603	11-Deoxy-17-hydroxy-corticosterone	28

HPLC methods available for measuring MP and/or MPHS are presented for comparison in Table I.

MATERIALS AND METHODS

Apparatus

A Varian Model 5010 liquid chromatograph was used with a Varian Model UV-50 variable wavelength detector. A stainless-steel column 100 mm × 5 mm I.D. (Shandon, Cheshire, U.K.) was packed with 5-μm Spherisorb S5 ODS 2 (HPLC Technology, Cheshire, U.K.) using a Shandon column packing instrument. The analytical column was surrounded by a Varian heating block. Samples were injected using a Valco injection valve fitted with a 10-μl loop. Chromatograms were recorded, and results calculated, by a Spectra-Physics SP 4270 Integrator.

Reagents

Acetonitrile (HPLC grade) was obtained from BDH (Poole, U.K.). Ethyl acetate, ammonium acetate, methanol, sulphuric acid and glacial acetic acid were AnalaR grade. 0.05 *M* acetate buffer was prepared by dissolving 3.85 g ammonium acetate in 1 l distilled water, the pH was then adjusted to 3.0 with glacial acetic acid.

Standards

Solu-Medrone, methylprednisolone and 11-desoxy-17-hydrocorticosterone

(internal standard) were gifts from Upjohn (Crawley, U.K.).

Stock standards of MP and internal standard, 1 mg/ml in methanol, were prepared, stored at 4°C and found to be stable over several months. MPHS stock standard, 1 mg/ml in distilled water, was prepared immediately before each assay as degradation to MP starts to occur after 24 h at 4°C. Subsequent dilutions for serum working standards were prepared in Technicon SMA reference serum (human) for the analysis of plasma samples, followed by the addition of 0.1 ml 3 *M* sulphuric acid/ml plasma. Urine working standards were diluted in urine from volunteers not receiving MPHS and adjusted to pH 2.0 with 3 *M* sulphuric acid. The internal standard (I.S.) was diluted to 50 μg/ml in 5% acetic acid.

Sample collection

Blood samples were collected at 0, ½, 1, 1½, 2, 3, 4, 6, 8, 24 h post infusion into heparinised tubes, centrifuged immediately and acidified by adding 0.1 ml 3 *M* sulphuric acid per ml of plasma. Urine samples collected at 0—2, 2—4, 4—8 and 8—24 h post infusion contained 0.5 ml 3 *M* sulphuric acid per 20 ml urine. All samples were stored at −20°C until analysed (usually within one week of collection).

Extraction

Extraction columns were prepared by placing a glass ball (3 mm diameter) into a 5-ml Macrotip (Laboratory, Industrial and Pharmaceuticals, Equipment and Services, West Yorkshire, U.K.) and adding Extrelut® (Merck) to a height of approximately 4 cm. 200 μl I.S. were added to 500 μl standard or test (plasma or urine), vortex mixed and applied to the Extrelut columns. After 10 min the columns were eluted with 5 ml ethyl acetate and the eluates evaporated to dryness under an air stream in a 40°C water bath. The residue was reconstituted in 100 μl mobile phase and 10 μl injected. Macrotips and glass balls were reused after washing, but the Extrelut was discarded.

Chromatography

The optimum mobile phase was found to be acetonitrile—acetate buffer (32.5:67.5, v/v) at a flow-rate of 1.5 ml/min. Chromatography was performed at 30°C with detection at 251 nm.

RESULTS

Fig. 1 shows the separation of MPHS, MP, I.S. and related compounds chromatographed using the described system. Representative chromatograms of patient samples who received MPHS are presented for plasma (Fig. 2a) and urine (Fig. 3a). No interfering peaks were detected in blood and urine samples taken immediately before infusion of MPHS (Figs. 2b and 3b). The following drugs may also be administered to patients receiving MPHS infusions and none was found to produce any chromatographic interference: prednisolone, prednisone, dexamethasone, cortisol, paracetamol, dextropropoxyphene, salicylic acid, oxyphenbutazone, indomethacin, penicillamine, piroxicam,

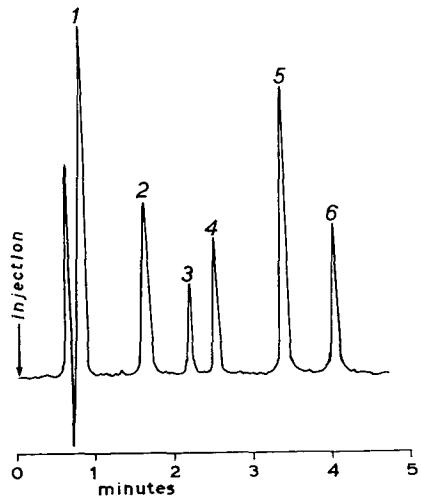

Fig. 1. Separation of cortisol (1), prednisone and prednisolone (2), methylprednisolone (3), dexamethasone (4), 11-desoxy-17-hydroxycorticosterone (5), methylprednisolone-21-hemisuccinate (6).

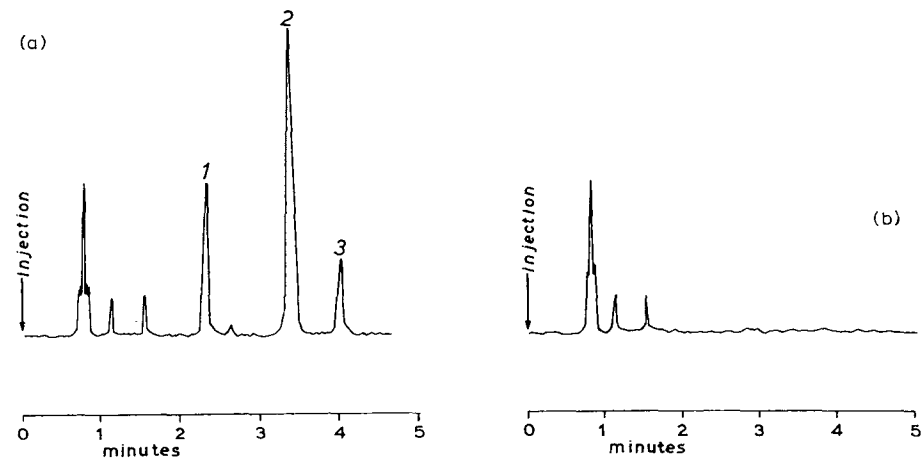

Fig. 2. (a) Chromatogram of a plasma sample from a patient with rheumatoid disease taken 30 min following infusion of 1 g MPHS, MP (1), I.S. (2), MPHS (3). (b) Chromatogram of a plasma sample from a patient taken immediately before infusion of 1 g MPHS.

flurbiprofen, naproxen and fenclofenac. Furthermore, no interference from endogenous compounds or drugs has been observed during the analysis of over 200 plasma and 150 urine samples.

Standard curves for MPHS and MP in serum and urine were linear up to 201 μmol/l and 267 μmol/l, respectively.

Recovery of MPHS and MP from serum and urine is shown in Table II. The recovery of I.S. at the concentration used was found to be 83.9%. These values were calculated by comparing the peak heights of extracted drugs against peak heights of the corresponding unextracted aqueous standards. It is important to

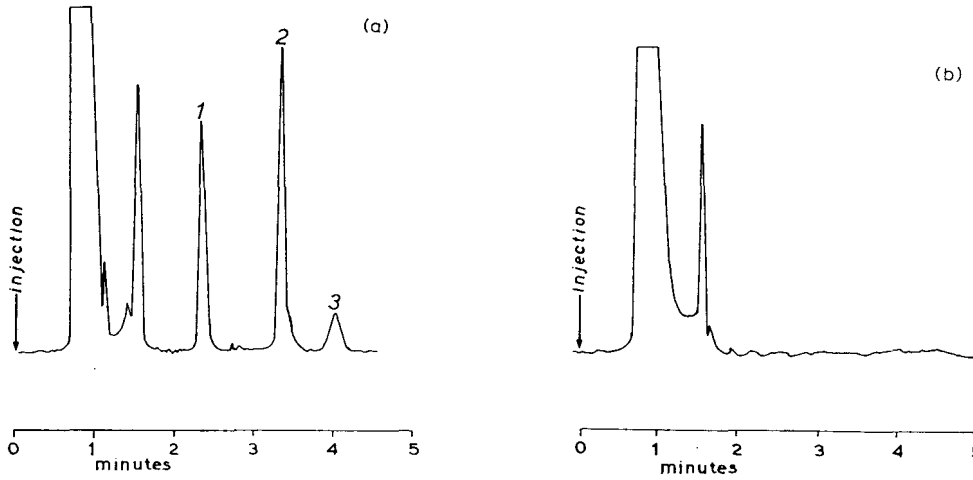

Fig. 3. (a) Chromatogram of a urine sample from a patient with rheumatoid disease collected 2—4 hours following infusion of 1 g MPHS, MP (1), I.S. (2), MPHS (3). (b) Chromatogram of a urine sample from a patient collected immediately before infusion of 1 g MPHS.

TABLE II

RECOVERY OF MPHS AND MP FROM SERUM AND URINE

n = number of samples.

		Amount of drug added (μmol/l)	n	Recovery (%)
Serum	MPHS	10.1	10	52.4
		40.2	10	56.4
		100.5	9	54.9
	MP	13.4	10	62.1
		53.4	10	63.9
		133.5	10	64.7
Urine	MPHS	2.0	9	89.2
		10.1	10	96.8
		100.5	10	92.4
	MP	2.7	10	93.0
		13.4	10	89.3
		133.5	10	83.0

note that the presence of acid in samples had little effect on the extraction efficiency of MP and I.S. but was crucial for MPHS. Without acid, recovery of MPHS from serum fell to less than 10%. The within- and between-batch coefficients of variation for serum and urine are shown in Table III. It was impossible to calculate between-batch variation for MPHS due to its slow degradation to MP over several weeks, despite being stored frozen with added acid. The lowest measurable levels of MPHS and MP were considered to be 603 nmol/l and 267 nmol/l, respectively. These levels represent approximately five times baseline noise.

MPHS and MP were measured in patients with severe renal failure, some of

TABLE III

PRECISION STUDIES FOR SERUM AND URINE

		Amount of drug added (μmol/l)	Within assay coefficient of variation	Between assay coefficient of variation
Serum	MPHS	2.0	13.7 (7*)	—**
		10.1	12.6 (10)	—**
		100.5	4.3 (10)	—**
	MP	2.7	7.6 (6)	15.1 (9)
		13.4	5.6 (10)	8.2 (10)
		133.5	5.7 (10)	8.6 (10)
Urine	MPHS	2.0	0.2 (10)	—**
		10.1	3.9 (9)	—**
		100.5	1.6 (9)	—**
	MP	2.7	9.6 (9)	9.6 (10)
		13.4	1.9 (10)	2.7 (10)
		133.5	1.4 (10)	3.4 (10)

*Number of samples.
**See text.

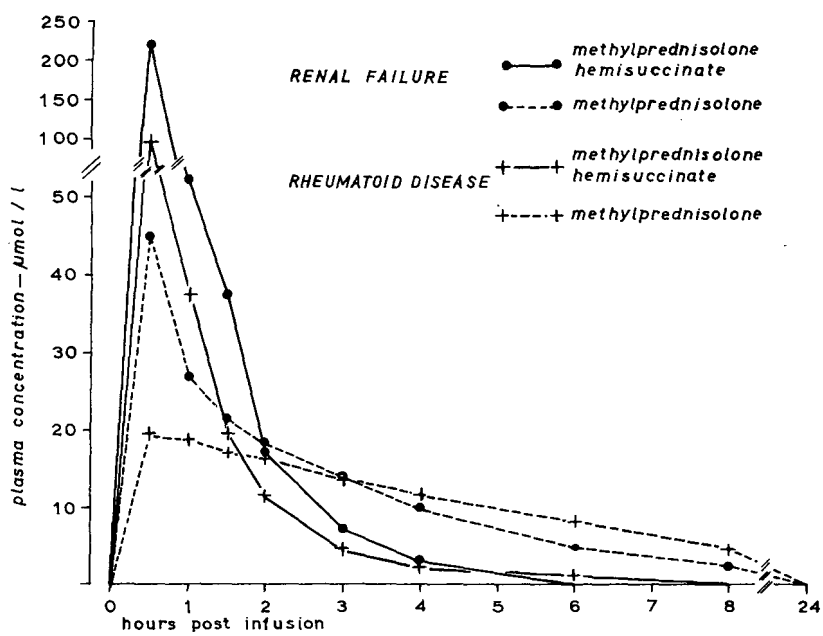

Fig. 4. Clearance of MPHS and MP from the plasma of a patient with rheumatoid disease and another with renal failure following a 1-g infusion of MPHS.

whom had received transplants, and those suffering from rheumatoid disease. Fig. 4 shows a typical example of the clearance of MPHS and MP from plasma following a 1-g infusion of MPHS given to a renal and a rheumatoid patient. Peak plasma levels were found at 30 min for MPHS and 30 min to 1 h for MP,

TABLE IV

MEAN PEAK PLASMA MPHS AND MP LEVELS (μmol/l) FOLLOWING INFUSION OF MPHS

Dose (g)	Rheumatoid disease		Renal failure	
	MPHS	MP	MPHS	MP
0.5	5.6 (3*)	15.5 (3)	67.6 (3)	18.7 (3)
1.0	23.7 (9)	26.7 (9)	189.0 (11)	36.1 (11)
2.0	55.9 (2)	36.6 (2)	—	—

*Number of patients.

TABLE V

MEAN % DOSE EXCRETED IN URINE AS FREE DRUG (MPHS + MP) PER 24 h FOLLOWING INFUSION

Dose (g)	Rheumatoid disease	Renal failure
0.5	22.2 (2*)	—
1.0	21.0 (6)	4.7 (7)
2.0	26.0 (2)	—

*Number of patients.

post-infusion of MPHS in both sets of patients (Table IV). MPHS was cleared more rapidly from plasma than MP; neither drug was detected in plasma 24 h after infusion. The amount of MPHS and MP excreted in the urine as the free drug during the 24 h following infusion is shown in Table V.

DISCUSSION

The method described was found to be selective for MPHS and MP with baseline separation from endogenous compounds and co-administered drugs. Sample preparation was easier and less time-consuming than previously reported methods, which use more classical extraction techniques [7—11]. This, coupled with faster chromatography enabled fifty patient samples to be analysed per day (Table I). The assay was developed for measuring MPHS and MP in biological fluids following "pulse" doses of MPHS. The high blood and urine levels of the drugs which result can easily be measured and therefore a smaller sample volume can be used when compared to other methods. This is important when multiple samples are taken from individual patients for pharmacokinetic studies. If a higher sensitivity is required, this can be achieved by increasing sample volume and using a larger injection loop. The present study is the only one in which MPHS and its hydrolysis product MP are measured in plasma and urine following "pulse" MPHS infusion. The other group to examine blood and urine following pulse therapy only measured MP, using a slower normal-phase chromatographic procedure [9]. However, their results may possibly be misleading since the analyses are based on the assump-

tion that MPHS is stable in human blood in vitro under their conditions of handling and storage (−20° C). In my experience, complete hydrolysis of MPHS to MP takes place after several weeks storage at −20° C, and even when preserved with acid very slow degradation takes place [1]. The findings of the present study also vary with those of Assael et al. [9] regarding the solubility of MPHS in ethyl acetate.

Peak plasma levels of MPHS and MP reflected the dose of MPHS administered. Patients with renal failure had consistently higher peak plasma levels of MPHS and MP than those with rheumatoid disease, which probably reflects the impaired renal function of the former. This could also explain the difference in urinary excretion of the free drug between the two patient groups and, in turn, would mean that there would be more opportunity for metabolism, further decreasing the amount of free drug excreted by renal patients.

However, a reduced clearance implies that the drugs would be detected in the plasma of renal patients for longer than those with rheumatoid disease but this was not found to be the case, even in patients given repeated infusions at 48-h intervals. The unaccounted for drug must have been metabolised, excreted into the urine as a compound at present undetectable or via some other route (e.g. faecal), or taken up into the general lipid depot and excreted at levels below the limit of detection of this assay over several days. Another study has reported less than 10% of the free drug recovered in urine [9] following pulse-dose MPHS. However, it is difficult to draw comparisons with this study as the conditions of sample collection and storage, extraction and chromatography are different. Slaunwhite and Sandberg [12] injected 0.4 mg of radioactively labelled MP intravenously and found 75% of the dose in the urine, of which 2.5% was unconjugated.

The ease and speed of sample preparation and chromatography make this method practical, while bulk purchase of Extrelut and reusable pipette tips for extraction columns contribute to low running costs. It is hoped that this method will help in the introduction of high-dose pulse MPHS infusions as an adjunct to established regimes for treating rheumatoid disease, or possibly as a more efficacious alternative to conventional oral low doses of steroids [13].

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CHROMBIO. 2648

DETERMINATION OF DOPAMINE-3- AND -4-O-SULPHATE IN HUMAN PLASMA AND URINE BY ANION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORIMETRIC DETECTION

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SUMMARY

A high-performance liquid chromatographic (HPLC) method is reported for the determination of dopamine-3- and -4-O-sulphate isomers in human plasma and urine using an anion exchanger coupled with post-column hydrolysis and fluorimetric detection. Samples of plasma or urine are partially purified on Dowex 1 and Dowex 50 columns and separated using HPLC. These compounds are then hydrolysed and determined automatically by the *p*-aminobenzoic acid method in a continuous-flow reaction system. As the *p*-aminobenzoic acid method is very specific for dopamine, it is also possible to determine the isomers by injecting 5—20 μl of urine or 100—200 μl of deproteinized plasma directly into the HPLC system without clean-up.

The detection limit of the method for both isomers is 0.3 pmol. In normal subjects, the plasma levels of dopamine-3- and -4-O-sulphate are 26.5 (S.D. 11.1) and 2.68 (S.D. 0.34) pmol/ml, and their urinary excretion rates are 1.73 (S.D. 0.56) and 0.27 (S.D. 0.04) nmol/min, respectively. Thus the two isomers are present in both plasma and urine and their urinary excretions reflect directly their plasma levels.

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INTRODUCTION

Circulating dopamine is mainly present in conjugated forms, and its sulphate esters are the predominant conjugates in human plasma [1—3]. It is still controversial whether these dopamine conjugates are simple inactivated products of dopamine, whether they are intermediates in the biosynthesis of catecholamines [4, 5] or whether they themselves have physiological activity [6, 7]. The examination of their possible roles requires the use of a sensitive and specific method. Although the sulpho-conjugated dopamines have been determined by enzymatic hydrolysis [1—3], it is impossible to distinguish the two isomers by this method. Recently, Arakawa et al. [8] and Elchisak [9] reported a high-performance liquid chromatographic (HPLC) separation of the two isomers and demonstrated the presence of both the 3- and 4-O-sulphates in human urine using a photochemical and electrochemical detector coupled with post-column hydrolysis, respectively. However, a more sensitive and specific method is required for the determination of their plasma levels.

Hamaji and Seki [10] reported a highly sensitive and specific method for the fluorimetric detection of free dopamine in plasma. By combination of this fluorimetric method with anion-exchange HPLC and post-column hydrolysis, we have developed a direct assay that is sufficiently sensitive for the measurement of the plasma levels of dopamine sulpho-conjugated isomers. Using this method, we have demonstrated the presence of both dopamine-3- and -4-O-sulphate in plasma and urine from normal human subjects.

EXPERIMENTAL

Dopamine-3- and -4-O-sulphate

Dopamine-3- and -4-O-sulphate were synthesized by the method of Jenner and Rose [11] with some modifications as follows.

Dopamine hydrochloride (0.4 g) (Sigma, St. Louis, MO, U.S.A.) was added with stirring during 20 min to 1.1 ml of sulphuric acid (analytical-reagent grade) (Wako, Osaka, Japan) in a small test-tube in an ice-bath. The reaction mixture was then quickly poured into 10 ml of ice-cold water with vigorous stirring. The diluted solution was promptly applied to a Dowex 50-X8 column (40 × 1.2 cm I.D., 200—400 mesh, H^+) and eluted with distilled water. The absorbance of the eluate was monitored spectrophotometrically at 280 nm. After discarding about 40 ml of the eluate, the sulphated dopamines were eluted in a volume of about 30 ml of eluate. This fraction was reduced to 10 ml in vacuo at 30°C and then applied to a Dowex 1-X2 column (30 × 2.0 cm I.D., 200—400 mesh, acetate form) and eluted with 0.2 *M* acetic acid. A 20-μl portion of each fraction (9 ml) of the eluate was analysed by reversed-phase HPLC [9] with a UV detector to determine the elution profiles of the two isomers. Fig. 1 shows typical elution patterns of the 3- and 4-O-sulphates on a Dowex 1 column. Fractions corresponding to the 3- and 4-O-isomers were evaporated to dryness and then recrystallized from hot ethanol—water (4:1, v/v) and 0.05 *M* acetic acid, respectively. Structural assignment of the final products was achieved by high-resolution ^1H NMR spectroscopy (Nicolet Model NT-360) [12]. The purity of the final products was over 95%, as judged by HPLC and NMR spectrometry.

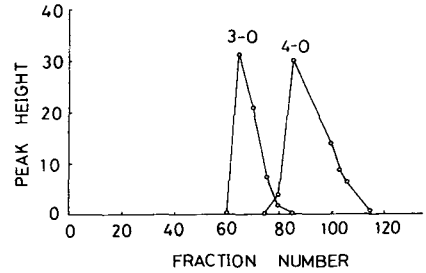

Fig. 1. Separation and purification of chemically synthesized dopamine sulphates by Dowex 1 column chromatography. Partially purified sulphated dopamines from a Dowex 50-X8 column were applied to a Dowex 1-X2 column (30 × 2.0 cm I.D.). A portion of each 9-ml fraction was injected into a reversed-phase HPLC column coupled with a UV detector, and the absorbance at 280 nm was monitored. The peak heights corresponding to dopamine-3-(3-O) and -4-O-sulphate (4-O) on the chromatogram were plotted against the fraction number. The isomers were identified by ^{1}H NMR analysis of the final purified preparations.

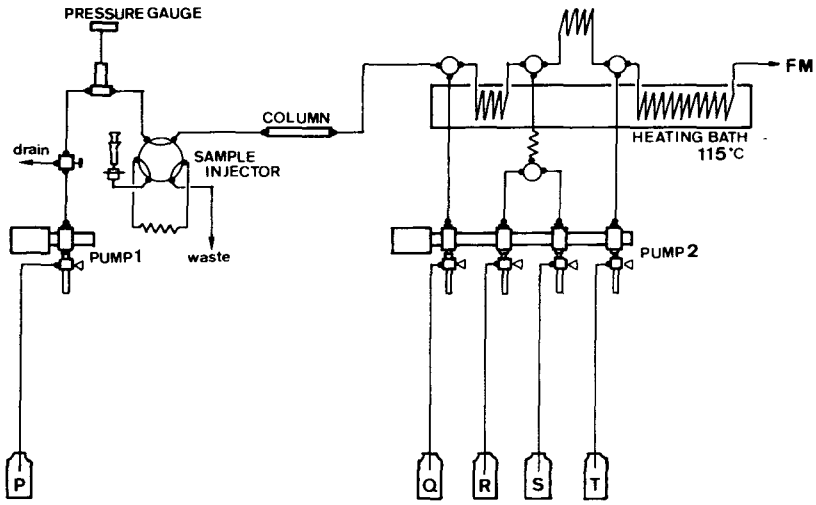

Fig. 2. Flow diagram of the HPLC system. Column: TSK-gel DEAE2SW (5 μm, 25 cm × 4.6 mm I.D.). Mobile phase: (P) 25 m*M* sodium phosphate buffer, pH 6.5, 0.5 ml/min. Reagents: (Q) 0.92 *M* perchloric acid, 0.15 ml/min; (R) 0.6% *p*-aminobenzoic acid, pH 6.3, 0.20 ml/min; (S) 1.3 *M* sodium hydroxide—0.4 *M* boric acid mixture, 0.20 ml/min; (T) 0.7% potassium hexacyanoferrate(III), pH 9.3, 0.20 ml/min. FM = Fluoromonitor. The excitation and emission wavelengths were 475 and 515 nm, respectively.

Chromatographic apparatus and conditions

Fig. 2 shows a schematic diagram of the chromatographic system. The mobile phase (25 m*M* sodium phosphate buffer, pH 6.5) was delivered at 0.5 ml/min using a constant-flow pump (pump 1) (LDC Constametric II; ATTO, Tokyo, Japan). The sample injector was a Rheodyne Model 7125 valve; the stainless-steel column was pre-packed with an anion exchanger (TSK-gel DEAE2SW, 5 μm, 25 cm × 4.6 mm I.D.; Toyo Soda, Tokyo, Japan). The plungers of a quadruple plunger pump (pump 2 in Fig. 2) (Model SF-4; Toyo Soda) served to deliver reagents Q, R, S and T independently. The column

eluate was first mixed with 0.92 *M* perchloric acid (Q, 0.15 ml/min) through polytetrafluoroethylene tubing (10 m × 0.5 mm I.D.) at 115°C in a closed water-bath. To the acid-hydrolysed eluate, 0.6% (w/v) *p*-aminobenzoic acid solution (R, 0.20 ml/min, adjusted to pH 6.3 with 6 *M* sodium hydroxide) and 1.3 *M* sodium hydroxide containing 0.4 *M* boric acid (S, 0.20 ml/min) were added with mixing in tubing (2 m × 0.5 mm I.D.). Finally, 0.7% (w/v) potassium hexacyanoferrate(III) solution (T, 0.20 ml/min, adjusted to pH 9.3 with 6 *M* sodium hydroxide) was added with mixing in a long reaction tube (20 m × 0.5 mm) at 115°C to develop the fluorophore. The pH of the final reaction mixture was 9.3.

The fluorescence intensity was measured at 515 nm using an excitation wavelength of 475 nm in a spectrofluorimeter (Model 650-10LC; Hitachi Seisakusho, Tokyo, Japan) equipped with a 90-μl square flow cell and a recorder (Model 056; Hitachi Seisakusho).

Sample preparation and clean-up

Blood was drawn from the antecubital vein of healthy adults into a heparinized syringe at the mid-point during collection of a 1-h urine sample. The blood was promptly transferred into an ice-chilled centrifuge tube containing a mixture of 0.2 *M* disodium EDTA and 0.2 *M* $Na_2S_2O_5$ (10 μl/ml of blood; adjusted to pH 6—7 with 6 *M* sodium hydroxide). After gentle mixing by inversion of the tube, plasma was separated by centrifugation at 4000 *g* for 10 min at 4°C. To 1.0 ml of the plasma, 0.5 ml of 2.5% perchloric acid was added and the mixture was stored at −80°C until taken for analysis. Under these conditions the two isomers were stable for at least one month. The mixture was centrifuged at 10 000 *g* for 30 min at 4°C and then the deproteinized supernatant (1.0 ml) was adjusted to pH 7.0 by dropwise addition of a mixture of 2 *M* potassium hydroxide and 1 *M* potassium dihydrogen phosphate. Insoluble potassium perchlorate was removed by brief centrifugation, and the supernatant was applied to a small column of Dowex 1-X2 (20 × 4 mm I.D., 200—400 mesh, acetate form). The column was washed with 2.0 ml of distilled water and then material was eluted with 4.0 ml of 0.5 *M* acetic acid. The eluate was introduced directly into a second column of Dowex 50-X8 (20 × 4 mm I.D., 200—400 mesh, H^+) and the flow-through fraction was collected and evaporated to dryness. The residue was dissolved in 250 μl of distilled water and 50—200 μl of the solution were injected into the HPLC system.

The procedure for partial purification of urine samples (1.0 ml) was the same as that for plasma, except that deproteinization and final evaporation were not required, and 100 μl of the eluate from the Dowex 50 column were injected directly into the HPLC system.

The recoveries of the partial purification procedure were determined from the peak heights of samples spiked with known amounts of the two isomers and subjected to the same procedure.

RESULTS AND DISCUSSION

Figs. 3 and 4 show typical chromatograms of dopamine-3- and -4-O-sulphate

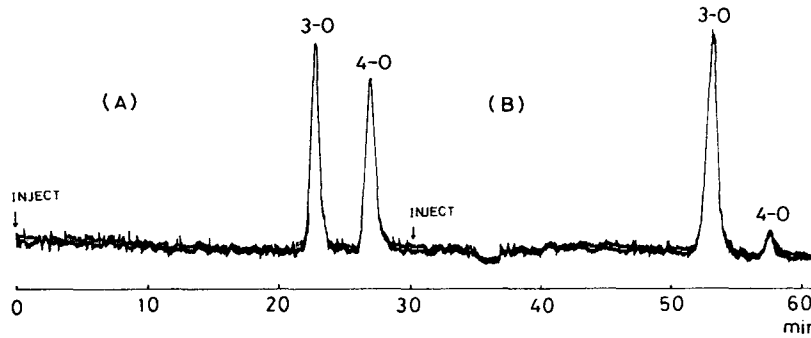

Fig. 3. Chromatograms of standard and plasma samples. (A) Elution pattern of the standard mixture containing 2.5 pmol each of dopamine-3- (3-O) and -4-O-sulphate (4-O). (B) Elution pattern of a plasma sample. The volume of sample injected corresponds to 133.3 μl of plasma and the plasma concentrations corrected for the recovery rates are 24.9 pmol/ml for 3-O and 2.9 pmol/ml for 4-O, respectively.

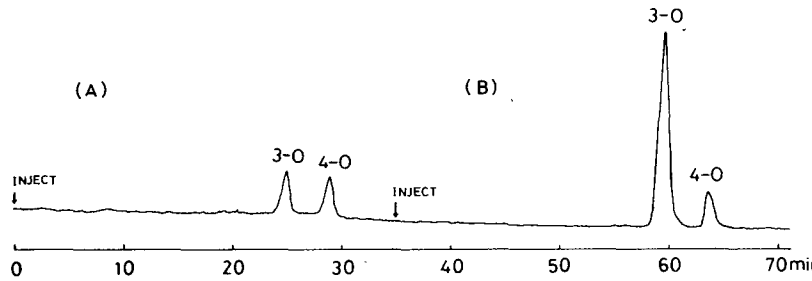

Fig. 4. Chromatograms of standard and urine samples. (A) Elution pattern of the standard mixture containing 5 pmol each of dopamine-3- (3-O) and -4-O-sulphate (4-O). (B) Elution pattern of a urine sample. The amount of sample injected corresponds to 20 μl of urine and the urinary concentrations corrected by the recovery rates are 4.46 nmol/ml for 3-O and 0.86 nmol/ml for 4-O, respectively.

in normal human plasma and urine. The two isomers were well separated and no interfering peaks appeared on the chromatogram. As the fluorimetric detection method using *p*-aminobenzoic acid is very specific for dopamine [7], it is also possible to determine the isomers by injecting 10 μl of the urine or 100—200 μl of the deproteinized plasma directly into the HPLC system without any purification procedures (Fig. 5), although with this procedure the column lifetime is decreased.

The amounts of the two isomers injected in the range 1—20 pmol are linearly related to the fluorescence intensities expressed as peak heights. The linear regression equations and correlation coefficients for dopamine-3- and -4-O-sulphate were $y = 0.379x + 0.129$ ($r = 0.99962$) and $y = 0.368x - 0.004$ ($r = 0.99983$), respectively. The detection limit (signal-to-noise ratio = 2) was 0.3 pmol for each isomer.

When 12.5 pmol of each isomer were added to 1.0 ml of pooled plasma, the overall recoveries were 78.3 ± 3.2% for dopamine-3-O-sulphate and 79.9 ± 1.3% for dopamine-4-O-sulphate ($n = 5$). The between-day (days 1, 2, 3, 7 and 14) coefficient of variation was 9.6% for dopamine-3-O-sulphate and 5.0%

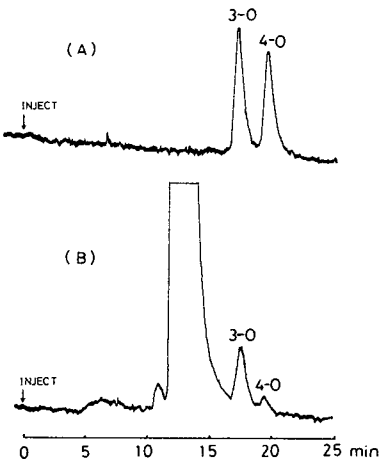

Fig. 5. Chromatogram of plasma sample injected directly into the HPLC column without any clean-up. (A) Elution pattern of the standard mixture containing 5 pmol each of dopamine-3- (3-O) and -4-O-sulphate (4-O). (B) Elution pattern of 200 μl of the deproteinized plasma injected directly into the HPLC column. The plasma concentrations for 3-O and 4-O are 21.8 and 4.4 pmol/ml, respectively.

TABLE I

PLASMA LEVELS AND URINARY EXCRETION RATES OF DOPAMINE-3- AND 4-O-SULPHATE IN NORMAL HUMAN SUBJECTS

Values are means ± S.D. (n = 10 for plasma; n = 5 for urine).

Dopamine sulphate	Plasma level (pmol/ml)	Urinary excretion rate (nmol/min)
3-O-Sulphate	26.5 ± 11.1	1.73 ± 0.56
4-O-Sulphate	2.68 ± 0.34	0.27 ± 0.04

for dopamine-4-O-sulphate, and the within-day coefficient of variation was less than 5% for both isomers (n = 5).

As shown in Table I, the mean concentrations of dopamine-3- and -4-O-sulphate in normal human plasma were 26.5 ± 11.1 and 2.68 ± 0.34 pmol/ml (n = 10), respectively. The mean urinary excretion rates in normal subjects were 1.73 ± 0.56 for the 3-O-sulphate and 0.27 ± 0.04 nmol/min for the 4-O-sulphate. These data are in good agreement with those determined by enzymatic hydrolysis [1—3] (plasma levels) and with an HPLC—photochemical detector [8] (urine levels).

The ratios of dopamine-3- to -4-O-sulphate in the plasma and urine obtained in this work were almost the same. Further, the clearance rates of the two isomers calculated from the present data (85—100 ml/min) are nearly equal to that of creatinine. These results suggest that the urinary levels of dopamine sulphates directly reflect the plasma concentrations of the isomers in man.

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CHROMBIO. 2637

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF GUANIDINO COMPOUNDS USING NINHYDRIN REAGENT

II. GUANIDINO COMPOUNDS IN BLOOD OF PATIENTS ON HAEMODIALYSIS THERAPY

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SUMMARY

An automated high-performance liquid chromatographic method using alkali-ninhydrin reagent for the post-column derivatization of guanidines has been developed. This procedure was applied to measurements made before and after haemodialysis. Among the guanidino compounds found in human blood, methylguanidine showed the lowest removal rate. The removal rate of guanidinosuccinic acid correlated with the plasma α_1-globulin fraction. The removal rate of each guanidino compound decreased with the period of dialysis.

INTRODUCTION

Guanidino compounds in body fluids are important markers of renal disorder, and the measurement of these compounds gives useful information about renal function and the effect of haemodialysis [1—10].

As guanidino compounds are very similar in chemical properties, and their amounts in biological fluids are very small, complete separation and sensitive detection have been investigated using high-performance liquid chromatography (HPLC) coupled with fluorometric detection. The 9,10-phenanthrenequinone method [9—12] has been developed for this purpose, but the reagent is sparingly soluble in water, and large amounts of organic solvents are required in order to prevent precipitation. We have developed the ninhydrin method [13], and Kai and co-workers [14—16] have developed the benzoin method to overcome this problem.

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This paper describes a fully automated HPLC system for the analysis of guanidino compounds using the ninhydrin reagent. This system was applied to the determinination of guanidino compounds in the sera of haemodialysed patients at the stable stage before and after dialysis. Relationships between the amounts of guanidino compounds and the period of haemodialysis or the clinical chemical data are also discussed.

MATERIALS AND METHODS

Reagents and standards

A standard solution for chromatography was prepared by adding 2 ml of aqueous creatine solution (20.74 μg/ml) and 2 ml of 0.1 *M* hydrochloric acid to the standard mixture of guanidino compounds purchased from Wako (Tokyo, Japan). Ninhydrin, sodium hydroxide, sodium citrate and boric acid were of amino-acid-analysis grade. Reagent-grade sodium chloride and analytical-grade 60% perchloric acid were used. All the reagents were purchased from Wako.

Chromatography

Fig. 1 shows the flow diagram of the automated HPLC system for the determination of guanidino compounds. A sample was injected into the chromatograph from an autosampler, Model SIL-2A (Shimadzu, Kyoto, Japan) on which 48 samples can be loaded at one time. Stepwise gradient elution was begun as the sample was injected, and was carried out using the following mobile phases: elution buffer 1, 0.05 *M* trisodium citrate (pH 3.5); elution buffer 2, 0.12 *M* trisodium citrate (pH 5.0); elution buffer 3, 0.12 *M* trisodium citrate (pH 6.0); elution buffer 4, 0.12 *M* trisodium citrate solution containing 0.5 *M* sodium chloride and 0.1 *M* boric acid (pH 11.4). Eluents 5 and 6, for column-washing, were 0.2 *M* sodium hydroxide and redistilled water, respectively. The pH of eluents 1, 2 and 3 were adjusted with 60% perchloric acid, and that of eluent 4 was adjusted with 1.0 *M* sodium hydroxide. All the eluents were filtered with microfilter FM 22 (pore size, 0.22 μm; Fuji Film, Tokyo, Japan) prior to use. The flow-rate of the mobile phase was 0.7 ml/min. The gradient programme was as follows: eluent 1 for 7 min; eluent 2 for 7 min; eluent 3 for 3 min; eluent 4 for 15 min; eluent 5 for 2 min; eluent 6 for 3 min. The

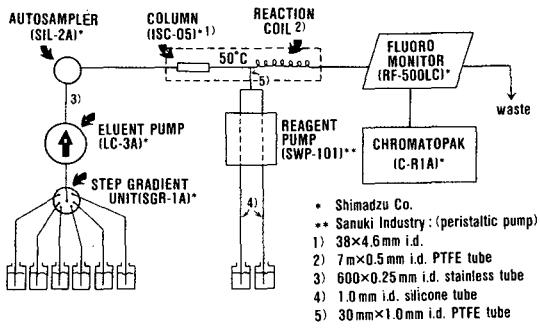

Fig. 1. Flow diagram of the HPLC system.

guanidino compounds were separated on an ion-exchange column ISC-05 (Shimadzu, Kyoto, Japan). Post-column derivatization was accomplished as follows. Aqueous 0.6% ninhydrin solution filtered with microfilter FM 22 and 1.5 *M* sodium hydroxide filtered with microfilter FR 20 (pore size, 0.20 μm; Fuji Film) were delivered with a peristaltic pump (Sanuki Industry, Tokyo, Japan) at a flow-rate of 0.4 ml/min and pre-mixed immediately before they were mixed with the eluate from the column. The resultant mixture was then heated in a reaction coil (7 m × 0.5 mm I.D.) placed in a water-bath kept at 50°C, and the fluorescence intensity was measured at 395 nm (excitation) and 500 nm (emission) with a fluorescence spectrophotometer RF-500-LC (Shimadzu). Quantitation was carried out with a data processor, Chromatopak C-R1A (Shimadzu). Retention times, peak height and the name and amount of each compound were recorded.

Samples

Sera of uraemic patients (31 male and 26 female) before and after haemodialysis were supplied from the Sagamidai Hospital. All haemodialysis was carried out with hollow fibres. Of the dialyses, 30% were by bicarbonate dialysis and the others by acetate dialysis. Hyland Q-PAK® Control serum I (Travenol, Tokyo, Japan) was used as a reference serum.

The sera were deproteinized prior to use as follows. To 100 μl of a serum were added 20 μl of 60% trichloroacetic acid. The resultant mixture was stirred then centrifuged at 3000 *g* for 5 min. To a 60-μl aliquot of the supernatant were added 25 μl of 0.8 *M* sodium hydroxide to bring the pH of the solution to between 2.0 and 2.5. The mixture was then chromatographed.

RESULTS AND DISCUSSION

Automated system for the determination of guanidino compounds

Ten guanidino compounds, taurocyamine (TAU), guanidino succinic acid (GSA), creatine (CT), guanidinoacetic acid (GAA), guanidinopropionic acid (GPA), creatinine (CTN), guanidinobutyric acid (GBA), arginine (ARG), guanidine (G), methylguanidine (MG), were separated in 37 min by the automated HPLC system shown in Fig. 1. Table I shows the day-to-day precision of the amount of guanidino compounds obtained from determination of the standard mixture. Coefficients of variation (n = 10) were in the range 0.7–3.0%. Table II shows the recovery of guanidino compounds from serum when the standard mixture added to the control serum was analysed. The recoveries were in the range 93.85—107.97%, and the coefficients of variation ranged from 0.37 to 1.28%.

Analysis of guanidino compounds in the sera of patients

Guanidino compounds in the sera of 57 haemodialysed patients in the stable stages were determined using the automated system. The data were used to calculate the reduction rate of guanidines as follows:

$$\text{rate (\%)} = 100 \times \frac{[G]_b - [G]_a}{[G]_b} \qquad (1)$$

TABLE I

DAY-TO-DAY PRECISION OF THE PRESENT METHOD FOR STANDARD SOLUTIONS OF GUANIDINO COMPOUNDS

n = 10 (for ten days) in all cases.

Compound	Amount added (pmol)	Amount found (mean ± S.D.) (pmol)	C.V. (%)
TAU	893.3	876.8 ± 5.964	0.680
GSA	893.3	915.9 ± 18.31	1.999
CT	1242.1	1248.8 ± 20.27	1.623
GAA	446.6	429.5 ± 8.160	1.899
GPA	446.6	428.6 ± 8.221	1.918
CTN	4466.3	4428.6 ± 66.22	1.495
GBA	893.3	893.5 ± 17.36	1.943
ARG	1786.5	1732.1 ± 23.15	1.337
G	1786.5	1663.9 ± 40.52	2.435
MG	446.6	425.8 ± 12.70	2.983

TABLE II

RECOVERIES OF GUANIDINO COMPOUNDS FROM HUMAN SERUM AND THEIR PRECISIONS WITHIN DAY

n = 10 in all cases.

Compound	Amount added (pmol)	Percentage recovery (mean ± S.D.)	C.V. (%)
TAU	893.3	107.97 ± 0.852	0.79
GSA	893.3	97.21 ± 0.553	0.57
CT	1242.1	100.59 ± 1.016	1.01
GAA	446.6	97.57 ± 0.850	0.87
GPA	446.6	96.06 ± 0.338	0.37
CTN	4466.3	99.95 ± 0.728	0.73
GBA	893.3	96.77 ± 1.126	1.16
ARG	1786.5	93.85 ± 0.730	0.78
G	1786.5	99.00 ± 1.040	1.05
MG	446.6	99.82 ± 1.279	1.28

where $[G]_b$ and $[G]_a$ represent the concentration of a guanidino compound before and after haemodialysis, respectively. GSA showed the highest reduction rate, 78.2—82.4%, and MG the lowest, 37.1—46.0%.

Table III shows the relationship between the reduction rate of guanidino compounds and the period of haemodialysis. The reduction rates did not fluctuate during the first ten years. However, the rate decreased for the sera of patients who had been undergoing prolonged haemodialysis therapy. After haemodialysis for over ten years, the rate decreased by 20—55% compared with that at the first stage (one to two years) of dialysis. The degree of decrease in the rate varied among the guanidino compounds. The removal rate of GSA did not decrease after haemodialysis over ten years, whereas the rate of removal of MG apparently did decrease.

TABLE III

COMPARISON OF MEAN REDUCTION RATE OF SERUM GUANIDINO COMPOUNDS IN THE CASES OF FOUR GROUPS CLASSIFIED BY THE PERIOD OF HAEMODIALYSIS TREATMENT

The reduction rates were calculated with eqn. 1.

Compound	Reduction rate (%) (mean ± S.D.)			
	Period (years)			
	<1 (n=4)*	1—5 (n=25**)	5—10 (n=25***)	>10 (n=3§)
TAU	51.24 ± 11.62	40.23 ± 14.97	38.93 ± 8.06	23.72 ± 15.97
GSA	79.94 ± 5.58	78.19 ± 8.72	79.83 ± 5.39	82.37 ± 2.55
CT	29.25 ± 23.87	31.93 ± 16.72	31.70 ± 25.51	15.55 ± 11.58
GAA	49.95 ± 19.07	46.77 ± 17.87	51.89 ± 9.86	36.82 ± 22.52
GPA	—	43.16 ± 6.53 (n=3)	45.64 ± 21.79 (n=6)	43.83 ± 19.72 (n=2)
CTN	55.67 ± 10.46	58.55 ± 7.60	59.15 ± 6.73	43.47 ± 22.40
GBA	41.25 (n=1)	46.31 ± 10.13 (n=7)	52.81 ± 19.05 (n=9)	32.65 (n=1)
ARG	65.56 ± 4.11	54.12 ± 8.96	57.19 ± 14.26	47.16 ± 20.70
G	47.82 ± 34.00	52.30 ± 10.35	49.05 ± 13.98	54.39 ± 12.10
MG	40.94 ± 9.14	45.95 ± 10.32	44.11 ± 9.41	37.13 ± 11.64

*One male, three females.
**Ten males, fifteen females.
***Eighteen males, seven females.
§Two males, one female.

TABLE IV

COMPARISON OF MEAN CONCENTRATION VALUES OF SERUM GUANIDINO COMPOUNDS IN THE CASES OF FOUR GROUPS CLASSIFIED BY THE PERIOD OF HAEMODIALYSIS TREATMENT

Compound	Concentration (μg/dl) (mean ± S.D.)			
	Period (years)			
	<1 (n=4*)	1—5 (n=25**)	5—10 (n=25***)	>10 (n=3§)
TAU	252.4 ± 68.2	335.2 ± 74.2	375.9 ± 66.9	365.8 ± 151.4
GSA	415.9 ± 299.0	400.1 ± 233.5	465.6 ± 161.1	531.1 ± 145.6
CT	546.0 ± 227.1	560.3 ± 254.4	399.4 ± 164.1	423.4 ± 121.1
GAA	37.23 ± 10.42	36.90 ± 17.59	38.98 ± 16.93	39.56 ± 14.46
GPA	12.85 ± 2.80 (n=2)	15.32 ± 3.15 (n=17)	14.83 ± 10.31 (n=14)	20.90 ± 5.75 (n=2)
CTN§§	8.12 ± 6.59	12.72 ± 3.36	13.47 ± 2.07	13.27 ± 3.80
GBA	9.87 (n=1)	12.05 ± 5.81 (n=19)	10.72 ± 3.12	9.03 ± 0.72
ARG	2.210 ± 0.796	2.534 ± 0.821	2.288 ± 0.348	2.070 ± 0.223
G	13.26 ± 4.55	17.65 ± 6.15	23.03 ± 10.00	32.18 ± 17.75
MG	25.66 ± 20.64	48.97 ± 24.97	61.05 ± 15.20	58.00 ± 17.07

*One male, three females.
**Ten males, fifteen females.
***Eighteen males, seven females.
§Two males, one female.
§§Concentration of CTN in mg/dl.

Table IV shows the relationship between the concentration of guanidino compounds in sera immediately before dialysis and the period of haemodialysis. The concentration increased with the period of haemodialysis. The rate of removal depends on several external factors, such as the method or equipment for dialysis.

The present study examined the relationship between the reduction rate of guanidino compounds and the clinical chemical data of the patients. Several chemical data that showed significant correlation coefficients ($p < 0.01$) are summarized in Table V. The α_1-globulin value showed a correlation with GSA

TABLE V

CORRELATION COEFFICIENTS OF VARIOUS CHEMICAL DATA

Dependent	Parameter	Correlation coefficient ($P < 0.01$)
TAU (%)	Urine volume (l per 24 h)	0.4272
GSA (%)	Age	−0.3583
	Haemodialysis (h/week)	0.4561
	Flow-rate	0.3150
	α_1-G	−0.6007
	P-lipid	−0.3404
	LDH	−0.3607
CT (%)	—	—
GAA (%)	α_1-G	−0.3341
	CPK	0.3403
GPA (%)	α_2-G	−0.3393
CTN (%)	Haemodialysis (h/week)	0.4944
	α_1-G	−0.3050 ($P < 0.05$)
GBA (%)	ALP	0.3055 ($P < 0.05$)
ARG (%)	—	—
G (%)	Urine volume (1 per 24 h)	−0.4106
	Haemodialysis (h/week)	0.3760
MG (%)	α_1-G	−0.3872

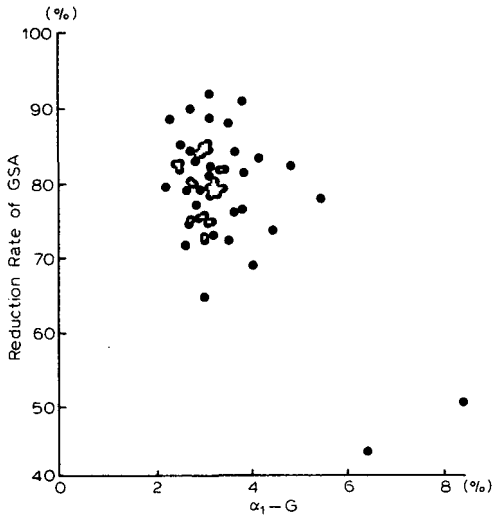

Fig. 2. Correlation between α_1-globulin fraction and reduction rate of GSA: $y = -0.5042x$ (± 1.813) + 95.28 (± 6.255); $n = 57$, $r = 0.601$, S.D. = 6.755, $F = 31.36$ ($> F_0^{0.05} = 4.018$).

and MG (e.g. Fig. 2). This indicates that an increase in the amount of α_1-globulin in blood results in a reduction of the removal of GSA and MG from blood. A number of basic drugs have been shown to have a strong affinity for α_1-acid glycoprotein. α_1-Acid glycoprotein, one of the α_1-globulin fractions, is an acute phase reactant [18, 19], and this fraction, as well as albumin, may play an important role in the protein binding of guanidino compounds, which are strongly basic compounds.

Native fluorescence of sera of uraemic patients

Some of the sera of patients suffering from renal disfunction was found to show strong fluorescence [20], with excitation and emission maxima at 330 and 425 nm. We have examined the peak with native fluorescence by replacing the post-column derivatization reagent (ninhydrin and sodium hydroxide) with redistilled water. When the wavelengths of the fluorometer were set at 330 nm (excitation) and 425 nm (emission), many peaks were detected. These peaks may interfere with the analysis of guanidines with benzoin, the reaction product of which shows excitation and emission maxima at 325 and 425 nm. In contrast, these naturally fluorescent materials were found not to interfere with the determination of guanidino compounds with the ninhydrin reagent, because the reaction product showed the excitation and emission maxima at 395 and 500 nm. The use of ninhydrin reagent in the present system was thus shown to be effective for samples with natural fluorescence.

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STABILITY OF PYRIDOXAL-5-PHOSPHATE SEMICARBAZONE: APPLICATIONS IN PLASMA VITAMIN B_6 ANALYSIS AND POPULATION SURVEYS OF VITAMIN B_6 NUTRITIONAL STATUS

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SUMMARY

The determination of pyridoxal-5-phosphate (PLP) and pyridoxal (PL) in plasma requires the availability of dark room facilities, due to the photosensitivity of these vitamin B_6 vitamers. The fact that the semicarbazone forms of PL and PLP are more strongly fluorescent than the underivatized B_6 vitamers has been exploited in plasma analyses, but it was not previously realised that these semicarbazone forms are also very stable even under conditions that lead to rapid decomposition of free PL and PLP. The stabilisation of PLP and PL obtained in this manner is sufficient and fully adequate to meet the practical requirements of clinical field studies. We report a high-performance liquid chromatographic method for plasma PLP and PL determinations based on precolumn semicarbazone formation and fluorescence detection. The method is sensitive enough for quantitative plasma PLP determinations even in B_6-deficient patients.

INTRODUCTION

The analytical problems associated with the determination of vitamin B_6 (B_6) vitamers in biological material are many and varied and these have been studied for decades. The disadvantages of microbiological assays, which includes the lack of selectivity, have been summarised recently [1].

Since pyridoxal-5-phosphate (PLP) is the physiologically active form of vitamin B_6 and since plasma PLP measurements afford a good indication of B_6 nutritional status [2], several alternative methods have been developed for PLP estimations in plasma or whole blood. A complicating aspect of such analyses is the fact that the B_6 vitamers are water-soluble, which prohibits the use of extraction procedures as a means of purification. Direct fluorimetric

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determination of PLP and pyridoxal (PL) in plasma is either unreliable due to interfering fluorescent plasma compounds [3, 4], or tedious due to the requirement of sample clean-up procedures like column and/or thin-layer chromatography [5—7].

Several methods based on high-performance liquid chromatography (HPLC) for the separation of some or all B_6 vitamers have been described [8—12] but these are not suitable for routine measurement of blood levels, due to lack of sensitivity [13]. Direct HPLC analyses of plasma samples and fluorimetric quantification of all six B_6 vitamers [14, 15] have been reported, but these methods require sophisticated HPLC equipment and/or complicated column-switching procedures and are thus in general not suitable for clinical applications where a simple but highly sensitive method for the determination of PLP is required. Schrijver et al. [1] reported a rapid and sensitive HPLC method based on post-column semicarbazone formation of PLP and PL followed by fluorescence detection, but this method suffers from the disadvantage that no internal standard was used.

All of the above mentioned HPLC methods suffer from the further disadvantage that the analyses have to be carried out in a specially equipped dark room [14, 15], due to the photosensitivity of PLP and PL. This requirement has obvious disadvantages in the application of these methods in an ordinary laboratory. Moreover, the recent upsurge of interest in B_6 as a possible aetiological agent in the development of coronary artery disease [16, 17] requires the availability of a practical procedure to determine the B_6 status of various population groups. The instability and photosensitivity of the B_6 vitamers is thus a serious problem in conducting field studies and population screening surveys. In this study we show that PLP semicarbazone (PLPSC) is sufficiently stable under ordinary conditions of light and temperature to form the basis of a new isocratic HPLC method, based on previous work by Gregory [18], which fulfills all of the above-mentioned requirements.

An important and distinguishing feature of the method is the introduction of 6-methyl-2-pyridine carboxaldehyde semicarbazone (MPCSC) as an internal standard. This compound is a suitable internal standard which greatly improves the utility and precision of the method under practical conditions.

EXPERIMENTAL

Reagents

PL and PLP were obtained from Merck (Darmstadt, F.R.G.). The internal standard, 6-methyl-2-pyridine carboxaldehyde (MPC), was bought from Aldrich (Milwaukee, WI, U.S.A.). Chromatography-grade dichloromethane and acetonitrile were supplied by Merck. All other chemicals were analytical-reagent grade and obtained from Merck.

Purification of PL and PLP

The commercial PLP and PL preparations were purified by reversed-phase HPLC (Whatman Partisil 10 ODS-3 column; mobile phase: 10% methanol and 0.1% glacial acetic acid in water) and then lyophilized. Purified PLP and PL were checked for impurities by reversed-phase ion-pair chromatography [10],

UV detection (290 nm) and wavelength scanning (210—360 nm) at different stages of peak elution. UV absorption spectra of the purified PLP and PL were determined in 0.1 *M* hydrochloric acid, 0.1 *M* sodium hydroxide and 0.1 *M* sodium phosphate buffer, pH 7.0.

Standards

Purified PLP and PL were used to prepare working standards containing 1—20 ng PLP and PL per ml. To prepare the internal standard, 6-methyl-2-pyridine carboxaldehyde was dissolved in 25 ml of 0.1 *M* sodium dihydrogen phosphate; 20 ml of 0.5 *M* semicarbazide solution was added and the mixture was heated for 20 min at 40—45°C. After cooling, 0.1 *M* disodium hydrogen phosphate was added to adjust the pH to 7.0; the volume was adjusted to 500 ml using Na_2HPO_4—NaH_2PO_4 buffer (0.1 *M*; pH = 7.0). The MPCSC solution was then divided into 2.0-ml aliquots, freeze-dried, sealed under nitrogen and stored at −20°C.

Sample preparation

To 1.0 ml plasma, 50 μl reconstituted MPCSC solution as internal standard were added. Plasma proteins were precipitated by the addition of 0.5 ml 10% trichloroacetic acid (TCA) while mixing vigorously. To the clear supernatant obtained after centrifugation, 50 μl of 0.5 *M* semicarbazide were added and the mixture was incubated at 40°C for 10 min. Prior to injection, the supernatant was extracted twice with 3.0 ml diethyl ether; the diethyl ether was aspirated and the water phase was extracted with 3.0 ml dichloromethane. A 100-μl aliquot of the supernatant was injected onto the column for analysis.

Instrumentation

A Perkin-Elmer Series 2 liquid chromatograph was slightly modified so that one pump was used for solvent delivery and the other pump for post-column reagent addition. An LS 4 Perkin-Elmer fluorescence spectrometer (excitation wavelength: 367 nm; emission wavelength: 478 nm) was coupled to a Perkin-Elmer Sigma 10 chromatography data station.

Columns

A Whatman (Clifton, NJ, U.S.A.) Solvecon pre-column (25 cm × 4.6 mm) installed on-stream and before the injector, was followed by a Brownlee Labs. (Santa Clara, CA, U.S.A.) RP-18 Spheri-5 guard column (4 cm × 4.6 mm) and a Whatman Partisil 10 ODS-3 analytical column (25 cm × 4.6 mm).

Chromatographic conditions

A solution of 0.05 *M* potassium dihydrogen phosphate (pH adjusted to 2.9 with concentrated orthophosphoric acid) containing 7% acetonitrile was used as mobile phase. Sodium hydroxide (4%, w/v) was introduced for post-column alkalinisation. The flow-rates of the solvent delivery pump and the post-column reagent pump were 1.1 and 0.1 ml/min, respectively.

Sample stability

The following preparations were placed (15 cm) under a 100-W electric light

bulb, and at certain time intervals (t = 0, 1, 2, 3, 5, 6 and 24 h) a 1.0-ml aliquot was withdrawn and analysed as described under sample preparation. (a) $1.2 \cdot 10^{-7}$ M PLP; (b) a $1.2 \cdot 10^{-7}$ M PLP solution containing $2.5 \cdot 10^{-2}$ M semicarbazide; (c) $1.2 \cdot 10^{-7}$ M PLP in 3% TCA; (d) $1.2 \cdot 10^{-7}$ M PLP in 3% TCA containing $2.5 \cdot 10^{-2}$ M semicarbazide; (e) human plasma; (f) supernatant after the same human plasma sample had been treated with TCA to precipitate plasma proteins (TCA-supernatant); (g) TCA-supernatant containing $2.5 \cdot 10^{-2}$ M semicarbazide; (h) $1.65 \cdot 10^{-4}$ M MPC; and (i) MPC solution containing $2.5 \cdot 10^{-2}$ M semicarbazide.

Samples containing semicarbazide were pre-heated at 40°C for 20 min before exposure to light.

Plasma and TCA-supernatant were also kept at both 4°C and 25°C in the dark and 1.0-ml aliquots were analysed for PLP at time intervals described above.

A solution of TCA-supernatant, reacted with semicarbazide, was kept at 4°C in the dark and analysed daily to test the long-term stability of PLP.

RESULTS

The commercial preparations of PLP and PL were not completely pure, but reversed-phase HPLC was very effective in separating PLP and PL from minor impurities. Purified PLP and PL eluted as single peaks during reversed-phase ion-pair chromatography [10], and peak homogeneity was confirmed by wavelength scanning at different stages of peak elution. UV absorbance spectra of purified PLP and PL were in close agreement with previously published results [19].

Fig. 1 shows a typical separation of (a) a standard mixture of PLP and PL; (b) a normal human plasma sample pre-treated as described under Experimental; (c) the same plasma spiked with 4.0 ng PL and 6.0 ng PLP per ml plasma; and (d) plasma from a person taking an oral pyridoxine supplement of 20 mg per day.

The retention times of all three peaks shown were found to be very sensitive to the acetonitrile concentration in the solvent. The optimum acetonitrile concentration in the solvent differed when different columns were used and also changed with column ageing. Serum contains two background peaks eluting before the PLP peak, and the solvent acetonitrile concentration was chosen such that baseline resolution between the PLP peak and the background peaks was achieved.

The within-day precision of the method was determined by dividing a serum pool into fifteen 1-ml aliquots, and determining the PL and PLP concentrations in each aliquot. The coefficients of variation (C.V.) for PLP and PL were 5.9% and 8.1%, respectively. The day-to-day variation was determined by freeze-drying a serum pool in 1.0-ml aliquots. For twenty successive days a 1-ml aliquot was reconstituted for PLP and PL determination as previously described. The C.V. values of 11.8% and 12.5% which were found for PLP and PL, respectively, compares well with the within-day variation found.

