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BIOMEDICAL APPLICATIONS

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JOURNAL OF CHROMATOGRAPHY

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CHROMBIO. 2383

DETERMINATION OF TESTOSTERONE PROPIONATE IN HUMAN PLASMA BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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(First received July 24th, 1984; revised manuscript received September 6th, 1984)

SUMMARY

A specific, sensitive and accurate quantitative analysis of testosterone propionate in human plasma was developed using gas chromatography—mass spectrometry—selected-ion monitoring. For the calculation of testosterone propionate in plasma, peak height ratios were measured by selected-ion monitoring performed on the molecular ions of the trifluoroacetyl derivative of testosterone propionate (*m/z* 440) and testosterone propionate-19,19,19-d_3 (*m/z* 443). The sensitivity of the method was judged from the lower limit of the detection of the mass spectrometer which was at 20 pg. The inter-assay coefficients of variation and relative error at a concentration of 1.31 ng/ml of plasma were 5.47% and —2.3%, respectively. The method described was applied to the determination of plasma concentrations of testosterone propionate-19,19,19-d_3 following an intramuscular dose of testosterone propionate-19,19,19-d_3 in a healthy male volunteer.

INTRODUCTION

Testosterone propionate is considered to be a short-acting parenteral form of testosterone used primarily in the treatment of hypogonadism, oligospermia and impotence. Although the clinical applications of testosterone propionate have been investigated, pharmacokinetic studies have been limited by the lack of a specific and sensitive assay. Testosterone propionate in pharmaceutical preparations has been measured by several methods involving gas chromatography (GC) [1—3], high-performance liquid chromatography (HPLC) [4], high-performance thin-layer chromatography (HPTLC) [5], infrared spectrophotometry [6] and colorimetry [7]. However, all these methods were not successful in measuring testosterone propionate in biological fluids because of low sensitivity and low selectivity. In an attempt to examine the disposition of testosterone propionate, radioimmunoassay (RIA) [8—12] or competitive protein-binding assay (CPBA) [13, 14] have been employed to detect variations of unesterified testosterone concentration in plasma after

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intramuscular administration of testosterone propionate. However, no direct evidence is available regarding the pharmacokinetic analysis of intramuscular-administered testosterone propionate. RIA and CPBA techniques cannot address this problem, since these types of assay cannot differentiate between endogenous testosterone and testosterone derived from administered testosterone propionate.

We have initiated studies designed to characterize pharmacokinetic properties of intramuscular administered testosterone propionate and to clarify the influence of testosterone propionate on endogenous testosterone levels. We have already reported the determination of plasma testosterone levels by gas chromatography—mass spectrometry—selected-ion monitoring (GC—MS—SIM) using deuterated testosterone as an internal standard [15] and the time course of deuterated testosterone levels after oral administration of the labelled testosterone [16, 17]. In the present study, a sensitive and specific GC—MS—SIM technique was developed for the quantitation of testosterone propionate in biological fluids. The method was applied to determine the plasma levels of deuterated testosterone propionate after the intramuscular administration of labelled testosterone propionate to a healthy male volunteer.

MATERIALS AND METHODS

Chemicals

Testosterone-19,19,19-d_3 (testosterone-19-d_3) was synthesized in our laboratory as described previously [18]. The isotopic composition was 99.0% deuterium atoms (d_3, 97.8%; d_2, 2.2%; d_1, 0.0%). Non-labelled testosterone propionate (reagent grade) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan) and was recrystallized from *n*-hexane before use. Testosterone propionate-19-d_3 intramuscular preparation was prepared by dissolving 25 mg of testosterone propionate-19-d_3 in sesame oil containing 20% benzyl benzoic acid. The preparation was sterilely filtered through the membrane filter (HA, pore size 0.45 μm; Millipore, Bedford, MA, U.S.A.). All other chemicals and solvents were analytical grade and used without further purification.

Synthesis of testosterone propionate-19,19,19-d_3

To a solution of 0.258 g (0.887 mmol) of testosterone-19-d_3 in 4.5 ml of dry toluene were added 0.193 g (1.483 mmol) of anhydrous propionic acid and 0.321 g of dry pyridine. The mixture was then heated under reflux for 2.5 h. After cooling the mixture, chloroform and water were added, separated and the aqueous layer was re-extracted. The combined extract was washed with water and dried over anhydrous sodium sulphate. After evaporating the solvent, the residue was subjected to thin-layer chromatography (TLC) (Kieselgel 60 F_{254} plates, 0.25 mm thick; E. Merck, Darmstadt, F.R.G.) and the zone corresponding to testosterone propionate (R_F 0.45, chloroform—ethyl acetate, 4:1, as developing solvent) was scraped off. After extraction with chloroform, the product was obtained as colourless needle crystals which were recrystallized from *n*-hexane (0.220 g, 71.5%). m.p. 120—121°C. NMR: 0.84 (3H, s, 18-CH_3), 1.14 (3H, t, J = 7.0 Hz, —CO—CH_2—CH_3). MS: m/z 347 (M^+). Anal. calc. for $C_{22}H_{29}{}^2H_3O_3$: C, 76.03; H, 9.28. Found: C, 76.02; H, 9.33. The isotopic composition was 99.0% deuterium atoms (d_3, 97.8%; d_2, 2.2%; d_1, 0.0%).

Gas chromatography—mass spectrometry—selected-ion monitoring

GC—MS—SIM measurements were made with a Shimadzu LKB-9000B gas chromatograph—mass spectrometer equipped with Shimadzu high-speed multiple ion detector—peak matcher 9060S. The GC column was a glass column (2 m × 3 mm I.D.) packed with 1.5% OV-1 on Shimalate W (80—100 mesh). The injector, column, and ion source temperatures were 260°C, 230°C and 270°C, respectively. Helium was used as the carrier gas at a flow-rate of 30 ml/min. The electron energy was set to 20 eV and the trap current to 60 μA. The multiple-ion detector was focused on the ions at m/z 440 and 443 to obtain peak height ratios.

Preparation of calibration curve

To each of four standards containing 2.62, 6.56, 13.12 and 26.24 ng of testosterone propionate in 20 μl of ethanol, 11.84 ng of testosterone propionate-19-d_3 in 20 μl of ethanol were added. After evaporation of the solvent to dryness, to each sample were added 200 μl of trifluoroacetic anhydride (reagent grade; Nakarai Chemicals, Kyoto, Japan). After standing for 30 min at room temperature, excess trifluoroacetic anhydride was removed under a stream of nitrogen and the residue was dissolved in 20 μl of *n*-hexane. A 1—3 μl volume of the *n*-hexane solution was analysed by GC—MS.

Sample preparation for GC—MS—SIM

Frozen plasma samples were thawed at room temperature. To a PTFE-lined screw-cap culture tube (100 × 16 mm) were added 1.0 ml of plasma sample and 13.12 ng of testosterone propionate dissolved in 20 μl ethanol as an internal standard. The plasma sample was allowed to stand for 30 min at room temperature. The sample was cooled in an ice-bath. Immediately after adding 40 μl of 3 *M* sodium hydroxide the sample was extracted with 3 × 3 ml of *n*-hexane using a vortex mixer for a few seconds, followed by centrifugation at 1000 *g* for 5 min at 4°C. The organic phase was carefully pipetted out into a 10-ml conical centrifuge tube and washed with 1 ml of 5% acetic acid and then with 1 ml of water. After evaporating the solvent, the residue was dissolved in 5 ml of 70% aqueous methanol and stored at −15°C for 1 h. After centrifugation, the upper layer was decanted and evaporated to dryness in a 50°C water bath under vacuum. The trifluoroacetate (TFA) derivative was formed by reacting the residue with trifluoroacetic anhydride as described above and 1—3 μl of the sample were subjected to GC—MS.

Stability studies

The first stability study evaluated the effect of the pH and the temperature in the extraction. To 10 ml each of 0.04 *M* Britton—Robinson buffer pH 7.4 and 12.0 were added 118.4 ng of testosterone propionate-19-d_3 dissolved in 20 μl of ethanol. The samples were incubated at 4°C, 25°C and 37°C. An aliquot of the sample (1 ml each) was assayed at various time intervals up to 4 h.

The second stability study evaluated the effect of freezing, storage at −20°C and thawing on control plasma spiked with the reference compound. Seven spiked plasma samples (1 ml each) were prepared by mixing 1-ml aliquots of

drug-free pooled human plasma with 11.84 ng of testosterone propionate-19-d_3 dissolved in 20 μl ethanol. One sample was immediately analysed. The remaining six samples were frozen at −20° C and one sample was analysed after 7, 14, 21, 30, 60 and 90 days of storage.

Intramuscular administration of testosterone propionate-19-d_3

A healthy male volunteer (67 kg, 27 year) was intramuscularly administered a single dose of 25 mg of testosterone propionate-19-d_3. Heparinized blood samples (10 ml) were taken 5 min before and 0.5, 1, 2, 3, 4, 6, 10, 14, 24, 30, 36, 48, 54, 60, 72, 78, 84, and 96 h after dosing. Plasma was separated by centrifugation and kept in a frozen state at −20° C until analysis.

RESULTS AND DISCUSSION

Choice of derivatives and sample preparation

Fig. 1 shows the electron-impact mass spectra of testosterone propionate-19-d_3 and testosterone propionate-19-d_3-TFA. In the mass spectrum of testosterone propionate-19-d_3 (Fig. 1A), the relative abundance of the molecular ion (m/z 347) was about 7.3%. In the mass spectrum of testosterone propionate-19-d_3-TFA (Fig. 1B), on the other hand, the relative abundance of the molecular ion (m/z 443) was as high as 25%. Therefore it would be an advantage to make the TFA derivative for the SIM analysis by measuring the abundant molecular ions to obtain higher sensitivity. The preparation of testosterone propionate-TFA required only a simple derivatization step.

In the extraction procedure of plasma samples it was necessary to eliminate lipids present in plasma, since these interfered with the SIM. This required the

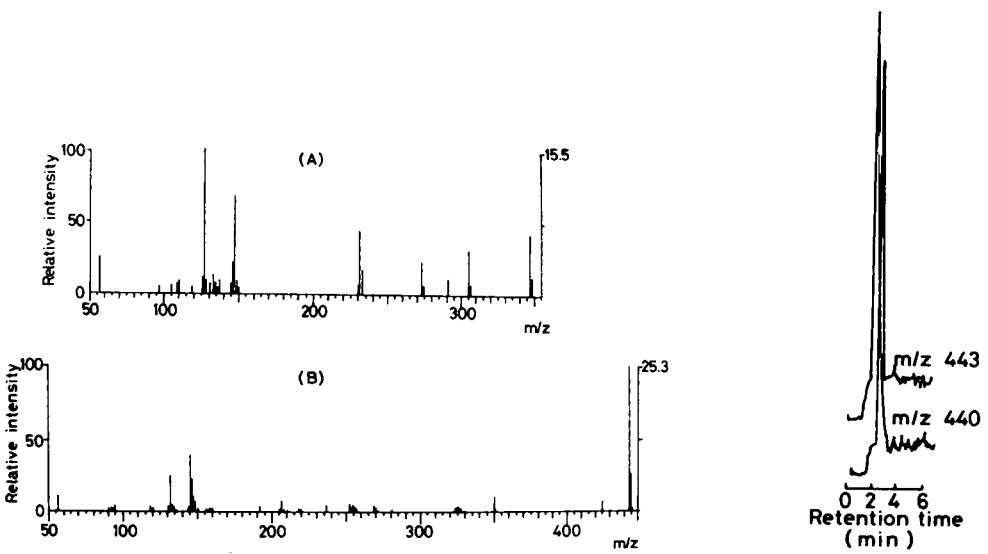

Fig. 1. Electron-impact mass spectra; (A) testosterone propionate-19-d_3; (B) TFA derivative of testosterone propionate-19-d_3.

Fig. 2. Selected-ion monitoring of testosterone propionate-TFA and testosterone propionate-19-d_3-TFA after processing from plasma sample.

treatment of a 70% methanol solution of the hexane extract at −15°C for 1 h. The total absolute recovery of testosterone propionate from plasma was about 65%.

Fig. 2 shows the selected-ion monitoring for blank plasma containing 13.12 ng/ml testosterone propionate and 11.84 ng/ml testosterone propionate-19-d_3. The retention times of testosterone propionate-TFA and testosterone propionate-19-d_3-TFA were the same (about 2.0 min) and there was no interference in the molecular-ion peaks by contributions from other materials in the plasma extract at these masses.

Stability

It has been reported that testosterone propionate is hydrolysed to testosterone by endogenous esterase [19] or by heating with catalytic amounts of base in aqueous solution [20]. In the present work, the testosterone propionate was extracted under alkaline conditions (pH 10—12) since many endogenous substances which interfere with the SIM analysis are acidic and not extractable at a higher pH. Therefore, the stability of testosterone propionate was studied at pH 12.0 and pH 7.4 (physiological pH). The results (Fig. 3A) show that testosterone propionate is stable at physiological pH. On the other hand, at pH 12.0 the stability of testosterone propionate was temperature-dependent (Fig. 3B). However, at 4°C testosterone propionate was stable for 4 h, so that plasma samples should be extracted at 4°C.

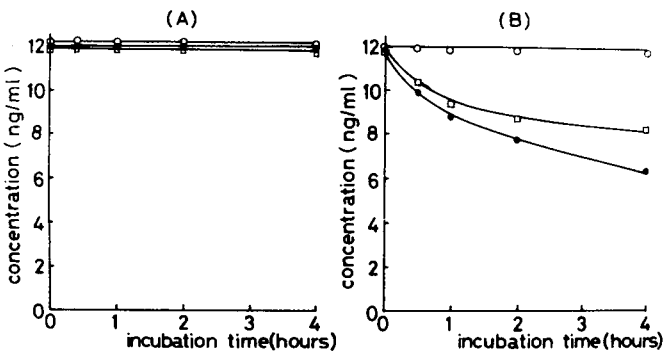

Fig. 3. Stability of testosterone propionate in Britton—Robinson buffer (A, pH 7.4; B, pH 12.0). (○) 4°C; (□) 25°C; (●) 37°C.

Secondly the effect of freezing, storage at −20°C and thawing on control plasma spiked with testosterone propionate was investigated. The data (Table I) showed that freezing and thawing do not have a significant effect on the stability of testosterone propionate and plasma samples could be stored at −20°C for 90 days without significant change.

Sensitivity

The sensitivity of the determination procedures described here was judged on the basis of the signal-to-noise ratio (S/N). The lower limit of detection of the mass spectrometer was 20 pg for testosterone propionate as shown in Fig. 4.

TABLE I

STABILITY OF TESTOSTERONE PROPIONATE IN PLASMA AT −20°C

Day	Plasma concentration (ng/ml)
0	12.34
7	12.08
14	12.24
21	11.72
30	11.65
60	11.53
90	11.45

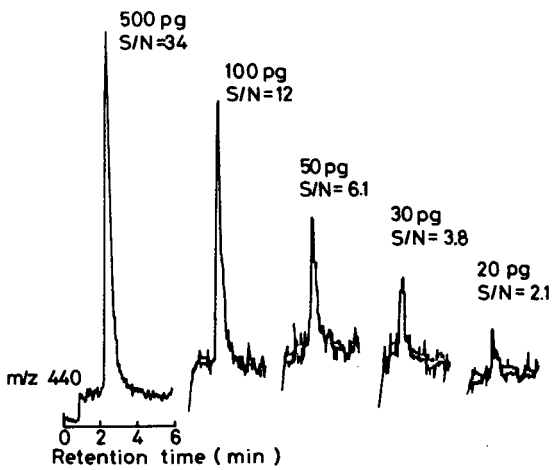

Fig. 4. Sensitivity of the determination on the basis of the signal-to-noise ratio (S/N).