Spiking of plasma with different amounts of PL and PLP indicated that the recovery of both vitamers was good. Table I shows that the recovery of PLP

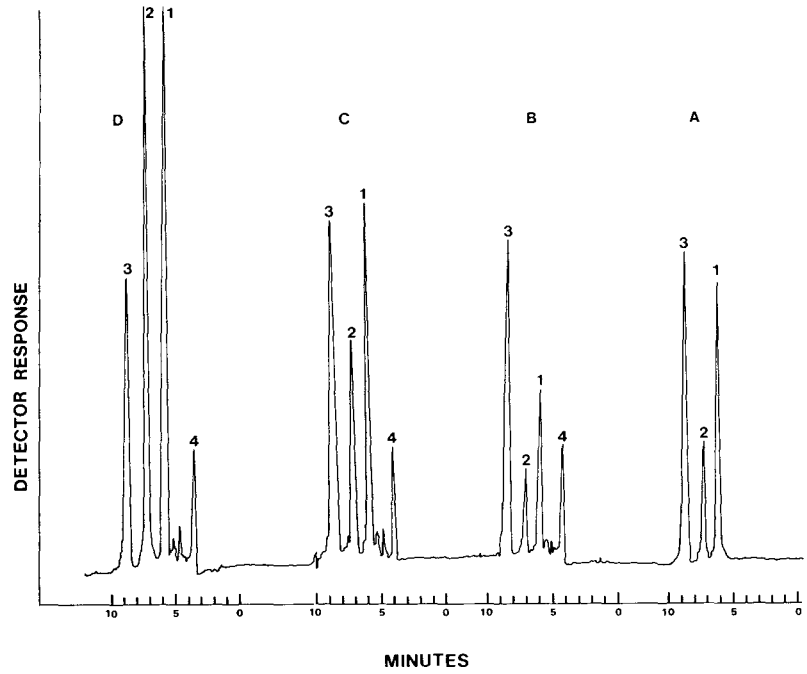

Fig. 1. The determination of PL and PLP in plasma using HPLC. (A) Standard, containing 10.0 ng PLP, 4.0 ng PL and 5.0 μg MPCSC per ml; (B) plasma sample from a normal, healthy person (PLP 5.7 ng/ml and PL 2.8 ng/ml); (C) the same plasma sample from B spiked with 6 ng PLP and 4 ng PL per ml plasma; (D) plasma sample from a person using oral pyridoxine supplements of 20 mg per day. Each sample was injected at t = 0 min. Peaks: 1 = PLPSC; 2 = PLSC; 3 = internal standard, MPCSC; 4 = plasma background.

TABLE I

RECOVERY OF PL AND PLP ADDED TO PLASMA

Concentration of vitamer added (ng/ml)	PLP found (ng/ml)	Percentage recovery	PL found (ng/ml)	Percentage recovery
0	6.8	—	0	—
4	10.5	92.5	3.6	90.0
5	11.7	98.0	5.1	102.0
8	14.9	101.3	6.5	81.3
10	16.9	101.0	8.8	88.0
20	26.0	96.0	20.0	100.0
Average (± S.D.)		97.8 ± 3.7		92.3 ± 8.6

was slightly better and more consistent than the recovery of PL, indicating that a small loss of PL may occur during the preparation procedure.

Routinely, 100-μl samples were used per injection, and reliable PLP detection at concentrations as low as 1 ng PLP per ml plasma was achieved. However, when using a 500-μl sample loop, the injection volume can be increased four-fold, thus setting the limit of sensitivity for PLP determinations at 0.25 ng PLP per ml plasma.

Plasma samples from 28 healthy, middle-aged (age ± 1 S.D.: 46.2 ± 9.6 years) men were analysed for PL and PLP and the plasma levels were 1.94 ± 1.12 and 11.44 ± 6.31 ng/ml, respectively, thus indicating that normal plasma PL and PLP levels are well within the sensitivity limit of this method.

The stability of PLP was studied under various conditions. In Figs. 2—4, the PLP concentration at $t = 0$ was assumed to be 100%. Fig. 2 indicates that PLP was stable in plasma for at least 6 h if the plasma was kept in the dark at either 4°C or room temperature. After 24 h, however, up to 10% loss of PLP from plasma was evident. However, direct light from a 100-W electric light bulb as described above results in a high loss of PLP from plasma due to PLP photodecomposition [15].

Fig. 3 shows that although a standard solution of PLP was prone to photodecomposition whether or not TCA was added to the solution, PLPSC was stable for a whole working day and after 24 h the loss was only 10% for a pure PLPSC solution. The same observation was made for the supernatant obtained after plasma protein precipitation (Fig. 4).

Fig. 4 indicates that supernatant treated with semicarbazide as described under Experimental, was stable under direct lighting conditions, while the PLP of an untreated supernatant was prone to photodecomposition. In fact, a supernatant treated with semicarbazide and stored at 4°C in the dark gave consistent results for PLP and PL determinations for at least 28 successive days.

As indicated in Fig. 5, the same principle applies in the case of internal standard: MPC concentration decreased when a solution containing MPC was placed directly under a 100-W electric light bulb, but the semicarbazide derivative concentration remained constant under these conditions.

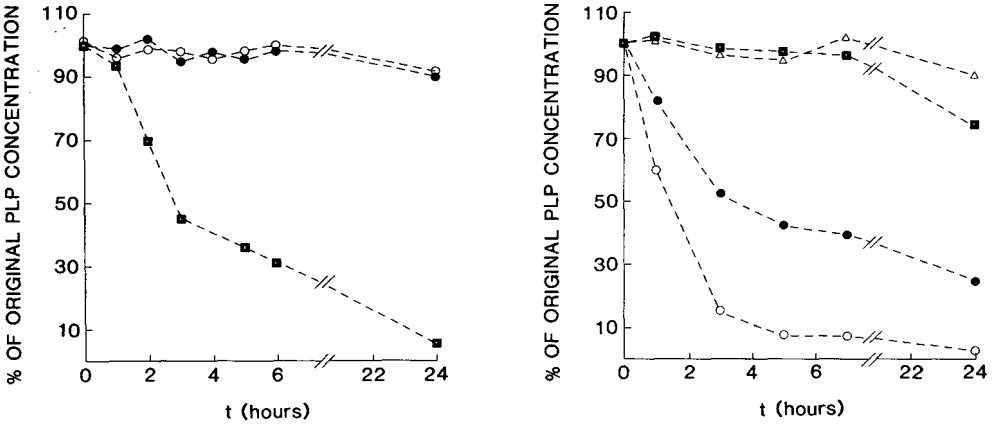

Fig. 2. The stability of PLP in heparin-plasma. The results presented are the average of three independent experiments. (•) Heparin-plasma stored in the dark at room temperature; (○) heparin-plasma stored at 4°C (dark); (▪) heparin-plasma stored under a 100-W electric light bulb (15 cm).

Fig. 3. Stability of different PLP solutions under a 100-W electric light bulb (15 cm). (○) $1.2 \cdot 10^{-7}$ *M* PLP; (△) $1.2 \cdot 10^{-7}$ *M* PLP in $2.5 \cdot 10^{-2}$ *M* semicarbazide solution; (▪) 3% TCA, containing $1.2 \cdot 10^{-7}$ *M* PLP and $2.5 \cdot 10^{-2}$ *M* semicarbazide; (•) 3% TCA, containing $1.2 \cdot 10^{-7}$ *M* PLP.

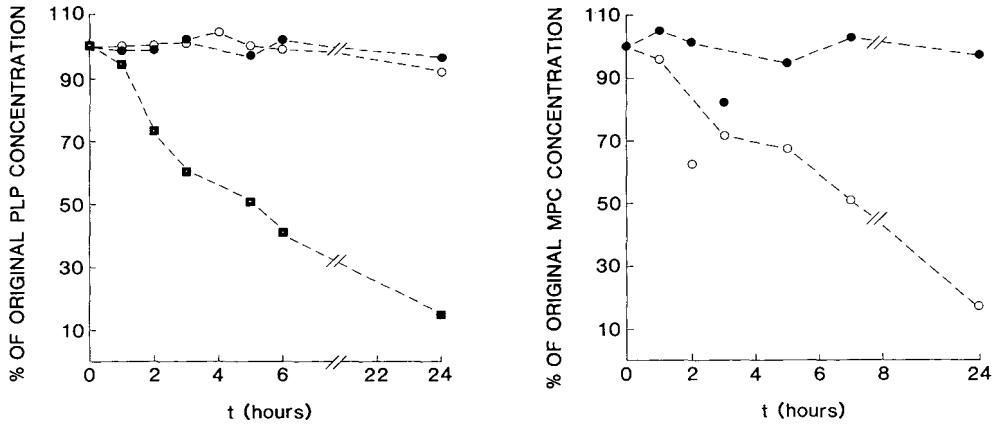

Fig. 4. Stability of PLP in the supernatant after precipitation of plasma proteins with 10% TCA. Supernatant stored at 4° C in the dark (○); at room temperature under a 100-W electric light bulb (15 cm) (■); and containing $2.5 \cdot 10^{-2}$ *M* semicarbazide, stored under a 100-W electric light bulb (15 cm) at room temperature (●).

Fig. 5. Stability of 6-methyl-2-pyridine carboxaldehyde (MPC) solutions under a 100-W electric light bulb. (○) $1.65 \cdot 10^{-4}$ *M* MPC; (●) $1.65 \cdot 10^{-4}$ *M* MPC in $2.5 \cdot 10^{-2}$ *M* semicarbazide solution.

DISCUSSION

The reaction of PL and PLP with semicarbazide as originally described by Cordes and Jencks [20] has frequently been utilized in the quantitative determination of PL and PLP in biological samples, because the semicarbazone derivatives show better fluorescence characteristics than the underivatized vitamers. Gregory [18] used pre-column derivatisation in determining tissue PLP levels as PLPSC. Schrijver et al. [1] in order to fully automate their HPLC system, used post-column derivatisation, while Vanderslice et al. [14] used on-column derivatisation.

Since a recent study indicated that regular laboratory light is extremely destructive to vitamin B_6 [21], our findings on the stability of PLPSC under ordinary laboratory light conditions stress the desirability of pre-column derivatisation. With pre-column derivatisation under the above mentioned conditions, HPLC analysis can be carried out in an ordinary laboratory, thus eliminating the need of a specially equipped dark room as used by others [14, 15]. Moreover, since PLPSC is stable under our conditions, TCA-supernatants reacted with semicarbazone can be kept in a fridge and analysed at a convenient time. It is therefore now possible to screen remote, rural populations with respect to their B_6 status, because only the most basic laboratory facilities are needed to prepare and store plasma PLP in the stable semicarbazone form.

The sensitivity of this method is very good. Since PLPSC is only moderately fluorescent in acidic to neutral solutions, Gregory [18] was unable to determine low plasma PLP levels. Schrijver et al. [1] using alkaline detection conditions, reported a sensitivity of up to 1.2 ng/ml. The sensitivity of our method is therefore as good as that described by Schrijver et al. [1] and considerably better than others. Although HPLC methods have been published

for the determination of all six B_6 vitamers and pyridoxic acid in one run [14, 15], these methods are generally less sensitive, because the fluorescence characteristics of the different B_6 vitamers differ widely [22], and the detection conditions adopted are usually a compromise between the optimum detection conditions of each vitamer. Thus Vanderslice et al. [14] reported the lower limit of sensitivity for PLP as 3.4 ng/ml, while Coburn and Mahuren [15] using a cation-exchange procedure not based on semicarbazone derivatisation, report that integration errors are significant at concentrations below 5 ng per injection. In our case the sensitivity of semicarbazone measurement in alkaline medium was such that reliable results were still obtainable at a level of 0.1 ng per injection.

The use of MPC as internal standard deserves comment. The semicarbazone derivative of MPC is less fluorescent than PLPSC and PLSC at the wavelengths selected and for this reason the MPC concentration chosen was much higher than that of PLP or PL concentrations in standard solutions. However, this concentration (0.5 μg per injection) is still well within the analytical capabilities of the column used and MPCSC elutes as a sharp, constant peak from the column under these conditions.

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CHROMBIO. 2620

ISOTACHOPHORESIS AS A USEFUL TOOL FOR MONITORING NEUROLOGICAL COMPLICATIONS OF ACUTE LEUKAEMIA IN CHILDREN

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SUMMARY

Cerebrospinal fluid proteins from 42 children with acute lymphoblastic leukaemia were analysed by isotachophoresis. The isotachopherograms of cerebrospinal fluid taken from patients undergoing central nervous system prophylaxis with neurological complications showed an increase of several peaks (albumin, prealbumin, and an unidentified peak), and changes in the globulin zone, compared with those from patients who had completed central nervous system prophylaxis for at least six months. The most striking finding was that these alterations were not associated with any other biochemical changes in the cerebrospinal fluid, as assayed by routine analysis. Isotachophoresis may be useful in the monitoring of therapy in children affected with acute lyphoblastic leukaemia.

INTRODUCTION

Leukaemia of the central nervous system (CNS) is the most common form of extramedullary relapse. Clinical features, the cerebrospinal fluid (CSF) cell count, the presence of blast cells and biochemical changes, have to be considered in an assessment of a diagnosis of meningeal leukaemia. Furthermore, increased

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attention is being given to the importance of biochemical changes in the detection of the potential toxicity and side-effects of antileukaemia therapy.

Analytical studies of CSF glucose, enzymes, fibrinogen, etc. have not lead to satisfactory conclusions. Electrophoretic fractionation of CSF proteins has been shown to be important in the diagnosis in various diseases of CNS [1]. Recently, isotachophoresis (ITP) has been demonstrated to be useful in the diagnosis of nervous diseases, such as multiple sclerosis [2, 3]. ITP has a concentrating effect, owing to a discontinuous electrolyte system where the leading ion (electrolyte) has a higher net mobility than the terminating ion (electrolyte). All molecules with a proper charge and intermediate mobility added to the system, will be concentrated between the leading and terminating ions [4]. The purpose of this study was to determine whether it was possible to detect characteristic abnormalities in the isotachophoretic pattern of CSF, in the presence of CNS complications, during the course of acute lymphoblastic leukaemia (ALL), and their therapeutic approach.

MATERIALS AND METHODS

The following reagents were used: hydroxypropylmethyl cellulose (HPMC, Down Chemical); 2-morpholinoethanesulphonic acid (MES, Merck); 2-amino-2-methyl-1,3-propanediol (AMMEDIOL, Merck); 6-aminohexanoic acid (Merck); barium hydroxide (Merck); ampholine pH 7–11 (LKB); glycine (Canalco); valine (Merck); β-alanine (Merck).

Patients

One hundred CSF samples were obtained from 42 patients who had ALL. The patients were categorized into five groups.

Group I consisted of 21 patients who had completed CNS prophylaxis for at least six months. Among these, ten patients were off therapy. These patients have been treated with "Roma 72" [5] and 7601 [6] protocols (Table I).

Group II consisted of twenty patients undergoing CNS prophylaxis. CSF was obtained during prophylactic treatment that was carried out according to 7902 or 7903A protocols [6] (Table I). The former was used for the low-risk leukaemia, the latter for the high-risk leukaemia.

Group III consisted of two patients with clinically overt signs and symptoms of somnolence syndrome. They had received cranial irradiation (1800 rads) and multiple doses of intrathecal methotrexate (12 mg per week, for six weeks) for CNS prophylaxis.

Group IV consisted of six patients with meningeal leukaemia. These patients were not receiving therapy for active meningeal leukaemia when CSF was obtained. All had received cranial irradiation and intrathecal methotrexate as prophylaxis. All presented leukaemic pleocytosis at the time of the study.

Group V consisted of two patients with clinically overt signs and symptoms of leukoencephalopathy. They had received cranial irradiation and multiple doses of intrathecal methotrexate for CNS prophylaxis. One of them had presented the somnolence syndrome before the onset of leukoencephalopathy and the other one had had the somnolence syndrome and a measles infection two months before.

TABLE I

SUMMARY OF THERAPY REGIMES

Abbreviations: *n* = number of patients; VCR = vincristine; PDN = prednisone; i.t. MTX = intrathecal methotrexate; RT = radiotherapy; 6-MP = 6-mercaptopurine; ASP = asparaginase; TG = thioguanine; ARA-C = cytosine arabinoside; MTX = methotrexate; ADR = adriamycin; i.t. ARA-C = intrathecal cytosine arabinoside; DRB = daunorubicin; CPM = cyclophosphamide; HUR = hydroxyurea; BCNU = bis(β-chloroethyl)nitrosourea.

Protocol	*n*	Induction	Consolidation	CNS prophylaxis	Maintenance
ROMA 72	13	VCR + PDN i.t. MTX		i.t. MTX RT 2400 r	MTX + 6-MP VCR + PDN
7601	9	VCR + PDN i.t. MTX	ASP	i.t. MTX RT 2400 r 6-MP	MTX + PDN
7902*	10	VCR + PDN i.t. MTX	ASP ARA-C + TG ADR	RT 1800 r	MTX + 6-MP VCR + PDN
7903**	10	VCR + PDN + ARA-C + MTX i.t. MTX + i.t. ARA-C	ASP ARA-C + TG ADR	i.t. MTX + i.t. ARA-C	TG + CPM HUR + DRB BTX + BCNU ARA-C + VCR i.t. MTX

*Used for low-risk leukaemia.
**Used for high-risk leukaemia.

Serum preparation

Blood was taken from the children affected by ALL. Serum was prepared from venous blood after clotting (2 h at room temperature) and centrifugation for 10 min at 1500 *g* at 4°C. Serum samples were immediately frozen (−20°C), until the assays were performed. After 1 h of incubation at 22°C, the serum, for ITP analysis, was diluted 1:10 with distilled water.

CSF preparation

CSF (1 ml) was obtained from patients by lumbar puncture at the time of routine clinical examination or immediately before scheduled intrathecal injection of antileukaemic drugs. CSF from patients affected by meningeal leukaemia was centrifuged at 1500 *g* at 4°C for 10 min before ITP assay. From a single lumbar puncture, two samples of CSF were taken: the first was used for routine analysis and the second for ITP analysis. All CSF samples were routinely analysed by cell count in a Nageotte chamber without dilution with Turk solution, by blast cell investigation using a cytocentrifuge for glucose, by the colorimetric method reported by Werner et al. [7] for total protein, by a colorimetric method reported by Weichselbaum [8]. The presence of haemoglobin and bilirubin was ruled out by spectrophotometric analysis of the CSF. All samples that did not contain red cells and haematic pigments were immediately frozen at −20°C until ITP was performed.

Isotachophoresis

The apparatus used in this investigation was the LKB 2127 Tachophor (LKB, Bromma, Sweden). Separation was carried out in a PTFE capillary tube (23 cm × 0.5 mm I.D.), kept at a constant temperature of 12°C. The apparatus was equipped with a UV detector set at 280 nm. The leading

electrolyte was 5 m*M* MES—10 m*M* AMMEDIOL—0.5% HPMC, and the pH 9.1. No adjustment to the pH was carried out. The terminating electrolyte was 10 m*M* 6-aminohexanoic acid—10 m*M* AMMEDIOL, adjusted to pH 10.8 with barium hydroxide. The initial current setting on the instrument was 200 μA, maintained until a potential of 15 kV had been reached. The current was then reduced to 40 μA. During detection, under a constant current of 40 μA, the voltage rose from 5 to 16 kV. The UV gain was 2. The separation time was less than 50 min. The samples were 9 μl of unconcentrated CSF plus 2 μl of spacer solution and 3 μl of serum (already diluted 1:10 with distilled water) plus 2 μl of spacer solution. To detect the sample compounds efficiently, spacer substances are often needed [9]. Spacer solutions were: 1.6 mg of glycine plus 1.6 mg of valine plus 1.44 mg of β-alanine plus 0.3 ml of ampholine (pH 7—9) plus 0.18 ml of ampholine (pH 9—11) [10].

RESULTS AND DISCUSSION

Unconcentrated CSF from children in Group I showed the albumin peak and an unidentified protein that migrated very rapidly (Fig. 1). The latter, which is also found in normal CSF specimens [11], was present in greater amounts in the samples from patients undergoing CNS prophylaxis (Fig. 2A) and somnolence syndrome (Fig. 3). This increase, also reported to occur in CSF from patients with chronic meningoencephalomyelitis, seems to reflect CNS damage [11].

ITP of CSF from patients undergoing CNS prophylaxis according to the 7902 protocol (Table I) also showed an increase in the albumin content and the presence of peaks migrating before glycine. Only immunoglobulin (Ig)

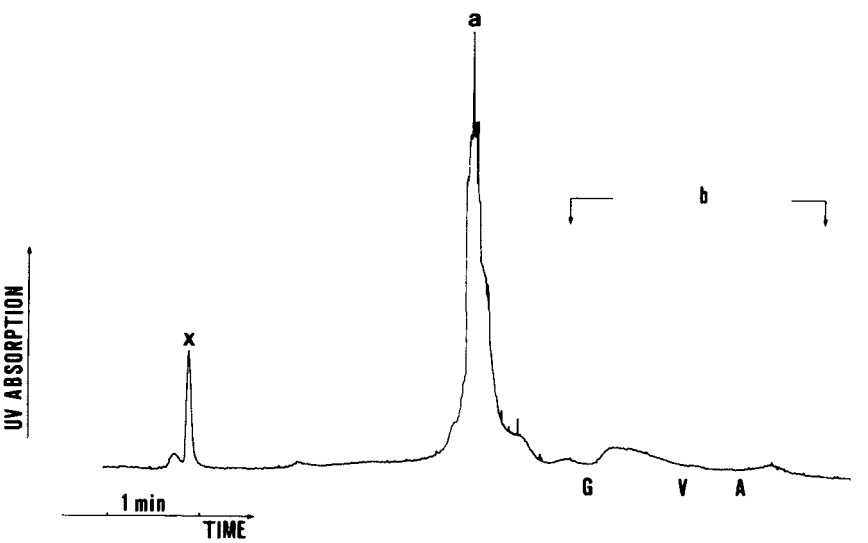

Fig. 1. Isotachopherogram showing the separation of CSF proteins, in the presence of spacer solution, from ALL patients off therapy. Peaks: X = unidentified; a = albumin; b = globulin zone; G = glycine; V = valine; A = β-alanine.

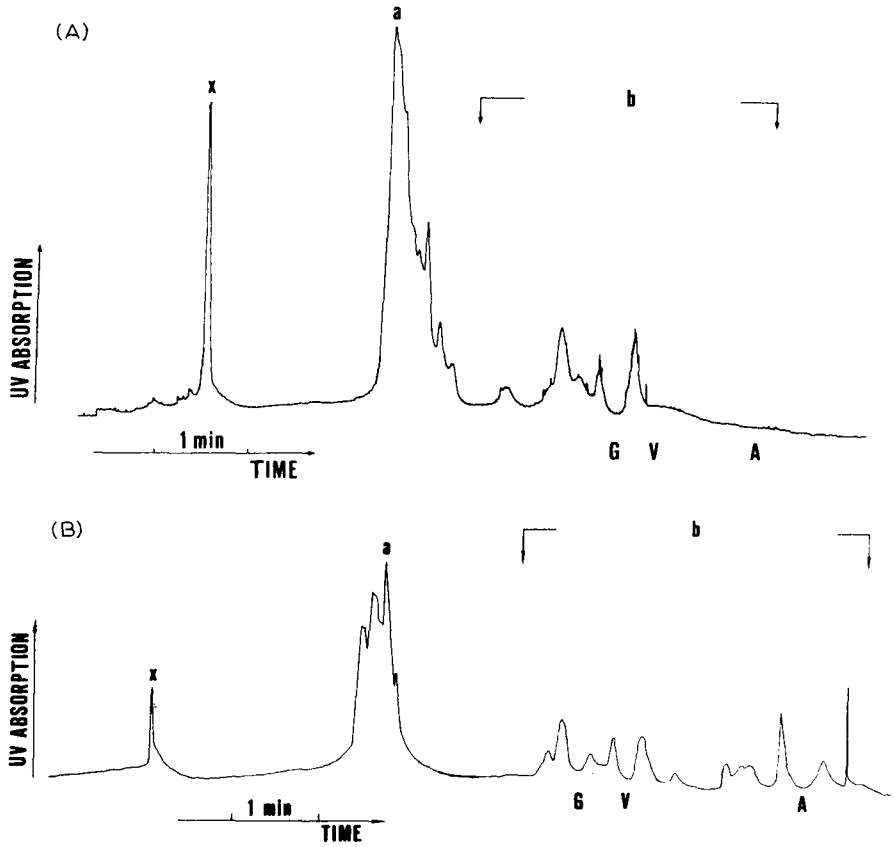

Fig. 2. ITP patterns of CSF from patients undergoing CNS prophylaxis, according to (A) protocol 7902, (B) protocol 7903A. For abbreviations, see Fig. 1.

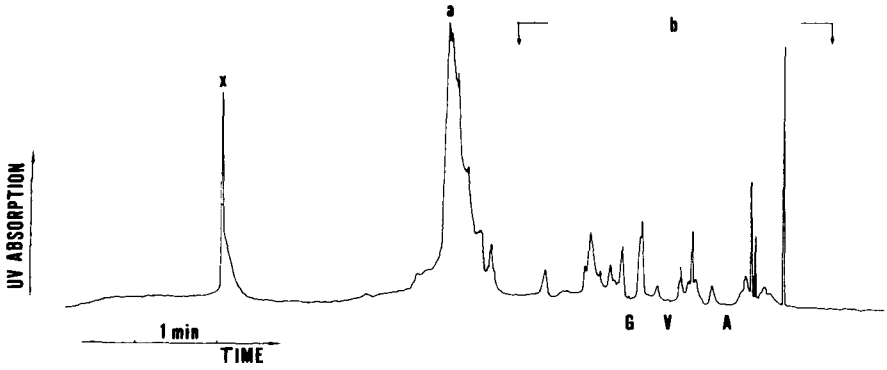

Fig. 3. ITP pattern of CSF from patients affected with somnolence syndrome. For abbreviations, see Fig. 1.

A appeared after the spacer [12] (Fig. 2A). This pattern suggests damage to the blood—brain barrier.

CSF from Group II patients, treated with the 7903A protocol (Table I), showed a change in the globulin zone after the amino acid valine (Fig. 2B). More marked alterations in this zone were observed in CSF from Group III patients (somnolence syndrome) (Fig. 3). The use of β-alanine allowed a separation into fast- and slow-moving globulins [13]. CSF specimens from patients with somnolence syndrome contained an increased percentage of slow-migrating IgG, corresponding to an IgG oligoclonal band in the high-alkaline region. It has been reported that this indicates an increased intrathecal

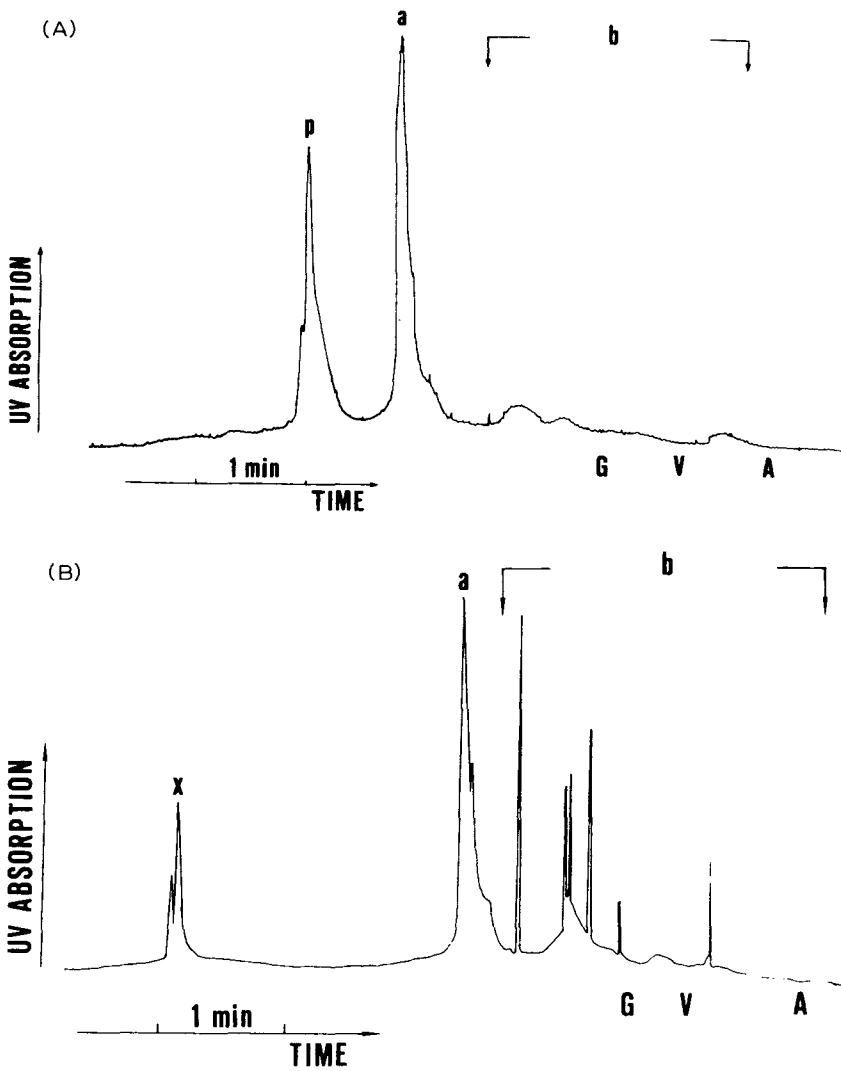

Fig. 4. (A) ITP trace of CSF from ALL patients affected with meningeal leukaemia, showing the presence of the prealbumin peak (p). (B) ITP trace of CSF from ALL patients showing an increase of the peak migrating before glycine. For abbreviations, see Fig. 1.

synthesis of IgG [2, 13]. Oligoclonal immunoglobulins have been found in several neurological disorders, accompanied by inflammatory reactions within the CNS, in patients with multiple sclerosis, and in cases of acute aseptic meningitis [14—18]. It has been reported that the somnolence syndrome may be an early indicator of permanent neurological damage, relative to the dosage of CNS irradiation [9, 20]. Our finding suggests that immunological processes, possibly following CNS prophylaxis, are involved in the pathogenesis of this syndrome [21].

ITP patterns of CSF from patients affected with meningeal leukaemia (Group IV) (Fig. 4A) showed the presence of prealbumin [22], which was not found in the serum. Similar patterns occurred in CSF from subjects with leuko-encephalopathy (Group V) (Fig. 5). In some Group IV patients more peaks were observed in the region between albumin and glycine (Fig. 4B). It is known that such an increase is found in the CSF of patients with neurological disease, perhaps owing to local synthesis [23]. It must be remembered that important acute-phase reactants migrate in the region between albumin and glycine, and that routine analysis of CSF from all patients was in the normal range. The six patients affected with meningeal leukaemia presented no CSF changes, except leukaemic pleocytosis.

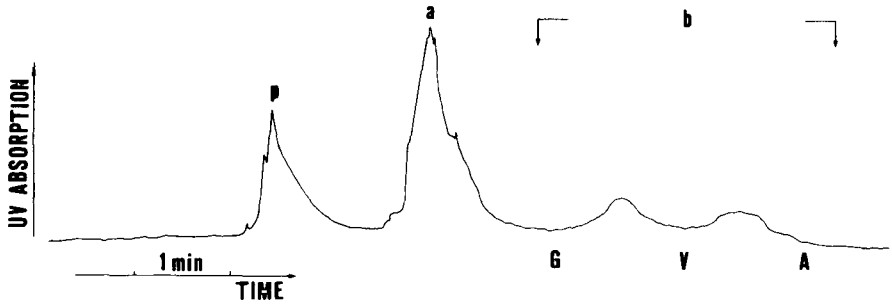

Fig. 5. ITP trace of CSF from ALL patients affected with leukoencephalopathy: note the enlarged prealbumin peak (p). For abbreviations, see Fig. 1.

In conclusion, ITP analysis could be a useful tool in the diagnosis of some CNS complications during ALL. In particular, it seems useful in the monitoring of CNS prophylaxis by intrathecal injections of antileukaemic drugs and/or irradiation [24]. It must be stressed that the presence of peaks other than albumin, in unconcentrated CSF, suggests pathological processes. ITP seems to be important in the investigation of possible markers of CNS damage (due to ALL per se or to the therapy), and of the pathogenesis of CNS complications. The advantages of analytical ITP are that very small samples of unconcentrated CSF can be examined in a short time (less than 50 min) and the results are immediately obtained on a recorder. Low- and high-molecular-weight compounds can be analysed. The method gives high resolution, is reproducible and easy to perform.

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CHROMBIO. 2633

DETERMINATION OF DRUGS IN UNTREATED BODY FLUIDS BY MICELLAR CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

Direct serum and urine injection, without sample extraction or protein precipitation steps, into a liquid chromatographic system using sodium dodecyl sulfate (SDS) with 10% added propanol as the mobile phase, is described for measurement of drug levels. The ability of SDS micelles to form soluble protein—SDS complexes, with no on-column precipitation, provides a simple, rapid method for routine determination of quinine, quinidine, propranolol, morphine and codeine at concentration levels found in serum and urine following administration of therapeutic doses. Absolute limits of detection ranged from 0.2 to 6 ng. Variation of the surfactants mobile phase concentration allows control of selectivity and analysis time, although a minimum concentration is required to prevent protein precipitation. Chromatographic efficiencies are improved by the addition of propanol to the micellar mobile phase, and sensitivities improved by use of fluorescence detection. The sensitivities are more than adequate for therapeutic drug monitoring of concentration ranges normally encountered in serum and urine.

INTRODUCTION

Aqueous solutions containing sodium dodecyl sulfate (SDS) at concentrations well above the critical micelle concentration (CMC) have been proposed as selective mobile phases in reversed-phase liquid chromatography [1, 2]. The effects of micellar mobile phases in high-performance liquid chromatography (HPLC) on the elution behavior of a series of neutral arenes and for the chromatographic behavior of ionizable eluents have been described [3, 4]. In micellar HPLC, there are two major equilibria whose constants govern chromatographic behavior, namely, solute—micelle described by K_{eq} and solute—stationary phase described by K_{sw}. The larger the K_{eq} value, the greater the effect of changes in surfactant concentration on the capacity factors.

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Consequently, relative retention times may be changed and selectivity can be obtained by varying the concentration of micelles in the mobile phase. This paper reports a simple, rapid, sensitive and direct technique which could be very useful for routine monitoring of drugs in biological fluids by micellar chromatography.

Development of selective and sensitive analytical techniques for the analysis of minute quantities of drugs in biological fluids have attracted considerable interest in analytical toxicology and therapeutic drug monitoring. Gas chromatography has been applied for determination of amphetamine and phentermine in biological fluids [5], morphine in opium [6] and heroin in illicit street preparations [7], but these methods require prior extraction of samples and, in most cases, derivatization of the drug before determination. Immunoassay methods such as enzyme-multiplied immunoassay techniques (EMIT®) have been the methods of choice for therapeutic drug monitoring because they are rapid, specific, sensitive and reliable [8], and biological fluids can be analyzed without prior extraction or protein precipitation. Unfortunately, EMIT is limited to only selected drugs. HPLC has also been applied to the determination of morphine and codeine in a variety of matrices [9, 10], and for other drugs in body fluids [11, 12]. For routine drug level monitoring in biological fluids, HPLC has drawbacks such as lengthy analysis time and tedious sample preparation, as it generally requires extraction of the drug from the protein-base sample or protein precipitation [13, 14]. These additional steps considerably increase the possiblity of error. Protein precipitation procedures, for example, may be incomplete resulting in column clogging [15].

In an attempt to eliminate these problems, pre-column technology has been used for partial sample clean-up, which allowed the direct injection of biological samples [16, 17]. Using this approach, no column deterioration or protein-binding effects were observed. Wahlund and Arvidsson [18] have shown that direct injection of blood plasma samples into reversed-phase columns resulted in skewed chromatographic peaks for the drug naproxen. The skew has been shown to be due to strong binding of naproxen to albumin present in the blood plasma.

Micellar HPLC provides an unique solution to these problems by solubilizing the protein components via a surfactant coating, making possible direct injection of biological fluids onto HPLC columns with no column clogging. In addition, the surfactant monomers appear to displace the drug bound to the protein, releasing it for partitioning to the stationary phase. In fact, Granneman and Sennello [19] have shown that surfactant monomers will competitively bind to proteins, thereby releasing protein-bound antibiotics. Also, Hirota and Kawase [20] have used SDS to improve the recovery of ubiquinone-10 in plasma samples. Therapeutic drug monitoring using UV and fluorescence detection for HPLC direct serum injection with micellar mobile phases has been reported recently and the results compared to the EMIT technique [21]. However, the moderate chromatographic efficiency prevented adequate sensitivity for determination of propranolol whose therapeutic range is 0.05–1.0 μg/ml. Dorsey et al. [22] demonstrated that efficiency approaching those with hydro-organic mobile phases can be achieved by addition of at least 3% propanol to the micellar mobile phase and by working at elevated temperature (40° C). In

the present study, SDS micellar mobile phases containing 10% propanol did not induce protein precipitation in serum and urine samples, but did exhibit improved chromatographic efficiencies. The sensitivity of the method was improved by using fluorescence instead of absorbance detection, allowing monitoring of additional drugs. The present study extends the use of micellar HPLC to the determination of quinine, quinidine, propranolol, morphine, and codeine in serum, and demonstrates for the first time the use of micellar HPLC for determination of drugs in urine by direct injection of the sample into the chromatograph.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Technicon FAST LC high-pressure pump (Technicon, Tarrytown, NJ, U.S.A.), a Model 7120 sample injector with a 20-μl injection loop (Rheodyne, Cotati, CA, U.S.A.), and a Model FS970 LC fluorometer (Kratos, Ramsey, NJ, U.S.A.). The HPLC analytical column was either a 10-μm μBondapak C_{18} (30 cm $\times$ 3.9 mm I.D.) (Waters Assoc., Milford, MA, U.S.A.) or a 5-μm Supelcosil LC-CN column (15 cm $\times$ 4.6 mm I.D.) (Supelco, Bellefonte, PA, U.S.A.). A pre-column (12.5 cm $\times$ 4.6 mm I.D.) packed with silica gel (25—40 μm) (Whatman, Clifton, NJ, U.S.A.) was located between the pump and sample injector in order to saturate the mobile phase with silica to minimize dissolution of the analytical column packing. All chromatograms were recorded on a Recordall Model 5000 strip chart recorder (Fisher Scientific, Springfield, NJ, U.S.A.).

Reagents

SDS was electrophoresis grade obtained from Bio-Rad Labs. (Rockville Center, NY, U.S.A.) and was used as received. Serum blank samples were obtained from General Diagnostics of Warner Lambert (Morris Plaines, NJ, U.S.A.) and used as received. Quinidine (Mallinckrodt, St. Louis, MO, U.S.A.), quinine, morphine and codeine alkaloids (S.B. Penic, Lyndhurst, NJ, U.S.A.), propranolol (Warner Lambert), and propanol and methanol (Fisher Scientific) were used as received.

Procedure

Micellar mobile phases were prepared by dissolving the appropriate quantity of SDS in distilled water containing 10% propanol. The mobile phase was filtered through a 0.45-μm Nylon-66 membrane filter (Rainin Instruments, Ridgefield, NJ, U.S.A.), and degassed under vacuum prior to use. A mobile phase flow-rate of 1.0 ml/min was used, and retention times were measured from the injection point to the peak maxima on the chromatogram. Stock solutions of 500 μg/ml of each drug in methanol were diluted to 10 μg/ml or 1 μg/ml with distilled water, and these were added in appropriate quantities to serum or urine. These serum- or urine-base standards were injected directly into the micellar HPLC system to prepare calibration curves of peak height versus concentration of drug, and to determine the precision of the measurements.

RESULTS AND DISCUSSION

Serum and urine blanks were mixed with the micellar mobile phase to determine if any protein precipitation was evident. For concentrations of SDS greater than 0.02 *M* containing 10% propanol, no precipitation was observed. When these blanks were injected into the chromatographic system, there was no evidence of pressure build-up owing to precipitated proteins at the head of the column or clogging of the injector port, even after more than 250 sample injections. However, there may be some strongly retained components binding to the stationary phase which could eventually cause column clogging, so the column was flushed overnight with mobile phase at 0.1 ml/min after analyzing serum or urine samples.

The blank serum or urine produced a rapid elution of unretained species at the solvent front, then returned completely to the baseline within 20 min. This is probably the result of the protein—SDS complex being excluded from the pores of the stationary phase support, preventing partitioning and retention. The background response level of the unretained species could be varied by changing the excitation wavelength along with appropriate changes in detector sensitivity range. Adjustments in SDS mobile phase concentration and detector sensitivity were made such that adequate resolution and sensitivity were obtained for quantitative determination of the drug content in the biological fluids. In most cases, drug elution occurred on the tail of the protein components, which prevented the use of the most sensitive detector ranges. Also, the sensitivity was adversely affected by the low intensity of the deuterium light source at the optimum absorption wavelength maxima of the drugs studied, and because a compromise excitation wavelength is necessary for the analysis of mixtures of drugs. The best sensitivity was obtained using 215—220 nm excitation, probably due to the high intensity of the deuterium light source at this wavelength, even though it does not correspond to the absorption wavelength maximum of any of the drugs studied. The fluorescence intensity was linear with drug concentration using this excitation wavelength. Sensitivity should be dramatically improved by using a light source with a high intensity at the drug's absorption wavelength maximum.

The detection and determination of morphine in body fluids is most frequently performed using urine because a large portion of this drug is excreted [23]. When using urine samples greater than 10 ml in total volume and obtained less than 48 h after ingestion of morphine, the sensitivity limit of 0.5 μg/ml has been recommended [24]. Fig. 1 shows the chromatogram of a urine blank and two urine samples containing 0.5 and 1.0 μg/ml of morphine, respectively. The fluorescence intensity increases linearly with morphine concentration, and the relative standard deviation (R.S.D.) of the calibration curve slope was 7.4%. Similar results were obtained for codeine. Table I lists the drugs examined in the present study, the calibration curve concentration range monitored in the urine samples, calibration curve linearity data, precision, and limits of detection for the drugs in urine.

Direct serum injection with unmodified micellar mobile phase HPLC for therapeutic drug monitoring of quinidine has been described, and the limit of detection was well below the therapeutic range normally monitored [21].

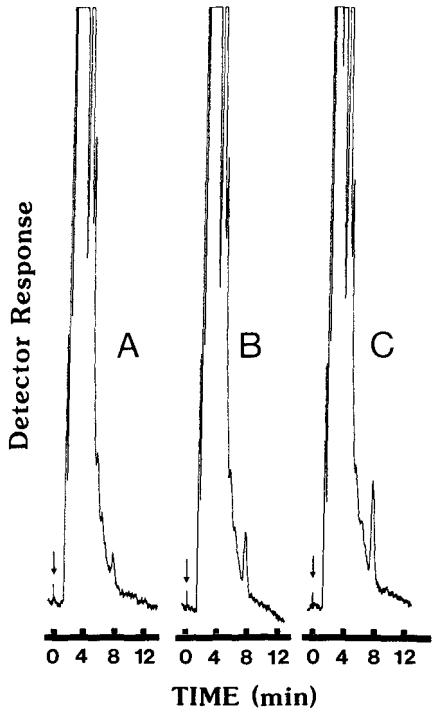

Fig. 1. Chromatograms of (A) urine blank, (B) urine blank with 0.5 μg/ml morphine, and (C) urine blank with 1.0 μg/ml morphine. Chromatographic conditions: column, 10-μm μBondapak C_{18}; mobile phase, 0.03 *M* SDS + 10% propanol; flow-rate, 1.0 ml/min; detector voltage, 700 V; sensitivity, 0.05 μA; excitation wavelength, 215 nm; emission cut-off filter, 300 nm.

TABLE I

CALIBRATION RANGE OF URINE DRUG STANDARDS, PERCENTAGE RELATIVE STANDARD DEVIATION (R.S.D.) OF SLOPE, PRECISION, AND LIMITS OF DETECTION (L.O.D.) FOR SELECTED DRUGS IN URINE SAMPLES

All concentrations in μg/ml except those listed for absolute L.O.D. values.

Drug	Calibration curve range in urine	Linearity* (% R.S.D. of slope)	Precision** (% R.S.D., $n = 5$)	Relative L.O.D.***	Absolute L.O.D. §
Morphine	0.4 —1.2	7.40	7.10	0.3	6
Codeine	0.5 —2.0	3.90	3.10	0.3	6
Propranolol	0.04—1.2	3.24	1.89	0.01	0.2
Quinidine	0.20—1.0	3.44	2.75	0.03	0.6
Quinine	0.20—1.0	10.36	6.27	0.03	0.6

*At least four different concentrations of urine drug standards were used.

**Concentrations of urine drug standards used for precision evaluation were 0.4, 1.0, 0.06, 0.6 and 0.2 μg/ml in order of each drug listed above.

***Limit of detection equal to concentration where signal = 3 × R.S.D. of noise. The noise is the standard deviation of several measurements of the response from blank serum measured at the retention time of the drug.

§ Absolute L.O.D. = (relative L.O.D.) (injection volume) reported in ng.

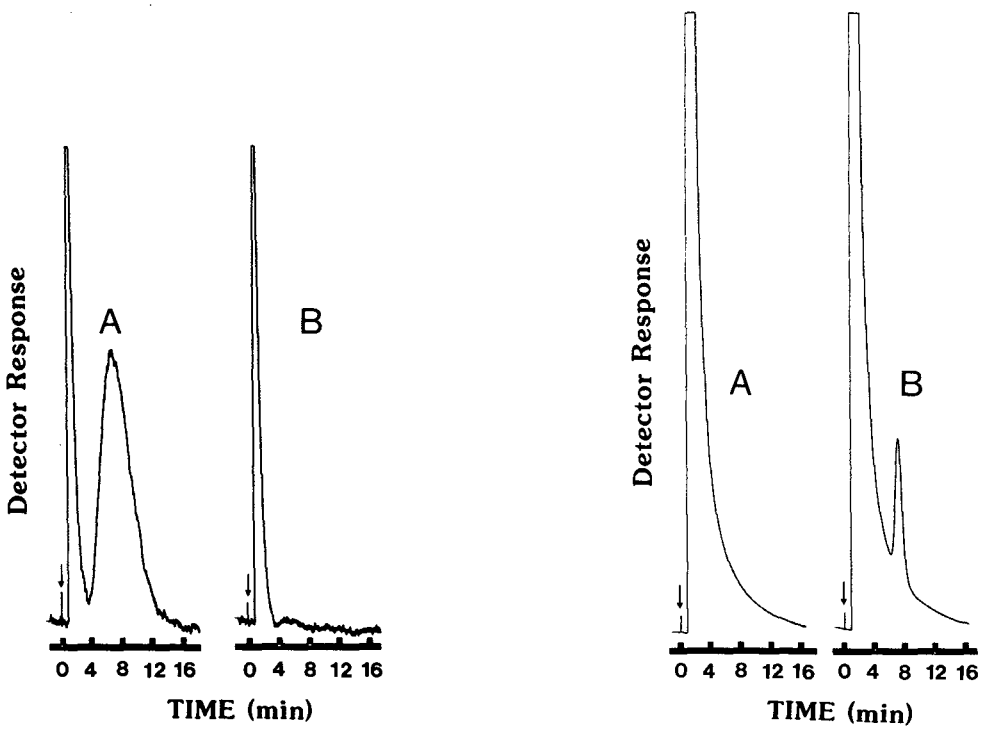

Fig. 2. Chromatograms of (A) serum with 4.0 μg/ml added quinidine and (B) serum blank. Chromatographic conditions: column, 5-μm Supelcosil LC-CN; mobile phase, 0.10 *M* SDS; flow-rate, 1.0 ml/min; detector voltage, 620 V; sensitivity, 0.02 μA; excitation wavelength, 336 nm; emission cut-off filter, 370 nm.

Fig. 3. Chromatograms of (A) serum blank and (B) serum with 3.0 μg/ml added quinidine. Chromatographic conditions: column, 5-μm Supelcosil LC-CN; mobile phase, 0.05 *M* SDS with 10% propanol; flow-rate, 1.0 ml/min; detector voltage, 700 V; sensitivity, 0.5 μA; excitation wavelength, 215 nm; emission cut-off filter, 300 nm.

However, the efficiency of micellar HPLC is moderate compared to hydro-organic reversed-phase HPLC unless a modifier such as propanol is added to the micellar mobile phase. Figs. 2 and 3 show the separation of serum containing known concentrations of quinidine using SDS solutions with and without added propanol, where considerable improvement in efficiency is obtained in the presence of 10% propanol. Note that the concentrations of SDS and quinidine, and the excitation wavelengths used to obtain the data in Figs. 2 and 3, were not the same, but similar retention times were observed. This strongly suggests that the efficiency is improved without drastic changes in selectivity. Although poor sensitivity for quinidine in serum using unmodified SDS mobile phases was found on C_{18} columns [21], good results (Table I) were obtained for determination of quinidine in urine using the propanol-modified mobile phase and C_{18} or cyano columns. Similar improvement in column efficiency with a concomitant decrease in the absolute limit of detection to 200 pg was observed for the determination of propranolol performed by direct injection of serum into the liquid chromatograph.

Figs. 4 and 5 provide a comparison of the separation of mixtures of propranolol and quinidine in serum and urine, respectively. The chromatograms shown in these figures were obtained using the same C_{18} column and 10% propanol in the SDS mobile phase, but the SDS concentrations and detector sensitivities were different. Inspection of the chromatograms in Figs. 1—5 clearly indicates that the body fluid background signal was the limiting factor in the limits of detection. Optimization of the signal-to-noise ratio could be performed by adjustment of detector sensitivity and surfactant concentration or by extraction of the drugs from the biological sample. The linearity of peak height response versus the drug concentration in urine samples, expressed as relative standard deviations of the slopes, ranged from 3.2 to 10.4%. The precision, given by the percentage R.S.D. of five replicate determinations of each drug in urine samples, was 1.9 to 7.1% (Table I). Although propranolol elutes on the tail of the serum protein components, obviating the use of the most sensitive detector range, a 200 ng/ml propranolol sample peak was clearly discerned whose height was proportional to drug concentration (Fig. 4). However, it has been found in other studies that the serum background can be completely eliminated by using a 470-nm cut-off filter [25]. Longer retention

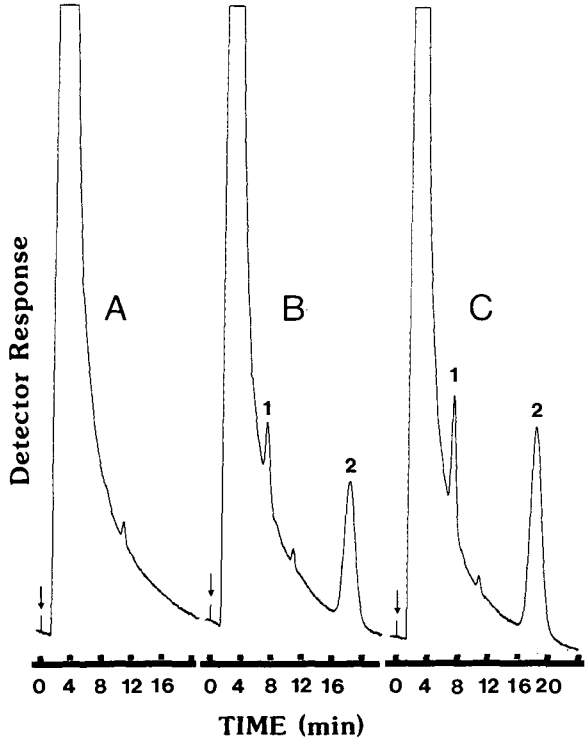

Fig. 4. Chromatograms of (A) serum blank, (B) serum blank with added 200 ng/ml propranolol (1) and 2 μg/ml quinidine (2), and (C) serum blank with added 400 ng/ml propranolol (1) and 3 μg/ml quinidine (2). Chromatographic conditions: column, 10-μm μBondapak C_{18}; mobile phase, 0.03 *M* SDS with 10% propanol; flow-rate, 1.0 ml/min; detector voltage, 700 V; sensitivity range, 0.5 μA; excitation wavelength, 215 nm; emission cut-off filter, 300 nm.

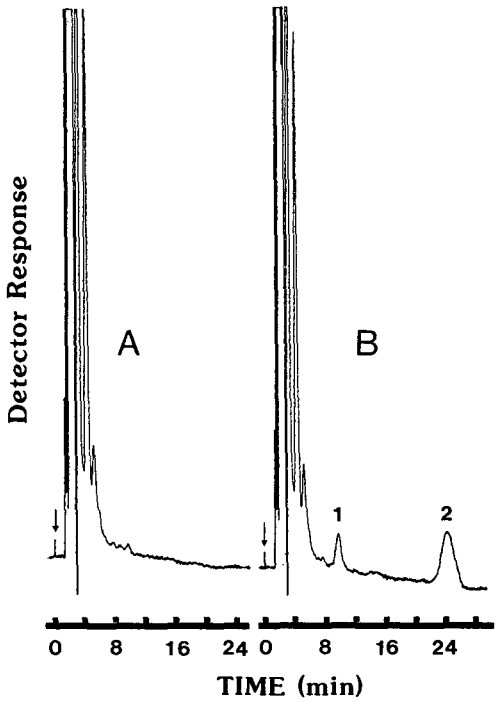

Fig. 5. Chromatograms of (A) urine blank and (B) urine with added 40 ng/ml propranolol (1) and 400 ng/ml quinidine (2). Chromatographic conditions: column, μBondapak C_{18}; mobile phase, 0.02 *M* SDS with 10% propanol; flow-rate, 1.0 ml/min; detector voltage, 700 V; sensitivity, 0.02 μA; excitation wavelength, 215 nm; emission cut-off filter, 300 nm.

times were observed at lower surfactant concentrations, as shown by comparing Figs. 4 and 5, illustrating the degree of selectivity obtainable by varying surfactant concentration at constant propanol content.

Modified micellar mobile phases give remarkably reproducible, sensitive, and rapid results for analysis of drugs in body fluids using direct injection of serum or urine samples. In addition to absorbance and fluorescence detection, it has been demonstrated recently that electrochemical detection works equally well in micellar chromatography [26]. Further studies in progress involve the use of different types of surfactants in the mobile phase for determination of other licit and illicit drugs in body fluids.

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ANALYSIS OF 5'-DEOXY-5-FLUOROURIDINE AND 5-FLUOROURACIL IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A relatively simple and sensitive high-performance liquid chromatographic (HPLC) method is described for measuring the two anticancer drugs 5'-deoxy-5-fluorouridine (5'dFUR) and 5-fluorouracil (5-FU) in human plasma and urine. The procedure for plasma includes solvent extraction using ethyl acetate—isopropyl alcohol (85:15) followed by silica gel column chromatography to separate these compounds from constituents normally occurring in plasma. The analysis by reversed-phase HPLC is performed on a phenyl column using an aqueous mobile phase with ultraviolet detection (280 nm). The overall recovery from plasma was 61% and 65% for 5'dFUR and 5-FU, respectively. The sensitivity limit of the assay for both compounds was 50 ng/ml of plasma. Analysis of these compounds in urine did not require the silica column chromatography isolation step.

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INTRODUCTION

The recently synthesized fluoropyrimidine, 5′-deoxy-5-fluorouridine (5′dFUR, doxifluridine, Ro 21-9738), is under investigation for use in the treatment of carcinomas of head and neck, ovary, breast and colon/rectum [1—5]. It is believed that 5′dFUR is a prodrug of 5-fluorouracil (5-FU), and is converted to 5-FU by intracellular enzymatic hydrolysis [1, 2, 6—9]. The enzyme responsible for the conversion, thymidine phosphorylase, is found in many normal tissues but appears to be present in higher activity in neoplastic cells [1, 2, 5, 8, 10]. This would be consistent with the fact that 5′dFUR exhibits a higher therapeutic index [1, 2, 5—7, 9, 11—15], and has been reported to be less immunosuppressive [16, 17] and cardiotoxic [18] than other fluorinated pyrimidines.

Detailed studies investigating the disposition kinetics of 5′dFUR and 5-FU following 5′dFUR administration are limited [4, 19—21]. This may be due to the lack of a simple and sensitive method to quantitate both compounds in biological fluids. Methods have been described for the determination of 5′dFUR in biological fluids using thin-layer chromatography [10] and high-performance liquid chromatography (HPLC) in conjunction with either labelled drugs [2, 7] or ultraviolet (UV) detection [22]. The novel HPLC method of Sommadossi and Cano [22] based on spectrophotometric detection was highly specific, however, the simultaneous quantitation of 5-FU was not possible according to the authors. In addition, its application to urine analysis has not been demonstrated.

Gustavsson et al. [23] recently reported a method for the simultaneous analysis of both compounds in plasma. The procedure involved deproteinization with picric acid followed by ion-exchange chromatography and analytical isotachophoresis. The complexity of the procedure and the instrumentation involved will probably result in the method having limited utility.

More recently, Malet-Martino et al. [24] have described a fluorine-19 NMR assay for measuring both compounds as well as major metabolites in whole blood, plasma and urine. While this method has the advantage of not requiring extraction, it appears to be less sensitive than reported HPLC procedures.

The purpose of this paper is to describe a relatively simple and sensitive HPLC method with UV detection for the quantitation of 5′dFUR and its metabolite, 5-FU, in both human plasma and urine.

EXPERIMENTAL

Reagents

All reagents were of analytical-reagent grade. They included methanol (HPLC grade, Fisher Scientific, Fair Lawn, NJ, U.S.A.), ethyl acetate (Baker Analyzed Reagent, J.T. Baker, Phillipsburg, NJ, U.S.A.), isopropyl alcohol (HPLC grade, Waters Assoc., Milford, MA, U.S.A.), orthophosphoric acid (BDH, Poole, U.K.), 50—100 mesh silica gel (Koch-Light, Colnbrook, U.K.) and dimethyldichlorosilane (Fluka, Buchs, Switzerland).

Analytical standards

5-Fluorouracil, 5'-deoxy-5-fluorouridine and other metabolites were kindly supplied by Hoffman-La Roche (Basel, Switzerland) and used without further purification. 5-Bromouracil (5-BrU) was purchased from Sigma (St Louis, MO, U.S.A.) and used as the internal standard (I.S.). Aqueous stock solutions (1 and 4 mg/ml 5-FU and 5'dFUR; 200 μg/ml 5-BrU) were prepared in silanized glassware and stored at 4°C. These solutions were stable for at least four months.

HPLC instrumentation

The chromatographic system consisted of a reciprocating piston pump (Model 6000A, Waters Assoc.), a syringe loading sample injector (Model U6K, Waters Assoc.), and a Spherisorb phenyl column (12.5 cm × 4.9 mm I.D., particle size 5 μm, Hichrom House, Berkshire, U.K.). All chromatography was performed at ambient temperature. The column effluent was monitored at 280 nm using a selectable-wavelength UV detector (Model 441, Waters Assoc.). The output from the detector was connected to a 10-mV potentiometric integrator (Model 3380A, Hewlett-Packard, Avondale, PA, U.S.A.) set at a chart speed of 1 cm/min.

Mobile phase

The isocratic mobile phase was distilled water which was purified through a Milli-Q system (Millipore, Bedford, MA, U.S.A.), filtered through a 0.45-μm Nylon 66 filter (Alltech, Deerfield, IL, U.S.A.) and degassed ultrasonically under vacuum. The mobile phase was pumped at a flow-rate of 1.5 ml/min (73 bar) and not recycled.

Analytical procedures

All centrifuge tubes and clean-up columns were silanized by placing them in a dessicator containing dichlorodimethylsilane (DMCS). Following exposure to DMCS vapor for 4—8 h, the tubes were placed in a methanol desiccator overnight, rinsed with water and then dried at 110°C.

Plasma

The flow diagram for the extraction procedure is shown in Fig. 1. Aliquots of plasma (0.5 ml) were pipetted into 15-ml silanized glass centrifuge tubes (Kimax, 100 × 16 mm, Kimble, Vineland, NJ, U.S.A.) fitted with PTFE-lined screw caps. After addition of internal standard (50 μl of 4 μg/ml aqueous stock for the 50—1000 ng/ml range; 50 μl of a 40 μg/ml aqueous stock for the 1—75 μg/ml range), 50 μl of 3% (v/v) aqueous orthophosphoric acid was added to adjust the pH to approximately 5.5. The tubes were gently shaken to ensure mixing and 5 ml of ethyl acetate—isopropyl alcohol (85:15) were added, then vortexed for 30 sec on a Vortex-Genie mixer (Model K-550-GE, Scientific Industries, Springfield, MA, U.S.A.). Following centrifugation (Model HN, International Equipment, Needham Heights, MA, U.S.A.) for 6 min to separate the phases, the entire organic layer was pipetted into a silanized, conical glass centrifuge tube and evaporated to dryness on an evaporator (N-EVAP, Organomation Assoc., Northborough, MA, U.S.A.) at 45—50°C under a gentle stream of nitrogen.

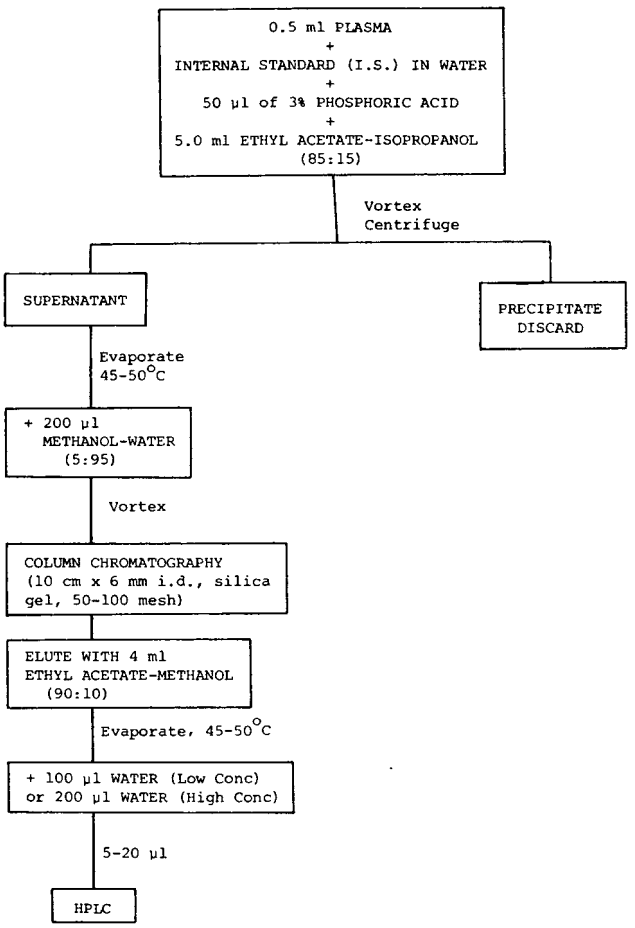

Fig. 1. Flow diagram for the isolation procedure of 5′dFUR and 5-FU from plasma.

The dried residue was dissolved in 200 µl methanol—water (5:95), vortexed for 30 sec and then subjected to column chromatography (10 cm × 6 mm I.D., silanized glass columns packed with 2.56 g 50—100 mesh silica gel). The gravity packed columns were rinsed with 10 ml of ethyl acetate—methanol (90:10) and then the entire residue mixture was placed on the top of the column using a micropipet (Pipetman, Gilson France, Villiers-le-Bel, France). The column was eluted with 4 ml of ethyl acetate—methanol (90:10) and the eluate fraction evaporated to dryness as described above. The resulting residue was dissolved in 100 µl (low concentration range) or 200 µl (high concentration range) deionized water, vortexed for 30 sec, and 5—20 µl injections were made into the HPLC system.

Urine

A 100-µl aliquot of a 1:100 dilution (5-FU) or 1:1000 dilution (5′dFUR) of urine was pipetted into a silanized glass centrifuge tube (Kimax, 100 × 13 mm, Kimble). A 40-µl (5-FU assay) or 50-µl (5′dFUR assay) volume of a

4 μg/ml aqueous solution of internal standard was added and the mixture acidified to a pH of approximately 5.5 with orthophosphoric acid. A 2-ml volume of ethyl acetate—isopropyl alcohol (85:15) mixture was added and the tubes were vortexed for 30 sec. After centrifuging for 6 min, the organic phase was removed with a Pasteur pipet and evaporated as described under plasma samples. The dried sample was redissolved in 100 μl deionized water and vortexed for 20 sec to facilitate dissolution. A volume of 10–20 μl was injected onto the column.

Calculations

Standard plasma calibration curves of peak height ratio versus plasma concentration were constructed using plasma samples to which increasing quantities of both drugs were added to give concentrations in the range 1—1000 ng/ml or 1—75 μg/ml. Similarly, urine calibration curves were prepared by adding 5-FU in concentrations of 0—250 μg/ml or 5'dFUR in concentrations of 0—10 mg/ml to blank urine. Concentrations of 5-FU and 5'dFUR were obtained from the peak height ratios and the regression equation of the appropriate calibration curve.

RESULTS AND DISCUSSION

Internal standard

5-Bromouracil was chosen as an internal standard because of its similarity in structure, maximum absorption wavelength and percentage recovery when compared to 5-FU. In addition, 5-BrU is not used as a therapeutic agent and is not a metabolite of either 5-FU or 5'dFUR.

Extraction and isolation procedure

Plasma. The performance of the extraction and isolation procedure was dependent on the nature of the extracting solvent mixture, extraction pH and inclusion of the additional silica column isolation step. The physical recovery of 5-FU and 5'-dFUR was determined by comparing the peak heights measured from the final extracts of plasma containing known concentrations (100 ng/ml) of both compounds with the peak heights measured from unextracted aqueous solutions supplemented with known concentrations of 5-FU and 5'dFUR. Recoveries of 5-FU and 5'dFUR determined in this manner using ethyl acetate—isopropyl alcohol (85:15) were found to be approximately 66 and 61%, respectively. Less satisfactory recoveries were found when ethyl acetate, diethyl ether, chloroform, pentane or lower percentages of isopropyl alcohol in ethyl acetate were used.

Since the pK_a values of these acidic compounds are approximately 8 [25], an extraction pH of approximately 5.5 was chosen to ensure that they would exist in the unionized form. Extraction at lower pH values (2 or 4) did not alter the extraction efficiency of 5-FU or 5'dFUR, but chromatograms of blank plasma resulted in additional peaks which interfered with 5-FU. In addition, these chromatograms demonstrated late peaks with retention times greater than 10 min. While these peaks did not directly interfere with analysis, they did prolong the time between injections.

Typical chromatograms from blank human plasma and plasma spiked with known amounts of 5-FU and 5'dFUR are shown in Fig. 2. While a small peak still eluted just before 5-FU, it did not interfere with the quantitation of 5-FU. Chromatograms using the silica column clean-up procedure were cleaner than those obtained by using the procedures of Christophidis et al. [26] or Sampson et al. [27].

The optimal quantity of silica gel for purifying 0.5 ml of plasma was approximately 2.6 g. Smaller amounts resulted in high blank readings while larger amounts did not result in cleaner chromatograms. Similarly, 4 ml of ethyl acetate—methanol (90:10) provided good recoveries of all compounds from the silica column. The use of larger volumes resulted in higher quantities of interfering substances.

Silica gel purchased from different sources demonstrated higher blank readings. This was apparently due to polar contaminants in the silica gel since pre-washing with methanol generally produced clean traces. However, with certain lots, use of 5 m*M* potassium dihydrogen phosphate, adjusted to pH 4, was required to separate unknown peaks from the compounds of interest. In addition, the use of silica gel which had been activated by heating at 110°C introduced interfering peaks. Therefore, unactivated silica gel was used for all analytical procedures.

The sample preparation and chromatography present many opportunities for selective loss of 5'dFUR and 5-FU. To avoid adsorption of 5-FU to glass [28, 29], all glassware was silanized as described previously. Similarly, the temperature used for evaporation was maintained at 45—50°C since temperatures higher than 60°C resulted in losses of up to 25% while temperatures lower than 40°C were not sufficient to permit acceptable evaporation times.

Urine. Typical chromatograms from blank human urine and urine spiked with known amounts of 5-FU and 5'dFUR are shown in Fig. 3. Since drug concentrations in urine are much higher than those found in plasma, no

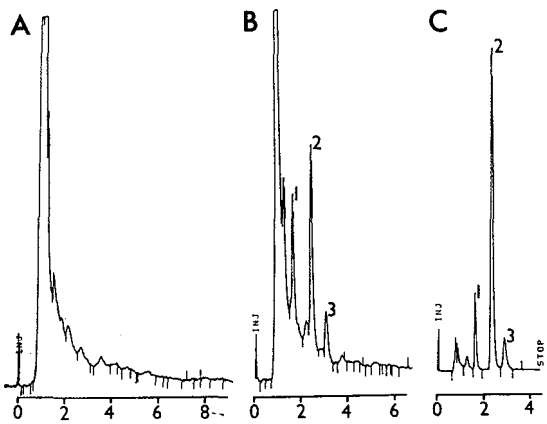

Fig. 2. Representative chromatograms from a 0.5-ml plasma extract showing retention times (min) for (1) 5-FU, (2) 5-BrU (internal standard) and (3) 5'dFUR. (A) Plasma control after extraction; (B) plasma spiked with 100 ng/ml 5-FU and 5'dFUR; (C) plasma spiked with 1 μg/ml 5-FU and 5'dFUR. The detector settings were 0.005 a.u.f.s. for A and B and 0.02 a.u.f.s. for C.

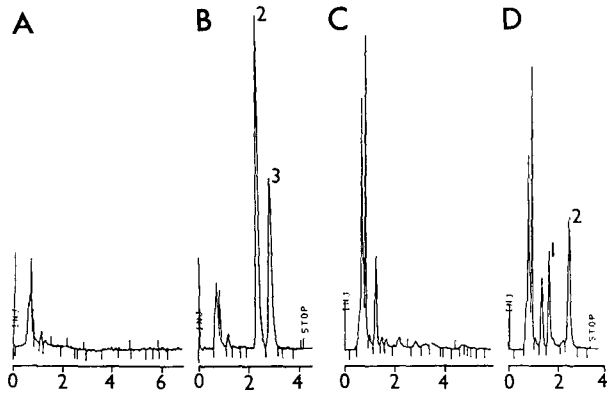

Fig. 3. Representative chromatograms from urine showing retention times (min) for (1) 5-FU, (2) 5-BrU and (3) 5'dFUR. (A) 1:1000 dilution of blank urine; (B) 1:1000 dilution of urine initially spiked with 2 mg/ml 5'dFUR; (C) 1:100 dilution of blank urine; (D) 1:100 dilution of urine initially spiked with 100 μg/ml 5-FU. The detector settings were 0.005 a.u.f.s. for A and B and 0.01 a.u.f.s. for C and D.

interference from normal urinary constituents were observed and thus, the silica column purification step was not necessary. The physical recovery of 5-FU (50 μg/ml) and 5'dFUR (2 mg/ml) from urine using this method was 83 and 85%, respectively.

Chromatographic behavior

The pH and ionic strength of the mobile phase had a profound effect on the resolution of 5-FU, 5'dFUR and I.S. from unknown plasma components. In general, as pH was lowered or ionic strength increased, the retention times of the three compounds were unaltered but blank chromatograms demonstrated peaks which could not be resolved from 5-FU. In contrast, when the pH of phosphate buffer was greater than 6.5, resolution was improved but column performance deteriorated rapidly. This may be related to precipitation of a plasma component in the column since flushing with 0.06% orthophosphoric acid (pH 2.3) and methanol restored column performance.

The Spherisorb phenyl (12.5 cm × 4.9 mm I.D.) column was chosen because it allowed adequate resolution of both 5'dFUR and 5-FU and provided optimal sensitivity since all peaks were eluted within 4 min. Other reversed-phase columns (C_8 and C_{18}) were tried but resulted in decreased sensitivity for 5'dFUR and longer assay times (20 min or longer per sample).

Linearity and precision

The standard curves for both compounds in plasma were linear over the concentration ranges studied, 50—1000 ng/ml and 1—75 μg/ml ($R^2 > 0.990$). The within-day coefficient of variation (C.V.), based on triplicate determinations, was less than 10% for both compounds at all concentrations. The between-day variation was calculated by performing triplicate analyses of plasma samples on three to four different days. This was done in samples containing both compounds at three or four concentrations. A summary of the

analysis is presented in Table I. Between-day C.V. values were all less than 10% except for the 100 ng/ml concentration of 5-FU for which we have no apparent explanation. Mean analytical recovery (accuracy), expressed as the ratio of compound added to that measured, was 102% (S.D. 3.8%) for 5-FU and 100% (S.D. 3.1%) for 5'dFUR.

Standard curves for 5-FU and 5'dFUR in urine over the range 50—250 μg/ml and 1—10 mg/ml, respectively, exhibited good linearity ($R^2 > 0.998$). Within-day variation was less than 4% for both compounds. Between-day variation was determined by analyzing triplicate samples on three different days (Table II). All C.V. values were less than 3%. Mean analytical recovery was 100% (S.D. 0.5%) for 5-FU and 100% (S.D. 0.4%) for 5'dFUR.

TABLE I

BETWEEN-DAY VARIABILITY OF PLASMA ASSAY

Concentration added	Mean concentration measured	*n*	Coefficient of variation (%)
5-FU			
50.0 ng/ml	53.9 ng/ml	4	3.7
100 ng/ml	107 ng/ml	4	13.5
400 ng/ml	401 ng/ml	4	4.3
1000 ng/ml	1012 ng/ml	4	1.9
1.00 μg/ml	0.99 μg/ml	3	4.4
5.00 μg/ml	4.98 μg/ml	3	2.0
50.0 μg/ml	49.6 μg/ml	3	1.7
5'dFUR			
50.0 ng/ml	50.1 ng/ml	4	9.6
100 ng/ml	98.4 ng/ml	4	8.7
400 ng/ml	389 ng/ml	4	6.3
1000 ng/ml	954 ng/ml	4	3.3
1.00 μg/ml	1.05 μg/ml	3	0.9
5.00 μg/ml	5.01 μg/ml	3	4.8
50.0 μg/ml	50.8 μg/ml	3	2.2
75.0 μg/ml	77.0 μg/ml	3	4.4

TABLE II

BETWEEN-DAY VARIABILITY OF URINE ASSAY

Concentration added	Mean concentration measured	*n*	Coefficient of variation (%)
5-FU (μg/ml)			
50.0	49.5	3	1.6
100	99.4	3	2.2
250	250	3	0.7
5'dFUR (mg/ml)			
1.00	1.00	3	0.9
2.00	2.01	3	0.7
10.0	9.99	3	0.7

Sensitivity

Using a signal-to-noise ratio of 3, the minimum detectable quantity on the column was 0.6 ng for 5-FU and 1.6 ng for 5'dFUR. By injecting 20 μl of the 100-μl reconstituted plasma extract at a sensitivity setting of 0.005 a.u.f.s., both of the compounds could be quantitated with acceptable precision at concentrations of 50 ng/ml in 0.5 ml of plasma.

Selectivity

The maximum UV absorbance of 5-FU, 5'dFUR and I.S. occurred at 266, 270 and 276 nm, respectively. Consequently, the chosen wavelength of 280 nm was near the maximum for all three compounds.

No interference was seen for the 5-FU anabolites 5-fluorouridine and 5-fluoro-2'-deoxyuridine or for the catabolic metabolite 2-fluoro-β-alanine. The inactive metabolite, 5,6-dihydrofluorouracil (DHFU), does exist at low μg/ml levels in plasma following the administration of 5-FU and 5'dFUR [20, 21, 30—32]. However, a pure sample of this compound in water does not demonstrate any absorbance at 280 nm, and thus cannot be detected using the present method.

Pharmacokinetic application

The application of the present method to the determination of 5'dFUR and 5-FU in plasma is shown in Fig. 4. The sensitivity of the assay was such that 5'dFUR and 5-FU plasma concentrations could be quantitated for 240 and 180 min, respectively, following the infusion of 2.0 g/m^2 5'dFUR to a patient with colorectal carcinoma.

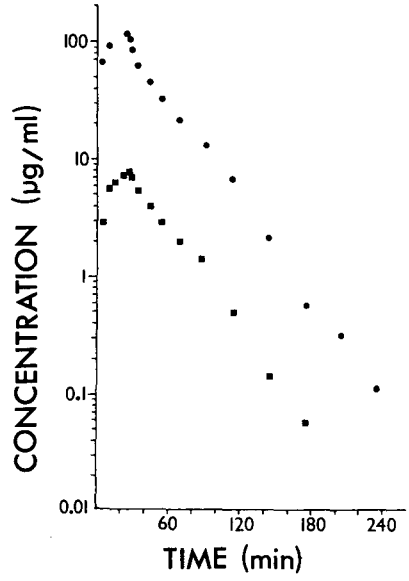

Fig. 4. Plasma concentration—time curves of 5'dFUR (•) and 5-FU (▪) in one patient after infusion of 2.0 g/m^2 5'dFUR over 25 min.

CONCLUSION

In summary, the present method enables the quantitation of both 5'dFUR and 5-FU in human plasma and urine. The sensitivity of the method is adequate for the analysis of both compounds following the administration of single doses of 5'dFUR. Consequently, the procedure should permit more detailed pharmacokinetic investigations of this novel fluoropyrimidine.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CERTAIN SALICYLATES AND THEIR MAJOR METABOLITES IN PLASMA FOLLOWING TOPICAL ADMINISTRATION OF A LINIMENT TO HEALTHY SUBJECTS

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SUMMARY

The liniment used is a topical analgesic and anti-inflammatory preparation containing two active constituents, 3-phenylpropylsalicylate and ethyl-5-methoxysalicylate, in solution in isobutyl decanoate. It is known that 3-phenylpropylsalicylate is metabolised to salicylic acid and salicyluric acid and ethyl-5-methoxysalicylate is metabolised to 5-methoxysalicylic acid and gentisic acid. In the present study the separation of the salicylates and their metabolites was carried out on a Waters μBondapak C_{18} column using two different mobile phases, methanol—water (80:20) for the parent drugs and methanol—5% aqueous acetic acid (27:73) for their metabolites. The salicylates and their metabolites were detected by absorption at 310 nm. The limits of detection for parent drugs and metabolites were respectively 0.2 and 0.1 μg/ml in plasma, using a 1-ml plasma sample and a 20-μl injection from a reconstituted volume of 250 μl. Mean percentage coefficients of variation for intra-assay and inter-assay precision were between 3.3 ± 1.9% to 9.1 ± 3.7% and 6.8 ± 2.2% to 15.7 ± 10.1%, respectively. Linearity, as measured by the correlation coefficient of intra-assay linear regression curves, was better than 0.998 in all cases.

INTRODUCTION

The liniment is composed of two active constituents, 3-phenylpropylsalicylate (PPS) and ethyl-5-methoxysalicylate; (EMS) in solution in isobutyl decanoate. Both PPS and EMS have anti-inflammatory and analgesic properties. The penetration of EMS through skin is rapid, while that of PPS is slower. The

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combination of EMS and PPS as a solution in isobutyl decanoate produces a synergistic analgesic and anti-inflammatory action.

The present study was undertaken to evaluate the percutaneous absorption of the liniment by determining PPS, EMS and its metabolites gentisic acid (GA), salicyluric acid (SU), 5-methoxysalicylic acid (MSA) and salicylic acid (SA) in blood plasma. Although a number of methods are available for quantifying salicylates in biological fluids [1—18], most of these methods lack sufficient sensitivity and only refer to assay of SA, SU and GA. In this paper a simple high-performance liquid chromatographic (HPLC) procedure for measuring PPS, EMS and their metabolites in plasma is described. The method was used to determine percutaneous absorption of the active constituents when 10 g of the linement were topically applied to the skin of healthy volunteers.

EXPERIMENTAL

Reagents

EMS, PPS and MSA were obtained from P.C.A.S. (France), SA from Baker, SU and GA from Aldrich, methanol and ethyl acetate (HPLC grade) from Fisons, and acetic acid (Aristar grade) and hydrochloric acid (1 *M*, analytical-reagent grade) from BDH (U.K.).

For the preparation of plasma standards, dried human plasma (from the Blood Transfusion Service Board, Ireland) was dissolved in nanopure water (deionised water obtained by reverse osmosis, Barnstead system). The blank plasma obtained was examined for the presence of endogenous components which might interfere with the salicylates and their metabolites in the assay system. The reconstituted plasma was stored at 4° C and used within two weeks of preparation.

Instrumentation

The HPLC system consisted of a Varian 5000 liquid chromatograph solvent delivery system equipped with a Waters U6K manual injector and fitted with a Waters Assoc. μBondapak C_{18} reversed-phase column (30 × 0.39 mm I.D., particle size 10 μm). A Pye-Unicam LC-3 variable-wavelength UV detector was used. Chromatograms were recorded on a Philips Model PM 8251 single-pen recorder.

Chromatography

The chromatographic conditions for separation of parent drugs were set as follows: mobile phase, methanol—water (80:20); flow-rate, 1.0 ml/min; recorder chart speed, 0.5 cm/min; detection wavelength, 310 nm; injection volume, 20 μl. Under the described chromatographic conditions the mean retention times for EMS and PPS were 3.6 min and 7.0 min respectively (Fig. 1A).

The chromatographic conditions for the separation of the metabolites were set as follows: mobile phase, methanol—5% aqueous acetic acid (27:73); flow-rate, 1.5 ml/min; recorder chart speed, detection wavelength and injection volume were the same as those set for the parent drugs. Under the described chromatographic conditions the mean retention times were 4.0 min for GA, 5.0 min for SU, 10.0 min for SA and 11.4 min for MSA (Fig. 1B).

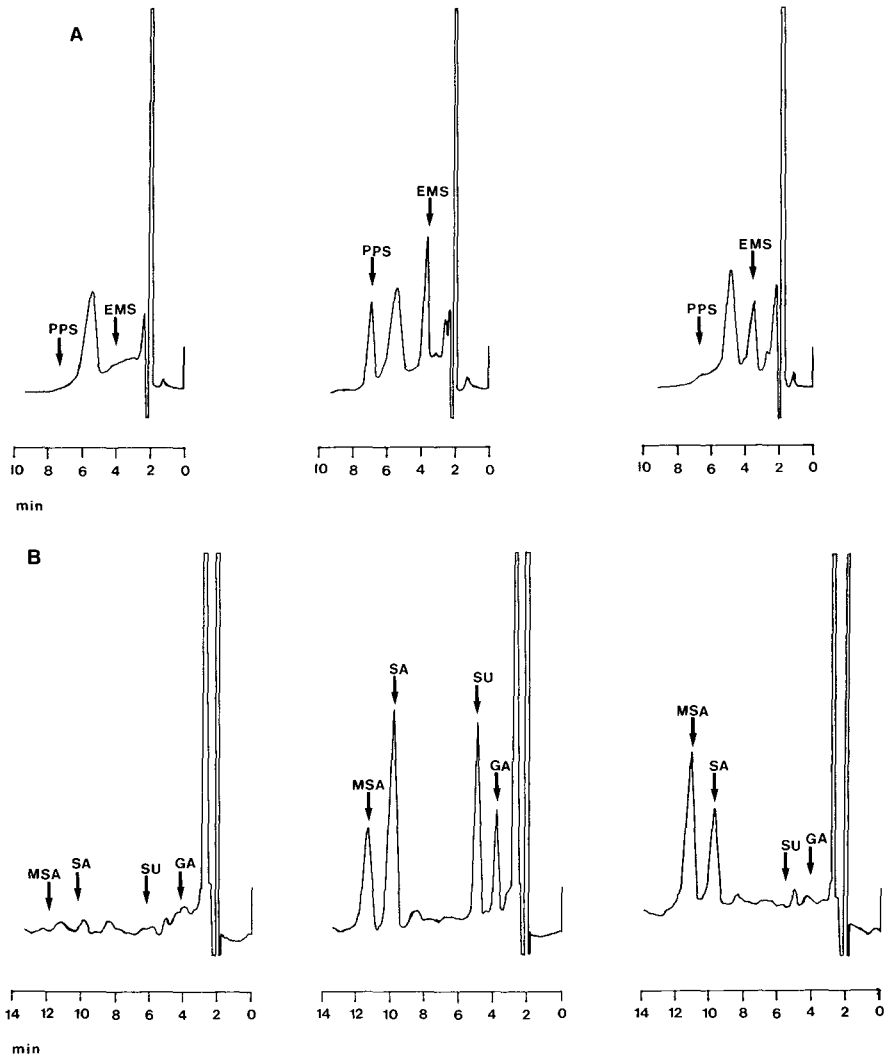

Fig. 1. (A) Chromatograms of extracts from the plasma of one of the volunteers (subject B.W.). Left: Pre-topical dose; middle: drug-free plasma spiked with 1 μg/ml EMS and PPS; right: 6 h post topical dose. (B) Chromatograms of extracts from the plasma of one of the volunteers (subject B.W.). Left: Pre-topical dose; middle: drug-free plasma spiked with 0.5 μg/ml of each of the metabolites; right: 6 h post topical dose.

Standards

From each of the salicylates and their metabolites 10.00 mg were accurately weighed and dissolved in 100 ml methanol to yield the stock standard solution (100 μg/ml). This stock solution was further diluted to give working standards ranging from 1 to 100 μg/ml. Spiked plasma standards were prepared each day by addition of 100 μl of the working standard solutions to 1 ml plasma, to provide standards ranging from 0.1 to 10 μg/ml of the drugs in plasma.

TABLE I

INTRA-ASSAY VARIATION FOR THE SALICYLATES AND THEIR METABOLITES

$\bar{x}$ = Mean concentration found; S.D. = standard deviation (n = 4); C.V. = coefficient of variation (%); r = correlation coefficient.

Concentration added (μg/ml)	Concentration found (μg/ml)											
	PPS		EMS		SA		MSA		SU		GA	
	$\bar{x}$ ± S.D.	C.V.	$\bar{x}$ ± S.D.	C.V.	$\bar{x}$ ± S.D.	C.V.	$\bar{x}$ ± S.D.	C.V.	$\bar{x}$ ± S.D.	C.V.	$\bar{x}$ ± S.D.	C.V.
0.50	0.59 ± 0.04	6.8	0.51 ± 0.07	13.7	0.60 ± 0.04	6.6	0.66 ± 0.13	19.6	0.54 ± 0.05	9.3	0.59 ± 0.02	3.4
1.00	0.94 ± 0.04	4.2	0.96 ± 0.13	13.5	0.98 ± 0.11	11.2	0.93 ± 0.04	4.3	0.98 ± 0.06	6.1	1.01 ± 0.09	8.9
2.00	2.02 ± 0.07	3.5	2.07 ± 0.23	11.1	1.96 ± 0.08	4.1	1.89 ± 0.09	4.8	2.05 ± 0.05	2.4	2.00 ± 0.06	3.0
4.00	3.94 ± 0.04	1.0	3.81 ± 0.25	6.5	3.92 ± 0.11	2.8	4.01 ± 0.09	2.2	3.88 ± 0.11	2.8	3.86 ± 0.18	4.7
6.00	6.04 ± 0.09	1.5	6.06 ± 0.49	8.1	6.19 ± 0.25	4.0	6.14 ± 0.17	2.7	6.16 ± 0.13	2.1	6.12 ± 0.09	1.5
8.00	7.94 ± 0.26	3.3	8.34 ± 0.34	4.1	7.66 ± 0.39	5.1	7.69 ± 0.35	4.4	7.79 ± 0.12	1.5	7.74 ± 0.18	2.3
10.00	10.06 ± 0.28	2.8	9.76 ± 0.64	6.5	10.19 ± 0.26	2.5	10.18 ± 0.49	4.8	10.11 ± 0.19	1.9	10.19 ± 0.23	2.3
Mean C.V. ± S.D.	3.3 ± 1.9		9.1 ± 3.7		5.2 ± 3.0		6.1 ± 6.0		3.7 ± 2.9		3.7 ± 2.5	
r	0.9999		0.9986		0.9987		0.9988		0.9994		0.9991	

TABLE II

INTER-ASSAY VARIATION FOR THE SALICYLATES AND THEIR METABOLITES

$\bar{x}$, S.D. and C.V. as in Table I.

Concentration added (μg/ml)	Concentration found (μg/ml)											
	PPS		EMS		SA		MSA		SU		GA	
	$\bar{x}$ ± S.D.	C.V.	$\bar{x}$ ± S.D.	C.V.	$\bar{x}$ ± S.D.	C.V.	$\bar{x}$ ± S.D.	C.V.	$\bar{x}$ ± S.D.	C.V.	$\bar{x}$ ± S.D.	C.V.
0.50	0.53 ± 0.18	33.9	0.51 ± 0.11	21.5	0.68 ± 0.06	8.8	0.55 ± 0.16	29.1	0.51 ± 0.08	15.7	0.49 ± 0.07	14.3
1.00	0.93 ± 0.22	23.6	0.99 ± 0.03	3.0	1.05 ± 0.07	6.7	0.98 ± 0.09	9.2	0.84 ± 0.06	7.1	0.90 ± 0.18	20.0
2.00	1.97 ± 0.23	11.6	2.05 ± 0.08	3.9	2.14 ± 0.13	6.1	1.95 ± 0.17	8.7	1.96 ± 0.10	5.1	1.99 ± 0.18	9.0
4.00	3.86 ± 0.52	13.5	3.95 ± 0.39	9.9	4.14 ± 0.11	2.6	3.96 ± 0.07	1.8	4.15 ± 0.13	3.1	4.10 ± 0.32	7.8
6.00	6.28 ± 0.72	11.5	6.02 ± 0.28	4.6	6.21 ± 0.41	6.6	6.18 ± 0.50	8.1	6.22 ± 0.44	7.1	6.13 ± 0.70	11.4
8.00	7.84 ± 0.19	2.4	7.87 ± 0.51	6.5	7.93 ± 0.64	8.1	7.84 ± 0.70	8.9	8.01 ± 0.58	7.2	8.02 ± 0.77	9.6
10.00	9.96 ± 1.35	13.5	10.10 ± 0.47	4.6	9.86 ± 0.88	8.9	10.06 ± 1.18	11.7	9.83 ± 0.80	8.1	9.88 ± 1.10	11.1
Mean C.V. ± S.D.	15.7 ± 10.1		7.7 ± 6.5		6.8 ± 2.2		11.1 ± 8.5		7.6 ± 3.9		11.9 ± 4.1	

Extraction procedure

Parent drugs (EMS and PPS). Plasma (1 ml) spiked with 100 μl working standards was mixed with 1 ml nanopure water in a glass-stoppered tube. After the addition of 7 ml ethyl acetate, using an all-glass dispenser, the drugs were extracted by rotating the tubes gently for 15 min on a mechanical rotator, followed by centrifugation for 15 min at 700 *g* at 0°C. Exactly 5 ml of the supernatant were transferred into a glass tube which was placed in a water bath at 40°C and the solvent was evaporated under a gentle stream of oxygen-free nitrogen. The residue was reconstituted in 250 μl methanol and 20 μl were injected for HPLC analysis.

Metabolites. The extraction procedure for the metabolites was exactly the same as that for the parent drugs except that a 2-ml aliquot of 0.05 *M* hydrochloric acid was added in place of nanopure water, before ethyl acetate extraction. Samples were extracted exactly as described above for standards except in place of the working standards an equivalent amount of methanol was added.

Calibration and calculation

Each calibration curve was obtained by linear regression of the peak heights of each compound versus concentrations of that compound (external standard method). These calibration curves were then used to interpolate the concentration of salicylates and their metabolites in plasma from the measured peak height of each individual compound.

RESULTS AND DISCUSSION

Limit of detection

Under procedural conditions the limits of detection, using a 1-ml plasma sample and 20-μl injections, were 0.2 and 0.1 μg/ml of the salicylates and their metabolites, respectively. The limit of detection was taken as the amount of compound giving a signal-to-noise ratio greater than 3:1. Higher sensitivity can be obtained by dissolving the extract residue in the mobile phase rather than in methanol. This will allow the injection of a larger sample volume. However, dissolution of extract residue in the mobile phase results in a cloudy solution which will have to be filtered prior to injection.

Reproducibility

The data presented in Tables I and II demonstrate the within-batch (intra-assay) and between-batch (inter-assay) variation of the method. Intra-assay variability was determined at seven concentrations in quadruplicate: 0.50, 1.00, 2.00, 4.00, 6.00, 8.00 and 10.00 μg/ml of each compound in plasma. Inter-assay variability was determined singly at the same seven concentrations in four replicate runs. The precision of the method (mean coefficient of variation ± standard deviation) for the values of recovered determinate standards, when calculated as "unknown" against the linear regression line were between 3.3 ± 1.9% to 9.1 ± 3.7% and 6.8 ± 2.2% to 15.7 ± 10.1% for intra-assay and inter-assay variation, respectively.

TABLE III

PLASMA LEVEL OF SALICYLATES AND THEIR MAJOR METABOLITES IN ONE OF THE VOLUNTEERS (SUBJECT B.W.)

N.D. = Not detectable.

Time	Concentration (μg/ml)					
	EMS	PPS	GA	SU	MSA	SA
Pre-Dose	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
1 h	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
2 h	0.5	N.D.	N.D.	N.D.	0.7	0.1
6 h	0.6	N.D.	N.D.	N.D.	0.7	0.3
12 h	0.3	N.D.	N.D.	N.D.	0.5	0.3
24 h	0.2	N.D.	N.D.	N.D.	0.7	0.2

Linearity

A measure of linearity, as defined by the correlation coefficient of the regression lines for each compound, is given under intra-assay variation (Table I). The correlation coefficients were better than 0.998 in all cases.

Recovery

Recovery was calculated by comparing the peak heights of parent drugs and metabolites after their extraction from plasma with the peak heights of series of unextracted reference standards. In the concentration range 0.5—10 μg/ml, the mean overall percentage recoveries were 81.2 ± 6.7% for EMS, 84.5 ± 8.2% for PPS, 62.1 ± 3.8% for GA, 66.7 ± 8.2% for SU, 73.4 ± 9.1% for SA and 67.3 ± 10.2% for MSA.

Plasma levels of salicylates and their metabolites

The purpose of the present study was to determine the disposition of the salicylates and their major metabolites following topical administration of the liniment to healthy volunteers. All subjects gave written informed consent to participate in the study, the protocol for which was approved by the Institutional Review Board. The liniment (10 g) was applied to the skin of the chest and back of the subjects. Blood (10 ml) was collected from an antecubital vein into pre-cooled (4°C) fluoride—oxalate vacutainers at the following times: pre-application and 1, 3, 6, 12 and 24 h after application. The plasma was separated within 15 min and stored at —20°C before analysis. Table III presents typical results from one of the volunteers using the described procedure. These data confirmed that percutaneous absorption of the active constituents of the liniment had occurred.

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URINARY DIHYDRODIOL METABOLITES OF PHENYTOIN: HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF DIASTEREOMERIC COMPOSITION*

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SUMMARY

Diastereomeric dihydrodiol metabolites of phenytoin, (5*S*)-5-[(3*R*,4*R*)-3,4-dihydroxy-1,5-cyclohexadien-1-yl]-5-phenylhydantoin, (*S*)-DHD, and (5*R*)-5-[(3*R*,4*R*)-3,4-dihydroxy-1,5-cyclohexadien-1-yl]-5-phenylhydantoin, (*R*)-DHD, have been resolved from each other and from urinary constituents with reversed-phase HPLC columns and acetonitrile—water gradients. Recoveries of DHD isomers from urine averaged 99.1% and it was demonstrated that known mixtures of DHD diastereomers added to blank urine were not altered by the assay procedures. The relative diastereomeric content of DHD was determined from integration of the chromatographic peaks. Assay of urine samples from patients on chronic phenytoin therapy and from volunteers indicated that both DHD isomers were present in all samples, and stereoselectivity favored the production of (*S*)-DHD.

INTRODUCTION

Metabolism of the prochiral antiepileptic drug phenytoin (5,5-diphenylhydantoin, PHT) to a dihydrodiol metabolite, 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin (DHD), was first demonstrated by Chang et al. [1]. Subsequent studies of DHD isolated from urines of dogs treated with PHT demonstrated the existence of two diastereomeric forms of the metabolite [2]. One of these isomers, designated as (*S*)-DHD, (5*S*)-5-[(3*R*,4*R*)-3,4-di-

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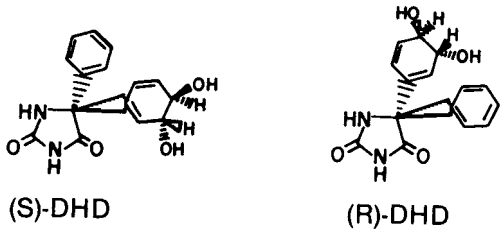

Fig. 1. Structures of (*S*)-DHD and (*R*)-DHD.

hydroxy-1,5-cyclohexadien-1-yl]-5-phenylhydantoin (Fig. 1), was found to be present in the urine of rats treated with PHT, and also in the urine of a patient on chronic PHT therapy. The existence of (*R*)-DHD, (5*R*)-5-[(3*R*,4*R*)-3,4-dihydroxy-1,5-cyclohexadien-1-yl]-5-phenylhydantoin (Fig. 1), in dog urine had been demonstrated, but minor amounts of (*R*)-DHD in rat and human urines could not be confirmed [2]. Previous reports of high-performance liquid chromatographic (HPLC) methodology for the study of DHD and other metabolites of PHT have used reversed-phase columns with methanol—water or acetonitrile—water eluents [3—6]. However, evidence of resolution of possible isomeric DHD by such methods has not been reported. We wish to describe methodology whereby DHD may be extracted from urine and its diastereomeric components successfully resolved and quantitated.

Our interest in the quantitation of the isomeric forms of DHD was to provide an indirect method of identifying the isomeric arene oxides which might be formed by the metabolism of the prochiral phenyl substituents of PHT. As arene oxides are transient metabolites, their presence can usually only be detected by monitoring rearrangement products (phenols) or *trans*-dihydrodiols, such as (*R*)- and (*S*)-DHD, which are derived from enzymatic hydration of putative (*R*)- and (*S*)-arene oxides of phenytoin, respectively.

EXPERIMENTAL SECTION

Chemicals

Samples of various mixtures of (*R*)- and (*S*)-DHD were available from previous studies [2], as were the isomeric racemic metabolites 5-(3-hydroxyphenyl)-5-phenylhydantoin (*m*-HPPH) and 5-(4-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH). The internal standard used for recovery studies, 5-ethyl-5-(4-hydroxyphenyl)hydantoin (EHPH) was available from a previous study [7]. A partially purified solid preparation of β-glucuronidase from *Helix pomatia* was purchased from Sigma (St. Louis, MO, U.S.A.). Acetonitrile and methanol, both HPLC grade, as well as all other analytical reagent grade chemicals were purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Apparatus

Two Altex Model 110A pumps (Berkeley, CA, U.S.A.) were used for solvent delivery and were coupled to a Rheodyne (Cotati, CA, U.S.A.), Model 7125 injector equipped with a 20-μl loop, and to an ISCO (Lincoln, NE, U.S.A.) Model V4 variable wavelength absorbance detector. Gradient programming and

system control were provided by an Axxiom Model 710 microprocessor from Cole Scientific (Calabasas, CA, U.S.A.). A Hewlett-Packard (Avondale, PA, U.S.A.) Model 3390A reporting integrator was used for measuring peak areas. A Millipore Norganic filter system from Waters (Milford, MA, U.S.A.) was used to produce HPLC grade water from distilled water. Sample preparation involved the use of Waters Sep-Pak C_{18} cartridges, Gelman (Ann Arbor, MI, U.S.A.) Acrodisc-CR 0.45-μm filters, and a Buchler (Fort Lee, NJ, U.S.A.) Evapo-Mix vortex evaporator.

Urine samples

Samples from pediatric patients on chronic PHT and other antiepileptic therapy were obtained during regularly scheduled pediatric neurology clinics at Duke University Medical Center (Durham, NC, U.S.A.) and at North Carolina Memorial Hospital (University of North Carolina School of Medicine, Chapel Hill, NC, U.S.A.). Informed consent of the parents was obtained and all studies were approved by the local institutional review boards. Samples from volunteers given a single 5 mg/kg i.v. dose of PHT were supplied by Dr. Gunnar Alván of the Department of Clinical Pharmacology of the Karolinska Institutet, Huddinge, Sweden. Samples of dog urine were obtained by the previously described procedure [2]. Rat urine was obtained following a 50 mg/kg i.p. dose to a female Sprague-Dawley rat, and collection of 0—48 h urine sample [2]. All types of urine sample were assayed for DHD and *p*-HPPH content by a gas—liquid chromatographic (GLC) method [8]. Samples containing 1 to greater than 50 μg DHD/ml of urine were selected for further assay of diastereomeric content of DHD.

Sample preparation

To 1.0 ml of urine in a 16 × 125 mm PTFE-lined screw-cap culture tube were added 1000 units of β-glucuronidase dissolved in 0.5 ml of 1.0 *M* sodium acetate buffer, pH 5.0. After incubation for 18 h at 37°C, the sample was transferred by disposable pipette onto a Sep-Pak cartridge which had previously been washed with 5 ml of methanol and then 5 ml of water. The urine sample was flushed through the cartridge, which was then washed with 5 ml of water. An Acrodisc-CR filter was attached to the bottom of the cartridge and 5 ml of 50% methanol—water was used to elute the sample. The bulk of the solvent was removed on the vortex evaporator, and the sample was completely dried using a stream of nitrogen gas. The residue was reconstituted in 50 μl of 50% methanol—water.

Chromatography

The standard chromatographic assay involved sample purification (Sep-Pak cartridges), gradient chromatography with collection of a DHD fraction (Fig. 2), followed by a second gradient chromatography of the purified DHD sample (Figs. 3 and 4). For both portions of the assay, the detection wavelength was 210 nm, the eluent was acetonitrile—water, and the columns were at ambient temperature (24—25°C).

A LiChrosorb RP-18 column (10 μm, 250 × 4.6 mm I.D.) from E.M. Reagents (Gibbstown, NJ, U.S.A.) and a 100 × 3.2 mm C_{18} guard column

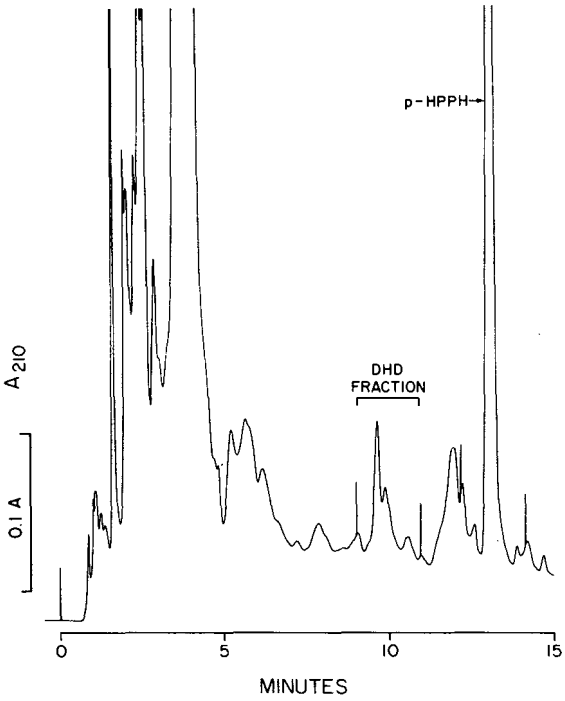

Fig. 2. Reversed-phase gradient separation (Program I) of DHD fraction from other urinary constituents and PHT metabolites after initial purification on a Sep-Pak cartridge. The urine sample contained 19 μg/ml of total DHD and was from a volunteer given a single dose of PHT.

(Whatman, Clifton, NJ, U.S.A.) were used for the initial chromatography (Fig. 2). The injection volume was 20 μl and the flow-rate was 2.0 ml/min. Gradient program I was as follows:

Step	Acetonitrile (vol. %)	Time (min)
1	10	0.00
2	10	4.00
3	50	10.00
4	5	1.00
5	5	2.00
6	10	2.00
7	10	End

A 4-ml fraction containing the DHD peaks (Fig. 2) was collected, evaporated under reduced pressure, and the residue reconstituted in 100 μl 50% methanol—water.

For the analytical determination a 0.5-μm in-line filter and a Microsorb C_{18} column (3 μm, 100 × 4.6 mm I.D.) from Rainin (Woburn, MA, U.S.A.) were used. The injection volume was 5 μl and the flow-rate was 1.0 ml/min.

Gradient program II was as follows:

Step	Acetonitrile (vol. %)	Time (min)
1	10	0.00
2	10	4.00
3	15	7.00
4	15	3.00
5	7	1.00
6	7	1.00
7	10	1.00
8	10	End

Peak verification and recovery studies

In order to verify the chemical content of DHD peaks, 1-ml fractions corresponding to (*S*)- and (*R*)-DHD from patient samples were collected during an analytical run. To each sample was added 0.25 ml of 12 *M* hydrochloric acid, and samples were placed on a steam bath for 30 min to dehydrate the dihydrodiols to their corresponding phenols (*p*-HPPH and *m*-HPPH). Upon cooling, the samples were neutralized with 0.25 ml 10 *M* sodium hydroxide and then extracted with 8 ml diethyl ether saturated with 0.25 *M* sodium phosphate buffer (pH 7.6). Samples (5 ml) of the extracts were evaporated and the residues reconstituted in 50 μl methanol. These samples, along with standard *p*- and *m*-HPPH solutions, were analyzed by HPLC on the 3 μm column using Program I. Retention times of *m*-HPPH and *p*-HPPH were 12.5 and 13.1 min, respectively.

For testing dihydrodiol recovery during sample preparation, two standard solutions were prepared to be 1:1 and 98:2 (*S*)-DHD/(*R*)-DHD, both of concentration 10 μg/ml water. From each of these solutions 1 ml was subjected to the sample preparation procedure while 1 ml was set aside to serve as control. Following the Sep-Pak cartridge elution step, 10 μl of an internal standard solution (100 μg EHPH/ml methanol) was added to both extracted and control samples. The samples were evaporated and the residues reconstituted in 100 μl 50% methanol—water prior to HPLC analysis (Program II). With this method, retention times of EHPH, (*S*)-DHD and (*R*)-DHD were 9.2, 12.5, and 13.4 min, respectively.

RESULTS

Assay development

Resolution of (*S*)-DHD and (*R*)-DHD from a 1:1 mixture of the purified metabolites [2] was achieved on a variety of ODS columns with acetonitrile—water eluents. (*S*)-DHD eluted first as verified with a sample known to contain largely (*S*)-DHD. Baseline resolution was achieved with 12% acetonitrile on a 10 cm, 3 μm spherical ODS column [resolution (R) = 2.2, k' = 14.1 for (*S*)-DHD, α = 1.12]. However, this isocratic system did not allow complete separation of DHD isomers from co-extracted endogenous compounds in human urine, and the following purification procedure was subsequently developed to circumvent this problem.

A urine sample was incubated with β-glucuronidase under conditions whereby all conjugated DHD should be released [8]. Metabolites were then extracted by a C_{18} Sep-Pak cartridge, and subsequent elution of the cartridge with 50% methanol gave a partially purified DHD fraction. This fraction was subjected to gradient chromatography (Program I) on a 25-cm, 10-μm irregular ODS column, and the DHD fraction was collected as indicated in Fig. 2. Subsequent gradient chromatography (Program II) of this concentrated DHD fraction on a 3-μm ODS column allowed separation of (*S*)- and (*R*)-DHD from interfering substances and from each other. Fig. 3A shows the chromatogram of the 1:1 standard mixture [2], and Fig. 3B the result obtained from the complete assay of blank human urine with 10 μg/ml of added 1:1 standard. It has been previously demonstrated that (*R*)- and (*S*)-DHD have identical UV spectra in the 240—270 nm range [2]. Integration of the chromatogram (Fig. 3A) of the 1:1 mixture at detection wavelengths from 210 to 270 nm gave identical 1:1 ratios, indicating the diastereomers have identical UV spectra in this range. The relative composition of DHD mixtures were thus determined from the ratio of peak areas measured by an electronic integrator. The chroma-

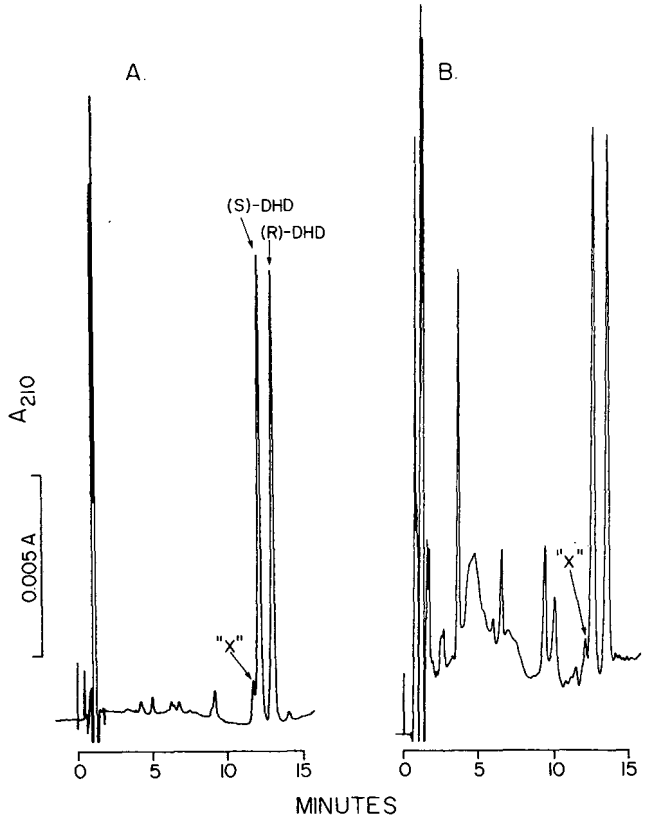

Fig. 3. (A) Chromatogram (Program II) of a 1:1 mixture of (*S*)-DHD/(*R*)-DHD previously isolated from dog urine [2]. "X" is an unidentified contaminant present in the original preparation. (B) Chromatogram of the complete assay of a blank human urine sample with 10 μg/ml of added 1:1 DHD mixture.

togram of an assayed blank urine sample, when monitored at either 210 or 254 nm, did not show any obvious interferences (Fig. 4A). The increased absorbance at 210 nm offered a 5—6 fold increased sensitivity over monitoring at 254 nm and this was the sole reason for chosing the lower wavelength.

Recovery and reproducibility of the assay

As it might be possible to alter inadvertently the ratio of diastereomers by incomplete extraction or elution, conditions were chosen such that maximal extraction of DHD from urine and recovery from the Sep-Pak cartridges were achieved. Recoveries of DHD from aqueous solutions were calculated by comparing peak area ratios of DHDs to an internal standard that was added to the aqueous DHD stock solution and to DHD fractions after Sep-Pak extraction/elution. When 10 μg/ml aqueous solutions of 1:1 and 98:2 mixtures of (*S*)-DHD/(*R*)-DHD were submitted to the Sep-Pak extraction/elution procedure, recoveries of 99.2 ± 0.5% (n = 4) and 99.0 ± 1.2% (n = 4), respectively, were observed. No detectable change in percentage (*S*)-DHD in the two samples could be observed after the extraction/elution procedure. With this procedure, it was not possible to calculate DHD recoveries from urine, as endogenous compounds coeluted with the internal standard. However, when blank urine samples with added 1:1 or 98:2 (*S*)-DHD/(*R*)-DHD mixtures, were assayed by the complete method, values of 50.5 ± 1.6% (*S*)-DHD (coefficient of variation (CV) = 3.2%, n = 4) and 98.4 ± 0.5% (*S*)-DHD (CV = 0.5%, n = 4), respectively, were obtained. These values were within experimental error of those of the corresponding aqueous DHD stock solutions. This evidence suggests that the recovery from urine is equivalent to that from water, and that extraction and collection of DHD through the assay procedure had not compromised the diastereomeric content of the DHD sample.

Evaluation of within-sample variability was performed with urine samples containing 82:18 and 98:2 mixtures of (*S*)-DHD/(*R*)-DHD. Repeated injections of DHD fractions from these two samples gave values of 81.9 ± 1.4% (*S*)-DHD (CV = 1.7%, n = 10) and 97.8 ± 0.8% (*S*)-DHD (CV = 0.8%, n = 10), respectively. Based upon the multiple assay of urines containing known DHD mixtures and the observation of within-sample variability, an error limit of ± 1% would routinely be applied to all samples assayed.

Verification of peak identity

In all human urine samples, both blank and with added DHD, little or no co-eluting endogenous substances have been found which interfere with quantitation of DHD. Fig. 4A is representative of a typical blank urine assay. Selected urine samples of patients or volunteers given PHT were carried through the assay procedure and collections of (*S*)- and (*R*)-DHD peaks were made. These samples were treated with acid to quantitatively dehydrate any DHD to a mixture of the isomeric phenols, *p*-HPPH and *m*-HPPH. The ratio of amounts of phenols produced from (*S*)-DHD to those produced by (*R*)-DHD fractions were within experimental error of the observed ratios for the intact DHD isomers, indicating that no UV-absorbing substances were interfering with DHD quantitation in these samples.

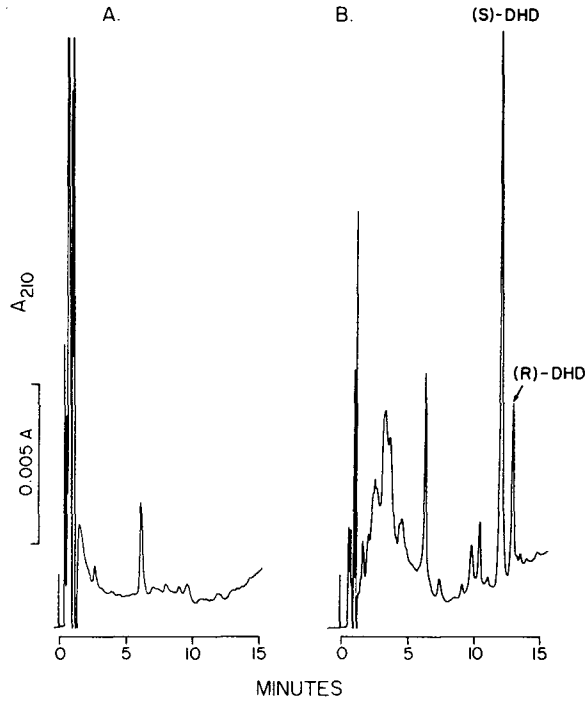

Fig. 4. (A) Chromatogram (Program II) of the complete assay of blank human urine. (B) Complete assay of a human urine sample containing 20 μg/ml of total DHD, which was found to contain 78% (*S*)-DHD.

Applicability of assay

Quantitation of relative amounts of DHD diastereomers was possible in urines of patients treated with PHT alone, and those treated with added valproic acid or phenobarbital. Examples of the stereoselectivity observed are shown in Table I. Patients treated with PHT and carbamazepine consistently had urinary product(s) with retention time(s) of approximately 14 min. Such materials interfered with the elution of (*R*)-DHD and made quantitation virtually impossible. It is assumed that the products represent metabolites of carbamazepine, although this has not been verified. Modification of the gradient elution conditions (longer initial hold time, 8 min) of Program I allowed improved separation of DHD from the urinary products, and the interference with the assay has largely been eliminated. Examples of the DHD composition obtained with the modified purification procedure are also shown in Table I.

It has also proved possible to assay DHD composition in the urine of other mammalian species dosed with PHT, with compositions from rat and dog urine having been found to be 98 and 43% (*S*)-DHD, respectively.

DISCUSSION

The existence of diastereomeric dihydrodiol metabolites of PHT, (*S*)-DHD and (*R*)-DHD (Fig. 1), has been confirmed by resolution of the purified

TABLE I

OBSERVED DIASTEREOMERIC COMPOSITION OF URINARY DHD IN PATIENTS (CHRONIC PHT THERAPY) AND VOLUNTEERS

VPA = Valproic acid; CBZ = carbamazepine; PB = phenobarbital.

Patient group	Average percentage (*S*)-DHD (range)
PHT alone (n = 7)	74 (69—77)
PHT, VPA (n = 3)	72 (68—78)
PHT, CBZ (n = 6)	73* (69—78)
PHT, PB (n = 2)	91 (86—96)
PHT, CBZ, PB (n = 3)	74 (57—88)
Volunteers (n = 7)	77 (75—79)

*Determined with the modified assay procedure (see *Applicability of assay*).

samples with the reversed-phase HPLC methodology reported here. The assayed composition of DHD from rat and dog urines has also provided evidence confirming the compositions that were previously assayed by chemical and instrumental methods [2].

The recovery of DHD isomers from urine and from Sep-Pak cartridges used for purification has been shown to be quantitative, and the assay procedures do not affect the diastereomeric content of known DHD mixtures added to blank human urine. This HPLC method is presently used for quantitation of relative amounts of DHD isomers in urine samples which have been previously assayed for total DHD content by a GLC method [8]. It is possible that the HPLC method could be expanded to allow quantitation of absolute amounts of the DHD isomers providing an appropriate internal standard could be found. The internal standard EHPH used in the recovery studies co-elutes with endogenous urinary constituents, however other elution conditions or other appropriate internal standards might be developed to allow quantitation of total amounts of DHD isomers.

Theoretically there should be four possible *trans*-dihydrodiol metabolites of PHT, (*S*)-DHD and its enantiomer, and (*R*)-DHD and its enantiomer. In the previous study, enantiomers of (*S*)-DHD and (*R*)-DHD were not detected, and this finding was reported to be consistent with the (*R*,*R*) stereochemistry that is prevalent in metabolic dihydrodiols of other aromatic compounds [2]. The chromatographic columns and conditions of this assay cannot differentiate between (*S*)-DHD and its enantiomer, or (*R*)-DHD and its enantiomer. While the results of the urinary DHD diastereomer assay are reported as % (*S*)-DHD (Table I), we cannot presently exclude the possibility that the peaks identified as (*S*)- and (*R*)-DHD may contain enantiomers of the respective compounds.

The results obtained from assay of human urinary DHD (Table I) suggest that both (*S*)- and (*R*)-DHD are being produced, with stereoselectivity favoring (*S*)-DHD. This provides the evidence of formation of two arene oxides of PHT

which are the precursors of the corresponding (*R*)- and (*S*)-DHD. Previous studies of stereoselective PHT metabolism have examined metabolic phenol (*p*-HPPH) stereochemistry and have suggested that the *pro-S*-phenyl substituent of PHT is preferentially metabolized in man [2, 9—11], with approximately 90% of the *p*-HPPH being of the *S*-configuration. The identification of (*S*)-DHD in human urine by this and a previous study [2] is consistent with an (*S*)-arene oxide being an intermediate in the formation of the (*S*)-phenol and (*S*)-DHD. The present study has demonstrated that a second arene oxide, the precursor to (*R*)-DHD, is also being formed in man. The stereoselectivity of DHD formation does not appear to vary for those patients on PHT alone, (74%(*S*)-), PHT and valproic acid (72%(*S*)-), or PHT and carbamazepine (73%(*S*)-) nor does it differ substantially from values obtained from volunteers (77%(*S*)-) given a single dose of PHT (Table I). Differences are observed in two patients on PHT and phenobarbital, and three patients on PHT, phenobarbital, and carbamazepine. This preliminary investigation suggests that some antiepileptic drugs may interfere with the stereoselective metabolism of PHT, and further such studies are in progress.

The HPLC methodology reported here allows the quantitation of diastereomeric content of DHD metabolites and can be used in conjunction with other assay methods for enantiomeric content of the phenolic metabolite (*p*-HPPH) [10, 11]. Application of such methods should allow a more complete study of stereoselective PHT metabolism in man and other species, and allow study of apparent differences in stereoselectivity observed for *p*-HPPH production [2, 9—11] and those presently observed for DHD production (Table I).

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CHROMBIO. 2640

SUB-NANOGRAM ANALYSIS OF YOHIMBINE AND RELATED COMPOUNDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive (50 pg/ml) method is described for the analysis of yohimbine in blood by high-performance liquid chromatography with fluorescence detection . The chromatographic behaviour of eserine (employed as internal standard), reserpine, corynanthine, yohimbinic acid, and yohimbine are examined on a series of reversed-phase and normal-phase chromatographic columns with methanol—water mobile phases.

INTRODUCTION

Yohimbine is an indole alkaloid obtained from a number of biological sources including Corynanthe Johimbe, Rubiaceae and related trees and Rauwolfia root. Pharmacologically classified as an alpha$_2$ adrenoceptor antagonist, it has recently been employed both as a means of identifying physiological responses mediated through alpha$_2$ receptors in vivo and in vitro, and clinically in the treatment of male impotence [1]. Although yohimbine has been studied for well over 100 years, the pharmacokinetics of this drug have received little attention, a situation due to the lack of suitable analytical methodologies for the drug.