Calibration curve

A calibration curve was prepared by adding known amounts of testosterone propionate (2.62—26.24 ng) to a fixed amount (11.84 ng) of testosterone propionate-19-d_3 and then assaying the mixture as the TFA derivative, monitoring the molecular ions at m/z 440 for testosterone propionate and m/z 443 for testosterone propionate-19-d_3. The peak height ratio was plotted against the molar ratio of testosterone propionate to testosterone propionate-19-d_3. The curve was linear for the molar ratio 0.2—2.2. A least-squares analysis gave a correlation coefficient of 0.999.

Accuracy

The accuracy of measurements was determined for testosterone propionate added to 1.0-ml aliquots of pooled plasma. The plasma samples contained 11.84 ng of testosterone propionate-19-d_3 and different amounts (1.31, 2.62, 6.56, 13.12 and 26.24 ng) of testosterone propionate. The amounts of testosterone propionate were measured by the present method. The amounts of testosterone propionate measured were in good agreement with the amounts of testosterone propionate spiked, the relative error being less than 3% (Table II).

TABLE II

ACCURACY OF SELECTED-ION MONITORING ANALYSIS OF TESTOSTERONE PROPIONATE IN PLASMA

Added (ng)	Found (ng)				C.V. (%)	Relative error (%)
	Individual values			Mean ± S.D.		
26.24	26.13	25.67	26.31	26.04 ± 0.33	1.27	−0.7
13.12	13.11	13.43	12.85	13.13 ± 0.24	1.83	0.7
6.56	6.44	6.51	6.48	6.48 ± 0.09	1.39	−1.2
2.62	2.48	2.67	2.66	2.60 ± 0.09	3.46	−0.8
1.31	1.36	1.29	1.19	1.28 ± 0.07	5.47	−2.3

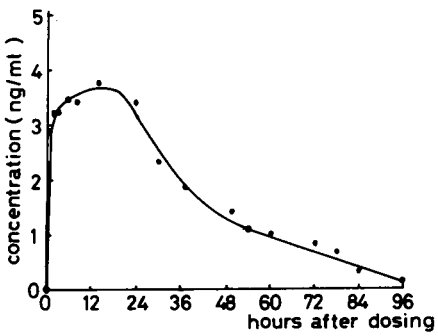

Fig. 5. Time course of testosterone propionate-19-d_3 after intramuscular administration of 25 mg of testosterone propionate-19-d_3 in a healthy male volunteer.

Intramuscular administration of testosterone propionate-19-d_3

The SIM method was applied to the determination of plasma levels of deuterated testosterone propionate in a healthy male volunteer after the intramuscular administration of the labelled testosterone propionate. Fig. 5 shows the time course of testosterone propionate-19-d_3. In the first blood samples, taken 30 min after administration of testosterone propionate-19-d_3, a plasma concentration of 2.94 ng/ml testosterone propionate-19-d_3 was detected. The plasma levels were kept constant (2—4 ng/ml) 24 h after administration and then decreased in monoexponential fashion. It became apparent that intramuscularly administered testosterone propionate-19-d_3 was gradually transferred from the site of injection in the muscle to the circulation.

CONCLUSION

In summary, the SIM method described here affords a specific, sensitive and accurate technique to determine testosterone propionate in plasma. The method makes it possible to examine the disposition of intramuscularly administered testosterone propionate by determining testosterone propionate in plasma. Pharmacokinetic and metabolic studies of testosterone propionate after the intramuscular administration of testosterone propionate are now in progress.

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CHROMBIO. 2393

GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC ANALYSIS OF VOLATILE AMINES PRODUCED BY SEVERAL STRAINS OF *CLOSTRIDIUM*

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SUMMARY

A gas chromatographic—mass spectrometric technique is proposed for the analysis of volatile amines which were isolated from *Clostridium* cultures by vacuum distillation and concentrated as hydrochloride salts. Headspace sampling after alkalinization of the salts under vacuum was the most suitable for subsequent gas chromatographic analysis. With ammonia-loaded helium as carrier gas, methylamines were separated on 4.8% PEG 20M + 0.3% potassium hydroxide on Carbopack B, and other volatile amines on 28% Pennwalt 223 + 4% potassium hydroxide on Gas-Chrom R. Bacterial volatile amines (dimethylamine, trimethylamine, isobutylamine, 3-methylbutylamine, etc.) were detected with a flame-ionization detector and identified by gas chromatography—mass spectrometry in electron-impact and chemical ionization modes.

INTRODUCTION

Volatile amines (VA) have been widely studied in foodstuffs [1—3] and in domestic or animal wastes [4—6], but their gas chromatographic (GC) analysis in bacterial growth media remains little documented.

GC analysis of VA bases is associated with analytical problems due to their high polarity, especially ghosting and severe tailing. These phenomena can be reduced with low reactive column packings such as porous polymers [7, 8] or alkali-coated packings [9—11] eventually pretreated with trimethylchlorosilane [4, 12]. Furthermore the addition of ammonia to the liquid sample [10, 11]

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or to the carrier gas [13] prevents the adsorption of VA and greatly improves their analysis.

When bacterial VA are present at trace levels, the formation of heptafluorobutyryl derivatives and electron-capture detection [14, 15] have been found valuable and avoid the above-mentioned problems. If derivatization is not expected, a vacuum distillation step is interesting to isolate VA from a complex matrix and to concentrate them as hydrochloride salts [16]. VA can be injected as their salts in an alkaline precolumn [17, 18] or as the corresponding bases after realkalinization and extraction [16]. The direct injection of a headspace sample of broth culture after salting out [7] or of an alkalinized culture supernatant fluid [8] has also been described.

A headspace gas chromatographic—mass spectrometric (GC—MS) technique was developed in this work for the analysis of VA produced by some strains of *Clostridium*, with a preliminary reduced pressure distillation step.

EXPERIMENTAL

Bacterial cultures

Nine strains of *Clostridium* were included in this study: *C. ghoni* ATCC 25757, *C. bifermentans* TM (Prévot), *C. sordellii* 82 (Prévot), *C. lituseburense* ATCC 25759, *C. mangenotii* ATCC 25761, *C. histolyticum* Tro 2E (Prévot), *C. perfringens* Lechien (Prévot), *C. difficile* ATCC 9689, and *C. cadaveris* ATCC 25783. A 50-ml volume of meat—liver medium (Infusion Viande-Foie, Institut Pasteur Production, 30 g in 1000 ml of bidistilled water), adjusted to pH 7.2 and contained in an Erlenmeyer flask fitted with two stopcocks (volume ca. 330 ml), was inoculated with 0.5 ml of a 24-h culture. Air was removed from the flask with a vacuum pump and the medium was incubated at 37°C for 120 h. Each strain was studied at least in triplicate along with sterile media processed in the same way.

Isolation of volatile amines

The broth culture was alkalinized with 10 g of sodium carbonate in a round-bottomed flask which was immediately connected to a glass distillation apparatus. Distillation was carried out at 35°C under 15—20 Torr until dryness (about 30 min), VA being trapped in two flasks containing 40 ml and 30 ml of 0.1 *M* hydrochloric acid, respectively. Both acidic solutions were pooled, evaporated and the residue of hydrochloride amine salts dried at 100°C in an air oven for 2 h.

Preparation of headspace sample

Hydrochloride salts, dissolved in 2 ml of bidistilled water, were introduced in a 35-ml flask (Fig. 1). After removing air with a vacuum pump, 1 ml of 6 *M* sodium hydroxide (analytical grade) was added. The flask was held at 50°C for 15 min, filled with helium and a headspace sample, collected in a 5-ml glass syringe through a lateral rubber septum, was injected into the column.

GC analysis

Analyses were performed on two gas chromatographs (Girdel 30 and Girdel

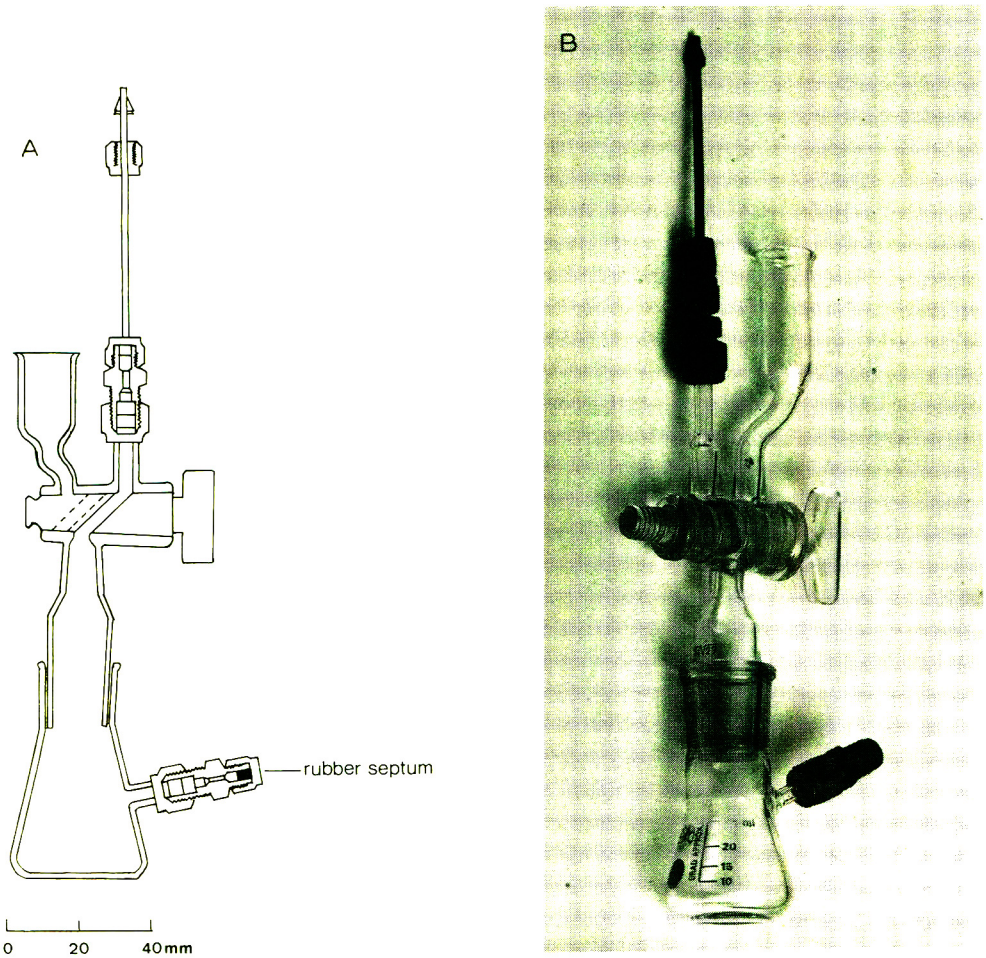

Fig. 1. Flask used for preparation of headspace samples. After alkalinization of volatile amine hydrochloride salts under vacuum, the flask is held at 50°C for 15 min, filled with helium and a headspace sample is collected in a 5-ml glass syringe through the lateral rubber septum.

300) equipped with flame-ionization detectors (air 350 ml min^{-1}, hydrogen 25 ml min^{-1}, sensitivity $5 \cdot 10^{-11}$ A f.s.). Injectors and detectors were heated at 220°C. Ammonia was added to the carrier gas (helium, flow-rate 17.6 ml min^{-1}) with a column conditioner as recommended by Dunn et al. [13] and built with Swagelok® fittings and a 30 mm × 6 mm O.D. glass tube, three-quarters filled with an ammonium hydroxide solution (Carlo Erba 30% RPE). Two borosilicate glass columns (2.10 m long, 6.35 mm O.D., 2 mm I.D.) were used, one packed with 28% Pennwalt 223 + 4% potassium hydroxide on 80—100 mesh Gas-Chrom R (Alltech; packing A) and operated isothermally at 80°C after conditioning overnight at 100°C, the other packed with 4.8% PEG 20M + 0.3% potassium hydroxide on 100—120 mesh Carbopack B (packing B, obtained from Professor Di Corcia, University of Rome) and operated isothermally at 50°C after conditioning overnight at 220°C. Retention times were

determined by co-chromatography with authentic standards of analytical grade: trimethylamine, dimethylamine and ethylamine hydrochlorides (Fluka); isopropylamine, isobutylamine and 2-methylbutylamine (Fluka); methylamine (Prolabo); *n*-butylamine, 3-methylbutylamine and *n*-pentylamine (Poly-Science); piperidine and pyrrolidine (Janssen-Chimica).

GC—MS analysis

A Riber R-10-10 quadrupole mass spectrometer coupled to a Girdel 30S gas chromatograph was operated in both electron-impact ionization mode (ionization potential 70 eV) and chemical ionization mode (ammonia 0.1 kPa, L'Air Liquide, purity ⩾ 99.96%). Chromatographic separation was performed on packing B with a temperature programme from 50°C to 170°C at 10°C min^{-1}. The operating mass range was 20—150 a.m.u. (*m/e* 28 and 32 excluded) and the scan rate was 245 a.m.u. sec^{-1}. Recording and calculation of mass spectra were done with a System Industries—Digital Equipment Corporation PDP/8M calculator. Mass spectra were compared with those of previously listed authentic standards and those of MS registers.

RESULTS AND DISCUSSION

Retention times of VA authentic standards in headspace GC analyses are given in Table I. Two different chromatographic techniques were necessary: Pennwalt 223 Amine Packing separated VA except methylamines as previously

TABLE I

RETENTION TIMES OF VOLATILE AMINE AUTHENTIC STANDARDS IN HEADSPACE GC ANALYSIS

Borosilicate glass columns (2.10 m long, 6.35 mm O.D., 2 mm I.D.). Injector temperature: 220°C. Detector: flame-ionization, temperature 220°C, attenuation $5 \cdot 10^{-11}$ A f.s. Carrier gas: helium (flow-rate 17.6 ml min^{-1}) loaded with ammonia.

	Retention time (min:sec)	
	28% Pennwalt 223 + 4% potassium hydroxide on 80—100 mesh Gas-Chrom R (80°C isothermal)	4.8% PEG 20M + 0.3% potassium hydroxide on 100—120 mesh Carbopack B (50°C isothermal)
Methylamine		0:54
Dimethylamine		2:00
Ethylamine		2:48
Trimethylamine	0:54	3:24
Isopropylamine	1:21	
n-Propylamine	1:54	
Isobutylamine	3:12	
n-Butylamine	4:09	
Pyrrolidine	6:30	
2-Methylbutylamine	6:45	
3-Methylbutylamine	7:00	
n-Pentylamine	8:30	
Piperidine	12:00	

reported by Dunn et al. [13]. For this latter purpose, packing B [9] was chosen. A preliminary study showed that a column packed with 4% Carbowax 20M + 0.8% potassium hydroxide on 60—80 mesh Carbopack B (commercially available from Supelco) could not be proposed for routine analysis of bacterial VA because of important instability of retention times in long-term use as sometimes noted [9, 19]. Furthermore, separation of C_1—C_6 VA on packing B would require temperature programming.

As ammonia prevents adsorption of VA in chromatographic columns, its addition to an amine solution is suitable for injection of liquids [10]. The headspace gas above an aqueous solution of VA and ammonia (1000 ppm) was sampled and injected but ghosting problems were still encountered. Alternative addition of ammonia to the carrier gas was much more efficient; this was attributed to the continuous presence of this base in the column and caused no analytical problem but a small and regular decrease of the baseline which was easily corrected with a second column in dual mode.