The separation of yohimbine from other Rauwolfia alkaloids by a variety of chromatographic techniques including paper [2—4], thin-layer [5, 6], gas—liquid (GLC) [7, 8] and high-performance liquid chromatography (HPLC) [9] is well established. Unfortunately, these reports have been concerned primarily with the chromatographic behaviour of indole alkaloids rather than drug analysis from biological samples. As a result, the sensitivity of these

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methods for the detection of yohimbine was either not determined or poorly estimated (approximately 2 μg/ml water) [7]. Recently, the development of an HPLC yohimbine assay with electrochemical detection has been reported by Goldberg et al. [10]. These authors have employed this assay procedure to examine the yohimbine plasma concentration–time profile in human volunteers on a continuous intravenous infusion of yohimbine (125 μg/kg bolus, 1 μg/kg min infusion). Under this dosage regimen, an assay of this sensitivity (minimum detectable quantity 10 ng/ml) is quite adequate for the quantitation of plasma yohimbine levels. However, the results of Goldberg et al. [10] suggest that for a clinically relevant dosage regiment (6–9 mg orally three times a day) [1], an analytical procedure capable of detecting less than 1 ng yohimbine/ml would be required for the study of human yohimbine pharmacokinetics. Human pharmacokinetic studies of reserpine, a Rauwolfia alkaloid closely related to yohimbine, support this estimate of required sensitivity (plasma concentrations of 160 pg/ml, 8 h following a 1-mg oral dose of reserpine) [9].

Although several techniques have been employed for the detection of yohimbine, including electrochemical oxidation [10] and UV absorbance [9], the native fluorescence of yohimbine (280 nm excitation, 360 nm emission) has been rarely exploited in the development of a sensitive assay for this compound. Utilizing a sample alkaline extraction into ethylene dichloride, Udenfriend et al. [11] were capable of detecting yohimbine in aqueous solution with a sensitivity of 10 ng/ml; however, the analysis of the drug in biological samples was not discussed. In this paper we describe an extraction and HPLC–fluorescence assay capable of quantitating 50 pg yohimbine per ml blood, plasma or urine. This assay is functional on normal-phase and a variety of reversed-phase chromatography packing materials with only minor variations in mobile phase composition. The chromatographic behaviour of yohimbine, reserpine, corynanthine, yohimbinic acid, and eserine (physostigmine) under alterations in mobile phase composition and with several stationary phases is described.

EXPERIMENTAL

Materials

Reagent-grade chemicals and HPLC-grade solvents were used throughout. Sigma (St. Louis, MO, U.S.A.) was the supplier for yohimbine · HCl, reserpine, eserine sulphate, and corynanthine · HCl. Yohimbinic acid was obtained from Aldrich (Milwaukee, WI, U.S.A.). All glassware was siliconized with 0.2% Aquasil (Pierce, Rockford, IL, U.S.A.) in water followed by three deionized-water rinses and oven drying (100°C, 1 h).

Apparatus

For the analysis of yohimbine, a high-performance liquid chromatograph was assembled from the following components: solvent metering pump (Model 110A, Beckman, Toronto, Canada), universal injection valve (Model C6U, Valco, Houston, TX, U.S.A.), and a spectrofluorometer (Model RF-530, Shimadzu, Kyoto, Japan) (excitation 280 nm, emission 360 nm). Analytical

chromatography columns (10 cm × 2.1 mm I.D. or 25 cm × 2.1 mm I.D.) were packed by a balanced density slurry method [12] with the following packing materials: Partisil-5® (5 μm particle size) (Whatman, Clifton, NJ, U.S.A.), μBondapak® C_{18} (10 μm particle size), Novapak® C_{18} (5 μm particle size) (Waters Assoc., Milford, MA, U.S.A.), or Spherisorb® C_{18} (5 μm particle size) (Phase Separations, Hauppauge, NY, U.S.A.). Unless otherwise indicated, assays were performed on a Partisil-5 column (25 cm × 2.1 mm I.D.) with methanol—water (95:5) as the mobile phase. Chromatogram peak heights and retention times were determined by an electronic integrator (Model HP-3390, Hewlett-Packard, Mississauga, Canada).

Sample preparation

Two methods of sample preparation were developed depending on the sample volume available and the sensitivity required. The first method is capable of detecting yohimbine with a sensitivity of 2 ng/ml utilizing 100 μl of sample. The second procedure requires a 5-ml sample but permits quantitation of yohimbine to 50 pg/ml. Both methods employ eserine as an internal standard.

Method 1. To a 1.5-ml polyethylene centrifuge tube (BelArt, Pequannock, NJ, U.S.A.) were added sample (100 μl blood, plasma or urine), buffer (50 μl, 0.5 *M* Na_2HPO_4, pH 11.0), eserine sulphate internal standard solution (50 μl, 1.5 μg/ml water), and ethyl acetate (100 μl). The tube was capped, mixed vigorously (1 min), and centrifuged (2 min at 13 000 *g*, Model 235A microcentrifuge, Fisher Scientific, Toronto, Canada). A 20-μl aliquot of the organic phase (upper) was injected onto the HPLC column.

Method 2. To a test tube were added sample (5 ml blood, plasma or urine), buffer (2 ml 0.5 *M* Na_2HPO_4, pH 11.0), eserine sulphate internal standard solution (100 μl, 2 μg/ml water), and ethyl acetate (5 ml). The tube was capped, mixed by slow rotation (5 min), and centrifuged (10 min, 250 *g*). The organic phase (upper) was transfered to a second tube containing 2 ml 0.05 *M* hydrochloric acid, the tube capped, mixed and centrifuged as before. After discarding the organic phase, pH 11.0 buffer (1 ml) and ethyl acetate (1 ml) were added to the yohimbine containing aqueous phase, followed by mixing and centrifugation as previously described. The organic phase was removed and evaporated in a 1.5-ml polyethylene centrifuge tube under a stream of nitrogen. The residue was redissolved in ethanol—methanol (85:15) (100 μl) and 20 μl were injected onto the column.

RESULTS AND DISCUSSION

Sample chromatograms for blood containing no yohimbine and for blood containing 300 pg yohimbine per ml are shown in Fig. 1. These samples were prepared from 5-ml blood samples by method 2. Similar chromatograms were obtained for 100-μl samples containing no yohimbine and 15 ng yohimbine per ml respectively when processed by method 1. As is frequently characteristic of techniques employing fluorescence detection, standard curves obtained by plotting the ratio of yohimbine to eserine peak heights against known sample concentrations of yohimbine were not linear throughout their entire range

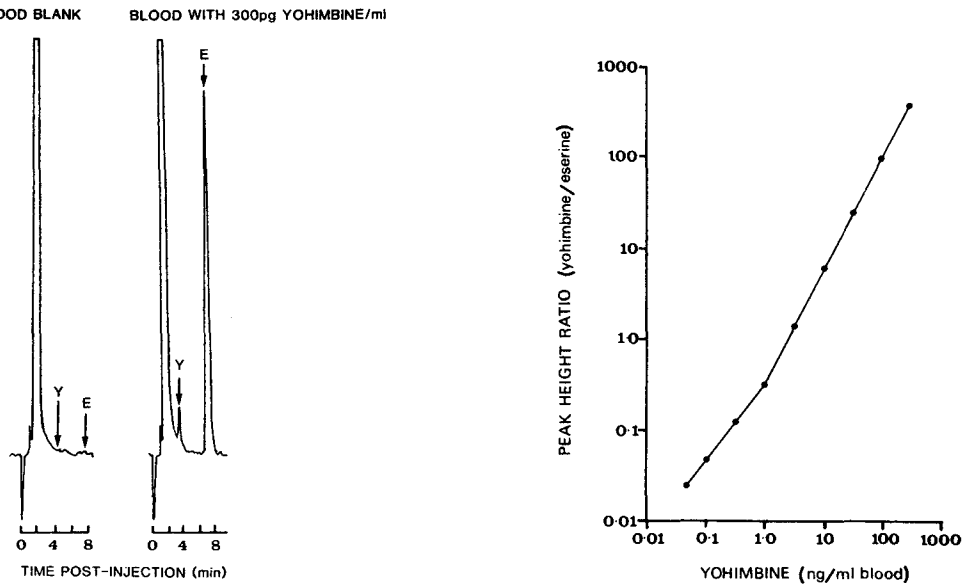

Fig. 1. Sample chromatograms for the HPLC—fluorescence analysis of a blank blood sample (no yohimbine (Y) or eserine (E) internal standard) and of a blood sample containing yohimbine (300 pg/ml) and eserine (40 ng/ml). Each 5-ml sample was processed according to method 2.

Fig. 2. A typical standard curve from blood samples containing known quantities of yohimbine processed according to method 2. Due to the extensive range of the standard curve, the relation of the yohimbine concentration to the ratio of yohimbine to eserine (internal standard) peak heights is depicted on log—log axes. In this case, yohimbine concentration and the yohimbine/eserine peak height ratio are linearily related from 50—1000 pg/ml and from 1—300 ng/ml blood.

(50 pg/ml to 1000 ng/ml) (Fig. 2). As a result blood yohimbine concentrations were determined by interpolation within linear sections (as determined by least-squares linear regression) of approximately a 100-fold concentration range of the standard curve. In this way accurate predictions of known concentration samples were obtained while maintaining predicted concentrations of blank samples not significantly different from zero. The between-day reproducibility over eight separate occasions for the estimation of samples containing known concentrations of yohimbine by each extraction method is shown in Table I.

Essential to the quantitation of yohimbine in a biological matrix is an initial separation of the drug from endogenous interfering compounds. For yohimbine and the related alkaloids raubasine and reserpine, the biological sample is commonly extracted with a chlorinated hydrocarbon (chloroform [7, 13], methylene chloride [14] or ethylene chloride [11, 15]) or acetone [16]. Unfortunately, the use of these solvents with protein-containing fluids frequently results in emulsion formation making phase separation difficult. The use of ethyl acetate as the extraction solvent circumvents this problem while maintaining an extraction efficiency greater than 99% for both yohimbine and eserine. While solvents less polar than ethyl acetate may provide cleaner extracts, the use of a highly polar solvent improves extraction efficien-

TABLE I

BETWEEN-DAY REPRODUCIBILITY OVER AN 8-DAY PERIOD FOR THE ANALYSIS OF BLOOD SAMPLES CONTAINING KNOWN CONCENTRATIONS OF YOHIMBINE

Known concentration (ng/ml)	Found concentration (ng/ml)	
	Mean ± S.D.	Coefficient of variation (%)
Extraction method 2		
0.05	0.049 ± 0.008	15.7
0.30	0.297 ± 0.021	7.2
3.00	2.98 ± 0.09	3.0
30.00	29.90 ± 1.24	4.1
Extraction method 1		
10.0	9.99 ± 0.59	5.9
50.0	51.5 ± 2.72	5.3
200.0	198.0 ± 8.30	4.2
1000.0	999.8 ± 25.8	2.6

cy and reduces sample adsorption to glass surfaces. In addition, red blood cells are completely solubilized with ethyl acetate allowing analysis of yohimbine in either blood or plasma with similar drug recovery.

For a drug assay there are several important criteria in the selection of an appropriate internal standard. These include similar chromatographic and extraction (pK_a, polarity, etc.) properties and comparable means of detection. The use of fluorescence detection in the analysis of yohimbine severely restricts the choice of an internal standard to one with similar excitation and emission spectra. Either reserpine or eserine are viable internal standards for this assay; however, eserine was selected since its retention time was longer (eserine 5.7 min, reserpine 2.0 min) than yohimbine (3.0 min) allowing the mobile phase composition to be optimized for the detection of yohimbine.

This assay procedure was developed to examine the pharmacokinetics of yohimbine in the treatment of male impotence. Accordingly, the potential for interference from drugs commonly prescribed to this population was examined. No interference was observed from the following drugs or their metabolites: propranolol, chlorothiazide, spironolactone, or acetylsalicylic acid. However, we have observed a potential interfering substance from volunteers who have taken quinine or consumed quinine-containing beverages. This interfering peak is due to a quinine metabolite and not the parent drug.

The combination of a normal-phase packing (silica gel) and a reversed-phase solvent system (methanol—water) represents a departure from procedures commonly used. However, it is apparent from Fig. 3 that the separation of yohimbine, reserpine, corynanthine, eserine, and yohimbinic acid by the normal-phase column is comparable to that afforded by three representative reversed-phase columns. Not only is the order of elution of these compounds identical and the capacity factors (k') similar, but the major shortcoming, poor separation of corynanthine and yohimbine, also persists amongst the columns examined. Under this binary solvent system, Partisil-5 was second only to Spherisorb in separating the above five substances. It is possible that

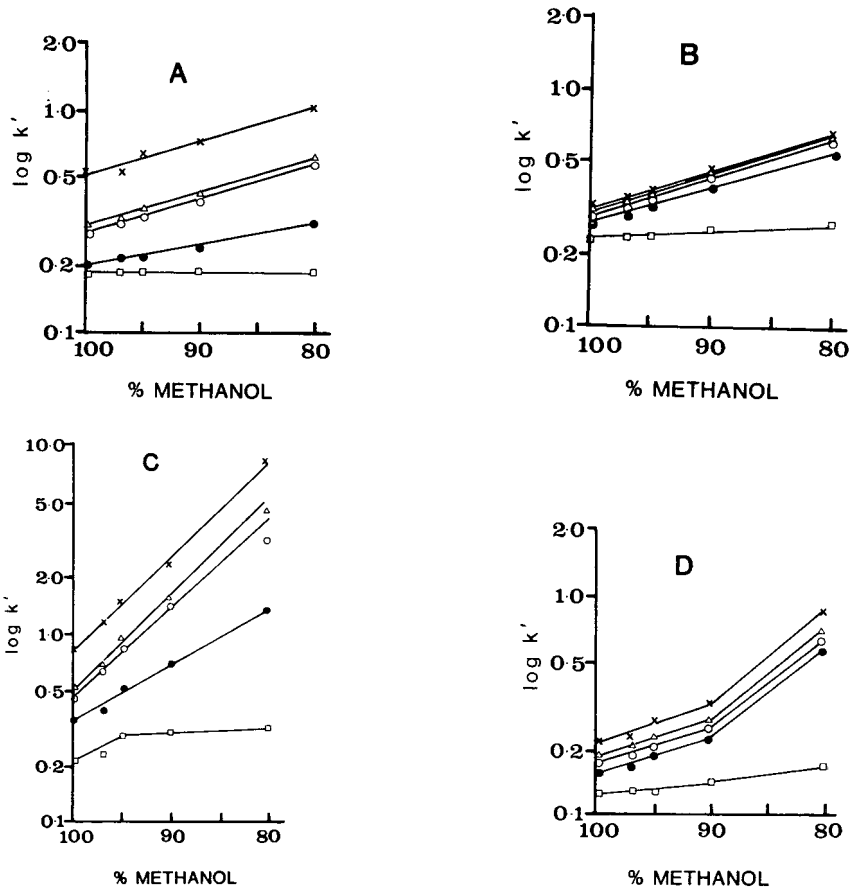

Fig. 3. Log k' on silica gel [Partisil-5 (A), Novapak C_{18} (B), Spherisorb C_{18} (C), and μBondapak C_{18} (D)] as a function of the mobile phase methanol composition. The compounds examined include eserine (×), corynanthine (△), yohimbine (○), reserpine (•), and yohimbinic acid (□).

the reversed-phase separation of certain classes of compounds with a methanol—water mobile phase is effected primarily by free silanol sites remaining exposed after bonding of the organic moiety. Accordingly, the separation of the five test compounds using Novapak C_{18}, a material with a high degree of end-capping to reduce the number of free silanol sites, was poorer than that with μBondapak C_{18}, a packing with a high proportion of free silanol sites. Thus, while maintaining a similar mobile phase, many drug analyses currently performed on reversed-phase materials may be viable on normal-phase columns with their added advantages of reduced cost, lot-to-lot consistency, and ready availability of small particle size (5 μm).

The usefulness of this method for the study of yohimbine pharmacokinetics was investigated by monitoring blood levels of yohimbine in human volunteers following a single 9-mg oral dose of the drug. A typical blood drug concentration—time curve from one volunteer is shown in Fig. 4. Clearly, both drug absorption from the gut and its clearance from the blood are rapid events. These

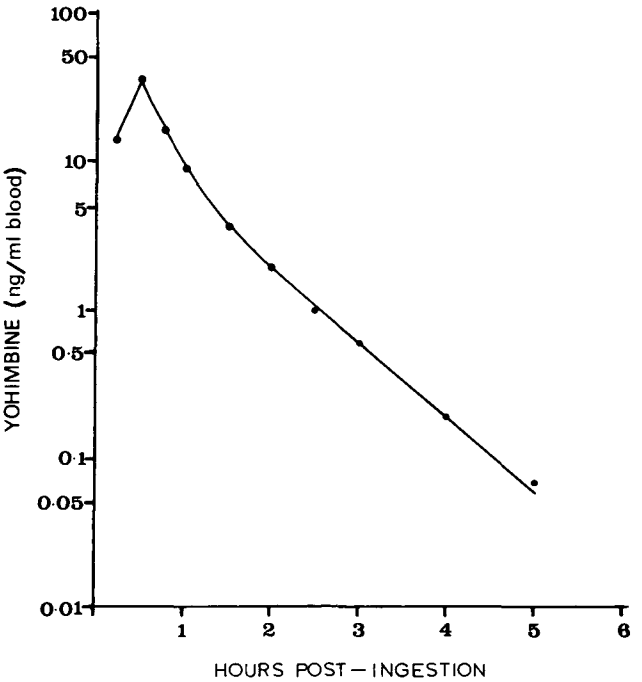

Fig. 4. Time course of blood concentrations of yohimbine following ingestion of a 9-mg oral dose of yohimbine to a human volunteer.

results demonstrate the necessity of a highly sensitive assay procedure for the study of yohimbine pharmacokinetics under a clinically relevant dosage regimen.

This method represents a highly sensitive HPLC fluorometric assay for yohimbine, reserpine and eserine in biological fluids including blood. Prepared samples are stable at 4°C (no noticeable degradation over 1 month) such that samples may be processed in batches and stored for subsequent analysis. Over 1000 analyses have been performed on one silica gel column without significant deterioration.

ACKNOWLEDGEMENTS

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SIMULTANEOUS DETERMINATION OF CARBAMAZEPINE AND ITS EPOXIDE METABOLITE IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple procedure for the simultaneous determination of carbamazepine and its major metabolite, carbamazepine epoxide, in plasma and urine is described. The assay involves two extractions of the drugs and an internal marker, clonazepam, from the alkalinized sample. The extract is evaporated to dryness at 45°C and the residue is redissolved in methanol (30 μl). A 25-μl aliquot is injected into the liquid chromatograph and eluted with acetonitrile—water (40:60, v/v) on a C_{18} pre-column linked to a 5-μm C_8 reversed-phase column. The eluent is detected at 215 nm. The method has been used to investigate the steady-state concentrations of carbamazepine and carbamazepine epoxide in the plasma and urine of a manic-depressive patient.

INTRODUCTION

Carbamazepine (CBZ), 5H-dibenzo[*b*,*f*]azepine-5-carboxamide, is an effective agent for the control of epileptic seizures and in the treatment of trigeminal neuralgia, and possesses acute and prophylactic antimanic effects in the control of manic-depressive illnesses [1]. Thirty-three metabolites of CBZ have been isolated and characterized in the urine from patients on oral CBZ [2]. Of these metabolites, carbamazepine 10,11-epoxide (CBZ-E) is both qualitatively and quantitatively the most important one from a clinical point of view. CBZ-E is pharmacologically as active (as an anticonvulsant) as the parent compound in experimental animals. In humans the variation of CBZ-E concentrations in the blood, both inter-individually and intra-individually, is greater than that of CBZ [3]. Monitoring the concentrations of CBZ and CBZ-E in plasma in the

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management of epilepsy has been proved useful. Very little is known about the plasma concentrations of these compounds in the treatment of manic depression by CBZ. The purpose of this paper is to report the development of a high-performance liquid chromatographic (HPLC) assay that can determine simultaneously the concentration of CBZ and CBZ-E in plasma and urine from a manic-depressive patient.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a Waters 6000A pump, a U6K injector with a 25-μl loop (Waters Assoc., Milford, MA, U.S.A.) and a variable-wavelength Hitachi 220-S UV detector with a chart recorder (Hitachi, Tokyo, Japan). Analyses were performed on a reversed-phase C_8 column (Hibar, LiChroCart RP-8, 250 mm × 4.6 mm I.D., 5 μm Merck, Darmstadt, F.R.G.) linked to a C_{18} pre-column (30 μm, 75 mm × 4.6 mm I.D., Serva, Heidelberg, F.R.G.). The operating conditions for the HPLC system were: mobile phase, acetonitrile—water (40:60); flow-rate, 1.2 ml/min; temperature, ambient (25 ± 1°C); UV detector wavelength, 215 nm; sensitivity scale, 0—0.01 a.u.f.s.

Other apparatus included 10-ml and 15-ml centrifuge tubes with well fitting screw caps (Sovirel, Levallois-Perret, France), and 15-ml stoppered evaporation tubes with finely tapered bases (50 μl capacity). All glassware was cleaned by soaking overnight in a 5% solution of Extran (Merck) in water, then rinsed thoroughly with methanol and hot tap water followed by distilled water. These tubes were subsequently silanized by rinsing with a 1% solution of Prosil-28 silanizing agent (PRC, Gainesville, FL, U.S.A.) followed by rinsing with distilled water and dried at 150°C overnight. This treatment of glassware was necessary to eliminate possible loss of drug owing to absorption on the glass walls [4]. Hamilton syringes, 10 μl and 25 μl, were used.

Materials

The following materials were used: dichloromethane, diethyl ether and methanol, all of Analar grade (Merck) were freshly distilled before use; sodium hydroxide solutions (5 *M* and 0.1 *M*); water was double-distilled in a glass apparatus; carbamazepine BP and carbamazepine 10,11-epoxide were gifts from Ciba Geigy (Basle, Switzerland) and clonazepam and a midazolam methanol analogue, 8-chloro-6-(2-chlorophenyl)-1-methyl-4H-imidazo-[1,5-*a*][1,4]-benzodiazepine-3-methanol (I, Ro 21-6962) were supplied by Roche (Basle, Switzerland).

Preparation of reagents and standards

Standard solutions calculated as mg base per ml in distilled methanol were made of CBZ and CBZ-E and diluted to the following calibration ranges: 0—5 μg/ml for CBZ and 0—800 ng/ml for CBZ-E. Dilutions were made up in drug-free plasma and urine (final volume, 0.5 ml) and to each were added 8 μl (100 μg/ml) of clonazepam as the internal standard. The midazolam methanol analogue, I, gave two peaks under the running conditions. The eluent of the second HPLC peak corresponding to pure I was collected and used to prepare standard solution.

The mobile phase consisted of a freshly prepared mixture of acetonitrile and distilled water, which was filtered before use through a Millipore filter type AA (pore size, 0.5 μm; Waters Assoc.). Further degassing was found not necessary immediately after filtration.

Extraction of CBZ and CBZ-E from plasma and urine

In a previous study on extraction of five antiepileptic drugs [5], CBZ was extracted under acidic conditions at pH 2. CBZ may be considered as a neutral substance with no basic or acidic functions. But the compound, with its tricyclic structure, is readily soluble in organic solvents. CBZ-E, on the other hand, is relatively polar. A selective extraction procedure was used to extract CBZ, CBZ-E and the internal standard (clonazepam) under alkaline conditions such that other acidic antiepileptic drugs were not co-extracted. The recovery of CBZ, CBZ-E and clonazepam from plasma and urine samples under alkaline conditions was studied. The midazolam analogue (I), with a longer retention time (10 min) could also be used as an internal standard if and when clonazepam was included in patients' therapy. Preliminary results suggested that two extractions with 7 ml of organic solvent mixture gave a better recovery.

General procedure

Into a 15-ml glass centrifuge tube, clonazepam (the internal standard, 800 ng) was added to the drug-containing plasma (0.5 ml) for assay. To precipitate the plasma proteins, methanol (200 μl) was added, followed by distilled water (1.5 ml) and 5 *M* sodium hydroxide (20 μl) to adjust the pH to 12. The basified solution was extracted twice with organic solvent [7 ml of a mixture of dichloromethane and diethyl ether (1:3)] by mixing with the aid of an automatic shaker for 15 min. After centrifugation for 10 min at 2500 *g* to break the emulsion, the organic extract was transferred into a 15-ml evaporation tube; the combined extract was then evaporated to dryness at 45°C in a water-bath. The residue was dissolved in distilled methanol (30 μl) and vortexed for 30 sec. An aliquot (25 μl) was injected into the liquid chromatograph.

To determine CBZ and CBZ-E in urine, 0.5-ml volumes were used and the same procedure was followed.

Quantitation

Calibration graphs were constructed by plotting the peak height ratio of the drugs to the internal standard, against the known concentrations of CBZ and CBZ-E added to drug-free plasma or urine to cover the ranges 0—5 μg/ml and 0—800 ng/ml, respectively. The drug or metabolite was quantitated by relating the respective peak height ratio to obtain the concentration from the calibration graph.

Recovery

To assess the recovery of CBZ and CBZ-E from plasma by the extraction procedure, the drugs were added to drug-free plasma or urine (2000 ng/ml for CBZ and 200 ng/ml for CBZ-E) and assayed with the internal standard as

described. For comparison, the same concentrations of CBZ and CBZ-E and internal standard were prepared in a diethyl ether—dichloromethane solution, evaporated and assayed, but with the extraction step omitted. The corresponding peak height ratios from the plasma and urine extractions and from the diethyl ether—dichloromethane solutions were compared.

Selectivity

Samples of plasma spiked with a variety of antiepileptic drugs [5] were analysed to find out if the latter produced peaks after chromatography that interfered with those of CBZ, CBZ-E and clonazepam.

Precision

Six replicate samples of CBZ and CBZ-E in plasma or urine at 2000 and 200 ng/ml, respectively, were assayed as described under *General procedure*, and the peak height ratios of the drug to the standard were calculated.

Stability on storage

Samples of plasma and urine spiked with drugs or samples from patients were analysed immediately and after storage at 4°C overnight, and at −20°C for seven days and three months.

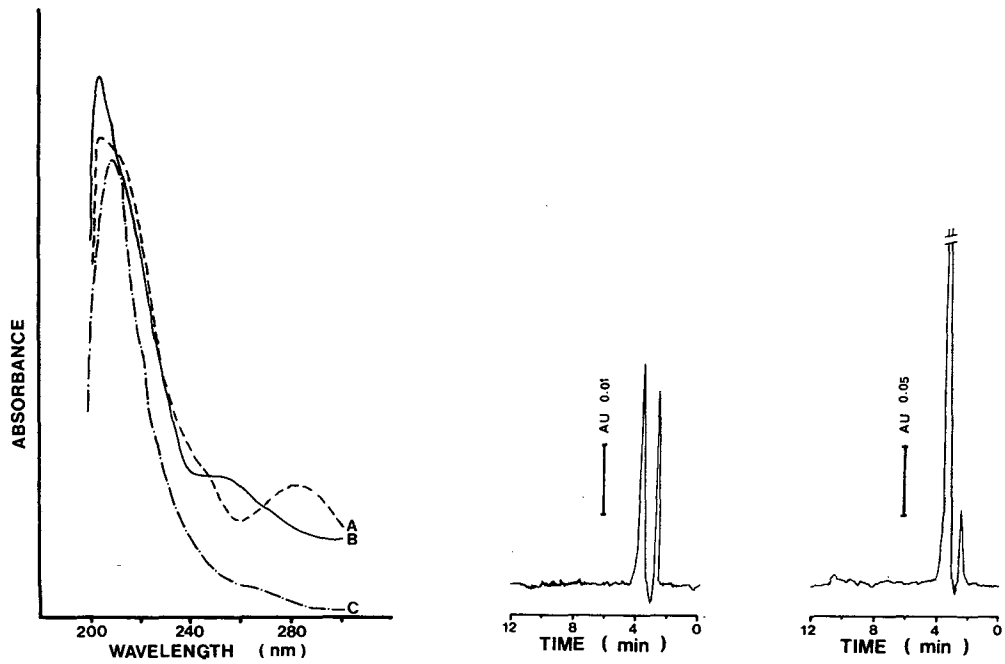

Fig. 1. UV spectra of carbamazepine (A) carbamazepine epoxide (C) and clonazapam (B) in methanol.

Fig. 2. Chromatograms of drug-free plasma (left) and urine (right) extracts.

RESULTS AND DISCUSSION

Performance of the HPLC system

Fig. 1 shows the UV spectra of CBZ, CBZ-E and clonazepam in methanol. The wavelength was set at 215 nm for optimal detection. Fig. 2 illustrates the chromatograms of a drug-free plasma and a drug-free urine extract. Fig. 3 shows the chromatograms of an extract of plasma spiked with CBZ, CBZ-E and internal standard, and plasma and urine samples from a patient treated with CBZ. The performance of the HPLC analytical system is summarized in Table I.

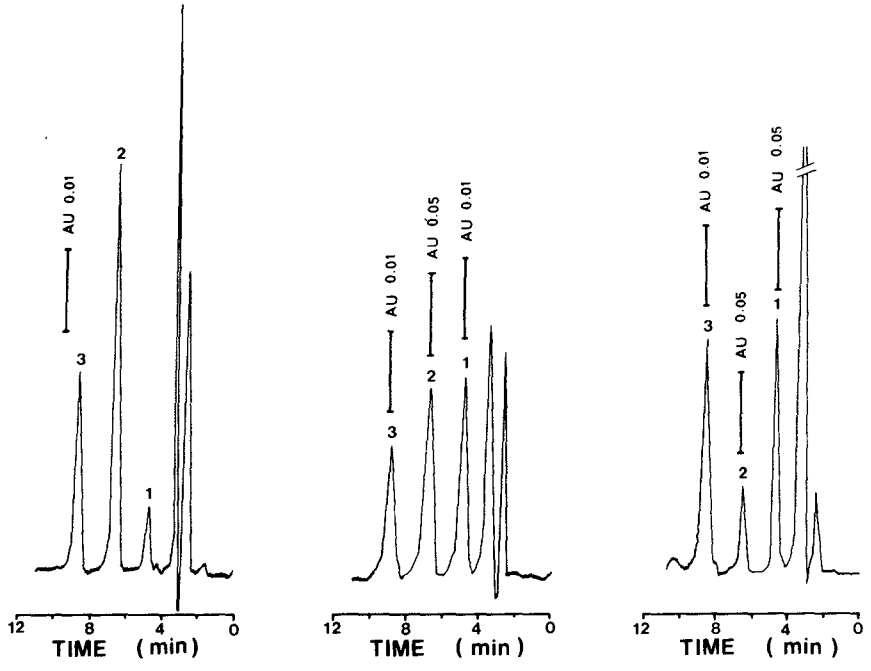

Fig. 3. Chromatograms of (1) carbamazepine epoxide (CBZ-E), (2) carbamazepine (CBZ) and (3) clonazepam (internal standard). Left: a plasma standard extract (CBZ 2.0 μg/ml, CBZ-E 0.2 μg/ml); middle: a plasma extract from a patient (CBZ 7.4 μg/ml, CBZ-E 1.4 μg/ml); right: a urine extract from the same patient (CBZ 1.7 μg/ml; CBZ-E 4.2 μg/ml).

TABLE I

PERFORMANCE OF THE HPLC SYSTEM

Injection volume, 25 μl; mobile phase, acetonitrile–water (40:60); flow-rate, 1.2 ml/min; column, RP C_{18} pre-column and C_8 analytical column; UV detection, 215 nm.

Drug	Retention time (min)	Symmetry factor (0.95—1.05*)	Resolution (> 1.0*)
Carbamazepine epoxide	4.8	1.0	—
Carbamazepine	6.6	0.97	1.38
Clonazepam	8.8	0.95	1.47

*Limits defined by British Pharmacopoeia (1980).

TABLE II

PERCENTAGE RECOVERY OF CARBAMAZEPINE AND CARBAMAZEPINE EPOXIDE FROM PLASMA AND URINE SAMPLES UNDER ALKALINE CONDITIONS

Drug	Plasma samples				Urine samples			
	One extraction	C.V.* (%)	Two extractions**	C.V. (%)	One extraction	C.V. (%)	Two extractions	C.V. (%)
CBZ (n = 6) (at 2000 ng/ml)	91.75	2.04	96.87	1.66	92.70	3.54	94.58	1.81
CBZ-E (n = 6) (at 200 ng/ml)	67.95	0.45	92.54	0.18	69.11	0.45	92.10	0.91

*C.V. = Coefficient of variation (n = 6).
**Plasma proteins were precipitated with 200 μl of methanol, followed by two extractions with dichloromethane–diethyl ether (1:3) at pH 12.

All analytical peaks are well resolved and their symmetry factors and resolution between adjacent peaks are within the British Pharmacopoeia limits [6], hence the peak height ratio technique for calibration is justified.

Recovery, extraction and selectivity

The addition of methanol (200 μl) to precipitate plasma proteins improved the recovery of CBZ and CBZ-E from the plasma. Extraction twice with organic solvent under alkaline conditions also improved the recovery (Table II). The extraction procedure has several advantages over published determinations of CBZ. Firstly, interference by acidic antiepileptic drugs, such as phenobarbital and phenytoin, is not encountered. Secondly, CBZ and CBZ-E are simultaneously extracted and assayed. Thirdly, the use of a solvent mixture [diethyl ether—dichloromethane (3:1)] improves recovery after two extractions. Finally, the procedure may possibly reduce the extraction of endogenous contaminants as no interfering peak appears in the chromatograms of a drug-free plasma and a drug-free urine extract (Fig. 2).

Reproducibility, linearity and storage

Repeated assays of plasma samples spiked with CBZ and CBZ-E indicated that the reproducibility of the procedure was satisfactory over the calibration ranges (Tables II and III). The calibration graphs relating the peak height ratios and concentrations of CBZ and CBZ-E added to plasma blanks were linear. The linear regression coefficients, r, were 0.9989 and 0.9999 (Table III).

Samples of plasma, urine and blood, whether fresh or stored at −20°C for one and four weeks, did not give peaks that would interfere with the measurement of peaks corresponding to CBZ, CBZ-E and internal marker (Fig. 2). There was no appreciable loss of the drugs from the samples after storage at 4°C overnight or at −20°C over a period of three months.

Application

The assay procedure was used to determine the plasma steady-state levels of CBZ and its major metabolites CBZ-E in a Chinese manic-depressive patient, who was stabilized on lithium carbonate (2.0 g daily) and carbamazepine (800 mg daily). The plasma concentrations of CBZ over a study period of 5 h were 7.8 μg/ml (overnight-fast value) to 9.5 μg/ml (4 h after oral dose), and the

TABLE III

CORRELATION BETWEEN PEAK HEIGHT RATIO AND CONCENTRATION OF CARBAMAZEPINE EPOXIDE AND CARBAMAZEPINE

Concentration (ng/ml)	CBZ-E		CBZ	
	Peak height ratio (mean ± S.D.)	C.V.* (%)	Peak height ratio (mean ± S.D.)	C.V. (%)
50	0.078 ± 0.005	0.19	—	—
100	0.138 ± 0.009	0.38	—	—
200	0.260 ± 0.011	0.44	—	—
400	0.516 ± 0.020	0.83	—	—
500	—	—	0.518 ± 0.018	0.75
600	0.758 ± 0.015	0.63	—	—
800	1.009 ± 0.023	0.93	—	—
1000	—	—	1.065 ± 0.042	1.70
2000	—	—	2.099 ± 0.035	1.41
3000	—	—	2.982 ± 0.110	4.49
4000	—	—	4.299 ± 0.15	6.12
5000	—	—	5.089 ± 0.14	6.11
Calibration graph	$y = 799.2x - 7.8$, $r = 0.9999$		$y = 968.3x - 6.24$, $r = 0.9989$	

*C.V. = Coefficient of variation ($n = 6$).

respective values for CBZ-E were 1.41 and 1.42 μg/ml. The ratio of CBZ-E to CBZ in the plasma was relatively constant: 0.18, 0.17, 0.17, 0.16 and 0.16 over various intervals during the study. Very little CBZ and CBZ-E were recovered in the 24-h urine (2300 ml): 1.7 and 4.2 μg/ml (ca. 0.5 and 1.25%, respectively, of a total daily dose of 800 mg). A programme on monitoring CBZ and CBZ-E in depressive patients of Chinese origin who are on CBZ therapy is being carried out.

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DETERMINATION OF MALOTILATE AND ITS METABOLITES IN PLASMA AND URINE

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SUMMARY

A method for the determination of malotilate (I), the corresponding monocarboxylic acid (II) and its decarboxylated product (III) in plasma is described. Plasma was extracted with chloroform spiked with internal standard. The residue, dissolved in methanol, was chromatographed on a reversed-phase column with a mobile phase of 60% acetonitrile and 1% acetic acid in water. The sensitivity limit for I, II and III was 50, 25 and 100 ng/ml of plasma, respectively. Compound I in the same plasma extract was also analysed by gas chromatography—electron-impact mass spectrometry. The base peaks *m/z* 160 for I and *m/z* 162 for internal standard (IV) were monitored; the sensitivity limit for I was 2.5 ng/ml of plasma.

The determination of the metabolites of I, II and its conjugate (V), and isopropyl-hydrogen malonate (VI) in urine by high-performance liquid chromatography is also described. The limit of quantification for VI was 2.0 μg/ml, and the overall coefficient of variation of VI was 4.7%. The limit of quantification for II in urine was 0.5 μg/ml and that for V was 1.0 μg/ml as total II (II + V). The overall precision of the method was satisfactory.

The method was used to determine plasma and urine concentrations in four dogs orally dosed with 100, 200 or 400 mg of malotilate.

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INTRODUCTION

Malotilate (I), diisopropyl 1,3-dithiol-2-ylidenemalonate (Fig. 1), was developed as a drug for chronic hepatitis and cirrhosis [1]. After oral administration, malotilate undergoes extensive first-pass metabolism. A major metabolite in plasma is the corresponding monocarboxylic acid (II) (Fig. 1) in humans [2], dogs [3] and rats [4]. Its decarboxylated product (III) (Fig. 1) is found in rat urine and is also a chemical degradation product of II (Fig. 1). In many species, isopropylhydrogen malonate (VI) (Fig. 1) is a major metabolite in urine [2—4]. In humans, the conjugate of II, compound V (Fig. 1), is a major metabolite in urine [2].

As compound II readily degrades to compound III, gas—liquid chromatography and spectrophotometry are not suitable for simultaneous determination. We describe here a high-performance liquid chromatographic (HPLC) method which allows a simple and precise analysis of plasma samples for I, II and III simultaneously, and an analysis of urine samples for V and VI. We also describe a sensitive mass fragmentographic method for I in plasma using the same sample preparation.

This method was used to determine malotilate and its metabolites in plasma and urine of dogs that had received 100, 200 or 400 mg of malotilate orally.

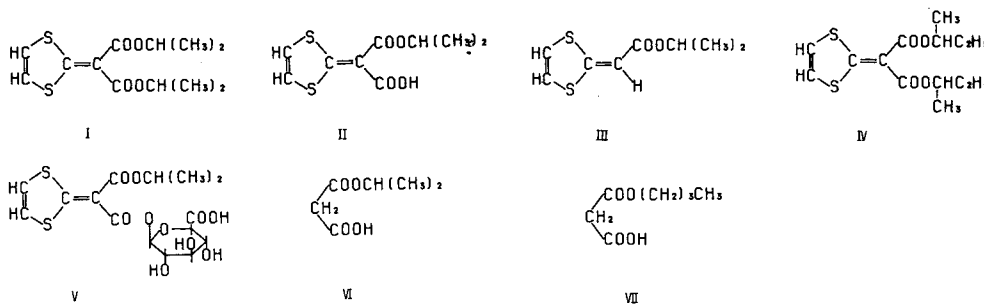


Fig. 1. Chemical structures of malotilate, its metabolites and internal standards.

EXPERIMENTAL

Materials

Malotilate, II, III, VI and internal standard for the plasma assay, di-*sec*.-butyl 1,3-dithiol-2-ylidenemalonate (IV) and *n*-butylhydrogen malonate (VII) for the urine assay, were synthesized at Nihon Nohyaku. *o*-4-Nitrobenzyl-N,N′-diisopropylisourea (NBDI) was obtained from Dohzinkagaku. Other chemicals were obtained commercially (reagent grade) and used without further purification.

The urine sample used for the evaluation of the HPLC assay for V was collected over an 8-h period from a male rabbit (weighing 2.4 kg) that had been given an oral dose of 200 mg of malotilate.

Plasma sample preparation

To 1.0 ml of plasma were added 1.0 ml of 1 *M* acetate buffer (pH 5.0), 1.0 ml of water and 5.0 ml of chloroform containing 1.0 μg of internal standard

(IV). The extraction mixture was agitated in a horizontal shaker for 10 min. After centrifugation, ca. 4 ml of organic phase were removed and evaporated to dryness. The residue was redissolved in 100 μl of methanol and aliquots of 10 μl were injected into the HPLC system as described below. Aliquots of 2 μl were also analysed by gas chromatography—mass spectrometry (GC—MS).

Urine sample preparation of VI

To 1.0 ml of urine were added 0.5 ml of 1 *M* hydrochloric acid and 5.0 ml of ethyl acetate containing 30 μg of the internal standard (VII). The mixture was shaken for 5 min and centrifuged, and 4.0 ml of the organic phase were pipetted into a centrifuge tube. The ethyl acetate extract was dried with anhydrous sodium sulphate (2 g). After centrifugation, 3.0 ml of the ethyl acetate extract were transferred to another tube. Then 1 ml of NBDI solution (5 mg/ml in ethylene dichloride) was added, and evaporated under a stream of nitrogen at 60°C to a volume of ca. 0.5 ml. The tube was stoppered and placed in a 70°C water-bath for 2 h, and the solvent was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 0.6 ml of *n*-hexane, and 1 ml of 0.01 *M* hydrochloric acid was added. The contents were vortexed and centrifuged. Then 10-μl aliquots of organic phase were analysed with the HPLC system.

Urine sample preparation of II and V

Aliquots (10 μl) of urine or diluted urine were injected into the HPLC system for assay of II. For assay of V, 0.5 ml of 1 *M* sodium hydroxide were added to 1 ml of urine, and the solution was allowed to stand for 1 h at room temperature. After addition of 0.5 ml of 0.8 *M* hydrochloric acid, aliquots of 10 μl were injected into the HPLC system.

HPLC system

A Hitachi 635 liquid chromatograph was used, equipped with a variable-wavelength UV—VIS detector. A modified stop-flow procedure was used to introduce the samples onto a Nucleosil $5C_{18}$ reversed-phase column (150 × 4.0 mm I.D.). The flow-rate was held constant at 1.0 ml/min.

For the assay of I, II and III in plasma, the mobile phase consisted of 10 ml of acetic acid and 600 ml of acetonitrile diluted to 1000 ml with water, and the effluent was monitored at 360 nm.

For the assay of VI in urine, the mobile phase consisted of 50 ml of acetic acid and 450 ml of methanol diluted to 1000 ml with water; the detector was set at 269 nm.

For the assay of II and V in urine, the mobile phase consisted of 50 ml of acetic acid and 400 ml of acetonitrile diluted to 1000 ml with water, and the effluent was monitored at 360 nm.

Gas chromatography—mass spectrometry

Samples were analysed by a JEOL gas chromatograph and a JEOL JMS-D300 combination instrument equipped with a chemical-ionization (CI) electron-impact (EI) ion source under the following conditions: ionization energy, 70 eV; ionization current, 300 μA; separator temperature, 290°C; ion source temperature, 200°C. The conditions of the gas chromatograph were as follows:

a coiled glass column (1 m × 2 mm I.D.) packed with 3% OV-1 on Gas-Chrom Q (80—100 mesh); injector and column temperatures of 230 and 200°C, respectively; flow-rate of helium as carrier gas, 75 ml/min.

Calculations

Peak height ratios of I, II, III or VI to that of internal standard were calculated, and calibration curves were constructed by plotting the peak height ratios versus concentration of I, II, III or VI (μg/ml) for spiked control plasma and urine samples.

For the urine assay of II, a calibration curve was constructed by plotting the peak height versus concentration of II spiked to control urine samples. For the assay of V, the data were obtained as total II and, from the difference of unhydrolysed and hydrolysed II, the concentration was reported as that of V.

Mass spectrometry

The solvent was evaporated from the eluted VI fraction under a stream of nitrogen, and the residue was redissolved in *n*-hexane. This solution was transferred to a capillary tube for direct insertion into the mass spectrometer. A JEOL JMS-01SG-2 mass spectrometer equipped with an EI ion source was used. Mass spectra were determined under the following conditions: ionizing energy, 75 eV; emission current, 200 μA; accelerating voltage, 10 kV; ion multiplier voltage, 2.5 kV; main slit, 200 μm.

Animal study

The studies were conducted in healthy male beagle dogs, weighing 10.5—13.0 kg. The dogs were fasted for 18 h prior to and 4 h after each received orally 100, 200 or 400 mg of malotilate powder with 20 ml of water. Blood samples were withdrawn from the foreleg vein. Urine samples were collected over 48 h after the administration. Throughout the experiments the animals were not restrained.

RESULTS AND DISCUSSION

HPLC assay of I, II and III in plasma

Typical chromatograms resulting from blank plasma and blank plasma spiked with I, II and III are shown in Fig. 2. Under these chromatographic conditions, I, II, III and internal standard (IV) had retention times of 8, 3, 5 and 15 min, respectively, and were resolved satisfactorily from the peaks due to endogeneous plasma components.

The calibration graphs were linear for samples over the concentration range studied here, 0.05—1.00 μg/ml. The least-squares linear regression line for I had an equation of $y = 4.34x - 0.08$ (y = peak height ratio, I:IV, and x = concentration of I), and those for II and III had equations of $y = 1.43x - 0.03$ and $y = 0.205x + 0.04$, respectively. The correlation coefficients of I, II and III were 0.999, 0.999 and 0.992, respectively ($n = 5$).

The coefficients of variation for intra-assay and inter-assay variability of I, II and III are given in Table I. The results demonstrate the high accuracy and reproducibility of the method.

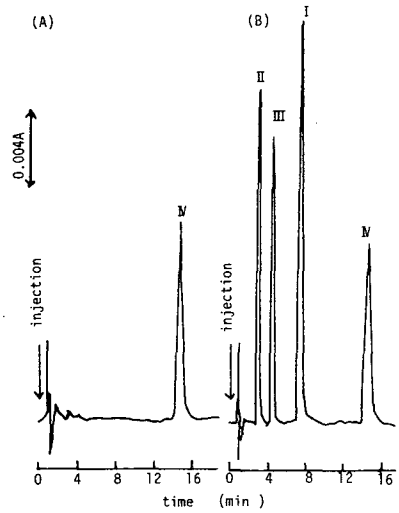


Fig. 2. HPLC chromatograms of (A) blank plasma and (B) blank plasma spiked with I (1.0 μg/ml of plasma), II (0.5 μg/ml of plasma) and III (0.94 μg/ml of plasma). Peak IV = internal standard.

TABLE I

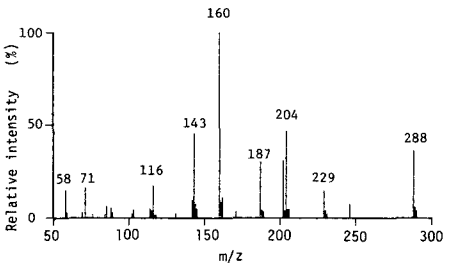
INTRA- AND INTER-ASSAY REPRODUCIBILITY OF HPLC FOR THE DETERMINATION OF I, II and III IN PLASMA

Compound	Concentration (μg/ml)	Coefficient of variation (%)	
		Intra-assay (n = 5)	Inter-assay (n = 5)
I	0.10	2.3	2.1
II	0.10	4.1	4.2
III	0.10	9.5	7.1

The recoveries of I, II and III from plasma were of the order of 98, 100 and 86%, respectively. The detection limit, defined on the basis of the amount of compound injected that caused an absorption five times greater than the baseline noise, was found to be at 4, 2 and 8 ng, equivalent to 50, 25 and 100 ng/ml plasma for I, II and III, respectively.

GC—MS analysis of I

For GC— MS analysis, compound I and internal standard have a common fragment ion at m/z 160, which was monitored for quantification. The detection limit, defined on the basis of the amount of I injected, was ca. 40 pg. The sample extracts for HPLC assay were also used for GC—MS assay. These samples, however, contained a large amount of internal standard, therefore in addition to monitoring the fragment ion at m/z 160, the corresponding isotope ion at m/z 162 due to S atoms was also monitored for the internal standard. Since the fragment ion at m/z 160 has to S atoms, the intensity ratio of the ion at m/z 160 and that at m/z 162 was ca. 10:1 (Fig. 3).



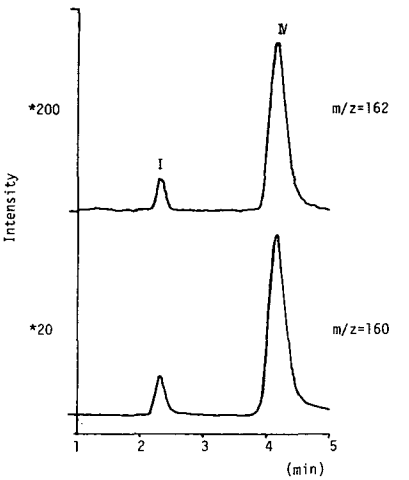


Fig. 3. Electron-impact (70 eV) mass spectrum of malotilate.

Fig. 4. Mass fragmentograms of I (0.8 μg/ml of plasma) and IV (internal standard).

A typical mass fragmentogram resulting from a blank plasma spiked with I, II and III is shown in Fig. 4. The retention times were ca. 2 and 3.3 min for I and internal standard, respectively. The sensitivity limit for I was 2.5 ng/ml of plasma.

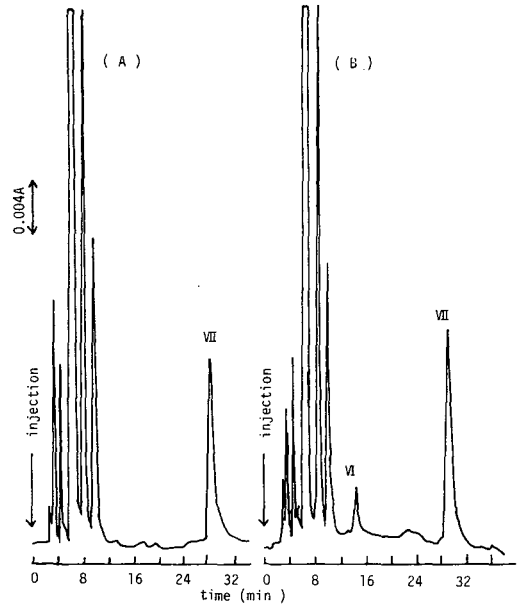
HPLC assay of VI

Fig. 5 shows typical chromatograms from blank urine and blank urine spiked with VI. Under these conditions, VI and the internal standard (VII) had retention times of 15 and 29 min, respectively, and were resolved satisfactorily from the peaks due to endogeneous urine components.

The calibration graph was linear for the samples over the concentration range studied here, 2.5—40 μg/ml. The least-squares linear regression line which represents the best fit of the data for VI had an equation of $y = 0.048x + 0.022$ (y = peak height ratio, VI:VII, and x = concentration of VI). The correlation coefficient was 0.999 (n = 5). The coefficients of variation for intra- and inter-assay variability of VI are given in Table II. The results demonstrate the high accuracy and reproducibility of the method.

The detection limit, defined on the basis of the urine concentration of VI that caused an absorption five times greater than the baseline noise, was found to be 2.0 μg/ml. The extraction efficiency was estimated as ca. 60%, but the coefficient of variation of the extraction efficiency was ca. 3%. The overall coefficient of variation of VI was 4.7%. In spite of the low absolute recovery, the overall precision of the method was satisfactory.

Various reaction times were compared to determine the best reaction time for the formation of the *p*-nitrobenzyl derivative; the results indicated that formation was complete within 2 h (Fig. 6). The derivatives of VI and the internal standard (VII) were stable for at least twenty days, if they are protected from light and stored at 5°C.



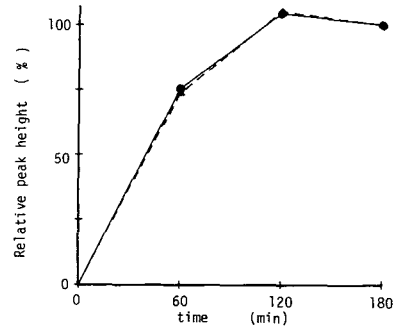


Fig. 5. Chromatograms of (A) blank urine and (B) blank urine spiked with VI (4.8 μg/ml of urine). Peak VII = internal standard.

Fig. 6. Rate of conversion of VI into *p*-nitrobenzyl derivative in ethyl acetate—ethylene dichloride (3:1). Data points: • = 8.66 μg/ml VI; ▲ = 34.64 μg/ml VI.

TABLE II

INTRA- AND INTER-ASSAY REPRODUCIBILITY OF HPLC FOR THE DETERMINATION OF II, TOTAL II (II + V) AND VI IN URINE

Compound	Concentration (μg/ml)	Coefficient of variation (%)	
		Intra-assay	Inter-assay
II	2.98	0.8 (n = 6)	1.7 (n = 5)
	5.95	4.0 (n = 6)	2.3 (n = 5)
Total II	3.68	2.1 (n = 5)	4.8 (n = 5)
	13.18	0.9 (n = 5)	5.2 (n = 5)
VI	9.60	7.2 (n = 6)	9.1 (n = 10)
	19.20	4.7 (n = 8)	4.8 (n = 9)

For identification of the *p*-nitrobenzyl ester of VI, the eluted HPLC fraction of VI was analysed by EI-MS. A satifactory spectrum was obtained (Fig. 7), and the fraction showed the molecular ion m/z 281.

Assay of II and V

For the evaluation of hydrolysis condition of urine samples, the sample from a rabbit given an oral dose of malotilate was used. To 1 ml urine were added 0.2, 0.3, 0.4 or 0.5 ml of 1 *M* sodium hydroxide, and the solution was allowed to stand at room temperature. The mixtures were neutralized with 0.8 *M*

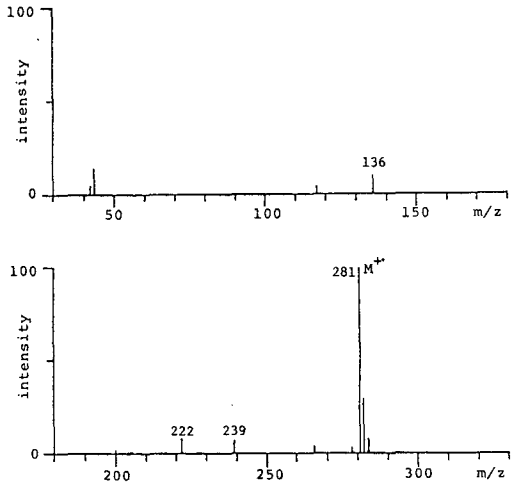


Fig. 7. Electron-impact mass spectrum of the *p*-nitrobenzyl ester of VI.

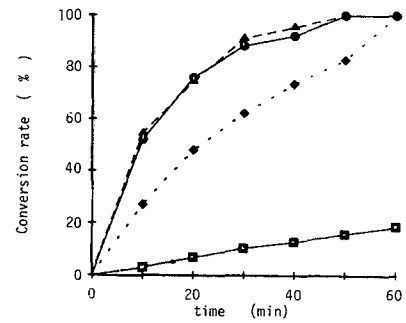


Fig. 8. Rate of hydrolysis of V to II under various alkaline conditions. Data points: ■ = 0.2 ml; ◆ = 0.3 ml; ▲ = 0.4 ml; ● = 0.5 ml of 1 *M* sodium hydroxide.

hydrochloric acid at 10-min intervals and analysed by HPLC. The hydrolysis conditions were thus determined to be 1 h with 0.5 ml of 1 *M* sodium hydroxide (Fig. 8).

Typical chromatograms for II (aqueous standard) and hydrolysed urine sample of a rabbit given an oral dose of malotilate are shown in Fig. 9. Urine components did not interfere, and the retention time of II was 9 min.

The least-squares linear regression line that represents the best fit of the data for II had an equation of $y = 1.09x + 0.4$ (y = peak height, and x = concentration of II). The correlation coefficient was 0.995 ($n = 5$). The coefficients of variation for intra-assay and inter-assay variability of II and total II (II + V) are given in Table II. The results demonstrate the high accuracy and reproducibility of the method.

The detection limit, defined on the basis of the amount of compound injected that caused an absorption five times greater than the baseline noise, was found to be 5 ng, equivalent to 0.5 μg/ml for II and 1 μg/ml for total II (II + V) in urine.

Animal study

The method was applied to pharmacokinetic studies of malotilate in beagle dogs. Four dogs were dosed orally with 100, 200 or 400 mg of malotilate powder. Plasma malotilate concentrations were very low. Compound III was not detected in any plasma samples. Mean concentrations of II in plasma after single oral administration of malotilate are shown in Fig. 10. The area under the curve (AUC), calculated by the trapezoidal rule, was 1.04 (100 mg per dog), 1.90 (200 mg per dog) and 3.91 μg/ml (400 mg per dog).

The assay procedure described for malotilate and its metabolites in plasma offers easy and rapid sample preparation, selectivity for both drug and metabolites, and adequate sensitivity for pharmacokinetic studies.

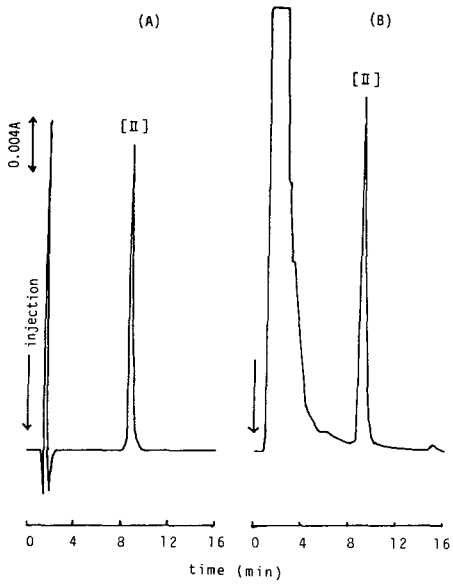


Fig. 9. Chromatograms of (A) standard solution containing 10 μg/ml II and (B) alkali-hydrolysed urine of the rabbit given an oral dose of malotilate.

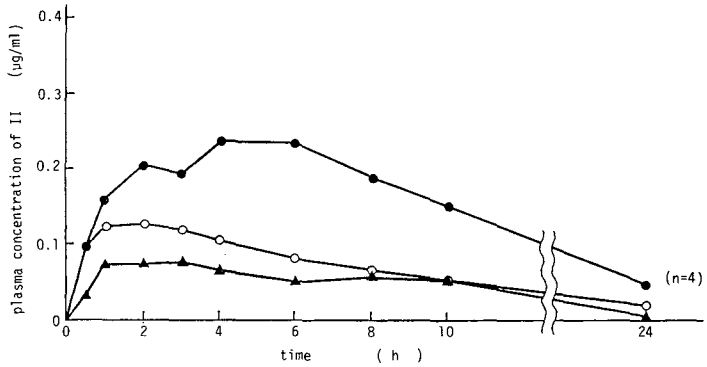


Fig. 10. Average plasma concentration—time curves of II after single oral administration of malotilate to dogs. Data points: ● = 400 mg per dog; ○ = 200 mg per dog; ▲ = 100 mg per dog.

TABLE III

CUMULATIVE URINARY EXCRETION OF TOTAL II AND VI FOLLOWING DOSAGE OF 100, 200 OR 400 mg OF MALOTILATE TO DOGS

Dose (mg)	Cumulative excretion within 48 h (% of dose)	
	Total II (mean ± S.D.)	VI (mean ± S.D.)
100	0.065 ± 0.047	19.11 ± 3.30
200	0.045 ± 0.064	13.75 ± 8.66
400	0.133 ± 0.079	12.74 ± 12.62

Cumulative urinary excretion data for VI (major metabolite) and other metabolites are presented in Table III. Unconjugated II accounted for less than 0.05% of the dose. Compound VI was excreted over the 48-h collection period and accounted for ca. 15% of the dose. A small amount of V (conjugated II) was excreted in urine, but urinary excretion of II or V was a minor route of malotilate elimination in the dog.

The HPLC method described here for the quantitative determination of II, V and VI in urine is not only convenient but also specific, and its accuracy and sensitivity are satisfactory for most pharmacokinetic studies.

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Note

Fatty acid analysis in phosphatidylethanolamine subclasses of human erythrocyte membranes by high-performance liquid chromatography

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Erythrocyte membrane lipid are composed of cholesterol, phospholipids and a small amount of glycolipids [1], and the phospholipids contain mainly sphingomyelin and three glycerophospholiplid classes: phosphatidylcholine, phosphatidylethanolamine (PE) and phosphatidylserine. Erythrocyte membranes are known to include plasmalogen form in glycerophospholipids, especially in PE [2—4].

Human erythrocyte membrane fatty acids in phospholipid classes have been described in detail [3, 5—9], but there have been only a few reports of fatty acid analysis on phospholipid subclasses; plasmalogen form and diacyl form [4—6]. Therefore, we studied fatty acids of PE subclasses of human erythrocyte membranes by high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Heparinized blood samples were obtained from seven healthy adults fasted overnight, and were immediately cooled in ice-water for ca. 1 h. The erythrocyte membranes were prepared as described previously [10] and suspended in 10 m*M* Tris—HCl buffer solution (pH 7.4) to bring the protein concentration to ca. 4 mg/ml. They were stored at —80°C until use.

Lipids were extracted from 0.5 ml of the membrane suspension by the method of Folch et al. [11], and the lower chloroform phase was evaporated to dryness in a stream of dry nitrogen. The lipid extracts were separated into phospholipid classes on thin-layer plates coated with silica gel G (Uniplate, Analtech, Newark, NJ, U.S.A.; chloroform—methanol—28% ammonia—water,

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65:25:0.5:2). The silica gel plates were exposed to the fumes from 36% hydrochloric acid and dried in a stream of ambient air for 10 min [12]. Phospholipid spots were located with iodine vapour. Scraped PE was further separated into diacyl-PE and lyso-PE (cleavage product from ethanolamine plasmalogen) by thin-layer chromatography (Uniplate, Analtech; chloroform–methanol–28% ammonia, 100:50:12). The PE subclass spots were located with iodine vapour and scraped. Scraped PE subclasses were saponified by heating at 98°C for 30 min in 2 ml of potassium hydroxide–methanol (50 g of potassium hydroxide, 500 ml of methanol, 450 ml of water). After acidification with hydrochloric acid, the fatty acids were extracted in chloroform, dried in a stream of nitrogen, and were esterified with a fluorescent marker, 9-anthryldiazomethane (Funakoshi, Tokyo, Japan) [13–15]. An aliquot of the esterified fatty acids was subjected to HPLC.

HPLC was performed with a Shimadzu LC-4A system equipped with a fluorescence spectrophotometer monitoring at 412 nm with excitation at 365 nm (Shimadzu, Kyoto, Japan). The column was a 25 cm × 4.6 mm I.D. stainless-steel tube prepacked with Zorbax C_8 (particle size, 5–6 μm; Shimadzu). The column temperature was maintained at 60°C. Fatty acids were eluted by a programmed linear gradient elution with acetonitrile–water (0 min, 80:20; 36 min, 80:20; 80 min, 100:0; 90 min, 100:0) within 90 min, and the flow-rate was 1.6 ml/min. Each fatty acid was identified by the retention time of standards, purchased from Serdary Research Lab. (London, Canada) or Funakoshi.

All organic solvents and water (Wako, Osaka, Japan) were commercial HPLC-grade materials, and butylated hydroxytoluene (an antioxidant) was added to methanol and chloroform (5 mg per 100 ml).

RESULTS

Nine fatty acid peaks were identified (Fig. 1). Major fatty acid components were $C_{16:0}$, $C_{18:1}$ and $C_{20:4}$ in diacyl-PE, and were $C_{20:4}$, $C_{22:6}$ and $C_{18:1}$ in

TABLE I

FATTY ACID COMPOSITIONS OF PHOSPHATIDYLETHANOLAMINE SUBCLASSES IN ERYTHROCYTE MEMBRANES

Values are area percentages (means ± S.D.).

Fatty acid	Diacyl form (n = 7)	Plasmalogen form (n = 7)
$C_{16:0}$	30.27 ± 1.39	11.78 ± 2.68
$C_{18:0}$	10.81 ± 0.89	4.04 ± 0.89
$C_{18:1}$	24.07 ± 2.75	16.36 ± 3.29
$C_{18:2}$	10.19 ± 1.06	11.15 ± 1.42
$C_{20:3}$	1.01 ± 0.14	0.97 ± 0.17
$C_{20:4}$	14.54 ± 1.14	29.99 ± 1.47
$C_{20:5}$	1.40 ± 0.58	4.47 ± 2.05
$C_{22:4}$	2.25 ± 0.73	4.58 ± 1.32
$C_{22:6}$	7.81 ± 1.24	17.63 ± 3.70

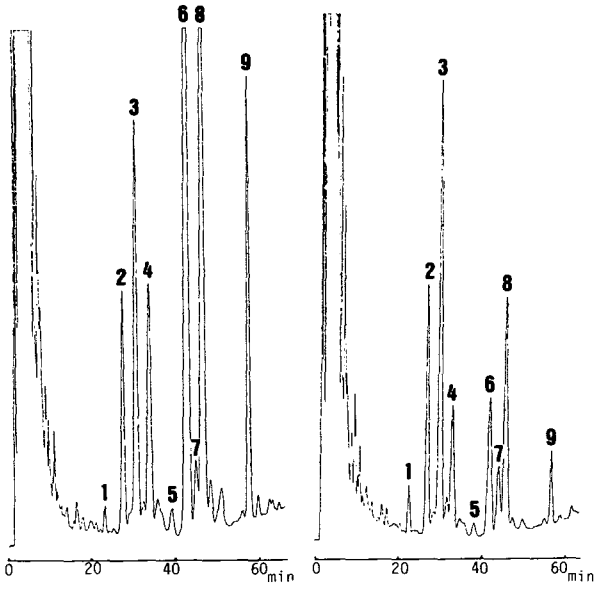

Fig. 1. Separations of fatty acids from diacyl (left) and plasmalogen (right) forms in PE of human erythrocyte membranes by HPLC. Peaks: 1 = $C_{20:5\omega9}$; 2 = $C_{22:6\omega3}$; 3 = $C_{20:4\omega6}$; 4 = $C_{18:2\omega6}$; 5 = $C_{20:3\omega6}$; 6 = $C_{16:0}$; 7 = $C_{22:4\omega6}$; 8 = $C_{18:1\omega9}$; 9 = $C_{18:0}$.

plasmalogen form of PE (Table I). Saturated long-chain fatty acids ($C_{20:0}$, $C_{22:0}$, $C_{24:0}$ and $C_{26:0}$) were trace components in both subfractions.

DISCUSSION

Human erythrocyte membrane fatty acids have been analysed in phospholipid fractions by gas chromatography (GC) [3, 5—9], and there have been also several reports on fatty acids in phospholipid subfractions [4—6]. Recently, HPLC has been introduced into fatty acid analyses [13—15]. There has been no study on fatty acid compositions of phospholipid subclasses in human erythrocyte membranes by HPLC, as far as we know.

Our study showed a dramatic difference in the fatty acid composition between diacyl and plasmalogen forms in PE. The fatty acids in plasmalogen form have more unsaturation than those in diacyl form, which agrees with the GC data obtained by previous authors [4—6]. There are some differences in the fatty acid compositions of both PE subclasses between our data and those in the previous reports [4—6]. In particular, the content of polyunsaturated fatty acids in both PE subclasses of the present study is less than that reported previously [4—6]. This discrepancy may be attributable to a difference in the preparation procedures rather than to a difference in the analytical modes (HPLC and GC), because the treatment with 36% hydrochloric acid used in our study may have destroyed not only vinyl ethers but also polyunsaturated fatty acids in part.

Erythrocyte membranes may be used as purified plasma membrane samples for studying membrane components and their metabolism. Therefore, it seems

important to bear in mind the facts that phosphlipid classes contain their subclasses and that there are marked differences between their fatty acid compositions. HPLC may be useful for studying fatty acids of biological samples.

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Note

Estimation of adrenal catecholamines by elevated-temperature liquid chromatography with amperometric detection

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High-performance liquid chromatography (HPLC) using amperometric detection is now widely used to measure catecholamines (CAs) and their congeners in biological tissues [1, 2]. The simultaneous determination of CAs in adrenal extracts presents a challenge to the chromatographer because of the very wide difference in the amounts of dopamine (DA) and epinephrine (EP) [3]. Published HPLC procedures [4, 5] for adrenal CAs have the disadvantages of lengthy chromatographic run time and/or suboptimal chromatographic conditions with respect to the amperometric detector response [6]. We report that adrenal DA content can be reliably measured along with EP and norepinephrine (NE) under optimized conditions by elevating the column temperature to shorten the DA retention time. Furthermore, the recommended conditions permit resolution of these CAs from some twenty other catechol compounds that might be present in adrenal tissue.

EXPERIMENTAL

Sample preparation

The adrenal glands of male Sprague—Dawley rats were removed under

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sodium methohexital sedation (Brietal, Eli Lilly, Toronto, Canada; 65 mg/kg body weight, subcutaneously). The glands were freed from fat and connective tissue, blotted on paper, weighed and homogenized together in 0.9 ml (or separately in 0.5 ml) of cold 0.3 *M* sucrose with a PTFE homogenizer. This medium allows one to measure concurrently adrenal CA biosynthetic enzymes, steroids and proteins [7]. Aliquots of 0.05 ml of homogenate were mixed with 0.10 ml of 3.9 m*M* sodium metabisulfite in acid-washed Eppendorf tubes and stored temporarily at −70° C.

Sample preparation was modified from that of Anton and Sayre [8] and Felice et al. [9] and involved initial deproteinization with cold 0.1 *M* perchloric acid for 5 min. The final sample volume of 0.25 ml contained also 300 ng of the internal standard, 3,4-dihydroxybenzylamine (DHBA; Sigma, St. Louis, MO, U.S.A.), dissolved in 0.1 *M* perchloric acid and 2.5 m*M* sodium metabisulfite. After centrifugation at 15 600 *g* for 25 min, CAs were adsorbed by shaking the supernatant fraction for 10 min with 20 mg of acid-washed alumina (Woelm neutral activity, grade I; ICN, Montreal, Canada) [8] previously buffered with 1.0 ml of a solution containing 0.5 *M* Tris—HCl, 27 m*M* disodium ethylenediaminetetraacetate (EDTA) and 2.6 m*M* sodium metabisulfite, pH 8.6 at 4°C. After the alumina had been washed twice with 0.5 ml of deionized water, the adsorbed amines were eluted by shaking for 15 min with 0.15 ml of 0.05 *M* phosphoric acid and 0.1 m*M* sodium metabisulfite; the eluates were centrifuged for 5 min to pellet alumina fines, and were stored at −70°C for at the most a few days.

Chromatographic system

A few microlitres of eluate were injected directly into the chromatographic system, which consisted of a Model 45 solvent delivery system a Model U6K manual injector and a μBondapak C_{18} column, 30 × 0.39 cm, obtained from Waters Scientific (Mississauga, Canada). A Co:Pell ODS guard column (Whatman, Clifton, NJ, U.S.A.) was included. The analytical column was enclosed in a water jacket (Alltech, Deerfield, IL, U.S.A.) thermostated at 30°C with a Lauda Model RM-3S circulator (Brinkmann Instruments, Rexdale, Canada).

The mobile phase was modified from that of Moyer and co-workers [6, 10]. It consisted of 8 parts of methanol (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) and 92 parts of a solution containing 0.1 *M* sodium phosphate (monobasic), 0.1 m*M* EDTA and 1.0 m*M* sodium octyl sulfate (Eastman Kodak, Rochester, NY, U.S.A.), pH 5.5, prepared in deionized water (Continental Waters Systems, El Paso, TX, U.S.A.) free of organic residues, filtered through 0.45-μm filters (Millipore, Bedford, MA, U.S.A.) and deaerated under vacuum. This solvent was sparged continuously with helium and pumped at a flow-rate of 3.0 ml/min. The inconvenience of column equilibration with the ion-pairing reagent was avoided by pumping overnight at 0.1 ml/min.

The CAs were detected by means of an oil-impregnated carbon paste electrode (in a Model TL-4 5-mil thin-layer cell) set, with a Model LC-4 electronic controller (Bioanalytical Systems, West Lafayette, IN, U.S.A.), at a sensitivity of 5 nA/V and at +0.70 V against a silver/silver chloride reference electrode. Quantitation [9] was done with a Model SP4100 computing integrator (Spectra-Physics, Santa Clara, CA, U.S.A.) by determination of peak

area ratio to the internal standard and by inverse linear regression analysis of a calibration curve constructed with standards taken through sample preparation. Levels of unconjugated CAs were expressed in terms of the free base as μg of EP and NE, or ng of DA per single adrenal or pair of adrenals.

Chemicals

Many catechol compounds were purchased from Sigma, but 3,4-dihydroxyphenylserine was from Calbiochem (Los Angeles, CA, U.S.A.), 3,4-dihydroxyphenylethanol from Regis (Morton Grove, IL, U.S.A.), dobutamine from Eli Lilly and apomorphine from MacFarland-Smith (Edinburgh, U.K.). Dihydroxyphenylalanine, carbidopa and α-methyldopamine were donated by Merck Sharp & Dohme (Kirkland, Canada), the α-methyl and α-ethyl derivatives of norepinephrine by Sterling-Winthrop Institute (Rensselaer, NY, U.S.A.), N-methylepinephrine by Dr. R.W. Fuller of Eli Lilly (Indianapolis, IN, U.S.A.) and fusaric acid (5-butylpicolinic acid) by Schering (Bloomfield, NJ, U.S.A.).

RESULTS AND DISCUSSION

Moyer and co-workers [6, 10] have optimized the chromatographic conditions for analysis of CAs with respect to both their resolution and detector response. We have raised the proportion of methanol to 8% to resolve the DA peak from that of a contaminant present in low amount in the internal standard (Fig. 1). Because the run-time had to be shorter in order to detect the DA peak reliably we had to choose between two further measures. (A) Additional supplementation with methanol to shorten the retention time. This

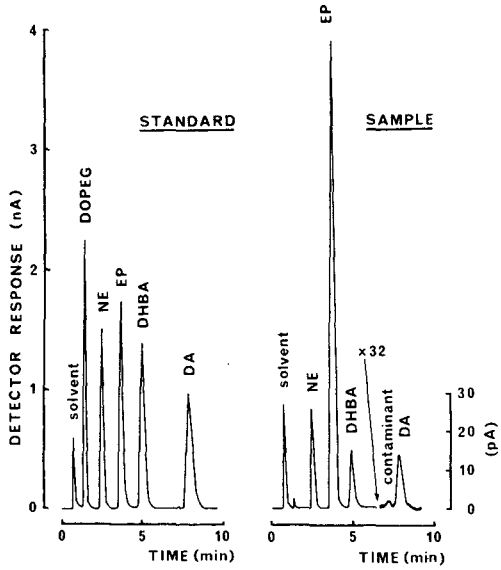

Fig. 1. Chromatogram of CAs from standards taken through sample preparation and from an adrenal extract with 300 ng of DHBA added to it. The arrow indicates when the recorder scale was magnified 32 times. This sample contained 4.9 μg of NE, 19.4 μg of EP and 120 ng of DA per adrenal.

would have required the use of glassy carbon electrodes or carbon pastes other than the oil-impregnated one, with which the highest sensitivities are achieved. (B) In view of that limitation, we found it advantageous to operate above ambient temperature because this not only decreased the retention times but also produced sharper and higher peaks, thereby lowering the detection limits of the method. Thus, with the present method, run-times can be reduced by 20% by simply raising the column temperature from ambient to 30°C (Fig. 2). At the higher temperature DA is eluted within 9 min (Fig. 1). This run-time is 45% shorter than that of another procedure for adrenal CAs [4]. For a large number of samples to be analyzed at one time, such a reduction in run-time is especially useful. Even higher temperatures, up to 60°C, can be used in assaying 3,4-dihydroxyphenylacetic acid and homovanillic acid [11]. As the column temperature is raised, the mobile phase must be pre-heated to this temperature to preserve resolution and peak symmetry [12]. On the other hand, lowering the temperature can compensate for the decrease of capacity factors after months of column use.

A linear detector response for peak area ratios of DA, NE and EP was observed between 0.3 and 150 ng (correlation coefficients greater than 0.9998), the curves for injected standards being co-linear with those obtained from spiked homogenates (not shown). The detector response for these substances was two- to five-fold greater [6] when the pH of the mobile phase was 5.5, as used here, than at pH 2.8 as in the other procedures for adrenal CAs [4, 5], therefore increasing the sensitivity of the method. Lower noise level and better

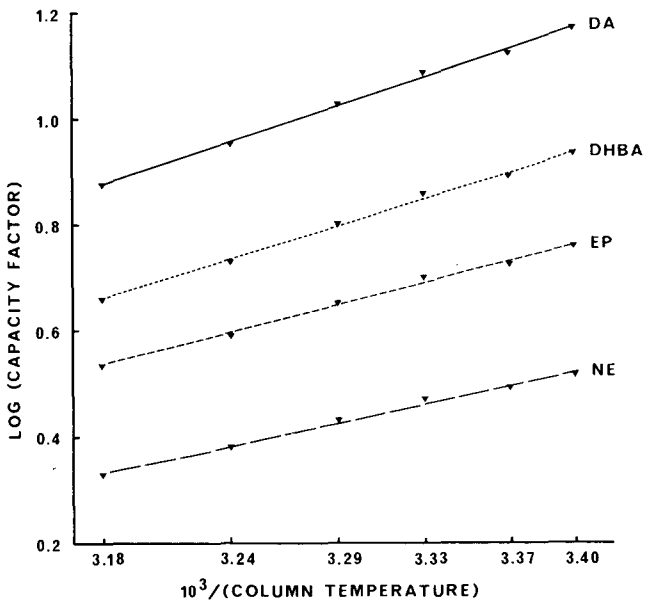

Fig. 2. Van't Hoff plots depicting the effect of temperature on the capacity factor for CAs. The abscissa represents temperatures between 20 and 40°C, graphed as 10^3 °K^{-1}. The capacity factors on the ordinate are a measure of the time spent by a solute on stationary phase divided by that in mobile phase. Standard errors were smaller than the data points; the slopes for NE, EP, DHBA and DA were 0.87, 1.03, 1.25 and 1.35, respectively.

sensitivities were also achieved by helium sparging of the mobile phase and by electrical grounding of the chromatographic column.

No interference may be expected for major sample components and DHBA, as evidenced from the capacity factors of the many catecholic and related compounds listed in Table I. This table also shows that the relative detector response, related to the oxidation current produced by various catechols, differs by two-fold at the most.

Data in Table II confirm the identity of DA, NE and EP peaks after powerful inhibition of DA hydroxylation by fusaric acid [13]. The presence of 3,4-dihydroxyphenylglycol (about 300 ng per adrenal in 425-g rats, Fig. 1) and of traces of N-methyldopamine (epinine) [14] was suggested by co-elution experiments but their amounts were not affected by administration of fusaric acid.

TABLE I

RETENTION AND DETECTION DATA OF CATECHOLS AND RELATED COMPOUNDS

These data were obtained by duplicate injections of 30 pmol (about 5 ng) of compounds with 15 pmol of DHBA. The mobile phase was thermostated at 22° C and pumped at 2.5 ml/min.

Compound	Retention time (min)	Capacity factor	Relative detector response*
DL-3,4-Dihydroxymandelic acid (DOMA)	1.2	0.22	(0.52)
DL-*threo*-3,4-Dihydroxyphenylserine (DOPS)	1.3	0.36	(0.66)
3,4-Dihydroxyphenylacetic acid	1.6	0.66	0.79
L-3,4-Dihydroxyphenylalanine	1.8	0.81	0.75
DL-3,4-Dihydroxyphenylglycol	2.0	1.0	0.87
L-α-Methyldopa	2.4	1.4	0.67
L-Carbidopa	2.8	1.9	1.4
L-Norepinephrine (NE)	3.8	2.9	0.96
L-α-Methylnorepinephrine	5.8	5.0	1.2
3,4-Dihydroxyphenylethanol	5.9	5.1	1.0
L-Epinephrine (EP)	6.0	5.2	1.1
N-Methylepinephrine (LYO 18839)	7.1	6.4	1.5
6-Hydroxydopamine	7.9	7.2	0.78
3,4-Dihydroxybenzylamine (DHBA)	8.6	7.8	1
N-Acetyldopamine	9.9	9.2	0.84
α-Ethylnorepinephrine (butanephrine)	13.0	12.3	0.77
Dopamine (DA)	14.2	13.7	1.1
N-Methyldopamine (epinine)	18.5	18.0	0.92
L-Isoproterenol	27.3	27.2	0.83
5,6-Dihydroxytryptamine	27.6	27.7	1.3
L-α-Methyldopamine	35.3	35.6	0.96
DL-α-Propyldopacetamide	>60		
Dobutamine	>60		
Apomorphine	>60		

*The relative detector response is the peak area ratio of a given substance compared to an equimolar amount of internal standard. The DOMA and DOPS peaks were, respectively, seven and twenty times larger than co-eluting solvent front peaks; their response factors are obtained after substracting the area of these solvent peaks.

TABLE II

ADRENAL CATECHOLAMINE LEVELS FOLLOWING INHIBITION OF DOPAMINE-β-HYDROXYLASE

Rats weighing 325—340 g were given a single intraperitoneal injection of fusaric acid, 100 mg/kg body weight, dissolved in saline pH 6.0, and their adrenals were removed 3 h later. Mean values ± standard errors are presented; data were subjected to logarithmic transformation in order to minimize the heterogeneity of their variances [18], before Student's *t*-tests were carried out: $p < 0.001$ for DA and EP.

Group	Number of rats	DA (ng/pair of adrenals)	NE (μg/pair of adrenals)	EP (μg/pair of adrenals)
Saline	4	287 ± 57	5.44 ± 0.58	26.4 ± 2.2
Fusaric acid	6	1210 ± 188 (420%)	4.90 ± 0.45 (90%)	14.9 ± 1.7 (56%)

Many precautions have been taken during sample preparation. The adrenals were removed under anesthesia with sodium methohexital because barbiturates do not raise the concentrations of plasma CAs [15] and, therefore, probably do not deplete the adrenals of their CA contents. The final perchloric acid concentration, 0.1 *M*, was insufficient to hydrolyse conjugated DA, which amounts to 5% of DA in the rat adrenal [4]. The purification of CAs on alumina was mandatory because adrenal steroids would otherwise cause deterioration of the reversed-phase HPLC column. This adsorption was done in the presence of EDTA, a condition that improves markedly the extraction of DA from tissues [8]. The use of phosphoric acid rather than perchloric acid provides 92—98% elution of all CAs tested [9, 16]. However, we obtained only 68—71% recovery of CAs, whether tissue matrix was present or not; differences in the preparation or type of alumina used could account for this discrepancy. Phosphoric acid also produced smaller void volume detector response and better resolution of CAs with short retention times [16].

The present method compares favorably with the hydroxyindole fluorometric technique of Laverty and Taylor [17]. In experiments [7] with intact rats weighing 340—450 g, the values for DA by liquid chromatography range from 184 to 352 ng/pair of adrenals, those for NE and EP from 8.0 to 11.0 and from 33.5 to 43.5 μg/pair of adrenals, respectively. Using the hydroxyindole method in separate experiments, we found contents of 171—245 ng/pair of adrenals for DA, 10.3—13.3 μg/pair of adrenals for NE, and 27.8—34.2 μg/pair of adrenals for EP in rats weighing 370—410 g killed by diethyl ether overdose. The minimal detectable quantity of DA (at a signal-to-noise ratio of 5) was about 75 ng per adrenal for the present method, 625 ng [17] for the other. The precision of duplicate sample measurement was greater than 2, 2 and 7% for NE, EP and DA, respectively; corresponding figures with the fluorometric assay were 5, 8 and over 10%. The improved precision and sensitivity are important for the measurement of very low amounts of DA in adrenal extracts.

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Note

Alkyl boronates as catechol-specific mobile phase pairing agents

Application to high-performance liquid chromatographic analysis of amines, precursors and metabolites in brain tissue

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(First received November 20th, 1984; revised manuscript received March 13th, 1985)

Reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection provides a simple and sensitive approach to the analysis of the principal biogenic amines, noradrenaline (NA), dopamine (DA) and serotonin (5HT), and their precursors and metabolites. In principle all of them can be separated and determined in a single chromatographic run, using suitable eluting buffer conditions with respect to pH, proportion of organic modifier, and nature and concentration of buffer anion [1—3]. Buffer pH can be used to control the retention of acidic monoamine metabolites, since their pK_a values lie within the range compatible with reversed-phase packings (pH 2—8) [4, 5]. The parent amines, however, are positively charged throughout this range; their retentions can then be controlled by addition of ion-pairing agents (usually alkyl sulphates or sulphonates such as sodium octyl sulphate) to the running buffer (see ref. 6).

In practice, it is often difficult to combine sufficient retention of noradrenaline (one of the most hydrophilic species of interest), to separate it from the unretained peak, with an acceptably short overall run time, because of unduly long retention of 5HT. Preliminary spearation of catecholamines (NA, DA and other catechols) (see for example ref. 7) using alumina is time-consuming and vitiates the advantages of single-chromatogram determination of many metabolites. Alkyl boronates are used as derivatisation reagents for gas-

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liquid chromatographic analysis of molecules with hydroxyl groups on adjacent carbon atoms such as carbohydrates, steroids and catecholamines [8]. Boric acid has been used in the form of gel columns for low-pressure separation of catechols [9], and in buffers to stabilise catechols against oxidation. The use of phenyl boronic acid bound to agarose [10] or silica [11] matrices for the high-pressure separation of catechols has also been recently described. This communication demonstrates that addition of alkyl boronates to the mobile phase retards the elution of catechols on a C_{18} reversed-phase column. This permits selective retention of the catecholamines and their catecholic metabolites versus the methoxy-hydroxy metabolites and 5HT and its metabolites on the C_{18} columns already used for most HPLC work.

EXPERIMENTAL

Butane boronic acid (BuB; Sigma or Aldrich) was added to 0.05 or 0.1 *M* sodium phosphate buffers. HPLC was carried out on a Gilson gradient system, using a Rheodyne injector with 200-μl sample loop, and a 15 cm × 4.6 mm column of Hypersil ODS 5 μm with a 5 cm × 2.1 mm guard column, packed with pellicular ODS silica (Chrompack). Fluorescence (Kratos 950; mercury line excitation, interference filter at 254 nm; emission at 365 nm band-pass) and electrochemical (Bioanalytical Systems, LC4 with TL5) detectors were used in series as previously described [12]. Elution was either isocratic, without addition of organic modifier, or a gradient of methanol was run. Buffer A contained no methanol; a linear gradient of 0—100% buffer B (containing 20% methanol) was imposed between 5 and 15 min post-injection and the methanol concentration was returned to zero between 15 and 20 min.

Standards were made up to final dilution in the initial running buffer. Brain tissue was deproteinised in about 10 vols. of 0.1 *M* phosphoric acid and the supernatant neutralised with an equal volume of 0.1 *M* phosphate buffer containing 10 m*M* BuB. This gave a final pH and BuB concentration equal to that of the initial eluting buffer (see below); after a further brief centrifugation, 200 μl of the neutralised extract were injected into the HPLC system.

RESULTS AND DISCUSSION

As would be expected from previous work [9] the effect of BuB was modest at pH 3—6, in the range usually used for HPLC of amine metabolites. The effect became much more marked around neutral pH (7.0—7.5). At this pH, acidic metabolites are less well retained, but by omitting methanol from the running buffer good resolution was achieved. Fig. 1 shows that increasing concentrations of BuB in phosphate buffer, pH 7.5, led to increased retention of catechols, but decreased retention of 5-hydroxyindoles, presumably due to BuB acting as an organic modifier with respect to the latter. 5 m*M* BuB and 0.1 *M* phosphate were selected as conditions to study a larger series of standards (Table I). The retarding effect seems to be greatest for the catecholamines, followed by the catechol metabolite 3,4-dihydroxyphenylacetic acid (DOPAC). The retention of other compounds is reduced, by a factor of about two thirds in most cases. The retention of methylated metabolites [homovanillic acid

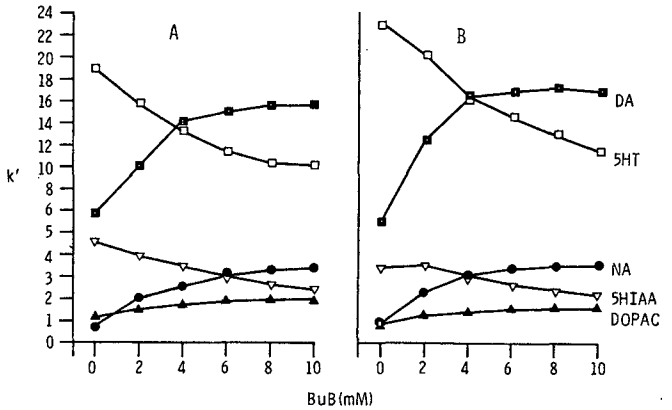

Fig. 1. Effect of increasing concentrations of butane boronic acid on the retention of catechols and 5-hydroxyindoles. Capacity factors (k') are plotted against final BuB concentration in phosphate eluting buffer, pH 7.5, at (A) 0.1 *M* and (B) 0.05 *M*.

TABLE I

EFFECT OF ADDITION OF BUTANE BORONIC ACID (5 m*M*) TO 0.1 *M* PHOSPHATE BUFFER pH 7.5 ON CAPACITY FACTORS FOR CATECHOLS AND OTHER COMPOUNDS

Compound	Capacity factor		Ratio $\frac{\text{with BuB}}{\text{without BuB}}$
	0 m*M* BuB	5 m*M* BuB	
Noradrenaline	0.4	2.15	5.38
Dopamine	3.35	11.8	3.52
3,4-Dihydroxyphenylacetic acid	0.93	1.55	1.68
Vanillylmandelic acid	0.5	0.8	1.60
3-Methoxy-4-hydroxyphenylglycol	7.0	6.18	0.882
Homovanillic acid	5.0	3.6	0.720
Tyrosine	1.55	1.03	0.661
Tryptophan	14.15	9.6	0.678
5-Hydroxyindoleacetic acid	4.2	2.8	0.666
Serotonin	15.0	9.35	0.623
5-Hydroxytryptophol	37.4	22.75	0.608

(HVA), 4-methoxy-4-hydroxyphenylglycol (MHPG)] is reduced less, and vanillylmandelic acid (VMA) is paradoxically retarded as strongly as DOPAC, perhaps due to its α-hydroxy acid structure. Phenyl boronic acid (Sigma) was also tried as a pairing agent, but gave a high electrochemical background.

The method was then applied to the analysis of areas dissected from rat brain. The analysis of NA, DA, 5HT, DOPAC and 5-hydroxyindoleacetic acid (5HIAA) could be carried out in two ways using conditions derived from Fig. 1. At 6 m*M* BuB in 0.05 *M* phosphate with no methanol, isocratic elution, these compounds elute in the order DOPAC, 5HIAA, NA, 5HT, DA. Alternatively, better peak shape for 5HT and DA can be obtained by using 5 m*M* BuB in 0.1 *M* phosphate and running a gradient of 0–20% methanol, as described under Experimental. Re-equilibration takes 15 min, probably because of disturbance of the equilibrium between the pairing agent and the column.

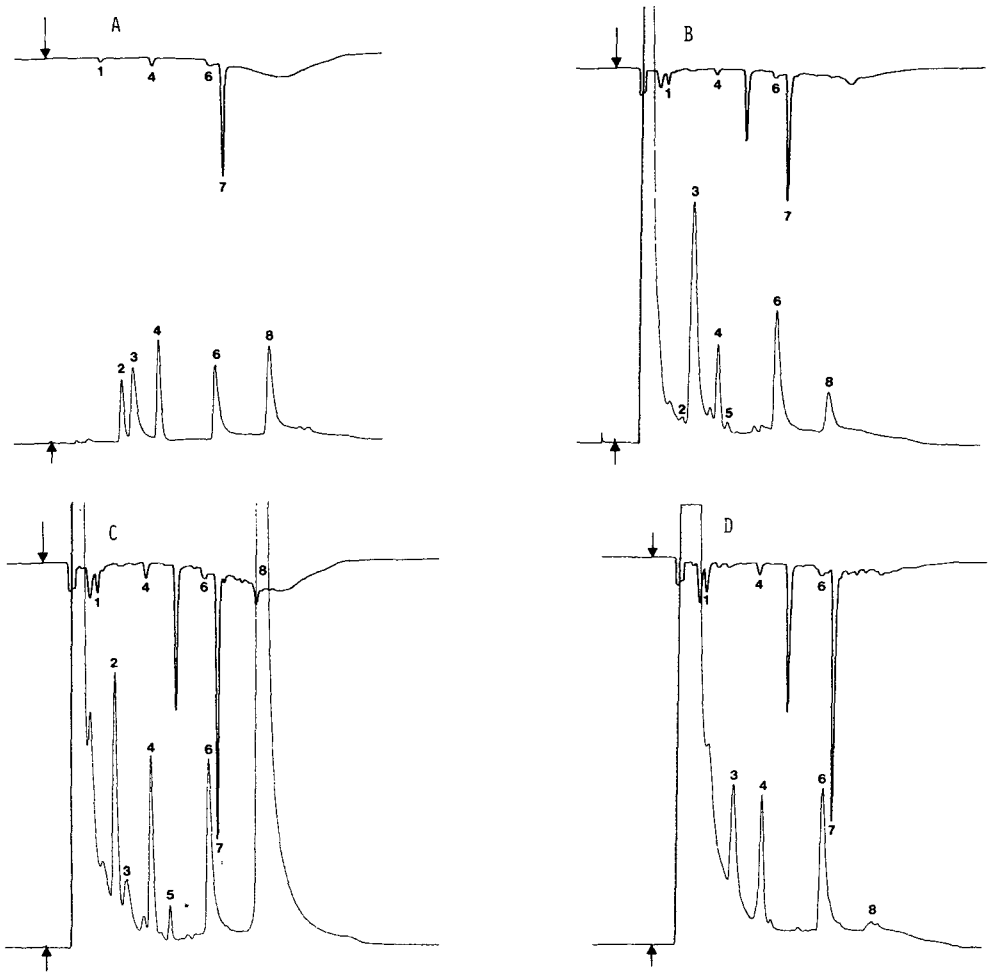

Fig. 2. Separation of amines and related compounds, standards and brain samples. Runnning buffers were 0.1 *M* phosphate, pH 7.5, containing 5 m*M* BuB (final). A gradient of 0—20% methanol was run as described in Experimental. The upper of each pair of traces is obtained by fluorescence detection, sensitivity 0.1, 254/365 nm. The lower is obtained by electrochemical detection in series at 0.4 V, 20 nA full scale. Injection is indicated by arrows. (A) Standards corresponding to 250 ng/g amines and metabolites and 2.5 μg/g amino acids carried through the method. (B) Rat brain hypothalamus. (C) Rat brain striatum. (D) Rat brain hippocampus. Peaks: 1 = tyrosine; 2 = DOPAC; 3 = NA; 4 = 5HIAA; 5 = HVA; 6 = 5HT; 7 = tryptophan; 8 = DA.

This is minimised by keeping the BuB concentration in the second buffer at 5 m*M* final. The elution order is slightly changed, NA eluting between DOPAC and 5HIAA (Fig. 2A). It is important to inject samples in the equivalent of the running buffer (see Experimental), since otherwise a reduction in retention and resolution, especially of NA, is seen, presumably due to the disturbance of the equilibrium between the BuB and the column. The presence of BuB will also help to stabilise catechols against spontaneous oxidation at this relatively high pH.

The working voltage of the electrochemical detector was reduced to 0.4 V

TABLE II

LEVELS OF AMINES, PRECURSORS AND METABOLITES IN RAT BRAIN AREAS

n = 3 in each case.

Compound	Level (μg/g wet weight)		
	Hippocampus	Striatum	Hypothalamus
Tyrosine	13.4	15.6	15.0
Noradrenaline	0.343	0.100	1.528
Dopamine	N.D.*	10.62	0.388
3,4-Dihydroxyphenylacetic acid	N.D.	1.288	N.D.
Tryptophan	5.11	5.67	4.68
Serotonin	0.490	0.478	0.573
5-Hydroxyindoleacetic acid	0.414	0.476	0.463

*N.D. = Not detectable.

so that the unretained peak did not interfere with DOPAC detection. Note that use of a buffer at higher pH results in a downward shift of the oxidation potentials relative to that in the usual pH range [13]. Thus HVA and MHPG, which are also resolved with this system (as expected from Table I), can be detected even at this potential, although sensitivity is greater if a higher working voltage (ca. 0.7 V) is used. Peaks for tyrosine, 5HIAA, 5HT and tryptophan are observed on the fluorescence detector (the amino acids being electrochemically inactive at these working potentials). Fig. 2B—D demonstrates the application of the method to various areas of rat brain. The marked variations, particularly in catecholamine and metabolite levels, between the brain areas analysed (Table II) are in general agreement with those in the literature [1—3, 14].

CONCLUSIONS

Butane boronic acid can be used as a pairing agent in an HPLC mobile phase at around neutral pH to selectively retard the elution of catechol compounds, in particular the catecholamines, from ODS silica. It is likely that the series of alkyl boronic acids will have similar properties. This provides a simple and reversible modification to the properties of these widely used reversed-phase columns, in contrast to the special columns needed if bonded organoboronate phases are used. It thus provides an extra parameter of mobile phase composition which can be manipulated to optimise the separation of complex mixtures of biogenic amines, precursors and metabolites such as are obtained from biological materials. As an example of a group-specific, rather than charge-specific, pairing agent being used in an HPLC mobile phase, BuB may also provide a model for a wide range of applications of other group-specific modifiers in reversed-phase chromatography.

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Note

High-performance liquid chromatographic support for the baseline separation of all phenylthiohydantoin amino acids

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Resolution of the phenylthiohydantoin (PTH) amino acid derivatives by high-performance liquid chromatography (HPLC) is essential for the sequence analysis of proteins by the Edman degradation. The PTH-amino acid derivatives must be analyzed in a short time, be resolved in a single run with a maximum of peak sharpness, and be quantified at low levels (< 10 pmol).

Separations of the PTH-amino acid derivatives by HPLC have been reported for several supports [1—5]. These separations generally show at least one pair of poorly resolved peaks or broad peaks. We report here the results using a support, diphenyl modified silica, which has been demonstrated to have different elution characteristics than straight-chain alkanes [6]. This support provides superior resolution of the difficult to separate PTH-amino acids.

MATERIALS AND METHODS

Reagents

Acetonitrile (HPLC grade), methanol (HPLC grade), acetic acid, sodium acetate and sodium hydroxide were obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). Trifluoroacetic acid and the PTH-amino acid standards were purchased from Pierce (Rockford, IL, U.S.A.). A stock PTH-amino acid mixture containing 200 nmol of each was prepared in 1 ml acetonitrile and stored at −80°C until use. The water used in the buffer preparations was HPLC grade prepared by a Hydro Services system (Research Triangle, NC, U.S.A.).

The buffer system used with the cyano column was prepared according to Hunkapiller and Hood [5]. The buffer system used with the C_{18} support was prepared likewise, except the pH of buffer A was varied in order to obtain

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better resolution of the PTH-amino acids. The trifluoroacetate—acetate buffer system of Hawke et al. [1] was used with the Altex column, with the exception that the pH of buffer A was varied from 4.8 to 6.0 in order to obtain resolution of certain PTH-amino acids.

A modified version of the trifluoroacetate—acetate buffer system of Hawke et al. [1] was used with the diphenyl support. Buffer B was prepared as above [1], however, buffer A was prepared as follows: 66 m*M* trifluoroacetic acid and 4 m*M* acetic acid adjusted to various pH values (5.6—6.2) with 1 *M* sodium hydroxide and dilute ammonium hydroxide. Sodium hydroxide was used to take the buffer to pH 4.9 and then ammonium hydroxide to the desired pH.

HPLC equipment

A Beckman Model 421 controller, two Beckman Model 112 solvent delivery modules, a Beckman Model 340 organizer (Beckman Instruments, Berkeley, CA, U.S.A.), and a Spectroflow Model 757 absorbance detector (Kratos, Westward, NJ, U.S.A.) were used.

The columns used in the separation of the PTH-amino acids were: (a) an Altex Ultrasphere-ODS column (5 μm particle size, 250 × 4.6 mm) obtained from Rainin Instrument (Woburn, MA, U.S.A.), (b) a Cyano (5 μm particle size, 250 × 4.6 mm), (c) a Bakerbond C_{18} (5 μm particle size, 250 × 4.6 mm) and (d) a Bakerbond wide-pore diphenyl column (5 μm particle size, 250 × 4.6 mm) all obtained from J.T. Baker.

RESULTS

The twenty PTH-amino acid derivatives could not be completely resolved using a Bakerbond cyano column even with extensive variation of the gradient. The PTH-amino acids that could not be well resolved were valine/proline/methionine and tryptophan/isoleucine/phenylalanine.

The best gradient using the Altex ODS column also yielded incomplete resolution of some PTH amino acids. Valine/proline could not be resolved and complete separation of glutamine/glycine and tryptophan/isoleucine/phenylalanine could not be obtained. In addition, the resolution of tryptophan/isoleucine/phenylalanine was not reproducible from run to turn.

The best gradient using the Bakerbond C_{18} column showed no resolution of valine/methionine and incomplete resolution of glutamic acid/carboxymethyl cysteine, isoleucine/phenylalanine, and valine/proline.

Three Bakerbond wide-pore diphenyl columns from different lots were used to resolve the twenty PTH-amino acids and all gave complete separation. One support was pre-treated with a pyridine—acetate buffer prior to use. The resolution of the PTH amino acid derivative mixture using this support is shown in Fig. 1A. Incomplete separation of carboxymethyl cysteine/asparagine/serine, proline/methionine and phenylalanine/tryptophan was obtained. The separations, however, are easily sufficient to clearly identify the twenty amino acid derivatives. The same separations were obtained using samples of 10 pmol of each amino acid (data not shown).

The other two columns had no pre-treatment with pyridine—acetate buffer, and were used as they came from the manufacturer. The resolution of the PTH-

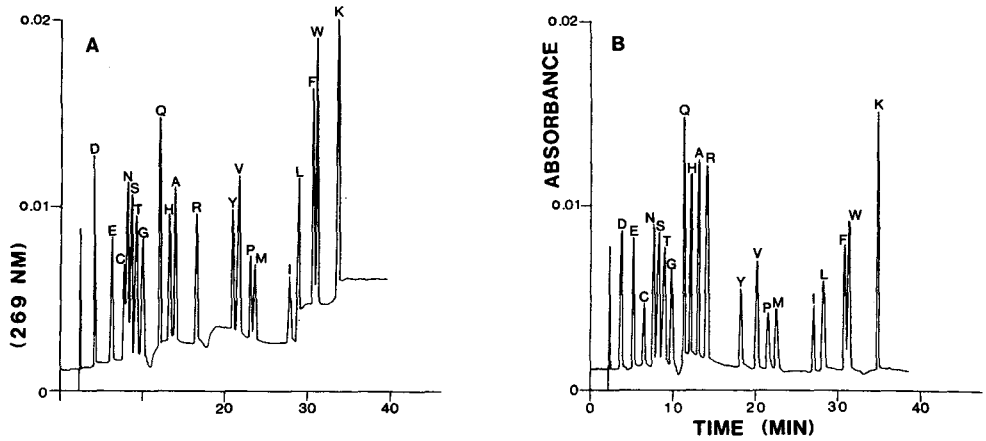

Fig. 1. Separation of PTH-amino acids on Bakerbond wide-pore diphenyl columns. Each peak represents 300 pmol (15 μl of stock solution injected) of the respective amino acid. For each chromatographic run the recorder range was set at 10 mV, the detector at 0.02 a.u.f.s. and the flow-rate was 1.5 ml/min. (A) Buffer A is 66 m*M* trifluoroactate—4 m*M* acetate, pH 5.6 and buffer B is acetonitrile—35 m*M* trifluoroacetate, pH 3.6 (75:25). The program (time in min) is as follows: (0) 5% B, (7) 18% B, (14) 28% B, (25) 45% B, (30) 50% B, (32) 100% B, (36) 5% B. The column temperature was 30°C. (B) Buffer A is 66 m*M* trifluoroacetate—4 m*M* acetate, pH 5.8 and buffer B is acetonitrile—35 m*M* trifluoroacetate, pH 3.6 (75:25). The program (time in min) is as follows: (0) 8% B, (7) 26% B, (20) 32% B, (25) 40% B, (30) 50% B, (36) 100% B, (40) 8% B. The column temperature was 27°C. Peaks: D = aspartic acid; E = glutamic acid: C = carboxymethyl cysteine; N = asparagine; S = serine; T = threonine; G = glycine; Q = glutamine; H = histidine; A = alanine; R = arginine; Y = tyrosine; V = valine; P = proline; M = methionine; I = isoleucine; L = leucine; F = phenylalanine; W = tryptophan; K = lysine.

amino acid mixture is shown in Fig. 1B. As with the first diphenyl column, the same separations were achieved with an amino acid mixture containing 10 pmol of each. A slightly different gradient than for the other diphenyl column was found to give the best resolution. Complete separation of all PTH derivatives was obtained. Although the gradient can be adjusted to provide a shorter run time we have chosen this gradient to provide a separation that prevents any possible ambiguity in the assignment of unknowns from the sequencer.

The pH of buffer A plays a major role in the elution times of arginine and histidine in this gradient. At a high pH (6.2) both elute early, histidine eluting with glutamine and arginine eluting with alanine. As the pH is lowered to 5.8 both arginine and histidine elute later, at the positions shown in Fig. 1B. Changing the pH of buffer A has essentially no effect on the elution times of the remaining eighteen PTH-amino acids.

The buffer A system used, that is adjusting the pH with both sodium hydroxide and ammonium hydroxide, was found to be necessary to give the best resolution of the PTH-amino acids at the beginning of the gradient elution. Without the presence of the ammonium ion, the resolution of asparagine/serine is not possible and the use of ammonium ion alone results in loss of resolution of other PTH amino acids.

Lower column temperatures also enhance the resolution of the PTH-amino acids. A column temperature of 27°C was found to give optimum resolution.

As the temperature was increased to 46.5°C, the resolution decreased substantially (data not shown).

DISCUSSION

In our efforts to obtain complete separation of all twenty PTH-amino acids we used a number of published methods [1—5] and modifications of these methods. In all cases one or more pairs of PTH-amino acids were poorly resolved or not resolved at all. In order to achieve a different selectivity than the cyano or C_{18} columns previously used, a diphenyl support was used. With this column we were able to achieve the complete separation of all twenty PTH-amino acids.

The reasons for the lack of success using the published methods may be due to variations in the column packings, the HPLC systems, or mobile phase components. In order to test the variation in the diphenyl column packings we tested three different columns from three separate lots and all were capable of achieving the resolution shown in Fig. 1. Although the separations shown in Fig. 1 are using conditions optimal for our work we have used other flow-rates, gradients, and pH values which provided complete separation of all peaks as well.

The key features of the separation described here are: (1) using a pH value that allows PTH-histidine and PTH-arginine to elute in a proper position, (2) using both NH_4^+ and Na^+ ions, and (3) the diphenyl packing. This separation system has been in virtually continuous use in our laboratory for over six months with little change in elution times or peak shapes. The use of this HPLC support, diphenyl modified silica, provides an easy method to achieve the complete separation of all twenty PTH-amino acids in 34 min and should be particularly useful for those laboratories that have had difficulties with other methods.

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Note

Rapid isocratic procedure for the separation of platelet-activating factor from phospholipids in human saliva by high-performance liquid chromatography

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(First received May 16th, 1984; revised manuscript received February 25th, 1985)

Platelet-activating factor (PAF) is an extremely potent ether-linked phospholipid which can initiate a wide range of biological activities including platelet activation, the cardiovascular and pulmonary changes of anaphylaxis and systemic hypotension [1—3]. The structure of PAF as derived from rabbit basophils [4], dog leukocyte [5] and rat medullary hypotensive lipid [3] has the structure 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (AGEPC, alkyl acetyl-GPC, PAF-acether).

The separation of PAF from other phospholipids has been done using thin-layer chromatography (TLC) [4]. Recently, high-performance liquid chromatographic (HPLC) methods, using silica gel columns, for the separation of PAF from other phospholipids have been reported [6, 7]. The advantage of HPLC over TLC includes higher resolution of phospholipids, excellent recovery of individual phospholipids, and there is no need to elute material from TLC plate zones for subsequent analysis. These HPLC methods involve gradient elution with two different solvent systems [6, 7], but UV detection of phospholipid peaks can be used with only one [7].

The method we employ is a technique based on the procedure described by Chen and Kou [8]. This system has the advantage of an isocratic gradient, good separation of PAF from other phospholipids, a short run time and UV detection of phospholipid markers since PAF has low UV absorbance.

EXPERIMENTAL

Materials

Soybean phosphatidylinositol (PI), bovine brain phosphatidylserin (PS),

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egg yolk phosphatidylethanolamine (PE), phosphatidylcholine (PC) and lysophosphatidylcholine (LPC), synthetic PAF (1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, AGEPC) and lyso-PAF (1-O-alkyl-2-hydroxy-*sn*-glycero-3-phosphocholine, LAGEPC) were purchased from Avanti Polar Lipids (Birmingham, AL, U.S.A.). [^{3}H-Cholinemethyl] phosphatidylcholine (^{3}HPC), [^{14}C-1-dipalmitoyl] phosphatidylethanolamine (^{14}CPE), [^{14}C-cholinemethyl] sphingomyelin (^{14}CSPH) and [^{3}H-1',2'-alkyl] 1-O-alkyl-2-acetyl-*sn*-glyceryl-3-phosphocholine (^{3}HAGEPC) from New England Nuclear (Boston, MA, U.S.A.). [^{14}C-1]-L-α-Phosphatidylserine (^{14}CPS), [^{3}H-2] L-α-phosphatidylinositol (^{3}HPI), [^{14}C-1] L-α-lysophosphatidylcholine (^{14}CLPC) and [^{3}H-1',2'-octadecyl] lyso-PAF (^{3}HLAGEPC) were purchased from Amersham (Arlington Heights, IL, U.S.A.). HPLC-grade acetonitrile, chloroform, methanol and 85% phosphoric acid were purchased from Fisher (St. Louis, MO, U.S.A.); alternatively HPLC-grade acetonitrile and methanol were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Preparation of salivary lipids

Collection and extraction into chloroform of unstimulated mixed human saliva was performed as described elsewhere [9]. The chloroform phases of the saliva extracts were pooled and dried to a 1-ml volume in a Buchler vortex-evaporator (Bucki-Brinkman Instruments, Westbury, NY, U.S.A.). The sample was then filtered through a MSD cameo II, 0.45-μm filter (Fisher Scientific, St. Louis, MO, U.S.A.) into a clean 75 × 12 mm glass tube and then taken to dryness in the vortex-evaporator. The dried saliva extract was then resuspended in 250 μl of HPLC-grade chloroform and chromatographed as described below.

Chromatographic conditions

The equipment used was a Waters Assoc. (Milford, MA, U.S.A.) liquid chromatography system consisting of a Model 6000 solvent delivery system, a Model U6K injector, a Model 450 variable-wavelength detector and a Model M730 data module. The chromatographic columns used were a 30 cm × 4 mm I.D. prepacked stainless-steel Micro-Pak SI-10 column (Varian Assoc., Palo Alto, CA, U.S.A.), a 25 cm × 4 mm I.D. prepacked stainless-steel Hibar LiChrosorb Si 60 column (Rainin, Woburn, MA, U.S.A.), or a 30 cm × 4 mm I.D. prepacked stainless-steel μPorasil column (Waters Assoc.). All three contained 10 μm particle size silica gel. The mobile phase was acetonitrile—methanol—85% phosphoric acid (130:5:1.5) using initially Fisher solvents followed by re-equilibration of the column and testing using Burdick & Jackson Labs. solvents (acetonitrile and methanol with Fisher phosphoric acid). Flow-rate through the column was 1 ml/min. All phospholipid standards and salivary lipids were dissolved in HPLC-grade chloroform and 1.5 and 10 μl, respectively, were injected into the column. Sample mixtures and recorder response are indictated in figure legends.

Sample testing

Volumes of 1 ml, representing 1-min fractions, were collected in glass 75 × 12 mm test tubes from the HPLC column. To each tube 2 ml of chloroform—methanol—water (1:2:0.8) were added. The test tubes were vortexed and 0.5

ml each of chloroform and water were added to effect phasing. The lower phase was removed and dried completely in a glass 75 × 12 mm test tube using a vortex-evaporator. The dried sample was resuspended in 100 μl of pH 7.2 0.15 *M* phosphate-buffered saline (PBS) containing 5 mg/ml human serum albumin fraction V (Sigma, St. Louis, MO, U.S.A.). The samples were tested for PAF activity using platelet aggregometry as described elsewhere [9]. Fractions containing PAF are indicated on the figures.

RESULTS

Synthetic PAF (AGEPC) was completely separated from other phospholipids by this system. As indicated by 203-nm absorbance in Fig. 1, AGEPC eluted between PC and LPC/SPH with a retention time of 13—14 min. The same elution pattern and retention times were found when using ^{3}HAGEPC and radioactive phospholipid standards (Fig. 2). Recovery of radioactive phospholipid standards and ^{3}HAGEPC from the HPLC column typically were in excess of 97% of the amounts injected. No difference in retention times were noted with any of the three columns used or with solvents from either Fisher or Burdick & Jackson Labs.

HPLC fractions of human saliva extracts containing PAF activity corresponded to two small peaks having retention times of 13 and 14 min (Fig. 3 A and B).

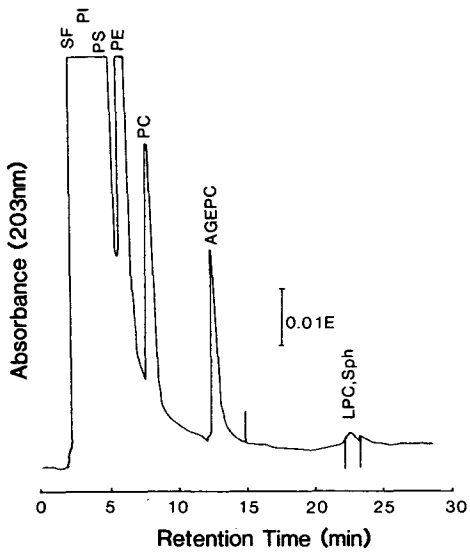

Fig. 1. Chromatogram of phospholipid standards. The amount injected was 1.5 μl of chloroform containing 0.5 μg each of PS, PE and PL, 2.5 μg each of PI and SPH, 5 μg each of LPC and LAGEPC and 10 μg of AGEPC. Chromatographic conditions: flow-rate 1 ml/min; mobile phase, acetonitrile—methanol—85% phosphoric acid (130:5:1.5); UV detection at 203 nm; recorder response 0.1 a.u.f.s.; and ambient temperature. Peaks: SF = solvent front; PI = phosphatidylinositol; PS = phosphatidylserine; PE = phosphatidylethanolamine; PC = phosphatidylcholine; AGEPC = alkyl glyceryl phosphorylcholine or PAF; LPC = lysophosphatidylcholine; LAGPEC = lyso-AGEPC or lyso-PAF; Sph = sphingomyelin.

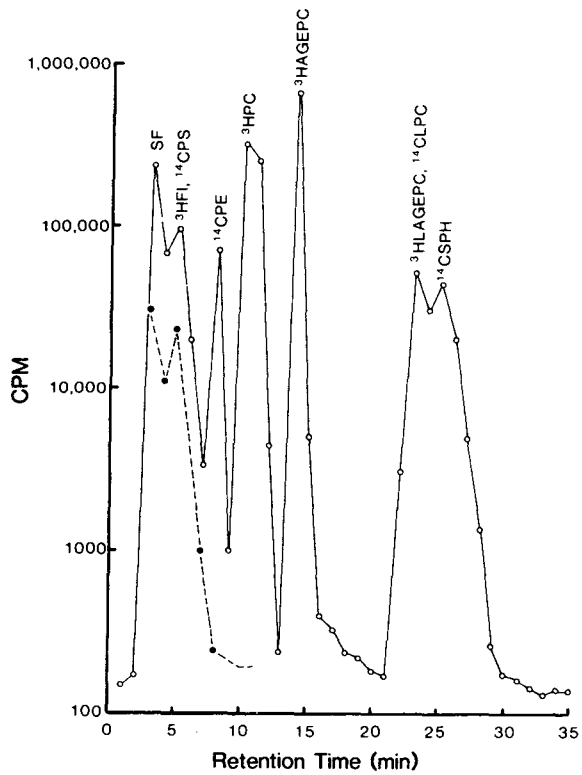

Fig. 2. Elution profile of different ^{3}H- or ^{14}C-labeled phospholipids separated by HPLC. Radioactivity in individual fractions collected at 1-min intervals and measured by liquid scintillation counting. For the first run (—), the amount injected was 1.5 μl of chloroform containing 18 ng of ^{14}CPS; 9 ng each of ^{14}CLPC, ^{14}CSPH and ^{14}CPE; 0.30 ng ^{3}HPC, 0.03 ng each ^{3}HPI and ^{3}HAGEPC; 0.01 ng ^{3}HLAGEPC. Other conditions are the same as in the legend to Fig. 1. Peaks: ^{3}HPI = [^{3}H-2]phosphatidylinositol; ^{14}CPS = [^{14}C-1]phosphatidylserine; ^{14}CPE = [^{14}C-1]phosphatidylethanolamine; ^{3}HPC = [^{3}H-cholinemethyl]phosphatidylcholine; ^{3}HAGPEC = [^{3}H-1',2'-alkyl]AGEPC or PAF; ^{14}CLPC = [^{14}C-1]lysophosphatidylcholine; ^{3}HLAGEPC = [^{3}H]-1',2'-alkyl]lyso-AGEPC or lyso-PAF, ^{14}CSPH = [^{14}C-cholinemethyl]sphingomyelin. For the second run (- - -), the amounts of ^{3}HPI and ^{14}CPS were reduced to 0.025 and 15 ng, respectively.

DISCUSSION

The presence of a lipid with characteristics of PAF in human saliva has been previously reported [9]. Fractionation of phospholipid species from human saliva indicates at least two species having platelet aggregating ability. These phospholipids had retention times very similar to synthetic PAF (AGEPC), confirming previous studies [9] that human salivary PAF (HS-PAF) and AGEPC have structural similarities.

The concentrations of HS-PAF in normal human mixed saliva (< 4 fmol/ml of saliva) is many times lower than other phospholipid species present [10—12]. These low concentrations of HS-PAF make normal detection and isolation extremely difficult. The use of HPLC silica columns with the solvent

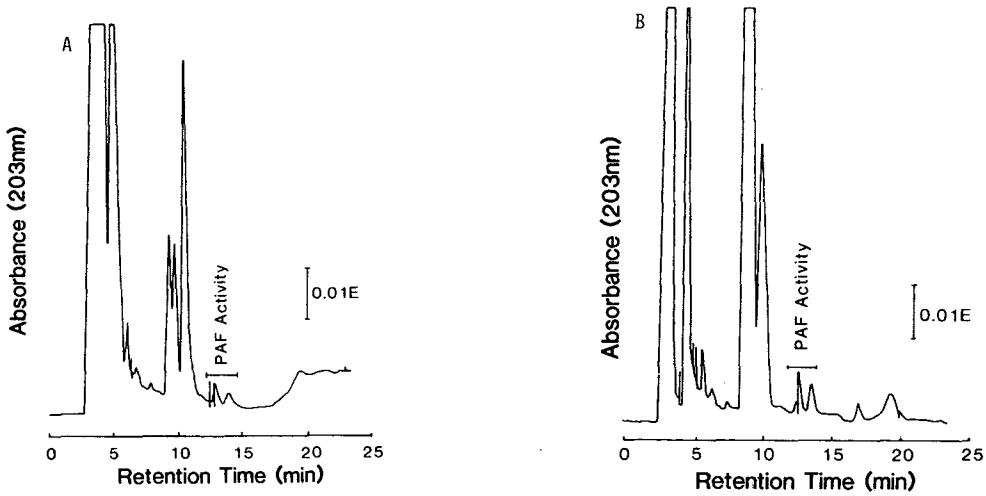

Fig. 3. Examples of chromatograms of human saliva extracted from two normal subjects (A and B) separated by HPLC. The amount injected was 10 μl of extract in chloroform. Chromatographic conditions were as in Fig. 1. PAF activity was determined by collecting fractions at 1-min intervals and testing as described in text.

system of Chen and Kou [8] results in the separation of synthetic PAF and HS-PAF from other common phospholipids with excellent recovery.