Results of analysis of VA produced by nine strains of *Clostridium* are summarized in Tables II and III. Chromatograms obtained with *C. sordellii* 82 are shown in Fig. 2. In previous studies of cultures of *Clostridium* by direct reduced-pressure headspace GC [20, 21], only trimethylamine was found as a VA. Even when replacing the flame-ionization detector by a thermoionic specific detector, no VA other than trimethylamine was detected in this work. This could have been due to the low concentration in the cultures and to the hydrogen-bonding ability of primary and secondary amines in the GC system and the aqueous medium. Problems associated with this phenomenon did not appear with trimethylamine. Thus it was thought advisable to isolate and concentrate VA as hydrochloride salts by vacuum distillation, a suitable method for such volatile compounds [16]. After alkalinization of the corresponding salts, injection of VA bases was tested in three modes: after extraction with a solvent (dodecane, standard for GC, Carlo-Erba), in aqueous solution and by headspace sampling. In the first modality, detection of late eluted dodecane did not interfere with that of low-molecular-weight VA but

TABLE II

VOLATILE AMINES PRODUCED BY NINE STRAINS OF *CLOSTRIDIUM* AND ANALYSED BY HEADSPACE GC ON A PENNWALT 223 COLUMN

Packing: 28% Pennwalt 223 + 4% potassium hydroxide on 80—100 mesh Gas-Chrom R. Other analytical conditions as in Table I. Results are arbitrarily expressed as peak area (mm^2), and are given as mean (n = 3 for each bacterial strain, n = 2 for uninoculated medium) with standard deviation in parentheses. For retention times of amines see Table I.

Bacterial strain	Trimethylamine	Unidentified compound*	Isobutyl-amine	3-Methylbutyl-amine	Unidentified compound**
C. ghoni ATCC 25757	8085 (1534)	1323 (90)	152 (62)	727 (184)	693 (104)
C. bifermentans TM	166,400 (7259)		1507 (496)	1140 (145)	
C. sordellii 82	87,040 (11,809)		237 (95)	24,309 (17,238)	
C. lituseburense ATCC 25759	5056 (832)		201 (44)	705 (359)	
C. mangenotii ATCC 25761	209,333 (15,123)	11,147 (760)	429 (105)	1592 (253)	1591 (362)
C. histolyticum Tro 2E	1495,040 (355,314)		859 (280)	1214 (130)	
C. perfringens Lechien	5679 (1222)		218 (113)	887 (296)	
C. difficile ATCC 9689	5957 (1104)		187 (61)	637 (146)	
C. cadaveris ATCC 25783	425,600 (56,708)		285 (41)	2007 (605)	
Uninoculated medium	5688 (577)		91 (16)	452 (6)	

*Retention time 1 min 48 sec.
**Retention time 10 min 48 sec.

TABLE III

VOLATILE AMINES PRODUCED BY NINE STRAINS OF *CLOSTRIDIUM* AND ANALYSED BY HEADSPACE GC ON A PEG 20M COLUMN

Packing: 4.8% PEG 20M + 0.3% potassium hydroxide on 100—120 mesh Carbopack B. Other analytical conditions as in Table I. Results are arbitrarily expressed as peak area (mm^2), and are given as mean (n = 3 for each bacterial strain, n = 2 for uninoculated medium) with standard deviation in parentheses. For retention times of amines see Table I.

Bacterial strain	Methylamine	Dimethylamine	Ethylamine	Trimethylamine
C. ghoni ATCC 25757		215 (129)	517 (7)*	9755 (2019)
C. bifermentans TM		83 (17)	372 (6)	139,520 (37,989)
C. sordellii 82	6875 (947)	1984 (816)		135,453 (8032)
C. lituseburense ATCC 25759	109 (12)	89 (8)	787 (45)	8176 (1343)
C. mangenotii ATCC 25761	272 (68)*	2500 (399)		144,043 (13,899)
C. histolyticum Tro 2E	88 (14)	107 (10)	1988 (162)	1132,893 (137,116)
C. perfringens Lechien	2107 (158)	102 (16)	254 (30)	8128 (356)
C. difficile ATCC 9689		104 (18)	1053 (724)	6955 (2947)
C. cadaveris ATCC 25783			645 (263)	302,933 (51,177)
Uninoculated medium	35 (7)	61 (27)	2610 (127)	5640 (509)

*The peak area could be determined only from two chromatograms.

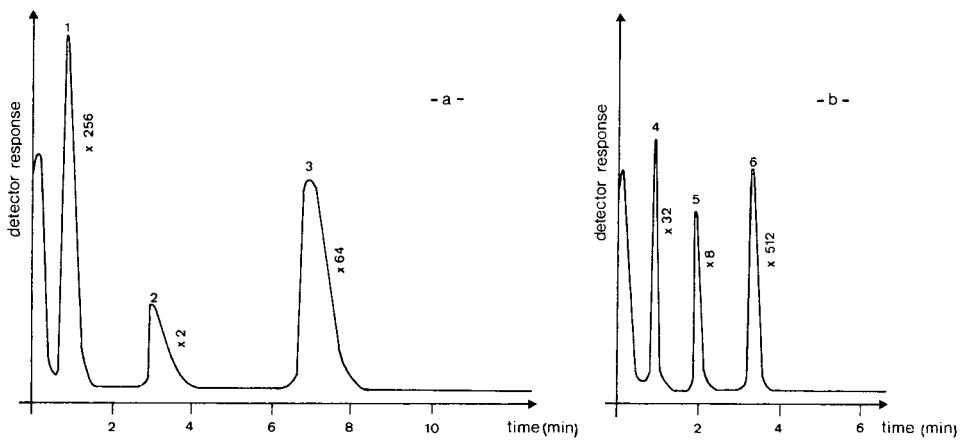

Fig. 2. Chromatograms of volatile amines produced by *C. sordellii* 82. Borosilicate glass columns (2.10 m long, 6.35 mm O.D., 2 mm I.D.) packed with 28% Pennwalt 223 + 4% potassium hydroxide on 80—100 mesh Gas-Chrom R (a) (80°C isothermal) or with 4.8% PEG 20M + 0.3% potassium hydroxide on 100—120 mesh Carbopack B (b) (50°C isothermal). Other analytical conditions as in Table I. Peaks: 1 and 6 = trimethylamine; 2 = isobutylamine; 3 = 3-methylbutylamine; 4 = methylamine; 5 = dimethylamine.

made repetitive analyses impossible. In the second modality, the injected volume was necessarily small, interfering peaks due to water appeared and crystallization of sodium chloride in the head of the column was bothersome. In the third modality, preliminary reduced-pressure headspace GC analyses were performed but adsorption of VA in the stainless-steel gas sampling valve could not be minimized. Therefore the direct injection of helium-diluted headspace gas, sampled with a glass syringe, was preferred.

In addition to co-chromatography, identification of VA was carried out by GC—MS. Electron-impact ionization of primary VA yields a small number of fragment ions (mainly *m/e* 30) and a low intensity molecular peak. Chemical ionization with ammonia as reagent gas was helpful for the determination of

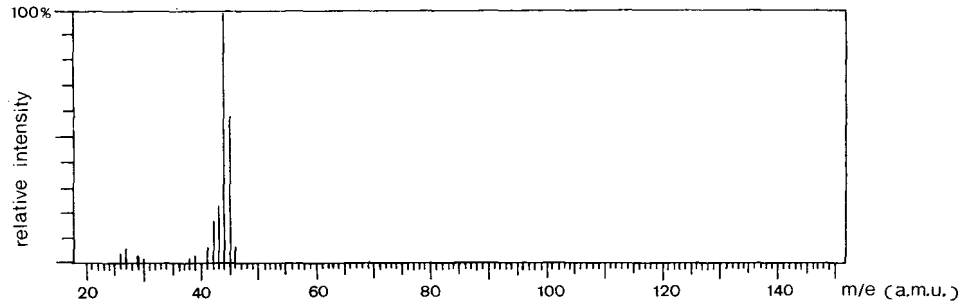

Fig. 3. Electron-impact mass spectrum of dimethylamine (molecular mass M = 45) from a sample of *C. mangenotii* ATCC 25761 (ionization potential 70 eV).

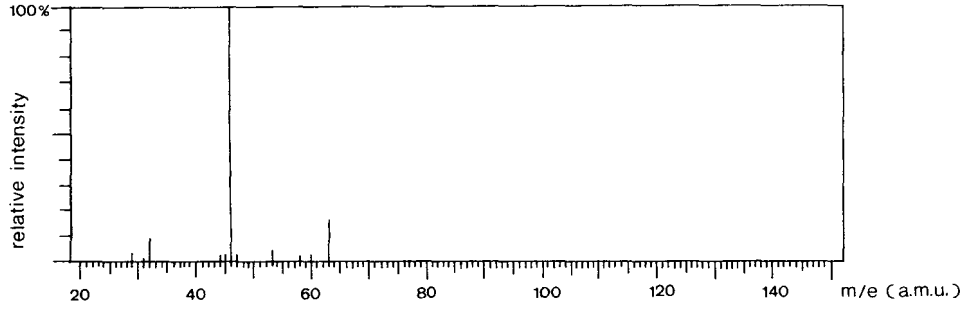

Fig. 4. Chemical ionization mass spectrum of dimethylamine (molecular mass M = 45) from a sample of *C. sordellii* 82, showing characteristic peaks M + 1 = 46 and M + 18 = 63 (ammonia 0.1 kPa).

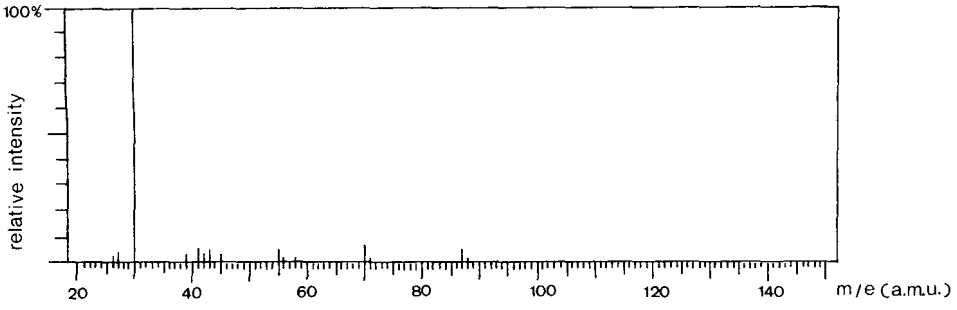

Fig. 5. Electron-impact mass spectrum of 3-methylbutylamine (molecular mass M = 87) from a sample of *C. mangenotii* ATCC 25761 (ionization potential 70 eV).

molecular mass. Mass spectra of dimethylamine (Figs. 3 and 4), trimethylamine, isobutylamine and 3-methylbutylamine (Figs. 5 and 6) were obtained with samples of *C. sordellii* or *C. mangenotii*. The profile of the ion *m/e* 30 showed a characteristic peak at the retention time of methylamine and a small peak of 2-methylbutylamine, eluted just before 3-methylbutylamine, which were difficult to discern on the total-ion current chromatogram. Another compound was detected which could not be identified as diethylamine or methylisopropylamine because of the similarity of their mass spectra.

GC analysis of bacterial amines has been proposed as a tool for identification

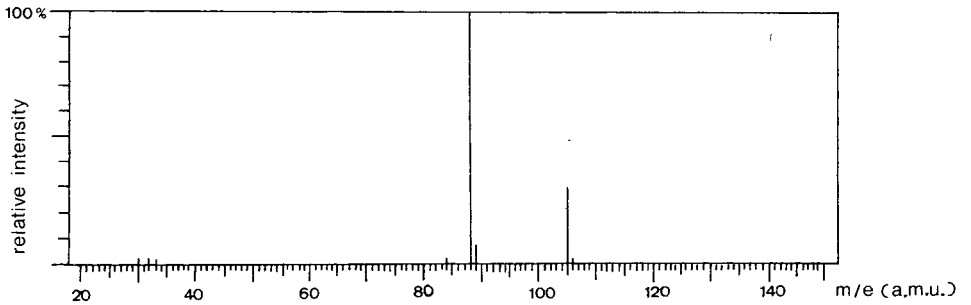

Fig. 6. Chemical ionization mass spectrum of 3-methylbutylamine (molecular mass M = 87) from a sample of *C. sordellii* 82, showing characteristic peaks M + 1 = 88 and M + 18 = 105 (ammonia 0.1 kPa).

of *Proteus* [7, 8, 14, 22], *Clostridium* [7, 23] and other pathogenic bacteria [24]. It was especially developed for the differentiation between *C. sordellii* and *C. bifermentans*: analysis of amine derivatives showed that β-phenylethylamine and tryptamine were produced by *C. bifermentans* [25, 26] whereas 3-methylbutylamine (isopentylamine) was produced by *C. sordellii* [15] as previously reported [27]. In this work, GC—MS analysis of concentrated bacterial VA confirmed 3-methylbutylamine with *C. sordellii* 82. Other decarboxylation amines such as the isomer 2-methylbutylamine and isobutylamine could be detected along with methylamines. Occurrence of methylamine and dimethylamine with *C. sordellii* 82 and of isobutylamine mainly with *C. bifermentans* TM may be mentioned as new distinctive characters of these strains. A preliminary distillation step is required in this work but seems necessary for recovery and concentration of VA traces. The GC technique proposed here presents all the advantages attributed to headspace analysis of amines [12], classical problems of VA analysis being overcome with ammonia conditioning of the column.

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ASSESSMENT OF HEAD-SPACE GAS—LIQUID CHROMATOGRAPHY FOR THE RAPID DETECTION OF GROWTH IN BLOOD CULTURES

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SUMMARY

Blood for transfusion was inoculated with between 10^0 and 10^2 colony-forming units (CFU) per ml of each of 59 microbial isolates and added to cooked meat broth. At intervals up to 72 h incubation, the cultures were examined by conventional visual inspection and automated head-space gas—liquid chromatography (HS-GLC). Forty-six isolates including all those examined of *Staphyloccoccus aureus, Streptococcus pyogenes, S. pneumoniae, S. faecalis, S. milleri, S. mitior, S. mitis, S. salivarius, S. sanguis, Escherichia coli, Klebsiella pneumoniae, K. oxytoca, Proteus mirabilis, Morganella morganii, Serratia* sp., *Enterobacter cloacae, Bacterioides fragilis, Clostridium perfringens, Candida albicans, C. krusei* and *Torulopsis glabrata,* and three isolates of *Staphylococcus epidermidis,* were detected by HS-GLC. HS-GLC failed to detect the growth of eleven isolates including all those of *Pseudomonas aeruginosa, Acinetobacter calcoaceticus, Haemophilus influenzae, Corynebacterium* sp. and two isolates of *S. epidermidis.* The growth of all 59 isolates was detected by visual inspection. No significant difference was found between HS-GLC analysis and visual inspection in the speed of detection of bacterial isolates. All the yeast isolates were detected by HS-GLC after 24 h incubation, indicating that it may be possible to detect fungemias earlier by HS-GLC analysis than by other methods.

INTRODUCTION

One of the highest priorities of bacteriology today is the early detection of bacteremia and septicemia. In most clinical laboratories the detection of growth in blood cultures still depends on the classical method of visual inspection. Despite close attention to macroscopic changes, Gram staining and subculturing, only 50% of positive blood cultures are detected within 24 h of blood collection [1], and therefore results of positive cultures may not be available to the clinician until 72 h after the blood was taken for culture.

Various techniques such as radiometry [2, 3], impedance measurement [4] and centrifugation—filtration [5] have been recently developed for the rapid

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diagnosis of bacteremia and septicemia. Analysis by head-space gas—liquid chromatography (HS-GLC) to detect volatile microbial metabolic products has been proposed as a method for the diagnosis of bacteremia [6, 7], an approach that has already proved useful for the rapid detection of urinary tract infection [8].

The present report compares the rapidity and sensitivity of the HS-GLC method with that of visual inspection for detecting growth in simulated blood cultures.

MATERIALS AND METHODS

Microorganisms

A total of 59 isolates was used in this investigation including five strains each of *Staphylococcus aureus*, *S. epidermidis*, *Streptococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*, four each of *Haemophilus influenzae* and *Proteus mirabilis*, three each of *Klebsiella pneumoniae* and *Candida albicans*, two each of *Streptococcus pyogenes*, *S. pneumoniae* and *Klebsiella oxytoca*, and one each of *Streptococcus milleri*, *S. mitior*, *S. mitis*, *S. salivarius*, *S. sanguis*, *Corynebacterium* sp., *Morganella morganii*, *Serratia* sp., *Enterobacter cloacae*, *Acinetobacter calcoaceticus*, *Bacteroides fragilis*, *Clostridium perfringens*, *Candida krusei* and *Torulopsis glabrata*. These microorganisms were identified using standard methods [9] and the API 20E system (Analytab Products, Plainview, NY, U.S.A.). The microorganisms were then cultured on appropriate solid media and stored at 4° C.

Simulated blood cultures

The medium used was brain—heart infusion broth (OXOID) containing 0.05% sodium polyanethol sulphonate and cooked bullock's heart. The medium was dispensed to give a final volume of 35 ml in 60-ml capacity glass screw-capped bottles and autoclaved at 121° C for 30 min. Immediately before incubation, 5 ml of whole human blood containing 0.327% (w/v) citric acid, 2.63% (w/v) sodium citrate, 0.251% (w/v) sodium acid phosphate and 2.32% (w/v) dextrose (Red Cross Blood Bank, Melbourne, Australia) were inoculated with one of the 59 microbial isolates and added to the culture medium. Inoculated blood media and control media containing uninoculated blood were incubated at 37° C in air and examined by both HS-GLC and visual inspection after 6, 9, 12, 15, 18, 24, 48 and 72 h incubation.

Quantitation of inoculum sizes

Viable counts on overnight cultures in brain—heart infusion broth of the microorganisms under test were performed to determine the inoculum size necessary to provide 10^0—10^2 colony-forming units (CFU) per ml blood in the simulated blood cultures. On the basis of these experiments, each 5-ml aliquot of blood for blood cultures was inoculated with 50 μl of a similar overnight culture diluted 10^{-6} for bacteria and 10^{-4} for yeasts. Confirmatory viable counts to determine the actual size of each inoculum were performed on the culture dilutions used to inoculate blood.

Analysis by HS-GLC

The GLC conditions were as described previously [8], except that the analysis time was 1.5 min. Head-space vapour was released from simulated blood cultures for GLC analysis by adding 2 ml blood culture supernatant to 3 g potassium carbonate in a glass vial provided with the automatic head-space injector, sealing immediately, shaking on a vortex mixer and holding in the injector turn-table at 60°C for injection. The column packed with 0.4% Carbowax 1500 on graphite 60—80 mesh was a new one and the retention times of ethanol, *n*-propanol and trimethylamine were 0.4 min, 0.675 min and 0.55 min, respectively.

Method of visual inspection

The inoculated blood cultures were inspected for the following signs of macroscopic growth: (1) gas bubbles; (2) lysis of blood; (3) reduction of hemoglobin; (4) turbidity; and (5) digestion of meat. Cultures exhibiting any of these features were stained by Gram's stain and subcultured onto appropriate media.

RESULTS

HS-GLC analysis was assessed by comparison with visual inspection for (a) its effectiveness in detecting a range of species (sensitivity) and (b) its speed in detecting the species (rapidity).

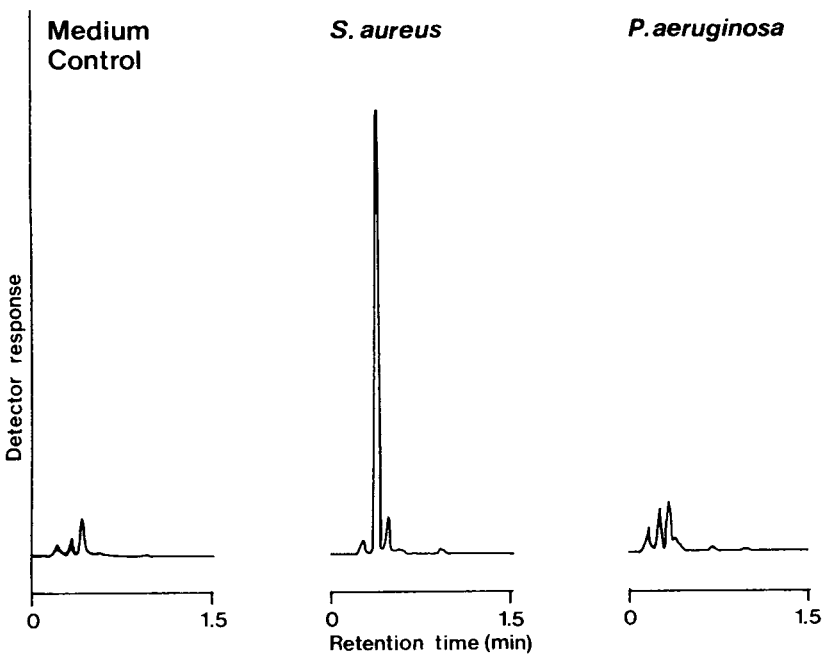

Fig. 1. Chromatograms of incubated, uninoculated blood culture medium (medium control) and of simulated blood cultures inoculated with *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The principal peak in the chromatogram of the *S. aureus* blood culture is ethanol, with a retention time of 0.4 min.

Range of species detected by HS-GLC

Simulated blood cultures from all 59 microbial isolates, comprising 22 species, showed growth by visual inspection. HS-GLC analysis of uninoculated simulated blood culture media yielded chromatograms in which the peaks were small both before and after incubation (Fig. 1) compared with the size of the ethanol peaks in chromatograms of cultures in which growth was detected by HS-GLC (Fig. 1). Ethanol was detected in 46 of the 59 cultures (Table I) viz. 22 isolates of Gram-positive cocci, including three isolates of *S. epidermidis*, all seventeen isolates of *Enterobacteriaceae*, both isolates of anaerobes and all five isolates of yeasts. Ethanol was not detected in thirteen cultures including all five isolates of *P. aeruginosa* (Fig. 1), one isolate of *A. calcoaceticus*, all four isolates of *H. influenzae*, one isolate of *Corynebacterium* sp. and two isolates of *S. epidermidis*.

After 24 h incubation, a peak with the same retention time as *n*-propanol

TABLE I

RANGE OF SPECIES DETECTED BY HS-GLC IN SIMULATED BLOOD CULTURES

Ethanol in cultures	Species of microorganism	No. of isolates
Produced	*Staphylococcus aureus*	5
	Staphylococcus epidermidis	3
	Streptococcus pyogenes	2
	Streptococcus pneumoniae	2
	Streptococcus faecalis	5
	Streptococcus milleri	1
	Streptococcus mitior	1
	Streptococcus mitis	1
	Streptococcus salivarius	1
	Streptococcus sanguis	1
	Escherichia coli	5
	Klebsiella pneumoniae	3
	Klebsiella oxytoca	2
	Proteus mirabilis	4
	Morganella morganii	1
	Serratia sp.	1
	Enterobacter cloacae	1
	Bacteroides fragilis	1
	Clostridium perfringens	1
	Candida albicans	3
	Candida krusei	1
	Torulopsis glabrata	1
Not produced	*Pseudomonas aeruginosa*	5
	Acinetobacter calcoaceticus	1
	Haemophilus influenzae	4
	Corynebacterium sp.	1
	Staphylococcus epidermidis	2
	Uninoculated culture medium	10

was detected in twelve cultures, including all five isolates of *E. coli*, all three isolates of *K. pneumoniae*, both isolates of *K. oxytoca* and the isolates of *M. morganii* and *C. perfringens*. After 24 h incubation, a peak with the same retention time as trimethylamine was detected in all four isolates of *P. mirabilis*.

Speed of detection by HS-GLC

The inoculum sizes (10^0—10^2 CFU/ml of blood) were chosen to simulate the low order of magnitude of microorganisms generally found in the blood of patients with bacteremia [10].

TABLE II

HS-GLC COMPARED WITH VISUAL INSPECTION FOR THE DETECTION OF GROWTH IN SIMULATED BLOOD CULTURES

	Detection time (h)				No. of isolates
	HS-GLC		Visual		
	Range	Median	Range	Median	
Enterobacteriaceae	9—15	9	9—12	12	17
Bacteroides fragilis		48		48	1
Clostridium perfringens		12		12	1
Candida albicans		24		48	3
Candida krusei		24		72	1
Torulopsis glabrata		24		72	1
Staphylococcus aureus	15—18	15	12—48	18	5
Staphylococcus epidermidis	18—24	18	18—24	18	3
Streptococcus pyogenes		15, 18		18	2
Streptococcus pneumoniae		15, 18		18	2
Viridans *Streptococci*	15—24	18	12—15	12	5
Streptococcus faecalis	9—12	9	9—12	9	5

The times at which growth was detected by an ethanol peak in HS-GLC analysis and by visual inspection, are shown in Table II. HS-GLC analyses and visual inspections were done at 3-h intervals between 6 and 18 h. Consequently, if times of detection by HS-GLC and visual inspection are recorded in Table II as differing by 3 h, i.e. one inspection interval, this was the maximum difference and a smaller difference might have been found if the inspection intervals had been shorter. Further analyses and inspections were done at 24 h, i.e. an inspection interval of 6 h, and at 48 and 72 h, i.e. inspection intervals of 24 h. Consequently, differences of 6 h, between 18 and 24 h, or 24 h in times of detection after 24 h, by HS-GLC and visual inspection were maximum differences.

Of the 41 bacterial isolates that showed growth by both HS-GLC analysis and visual inspection, 17 were detected at the same time by both methods, 15 were detected earlier by HS-GLC analysis and 9 earlier by visual inspection. In all but three instances of earlier detection, by one method or the other, the difference was only one inspection interval, usually a maximum of 3 h. In cultures of one isolate of *S. aureus*, growth was detected by HS-GLC analysis

more than one inspection interval earlier than visual inspection; and in the cultures of *S. mitis* and *S. sanguis*, growth was detected by visual inspection more than one inspection interval earlier than by HS-GLC analysis.

The five yeast isolates showed growth by both HS-GLC analysis and visual inspection. All were detected earlier by HS-GLC analysis, three by a maximum of 24 h (one inspection interval) and two by more than 24 h.

DISCUSSION

The assessment of HS-GLC analysis was based upon the sensitivity and rapidity of the technique compared with that of the conventional blood culture method. No false positive results were obtained in our investigation. With HS-GLC analysis, the detection of ethanol production during incubation of blood cultures always indicated microbial growth. The likely source in the blood culture medium from which ethanol is produced is glucose, a carbohydrate that is known to be fermented by all of the detected isolates [9]. However, other compounds present in both the brain—heart infusion broth and the transfusion blood may also be possible sources from which ethanol could be produced. It should be noted that the transfusion blood used for the simulated blood cultures was buffered and contained added dextrose and citrate. Both additives are energy sources for microbial growth. In addition, the inocula for the simulated blood cultures were organisms that had been maintained in the laboratory and thus may have been physiologically different from organisms in the blood of a bacteremic patient. Therefore the sensitivity and rapidity of HS-GLC analysis and the rapidity of visual inspection for the detection of growth in simulated blood cultures may differ from routine blood cultures.

The sensitivity of HS-GLC in detecting a range of species when compared to visual inspection, was 78%. The variety of species detected was similar to those of previous studies [6, 7]. Species detected included *S. aureus*; eight species of *Streptococcus*; seven species of *Enterobacteriaceae*; three species of yeast including *C. albicans*, *C. krusei* and *T. glabrata*; and two anaerobic species, *B. fragilis* and *C. perfringens*.

Production of *n*-propanol by *E. coli* or a related species, and trimethylamine by *P. mirabilis*, have been previously reported [7]. In this investigation, small amounts of *n*-propanol and trimethylamine, compared with ethanol, were detected in 24-h cultures of *E. coli* and *P. mirabilis*, respectively, and may have been detected in early cultures if an integrator had been used. It appears that *n*-propanol and trimethylamine could be useful as markers for the presumptive identification of certain species of *Enterobacteriaceae*.

Species not detected by HS-GLC analysis included two established pathogens, *P. aeruginosa* and *H. influenzae*; an opportunistic pathogen, *A. calcoaceticus*; and a non-pathogenic *Corynebacterium* species. Detection of *S. epidermidis* by HS-GLC analysis was variable, confirming the results of previous studies [6, 7].

Overall, there was no significant difference in rapidity between HS-GLC and visual inspection. Detection of the *Enterobacteriaceae* and anaerobes by HS-GLC was either marginally quicker than, or at the same time as, visual inspection. With the exception of the viridans *Streptococci*, which were

detected earlier by visual inspection than by HS-GLC analysis, there was no significant difference in the detection times of the Gram-positive cocci by either method.

The most promising results in terms of rapidity occurred with the yeast isolates. Systemic fungal infections have become an increasing problem in immuno-compromised patients. A short detection time is very important for better management of this type of patient, since fungemia is often detected late in the course of the disease or after death [11]. In this investigation all the yeast isolates were detected by HS-GLC analysis after only 24-h incubation of blood cultures. Bille et al. [11], in a ten-year study on the detection of yeast in blood cultures, found that the recovery time of *Candida* sp. from vented biphasic brain—heart infusion blood culture medium ranged from 1 to 28 days, with mean recovery time of 3.5 days for *C. albicans*, 2.8 days for *C. krusei* and 8.3 days for *T. glabrata*. The average detection times of *C. albicans* and *T. glabrata* by the BACTEC radiometric system have been reported as 2.7 and 4.7 days, respectively [12].

Attempts to detect bacteremia rapidly by other gas chromatographic techniques, including analysis of acidified blood cultures [13] and direct analysis of patients' sera [14], have not been an improvement on traditional methods. Perhaps the use of a more sensitive technique, such as frequency-pulsed, electron-capture GLC [15], might expedite the detection of growth in blood cultures.

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CHROMBIO. 2373

STRUCTURAL ANALYSIS OF UNDERIVATIZED SIALIC ACIDS BY COMBINED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

Mass spectra of chemically ionized, positive ions of underivatized N,O-acylated sialic acids, 2-deoxy-2,3-didehydro-N-acetylneuraminic acid and sialyl-α(2-3)-lactose were obtained by combined high-performance liquid chromatography—mass spectrometry, using a direct liquid inlet system. The mass spectra of the different compounds for which fragmentation schemes are proposed enable the differentiation between sialic acids, although the localization of O-substituents is not possible. However, since the various sialic acids separated well on high-performance liquid chromatography, combined high-performance liquid chromatography—mass spectrometry allowed their unequivocal characterization.

INTRODUCTION

Structural analyses of sialic acids have so far been performed by combined gas—liquid chromatography—mass spectrometry (GLC—MS) [1] or by nuclear magnetic resonance spectroscopy [2]. The general application of these methods, however, suffers from the facts that (i) relatively large amounts of carefully purified sialic acids (50—500 μg) are required, (ii) derivatization is necessary for GLC—MS analysis of sialic acids, and (iii) extensive purification and derivatization steps may lead to intramolecular migration of O-acetyl

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groups or to loss of sialic acids [3, 4]. Recently, a high-performance liquid chromatographic (HPLC) method has been developed for the sensitive analysis of different, naturally occurring N,O-acylated sialic acids and for studies of their metabolic reactions [5, 6]. A major advantage of this method is that derivatization and, in most cases, extensive purification of sialic acids are not required.

In the present study it is shown that HPLC, combined with chemical-ionization mass spectrometry, represents a powerful tool for the characterization of different underivatized sialic acids. It may be useful for the rapid analysis of micro-quantities of sialic acid mixtures, isolated from biological material available only in small amounts, or originating from enzyme reactions metabolizing sialic acids [3].

MATERIALS AND METHODS

Materials

Sialic acids were isolated from bovine, equine and porcine submandibular gland glycoproteins as described in ref. 7. Sialyllactose (Neu5Ac-α(2-3)-lactose)* was prepared from bovine colostrum [8]. Neu5Ac2en was purchased from Boehringer.