The use of the Chen and Kou [8] solvent system and silica HPLC columns should also be applicable to the separation and purification of PAF from contaminating phospholipids in other biological samples.

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Note

Purification of the peracetylated glycosphingolipids of the Gala series (galactosyl- and galabiosylceramides)

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The presence of galabiosylceramides in the ceramide dihexoside fraction of the neutral glycolipids has been reported in humans in Fabry's kidney disease [1], in neutrophils [2] and meconium [3]. On thin-layer chromatography, lactosyl- and galabiosylceramides have the same migration rate [4]. In the course of our study of neutral glycolipids of human thyroid [5], the preliminary analysis of glycolipid fractions suggested such a mixture in the ceramide dihexoside. The separation of these two ceramides was achieved by McCluer et al. [6] by high-performance liquid chromatography of derivatized glycosphingolipids. In an attempt to separate these two compounds with a simplified method, we have compared the results obtained with the ceramide dihexoside fraction, with or without peracetylation, on silica gel high-performance thin-layer plates with and without borate impregnation. The investigation was extended to the cerebroside fraction in order to define the best possible procedure.

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EXPERIMENTAL

Thin-layer chromatography

Silica gel 60 glass plates for high-performance thin-layer chromatography (HTPLC) and silica gel 60 plastic-backed plates were obtained from Merck (Darmstadt, F.R.G.).

The solvents were of analytical-reagent grade (Merck). Three solvent systems were used: (1) chloroform–methanol–water (65:25:4); (2) dichloroethane–methanol (98:2); (3) chloroform–methanol–water–28% ammonia solution (40:10:0.9:0.15).

The plates were run at room temperature for 20 cm with solvents 1 and 2 and for 10 cm with solvent 3 in 20 × 20 × 10 cm chambers (Desaga, Heidelberg, F.R.G.) lined with filter-paper.

Purification of the neutral glycolipids

Neutral glycolipids from human thyroids were extracted and characterized using the procedure described earlier [5]. Briefly, after removal of thyroglobulin, the tissue was homogenized and the lipids were extracted into chloroform–methanol–water (1:2:0.7) [7]. After desalting on a Sephadex G-25 column [8] and removal of the acidic lipids on DEAE-Sephadex [9], the non-acidic lipids were acetylated and applied to a Florisil column according to Saito and Hakomori [10]. The neutral glycolipids were eluted with dichloroethane–acetone (1:1). After deacetylation with 1% sodium methoxide in methanol, the salts were removed on a Sephadex G-25 column. The glycolipids were separated on a silicic acid column by a gradient of methanol in diisopropyl ether as described elsewhere [5]. Elution was monitored on silica gel 60 high-performance thin-layer plates developed in solvent 1 and detected with orcinol–sulphuric acid reagent at 120°C.

Isolation of the neutral glycolipids

The different compounds of the mono- and dihexosylceramide fractions recovered from the silicic acid column were isolated by TLC using two methods. Glucosylceramide from Gaucher spleen (Sigma, St. Louis, MO, U.S.A.), galactosylceramide from rat brain and lactosylceramide from human liver were used as standards.

Separation following peracetylation [10]. The acetylated fractions were applied to a silica gel 60 plate (Merck) developed with solvent 2. Part of the plate was sprayed with orcinol–sulphuric acid reagent in order to localize the spots and the glycolipids were recovered from the scraped gel by sonication in chloroform–methanol (1:1). The glycolipids were deacetylated as described above.

Separation on borated silica gel plates according to Kean [11]. Plastic-backed silica gel 60 plates (Merck) were dipped in a 1% methanolic solution of sodium tetraborate. After removal of the solvent, the plates were dried and kept at room temperature. They were then used for separation of the deacetylated glycolipids in solvent 3. Part of each developed plate was sprayed with orcinol–sulphuric acid reagent at 120°C and the gel corresponding to the located spots was recovered. Each compound was eluted and purified as described above.

Gas—liquid chromatography

The samples were hydrolysed with either 0.5 *M* hydrochloric acid in dry methanol for 18 h at 80°C (for fatty acid and carbohydrate analysis) or with methanol—HCl—water (83:8.6:9.4) (for long-chain base analysis) and subjected to gas—liquid chromatography on a Packard 427 chromatograph as described previously [12].

Carbohydrate analysis. The carbohydrates were analysed as trifluoroacetate derivatives [13]; inositol (Merck) was added to the samples as an internal standard and analysis was carried out on a 3% SP 2401 on 100– 200 mesh Supelcoport (Supelchem, Paris, France) glass column (200 × 0.2 cm I.D.) with nitrogen as the carrier gas. The injection port was heated at 200°C, the flame-ionization detector at 250°C and the column from 100 to 210°C at 2°C/min. Identification and quantification were accomplished by comparison with known standard sugars under the same conditions.

Fatty acid analysis. After acid hydrolysis, fatty acid methyl esters were extracted with *n*-hexane and fractionated on a Florisil (100– 120 mesh) column into non-hydroxy and hydroxy fatty acid methyl esters [14]. $C_{21:0}$ fatty acid methyl ester (Sigma) was added as an internal standard to both fractions to quantify the relative proportions of non-hydroxy and hydroxy fatty acids. Analyses were performed on a 3% OV-1 on 100—200 mesh Supelcoport column (200 × 0.2 cm I.D.) (Supelchem). The column was heated from 175 to 310°C at 3°C/min. The fatty acid methyl esters were identified by comparison with standard hydroxy and non-hydroxy fatty acids (Applied Science Europe, Oud-Beijerland, The Netherlands). In some instances, the hydroxylated fatty acids were also identified after silylation according to Bouhours and Glickman [15].

Long-chain base analysis. Long-chain bases were extracted from the methanolysate in diethyl ether after addition of sodium hydroxide [16], silylated [17] and the trimethylsilyl (TMS) derivatives were analysed on a 3% OV-1 on 100—200 mesh Supelcoport column. The column was heated from 230 to 290°C at 3°C/min. Identification was performed by comparison with standard long-chain bases (Serva, Heidelberg, F.R.G.).

RESULTS AND DISCUSSION

The pattern of neutral glycolipids of human thyroid revealed the presence of lipids co-migrating with ceramide monohexoside (CMH), ceramide dihexoside (CDH), ceramide trihexoside (CTH) and globoside. Their complete analysis will be reported elsewhere [5].

The deacetylated glycolipid fractions eluted from the silicic acid column were homogeneous on thin-layer plates and their carbohydrate composition was determined by GLC. The molar ratios obtained were Gal:Glc = 0.9:1 for CMH and 2:1 for CDH and CTH. These results suggested a mixture of glucosyl- and galactosylceramides in CMH, and also a mixture of lactosyl- and galabiosyl-ceramides in CDH. An additional point of interest was the presence of both sphingosine and phytosphingosine, as well as hydroxy and non-hydroxy fatty acids in these fractions. Therefore, several methods were used in an attempt to isolate the different compounds present in CMH and CDH.

TABLE I

CARBOHYDRATE AND LONG-CHAIN BASE COMPOSITION OF THE DIFFERENT BANDS SEPARATED BY THIN-LAYER CHROMATOGRAPHY ON SILICA GEL 60, UNLESS STATED OTHERWISE

For the solvent systems, see Experimental. Means of three separate analyses with a range of variation of 3—5%.

Type	Component*	Ceramide monohexoside							Ceramide dihexoside: CDH (human thyroid)			
		CMH (human thyroid)					Acetylated CMH (rat brain)		Non-acetylated		Acetylated	
		Silica gel 60		Borate-impregnated silica gel 60								
		UB**	LB**	UB	IB**	LB	UB	LB	UB	LB	UB	LB
Carbohydrates (molar ratios)	Gal	0.4	1.2	0	0.1	1	1	1	1.1	2.7	2	0.9
	Glc	1	1	1	1	0.1	0.2	0.1	1	1	0.2	1
Long-chain bases (%)	d 18:1	78	76	98	44	95	N.D.***	N.D.	91	95	85	99
	t 18:0	22	24	2	56	5	N.D.	N.D.	9	5	15	1

*Gal = galactose; Glc = glucose; d 18:1 = C_{18} sphingosine; t 18:0 = 4D-hydroxysphinganine.
**UB = upper band; IB = intermediate band; LB = lower band.
***N.D. = Not determined.

TABLE II

FATTY ACID DISTRIBUTION OF THE DIFFERENT BANDS SEPARATED BY THIN-LAYER CHROMATOGRAPHY

Means of three separate analyses with a range of variation of 3—5%. Each band is expressed as a percentage of the total, normal (N) or hydroxylated (OH) fatty acids.

Fatty acid	Ceramide monohexoside														Ceramide dihexoside: CDH (human thyroid)							
	CMH (human thyroid)										Acetylated CMH (rat brain)				Non-acetylated				Acetylated			
	Silica gel 60				Borate-impregnated silica gel 60						UB		LB		UB		LB		UB		LB	
	UB*		LB*		UB		IB*		LB													
	N 96%	OH 4%	N 26%	OH 74%	N 92%	OH 8%	N 67%	OH 33%	N 48%	OH 52%	N 55%	OH 45%	N 95%	OH 5%	N 85%	OH 15%	N 88%	OH 12%	N 82%	OH 18%	N 89%	OH 11%
16:1	2	—	10	—	1	—	2	—	3	—	7	—	4	—	15	—	8	—	14	—	12	—
16:0	5	—	20	—	14	—	16	—	17	—	32	—	19	—	31	—	26	—	37	—	36	—
18:1	2	—	10	—	7	—	4	—	5	—	26	—	12	—	15	—	14	—	10	—	12	—
18:0	2	—	10	8	7	—	7	13	10	4	20	1	14	—	12	23	15	20	14	14	12	14
20:0	12	20	12	15	12	9	6	24	8	8	1	2	3	12	4	15	6	10	2	6	3	8
22:0	31	—	20	25	33	21	22	20	20	25	4	28	12	25	8	10	11	13	8	15	9	12
23:0	—	—	—	—	6	—	8	—	5	—	1	—	2	—	1	—	2	—	2	—	2	—
24:1	21	40	5	25	7	49	18	28	15	25	2	29	9	50	2	33	4	41	5	30	5	41
24:0	25	40	15	28	13	21	18	16	16	37	6	40	25	13	11	18	14	16	8	35	9	25

*UB = upper band; IB = intermediate band; LB = lower band.

Each band of the doublet migrating on TLC as CMH and CDH in solvent 1 was analysed by GLC. As can be seen in Tables I and II, all bands contained galactose and glucose, although the lower band of each doublet seemed to be enriched in galactose. The variations in migration could not be accounted for by clear differences in the ceramide portion. Kean [11] reported the separation of gluco- and galactocerebrosides by means of borate TLC. His method was applied, using borate-impregnated HPTLC plates, to the cerebrosides of human thyroid. As shown in Fig. 1, we were able to separate three bands. The upper band co-migrated with authentic glucosylceramide from Gaucher spleen and contained only glucose, C_{18} sphingosine (Table I) and a small amount (8%) of hydroxy fatty acids (Table II). The intermediate band contained glucose, an equal amount of C_{18} sphingosine and phytosphingosine and a significant proportion (33%) of hydroxy fatty acids. This band appears to be still heterogeneous. The lower band that co-migrated with galactosylceramide from

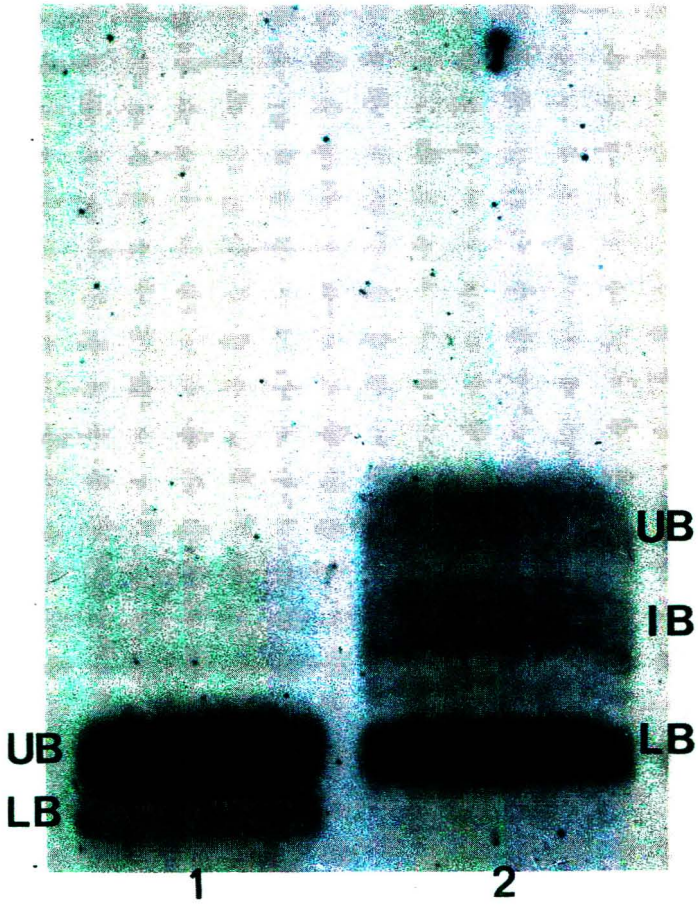

Fig. 1. Thin-layer chromatography of neutral glycolipids on borate-impregnated silica gel 60. Lane 1, rat brain CMH; lane 2, human thyroid CMH. UB, upper band; IB, intermediate band; LB, lower band. Solvent system 3: chloroform—methanol—water—28% ammonia solution (40:10:0.9:0.15).

rat brain (Fig. 1) consisted of galactose, C_{18} sphingosine and a large amount (52%) of hydroxy fatty acids. The separation obtained with cerebrosides of human thyroid was consistent with the results obtained by Karlsson et al. [18] for bovine kidney cerebrosides by means of column chromatography on borate-impregnated silica gel G.

A second method of separation involved TLC after peracetylation of the cerebroside fraction. As shown in Fig. 2, a double band was obtained with the cerebrosides extracted from both human thyroid and rat brain. Because of the small amount of material available, the analysis of the bands eluted from silica gel was carried out on rat brain cerebrosides. The results revealed that both bands contained galactose and the upper band seemed to be enriched in hydroxylated fatty acids (Table II). Therefore, the use of borate-impregnated silica gel appears to be the best method, yielding a satisfactory separation of subfractions from cerebrosides.

As reported by Kean [11], TLC was ineffective in separating lactosyl- and galabiosylceramides, whether the plates were prepared in the presence of borate (data not shown) or not. However, following peracetylation, two bands were obtained by TLC (in solvent 2) of the ceramide dihexoside fraction extracted from human thyroid, in contrast to authentic lactosylceramide, which gave only one band, as shown in Fig. 2. The fast-migrating band yielded only galactose, as would be expected from pure galabiosylceramide, whereas the lower band had the carbohydrate composition of lactosylceramide. Moreover, all the phytosphingosine found in the CDH fraction was in the upper band.

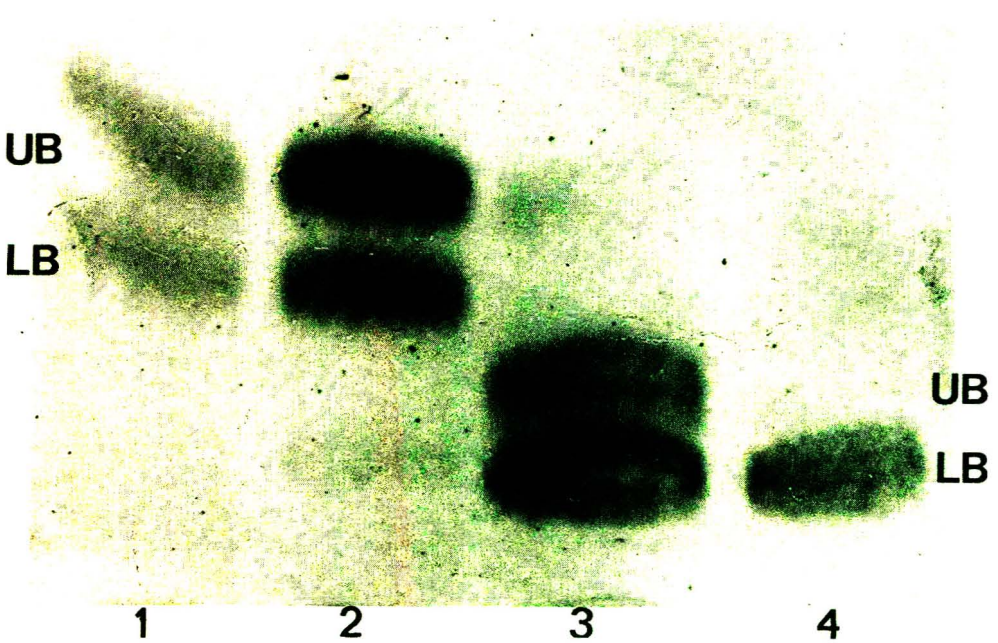

Fig. 2. Thin-layer chromatographic separation of peracetylated neutral glycolipids. Lane 1, human thyroid CMH; lane 2, rat brain CMH; lane 3, human thyroid CDH; lane 4, standard CDH (human liver). UB, upper band; LB, lower band. Solvent system 2: dichloroethane–methanol (98:2).

Both contained some hydroxylated fatty acids. Hence lactosyl- and galabiosylceramides are readily separated by TLC after peracetylation. An alternative procedure using column chromatography on Florisil with gradient elution is currently being investigated in our laboratory, which should avoid any contamination of the samples when eluting the glycolipids from thin-layer plates. In any case, the method described in this paper allows the reliable purification of galabiosylceramide from a ceramide dihexoside fraction in a very simple way compared with the sophisticated procedure described by McCluer et al. [6], involving high-performance liquid chromatography of perbenzoylated glycosphingolipids.

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Note

Antibody-mediated extraction of the main tetrahydrocannabinol metabolite, 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid, from human urine and its identification by gas chromatography—mass spectrometry in the sub-nanogram range

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In forensic chemistry, large numbers of urine samples must be analysed for cannabis content. For this purpose, immunological assays such as enzyme-multiplied immunoassay technique (EMIT) [1, 2] and radioimmunoassay (RIA) [3—5] are widely used. These methods are very rapid, and economical; they can also be automated to a high degree. As they suffer from a lack of substrate specificity, however, a positive EMIT or RIA result must be confirmed by means of a second independent method, in order to comply with standard forensic requirements.

For the detection of 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid (THC-COOH), the main urinary metabolite of Δ^9-tetrahydrocannabinol (THC), several techniques have been described, including high-performance liquid chromatography [6, 7], gas chromatography [8], gas chromatography—mass spectrometry (GC—MS) [9—12], and thin-layer chromatography (TLC) [13, 14]. The detection limits of these methods depend mainly on the clean-up procedure used during extraction. Even after bonded-phase adsorption chromatography, a detection limit no better than 20—50 ng/ml was achieved, using

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TLC [15] and GC—MS [10]. These techniques are sufficient for the confirmation of positive EMIT results. Because RIA has a higher sensitivity (5 ng/ml, Immunalysis, U.S.A.) than EMIT (20 ng/ml), only some of the positive RIA results can be confirmed by the methods cited above.

We applied a new analytical principle for the detection of THC-COOH in urine combining the features of immunoassay and GC—MS [16, 17]. A simple antibody-mediated extraction procedure for cannabinoids yielded a highly pure extract, which led to a considerable improvement of the detection limit with GC—MS.

MATERIALS AND METHODS

Acetone, isooctane and methanol (nanograde) were obtained from Mallinckrodt (St. Louis, MO, U.S.A.) and used without further purification. Dimethyl sulphoxide and methyl iodide were purchased from Merck (Darmstadt, F.R.G.) and tetramethylammonium hydroxide from Fluka (Neu-Ulm, F.R.G.). CNBr-activated Sepharose 4B was obtained from Deutsche Pharmacia (Freiburg, F.R.G.) and the THC antibody from Miles (Munich, F.R.G.). [^{3}H]THC (specific activity 30 mCi/mg) and [^{3}H]flunitrazepam (specific activity 263 mCi/mg) were purchased from Amersham (Buckinghamshire, U.K.). Water was distilled twice before use.

For the coupling procedure, 1 g of Sepharose was washed for 15 min with 500 ml of 1 m*M* hydrochloric acid on a sintered glass filter, and 4.5 mg of THC antibody, dissolved in 0.1 *M* sodium hydrogen carbonate containing 0.5 *M* sodium chloride was then added. After end-over-end rotation for 2 h at room temperature, the excess ligand was washed away with coupling buffer. Inactivation of remaining active groups was performed with Tris—HCl buffer (0.1 *M*, pH 8). The product was washed three times with 0.1 *M* acetate buffer (pH 4) and 0.1 *M* Tris buffer (pH 8), each containing 0.5 *M* sodium chloride. The Sepharose-coupled antibody was stored in 10 ml of 0.1 *M* phosphate buffer (pH 7) at 4°C in the dark.

For extraction, 1 ml of the phosphate buffer (containing 0.45 mg of immobilized antibody) was placed in a Pasteur pipette and held in place by a swab of cotton wool. The cotton wool, as well as the gel, was washed extensively with acetone and water before use. After the extraction procedure, the antibody columns were washed with water and then immersed in 1—2 ml of 0.1 *M* phosphate buffer (pH 7) and stored at 4°C in the dark. The columns were reuseable up to 50 times [16, 17]. The maximum binding capacity was 300 ng of THC-COOH.

For hydrolysis, 1 ml of 10 *M* potassium hydroxide and 1 ml of methanol were added to a 5-ml aliquot of the urine. This mixture was incubated for 15 min at 50°C. After cooling, 2 ml of 0.1 *M* phosphate buffer (pH 7) were added, and the mixture was adjusted to pH 7 with concentrated hydrochloric acid.

A 1—5 ml volume of the hydrolysed and filtered urine was passed through the column, which was then washed with 15 ml of water. Elution of the drug was performed with 10 ml of acetone—water (95:5). The solvent was removed with a dry stream of nitrogen.

For GC—MS analysis, the residue was dissolved in 10 μl of a mixture of 1 g

of tetramethylammonium hydroxide, 1 ml of water and 20 ml of dimethyl sulphoxide [8]. After 2 min, 20 μl of methyl iodide were added. The reaction mixture was incubated at room temperature for 10 min and then extracted with 1 ml of isooctane. The isooctane was removed by a dry stream of nitrogen, and the residue was dissolved in 10 μl of isooctane. A 1-μl aliquot was used for GC—MS analysis.

Mass spectra were run on a Finnigan 4021 GC—MS system: injection port temperature, 280°C; SE 54 chemical-bonded fused-silica capillary column (25 m × 0.23 mm I.D.) directly coupled to the ion source (250°C); column temperature, 75—300°C; rate 15°C/min. The mass spectrometer was run in the electron-impact ionization mode. The main fragments of THC-COOH, *m/z* 313 (100%), *m/z* 357 (50%), and *m/z* 372 (35%), were registered in the multiple-ion detection (MID) mode. The retention time was 15.9 min.

RESULTS AND DISCUSSION

The THC antiserum coupled to Sepharose binds THC as well as THC-COOH [3, 18]. Therefore, the recovery was checked with tritium-labelled THC, as labelled THC-COOH was not available. Unspecific binding was tested with tritium-labelled flunitrazepam. [^{3}H]THC and [^{3}H]flunitrazepam dissolved in urine were submitted to the extraction procedure. The radioactivity in the extracted urine and in the aqueous acetone eluates was measured. The results show high specificity for [^{3}H]THC and low unspecific binding for [^{3}H]-flunitrazepam (Table I).

Extracts of blank urines with the Sepharose-coupled antibody gave no hint

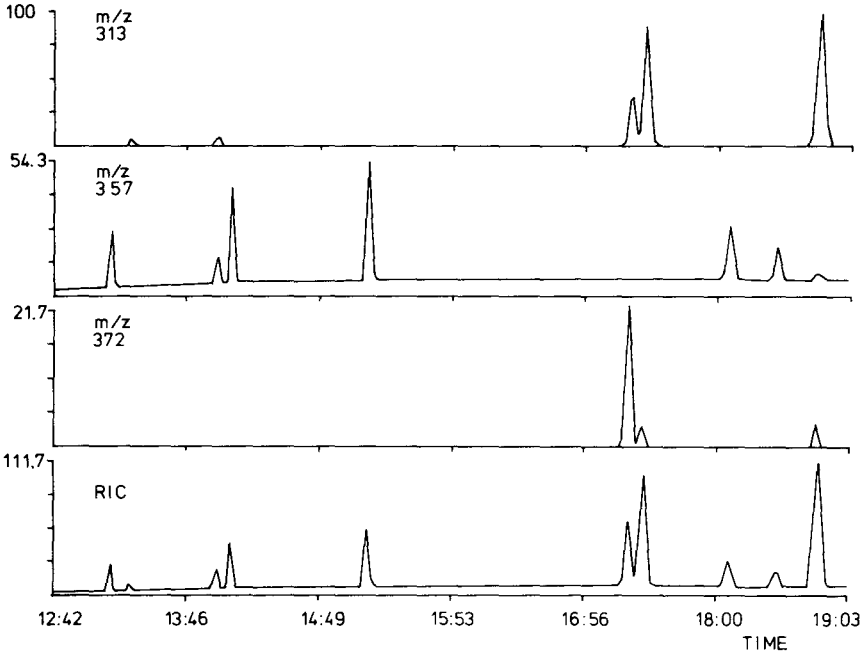

Fig. 1. Mass chromatogram of blank urine extract.

of interfering substances (Fig. 1). For the determination of the detection limit, urine was spiked with THC-COOH. In the MID mode, THC-COOH could be identified at a concentration of 0.5 ng/ml (Fig. 2; only the relevant range of the retention time, 14.49 to 16.56 min, is represented). This is a factor of 10 lower than the detection limit of RIA. A full mass scan could be obtained from samples with 20 ng/ml THC-COOH. The total-ion current chromatogram is shown in Fig. 3, and the mass spectrum of THC-COOH in Fig. 4.

The method presented here permits the routine analysis of a large number of urine samples. It is simple, rapid and cost-effective, because the antibody columns may be used many times. In addition, the high level of purification reduces the contamination of the ion source of the GC—MS. A considerable improvement of sensitivity and specificity is achieved by the strong affinity of THC-COOH for the antibody and the separation of cross-reacting substances by a second analytical step (GC—MS).

TABLE I

RECOVERY (n = 4) OF [^{3}H]THC AND [^{3}H]FLUNITRAZEPAM IN URINE AND AQUEOUS ACETONE ELUATES AFTER PASSAGE THROUGH SEPHAROSE-COUPLED ANTIBODY COLUMNS

Substance added	Percentage recovery in urine after extraction (mean ± S.D.)	Percentage recovery in aqueous acetone fraction (mean ± S.D.)
[^{3}H]THC (100 ng)	6.6 ± 0.8	92.3 ± 3.9
[^{3}H]Flunitrazepam (100 ng)	99.5 ± 3.5	0.9 ± 0.3

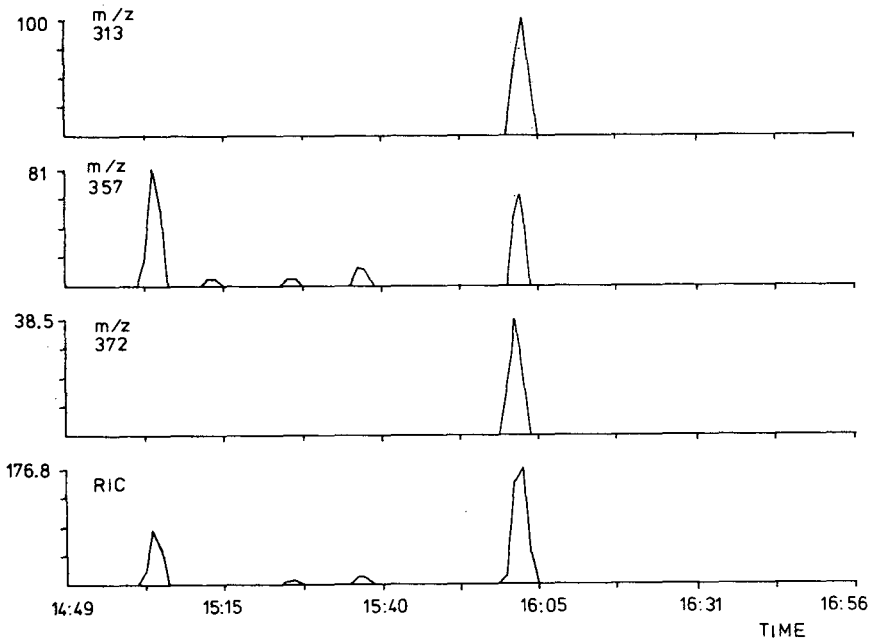

Fig. 2. Mass chromatogram of urine spiked with 0.5 ng/ml THC-COOH.

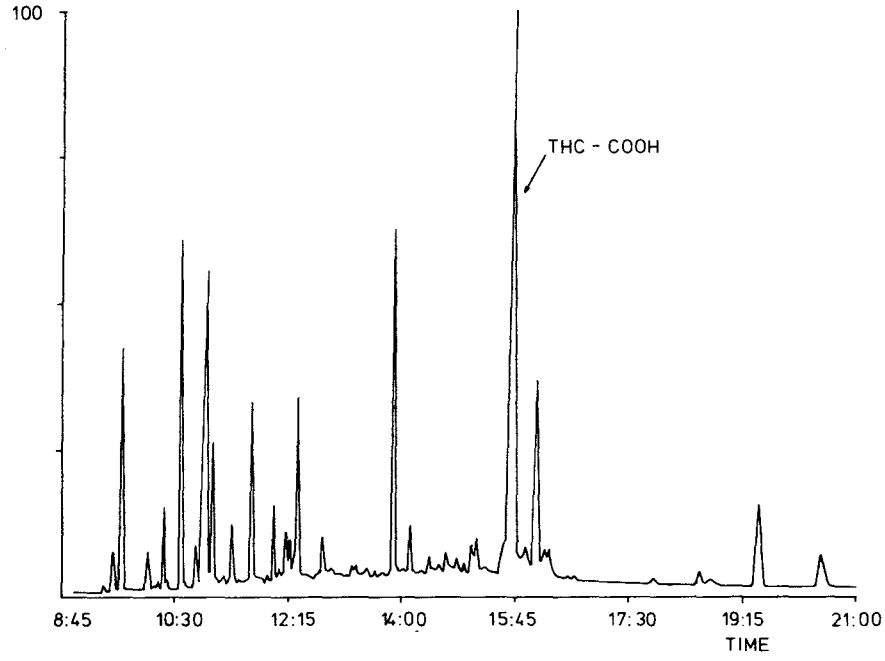

Fig. 3. Total-ion current chromatogram of urine with 20 ng/ml THC-COOH.

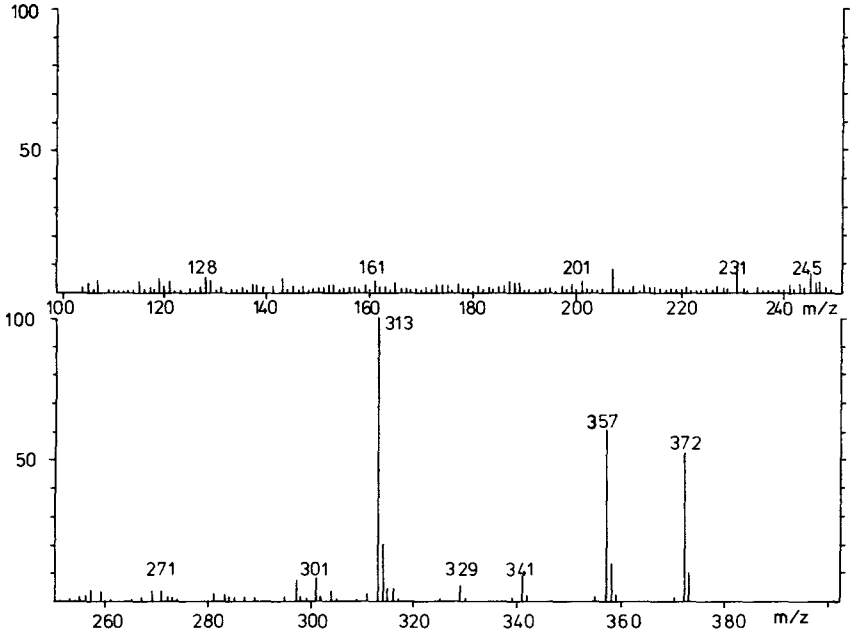

Fig. 4. Mass spectrum of 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid from urine with 20 ng/ml THC-COOH.

The proposed procedure could also be used in the near future for the specific extraction of other drugs from biological fluids, thus opening a broad field of application in forensic and toxicological chemistry.

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Note

Determination of di- and mono(2-ethylhexyl) phthalate in plasma by gas chromatography

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Di(2-ethylhexyl) phthalate (DEHP) is used as a plasticizer in the manufacture of PVC plastics, sometimes up to a concentration of 30—40%. Many studies have shown that DEHP can be leached from the PVC matrix and contaminate the environment. For example, it can accumulate in blood stored in plastic blood bag assemblies [1]. There is therefore major concern about the possible health hazards associated with the use of DEHP, especially as it has been shown to induce liver tumours and testicular atrophy in rats and mice [2—4]. Mono(2-ethylhexyl) phthalate (MEHP), which is formed by hydrolysis of one ester linkage, is a major metabolite of DEHP [5] and is suspected to play an important role in the induction of the toxic effects observed after administration of DEHP [6].

Several methods have been proposed for the determination of DEHP and MEHP in plasma, including gas chromatography (GC) with flame ionization [7, 8] and electron-capture detection [9—11] and high-performance liquid chromatography [12]. The time-consuming sample preparation [9], the use of temperature programming [7], the absence of an internal standard [7, 12] or the use of only one internal standard for both DEHP and MEHP [8, 9], and the use of large amounts of solvents [7] make these methods unsuitable for routine purposes. Moreover, the alkylation of MEHP was performed previously by treating the extract with diazomethane [7, 8, 10], a hazardous compound, or by a time-consuming solid—liquid phase-transfer catalysis, giving rise to the hexyl ester [9]. Finally, the use of large plasma samples precludes the application of these methods to the study of the toxicokinetics of DEHP in rats.

This paper presents a rapid, simple and sensitive GC method, using flame ionization detection, for the determination of DEHP and MEHP in small

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plasma samples. Preliminary data are given on the plasma concentrations of DEHP and MEHP after oral administration of DEHP in rats.

EXPERIMENTAL

Chemicals and reagents

DEHP (Essochem Europe, Machelen, Belgium) was used as received and MEHP (British Petroleum, Sully, U.K.) was purified before use [13]. Mono- and di-*n*-octyl phthalate were synthesized according to the method of Albro et al. [13]. The purities (> 99%) of all compounds were checked by GC and thin-layer chromatography. *n*-Hexane (UCB, Brussels, Belgium) was of pesticide grade. Silicic acid (Sigma, St. Louis, MO, U.S.A.) was rinsed with *n*-hexane before use. The derivatization reagent, tetrabutylammonium hydroxide (Chrompack, Merksem, Belgium), was used as a 0.05 *M* solution in methanol—chloroform (1:3). All other reagents and solvents used were of analytical-reagent grade.

Glassware

All glassware was silanized by soaking for 1 h in a 5% solution of trichloromethylsilane in xylene, rinsed with xylene and methanol, dried for 1 h at 100°C and rinsed with *n*-hexane before use.

Gas chromatography

A Varian 2100 gas chromatograph, equipped with a dual flame ionization detector, was used. GC was performed on 1.8 m × 2 mm I.D. glass columns packed with Gas-Chrom Q coated with SE-30 (Alltech Europe, Eke, Belgium), either at 2% for DEHP or at 5% for MEHP, with a nitrogen flow-rate of 30 ml/min. The temperatures for both compounds were: column 190°C, injection block 270°C and detector 250°C. The hydrogen and air flow-rates were 60 and 300 ml/min, respectively. The peak areas were recorded on a Hewlett-Packard 3380A recording integrator. Both columns were kept in the gas chromatograph throughout.

Extraction and derivatization

A 300-μl volume of acetonitrile containing 10 μg of both internal standards (di-*n*-octyl phthalate for DEHP, and mono-*n*-octyl phthalate for MEHP) and 200 μl of water were added to 100 μl of plasma in a 10-ml glass-stoppered centrifuge tube.

For the determination of DEHP, the mixture was extracted with 3.0 ml of a 1.5 g% suspension of silicic acid in *n*-hexane by shaking horizontally for 5 min. After centrifugation for 5 min at 3000 *g*, the organic layer was transferred with a Pasteur pipette into a 6-ml glass-stoppered conical tube, and the organic layer was removed under a gentle stream of nitrogen at room temperature. The walls were washed with 300 μl of *n*-hexane and the solvent was evaporated under nitrogen. For GC analysis, the residue was dissolved in 10 μl of chloroform and an aliquot was injected into the gas chromatograph.

For the determination of MEHP, 2 ml of 0.1 *M* citrate buffer (pH 2) were added to the remaining aqueous phase, which was then extracted twice with

3.0 ml of *n*-hexane. The sample was processed further as for DEHP. For GC analysis the residue was dissolved in 10 μl of a 0.05 *M* tetrabutylammonium hydroxide solution in methanol—chloroform (1:3) and 1 μl was injected into the gas chromatograph.

Calibration

Di- and mono-*n*-octyl phthalate were selected as internal standards for DEHP and MEHP, respectively, because of their close structural relationship. For the calibration graph, plasma samples were spiked with increasing amounts (0.5—20 μg) of DEHP and MEHP and a constant amount (10 μg) of both internal standards using standard solutions of 0.2 μg/μl in acetonitrile. These calibration samples were taken through the extraction and chromatographic procedures described above. The peak area ratios of DEHP to di-*n*-octyl phthalate and MEHP to mono-*n*-octyl phthalate were plotted as a function of the concentration of DEHP and MEHP, and an unweighted least-squares regression analysis was performed.

Absolute recovery

The absolute recovery of DEHP and MEHP was determined by adding the internal standard after the extraction and comparing the peak area ratios with those of a calibration graph obtained by injecting various amounts of DEHP and MEHP to which a constant amount (10 μg) of internal standard had been added. For DEHP, the extracted samples were corrected for the contamination with DEHP originating from the extraction.

RESULTS AND DISCUSSION

Extraction conditions and gas chromatography

Mono- and diesters of phthalic acid were isolated from plasma by two consecutive extractions at different pH; during an extraction at neutral pH the diesters and cholesterol were removed, while acidic monoesters were isolated after acidification of the same sample to pH 2. The use of a suspension of silicic acid in *n*-hexane for the extraction produced a reduction in the background interference peaks in plasma samples and prevented emulsification of the reagents during the extraction.

DEHP and MEHP were detected by GC using flame ionization detection. In order to improve the retention times and peak shapes, two different columns were used, both of which were kept at the same oven temperature, which allowed concurrent determination of DEHP and MEHP using two recording integrators. It is possible to separate DEHP and MEHP and their respective internal standards in one run, using a 5% SE-30 column at 200°C; however, an interfering peak elutes together with the internal standard of MEHP, and the retention time of cholesterol under these chromatographic conditions is very long.

Esterification of MEHP

MEHP was derivatized to its butyl ester by pyrolysis of tetrabutylammonium hydroxide in the heated inlet zone of the gas chromatograph, with methanol

catalysis. This alkylation method provides a safe alternative to derivatization with diazomethane [7, 8, 10], and does not require any preparative work. Moreover, in this procedure the diesters are not exposed to the alkylating reagent; such an exposure results in a large standard deviation for the diesters [9].

Derivatization of a compound by esterification for the purpose of analytical determination involves a chemical reaction. For this reason, the addition of a structurally related internal standard before the extraction, undergoing the same derivatization as the product to be quantitated, is necessary. In this study mono-*n*-octyl phthalate, a structural isomer of MEHP, was chosen as the internal standard.

Sensitivity and selectivity

Figs. 1 and 2 show representative chromatograms of plasma extracts. As shown in Fig. 1A, contamination with DEHP originating from the extraction could not be avoided entirely. Many reports have dealt with the problem of contamination with DEHP of solvents and materials used in the laboratory [11, 14]. A value of 0.37 ± 0.16 μg DEHP per 100 μl of blank sample (n = 34)

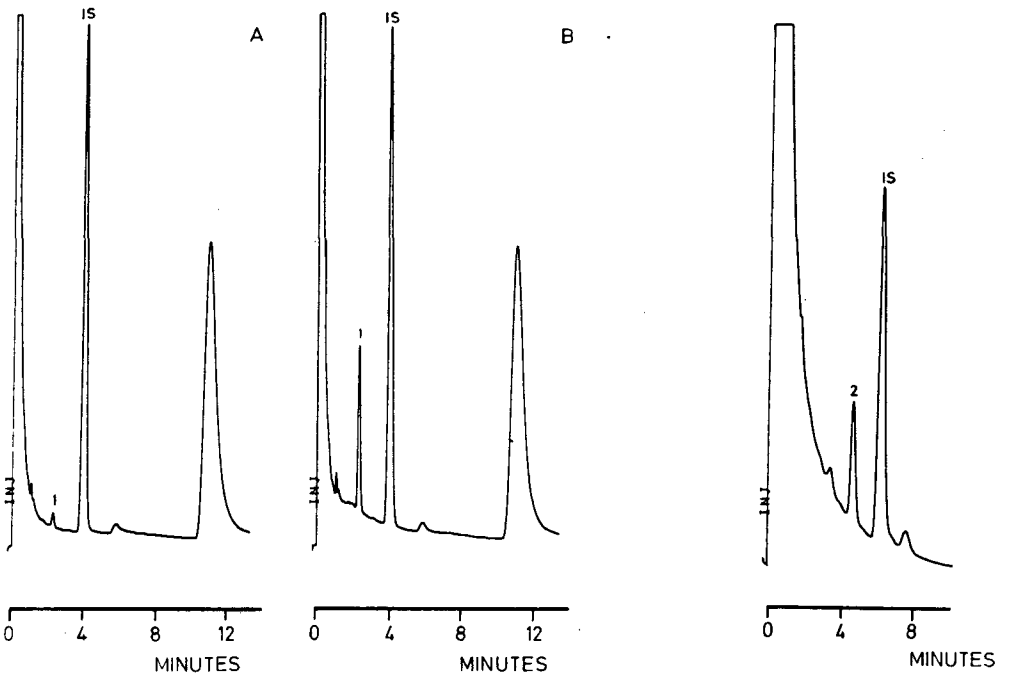

Fig. 1. Gas chromatograms of extracts of 100 μl of plasma. (A) Blank plasma sample spiked with 10 μg of di-*n*-octyl phthalate (retention time, t_R = 4.15 min) as internal standard (IS); a small degree of contamination with DEHP (1) can be seen (t_R = 2.46 min). (B) Plasma spiked with 2 μg of DEHP (1) (t_R = 2.46 min) and 10 μg of di-*n*-octyl phthalate (IS) (t_R = 4.15 min). The peak with t_R = 11.30 min is cholesterol.

Fig. 2. Gas chromatogram of an extract of 100 μl of plasma spiked with 2 μg of MEHP (2) (t_R = 4.63 min) and 10 μg of mono-*n*-octyl phthalate (IS) as internal standard (t_R = 6.20 min).

was obtained by limiting the extraction procedure of DEHP to a single step, by using a pesticide-grade extraction solvent and by rinsing all glassware with *n*-hexane before use.

The level that still allowed the quantitation of both MEHP and DEHP was 5 μg/ml, although lower levels were detectable. By increasing the sample volume it is possible to increase the sensitivity. Extraction of 0.5 ml of plasma enabled us to detect 1.5 μg/ml of both DEHP and MEHP.

The sensitivity and selectivity of the proposed method for DEHP are similar to those reported using electron-capture detection [11]. A higher sensitivity is achieved for MEHP by this method [10].

Precision, recovery and linearity

The extraction gave good recoveries of both DEHP and MEHP from plasma (Table I). Plasma calibration graphs were linear from 5 to 200 μg/ml. The average slope of 34 calibration graphs constructed over a period of six months for DEHP was 0.00958 ± 0.00078 (mean ± standard deviation), with an intercept of 0.0498 ± 0.0379 and a correlation coefficient of 0.9990 ± 0.0009. For MEHP the average slope was 0.01293 ± 0.00137 with an intercept of 0.0176 ± 0.0155 and a correlation coefficient of 0.9983 ± 0.0014. The within- and between-assay accuracy and precision were acceptable (Table II).

TABLE I

ABSOLUTE RECOVERIES OF DEHP AND MEHP FROM PLASMA (n = 5)

Amount added to 100 μl of plasma (μg)	DEHP		MEHP	
	Mean (%)	R.S.D. (%)	Mean (%)	R.S.D. (%)
0.8	90.6	2.2	81.2	7.9
2	81.8	8.5	74.5	5.5
10	88.0	4.4	78.2	3.1
18	89.7	1.4	82.9	2.0

TABLE II

WITHIN- AND BETWEEN-RUN ACCURACY AND PRECISION FOR DEHP AND MEHP

Amount added to 100 μl of plasma (μg)	DEHP		MEHP	
	Relative error (%)	R.S.D. (%)	Relative error (%)	R.S.D. (%)
Within-run (n = 5)				
0.5	−6.4	10.4	+4.6	15.4
1	−3.6	4.8	−3.7	5.2
2	−4.5	4.7	−2.0	4.6
10	+3.8	2.5	−4.0	0.9
18	−2.2	4.7	−3.1	1.4
Between-run (n = 38)				
10	−0.2	4.4	−0.3	5.4

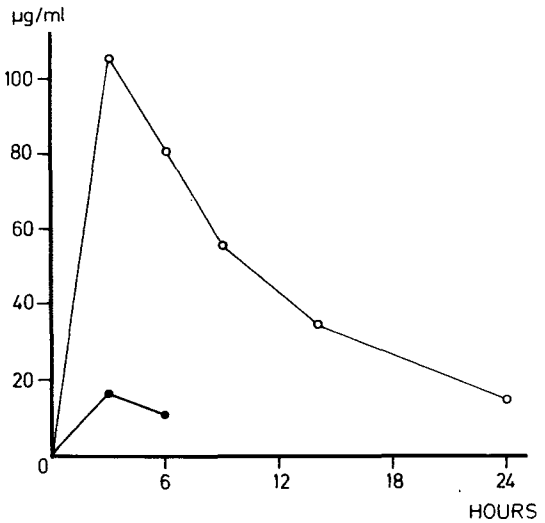

Fig. 3. Concentration—time curves of DEHP (•) and MEHP (○) in the plasma of an immature male rat after oral administration of 2.8 g/kg DEHP dissolved in corn oil (total volume: 5 ml/kg).

Preliminary toxicokinetic investigation

The small volume of sample required allows the study of plasma concentrations of DEHP and MEHP in rats after administration of DEHP. Fig. 3 shows plasma levels of DEHP and MEHP in a rat treated orally with 2.8 g/kg DEHP. A more detailed study of the plasma levels of DEHP and MEHP in rats will be published elsewhere [15].

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Note

Rapid gas—liquid chromatographic method for plasma verapamil level determination

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Verapamil is a newly marketed antiarrhythmic drug. It has a significant presystemic elimination when administered orally and, therefore, intravenous therapy is often recommended. Optimum therapeutic effect is obtained when verapamil plasma concentrations are between 100 and 400 ng/ml.

Several chromatographic procedures for the determination of verapamil in biological fluids have been described in the literature [1—11]. Some of the procedures have also quantitated verapamil metabolites [1, 6, 7]. The high-performance liquid chromatographic (HPLC), as well as the gas—liquid chromatographic (GLC) procedures are sensitive but require lengthy and tedious multiple extraction steps especially when the metabolites are quantitated.

This report describes a rapid, sensitive and accurate GLC procedure with thermionic specific detection for the determination of verapamil in dog plasma. This procedure also has a potential use in controlled clinical pharmacokinetic studies of this antiarrhythmic agent in humans.

MATERIALS AND METHODS

Reagents

Ethyl acetate, diethyl ether, pentane (Burdick & Jackson Labs., Muskegon,

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MI, U.S.A.), sodium hydroxide (Fisher Scientific, Fairlawn, NJ, U.S.A.), verapamil hydrochloride (Knoll Pharmaceuticals, Whippany, NJ, U.S.A.), and 1-(diethylaminoethyl)-2-(*p*-ethoxybenzyl)-5-nitrobenzimidazole·HCl, the internal standard, were used.

Stock solutions

Verapamil stock solution. Verapamil · HCl (10.0 mg) was dissolved in 100 ml methanol to yield a stock solution containing 100 μg/ml verapamil · HCl.

Internal standard stock solution. Internal standard (25.0 mg) was dissolved in 25 ml ethyl acetate to yield a stock solution containing 1.0 mg/ml internal standard.

Standards

Verapamil standard solution. The verapamil stock solution was diluted 1:5 to yield a 20 μg/ml verapamil · HCl standard solution.

Internal standard solution. The internal standard stock solution (50 μl) was diluted to 5 ml to yield a 10 μg/ml internal standard solution.

Quality-control samples

To a 25-ml volumetric flask, 750 μl of the verapamil standard solution (20 μg/ml) were added and the final volume adjusted to 25 ml by addition of blank plasma. After mixing, 1.25-ml aliquots were transferred to glass tubes, tightly capped and stored frozen at −20°C until use. Each quality-control sample contained 600 ng/ml verapamil. Of the sample 1 ml was used for analysis.

Chromatographic conditions

The gas chromatograph (Varian Model 3700) was equipped with a thermionic specific detector and a deactivated, silylated glass column (1.2 m × 2 mm I.D., 6 mm O.D.) packed with 1% OV-1 on 60—80 mesh Gas-Chrom Q. The carrier gas was pre-purified nitrogen set to a flow-rate of 30 ml/min. The air flow-rate to the detector was adjusted to 175 ml/min and the hydrogen flow-rate was set to 4.5 ml/min. The bead current and bias voltage settings were 470 and 4, respectively. The electrometer attenuation was set at $2—8 \cdot 10^{-11}$. The gas chromatograph operating conditions were: injector temperature, 280°C, column temperature, 250°C, and the detector temperature, 280°C. The column was conditioned at 270°C for 24 h.

Sample preparation

To glass tubes (15 ml capacity) were added 1 ml plasma, 50 μl internal standard solution, 50 μl sodium hydroxide (10 *M*) and 7.5 ml pentane—diethyl ether (70:30). The tubes were tightly capped and shaken for 15 min. The tubes were then centrifuged at 1000 *g* for 10 min (room temperature) and 6.0 ml of the organic phase (upper layer) were transferred to clean glass tubes and evaporated to dryness at 40°C with a stream of air. The residue was then reconstituted in 100 μl ethyl acetate and 2.0 μl were injected for GLC analysis.

RESULTS AND DISCUSSION

Typical chromatograms of a calibration standard (100 ng/ml verapamil), a pre-dose (blank) sample from an experimental subject (dog), and a plasma sample obtained from that subject after the intravenous (i.v.) administration of verapamil are shown in Fig. 1. Blank plasma did not have any interfering peaks as is evident from Fig. 1a. Retention times of verapamil and the internal standard were 1.7 and 2.5 min, respectively. The validity of the assay procedure was established through a study of linearity of response, reproducibility, accuracy and precision.

Standard calibration curves corresponding to verapamil concentrations of 0.0, 50.0, 100.0, 200.0, 400.0, 600.0, 800.0, and 1000.0 ng/ml of plasma were prepared by adding appropriate volumes of the verapamil standard solution to tubes containing 1.0 ml blank plasma. The standard calibration curve was prepared by plotting peak height ratios (verapamil:internal standard) as a function of the verapamil concentration. The calibration curve was found to be linear over the verapamil concentration range of 50—1000 ng/ml. The best-fit least-squares line was obtained by using a calibration curve program on the Apple II plus microcomputer. The data best fit a straight line. The correlation coefficients for inter-day standard calibration curves ranged from 0.9955 to 0.9989. The coefficients of variation (C.V., %) calculated from the inversely estimated concentrations for inter-day standard calibration curves ranged from 4.87% to 6.82%. The intra-day reproducibility of the calibration samples had C.V. values ranging from 1.11% to 9.31% as shown in Table I.

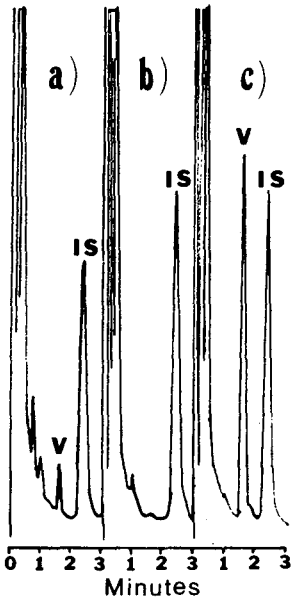

Fig. 1. Typical chromatograms representing (a) a calibration standard containing 100 ng/ml verapamil in plasma, (b) a pre-dose (blank) sample from the experimental subject (dog), and (c) a plasma sample obtained from the same subject following the i.v. infusion of verapamil (0.267 mg/kg/min for 3.0 min). Peaks: V, verapamil; IS, internal standard.

TABLE I

INTRA-DAY REPRODUCIBILITY OF THE ASSAY

Each peak height ratio was obtained from an independently prepared calibration sample; all samples were assayed on the same day.

Concentration (ng/ml)	Peak height ratio			Mean ± S.D.	Coefficient of variation (%)
50	0.147	0.176	0.170	0.1643 ± 0.0153	9.31
100	0.300	0.300	0.294	0.298 ± 0.003	1.11
200	0.500	0.609	0.579	0.582 ± 0.0247	4.24
400	1.154	1.224	1.25	1.209 ± 0.049	4.10
600	1.704	1.818	1.817	1.779 ± 0.065	3.68
800	2.385	2.481	2.387	2.417 ± 0.0548	2.26
1000	2.758	3.08	2.963	2.933 ± 0.163	5.5

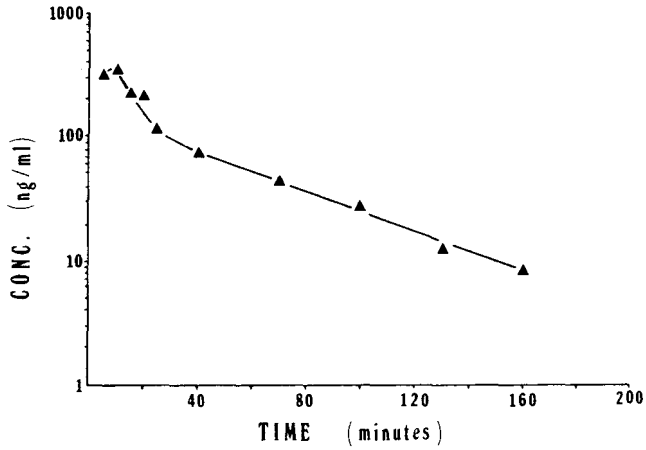

Fig. 2. The plasma concentration–time profile in a dog following a short i.v. infusion of verapamil (0.267 mg/kg/min for 3.0 min).

The accuracy of the method was assessed by analyzing quality-control samples on each assay day. The quality-control sample variability was found to have a C.V. of 9.26% and a mean concentration of 592.7 ng/ml with the theoretical concentration being 600 ng/ml.

Application of the method developed was demonstrated by measuring plasma levels of verapamil in dogs after a short i.v. infusion of verapamil. The plasma concentration–time profile of verapamil obtained in one such pharmacokinetic study is shown in Fig. 2. The post-infusion data are well described by the following polyexponential equation:

$$C = 244.0e^{-0.1583t} + 125.4e^{-0.0177t}$$

The distribution half-life ($t_{1/2\alpha}$) of verapamil was 4.4 min and the terminal half-life ($t_{1/2\beta}$) was 39 min.

Several analytical methods for verapamil determination require multiple extraction steps [5–7, 9, 10] and are, therefore, time-consuming. The HPLC

procedures described in the literature [5, 6, 8—10] require longer chromatographic separation time as compared to the GLC method reported here. Several HPLC methods allow the determination of verapamil and some of its metabolites. However, only norverapamil has been shown to possess some biological activity. Although this method has not been applied for the determination of verapamil levels in clinical studies involving humans, the calibration samples and assay validation studies utilized both human and dog blank (control) plasmas interchangeably, and no interfering peaks were seen. The authors will be using this procedure for the determination of verapamil levels in plasma samples from a controlled clinical trial. Also, the sensitivity, precision and accuracy of this procedure compares well with those reported in the literature.

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Note

Quantitative determination of cantharidin in biological materials using capillary gas chromatography with flame ionization detection

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Cantharidin (Fig. 1), a terpenoid produced by blister beetles (*Coleoptera, Meloidae*) for defense against predators [1], is a potent vesicant and poison. Painful skin blisters develop after people accidentally coat their skin with droplets of cantharidin-laden blood discharged reflexively by disturbed blister beetles [2]. This problem is common in warm regions where many blister beetles aggregate on crops and flock to lights at night [3]. Fatalities in humans resulting from the ingestion of cantharidin are rare now that various formulations, most notably the aphrodisiac "Spanish fly" made from powdered blister beetles, have become outmoded [4]. By contrast, cantharidin-poisoning in

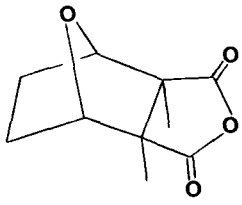

Fig. 1. Chemical structure of cantharidin.

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livestock, particularly horses, has grown recently because farm animals are increasingly being fed quickly baled leguminous hay contaminated with blister beetles that were trapped inadvertently while feeding on the foliage [5, 6].

Determination of cantharidin in biological materials is difficult. Recent high-performance liquid chromatographic (HPLC) techniques [7] can detect 1 ng of derivatized cantharidin, a sensitivity far better than blistering bioassays [8] and conventional gas chromatographic (GC) procedures [9]. But these HPLC methods are time-consuming; they require more than 20 h alone for preparative chromatography and derivatization after extraction of cantharidin. We here describe a quick capillary GC method that detects as little as picogram amounts of underivatized cantharidin in crude extracts of biological materials, including the tissues of blister beetles.

EXPERIMENTAL

Solvents, reagents and standards

Reagent-grade acetone was obtained from Eastman Kodak (Rochester, NY, U.S.A.). Spectro-grade methylene chloride and chloroform and reagent-grade hydrochloric acid were from Fisher (Fair Lawn, NJ, U.S.A.). Reagent-grade sodium bicarbonate and sodium sulfate were from Baker (Phillipsburg, NJ, U.S.A.) and from Merck (Rahway, NJ, U.S.A.). Standard cantharidin was purchased from Inland Alkaloid (St. Louis, MO, U.S.A.). Standard benzophenone was from Aldrich (Milwaukee, WI, U.S.A.).

Apparatus

The gas chromatograph was a Varian Model 3700 equipped with a split/splitless injector and a flame ionization detector. Helium was used as the carrier gas at a flow-rate of approximately 1 ml/min, with a make-up helium gas flow-rate of 30 ml/min. Hydrogen and air flow-rates were 30 and 300 ml/min, respectively. The range and attenuator settings corresponded to $6 \cdot 10^{-12}$ A for full scale deflection. The column oven temperature was programmed from 100°C upon injection to 270°C at 20°/min. The injector and the detector were at 190°C and 320°C, respectively. The splitter (ratio 100:1) was turned on 0.5 min after injection to purge the inlet of solvent. A Hewlett-Packard Model 3390A integrator was used to quantitate the chromatograms. Samples (0.4 μl each) were injected using the splitless mode with a 1-μl syringe.

The fused-silica capillary column (27 m × 0.329 mm I.D.) was coated with 0.25-μm DB-5 (J. & W. Scientific, Rancho Cordova, CA, U.S.A.).

Extraction procedure

Cantharidin was extracted from animal tissues using a modified version of a published technique [1]. Wet biological samples ($\leqslant$ 1 g) were cut into small pieces or, in the case of liquids, were absorbed into cotton swabs or filter paper, then they were placed individually into microsoxhlet thimbles (Whatman Catalogue no. 2800-105) which had been pre-extracted for three days with acetone.