High-performance liquid chromatography

HPLC of sialic acids and sialyllactose was performed on a stainless-steel column (40 × 4.6 mm) filled with Aminex A-29, using a HPLC pump (Milton Roy, Model ConstaMetric III). Sialic acids were eluted with ammonium formate (20 m*M*, pH 6) in water—acetonitrile (4:1, v/v) at a flow-rate of 0.5 ml/min and 15—20 bars. Eluted sialic acids were monitored at 200 nm. For further details of this method see ref. 5.

High-performance liquid chromatography—mass spectrometry

For the direct liquid inlet technique, a Hewlett-Packard mass spectrometer (Model 5985 B) equipped with an LC—MS interface was used. HPLC conditions were the same as above, except that 3% of the HPLC effluent was transferred to the mass spectrometer source. At the source temperature of 250°C the buffer components are volatile and act as chemical-ionization reagent gas. In some cases pure sialic acids dissolved in water (5—10 μg per 10 μl) were applied directly without column, on-line to the mass spectrometer.

RESULTS AND DISCUSSION

Since in the combined HPLC—MS system only volatile salts can be used, the previously described sodium sulphate solution [5] was replaced by a water—acetonitrile mixture containing ammonium formate (or acetate). This buffer

*Abbreviations: Neu5Ac = N-acetylneuraminic acid; Neu5Gc = N-glycolylneuraminic acid; Neu5,9Ac_2 = N-acetyl-9-O-acetylneuraminic acid; further abbreviations for mono-, di-, and tri-O-acetylated sialic acids are used correspondingly; Neu5Ac2en = 2-deoxy-2,3-didehydro-N-acetylneuraminic acid. The sialic acid nomenclature corresponds to a proposal made at the Vth International Symposium on Glycoconjugates, held at Kiel-Damp in 1979.

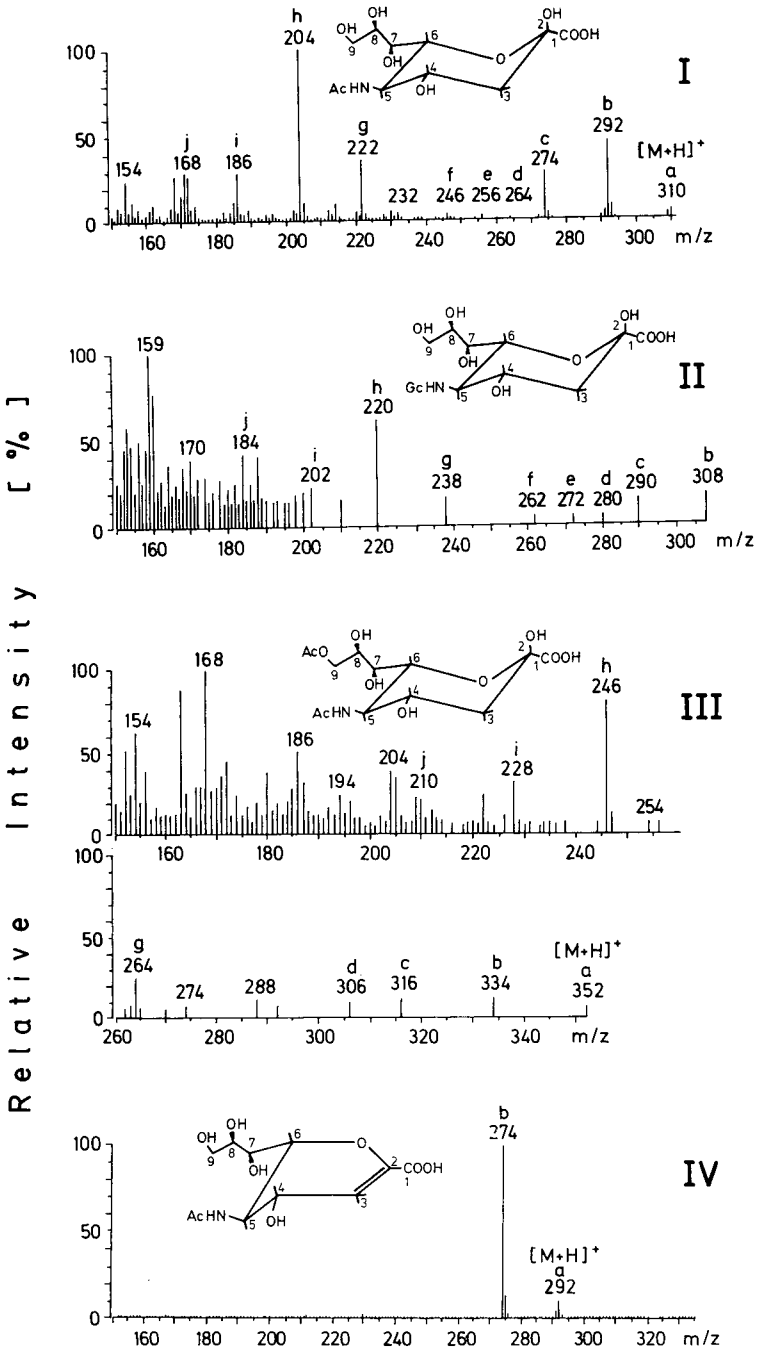

Fig. 1. Mass spectra of chemically ionized, positive ions of underivatized sialic acids obtained by combined HPLC—MS. I, Neu5Ac; II, Neu5Gc; III, Neu5,9Ac$_2$; and IV, Neu5Ac2en. The structural formula and [M + H]$^+$ fragments of sialic acids are shown. The [M + H]$^+$ fragment of Neu5Gc was not recorded in this spectrum. For other sialic acids and for the explanation of fragments see Figs. 2 and 3, and Table I.

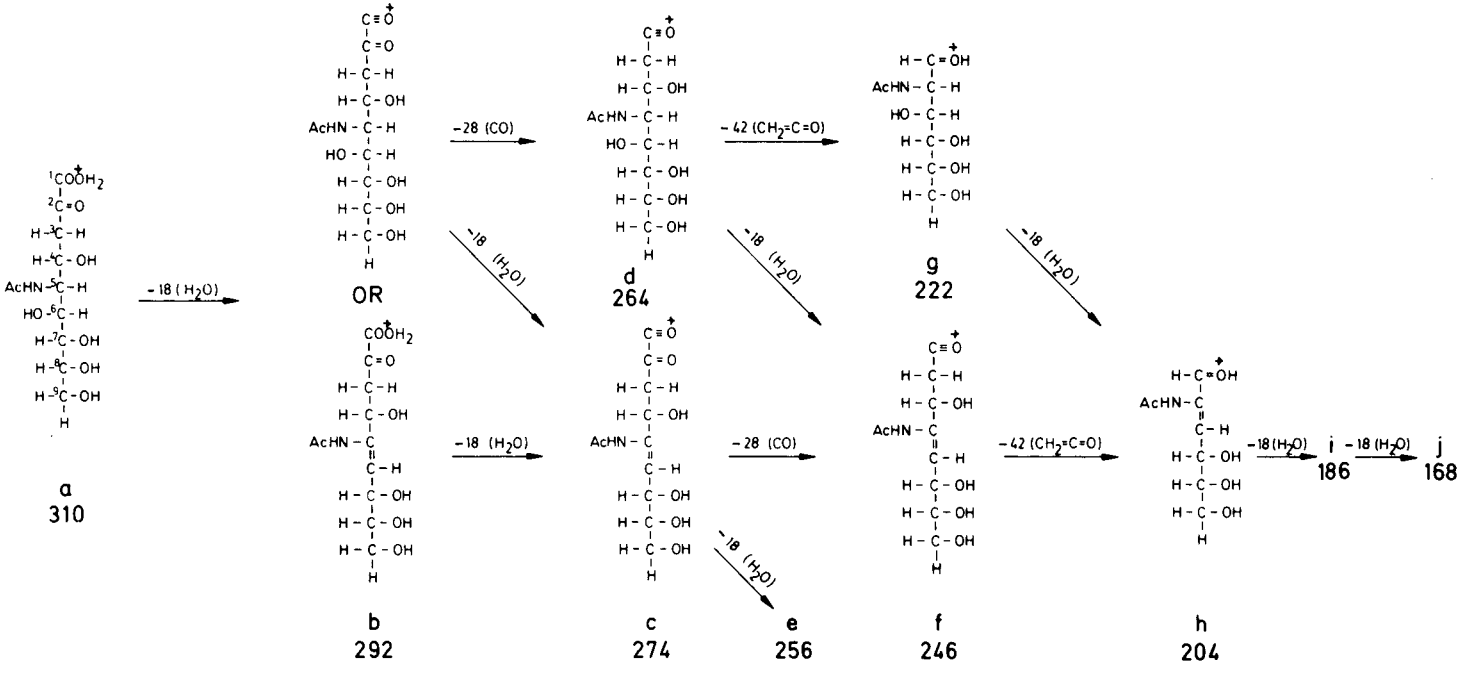

Fig. 2. Mass fragmentation pathway proposed for chemically ionized, positive ions of underivatized Neu5Ac (open-chain form) by combined HPLC—MS.

proved to be most suitable for the recording of mass spectra of underivatized sialic acids, as it volatilizes at the source of the mass spectrometer at a temperature of 250°C and thus acts as chemical-ionization gas. A disadvantage of these volatile salts, as compared to the sodium sulphate solution, however, is a reduction of the sensitivity of the photometric determination of sialic acids by a factor of 10—50.

The mass spectra of Neu5Ac, Neu5Gc, Neu5,9Ac_2 and Neu5Ac2en are shown in Fig. 1. The mass fragments of these and other O-acetylated sialic acids are given in Table I. In the spectra of saturated sialic acids the prominent peak is fragment h, which is considered as the base peak, allowing discrimination between different sialic acids and determination of the degree of O-acetylation of sialic acids. Thus, the *m/z* values of fragment h for Neu5Ac, Neu5,9Ac_2, Neu5,7,9Ac_3 and Neu5,7,8,9Ac_4 differ by 42 units each, corresponding to the difference of one O-acetyl residue each (Table I). Similar differences are exhibited by the other fragments of these sialic acids. The fragmentation patterns of the mono-O-acetylated sialic acids Neu4,5Ac_2, Neu5,7Ac_2 and Neu5,9Ac_2 are qualitatively identical and only slight differences in the intensities of the peaks are seen. Therefore, the position of the O-acetyl groups cannot be elucidated by this method. However, different O-acetylated sialic acids separate well on HPLC, thus allowing the localization of the position of O-acetyl groups indirectly by the use of standards. Neu5Ac and Neu5Gc can readily be discriminated by their mass fragmentation patterns, all *m/z* values differing by 16 mass units. The simplicity of the mass spectrum of Neu5Ac2en showing only two ions at *m/z* = 292 $[M + H]^+$ and 274 (292 − 18) is remarkable (Fig. 1).

Fragmentation reactions induced by chemical ionization of saturated sialic acids and of Neu5Ac2en, which are assumed to lead to the fragments of Fig. 1 and Table I, are shown in Figs. 2 and 3, respectively. This fragmentation pattern can be explained as follows. As most fragments of one sialic acid differ by 18 mass units, it is concluded that the $[M + H]^+$ ions lose several water

TABLE I

MASS FRAGMENTATION OF UNDERIVATIZED SIALIC ACIDS SUBJECTED TO COMBINED HPLC—MS

Compound analysed	Characteristic ions (a—j)* at *m/z*									
	j	i	h	g	f	e	d	c	b	a
Neu5Ac	168	186	204	222	246	256	264	274	292	310
Neu5Ac2en	—	—	—	—	—	—	—	—	274	292
Neu5Gc	184	202	220	238	262	272	280	290	308	326
Neu4,5Ac_2 / Neu5,7Ac_2 / Neu5,9Ac_2	210	228	246	264	—	—	306	316	334	352
Neu5,7,9Ac_3	—	—	288	306	—	—	348	358	376	394
Neu5,7,8,9Ac_4	—	—	330	348	—	—	390	400	418	436

*Ion a corresponds to $[M + H]^+$ and fragments b—j derive from ion a by the loss of water (18 mass units), carbon monoxide (28 mass units) or ketene (42 mass units). See also Figs. 2 and 3.

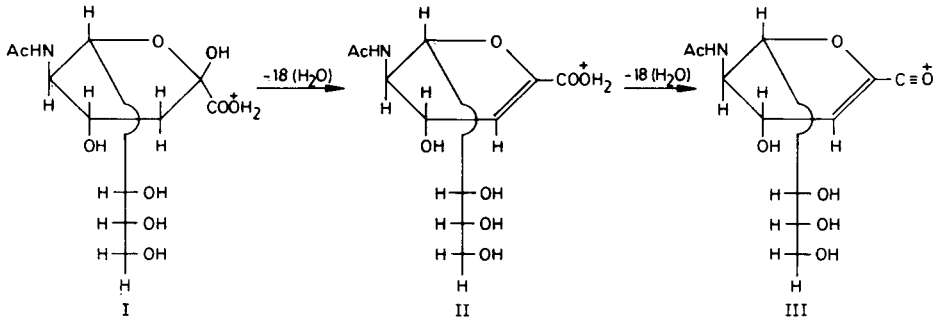

Fig. 3. Mass fragmentation pathway proposed for chemically ionized, positive ions of underivatized Neu5Ac (ring form) and Neu5Ac2en by combined HPLC—MS. I, $[M + H]^+$, m/z = 310 for Neu5Ac; II, $[M + H]^+$, m/z = 292 for Neu5Ac2en, or $[M + H - H_2O]^+$, m/z = 292 for Neu5Ac; III, $[M + H - H_2O]^+$, m/z = 274 for Neu5Ac2en, or $[M + H - 2H_2O]^+$, m/z = 274 for Neu5Ac.

molecules until a stable ion is formed. Fragment h, for example, with m/z = 204 for Neu5Ac, seems to be a most favourable ion as it is the promiment peak. This is also true for the corresponding fragments h of Neu5Gc (m/z = 220) and Neu5,9Ac_2 (m/z = 246) (Fig. 1). A scheme for the formation of the h fragment from Neu5Ac including the elimination of two water molecules and of CO and CH_2=C=O groups is shown in Fig. 2. It is imaginable that for Neu5Ac the h fragment (m/z = 204) can also be formed by the removal of the acetyl group (43 mass units) from the nitrogen atom of fragment b, in addition to the carboxyl residue (45 mass units; not shown in Fig. 2). Assuming this fragmentation pathway for Neu5Gc, the removal of the glycolyl group (59 mass units) and of the carboxyl group from fragment b would result in a h fragment of m/z = 204. As such a peak is missing in the mass spectrum of Neu5Gc and the prominent h fragment is m/z = 220 (Fig. 1), the N-acyl groups seem not to be removed during the fragmentation of sialic acid (Fig. 1). Thus, the formation of the h fragments appears only to be possible by the removal of the C-1 to C-3 part of the nine-carbon chain of sialic acids according to the fragmentation pattern shown in Fig. 2. The assumption of the removal of a H_2O molecule between C-5 and C-6 from $[M + H]^+$ or $[M + H - H_2O]^+$ ions resulting in fragment c as depicted in Fig. 2 is supported by the observation that sialic acids with O-acetyl groups at C-4, C-7, C-8 or C-9 form ion c without losing the acetyl group (see Table I). The fragments discussed so far, with the exception of the upper form of fragment b of Fig. 2, can be explained by the existence of the open-chain configuration of the sialic acids. The existence of this form of free, underivatized sialic acid has been demonstrated by proton-NMR spectoscopy [2]. Therefore, as expected, the fragmentation of underivatized sialic acid is different from the well known fragmentation scheme observed for the electron-impact mass spectra of the methyl ester, per-O-trimethylsilyl ether derivatives of sialic acids having as glycosides ring structures [1].