Each sample, contained in a Soxhlet thimble inside a glass test tube capped with a condenser, was hydrolyzed by treatment with three to four drops of

concentrated (12 *M*) hydrochloric acid and 1—2 ml of acetone for 4 h at 120° C. After hydrolysis, the thimble and the liquid remaining in the test tube were transferred to a microsoxhlet extractor (Corning Glass Works, Corning, NY, U.S.A.). The hyrolysis tube was rinsed four times with methylene chloride. The rinsings and additional methylene chloride were added to the extractor to bring the liquid to approximately 15 ml. The solvent was refluxed through the extraction apparatus for 12—15 h, and after cooling to room temperature, the solution in the extraction flask was concentrated to approximately 0.2 ml using a stream of nitrogen. The concentrate, neutralized and dried by passing it through a column packed with 0.8 g of anhydrous sodium bicarbonate layered on top of 0.8 g of anhydrous sodium sulfate, was collected in a 1-dram screw-capped vial. The extraction flask was rinsed four times with methylene chloride; each rinse was passed in sequence through the packed pipette into the vial. The pooled organic solution in the vial was concentrated to approximately 0.2 ml using a stream of nitrogen. Then the vial was capped with aluminum foil and stored at —20° C until just before analysis of its contents.

Analytical procedure

For GC analysis each extract was diluted to approximately 0.3 ml with chloroform. As an internal standard, 19.53 μg of benzophenone dissolved in 0.1 ml of chloroform were added to the diluted extract. After mixing, 0.4 μl of the benzophenone-containing chloroform solution of the extract was injected into the gas chromatograph.

Peak area ratios were calculated by dividing the area of each cantharidin peak by the area of the corresponding internal standard peak. A calibration curve was constructed by plotting peak area ratio as a function of the known cantharidin-to-benzophenone ratio in standard solutions containing pure cantharidin. This calibration curve was used subsequently to calculate unknown amounts of cantharidin in each extract of biological materials.

RESULTS AND DISCUSSION

Use of benzophenone as an internal standard in the quantitation of cantharidin is largely responsible for the success of the analytical method. An internal standard in general obviates the need to make highly accurate sub-microliter injections into the GC. The choice of benzophenone was made after attempts to use three compounds having closer structural resemblance to cantharidin, namely benzocantharidin [10], 3,6-endoxo-1,2,3,6-tetrahydrophthalic anhydride, and 1,2,3,6-tetrahydrophthalic anhydride, were unsuccessful. The latter compounds proved to have unsatisfactory GC behavior. Benzophenone is ideal in its GC behavior relative to cantharidin: the two compounds display similar retention times (5.74 and 5.20 min, respectively) but are completely resolved.

Fig. 2 illustrates the separation of cantharidin from blood of the margined blister beetle, *Epicauta pestifera*, along with the internal standard. Typical gas chromatograms obtained with the method using other tissues from this insect species are also shown in Fig. 2.

The calibration curve, constructed by adding known amounts of the

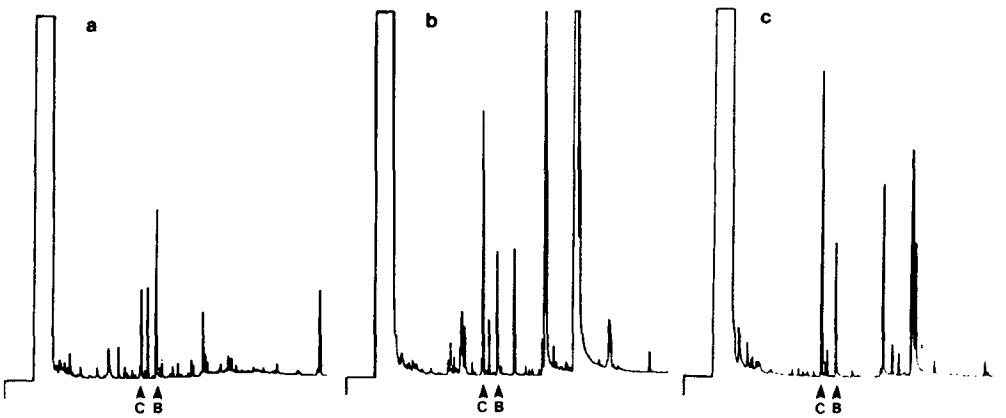

Fig. 2. Gas chromatograms showing the quantitation of cantharidin (C) in extracts of the margined blister beetle using benzophenone (B) as an internal standard. Peaks of interest are indicated by arrows. Retention times are 5.20 min for C and 5.74 min for B, using a 27-m 0.25-μm DB-5 column with an oven temperature increasing from 100°C to 270°C at 20°/min. Figures show injections containing 33.2 ng of C (a), 149 ng of C (b), and 140 ng of C (c), all with 40.7 ng of B added, in extracts of blood droplets discharged by a male beetle (a), somatic tissues of a female (b), and the testes of a male beetle (c).

cantharidin standard and a constant amount of the internal standard, was shown to be linear in the range 4 ng to 2 μg of cantharidin for a single injection. Under the best conditions, the maximal sensitivity of the analytical method for a single injection was found to be 30 pg of cantharidin. Under typical conditions in a biological matrix, we find it useful to determine as little as 500 pg of cantharidin.

Measurements made for standards and for various insect tissues, including mealworms (larval *Tenbrio molitor* that lack cantharidin) and female blister beetles [adult *Epicauta pestifera* which have at most a small amount (35 μg) of cantharidin], spiked with 1—300 μg of cantharidin showed that the accuracy of the technique is greater than 95% and that its precision is within 2—3%. Overall recovery of cantharidin from spiked and unspiked samples, which was determined by submitting them to hydrolysis and extraction a second and third time, is approximately 99%.

We have experienced little long-term variability using this method to analyze cantharidin in a variety of biological matrices. In other work to be reported elsewhere in detail, we have found the intra-assay error to range from 1 to 7% across all animal samples examined, including tissues and excrement from vertebrates poisoned by cantharidin.

The analytical method is relatively quick. Neither preparative chromatography nor derivatization is required before sub-nanogram amounts of cantharidin can be detected. An analysis of cantharidin in a sample can be completed in one day if the extraction is performed overnight.

This analytical method is being applied to a number of projects, including the biosynthesis of cantharidin in blister beetles and the uptake and systemic distribution of the substance in animals that have eaten blister beetles.

ACKNOWLEDGEMENTS

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Note

Colchicine quantitation by high-performance liquid chromatography in human plasma and urine

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The clinical features of colchicine poisoning have been well documented over the past 40 years [1—4]. Most reported cases were associated with the

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ingestion of doses of colchicine of 50 mg or more. Other reports have described severe toxic side-effects and some fatalities following doses of 5—15 mg.

Colchicine is given under medical supervision for the treatment of malignancy or gout [5—7]. In a few instances the toxicity was attributed retrospectively to poor renal function, but in others the possibility of individual sensitivity to colchicine has been raised [5].

Despite the awareness of the low therapeutic index of colchicine, little has been done, until recently, to study its pharmacokinetics, largely due to the lack of a suitable method for the estimation of colchicine in body fluids.

Colchicine has been determined using colorimetric methods [8, 9], which were insufficiently sensitive for use in humans taking therapeutic doses. Bourdon and Galliot [10] developed a fluorimetric method, and colchicine has also been determined in body fluids by gallium chelate formation [11]. The pharmacokinetic parameters of colchicine, the half-life and the volume of distribution, have been determined in human volunteers by a radioisotope dilution technique [12] and later by radioimmunoassay [13—15].

More recently, Jarvie et al. [16] and Caplan et al. [17] have described a high-performance liquid chromatographic (HPLC) method for the estimation of colchicine in a poisoned patient.

A method that is sufficiently rapid and accurate at the relatively high levels found in cases of colchicine overdose has been developed in our laboratory, in order to provide some prognostic guidance to the clinicians with such patients. This method could assist in gaining an appreciation of the therapy used after an intoxication. Preliminary results obtained from a single case are reported.

EXPERIMENTAL

Chemicals and reagents

Colchicine was a gift from Laboratories Houde I.S.H., (Paris, France) and used directly. Morpholinopropylcolchicamide was used as internal standard

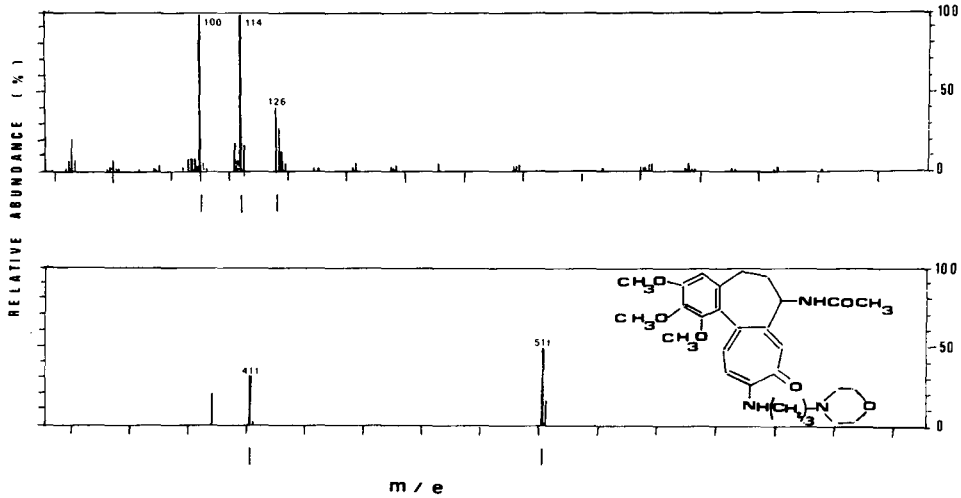

Fig. 1. Electron-impact mass spectrum of internal standard (morpholinopropylcolchicamide).

(Fig. 1). It was synthesized by refluxing 1 g of colchicine (2.5 m*M*) with 0.36 g of N-(3-aminopropyl)morpholine (2.5 m*M*) in 50 ml of pyridine for 4 h. The solution was concentrated to 20 ml in vacuo and cooled to give the expected amide, which can be crystallized from ethanol: 1.1 g (86%); m.p.: 145°C; M^+ (electron-impact) = 511; IR: 1670 cm^{-1} ($\nu_{C=O}$ amide), 2800–2820 cm^{-1} (ν_{CH_2} morpholinoalkyl). Acetonitrile was HPLC grade (Carlo Erba, Italy); all other chemicals and solvents were reagent grade. Distilled water was purified by passing it through a reverse-osmosis four-filter system (Millipore, Bedford, MA, U.S.A.).

Mass spectrometric analysis

The structure of the internal standard was assessed using a mass spectrometer–computer system (Riber-mag) with a 70-eV electron-impact ionization source.

Chromatographic conditions and instrumentation

A Varian (Palo Alto, CA, U.S.A.) Model 5000 liquid chromatograph equipped with a Valco sample injection valve and UV variable-wavelength detector (Varichrom) was used for the analysis. Chromatography was performed on a 300 × 4.0 mm I.D. stainless-steel Micropak MCH 10-μm column (Varian), with a precolumn (40 × 4 mm I.D.) that contained the same phase. The mobile phase was actonitrile–water (50:50) at a flow-rate of 2.0 ml/min and a pressure of ca. 140 bar. The separation was performed at ambient temperature. Colchicine and internal standard were detected at a wavelength of 245 nm.

Standard solutions

Standard stock solutions of colchicine and internal standard (morpholinopropylcolchicamide) were prepared in methanol at a concentration of 100 μg/ml. These were refrigerated at 4°C and found to be stable for several weeks in the dark.

The internal standard stock solution (100 μg/ml) was diluted 1:100 with methanol, and 100 μl of this solution were used for internal standardization (100 ng).

Plasma standards (calibration standards) were prepared at concentrations of 5, 10, 20, 25, 50, 75 and 100 ng/ml. The 100 ng/ml standard was prepared by adding 50 μl of the colchicine stock solution (100 μg/ml) to 50 ml of drug-free human plasma. The other standards were then prepared by stepwise dilutions with drug-free plasma. These calibration standards were stored deep-frozen (−20°C) in small portions until needed for analysis.

Urine standards (calibration standards) were prepared at concentrations of 0.25, 0.5, 1, 2.5, 3.75 and 5 ng/ml. The 5 ng/ml standard was prepared by adding 50 μl of the colchicine stock solution (100 μg/ml) to 50 ml of drug-free human urine to yield a concentration of 100 ng/ml. This solution was diluted 1:20 with drug-free urine to yield a concentration of 5 ng/ml. The other standards were prepared by stepwise dilutions with drug-free urine. Calibration standards are lower in urine than in plasma, because the extraction procedure is carried out with 20 ml of urine. The urine calibration standards were stored deep-frozen (−20°C) in small portions until needed for analysis.

Plasma extraction procedure

Calibration curve. To 1.0 ml of each solution of plasma standard in a 50-ml centrifuge tube containing 100 ng of internal standard (morpholinopropylcolchicamide) were added 1.0 ml of 8 *M* ammonium hydroxide and 15 ml of dichloromethane. The tube was mechanically shaken for 10 min and then centrifuged at 850 *g* for 5 min. The aqueous phase (upper) was transferred to another tube and re-extracted in a similar manner. The two organic phases were mixed and ethanol (10.0 ml) was added. The tube was vortexed for 5 min and then centrifuged at 850 *g* for 5 min. The supernatant was evaporated to dryness under nitrogen at 50°C. The residue was redissolved in 100 μl of mobile phase (acetonitrile—water, 50:50), and 50 μl were injected into the liquid chromatograph.

Samples. A suitable volume of plasma (up to 1 ml) was combined with 100 μl of internal standard (100 ng of morpholinopropylcolchicamide), 1 ml of 8 *M* ammonium hydroxide and 15 ml of dichloromethane. This mixture was further treated as described for the calibration curve.

Urine extraction procedure

Calibration curve. To 20 ml of each solution of urine standard in a 50-ml centrifuge tube containing 100 ng of internal standard were added 5 ml of 8 *M* ammonium hydroxide and 15 ml of dichloromethane, and extraction proceeded as described for plasma. The upper aqueous phase was re-extracted twice with 15 ml of dichloromethane. The organic phases were transferred to a tube containing anhydrous sodium sulphate and filtered through Whatman 1 paper into a clean tube. The solvent was evaporated to dryness under nitrogen at 50°C. The residue was redissolved in 1 ml of distilled water and passed through a Sep-Pak C_{18} cartridge (Waters). The cartridge was eluted with 5 ml of acetonitrile—water (50:50). The solution was evaporated to dryness under nitrogen at 50°C. The final residue was dissolved in 100 μl of mobile phase (acetonitrile—water, 50:50), and 50 μl were injected into the liquid chromatograph.

Samples. Urine samples of 20 ml were further treated as described for the calibration curve.

Quantitation

Calibration standards covering the anticipated concentration range (5—100 ng/ml) in methanol, plasma and urine were processed. Peak area ratios of colchicine to the internal standard were measured, and the calibration was obtained from linear regression of the peak area ratio against concentration. This line was then used to calculate the concentration of the drug in the unknown samples.

Recovery

Extracts from urine and from plasma, prepared as described above, were compared with a direct assay of standards in methanolic solution. These relative recoveries were determined for two different concentrations. The absolute recoveries were also determined for these two different concentrations from extracts of urine and plasma, treated using the procedure described

above, except that the internal standard was omitted. All extraction sample residues were reconstituted in 100 μl of the solution of internal standard (1 μg/ml) in mobile phase (acetonitrile—water, 50:50). In this recovery analysis, morpholinopropylcolchicamide served as external standard.

Interference

Interferences from endogenous material and from drugs commonly used in therapeutic treatment or often found in poisoned patients were researched (Table I). Drugs were tested at concentrations of 500 ng/ml.

TABLE I

DRUGS TESTED FOR POSSIBLE INTERFERENCE IN THE HPLC ASSAY OF COLCHICINE

Vinbarbital	Meprobamate
Amobarbital	Prazepam
Secobarbital	Chlordiazepoxide
Phenobarbital	Chlorazepam
Barbital	Medazepam
Butalbital	Diazepam
Butobarbital	Lorazepam
Thiopental	Clonazepam
Caffeine	Nitrazepam
Theophylline	Quinidine

Human toxicokinetic studies

The procedure was used to analyse the in vivo disposition of colchicine in a poisoned patient, found after absorption of 31 mg of drug. Blood was collected frequently into heparinized tubes over a period of 12 h after the admission. The plasma was separated, frozen and stored in the dark at −20°C until analysed. During the period of admission, 300 ml of urine were collected.

RESULTS AND DISCUSSION

Fig. 2 shows the separation and quantitation of colchicine in human plasma and urine using morpholinopropylcolchicamide as internal standard. In the chromatograms which were obtained after extraction of 1.0 ml of blank plasma or 20 ml of blank urine, no additional peaks that could interfere with the determination of colchicine and internal standard are present. Fig. 2A represents a chromatogram of a blank plasma. Fig. 2B is a chromatogram obtained after extraction of 1.0 ml plasma containing 5 ng/ml colchicine. Fig. 2C is a chromatogram obtained after extraction of 20.0 ml of urine containing 0.5 ng/ml colchicine. Drug and internal standard are well separated with retention times of 3.5 and 4.7 min, respectively.

The introduction of a N-(3-aminopropyl)morpholine chain makes the molecule more hydrophilic and thus modifies its retention time in the column with an acetonitrile—water mobile phase. Nevertheless, the polycyclic moiety of colchicine remains unchanged, involving only a slight difference of separation which permits a good comparison between the peaks of colchicine and its internal standard.

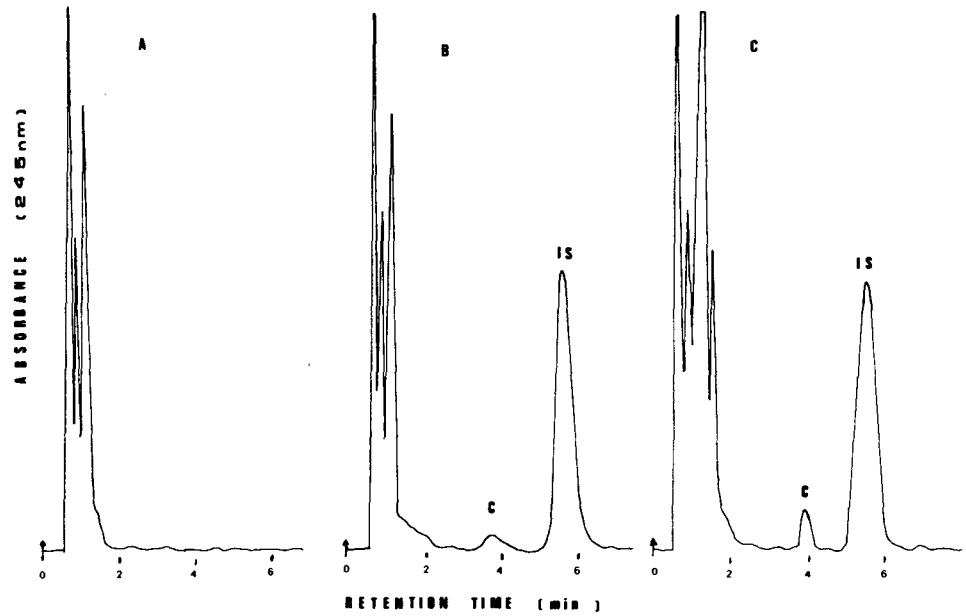

Fig. 2. HPLC profiles of (A) extracted serum blank, (B) human plasma (1 ml) containing 5 ng/ml colchicine and 100 ng/ml internal standard, (C) human urine (20 ml) containing 0.5 ng/ml colchicine and 100 ng/20 ml internal standard. Peaks: C = colchicine; IS = internal standard.

The calibration curves were obtained using methanolic solution of standards, human plasma or human urine spiked with 5—100 ng/ml colchicine and 100 ng/ml internal standard. There was a good correlation between the amount of colchicine added to the human plasma and urine and the amount detected in the samples of both 1.0 ml of plasma and 20.0 ml of urine. The linear regression equations of data are shown in Table II. Calibration curves in plasma and urine showed good linearity between peak area ratios and concentrations from 5 to 100 ng/ml, and the present method is able to detect 5 ng/ml colchicine. The intra- and inter-assay precision data for colchicine in both plasma and urine are summarized in Table III. There was little variation in colchicine determination with coefficients of variation below 9%.

Analytical relative and absolute recoveries of colchicine both in plasma and urine were determined at two concentrations (10 and 50 ng/ml) and are reported in Table IV. For absolute recovery, morpholinopropylcolchicamide was used as external standard.

TABLE II

LINEAR REGRESSION EQUATIONS FOR COLCHICINE

Methanolic solution	$y = 0.0190x - 0.0153$	($r = 0.9937$)
Plasma extraction	$y = 0.0176x - 0.0035$	($r = 0.9984$)
Urine extraction	$y = 0.0193x + 0.0041$	($r = 0.9985$)

y = peak area ratio colchicine to internal standard; x = colchicine concentration.

TABLE III

INTRA- AND INTER-ASSAY COEFFICIENT OF VARIATION FOR COLCHICINE

Sample	Concentration (ng/ml)	Within-run C.V. (%) (n^* = 15)	Day-to-day C.V. (%) (n = 16)
Plasma	10	8.73	7.70
	50	2.09	1.80
Urine	10	4.34	6.00
	50	4.30	6.00

*n = Number of determinations.

TABLE IV

RELATIVE AND ABSOLUTE RECOVERIES OF COLCHICINE

Sample	Concentration (ng/ml)	Relative recovery (%)	Absolute recovery (%)
Plasma	10	94 ± 4	78 ± 2
	50	98 ± 3	90 ± 1
Urine	10	94 ± 8	86 ± 3
	50	98 ± 3	90 ± 2

The assay was shown to be selective, without interference from endogenous material and from other drugs commonly used in therapeutic treatment or often found in poisoned patients (except for quinidine, which eluted at the same retention time as colchicine) (Table I).

The HPLC procedure described herein has been used for the assay of human plasma samples obtained from one poisoned subject. After ingestion of 31 mg of colchicine, the colchicine plasma levels were 720 ng/ml at 20 min, 212 ng/ml at 125 min, 132 ng/ml at 305 min and 120 ng/ml at 605 min. In these assays, only 100 μl of plasma sample were used. The level of colchicine found in the urine of the same patient was 5 μg/l.

In summary, this HPLC assay shows good reproducibility, sensitivity and selectivity. It has the advantage of being a relatively convenient, rapid and simple method. It was developed in response to a clinical problem and can provide an indication of the plasma colchicine concentration in as little as 10 min and an accurate answer within 1 h, once standards have been processed. This method was applied to the analysis of samples from poisoned patients and will be easily applicable to toxicokinetic studies in humans.

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Note

High-performance liquid chromatographic assay for methotrexate utilizing a cold acetonitrile purification and separation of plasma or cerebrospinal fluid

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Methotrexate (MTX, 4-amino-N^{10}-methylpteroglutamic acid) has been used in the treatment of acute lymphocytic leukemia (ALL) either alone or in combination with other antineoplastic agents for over thirty years [1]. MTX is not only a potent cytotoxic agent that attacks leukemic cells but also non-selectively causes toxic damage to normal tissue, especially the epithelial tissues of the gut, liver and kidney [2—4]. MTX has been shown to be involved in or be the toxic cause of myelosuppression, gastrointestinal mucositis, hepatitis, acute desquamative dermatitis, and renal dysfunction [5—7]. The occurrence and severity of toxicity is dependent on the MTX concentration and duration of exposure. Since both of these are important determinants of toxicity, monitoring of plasma concentration is essential to ensure that drug levels are reduced within an appropriate period of time to minimize toxic effects.

While there are several analytical methods currently employed to assay plasma MTX, including fluorometry [8], high-performance liquid chromatography (HPLC), ultraviolet techniques [9—12], radioimmunoassays (RIA) [13], enzyme inhibition assays (EIA) [14], and enzyme-multiplied immunoassays (EMIT) [15], these methodologies possess inherent difficulties that limit their applicability. Immunochemical methods lack specificity since metabolites, such as 7-hydroxymethotrexate (7-OHMTX) and 2,4-diamino-N^{10}-methylpteroic acid (DAMPA) can cross react with the antibody to produce spuriously high results [16]. Enzyme inhibition techniques are susceptible to interference by commonly used antibiotics such as trimethoprim [17]. The EMIT assay provides limited sensitivity. HPLC techniques utilizing either UV or

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fluorescence detection methods offer a means whereby both specificity and sensitivity can be simultaneously achieved.

Sample preparation techniques for the HPLC measurement of MTX in serum or plasma fall into two distinct categories. The first utilizes pre-column clean-up of a plasma or serum sample, does not require the precipitation of plasma or serum proteins and, therefore, serum or plasma may be directly injected. These non-extraction techniques have a rapid total analysis time (10—20 min) [18] but suffer from a decrease in column efficiency, the cost of replacement of pre-columns, lengthy washings of pre-columns, and variable recovery of the drug (80—110%) [19].

In the second category of preparation techniques, the drug is extracted in a more purified and concentrated state before analysis by HPLC. These extraction techniques also suffer from several serious disadvantages such as time required for extraction (up to 1 h), chromatography time (10—40 min) [20], low recoveries (40—50% [9] and detection limits generally greater than 50 ng/ml [18]. Most, if not all, extraction techniques utilize a protein precipitant (acid or acetonitrile) followed by a salt-saturated extraction into a volatile organic solvent such as ethyl acetate or diethyl ether. The technique described in this report eliminates the need for salt solvent extraction, yet allows for the purification and concentration of the drug by a cold separation of acetonitrile from serum, plasma, or cerebrospinal fluid (CSF). This allows for superior recovery with increased sensitivity while utilizing a small sample size, thus making it ideally suited for analysis of pediatric samples.

EXPERIMENTAL

Methotrexate was provided by the National Cancer Institute (Washington, DC, U.S.A.) as manufactured by Lederle Labs. (American Cyanamid, Pearl River, NY, U.S.A.). Monobasic sodium phosphate was purchased from Fisher (Fairlawn, NJ, U.S.A.). Tris(hydroxymethyl)aminomethane base, phosphoric acid, HPLC-grade methanol and acetonitrile were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). *p*-Aminoacetophenone (PAAP) was purchased from Eastman-Kodak (Rochester, NY, U.S.A.). Water was deionized and doubly distilled for HPLC use. All other reagents, equipment, and glassware were of standard laboratory quality.

The mobile phase consisted of 0.1 *M* monobasic sodium phosphate and 10 m*M* Tris phosphate (pH to 5.75 by the addition of a few drops of concentrated phosphoric acid), methanol, and acetonitrile (82:13:5). After filtration, the mobile phase was degassed under reduced pressure with continual sonication.

The chromatographic system consisted of a M-45 solvent delivery system, a Model 480 UV/VIS variable-wavelength detector, a Z module radial compression unit, and a 5-μm reversed-phase C_{18} Radial Pak column (all from Waters Assoc., Milford, MA, U.S.A.). The injector was a Reodyne Model 7125 (Berkeley, CA, U.S.A.) equipped with a 100-μl loop. A flow-rate of 2.3 ml/min at a pressure of 7 mPa (1000 p.s.i.) was used for all chromatography. Chromatography was carried out at ambient temperature. The column eluent was monitored for UV absorbance at 313 nm. Detector output was recorded and integrated by a Perkin-Elmer Model 15 (Perkin-Elmer, Norwalk, CT, U.S.A.) data station.

Stock solutions of internal standard (PAAP) and MTX were prepared in water at concentrations of $2.5 \cdot 10^{-4}$ *M* and $2.5 \cdot 10^{-5}$ *M*, respectively. MTX analytical and quality-control standards were prepared over a concentration range of $5 \cdot 10^{-8}$ *M* to $1 \cdot 10^{-5}$ *M* by adding 20 μl of an appropriate dilution of stock solution to 460 μl freshly pooled drug-free plasma obtained from healthy volunteers. Analytical standards for CSF assays were similarly prepared in Elliotts B solution.

Patient blood, 1 ml, was collected in tubes containing ethylenediaminetetraacetic acid (EDTA) as the anticoagulant. Plasma was transferred to tubes covered with aluminum foil and stored on ice or refrigerated until assayed. A 20-μl aliquot of internal standard stock solution was added to 480 μl plasma or CSF to yield a final concentration of 1352 ng/ml. Each tube was vortexed for 5 sec. Protein was precipitated by the slow dropwise addition of acetonitrile during vortexing. The supernatant and precipitate were transferred into a 1.5-ml Eppendorf microfuge tube with a wide-bore transfer pipet and centrifuged at room temperature for 5 min at 13 000 *g*. The supernatant was removed, placed into a new 75 × 12 mm disposable culture tube that was precooled on ice for 5 min, and kept on ice for at least an additional 10 min. The tubes were then centrifuged at 2175 *g* at 0°C for 5 min, after which they were returned to ice for an additional 5 min. The top layer of the acetonitrile supernatant was aspirated with a Pasteur pipette and discarded. Of the lower aqueous layer 360 μl were removed and transferred to a clean 75 × 10 mm disposable culture tube and taken to dryness at 50°C under nitrogen. The dried material was reconstituted in 100 μl of mobile phase and approximately 40 μl were injected onto the column.

In order to assess the linearity, precision, and accuracy of this procedure, standard curves were constructed in plasma or serum over a concentration range of $1 \cdot 10^{-5}$ *M* (4540 ng/ml) to $5 \cdot 10^{-8}$ *M* (22 ng/ml). Precision was determined with a medium range concentration of $1 \cdot 10^{-6}$ *M* (454 ng/ml) for both intra- and inter-assay variation. Accuracy was assessed by the daily determination of a plasma control known to contain $1 \cdot 10^{-6}$ *M* (454 ng/ml) MTX over a period of ten days.

RESULTS AND DISCUSSION

At ambient temperature acetonitrile is water miscible and separation of the organic and aqueous phases does not occur. However, at 0°C phase separation occurs allowing differential separation of MTX from interfering plasma constituents. This phenomenon provides the basis for the purification and concentration procedure used in this method.

Fig. 1 demonstrates typical chromatograms obtained from a plasma sample containing MTX at $1 \cdot 10^{-6}$ *M* (454 ng/ml) after the separation of the two phases. The upper acetonitrile layer (Fig. 1A) of the two-phase system is devoid of MTX (retention time = 5.00 min) while MTX remains exclusively in the lower aqueous phase (Fig. 1B).

While it can be seen that the internal standard separates between the two phases, this separation is reproducible and consistent so that a constant amount of the internal standard is always present in the phase containing MTX. The

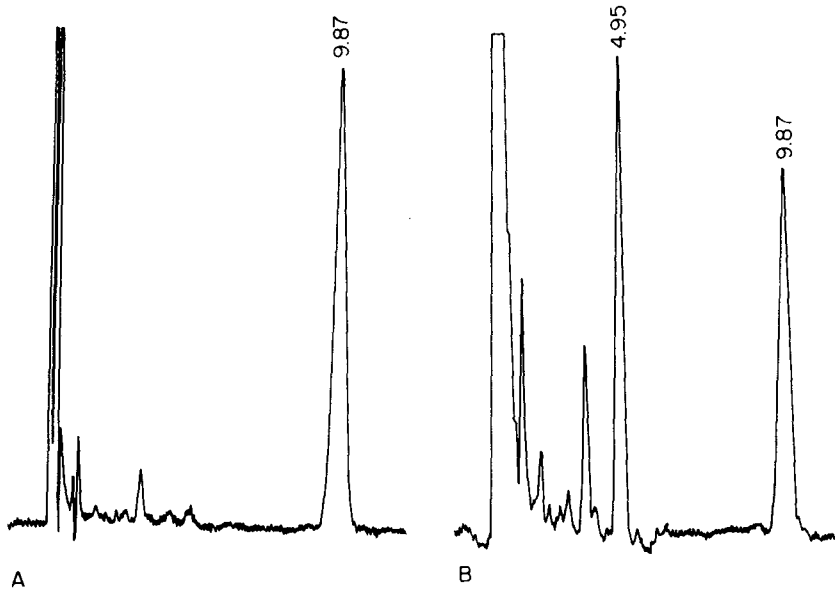

Fig. 1. Chromatograms of a control plasma sample. Methotrexate (MTX) was present at $1 \cdot 10^{-6}$ *M* (454 ng/ml). (A) Acetonitrile upper phase devoid of MTX with the internal standard, *p*-aminoacetophenone (PAAP), eluting at approximately 10 min; (B) aqueous lower phase with MTX eluting at approximately 5.00 min and PAAP at 10 min. Chromatographic conditions are detailed in the text.

TABLE I

REPRODUCIBILITY DATA FOR *p*-AMINOACETOPHENONE, INTERNAL STANDARD, IN AQUEOUS PHASE

Values represent the mean actual area counts for the internal standard peaks using day-to-day comparisons.

Integrator type	Group*	Mean ± S.D.	*n*	Coefficient of variation (%)
Waters Model 700	A	378874.3 ± 15314.7	28	4.10
	B	389392.1 ± 15603.2	14	4.01
(8-84)**	C	369593.9	50	4.20
Perkin-Elmer Model				
Sigma 15 data system	A	1.453 ± 0.0750	29	5.20
	B	1.4825 ± 0.1298	24	8.73
(5-84)**	C	1.3948 ± 0.0902	79	6.50

*Groups are designated as follows: A, calibration standards; B, quality controls, C, unknown patient samples.
**Month assayed.

actual area counts of the internal standard peaks of calibration standards, quality-control, and patient plasma samples from two different integrators during two different months are shown in Table I. It is apparent that the concentration of internal standard in the aqueous phase which contains MTX

is extremely reproducible and stable. Coefficients of variation (C.V.) on day-to-day assays were 4—6% throughout both months irrespective of the type of integrator used or the type of sample assayed (calibration standard, quality control, or patient sample).

Since the area counts directly reflect the concentration of a sample, these results indicate that although the internal standard does not fully partition into the aqueous phase, its concentration in that phase is constant.

The recovery of MTX was determined to be 96%. MTX had a retention time of 5.00 min, while that of the internal standard, PAAP, was approximately 10 min. A standard curve prepared in normal plasma was linear (linear regression correlation coefficient $r = 0.99$) between $1 \cdot 10^{-5}$ M (4450 ng/ml) and $5 \cdot 10^{-8}$ M (22 ng/ml). The linear dynamic range was $2 \cdot 10^{2}$. Within-assay precision was excellent (C.V. = 1.73%, $n = 6$) at a concentration of $1 \cdot 10^{-6}$ M (454 ng/ml) as was day-to-day precision for the same concentration (C.V. = 3.80%, $n = 10$) (Table II). The sensitivity limit was found to be $4.40 \cdot 10^{-8}$ M (20 ng/ml). This concentration yielded a response with a signal-to-noise ratio of at least 2.

TABLE II

REPRODUCIBILITY DATA FOR METHOTREXATE IN HUMAN PLASMA

Values are based on a midrange concentration of 454 ng/ml. Approximately 40 μl were injected at a sensitivity setting of 0.005 a.u.f.s.

Group	Mean ± S.D.	n	Coefficient of variation (%)
Within-day	484.9 ± 8.42	6	1.73
Between-day	437.1 ± 16.70	10	3.80

A comparison of the accuracy of this technique with other commonly used assays was assessed from College of American Pathologists (CAP) Therapeutic Drug Monitoring quality-control surveys obtained during the month of April, 1984. Accuracy was assessed at two target MTX concentrations, a low value of $6 \cdot 10^{-6}$ M and a high value of $1 \cdot 10^{-5}$ M. This method resulted in a relative error of —8.6 for the low concentration and +5.6% for the high concentration based on a comparison of mean assay values to the target value of the unknown samples. In contrast, mean relative errors for the assays currently in common clinical use (RIA, EIA, EMIT) were —15.4% and —13.26% for the low and high concentrations, respectively.

Fig. 2A demonstrates a control plasma sample at a MTX concentration of $1 \cdot 10^{-6}$ M (454 ng/ml) with the internal standard present. A chromatogram of plasma from a child with ALL is presented in Fig. 2B. Before drug administration, there is a stable baseline with no interfering peaks at the retention time of MTX. Fig. 2C illustrates a typical chromatogram for an ALL patient receiving MTX. MTX appears at its characteristic retention time along with a later eluting peak with a retention time of about 6.00 min which represents the

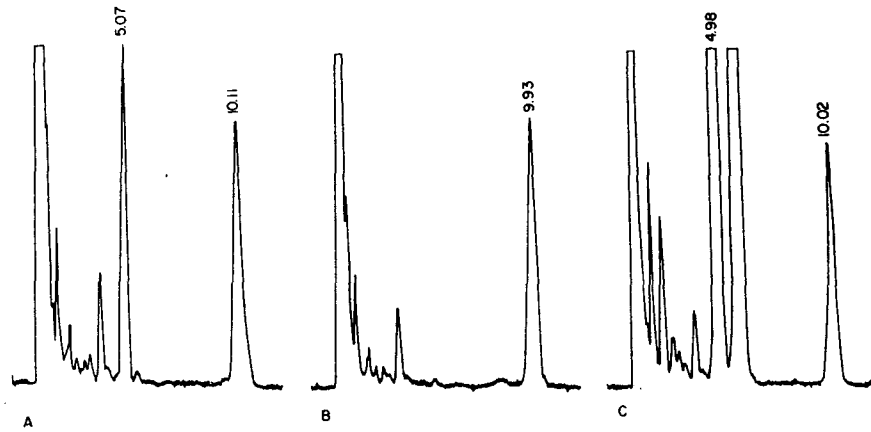

Fig. 2. Chromatograms of (A) a control plasma containing methotrexate (MTX) at $1 \cdot 10^{-6}$ *M* (454 ng/ml); (B) blank plasma of a typical pediatric acute lymphocytic leukemia patient before drug administration; (C) same patient as in (B) after 12 h of methotrexate infusion. Internal standard (PAAP) was present in all three cases.

7-OH metabolite of MTX. Hemolysis was not found to interfere. The assay may be used equally well with plasma, serum, and CSF samples.

An artifical CSF vehicle such as Elliott's B solution must be used to prepare standards and controls for the measurement of MTX in CSF. The acetonitrile and aqueous phases do not separate during the extraction procedure when water or serum albumin dissolved in water is used. Therefore, it appears that inorganic salts are essential for separation of the two phases.

Commonly used antineoplastic agents and adjuvants, such as cytosine arabinoside, hydrocortisone, cyclophosphamide, vinblastine, antibiotics, citrovorum factor, and adriamycin, do not interfere with the chromatography of MTX or PAAP in this system.

The method described here employs a simple cold acetonitrile separation of interfering plasma constituents from MTX, is highly sensitive, and relatively quick when compared to other extraction methods. It has several additional advantages over existing techniques. It allows for isocratic separation with a relatively short overall chromatography time (11 min), the injection volume is low (40 μl or less), sensitivity is high ($4.4 \cdot 10^{-8}$ *M*; 20 ng/ml), and small sample volumes (as little as 240 μl) are required. Recovery by this process is nearly complete, i.e. 96%. Dilutions for higher concentrations (10^{-4} or 10^{-5} *M*) are not required as in RIA or non-isotope immunoassays. Finally, the precision and accuracy is better than that of many of the previously existing techniques because of the use of an internal standard.

At present, clinical monitoring of serum MTX is essential to ensure that serious toxicity does not occur. This assay is applicable to both routine clinical and basic research work. Its sensitivity is well within the values required for clinical monitoring. MTX serum levels at 48 h of less than $9 \cdot 10^{-7}$ *M* and 72-h values of less than $1 \cdot 10^{-7}$ *M* have been found to correlate well with a lack of serious clinical toxicity [21—27]. While not as sensitive as the dihydrofolate reductase enzyme binding assay [28], this assay provides superior sensitivity to other published HPLC assays and offers greater specificity than RIA or other immunoassays currently available.

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Note

Determination of N-5-dimethyl-9-[(2-methoxy-4-methylsulfonylamino)phenylamino]-4-acridinecarboxamide in plasma by high-performance liquid chromatography

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(First received October 31st, 1984; revised manuscript received March 14th, 1985)

We have previously reported a high-performance liquid chromatographic (HPLC) method for the determination of the novel anticancer drug amsacrine, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide in plasma [1], which is suitable for pharmacokinetic studies in rabbits and patients [2, 3]. Although amsacrine is an effective clinical drug for the treatment of various haematological malignancies it has shown disappointing activity against most solid tumours [4]. A new analogue, N-5-dimethyl-9-[(2-methoxy-4-methylsulfonylamino)phenylamino]-4-acridinecarboxamide [I, CI-921, NSC 343 499) has been identified, which has significantly superior activity in various in vitro and in vivo solid tumour test systems, and which may offer a broader clinical antitumour spectrum [5]. This compound will enter phase 1 clinical trials early in 1985. We have developed an HPLC method for the determination of I in plasma which will allow the study of its pharmacokinetics during phase 1 trials, and also in animals.

MATERIALS AND METHODS

The apparatus and materials have been previously described [1]. The internal standard (I.S.) has the same structure as I, but with the methyl in the sulfonanilide group substituted by an ethyl group (Fig. 1). This substitution did

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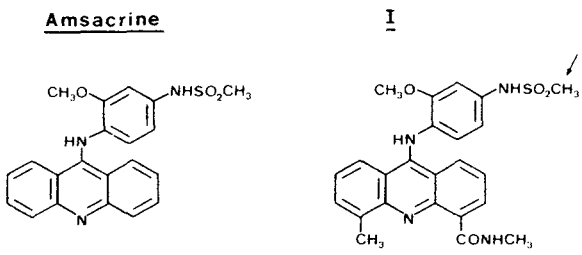

Fig. 1. Structures of amsacrine and analogue (I). The internal standard had an ethyl group substituted for the methyl group indicated on I.

not alter the pK_a of the molecule or its extraction properties. However, the greater lipophilicity will result in a longer retention time on the Radial-Pak C_{18} column. Pure I (the isethionate salt) and the I.S. (the hydrochloride salt) were supplied by Dr. B. Baguley (Cancer Research Laboratory, Auckland School of Medicine, Auckland, New Zealand). Stock solutions (2 mmol/l) were made up in methanol. This stock solution of I was further diluted 1:100 with blood-bank plasma to give a concentration of 20 μmol/l. Further serial dilutions with plasma were made to give the following concentrations: 10, 8, 6, 4, 2, 1, 0.8, 0.6, 0.4, 0.2 and 0.1 μmol/l plus a blank plasma. These concentrations were used to construct a calibration curve. The I.S. solution was further diluted 1:100 in methanol to 20 μmol/l and 100 μl were added to each 0.5-ml plasma sample. In addition, pure I was weighed out and added to four large plasma pools to give concentrations of 10, 5, 2 and 1 μmol/l, which were used to determine the accuracy and precision of the method. They were also included in each subsequent assay as quality controls, and to determine the inter-assay precision.

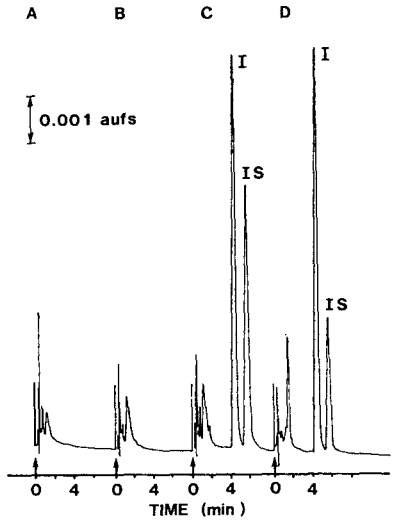

Fig. 2. HPLC traces of extracts of blank human plasma (A), blank rabbit plasma (B), human plasma quality control (5 μmol/l) (C), and post-infusion rabbit sample (D). IS = internal standard.

The clean-up and extraction procedure were the same as for amsacrine [1]. The HPLC mobile phase was prepared by adding 10 ml of stock triethylamine phosphate (TEAP) solution to 990 ml of acetonitrile—water (43:57) to give a final concentration of 0.01 mmol/l TEAP. The chromatographic separations were performed on a Waters 10 × 0.8 cm Radial-Pak C_{18} column (10 μm particle size) with a mobile phase flow-rate of 6.5 ml/min and detection by a Model 440 UV detector at 254 nm. Under these conditions the elution time of I and I.S. were 4.5 and 5.7 min, respectively, with baseline separation (Fig. 2).

RESULTS

A calibration curve for I in plasma ranging from 0.1 to 20 μmol/l was prepared by plotting peak area ratios to concentrations of I. An excellent linear relationship was obtained, represented by the equation $y = 0.242x - 0.055$ ($r = 0.9999$, $p < 0.001$). Using a 0.5-ml plasma aliquot, 0.1 μmol/l was the lowest concentration that could be measured with acceptable precision, that is a coefficient of variation of less than 7% for six repeated measurements within one assay. The accuracy of the measurement of this lowest concentration was also acceptable, lying between 85 and 115% of the true value, i.e. a 85—115% recovery. Estimates of the intra-assay precision and the accuracy of the method over the range 1—10 μmol/l are given in Table I. The precision of the method was good, with the coefficients of variation for six determinations of each concentration all being less than 2.7%. The accuracy of the method over this range was also acceptable, with the mean concentrations determined lying within 99—114% of their true values. The reproducibility of the method was tested over a three-month period by including aliquots of the four plasma pools in fifteen consecutive assays. The results are presented in Table II and indicate good reproducibility with the coefficients of variation for the fifteen determinations all being less than 5.3%. From this data, it also was apparent that I is stable in plasma stored frozen at −20°C for at least three months.

No peaks interfering with I or I.S. were observed on extraction of human or rabbit plasma (Fig. 2). A number of other anticancer drugs including adriamycin, chlorambucil, cytosine arabinoside, 5-fluorouracil, lomustine,

TABLE I

INTRA-ASSAY PRECISION AND ACCURACY IN PLASMA

$n = 6$.

Concentration added (μmol/l)	Concentration measured (mean ± S.D., μmol/l)	Coefficient of variation (%)	Recovery* (%)
10.0	10.21 ± 0.13	1.3	102
5.0	5.72 ± 0.09	1.6	114
2.0	1.99 ± 0.05	2.7	99
1.0	1.08 ± 0.02	2.2	108

*The accuracy of the method is expressed as the percentage recovery, calculated from the ratio of the mean concentration measured to the true value (i.e. the amount added) in plasma.

TABLE II

INTER-ASSAY PRECISION OF DETERMINATION OF I IN PLASMA

The reproducibility of the method was tested over a three-month period in fifteen consecutive assays.

Concentration added (μmol/l)	Concentration measured (mean ± S.D., μmol/l)	Coefficient of variation (%)
10	10.53 ± 0.56	5.3
5	5.98 ± 0.24	4.1
2	2.07 ± 0.07	3.6
1	1.10 ± 0.05	4.5

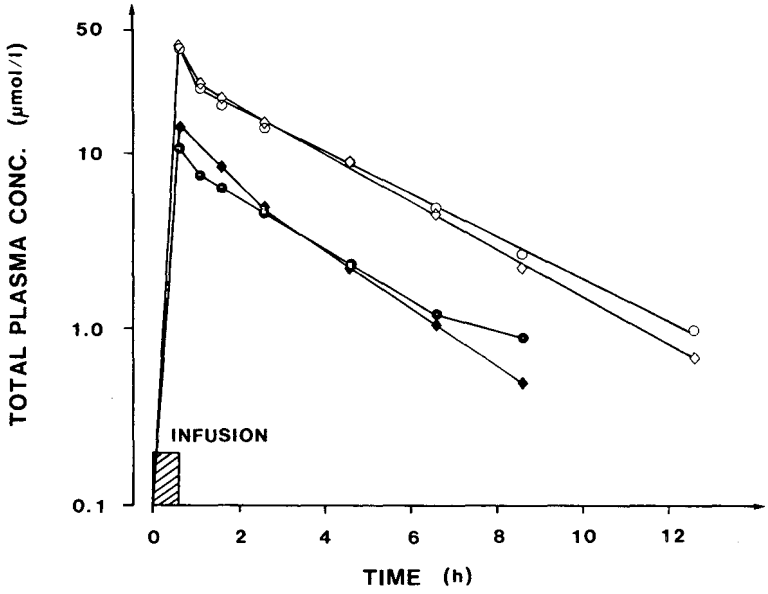

Fig. 3. Concentration—time profiles of I (open symbols) and amsacrine (closed symbols) after equimolar infusions in two rabbits. Each point is the mean of duplicate determinations.

melphalan, methotrexate, prednisolone, 6-thioguanine, vincristine and vinblastine did not interfere with the I or I.S. peaks with our extraction procedure and under these chromatographic conditions.

The assay method was applied to the measurement of I in rabbit plasma after a 35-min infusion of 12.7 μmol/kg. This dose and rate is approximately equivalent in molar terms to the amsacrine infusion received by our acute myelogenous leukaemic patients [3]. The concentration—time profile observed in two rabbits is illustrated in Fig. 3. Also included are the concentration—time profiles for an equimolar amsacrine infusion in the same rabbits.

In summary, we have extended our amsacrine assay to allow the determination of a new analogue in plasma. This method is relatively rapid and very reproducible and allows the drug to be estimated with good accuracy and precision down to a concentration of 0.1 μmol/l in 0.5 ml plasma which is sufficient for pharmacokinetic studies in patients and laboratory animals.

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Note

Determination of benzylpenicillin in plasma and urine by high-performance liquid chromatography

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(First received December 19th, 1984; revised manuscript received March 6th, 1985)

In spite of the introduction of newer β-lactam penicillins with either a wider spectrum of activity or increased activity against penicillinase-producing bacteria, benzylpenicillin is still frequently considered to be the drug of choice for the treatment of infections due to susceptible organisms [1, 2].

Several high-performance liquid chromatographic (HPLC) methods for benzylpenicillin in dosage forms or as pure drug have been reported [3—5]. In studies examining various aspects of the pharmacokinetics of benzylpenicillin the usual method for assay of the drug has been microbiological [6—9]. Microbiological assays are slow and also suffer from a lack of selectivity and low precision [10]. HPLC has the advantage of being rapid, highly selective and usually more precise than microbiological assays. HPLC has been developed for a number of penicillins in biological fluids [10—14] but only that of Westerlund et al. [10] assays benzylpenicillin. The method of Westerlund et al. [10] involves post-column derivatisation, a process which requires special equipment and techniques which are often not available.

The present study describes a simple, rapid and selective HPLC assay for the determination of benzylpenicillin in plasma and urine. This method is suitable for clinical and pharmacokinetic studies.

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MATERIALS AND METHODS

Chemicals

Benzylpenicillin was supplied by Commonwealth Serum Labs. (Australia). Methanol and acetonitrile were specially purified for HPLC and supplied by Waters Assoc. (Milford, MA, U.S.A.). All other chemicals were analytical-reagent grade. The water used was deionized, then glass-distilled. Benzylpenicilloic acid, the major metabolite of benzylpenicillin, was prepared according to the method of Cole et al. [8].

HPLC instrumentation and conditions

Reversed-phase HPLC was performed using a Waters M6000A solvent delivery system fitted with a U6K injector. A C_{18} μBondapak column (particle size 10 μm; 300 mm × 3.9 mm I.D.; Waters Assoc.), was used in combination with a guard column (μBondapak C_{18}/Porasil B; Waters Assoc., 23 mm × 3.9 mm I.D.) in all studies. UV absorbance at 214 nm was monitored with a Waters Model 441 UV absorption detector fitted with a zinc lamp. The absorbance was recorded on a dual-channel Omniscribe recorder (Houston Instruments, Austin, TX, U.S.A.). Injections were made with a 25-μl Hamilton syringe. The mobile phase was a 0.015 *M* phosphate buffer (pH 7.0 ± 0.1)—methanol (72:30) mixture. The flow-rate was 1 ml/min.

Sample preparation

Plasma and urine samples were processed by transferring a 200-μl aliquot of the sample into a glass tube (disposable borosilicate glass culture tubes; 50 × 6 mm; Kimble, IL, U.S.A.) and adding an equal volume of acetonitrile. The sample was vortexed for 1 min, then centrifuged at 1500 *g* for 5 min. An aliquot (20 μl for plasma, 10 μl for urine) of clear supernatant was injected onto the column.

Preparation of standard curves

Plasma standards were prepared at the start of each day by spiking drug-free plasma with known amounts of a freshly prepared aqueous solution of benzylpenicillin to produce concentrations of 0.5—50 mg/l. The standards were then analyzed in the described manner. Urine standards were prepared in a similar manner in drug-free urine to produce concentrations of 20—4000 mg/l. Standard curves were prepared by plotting the peak height of benzylpenicillin versus concentration.

RESULTS AND DISCUSSION

Typical chromatograms of blank plasma and plasma from a subject following intravenous administration of benzylpenicillin are shown in Fig. 1. The retention time for benzylpenicillin is 12.4 min. Fig. 2 shows chromatograms of blank urine and urine following administration of benzylpenicillin from the same subject.

Several HPLC methods for benzylpenicillin in non-biological fluids used UV detection at 254 nm [3, 5, 15]. It was found using the present system that a

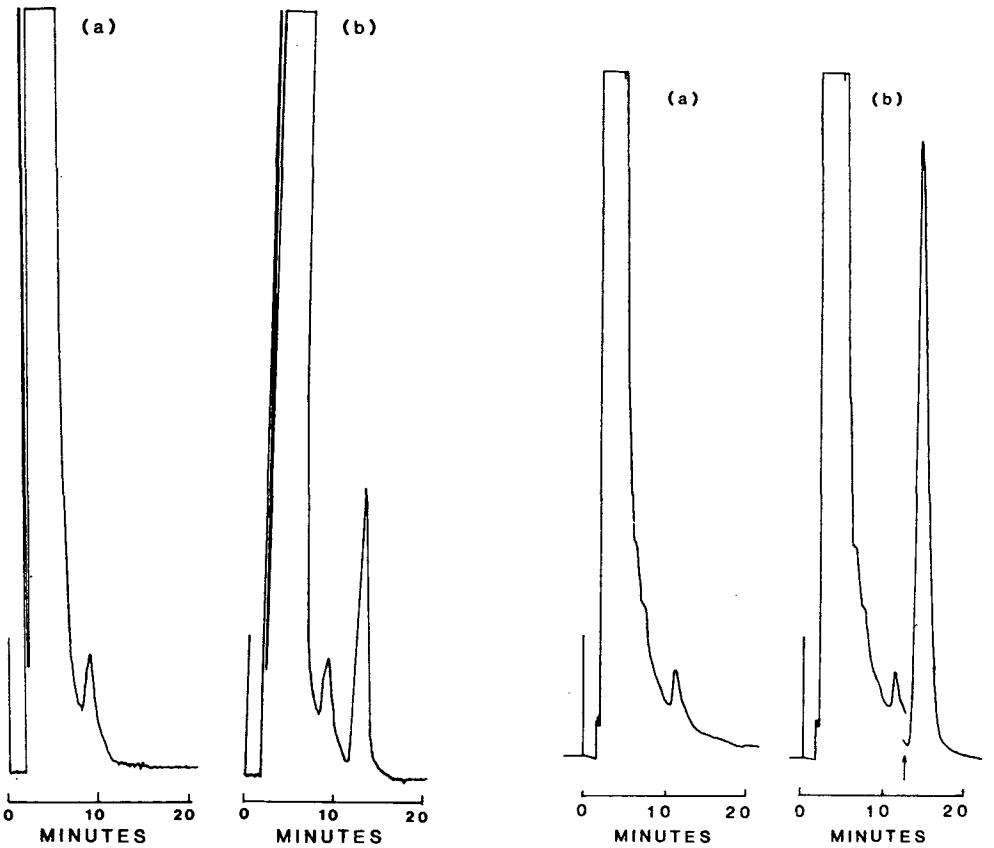

Fig. 1. Chromatograms of (a) blank plasma and (b) plasma from the same subject 30 min after intravenous administration of 600 mg benzylpenicillin. The plasma concentration of benzylpenicillin is estimated to be 7.3 mg/l.

Fig. 2. Chromatograms of (a) blank urine and (b) urine from the same subject collected from 0—8 h after intravenous administration of 600 mg benzylpenicillin. The urine concentation of benzylpenicillin is estimated to be 1310 mg/l. The arrow indicates change in absorbance scale.

greater than twenty-fold increase in sensitivity could be achieved by monitoring the effluent at 214 nm rather than at 254 nm without increased interference or baseline noise. UV detection in the range 210—230 nm has been used by other workers [11—14] to quantitate various other penicillins in biological fluids.

A number of agents may be used to precipitate plasma protein. In HPLC methods for other penicillins, perchloric acid [11] trichloroacetic acid [10] and acetonitrile [13] have been used. In the present study acetonitrile was found to completely precipitate plasma protein and provided the cleanest chromatogram. Acetonitrile was also added to urine samples to reduce the number of endogenous peaks.

Over the concentration ranges studied (0—50 mg/l for plasma and 0—4000 mg/l for urine), linearity of response was found to be good ($r > 0.99$) and consistently reproducible for standard curves based on peak height following injection of a known volume of supernatant.

Under the conditions of this assay, the detection limit for benzylpenicillin in plasma was 0.2 mg/l; 0.5 mg/l could be determined quite readily and reproducibly. In urine the detection limit was about 10 mg/l.

The usual method for the determination of benzylpenicillin in biological fluids is by microbiological means. Such assays have a low precision with an experimental error of ± 15% and their selectivity is reduced if active metabolites or other compounds with antibacterial action are present [10]. The intra- and inter-day reproducibility of the present assay for plasma are shown in Table I. The inter-day coefficient of variation could be reduced to that of the intra-day by the preparation of standard curves each day of assay. The intra-day coefficients of variation for benzylpenicillin in urine at concentrations of 2000, 200 and 20 mg/l were 2.74, 1.69 and 8.5% respectively (n = 6). The selectivity of this method was studied by measuring the retention times of other common penicillins. The results are shown in Table II. Modification of the polarity of the mobile phase could make this system suitable for the assay of these compounds.

The present method was used to follow the disposition of benzylpenicillin administered intravenously and intramuscularly to volunteers. Fig. 3 shows the plasma concentration—time profiles for benzylpenicillin in one subject following the administration of 600 mg of the drug intravenously and intramuscularly on separate occasions. The plasma benzylpenicillin concentration—time profiles for volunteers following intravenous administration showed levels similar to those obtained by Kates et al. [9] following intravenous administration of the same dose but using a microbiological assay.

TABLE I

INTRA- AND INTER-DAY VARIATION OF BENZYLPENICILLIN IN PLASMA

Spiked concentration (mg/l)	Intra-day coefficient of variation* (%)	Inter-day coefficient of variation** (%)
20	2.53	3.59
10	2.26	4.89
4	1.69	5.81
1	7.30	9.16

*n = 6.
**n = 5 over two weeks.

TABLE II

RETENTION TIMES OF VARIOUS PENICILLINS

Pencillin	Retention time (min)
Ampicillin	6.0
Methicillin	9.2
Benzylpenicillin	12.4
Amoxycillin	14.4
Oxacillin	26.0
Cloxacillin	37.2
Flucloxacillin	40.0

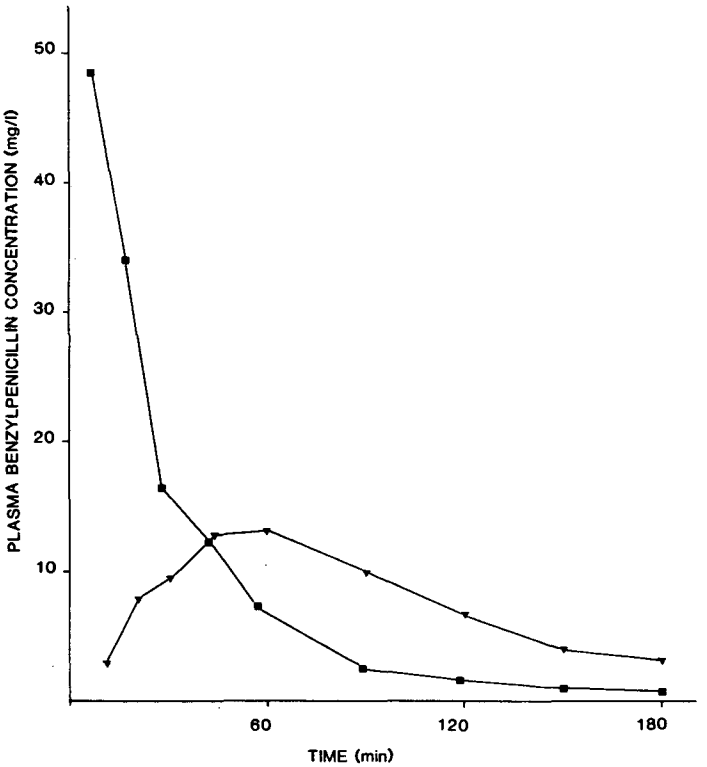

Fig. 3. Time course of plasma benzylpenicillin concentrations found in a subject following (■) intravenous and (▼) intramuscular administration of 600 mg benzylpenicillin.