As part of the Neu5Ac and the other saturated sialic acid molecules in aqueous solution are present in ring form, formation of fragment b from Neu5Ac may also occur as shown in Fig. 3. The first fragment formed

($[M + H - H_2O]^+$, m/z = 292) corresponds to Neu5Ac2en and the second ion represents $[M + H - 2H_2O]^+$. However, comparison of the peak intensities (fragments b and h of Neu5Ac) indicates that fragmentation of Neu5Ac mainly occurs in the open chain form. Neu5Ac2en, which only exists in ring form, shows a quite different fragmentation behaviour when compared with that of saturated sialic acids (Fig. 1). It forms almost exclusively the $[M + H]^+$ and $[M + H - H_2O]^+$ ions (Fig. 3).

The trisaccharide sialyllactose was also analysed by HPLC—MS without prior derivatization. The mass spectrum for sialyl-α(2-3)-lactose is presented in Fig. 4. It exhibits the fragments for lactose ($[M + H]^+$, m/z = 343), and for galactose or glucose (m/z = 180), and also shows the typical fragmentation pattern for Neu5Ac. However, the molecular ion peak for Neu5Ac is missing in this spectrum.

In conclusion, the analytical data presented demonstrate that the HPLC—MS technique is useful for the structural analysis of free or glycosidically bound sialic acids. The method does not require derivatization steps and amounts of 1—10 μg sialic acids can rapidly be analysed.

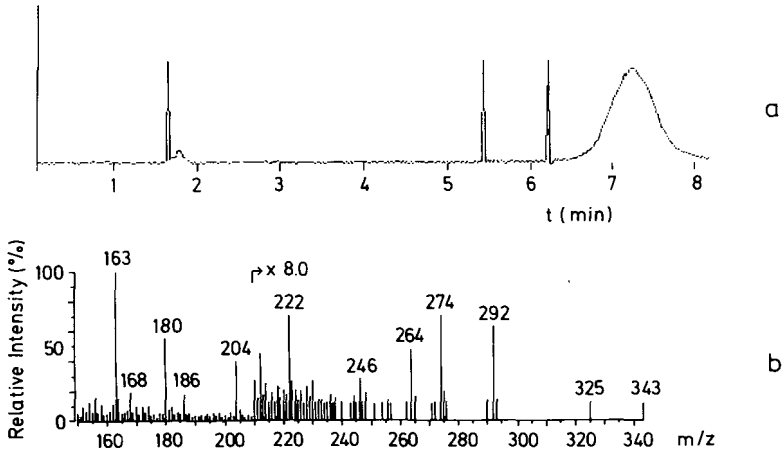

Fig. 4. Analysis of underivatized sialyl-α(2-3)-lactose by HPLC—MS in the positive-ion mode. (a) Total-ionization chromatogram (sharp peaks are due to air bubbles). (b) Mass spectrum; explanation of fragments: m/z = 343, $[\text{lactose} + H]^+$; m/z = 325, $[\text{lactose} + H - H_2O]^+$; m/z = 292, 274, 264, 246, 222, 204, 186 and 168 are typical fragments of Neu5Ac; $[M + H]^+$ of Neu5Ac is not visible; m/z = 180 represents glucose or galactose.

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CHROMBIO. 2385

SIMULTANEOUS ASSAY OF 3,4-DIHYDROXYPHENYLALANINE, CATECHOLAMINES AND O-METHYLATED METABOLITES IN HUMAN PLASMA USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

We devised a procedure for the simultaneous determination of 3,4-dihydroxyphenylalanine, catecholamines and O-methylated metabolites using a reversed-phase liquid chromatographic system. Detection is achieved by an electrochemical detector and a fluorescence detector connected in series. Sample preparation is kept to a minimum, and involves precipitation of proteins with trichloroacetic acid and perchloric acid, and subsequent neutralization, thus omitting the commonly adopted adsorption step. Chromatographic peaks were identified on the basis of retention behaviour and the ratio of responses at several oxidation potentials. The method was applied to the quantitative determination of 3,4-dihydroxyphenylalanine, catecholamines and O-methylated metabolites in human plasma.

INTRODUCTION

Analytical techniques for the catecholamines in tissues and body fluids are extremely useful in medicine and biochemistry. Analysis of compounds such as dopamine (DA) and 3,4-dihydroxyphenylalanine (DOPA), which are neurotransmitters in various tissues such as the brain, is important in the understanding of the central nervous system. Many analytical procedures have been devised for DOPA, catecholamines and their O-methylated metabolites such as 3-methoxy-4-hydroxyphenylalanine (3-O-methyl-DOPA), metanephrine (MN), normetanephrine (NMN) and 3-methoxy-4-hydroxyphenethylamine (3-O-methyl-DA). The quantitative methods for determining these compounds employ gas chromatography—mass spectrometry [1—4], radioenzymatic assay [5—7], and high-performance liquid chromatography (HPLC) with fluorescence detection [8—10] and electrochemical detection (ED) [11—17]. Since the metabolic pathways of DOPA and catecholamines have been

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established, their measurement has become clinically important for certain disease states; for example, patients with Parkinson's disease have lower levels of DA than normal [18]. The diagnosis of some tumours may be made via catecholamine analysis; a useful monitor for neuroblastoma is the increased secretion of DA or noradrenaline (NA) [19]. In addition, the O-methylated metabolites were recognized as major intermediates of DOPA and catecholamines, which play a part in their therapeutic action [20—22]. Therefore, it is necessary to measure these compounds simultaneously. However, no simple HPLC method exists for the simultaneous determination of DOPA, catecholamines and O-methylated metabolites in plasma. Further, with all these methods an extensive clean-up procedure, sometimes followed by a concentration step, is inevitable in order to obtain accurate and precise data.

The present report describes a method for the simultaneous determination of free and conjugated DOPA, catecholamines and O-methylated metabolites in plasma. The procedure requires minimal sample pretreatment utilizing a C_{18} reversed-phase column [23, 24]. Also, the sensitivities afforded by ED and fluorescence detection are compared.

EXPERIMENTAL

Reagents

Adrenaline (A), NA, MN, NMN, DOPA, DA and *o*-tyrosine were purchased from Nakarai, Kyoto, Japan. 3-O-Methyl-DA was obtained from Calbiochem, U.S.A. 3-O-Methyl-DOPA was purchased from Kyowa Hakko, Japan.

HPLC conditions

The HPLC system consisted of a Yanagimoto L-2000 high-speed liquid chromatograph equipped with a Yanagimoto VMD 101 electrochemical detector [25] and a Shimadzu RF-530 fluorescence spectrophotometer. The ED cell was a thin-layer design fitted with a glassy carbon working electrode and an Ag/AgCl reference electrode. The two detectors were connected in series, with the electrochemical detector downstream. The column consisted of a Yanapak ODS (Yanagimoto Manufactory, 10 μm) prepacked column (250 mm × 4.0 mm I.D.). A short precolumn (10 mm × 4.0 mm I.D.) packed with Nucleosil C_{18} (Macherey-Nagel, 10 μm) was used as a guard column. The temperature of the column was set at 25°C, the flow-rate was 0.54 ml/min. A working electrode potential of +0.6 V relative to the Ag/AgCl reference electrode was used for the first 17 min, and then increased to +0.9 V. The change in the working potential caused a significant change in the background current. Accordingly, prior to the analysis, this procedure was repeated several times until the baseline became stable within 6—7 min following the change of potential. The chromatographic system was programmed as follows: 0—6 min: 0.05 *M* phosphate buffer (pH 3.1); 6—36 min: linear gradient of increasing methanol concentration (up to 15%) in phosphate buffer; after 36 min: 15% methanol in phosphate buffer. The fluorescence was monitored using an excitation wavelength of 282 nm and emission wavelength of 322 nm. The mobile phase was passed through a 0.22-μm Millipore filter before introduction into the system and degassed in a vacuum prior to use. The retention of chromato-

graphic peaks was expressed as the capacity ratio, k', which was calculated by the formula $k' = (t_R - t_0)/t_0$, where t_0 and t_R are the retention of an unretained solute and of the solute in question, respectively. Retention times were measured with a Shimadzu C-RIA Chromatopac. The column's dead time, t_0, was measured by noting the first baseline disturbance after hydrochloric acid was injected.

Peak identification

The peaks of DOPA, catecholamines and O-methylated metabolites were identified by a combination of methods. Initially, peak identification of the compounds was performed on the basis of liquid chromatographic retention behaviour and co-injection with the reference compounds. Secondly, ratios of responses at several oxidation potentials (DOPA and catecholamines +0.3V to +0.6 V; metabolites +0.5 V to +0.9 V) were calculated for the reference compounds and compared with those of the peaks in the plasma samples.

Quantitative analysis

The ratios for peak height of compound to that of internal standard were calculated and calibration curves were constructed for each compound; in each case a linear relationship between compound concentration and peak height ratio was observed over the concentration ranges studied. The equations for the calibration curves were constructed by linear regression analysis. Calibration curves for each compound were linear over the range 0.25—100 ng/ml with ED and 1.0—100 ng/ml with fluorescence detection.

Plasma

The five subjects (three men, two women) were members of our university staff and students. The blood samples (venous blood) were obtained from the subjects between 10.00 and 11.00 a.m. Samples of blood (12 ml) were

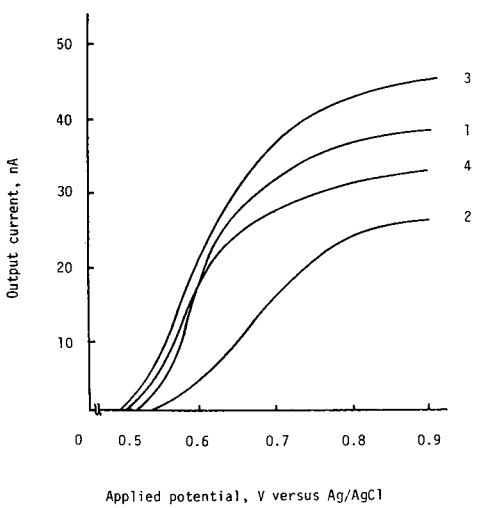

Fig. 1. Relationship between electrochemical detector response and applied potential curves. 1 = NMN; 2 = MN; 3 = 3-O-methyl-DOPA; 4 = 3-O-methyl-DA.

collected into a syringe containing 0.1 ml of sodium heparin (1000 U/ml) and centrifuged immediately at 1000 *g* for 5 min at 4° C.

Free DOPA, catecholamines and O-methylated metabolites. A 2-ml sample of plasma, 100 µl of 1 µg/ml *o*-tyrosine as internal standard and 0.45 ml of

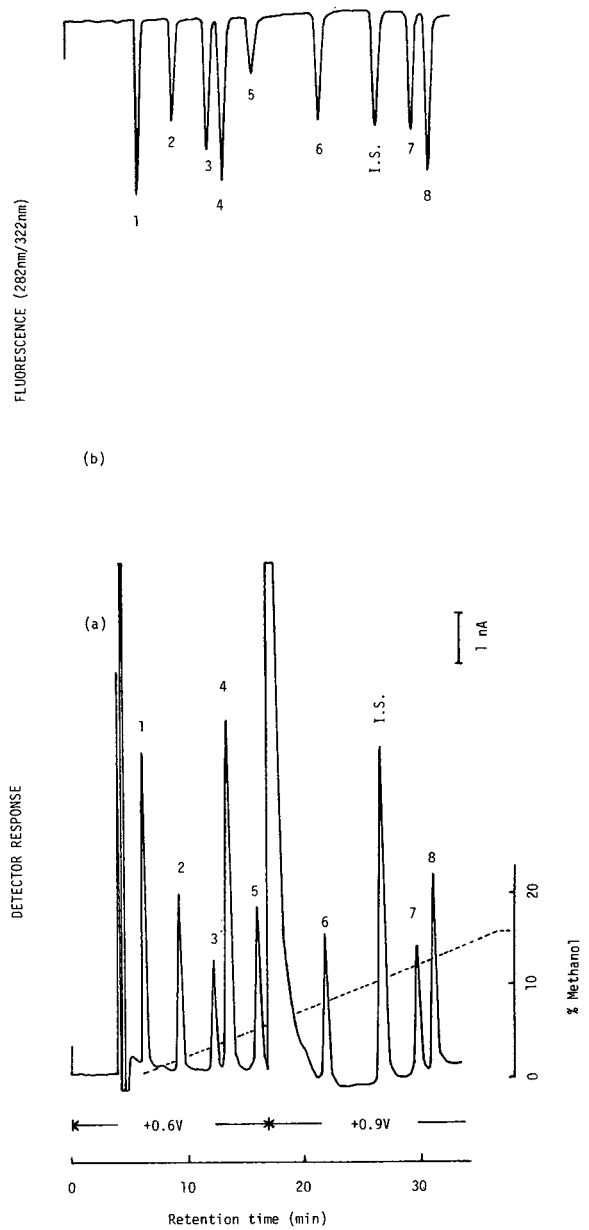

Fig. 2. Chromatograms of a standard solution obtained by HPLC with electrochemical detection (a) and native fluorescence (b). Injection sample: 100 µl of standard solution, containing 20 ng/ml of each compound, and 0.1 µg/ml of *o*-tyrosine. Chromatographic conditions as described in Experimental. Peaks: 1 = NA; 2 = A; 3 = NMN; 4 = DOPA; 5 = DA; 6 = MN; 7 = 3-O-methyl-DOPA; 8 = 3-O-methyl-DA; I.S. = *o*-tyrosine, internal standard.

1.0 *M* trichloroacetic acid were added to a centrifuge tube. The plasma was centrifuged at 10,000 *g* for 10 min. Next, the supernatant was treated with 0.05 ml of 5% potassium hydroxide and centrifuged again.

Total DOPA, catecholamines and O-methylated metabolites. A mixture of 2 ml of plasma containing internal standard and 0.3 ml of 9 *M* $HClO_4$ was heated in a boiling water bath for 20 min. Further, the supernatant was treated with 0.2 ml of 20% potassium hydroxide and centrifuged again.

RESULTS AND DISCUSSION

The response characteristics of the detector system with respect to the applied potentials over the range +0.3 V to +0.9 V versus nA of output current generated for the detection of O-methylated standards are shown in Fig. 1.

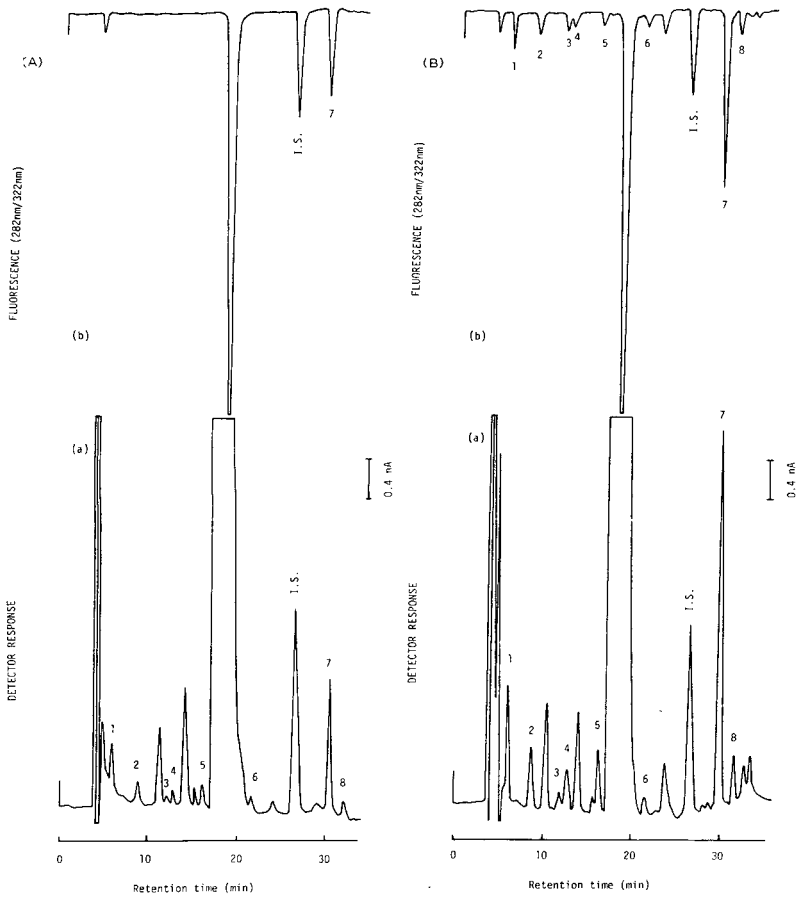

Fig. 3. Chromatograms of a free plasma sample (A) and a total plasma sample (B) obtained by HPLC with electrochemical detection (a) and native fluorescence (b). Injection sample 100 µl of plasma containing (ng/ml); (A) 1, 0.65; 2, 0.37; 3, 0.57; 4, 0.91; 5, 0.39; 6, 0.42; 7, 19.22; 8, 0.42; (B) 1, 2.08; 2, 1.44; 3, 2.55; 4, 2.33; 5, 2.14; 6, 1.88; 7, 45.42; 8, 2.76. Chromatographic conditions as described in Experimental. Peaks: 1 = NA; 2 = A; 3 = NMN; 4 = DOPA; 5 = DA; 6 = MN; 7 = 3-O-methyl-DOPA; 8 = 3-O-methyl-DA; I.S. = *o*-tyrosine, internal standard.

DOPA and catecholamines are easily oxidized at a potential of +0.5 V [13], while O-methylated standards are not readily oxidized at a low potential. Hence, a potential of +0.9 V provided sufficient sensitivity for the determination of O-methylated standards. However, the peak of NMN was rapidly eluted third and had an almost identical retention time to that of DOPA. In the present method, the working electrode operating at a potential of +0.6 V instead of +0.5 V proved to be optimal for the oxidation of DOPA and catecholamines; it was then increased to +0.9 V, the optimum potential for O-methylated metabolites. These settings provide peak/current ratios ranging from 0.5 for NMN to near 1.0 for DOPA and catecholamines. The reversed-phase HPLC separation of the synthetic mixture of DOPA, catecholamines and O-methylated standards, detected amperometrically and by measuring their native fluorescence, is shown in Fig. 2a and b, respectively. When the working potential changed from +0.6 V to +0.9 V, the potential caused a significant change in the background current. Complete separation of individual components of the mixture was obtained. Then, the separation and detection methods were tested in the analysis of plasma samples. Chromatograms of a normal plasma sample prior to, and after, hydrolysis at 100°C are shown in Fig. 3. *o*-Tyrosine served as an internal standard. The chromatogram of the plasma sample was recorded at the most sensitive setting of the electrochemical detector (4 nA full scale). The background seems to be almost free of interfering substances, thus allowing the determination of DOPA, catecholamines and O-methylated metabolites. As evident from Fig. 3A, only 3-O-methyl-DOPA could be detected using the fluorescence detector; its free concentrations in plasma of a normal subject are too low to be detected by the present system. The absolute detection limits for the compounds are listed in Table I. The electrochemical detector is more sensitive than the fluorescence detector. However, the total levels of DOPA, catecholamines and O-methylated metabolites can be determined by both detectors. However, when the working electrode was operated at a potential of +0.9 V from the start, NA in plasma samples could not be determined due to interference by the adjacent peak.

The capacity ratio and chromatographic peak height ratio of the eight

TABLE I

TYPICAL DETECTION LIMITS

Compound	Detection limit* (ng/ml)	
	Amperometric	Fluorescence
NA	0.10	0.50
A	0.10	0.50
NMN	0.20	0.95
DOPA	0.10	0.75
DA	0.15	0.75
MN	0.15	0.95
3-O-Methyl-DOPA	0.20	1.00
3-O-Methyl-DA	0.20	1.00

*Injected quantity giving a signal-to-noise ratio of 2.0.

TABLE II

HPLC CAPACITY RATIOS AND PEAK CURRENT RATIOS AT VARIOUS OPERATING POTENTIALS OF DOPA, CATECHOLAMINES AND O-METHYLATED METABOLITES

	NA	A	NMN	DOPA	DA	MN	3-O-Methyl-DOPA	3-O-Methyl-DA
Capacity ratio	0.6	1.3	2.1	2.4	3.1	4.5	6.5	6.8
300/600 mV	0.02	0.10	N.D.*	0.03	0.10	N.D.	N.D.	N.D.
350/600 mV	0.29	0.26	N.D.	0.38	0.33	N.D.	N.D.	N.D.
400/600 mV	0.90	0.92	N.D.	0.91	0.92	N.D.	N.D.	N.D.
500/600 mV	0.99	0.98	N.D.	0.98	0.99	N.D.	N.D.	N.D.
550/900 mV	1.01	1.01	0.19	1.01	1.01	0.08	0.22	0.20
600/900 mV	1.03	1.02	0.51	1.02	1.03	0.16	0.55	0.58
700/900 mV	1.02	1.01	0.89	1.01	1.02	0.64	0.88	0.89
800/900 mV	1.02	1.01	0.94	1.01	1.02	0.90	0.93	0.95

*N.D., Ratio is approximately 0.0.

reference compounds are shown in Table II. For electrodes operating at 800/900 mV, the ratios for all peaks are nearly unity. However, at 500/600 mV, DOPA and catecholamines gave ratios of 0.98, whereas O-methylated metabolites gave ratios of 0.0.

The amounts of free and total DOPA, catecholamines and O-methylated metabolites found in the plasma of five healthy subjects are listed in Table III. These levels agree with those reported in the literature, except for A and DA, which are slightly higher. This might be attributed to the manner in which the sample was taken, which may have influenced the actual concentration of A and DA in the plasma. The levels of O-methylated metabolites, particularly that of 3-O-methyl-DOPA, in the plasma of normal individuals are higher than those of the other compounds. Since the free levels were determined by ED, the absolute quantity of conjugated compounds present in a given specimen is the difference between the total (determined after hydrolysis) and free (determined prior to hydrolysis) compounds.

The recovery was measured using the free and total plasma procedure. The compounds were added to plasma at concentrations of 0.5 and 5.0 ng/ml and the recovery results are shown in Table IV. The present method gives reproducible and high recoveries (greater than 95%) when compared to those usually obtained (about 70%) after alumina or ion-exchange resin separation. Therefore, this method permits the elimination of the extraction step by direct injection of the deproteinized and neutralized plasma supernatant into the reversed-phase HPLC system. Hence, a possible source of error and a loss in sensitivity is eliminated. A drawback of the direct injection of biological materials is a shortened life of the column, especially the column top [26]: a remedy may be the insertion of a precolumn [27, 28]. In general, the columns used were adequate for about 500 injections, after which the resolution between NMN and DOPA and between 3-O-methyl-DOPA and 3-O-methyl-DA deteriorates.

Comparison of the two detectors indicates that the ED method is sensitive and specific for determining plasma levels of DOPA, catecholamines and their

TABLE III

CONTENTS OF DOPA, CATECHOLAMINES AND O-METHYLATED METABOLITES IN PLASMA DETERMINED USING ELECTROCHEMICAL AND FLUOROMETRIC DETECTORS

Results are expressed in ng/ml of plasma. Measurements made with the fluorometric detector are given in parentheses.

Subject		NA		A		NMN		DOPA	
Age (years)	Sex	Free	Total	Free	Total	Free	Total	Free	Total
37	M	0.65	2.08 (1.89)	0.37	1.44 (1.32)	0.57	2.55 (2.36)	0.91	2.33 (1.94)
32	M	0.41	1.86 (1.69)	N.D.*	0.84 (N.D.)	N.D.	1.81 (1.72)	0.82	2.19 (2.03)
21	M	0.39	1.48 (1.34)	0.29	1.03 (1.11)	0.51	2.24 (2.08)	0.48	2.01 (1.88)
23	F	N.D.	0.83 (N.D.)	N.D.	N.D. (N.D.)	0.42	1.07 (N.D.)	N.D.	1.16 (1.05)
20	F	0.27	1.18 (1.24)	N.D.	0.54 (N.D.)	0.33	1.54 (1.42)	0.44	1.62 (1.58)

*N.D., Not detectable.

TABLE IV

RECOVERY OF INVESTIGATED COMPOUNDS MEASURED BY ELECTROCHEMICAL DETECTION

Values represent mean ± S.D. of five experiments.

Compound	Recovery (%)	
	Free*	Total**
NA	95.1 ± 1.9	96.0 ± 2.1
A	95.8 ± 2.3	97.4 ± 2.7
NMN	95.3 ± 1.7	95.6 ± 1.8
DOPA	100.2 ± 2.1	101.2 ± 2.4
DA	102.4 ± 2.0	103.3 ± 1.9
MN	97.9 ± 2.4	96.8 ± 3.1
3-O-Methyl-DOPA	98.3 ± 1.3	98.4 ± 1.2
3-O-Methyl-DA	96.1 ± 1.8	95.3 ± 2.4
o-Tyrosine***	98.4 ± 2.0	97.8 ± 1.5

*Compounds were added at a concentration of 0.5 ng/ml.
**Compounds were added at a concentration of 5.0 ng/ml.
***Amount added was 0.1 μg/ml.

O-methylated metabolites. The fluorescence method was not as sensitive as ED, but it was less problematic in setting up and was found to be quite satisfactory for the measurement of high plasma concentrations of these compounds.

DA		MN		3-O-Methyl-DOPA		3-O-Methyl-DA	
Free	Total	Free	Total	Free	Total	Free	Total
0.39	2.14 (1.97)	0.42	1.88 (1.69)	19.22 (20.02)	45.42 (44.35)	0.42	2.76 (2.21)
0.41	2.96 (2.78)	0.37	1.43 (1.52)	20.36 (21.03)	52.18 (50.02)	0.56	3.01 (2.89)
N.D.	1.84 (1.91)	0.48	1.72 (1.63)	16.44 (15.98)	66.14 (63.82)	N.D.	1.33 (N.D.)
0.37	1.68 (1.56)	N.D.	0.98 (N.D.)	30.12 (28.97)	78.42 (77.56)	0.38	1.70 (1.56)
0.29	1.34 (1.27)	0.46	1.12 (1.21)	21.08 (20.48)	42.38 (40.14)	0.29	0.82 (N.D.)

CONCLUSIONS

The method reported gives a good separation of free and conjugated DOPA, catecholamines and O-methylated metabolites found in human plasma without a complex clean-up procedure. Also, it permits the simultaneous determination of these compounds by reversed-phase HPLC. The assay is suitable for research application and routine clinical analysis.

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QUANTITIES OF B_6 VITAMERS IN HUMAN MILK BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

INFLUENCE OF MATERNAL VITAMIN B_6 STATUS*

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SUMMARY

A rapid, sensitive procedure is described for the analysis of the B_6 vitamers pyridoxal, pyridoxamine, and pyridoxine in human milk from women taking and not taking supplements containing the vitamin using high-performance liquid chromatography with fluorometric detection. Vitamer values represent the sum of their phosphorylated and unphosphorylated forms. Minimum detectable quantities were 1—3 ng. Excellent recoveries of these vitamers in milk were obtained. Similar B_6 vitamer concentrations of milk were obtained using the developed high-performance liquid chromatographic and the accepted microbiological techniques. Pyridoxal, actually consisting of pyridoxal plus pyridoxal phosphate, was the predominant B_6 vitamer in human milk. The concentration of B_6 vitamers in milk was reflective of the maternal vitamin B_6 status.

INTRODUCTION

In recent years there has been increasing concern regarding the nutritional status of lactating women and nutrient composition of human milk. Deleterious effects of low vitamin B_6 intakes in the young have been observed in humans as well as in rats. There has been some evidence that the dietary intakes of vitamin B_6 of lactating women influence the concentration of the vitamin in their milk [1, 2].

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Vitamin B_6 exists in three interconvertible forms: pyridoxine (PN, also known as pyridoxol), pyridoxal (PL), and pyridoxamine (PM), each of which has a corresponding 5′-phosphate (P). The physicochemical properties of the B_6 vitamers, in particular their ionic nature, facilitate their assay by high-performance liquid chromatography (HPLC). The B_6 vitamer content of human plasma, animal tissues, urine, and selected foods as measured using HPLC techniques has been reported [3—15]. The B_6 vitamers present in milk from mothers supplemented with pyridoxine hydrochloride were recently quantitated by Vanderslice et al. [16] and Hamaker et al. [17] using HPLC techniques; the mothers in the first study received 0.5 or 4 mg of the vitamin daily and those in the second study 2.5 or 15 mg. To our knowledge, the concentrations of the B_6 vitamers PL, PM, and PN, in their unphosphorylated and/or phosphorylated forms, in milk from mothers not taking vitamin B_6 supplements have not been reported.

The objectives of this research were to determine the influence of the vitamin B_6 status of lactating women on the B_6 vitamer content of their milk as measured by microbiological methods as well as by a reversed-phase ion-pair HPLC method with fluorometric detection which had previously been developed in our laboratory [15]. The relationships between vitamin B_6 intakes, vitamin B_6 status measurements, and B_6 vitamer concentrations in milk were also examined. The yeast employed in the microbiological assay of the vitamers is not able to utilize the phosphorylated derivatives of the B_6 vitamers [18]; hence, in order to compare vitamer values obtained using microbiological and HPLC procedures, the phosphorylated B_6 vitamers were dephosphorylated and quantitated in their respective unphosphorylated forms. Consequently, these individual vitameric values represent the sum of both forms of the vitamer, the unphosphorylated and the phosphorylated. Since the unphosphorylated derivatives constitute less than 10% of the total vitamin B_6 content of mammalian tissue [19], the non-phosphate values reported in this research are considered to represent primarily the tissue values of the phosphorylated vitamers.

EXPERIMENTAL

A Waters Assoc. (Milford, MA, U.S.A.) HPLC system equipped with a fluorescence detector (300 nm excitation, 375 nm emission) and a μBondapak octadecylsilane column (30 cm × 3.9 mm I.D., 10 μm porous packing, C_{18}, Waters Assoc.) was utilized in this research. The mobile phase was a gradient of 85% methanol and PIC B-7 reagent (Waters Assoc.); the gradient conditions, vendors of standards, and instrumentation have previously been described in detail [15].

Deoxypyridoxine (DPN) was selected as the internal standard. Following chromatography of the individual vitamers PL, PN, and PM as well as DPN, a 250-μl aliquot of an aqueous combined standard solution (containing 50 ng of PL, 6.25 ng of PN, 6.25 ng of PM, and 118.75 ng of DPN per ml) was injected onto the column; satisfactory separation of the vitamers was achieved in about 15 min (Fig. 1). Identity was confirmed by standard addition (spiking) as well as by extra chromatographic spectrofluorometry on collected HPLC eluates.

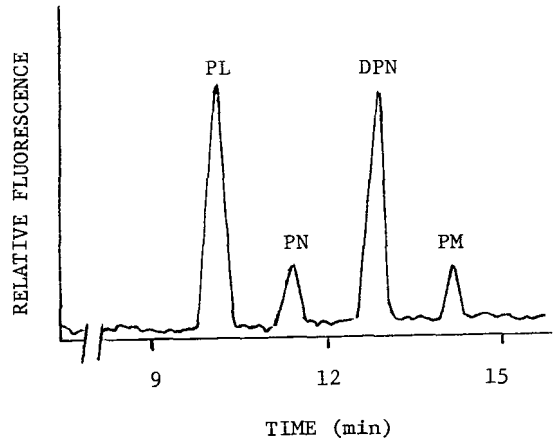

Fig. 1. Separation of B_6 standards by HPLC.

Linear calibration curves for PL, PM, PN, and DPN were obtained; minimum detectable quantities were 3 ng (18 ng/ml sample) for PL and 1 ng (6 ng/ml sample) for PM and PN.