In summary, the HPLC method presented here provides a selective, reliable and reproducible method for the rapid determination of benzylpenicillin in plasma and urine. The method does not require time-consuming or complex extraction or derivatisation techniques. We have found the method suitable for pharmacokinetic studies of benzylpenicillin.

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Note

Determination of trazodone in human plasma by liquid chromatography with fluorescence detection

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(First received January 14th, 1985; revised manuscript received March 19th, 1985)

Trazodone is one of the newly introduced antidepressant drugs. It has been claimed that unlike tricyclic antidepressants, trazodone does not have anticholinergic side-effects and is relatively less cardiotoxic [1, 2]. Optimal therapeutic range for this drug has not yet been established [3]. However, knowledge of plasma concentration of trazodone is required to check compliance and to decide treatment failures. Trazodone has been determined by gas chromatography in therapeutic [3, 4] and in toxic concentrations [5]. This drug has also been determined by liquid chromatography (LC) using UV detection at 254 nm [6], at 242 nm [7] or at 214 nm [8] and with electrochemical detection with an oxidation potential of 1.15 V [9]. Trazodone has been determined fluorometrically in the eluates of spots corresponding to the R_F value of trazodone obtained by the separation of plasma extracts by thin-layer chromatography [10]. We describe an LC procedure for sensitive determination of trazodone using its native fluorescence for its detection.

EXPERIMENTAL

Reagents

All reagents were of analytical-reagent grade. Solvents had been distilled in glass by the supplier (Caledon Labs., Georgetown, Canada). Deionized water was distilled in an all-glass still.

Standards

A 1 g/l stock solution of trazodone was prepared by dissolving 54.9 mg of

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trazodone hydrochloride (Bristol Meyer Canda) in 50 ml of methanol. The solution was stored at 4°C. A 100 mg/l solution was prepared by diluting 1 ml of stock trazodone to 10 ml with water. Plasma standard of 4 mg/l was prepared by adding 2 ml of diluted trazodone solution to 50 ml of drug-free pooled plasma: additional plasma standards of 2.0, 1.0, 0.5, 0.25, 0.125 and 0.62 mg/l were prepared by serial dilutions with drug-free plasma. In some cases bovine serum albumin solution (60 g/l) was used when drug-free plasma was not available. The standards were stored frozen at −15°C in 1-ml portions. The stock internal standard solution (100 mg/l) was prepared by dissolving 20 mg of harmine hydrochloride (Aldrich) in 200 ml of methanol and stored at 4°C. Working internal standard solution was prepared by diluting 10 μl of stock to 20 ml with 0.1 *M* sodium acetate solution.

Sample preparation

Bond-Elut C_{18} 1-ml disposable extraction columns (Analytichem International) were washed under suction twice with methanol, once with water and once with 0.1 *M* sodium acetate. Working internal standard (200 μl) and plasma sample (0.5 ml) were applied to the washed columns. Suction was adjusted so that the liquid passed through the columns in 30—50 sec. The columns were washed twice with water and twice with methanol—water (10:90). The columns were transferred to labelled 100 × 16 mm disposable glass tubes. Methanol (0.5 ml) was added to each column and the tubes were centrifuged to elute the columns. An aliquot (25 μl) of the eluate was injected into the chromatograph.

Chromatography

The chromatographic separation is performed isocratically at room temperature with a single-piston reciprocating pump (Model 110A; Beckman) Injections are made with a syringe-loading injector with a 20-μl loop (Model 7125, Rheodyne). The peaks are detected with a fluorescence detector (Model RF-530; Shimadzu) at an excitation wavelength of 320 nm and an emission wavelength of 440 nm. A 15 cm × 4.6 mm Ultrasphere octyl column packed with particles of average diameter of 5 μm (Beckman) is used. The column is protected with a guard column (70 × 4.6 mm) packed with Co:Pell ODS of particle size 30—40 μm (Whatman). The mobile phase is prepared by mixing 500 ml acetonitrile, 500 ml water, 0.5 ml tetramethylammonium hydroxide (20 g/l) (Sigma) and 0.5 ml of 70% perchloric acid. It is pumped at a flow-rate of 1 ml/min with 6.9 MPa as back-pressure. The peaks are recorded with a recording integrator (Model CR 3A, Shimadzu). The integrator is connected to 1V full scale output of the detector.

RESULTS AND DISCUSSION

Trazodone has been extracted from plasma with a variety of organic solvents at alkaline pH (6—8). Solid-phase extraction as described in the present report is rapid and simple. Trazodone is extracted in yields of 90—100%. There is no change in the ratio of drug to internal standard after extraction by the present procedure.

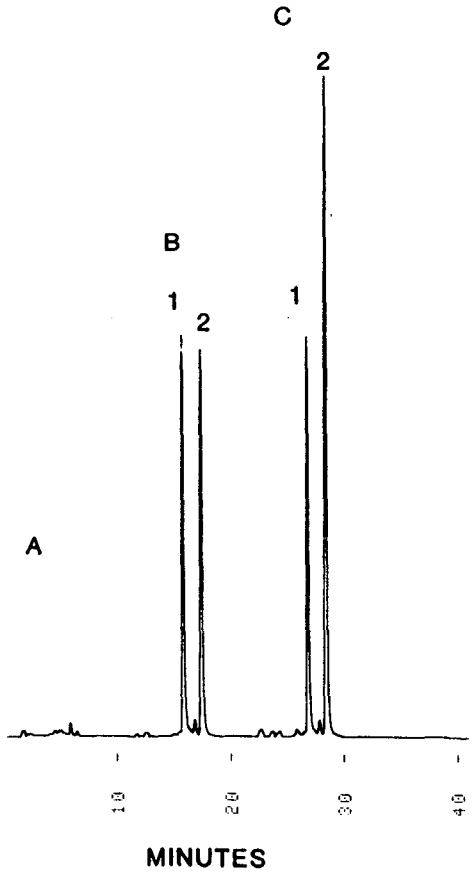

Fig. 1. Liquid chromatograms of (A) drug-free plasma, (B) plasma with added trazodone (0.3 mg/l), (C) plasma of a patient receiving a daily trazodone dose of 150 mg. The trazodone peak corresponds to 0.52 mg/l. Detector: sensitivity high, integrator attenuation 3, chart speed 5 min/cm. Peaks: 1 = harmine; 2 = trazodone.

Fig. 1A shows a chromatogram of the extract of drug-free plasma. There are virtually no peaks due to endogenous components of plasma. Fig. 1B shows a chromatogram of an extract of plasma standard. The peaks of drug and of internal standard are sharp and well separated. The standard curve is linear for the range tested (0.05—4 mg/l) and passes through the origin.

Detection of native fluorescence provides high sensitivity and selectivity. As low as 1 ng of trazodone injected on-column can be quantitated. Because of high sensitivity of detection and relatively high therapeutic concentrations of this drug, evaporation of the plasma extract is not required. Harmine used as internal standard has a structure different from that of trazodone. However, it behaves similar to trazodone during extraction and is fluorescent at the excitation and emission wavelength selected for trazodone. However, fluorescence response of harmine under the described conditions is fifteen times that of trazodone. Etoperidone, an analogue of trazodone, which has been used as internal standard for the determination of trazodone [9] lacks

TABLE I

INTERFERENCE STUDIES

Compound	Concentration in plasma (mg/l)	Retention time (min)
Harmine		5.6
Trazodone		7.7
Acetaminophen	100	—*
N-Acetylprocainamide	20	—
Amitriptyline	1.0	—
Clomipramine	1.0	—
Diazepam	1.0	—
Disopyramide	10	—
Doxepin	1.0	—
Imipramine	1.0	—
Lidocaine	10	—
Maprotiline	1.0	—
Procainamide	20	—
Propranolol	0.5	—
Protriptyline	1.0	9.1
Quinidine	5.0	9.3
Quinine	5.0	8.8
Salicylate	50	—
Trimipramine	1.0	—

*Dash signifies no peak between 2 and 20 min.

TABLE II

ESTIMATION OF PRECISION

n	Plasma trazodone level (mean ± S.D., mg/l)	Coefficient of variation (%)
Within-batch		
10	0.050 ± 0.003	5.1
10	0.501 ± 0.015	3.0
Between-batch		
12	0.050 ± 0.003	6.5
12	0.500 ± 0.030	6.1

fluorescence at the excitation and emission wavelengths used for the detection of trazodone. Plasma standards spiked with a number of drugs (Table I) were analyzed by the present procedure. No interference has been observed. Only quinines and protriptyline show any response. Analysis of plasma of a patient receiving trazodone does not show any additional peaks (Fig. 1C). The procedure is satisfactorily reproducible (Table II).

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CHROMBIO. 2649

Note

Determination of chlorambucil in plasma using reversed-phase high-performance liquid chromatography

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Chlorambucil is an alkylating agent which is used to treat chronic lymphocytic leukaemia (CLL) [1], lymphomas and ovarian carcinoma [2]. The major side-effect of this drug is bone marrow suppression. Therefore, a knowledge of the pharmacokinetics of chlorambucil may enable therapy to be optimised whilst safeguarding the patient from toxicity.

The early attempts to quantify chlorambucil were based on colorimetry [3], radiolabelling [4] and gas chromatography–mass spectroscopy [5]. More recently several methods for the determination of chlorambucil, using high-

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performance liquid chromatography (HPLC) have been described [6–9]. Whilst sufficiently sensitive to enable determination of pharmacokinetic data, disadvantages of existing procedures include the use of solvent extraction [6], the need for gradient elution [7] and relatively long retention times [8, 9]. Zakaria and Brown [9] have described a method for chlorambucil measurement by direct injection of plasma onto the column. However, the maximum volume of plasma reported permits detection to only 120 ng/ml.

This paper describes a reversed-phase HPLC assay for chlorambucil in plasma. Sample clean-up involves precipitation of plasma macromolecular components with concentrated perchloric acid, followed by extraction of chlorambucil from the supernatant with C_{18} Sep-Pak. The retention time for the drug is 4.2 min and the limit of detection is 10 ng/ml. In addition, the extraction and modified chromatographic procedures enables simultaneous determination of chlorambucil and melphalan. Although these drugs are rarely combined in therapeutic protocols, the technique may nonetheless facilitate pharmacokinetic studies of these alkylating agents.

MATERIALS AND METHODS

Instrumentation

HPLC equipment from the Pye Unicam PU 4000 system (Cambridge, U.K.) incorporated a dual reciprocating pulseless pump and variable-wavelength UV detector, set to 260 nm. Detector sensitivity was 0.08 a.u.f.s. Chromatograms were recorded on a Pye CDP 4 computing integrator with 100 mV f.s.d. The column was Spherisorb ODS 5 μm particle size, 250 × 4.6 mm I.D. (Phase Separations, Queensferry, U.K.). The column, guard column (Spherisorb ODS 5 μm, 30 × 4.6 mm I.D.), Rheodyne 7125 injection valve (Cotati, CA, U.S.A.) with a 200-μl loop, were mounted in a block heater (Jones Chromatography, Cardiff, U.K.) and maintained at 40°C.

Mobile phase

The mobile phase consisted of a mixture of methanol (Fisons, Loughborough, U.K.)–water (80:20). All materials and reagents were of HPLC grade and were filtered when appropriate with a 0.2-μm Millipore filter prior to use. The flow-rate of the mobile phase was 1.3 ml/min (150 bar).

Sample preparation and extraction

Peripheral blood samples (6 ml) were collected in lithium heparin tubes and stored at 2°C. After centrifugation (1300 *g*, 10 min, −6°C), 3-ml plasma aliquots were removed and the macromolecular components precipitated with 132 μl of cold concentrated perchloric acid (2°C). The mixture was vortex-mixed for 3 min prior to centrifugation (1300 *g*, 15 min, −6°C). The supernatant was removed and passed through a C_{18} Sep-Pak (Waters Assoc., Taunton, MA, U.S.A.). The Sep-Pak was washed with 10 ml of 15% methanol in water (2°C), and chlorambucil then eluted with 2 ml of methanol. The eluate was stored at −20°C prior to chromatography.

RESULTS AND DISCUSSION

In our assay for melphalan [10], the mobile phase was a mixture of methanol—water (80:20) containing 0.0135% (w/v) sodium dodecyl sulphate (BDH, Poole, U.K.). This was adjusted to pH 3.11, using sulphuric acid. The separation of chlorambucil from remaining plasma components is unaffected by ion-pairing chromatography, thus the mobile phase may be simplified to methanol—water (80:20).

The type of guard column used had a significant effect on drug resolution. A 5-cm guard column packed with Co:Pell ODS (particle size 30—38 μm) produced inadequate resolution. Effective removal of extraneous plasma contaminants was achieved using a LiChrosorb ODS 10-μm guard column (30 × 4.6 mm I.D.). However, the best results were obtained by a Spherisorb ODS 5-μm guard column (30 × 4.6 mm I.D.).

The coefficient of variation for ten concentration duplicates (20–1200 ng/ml) extracted from plasma was 0.71%; the correlation coefficient for the calibration graph was 0.998. Within-batch variability for standard solutions of 100, 500 and 1000 ng/ml was 1.67%, 0.96% and 0.18%, respectively, for each chromatography ten times. Recovery of drug from plasma was approximately 60%, with a limit of detection of 10 ng/ml.

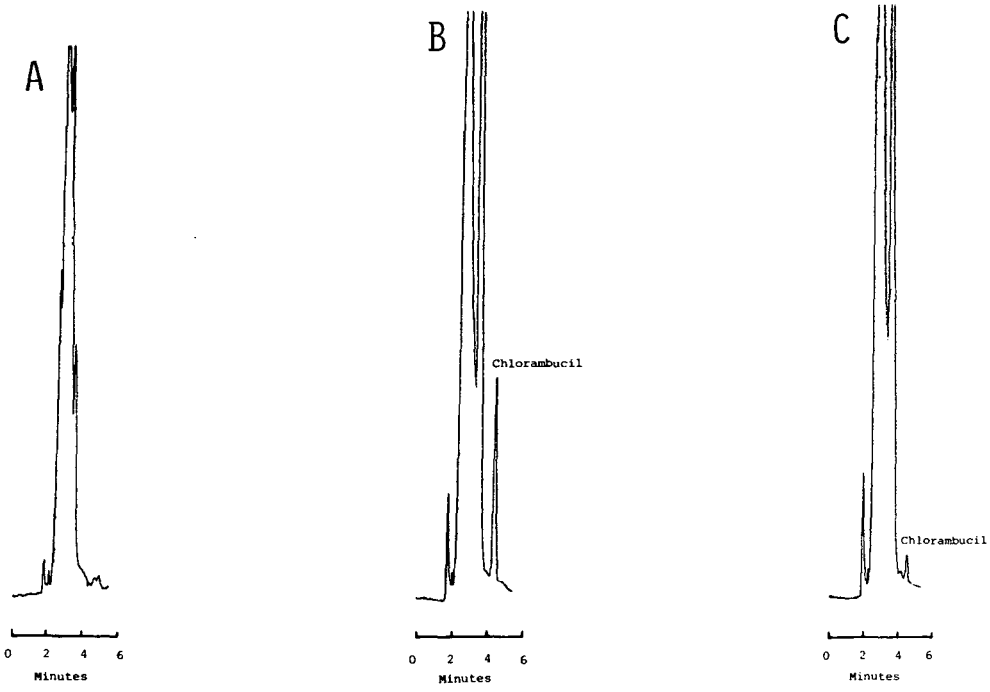

Fig. 1. Chromatograms of plasma samples from a patient (A) before drug administration, (B) 70 min after oral administration of 30 mg chlorambucil (plasma concentration 272 ng/ml), (C) 270 min after drug administration (plasma concentration 40 ng/ml). Samples were separated on a Spherisorb ODS 5-μm column (250 × 4.6 mm I.D.) with a Spherisorb ODS 5-μm (30 × 4.6 mm I.D.) guard column at 40°C. The mobile phase (flow-rate 1.3 ml/min) was a mixture of methanol—water (80:20). Detection was by UV absorption at 260 nm.

Acetonitrile and trichloroacetic acid (BDH) were examined as alternative precipitating reagents in an attempt to improve extraction efficiency and reduce plasma interference. Recovery of drug from plasma was 84% and 30%, respectively. However, both reagents increased plasma background. Thus, the best compromise between resolution and recovery was achieved using concentrated perchloric acid (Fig. 1).

Zakaria and Brown [9] noted the potential degradation of chlorambucil, in previous reports, during collection of plasma samples and storage of extracts prior to chromatography. In view of this we investigated the stability of the drug in methanol and plasma. Solutions were incubated at −20° C, 2° C and 37° C over a 6-h period, and samples removed and chromatographed at intervals. Chlorambucil was found to be stable in methanol at all temperatures investigated. In plasma (Fig. 2) the drug was not hydrolysed at −20° C and 2° C. However, at 37° C hydrolysis occurred, which has also been described by Ehrsson et al. [11]. Therefore, loss of chlorambucil by hydrolysis does not occur during the conditions reported here for collection of plasma samples and storage of methanolic extracts.

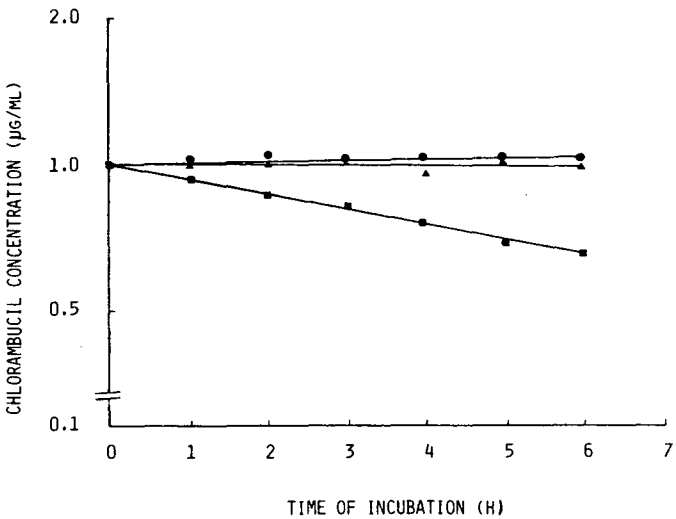

Fig. 2. Stability of chlorambucil in plasma over a 6-h period at −20° C (•), 2° C (▲), and 37° C (■).

We have determined plasma levels of chlorambucil in five patients (two CLL and three non-Hodgkin's lymphoma) receiving high-dose chemotherapy (25–40 mg daily for three days). Samples of peripheral blood were taken over a 6-h period and stored in ice (2° C) prior to analysis. Peak plasma concentrations ranged from 703 to 909 ng/ml occurring 25–120 min after drug administration. Methanolic solutions of prednisolone and prednisone were chromatographed to confirm that concomitant medication did not affect chlorambucil resolution.

CONCLUSION

In this paper we have described a rapid and simple assay for chlorambucil in plasma, with a limit of detection of 10 ng/ml (based on a peak height of twice baseline noise). We have shown that plasma samples may be collected in ice (2°C) over a period of 6 h without hydrolysis of drug. In addition, the methanolic extract of chlorambucil is stable under storage. We are currently using this assay to study the pharmacokinetics of chlorambucil in patients with CLL and lymphoma.

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CHROMBIO. 2646

Note

Quantification of *R*-(+)-7-chloro-8-hydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-methyl-3-benzazepine in brain and blood by use of reversed-phase high-performance liquid chromatography with electrochemical detection

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Antipsychotic drugs are believed to act by blocking dopamine receptors [1—6]. These have been divided into two classes on the basis of specific biochemical characteristics: the D_1 class is linked to the dopamine-mediated stimulation of cyclic adenosine 5′-monophosphate (cAMP) synthesis, whereas the D_2 class (labeled by [^{3}H]spiperone) is not [7]. On the basis of pharmacological data, it is the D_2 class that has been felt to mediate both antipsychotic effects in man and various antidopaminergic behavioral effects in laboratory animals or man (cf. refs. 2—6). Based principally upon relative potencies in

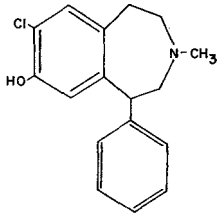

Fig. 1. Structure of *R*-(+)-7-chloro-8-hydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-methyl-3-benzazepine (I).

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several in vitro biochemical tests, *R*-(+)-7-chloro-8-hydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-methyl-3-benzazepine [SCH23390, I, Fig. 1] recently was reported to be the first selective D_1 antagonist [8—11]. The drug inhibited dopamine-sensitive adenylate cyclase with an IC_{50} (concentration required to get 50% inhibition) of ca. 1 n*M*, whereas concentrations three orders of magnitude higher were required to inhibit the binding of [^{3}H]spiperone. However, quite unexpectedly, I was found to be extremely potent in antidopaminergic behavioral tests, characteristics expected of D_2, but not D_1, dopamine receptor antagonists [12—14]. These data have spurred enormous interest in the pharmacology of I as a probe to study the neurobiology of dopamine receptors, and it is clear that quantification of the parent compound and any major metabolites will be an essential component of future studies.

This paper describes the application of reversed-phase high-performance liquid chromatography (HPLC) with electrochemial detection for the quantification of this drug in rat brain and serum. Using this method, intraperitoneal doses of I were found to be cleared from the circulation extremely rapidly, but to persist at significant concentrations in brain even when plasma concentrations were undetectable. This method should prove useful in understanding how pharmacokinetic events contribute to the pharmacodynamics of this extremely interesting drug.

EXPERIMENTAL

General

All glassware was pre-treated with a 5% solution of dichlorodimethylsilane in toluene, and then thoroughly rinsed with methanol. I was a gift of Schering (Bloomfield, NJ, U.S.A.) and chlorpromazine · HCl was a gift of Smith Kline and French (Philadelphia, PA, U.S.A.). Tetrabutylammonium chloride was purchased from Sigma (St. Louis, MO, U.S.A.), and HPLC-grade solvents and other reagents were supplied by Fisher (Pittsburgh, PA, U.S.A.).

Sample preparation

Rat serum. A 1-ml aliquot of plasma is transferred to a 10-ml round-bottom culture tube containing 100 ng of chlorpromazine which serves as an internal standard. The pH is adjusted by addition of 1 ml of 1 *M* sodium carbonate buffer (pH 9.5), and 3 ml of *tert.*-butyl methyl ether—hexane (1:1) are added. After mixing for 10 min on a wrist-action shaker (Burrel, Pittsburgh, PA, U.S.A.) and centrifugation at 2000 *g* for 5 min, the organic layer is transferred to a tube containing 1 ml of 0.1 *M* hydrochloric acid. Following shaking for another 10 min and centrifugation, the organic phase is aspirated and discarded. The aqueous phase is then washed with 2 ml of hexane and the hexane is discarded. The aqueous phase is made alkaline by addition of 1 ml of the carbonate buffer, and extracted again with 3 ml *tert.*-butyl methyl ether—hexane by shaking for 10 min. After centrifugation, the organic phase is transferred to a conical bottom glass tube and evaporated under a gentle stream of nitrogen at room temperature. The resulting residue is dissolved in 100 μl of mobile phase and 50—100 μl are injected into the HPLC system. A calibration

curve is prepared using 1-ml aliquots of drug-free serum to which varying (0, 0.5, 1.0, 2.5, 5.0, 10.0, or 20.0 ng) amounts of I and a fixed (100 ng) amount of chlorpromazine are added.

Rat brain. Each brain is homogenized in 2 ml of 0.2 *M* perchloric acid using a Brinkmann Polytron®, the homogenates are vortexed, and a 1-ml aliquot is added to 1.5-ml microcentrifuge tubes containing 50 ng of chlorpromazine (internal standard). After centrifugation (10 000 *g*) for 10—15 min at 4°C in a microcentrifuge (Fisher), the supernatant is transferred to a round-bottom tube and extracted as described above. For the quantification of I in brain tissue, a standard curve is prepared using brain tissue from untreated rats, which is homogenized in 0.2 *M* perchloric acid. Aliquots (1 ml) of the homogenate are transferred to microcentrifuge tubes containing 50 ng of internal standard (chlorpromazine) and I (0, 1, 2.5, 5, 10, or 25 ng/g), and extraced as above.

Chromatography

Mobile phase. The mobile phase used is a mixture of 0.1 *M* ammonium acetate (pH adjusted to 5.5 with glacial acetic acid)—acetonitrile (1:1). Tetrabutyl ammonium chloride is added to a final concentration of 1 m*M* to minimize the effects of secondary interactions between the protonated I (and also chlorpromazine) and unreacted silanol residues on the silica backbone of the adsorbent.

Equipment. A 250 mm × 4.0 mm I.D. stainless-steel column packed with 10-μm microparticulate silica (Si 100 RP-8, E. Merck, Darmstadt, F.R.G.) is used for the isocratic HPLC separations, which used a Laboratory Data Control Constametric III pump to generate a flow-rate of 1.5 ml/min. A glassy carbon working electrode (Bioanalytical Systems, TL-4) is maintained at a potential of +0.85 V (versus an Ag/AgCl reference electrode) by an LC-4 controller (Bioanalytical Systems). Cyclic voltammograms were obtained using a CV-1B appparatus (Bioanalytical Systems), a glassy carbon working electrode, and concentrations of each compound of 250 μ*M* in 0.1 *M* ammonium acetate buffer.

RESULTS

Cyclic voltammograms of I and chlorpromazine indicated peak anodic current responses at +730 and +810 mV, respectively, and supported the feasibility of electrochemical detection for recovery-monitored quantification of I. Typical chromatographic separations obtained with the procedure described above are shown in Fig. 2. Retention times of 3.1 min (I) and 5.5 min (chlorpromazine) permit a high daily throughput rate. No significant chromatographic interferences from any endogenous substances in either brain or blood were noted. The assay described has a lower working limit of detection of 0.5 ng I per ml of blood or brain supernatant. As is shown in Table I, the technique is sufficiently accurate and precise for routine use. In preliminary studies with this method, the absolute recovery of I across the whole working range of concentrations has averaged 82%.

An approximate time course of the absorption, distribution, and elimination of I was defined after intraperitoneal (i.p.) injection. As shown in Table II,

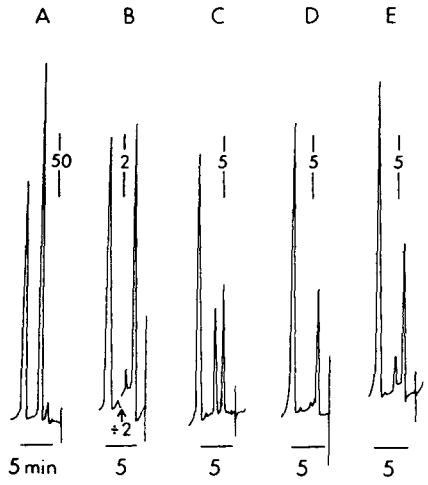

Fig. 2. Chromatogram of I and the internal standard chlorpromazine. The peaks for chlorpromazine (left; retention time ca. 5.5 min) and I (right; retention time ca. 3.5 min) can be noted in A. (A) 200 ng I and 200 ng chlorpromazine internal standard (500 nA/V scale). (B) Extraction of 0.5 ng I standard (20 nA/V scale). (C) Extraction of 10 ng I standard (50 nA/V scale). (D) Extracted plasma from drug-free rats. (E) Extracted plasma from rat sacrificed 60 min following administration of I.

TABLE I

RECOVERY, PRECISION AND ACCURACY OF HPLC MEASUREMENT OF I

These values represent data from five separate determinations made on the same day.

Concentration added (ng/ml)	Concentration found (ng/ml)	Coefficient of variation (%)	Absolute percentage recovery
2.5	2.9 ± 0.1	3.0	84.6
50	44.6 ± 1.3	3.0	78.3

TABLE II

TIME COURSE OF THE CONCENTRATION OF I IN BLOOD AND BRAIN AFTER INTRAPERITONEAL INJECTION

All rats were injected with 0.3 mg/kg I by intraperitoneal injection at various times before sacrifice. Each value represents the mean ± S.E.M. for three animals.

Time (min)	Concentration	
	Blood (ng/ml of serum)	Brain (ng/g of tissue)
15	13.5 ± 5.4	8.4 ± 0.6
30	9.6 ± 0.7	11.0 ± 1.8
60	16.5 ± 3.6	9.1 ± 1.3
120	1.3 ± 0.6	7.1 ± 0.8

there is a rapid disappearance of I from the blood, but not the brain. Although formal pharmacokinetic studies have not yet been performed, the i.p. studies indicate that after the absorption phase, the plasma half-life of I appears to be very short, in the order of 20 min. Of greatest interest, however, is the fact that the brain concentrations of I (Table II) are still near maximal at the latest time points we have examined (2 h), whereas the plasma concentrations are undetectable (< 0.5 ng/ml).

DISCUSSION

Two major pharmacological issues concerning I have been addressed by the use of this method. First, Iorio and co-workers [8, 9] reported that after oral administration I had an ID_{50} (dose required to get 50% inhibition) of ca. 2.5 mg/kg against apomorphine-induced behaviors. Conversely, we [12, 13] have found that I, given i.p. or intracerebroventricularly (i.c.v.), was extremely potent (ID_{50} = 0.03 mg/kg i.p. and 1 μg i.c.v.) in antagonizing the behavioral effects of apomorphine. The psychopharmacological studies suggest extensive first-pass metabolism of I, and the approximate plasma half-life determined here is consistent with that notion. A likely hypothesis is that formal pharmacokinetic experiments will find that I given orally has an extremely large volume of distribution. Of particular importance to the large psychopharmacological literature that is already emerging with this drug is that the plasma half-life of I after parenteral administration is ca. 20 min and that, after an i.p. dose of 0.1 mg/kg, neither the parent compound nor any electrochemically active metabolites [e.g., *R*-(+)-7-chloro-8-hydroxy-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine] are detectable in blood 2 h after drug administration.

Conversely, there are significant amounts of I still present in brain after the drug has disappeared from the blood. It is interesting that preliminary biological experiments have shown that after i.p. administration of I, there is significant inhibition of amphetamine-induced locomotion in vivo for at least 8 h after treatment [15]. Moreover, dopamine-stimulated cAMP synthesis is also inhibited in membranes from animals pretreated 12 h prior to sacrifice [15]. The data presented here support the hypothesis that it is the parent compound and not, as frequently occurs with other drugs [16, 17], a metabolite, that is responsible for the antidopaminergic effects of I.

In summary, we have described a simple and sensitive method for extracting and quantifying I from blood or brain. Since this compound is likely to be extremely important in the study and definition of dopamine receptors, the method described should be of value in thorough studies of the pharmacology of this drug.

ACKNOWLEDGEMENTS

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Note

Determination of pindolol in plasma and urine by thin-layer chromatography*

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Pindolol, DL-4-(2-hydroxy-3-isopropylaminopropoxy)indole, is a widely used non-cardioselective β-adrenoceptor blocking agent with intrinsic sympathomimetic activity. As the doses administered are usually very low and the plasma half-life is comparably short, sensitive methods are necessary for the determination of the drug in biological materials after single or chronic administration. A method for the fluorimetric determination of pindolol in plasma and urine was described by Pacha [1]. This procedure involves extraction from biological material and subsequent derivatization with *o*-phthaldialdehyde to a product that was described to have a 10^4 times higher fluorescence than the parent molecule. This method, however, does not include the separation of the pindolol derivatization product from metabolites or plasma and urine constituents, i.e., the fluorescence is measured in solution without previous chromatography.

Guerret [2] and Guerret et al. [3] suggested a gas- liquid chromatographic method for the determination of pindolol. This method involves a re-extraction step and a derivatization procedure, both of which are time-consuming. This also holds for a high-performance liquid chromatographic (HPLC) method described by Bangah et al. [4]. Mohamed et al. [5] proposed a spectrophotometric method for the determination of pindolol. This method evaluates pindolol in tablets, and therefore no extraction and separation procedures were suggested. Another method, described by Lefebvre et al. [6], is based on HPLC

*Part of the dissertation of M. Prinoth (Frankfurt/M.).

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separation and measurement of UV absorbance. The detection limit of this method is not sufficient for the determination of plasma levels.

A test for monitoring antihypertensive drug compliance with β-blockers was suggested by Jack and co-workers [7, 8]. This method involves an extraction step, which reduces the applicability of the test, and in addition a relatively large volume of urine is necessary.

In view of the above shortcomings, a qualitative test for pindolol that is simple, reliable and easy to apply was developed.

The quantitative method consists of an extraction step, thin-layer chromatographic (TLC) separation and densitometric evaluation of the intrinsic fluorescence. We also investigated whether treatment of the TLC plate after chromatography with a solution of *o*-phthaldialdehyde [9, 10], which has also been proposed as a TLC spray reagent, leads to a marked fluorescence enhancement and thus lowers the detection limit significantly.

EXPERIMENTAL

Chemicals and materials

Solvents (analytical-reagent grade) and pre-coated silica gel 60 TLC plates without fluorescence indicator (20 × 20 and 5 × 10 cm) were obtained from E. Merck (Darmstadt, F.R.G.). Pindolol and its metabolite 4-(2-hydroxy-3-isopropylaminopropoxy)oxindole (23-179) were supplied by Sandoz (Basle, Switzerland) and nadolol (internal standard) by Squibb (Princeton, NJ, U.S.A.).

Instruments

The TLC plates were scanned with a KM 3 chromatogram-spectrophotometer (Carl Zeiss, Oberkochen, F.R.G.) and a Model 56 recorder (Perkin-Elmer, Überlingen, F.R.G.). Solutions were applied on to TLC plates using a Linomat III (Camag, Muttenz, Switzerland).

Extraction from plasma samples

The extraction is performed in screw-capped glass centrifuge tubes, in which 0.5 g of sodium chloride, 1 ml of 5 *M* sodium hydroxide solution, 20 μl of a methanolic solution of nadolol (20 μl = 200 ng of nadolol) and 5 ml of methylene chloride—diethyl ether (20:80) are added to 2 ml of plasma. After shaking (15 min), using a mechanical shaker, the tubes are centrifuged (10 min, 4000 *g*) to separate the layers, then 4 ml of the organic phase are transferred into another tube and evaporated to dryness under a stream of nitrogen at 50°C.

Extraction from urine samples

A 50-μl volume of 1 *M* sodium hydroxide solution, 0.5 g of sodium chloride and 6 ml of *n*-butyl acetate are added to 0.5 ml of urine. The mixture is extracted for 20 min and, after centrifugation at 4000 *g* for 5 min, 5 ml of the organic layer are transferred into another glass tube and evaporated to dryness. The evaporation process is carried out in a vacuum centrifuge at 30°C (Speed Vac Concentrator; Bachofer Laboratoriumswerke, Reutlingen, F.R.G.).

Thin-layer chromatography

A 50-µl volume of methanol is added to the residue of the plasma extract and 40 µl of the resulting solution are then applied to a TLC plate in a 5-mm strip. A volume of 50 µl of *n*-butyl acetate is added to the residue of the urine extract, then 35 µl of this solution are applied to a TLC plate in a 5-mm strip.

To determine the concentration of unknown samples, three extracts from urine and plasma standards are also applied so that a calibration graph is included for each plate. Plasma standard concentrations are 50, 100, and 200 ng/ml and urine standard concentrations are 100, 300 and 500 ng per 500 µl.

The plate is developed at room temperature in an unlined glass tank (Desaga) containing 100 ml of chloroform–methanol–acetic acid (75:20:5). After developing the plate (12 cm, ambient temperature), it is air-dried. The R_F values of pindolol and nadolol are 0.45 and 0.24, respectively.

Fluorescence enhancement

(A) After drying, the TLC plate is dipped into a mixture of 4% paraffin liquid in cyclohexane.

(B) After complete drying (the acetic acid must be removed), the plate is sprayed with an *o*-phthaldialdehyde reagent solution [100 mg of *o*-phthaldialdehyde and 200 µl of mercaptoethanol in ethanol–buffer (pH 11) 1:1)] and heated at 40–50°C for 10 min.

Densitometric evaluation of the chromatogram

The spectrophotometer was operated in the fluorescence mode (arrangement: monochromator–sample). The light source was an ST 41 mercury lamp. For measuring the intrinsic fluorescence of the compounds an excitation wavelength of 265 nm was chosen. Emission was filtered with an M 313 monochromatic filter. The slit was 1 × 6 mm.

For measuring the fluorescence after *o*-phthaldialdehyde treatment, the excitation wavelength was 365 nm and an FL 43 filter was used to filter the emitted light (slit: 1 × 6 mm).

Unknown concentrations were measured by calculating the peak areas of the standards and the samples. If an internal standard was used, the peak area ratio was calculated.

Metabolite and drug interferences

The pindolol metabolite and several drugs and their metabolites were tested for interference with the assay. The substances tested belonged to the following groups of drugs: (a) antirheumatic/analgesic drugs (acetylsalicylic acid and the metabolites salicylic acid and salicyluric acid, azapropazone, phenylbutazone and oxyphenbutazone); (b) other β-adrenoceptor-blocking agents (propranolol, metoprolol and atenolol); and (c) quinidine.

Qualitative test for the urine samples

Depending on the dosage of pindolol, different volumes of urine (3–10 µl) are directly applied to a TLC plate (silica gel 60, 5 × 10 cm) with a disposable ring-marked micro-pipette. As a reference, 20 µl of a methanolic solution containing 10 mg of pindolol per 100 ml are also applied to the plate. The plate is developed in a glass tank containing chloroform–methanol (95:5; ammonia

atmosphere) at ambient temperature. After development, the plate is dried with a hair-dryer until the ammonia is completely evaporated.

For the detection of pindolol, approximately 50 μl of *p*-dimethylaminobenzaldehyde reagent (prepared by dissolving 1 g of *p*-dimethylaminobenzaldehyde in 100 ml of ethanol and adding 10 ml of concentrated hydrochloric acid) are applied to the area of the R_F value (R_F = 0.33), also using a micro-pipette. Pindolol turns blue immediately after the treated area dries.

RESULTS AND DISCUSSION

Fluorescence enhancement and detection limit

The detection limit for pindolol is 2–4 ng per spot. This can be improved by dipping the plate into paraffin—cyclohexane (A); the fluorescence intensity is then 3.4 times higher and a detection limit of 0.5–1 ng per spot results. The detection limit in plasma samples is about 2 ng/ml after paraffin—cyclohexane treatment. We tried to improve the sensitivity by spraying the plates with *o*-phthaldialdehyde solution (B), but better results could not be obtained; the detection limit after this treatment was 2.5 ng/ml if a 2-ml sample was used. This is due to the fact that in samples with low concentrations the fluorescence intensity of partially interfering peaks increases to the same extent as that of pindolol. Therefore, method A was used to enhance the fluorescence intensity. Using HPTLC plates we were able to detect 1 ng/ml in plasma samples. For the determination of pindolol in urine, 20 ng per 500 μl was the lowest limit using silica gel 60 plates (20 × 20 cm).

The investigations showed that the plates must be measured immediately after chromatography and not be exposed to light and air, as pindolol decomposes on the TLC plate (see Table I). The fluorescence was more intense and the detection limit, if the pure substance was used, increased after treatment of the plate with *o*-phthaldialdehyde solution. However, if pindolol

TABLE I

CHANGES IN THE FLUORESCENCE INTENSITY OF PINDOLOL (1000 ng PER SPOT) WITH TIME

(A) During storage of the TLC plates in the dark and under vacuum conditions; (B) while exposed to light and air.

Time (h)	Fluorescence intensity (%)		Intensity of violet colour of the decomposition product*	
	A	B	A	B
0.0	100	100	—	—
0.5	100	69	—	+
1.0	100	60	—	+
1.5	100	52	—	++
2.0	100	40	—	+++
5.0	90	28	(+)	++++

*—, No colour; (+), weakly visible; +, visible (the number of +-signs indicates the intensity of the colour).

was extracted from plasma samples, the detection limit was not improved, as the fluorescence of plasma constituents also increased. Further, the deviations between different plates were high, because trace amounts of acetic acid on the plate disturbed the reaction with *o*-phthaldialdehyde (although buffer of pH 11 was added). Hence the determination of the parent compound was more reliable.

Recovery and linearity

Recovery studies were performed by extracting and analysing spiked plasma and urine samples and comparing the resulting peaks with those of a methanolic solution of pindolol. Mean recoveries of 93% for plasma and 71% for urine were obtained. The calibration graphs for plasma and urine were linear up to 350 ng/ml and 2000 ng per 500 µl, respectively.

The coefficient of variation for plasma was 3.7% at a concentration of 50 ng/ml (n = 10) and 5.5% at a concentration of 10 ng/ml (n = 8) with nadolol as an internal standard. For urine the coefficient of variation was 5.5% at a concentration of 200 ng per 500 µl (n = 6).

Drug and metabolite interferences

Plasma constituents do not interfere with pindolol or nadolol, even at low concentrations, as can be seen in Fig. 1A and B, whereas urine constituents influence the nadolol peak (Fig. 1C). This is why nadolol cannot be used as an internal standard in urine samples.

No interference by the metabolite tested is observed.

If triamterene and hydrochlorothiazide are present at very high concentrations there might be interactions. All the other drugs tested do not interfere with pindolol or the internal standard.

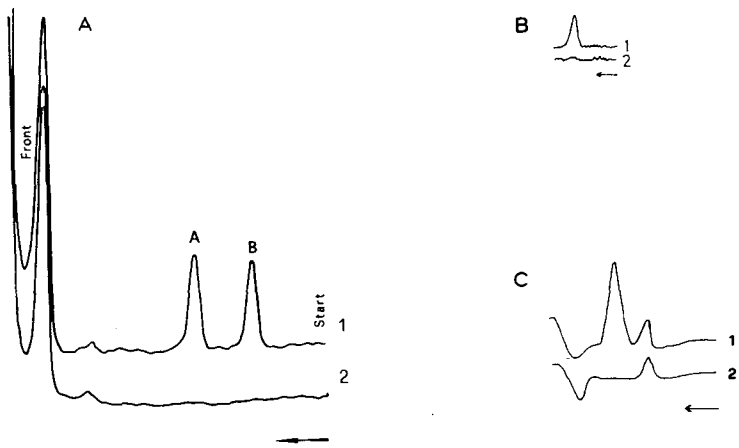

Fig. 1. (A) Thin-layer chromatograms of extracts from a plasma standard containing 50 ng/ml of pindolol (1) and from blank plasma (2). Peaks: A = pindolol; B = internal standard (nadolol). (B) Thin-layer chromatograms of extracts from a plasma standard containing 5 ng/ml pindolol (1) and from blank plasma (2) without an internal standard. (C) Thin-layer chromatograms of extracts from a urine sample obtained 8 h after oral application of 5 mg of pindolol (1) and from blank urine (2). Concentration of pindolol: 178 ng per 500 µl.

Determination of plasma levels

Fig. 2A and B show plasma levels of pindolol with time for samples obtained from two volunteers (multiple oral dosage, plasma levels after application of 10 ng of pindolol during 12 or 24 h). These data show the applicability of the method to pharmacokinetic studies.

Determination of urine levels

The applicability of the method was also tested by analysing urine samples from volunteers who took pindolol at different therapeutic concentrations (2.5, 5 and 10 mg) (Fig. 3). The concentrations found ranged from 20 to 2500 ng per 500 μl.

Urine samples from these volunteers were also analysed by the qualitative method. The value above which every test was positive was 3 ng per spot. This means that, after normal dosing, pindolol is detectable over a period of at least 8 h and consequently allows the assessment of patient compliance for every single day.

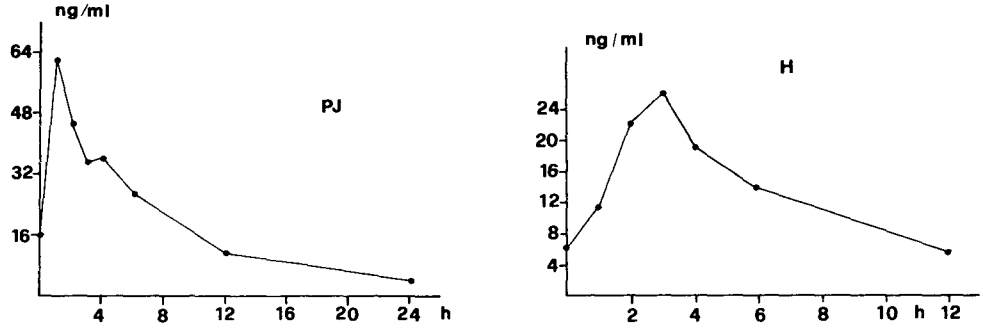

Fig. 2. Plasma levels of pindolol with time for samples obtained from two volunteers (PJ and H, after multiple oral administration of 10 mg of pindolol twice daily) on the seventh day of treatment after the morning dose (10 mg).

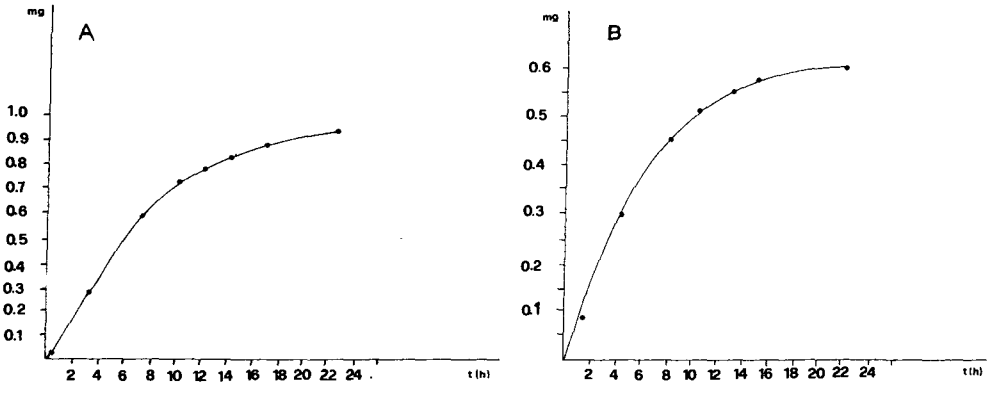

Fig. 3. Cumulative urinary excretion of pindolol in one volunteer after oral administration of (A) 5 mg and (B) 2.5 mg of pindolol.

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Letter to the Editor

Use and regeneration of Amicon ultrafiltration cones for deproteination of microsomal solutions before chromatographic analysis

Sir,

Recently, we have worked on the development of an assay for measuring quantitatively the formation of monohaloacetaldehyde and monohalooxirane from the microsomal metabolism of various carcinogenic haloaliphatics, such as vinyl chloride, 1,2-dibromoethane, 1,2-dichloroethane, and the clinically used N-mustards [1]. The assay is based on the fact that monohaloacetaldehyde [2, 3] and monohalooxirane [4] will react with adenine compounds to form highly fluorescent 1,N^6-ethenoadenines. Consequently, in our assay, typical microsomal metabolism is allowed to take place in the presence of an adenine, after which the solution is acidified and heated to maximise the efficiency of the fluorochromogenic reaction. Cyclic AMP has been found to be a suitable adenine in terms of its solubility and its stability in the post-metabolic steps. The product of the trapping, 1,N^6-etheno-cAMP, is then measured using high-performance liquid chromatography and a fluorescence detector.

A critical aspect in the development of this assay was the choice of the method for deproteinating the acidified microsomal solutions. It was anticipated that the typical experiment would have many samples that would need to be deproteinated before chromatographic analysis. Hence, it was desirable to have a quick method for deproteination. Hartwick et al. [5] have reviewed the various strategies for deproteinating biological samples and have compared several of the more popular methods. Ultrafiltration using Amicon Centriflo membrane cones CF25 was found to be easy, efficient and rapid. Also, the ultrafiltration method did not involve any dilution of the sample with solutions of salts or solvents, which also could be sources of interfering peaks during chromatography. Consequently, ultrafiltration was tried as the method for deproteination in our assay and has proven to be quite useful, provided several properties of the cones are appreciated. Given the growing use of ultrafiltration cones in the processing of biological materials for chromatographic analysis [5—7] as well as their potential use in microsomal studies such as our own, we thought that our experiences in using and regenerating these devices would be of interest to your readers.

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First, the cones should be pre-dried by centrifugation immediately before use so as to avoid a slight dilution of the sample because of their residual wetness (ca. 0.1 ml). We pre-dry cones by standing them inverted on paper towels for ca. 5 min before centrifuging them in their supports for 10 min at 750 *g* (Sorval RC-5 with a type SS-34 head).

Second, microsomal solutions (1 ml) with protein concentrations of $\leqslant$ 2 mg/ml have been deproteinated at 4°C by centrifuging for ca. 35 min at 750 *g*. The resulting filtrate is clear and suitable for immediate injection onto a column. If suspected, the presence of protein can be assessed qualitatively by the development of a blue colour when 0.1 ml of the filtrate is combined with 0.1 ml of undiluted Bio-Rad protein assay dye (Coomassie Brilliant Blue G-250) [8].

Third, the cost of the cones and the number of samples that could be expected in the course of an ongoing study of microsomal metabolism dictate that the cones be reusable. However, the methods recommended in the product literature for regenerating the cones, soaking in 0.1% sodium hydroxide or 3—5% sodium chloride, are designed to remove soluble proteins, but neither method is effective with microsomal protein solutions. What occludes the pores of a cone after ultrafiltration of a microsomal protein solution is not simply protein but rather the microsomes themselves, which are microspheres of intracellular membrane embedded with proteins. The lipid nature of the microsomes suggested that a lipase solution could be used to digest the occluded material. We have regenerated the cones with the following enzymatic procedure.

Cones in their supports were placed atop 50-ml polycarbonate tubes that had been filled with 100 m*M* potassium phosphate (pH 7.5). The cones were then filled with a solution of 1% (w/v) lipase (Sigma product number L3126) in the same buffer. Parafilm was stretched over the cones to prevent evaporation, and the cones in their polycarbonate tubes were incubated overnight (15—24 h) at 37°C. By the end of the incubation, the cones had developed an offensive odour due to some microbial growth. The contents of the cones and the polycarbonate tubes were discarded. The cones were rinsed in running tap water for 5 min, placed in 1-l beakers full of 1 m*M* sodium hydroxide in 50% aqueous ethanol, and agitated for a 30 min in a sonic bath (Heat Systems Ultrasonics; 60 Hz). The contents of the beakers were discarded, the cones were rinsed again in running tap water for 5 min, and the 30-min sonication in freshly made alkaline, aqueous ethanol was repeated. Finally, the cones were rinsed in running tap water for 5 min and stored in distilled water in a refrigerator at 4°C.

The enzymatic method described above has proved so successful that some of the cones have been used for over one year, during which time they were regenerated over twenty times. Only one cone in the forty cones that were actively used during our research was not regenerated. This failure occurred only after thirteen previous regenerations. This cone was easily recognized by a decrease in both the volume of its filtrate and in its ability to transmit an ethenoadenine standard. To date, none of the forty actively used cones has been observed to develop a leak, which would manifest itself by an increase in the volume of the filtrate as well as the presence of protein in the filtrate.

Fourth, using 1,N^6-etheno-cAMP, the product of the trapping reaction of our

TABLE I

THE LOSS IN TRANSMISSION OF 1,N^6-ETHENO-CYCLIC AMP BY ULTRAFILTRATION CONES THAT HAVE BEEN REGENERATED ENZYMATICALLY

A solution containing microsomes (1 mg/ml) and 1,N^6-etheno-cAMP was acidified to pH 4.0 and deproteinated by ultrafiltration using cones with different histories of use as indicated. Transmission of 1,N^6-etheno-cAMP into the filtrate was determined chromatographically [1]. The average transmission for unused cones is set arbitrarily to 1.00 and all values are reported relative to it as the average for four cones (± the standard deviation).

Number of previous regenerations	Relative transmission
0	1.00 ± 0.01
1	0.98 ± 0.02
10	0.91 ± 0.02
⩾20	0.93 ± 0.02

assay, we have noticed that there is a slight difference in terms of the transmission between new, unused cones and those that have been regenerated (Table I). This difference between cones, depending on their number of regenerations, contributes to the variance of our assay but the magnitude is such that it is inconsequential when cones are randomly assigned for deproteinating microsomal solutions. Therefore, in order to randomize cones and otherwise keep a history of their use, we have numbered our cones by a hole-punch scheme on their rims.

Finally, it should be noted that the cones (unused as well as regenerated) do not transmit all of an ethenoadenine standard into their filtrate. A similar situation was observed by Hartwick et al. [5] for the recovery of the purine theophylline from human serum. The loss in transmission in our assay has at least two components: adsorption on the cones and adsorption on the microsomes. The latter may include a pH-dependent binding to proteins [5].

ACKNOWLEDGEMENTS

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CHROMBIO. 2642

Letter to the Editor

Simultaneous determination of anticonvulsant drugs and metabolites in plasma by high-performance liquid chromatography

Sir,

The simultaneous determination of phenobarbital (PB), phenytoin (DPH), carbamazepine (CBZ) and its metabolite 10, 11-epoxycarbamazepine (ECBZ) by high-performance liquid chromatography (HPLC) is well documented [1—6]. However, with the exception of the procedure of Wad [6], none of the published methods includes *trans*-10,11-dihydroxy-10,11-dihydrocarbamazepine (DHCBZ), another metabolite of CBZ. Although mean plasma levels of DHCBZ are greater than those of ECBZ [7, 8], only two papers have dealt with its HPLC quantitation together with CBZ and ECBZ [6, 8]. Moreover, a new anticonvulsant, oxcarbazepine (OCZ), is under clinical evaluation. This last compound, found at very low levels in plasma, is extensively metabolized to DHCBZ and 10-hydroxy-10,11-dihydrocarbamazepine (HCBZ), which is its main metabolite in plasma [9, 10]. Methods for the determination of OCZ and its two metabolites have been described [8, 10—13].

We propose here a simple liquid chromatographic method for the simultaneous quantitation of PB, DPH, CBZ, ECBZ, DHCBZ and HCBZ in plasma.

A 500-μl aliquot of serum or plasma and 500 μl of a 0.3 mol/l phosphate buffer (pH 6.7) are mixed in a 10-ml stoppered glass centrifuge tube, then 2.50 ml of ethyl acetate R.G. containing 1.5 mg/l 9-hydroxymethyl-10-carbamoylacridane (HMCA) (Ciba-Geigy, Basle, Switzerland) and 5 mg/l 5-allyl-5-cyclopentenylbarbituric acid (ACB) (Siegfried, Zofingen, Switzerland) as internal standards are added. The tube is shaken on a rotary mixer for 5 min at 20 rpm and centrifuged. A 2-ml volume of the organic layer is transferred into a 10-ml conical glass centrifuge tube. The extract is evaporated to dryness at 50°C under a stream of nitrogen. The mobile phase described below (100 μl) is added to the dry residue. After vortex mixing, 40 μl are injected.

As the liquid chromatographic system, a Pye Unicam (Cambridge, U.K.) 4010 dual-piston pump, a Rheodyne (Berkeley, CA, U.S.A.) 7125 injection valve with a 500 μl-loop, an LKB Uvicord SII 2338 fixed-wavelength detector

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(Bromma, Sweden) and two Kipp and Zonen (Delft, The Netherlands) BD 40 recorders are used.

Analyses are performed on a reversed-phase 5-μm Spherisorb ODS column (150 × 4.6 mm I.D.) protected by a guard column packed with a pellicular reversed-phase material (Chrompack 28984 and 28623, Middelburg, The Netherlands). The mobile phase is water—methanol (R.G.)- acetonitrile (R.G.) (64:22:14, v/v), degassed by helium sparging. The operating conditions are as follows: mobile phase flow-rate 1.4 ml/min; ambient temperature; detector wavelength, 254 nm; time constant, 2 sec; sensitivity, 0.01 and 0.05 a.u.f.s. (simultaneous dual recording).

The following peak height ratios are calculated for each sample: PB/ACB, DPH/HMCA, CBZ/HMCA, DHCBZ/HMCA, HCBZ/HMCA and ECBZ/HMCA. The concentration of each compound is calculated by comparison with the ratio obtained for a calibration serum analysed under identical conditions. The latter is a drug-free human serum pool spiked with known amounts of the six compounds assayed.

The chromatograms show good resolution. The retention times are 3.4 (DHCBZ), 3.8 (PB), 4.4 (HCBZ), 5.6 (ACB), 6.2 (ECBZ), 7.9 (HMCA), 9.0 (DPH) and 13.4 min (CBZ). The plate heights range from 0.020 to 0.028 mm and the selectivities between two consecutive peaks (α) from 1.12 to 1.52.

Salicylate, theophylline, phenylethylmalonamide, ethosuximide, caffeine, primidone, oxcarbazepine and iminostilbene elute at 1.1, 1.7, 1.9, 2.1, 2.5, 2.8, 7.6 and 10.5 min, respectively. None of these substances interfere. Valproic acid is not detected. Endogenous constituents of the samples do not interfere.

The detection limits (mg/l) are 0.2 (PB), 0.4 (DPH), 0.05 (CBZ), 0.1 (ECBZ), 0.2 (DHCBZ) and 0.2 (HCBZ). The within-run and between-run coefficients of variation (%) estimated under routine conditions are 3.6 and 5.8 (PB), 2.3 and 3.7 (DPH), 2.1 and 3.0 (CBZ), 5.6 and 8.4 (ECBZ), 4.8 and 9.1 (DHCBZ) and 2.9 and 4.1 (HCBZ). This good precision results from the use of two internal standards, a barbiturate for PB [14] and an acridane derivative for the tricyclic substances and for DPH [13]. Of the extraction solvents tested (ethyl acetate, dichloromethane, chloroform, methyl isobutyl ketone, diethyl ether and diisopropyl ether), ethyl acetate yields the best compromise between drug recovery and extraction specificity [8, 13].

Compared with the only other procedure described for determining these six compounds [6], our method has two advantages, namely that lower retention times are obtained and that gradient elution and column heating are unnecessary, thus ensuring greater practicability and rapidity.

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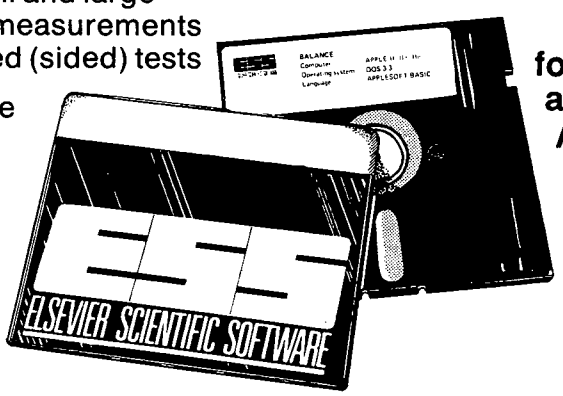

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Proceedings of Two Scientific Symposia held in Matrafüred, Hungary, October 17-20 and October 20-22, 1982

edited by E. PUNGOR, I. BUZAS *and* G.E. VERESS, *Institute for General and Analytical Chemistry, Technical University, Budapest, Hungary*

Two symposia were held in Matrafüred, Hungary, in October 1982 and the proceedings of both are contained in this book.

The first was the Symposium on Electrochemical Detection in Flow Analysis, the aim of which was to define the physical parameters of electrochemical detectors that are most important in flow applications, and to study how and under what conditions these detectors can be used in other fields in addition to direct flow analysis, e.g. in chromatography or clinical analysis.

The other meeting was the first international symposium to be held on Pattern Recognition in Analytical Chemistry. This was a particularly successful meeting and both lectures and discussions are presented in the second part of the book. The volume will provide much information and food for thought for many workers in various fields of analytical chemistry.

(Due to limitations of space, only the plenary and keynote lectures from the first conference and the topics of the second conference are listed below.)

CONTENTS

Part A - Electrochemical Detection in Flow Analysis
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Keynote Lectures: Enzyme reactors in analytical flow systems *(G. Johansson et al.).* Fundamentals of the electrochemical high sensitivity sensors for the detection of various contaminants in atmosphere *(U. Palm).* Some characteristics of flow and continuous analysis with ion-selective electrodes *(J.D.R. Thomas).* Automated polarographic and photometric system for serial analysis *(K. Tóth et al.).* Some aspects of application of ion-selective electrode detectors in flow analysis *(M. Trojanowitz).* Discussion Lectures *(15 papers).* Panel discussion. Subject Index.

Part B - Pattern Recognition in Analytical Chemistry
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