Extraction of B_6 vitamers in milk samples

A known quantity of DPN was added to a 3-ml aliquot of pooled milk and the solution mixed. Next, 0.6 ml of a 2 U/ml potato acid phosphatase (EC 3.1.3.2; orthophosphoric monoester phosphohydrolase; Sigma, St. Louis, MO, U.S.A.) in 0.2 *M* potassium acetate, pH 4.5, was added to the sample in order to hydrolyze the phosphate esters of the B_6 vitamers [20]; samples were incubated for 1 h in a 37°C shaker water bath. The protein was precipitated by adding 0.25 ml of 100% trichloroacetic acid (TCA); the sample was mixed by vortex and incubated for 15 min in a 50°C water bath. Methylene chloride, 3 ml, was added to the samples followed by vigorous shaking to remove lipids; samples were then centrifuged for 15 min at 4°C and 4000 *g*. The resulting supernatants were adjusted to pH 5.2 with 33% sodium hydroxide and filtered through a 0.45-μm filter with a syringe attachment prior to injection into the HPLC system.

A typical chromatogram of milk extract is depicted in Fig. 2. Peak identity was confirmed by comparison of standard retention times with sample retention times, use of relative retention times, spiking, and extra chromatographic spectrofluorometry of eluates. Vitamer recoveries were determined by spiking the samples before extraction. The recoveries were as follows: PL, 86%; PM, 105%; and PN, 83%; the recovery of DPN was 95%. Phosphorylated vitamers were recovered as their respective unphosphorylated forms as follows: PLP → PL, 96%; PMP → PM, 70%; and PNP → PN, 83%. Hence, vitamer values represent the sum of both the phosphorylated and unphosphorylated forms of the vitamer. The data were not corrected for percent recoveries. The coefficients of variation for B_6 vitamer concentrations of milk samples that were extracted and analyzed on different days were around 5%; the same was true for the microbiological assay. The B_6 vitamer content of samples from three women was determined freshly expressed as well as frozen for 2 h and for

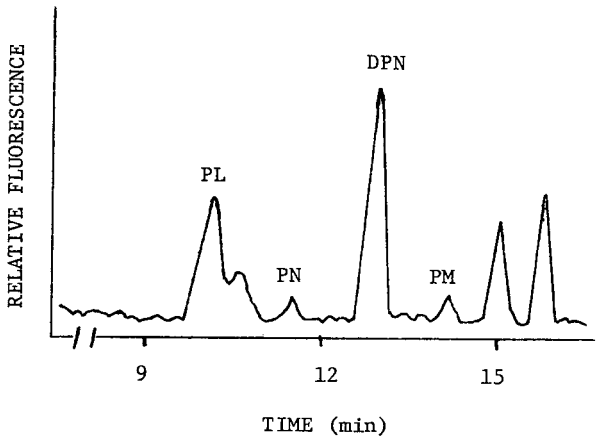

Fig. 2. Separation of B_6 vitamers in a representative milk extract by HPLC.

four months at −20°C; frozen and freshly expressed samples had similar vitamer contents thus indicating that human milk samples could be frozen for at least four months without loss of B_6 vitamers.

Subject description and dietary analysis

Twenty-one white lactating women in apparent good health, 21—35 years of age, and 3—7 months postpartum volunteered as subjects. The study was approved by the University's Human Volunteers Committee. Subjects were weighed and heights obtained. Information regarding food intake was obtained from each subject using one 24-h recall (with cross-checking and food models) and four days of food records; these dietary records were obtained and the protein and vitamin B_6 intakes were estimated as described previously [21]. These intakes were compared to the Recommended Dietary Allowances (RDA) for lactating women [22].

Milk sample collection and vitamin B_6 status analysis

Subjects manually expressed fore milk into vials on three consecutive mornings; the samples were frozen immediately by the subjects. West and Kirksey [1] found that the vitamin B_6 content in a fore sample of early morning milk was representative of milk expressed completely from the breast. Milk from the three vials was pooled for B_6 vitamer analysis.

The vitamin B_6 status of the subjects was determined via the two most commonly used parameters: coenzyme stimulation of erythrocyte alanine aminotransferase activity (EC 2.6.1.2; L-alanine:2-oxoglutarate aminotransferase, E-ALAT) and radioisotopically measured plasma pyridoxal phosphate (PLP) concentration. Approximately 20 ml of non-fasting blood was obtained by a Registered Medical Technologist on the morning following the final morning of milk collection and the five days of food intake records. These two methods used for status assessment have been described previously [21].

B_6 Vitamers via microbiological analysis

A modification of the Association of Official Analytical Chemists (AOAC)

procedures [23] for analyzing B_6 vitamers in food materials was used to determine the PL, PM, and PN content of the milk samples. The assay inoculum was prepared by incubating *Saccharomyces uvarum* (ATCC 9080, American Type Culture Company, Rockville, MD, U.S.A.) on Pyridoxine Y media (Difco Labs., Detroit, MI, U.S.A.). The protein in the pooled milk samples was precipitated using sulfosalicylic acid (0.1 g per 2 ml milk) [24]. The sample was then centrifuged for 2 min at 3000 *g* and 4° C; the supernatant was filtered and 6 ml of 0.2 *M* hydrochloric acid were added and the mixture was placed into a boiling water bath for 1 h; this step enabled the phosphate groups to be cleaved from the B_6 vitamers. The pH of the sample was adjusted to 4.5 with 10% potassium hydroxide. A glass column (250 mm × 17 mm O.D., 14.5 mm I.D., 250 ml reservoir) was used in the separation of the vitamers, the column separation and subsequent analyses for B_6 content were as described in the AOAC procedure [23]. In order to check vitamer recoveries, milk samples were spiked with each vitamer prior to extraction. The recoveries were as follows: PL, 93%; PM, 87%; and PN, 84%. Milk samples were also spiked with phosphorylated forms of the vitamers; these vitamers were dephosphorylated and recovered as their respective unphosphorylated forms as follows: PLP → PL, 83%; PMP → PM, 85%; and PNP → PN, 82%. The data were not corrected for percent recoveries.

Statistical analysis

The subjects were classified into adequate and inadequate status groups based upon their coenzyme stimulation values. Coenzyme stimulation values $\geqslant$ 16% [25] or > 25% [26] are considered to be indicative of vitamin B_6 inadequacy. The criteria used for defining vitamin B_6 inadequacy in the current study is a stimulation value $\geqslant$ 16%. All data were evaluated using analysis of variance procedures [27]; means ($\overline{X}$) and standard deviations (S.D.) were also calculated. Pearson *r* correlation coefficients were determined between data obtained for the various parameters.

RESULTS AND DISCUSSION

On the basis of their coenzyme stimulation of E-ALAT activities, the subjects were classified as having either adequate or inadequate status; other status parameters were not utilized in that values indicative of inadequate status have not yet been established. Six subjects had coenzyme values > 25% and one female had a value of 22.2%; these seven subjects were classified as having inadequate vitamin B_6 status. Fourteen subjects who had coenzyme values <16% were classified as having adequate vitamin B_6 status.

Anthropometric and dietary assessment

The age, height, and weight measurements of the subjects classified as having inadequate and adequate vitamin B_6 status were similar. These values for all subjects combined were as follows: 27.1 ± 3.4 years, 164.4 ± 7.1 cm, and 58.6 ± 8.7 kg ($\overline{X}$ ± S.D.). The subjects were 4.6 ± 1.6 months ($\overline{X}$ ± S.D.) postpartum.

No significant differences were observed between intake data obtained by

the 24-h recalls and the four-day records. The average daily protein intake of subjects classified as having inadequate and adequate vitamin B_6 status were similar; the intake for all subjects combined was 89.6 ± 21.4 g ($\overline{X}$ ± S.D.). The vitamin B_6 intake of the seven subjects classed as being inadequate in status was 1.16 ± 0.24 mg ($\overline{X}$ ± S.D.) daily; none of the subjects in this status group reported taking supplements containing vitamin B_6. The fourteen subjects in the adequate status group reported consuming 1.52 ± 0.34 mg ($\overline{X}$ ± S.D.) of the vitamin from food sources; their intake from food and supplements combined was 11.23 ± 16.32 mg daily (median was 5.17 mg; one subject reported taking a supplement containing 65 mg of the vitamin). All of the women in the group with adequate status reported taking nutrient supplements that contained vitamin B_6. Women in the inadequate status group reported consuming significantly less ($p < 0.01$) vitamin B_6 from food, as well as from food and supplements combined, than subjects in the adequate group. All of the subjects classified as having inadequate status and five of the women classed as adequate reported consuming a daily intake of vitamin B_6 from food sources that was less than two-thirds of the RDA for lactating women; none of the subjects reported consuming as much as 2.5 mg daily (the RDA) of the vitamin from food sources.

Vitamin B_6 status assessment

The coenzyme stimulation of E-ALAT activities of subjects classified as having inadequate and adequate vitamin B_6 status was 34.9 ± 8.7% and 5.4 ± 6.4% ($\overline{X}$ ± S.D.), respectively. Stimulation values of the group classed as being inadequate were significantly higher ($p < 0.0001$) than those classed as adequate. The radioisotopically measured plasma PLP concentrations of subjects classed as being inadequate ($\overline{X}$ ± S.D., 15.3 ± 5.9 ng/ml) were significantly lower ($p < 0.003$) than those classed as being adequate (39.5 ± 18.1 ng/ml). Rose et al. [28] suggested that a plasma PLP level of < 8.5 ng/ml represented inadequate vitamin B_6 status; Russ et al. [29] suggested that a value < 13 ng/ml was representative of inadequate status. All plasma PLP values of subjects in this study exceeded 8.5 ng/ml but four subjects in the inadequate classification group had values below 13 ng/ml. A Pearson correlation coefficient of −0.59 ($p < 0.005$) was obtained in this study between coenzyme stimulation of E-ALAT values and plasma PLP concentrations. Russ et al. [29] reported finding a Pearson correlation coefficient of −0.60 between these two parameters.

B_6 Vitamers in milk by HPLC assay

The B_6 vitamer content of milk from the subjects as measured by HPLC assay are given in Table I. Phosphorylated B_6 vitamers were dephosphorylated and quantitated in their respective unphosphorylated forms. The PL concentrations of milk of women having inadequate classification were significantly lower ($p < 0.01$) than values of the adequate group. The PM values for milk from two subjects classed as inadequate were below minimum detectable levels. The PN values for milk from five women classed as adequate and two subjects classed as inadequate were below minimum detectable limits. The minimum detectable limits for PM and PN were 1 ng per injection which may be equivalent to as

TABLE I

B_6 VITAMER CONTENT OF MILK BY HPLC ASSAY

Individual vitameric values represent the sum of both forms of the vitamer, the unphosphorylated and the phosphorylated. Values represent $\bar{X}$ ± S.D. and are given in pmol/ml.

Status classification	PL	PM	PN	Total B_6 vitamers
Inadequate	226 ± 71*	42 ± 33**	51 ± 40	320 ± 103*
Adequate	627 ± 324	102 ± 63	42 ± 38	770 ± 341

*Significantly different from values of adequate group at $p < 0.01$.
**Significantly different from values of adequate group at $p < 0.05$.

much as 6 μg/l. Non-detectable levels were statistically evaluated as being zero. The PM concentrations of milk from women classed as inadequate were significantly lower ($p < 0.05$) than those of the group classed as adequate. The PN concentrations of milk from both classes of subjects were similar. The total B_6 vitamer (TL) concentration of milk from women classed as having adequate status as measured by HPLC assay was significantly lower ($p < 0.01$) than for those in the adequate classification. Two significant Pearson correlation coefficients were obtained between HPLC-derived data; these were as follows: PL and TL, $r = 0.98$, $p < 0.0001$; PM and TL, $r = 0.53$, $p < 0.02$.

The predominant B_6 vitamer in milk from subjects in both status classifications as measured via HPLC techniques was PL (representing both PL and PLP). The percent distributions of B_6 vitamers in milk from all subjects were as follows: PL, 76.8 ± 14.3%; PM, 13.8 ± 8.9%; and PN, 9.4 ± 8.6% ($\bar{X}$ ± S.D.). Vanderslice et al. [16] and Hamaker et al. [17] also found PL (PL + PLP) to be the major vitameric form.

B_6 Vitamers in milk by microbiological assay

The B_6 vitamer content of milk from the subjects as measured by microbiological assay is given in Table II; total B_6 vitamers were calculated as a sum of the vitamers. PL values for milk from the group classed as inadequate were

TABLE II

B_6 VITAMER CONTENT OF MILK BY MICROBIOLOGICAL ASSAY

Individual vitameric values represent the sum of both forms of the vitamer, the unphosphorylated and the phosphorylated. Values represent $\bar{X}$ ± S.D. and are given in pmol/ml.

Status classification	PL	PM	PN	Total B_6 vitamers
Inadequate	645 ± 57*	54 ± 11	56 ± 24	755 ± 66**
Adequate	820 ± 85	66 ± 15	68 ± 32	955 ± 98

*Significantly different from values of adequate group at $p < 0.0001$.
**Approached being significantly different from values of adequate group at $p < 0.10$.

significantly lower ($p < 0.0001$) than those of women in the adequate classification. The PM and PN values for milk from the inadequate classification group were rather similar to those of the adequate women. The total B_6 vitamer concentration of milk from women in the inadequate classification group was significantly lower ($p < 0.0001$) than for the adequate group. The following Pearson correlation coefficients were obtained between microbiologically derived data: PL and total B_6 vitamers (TL), $r = 0.96, p < 0.0001$; PM and TL, $r = 0.51, p < 0.02$; PN and TL, $r = 0.43, p < 0.05$.

The predominant B_6 vitamer in milk from both inadequate and adequate status groups as measured by microbiological techniques was PL (representing PL + PLP). Approximately equal quantities of both PM and PN were present in milk from both status groups. The percent distributions of B_6 vitamers in all subjects were as follows: PL, 85.7 ± 3.3%; PM, 7.0 ± 1.5%; and PN, 7.2 ± 2.8% ($\overline{X}$ ± S.D.).

Comparison of B_6 vitamer data obtained by HPLC and microbiological assays

Correlations were calculated between data obtained by HPLC and microbiological techniques. The PL and TL (total B_6 vitamer) concentrations of milk of all subjects as measured by HPLC and microbiological techniques were significantly and highly correlated with each other ($r = 0.63$, $p < 0.003$, and $r = 0.64$, $p < 0.002$, respectively). PM and PN values obtained by HPLC assay were not significantly correlated with those obtained using microbiological techniques.

The concentrations of PL, PM, PN, and total B_6 vitamers in human milk as determined by HPLC procedures were rather similar to values obtained by microbiological assay in the current study. These two methods are quite diverse and have different methods of vitamer extraction. Excellent correlations between HPLC and microbiologically derived data were observed in the current study for PL and total B_6 vitamers but not for PM and PN where several values were observed to be below minimum detectable limits. Vanderslice et al. [16] reported finding satisfactory agreement between microbiologically measured total vitamin B_6 and HPLC-derived total B_6 vitamer (calculated by addition) concentrations. Excellent recoveries from spikes were obtained in the present study for both microbiological and HPLC assays including extraction procedures.

B_6 Vitamers in milk versus status assessment

Significant correlations were obtained between the milk B_6 vitamer values and data obtained by the two status parameters (Table III). The B_6 vitamer content of the human milk as determined by both HPLC and microbiological techniques was definitely highly correlated with the vitamin B_6 status of the lactating women as measured by both coenzyme stimulation of E-ALAT activities and plasma PLP levels. Correlations approaching significance were observed between estimated vitamin B_6 intakes and coenzyme stimulation activities ($r = 0.39$, $p < 0.08$) as well as plasma PLP values ($r = 0.42, p < 0.06$). Significant or near significant correlations were also obtained between estimated vitamin B_6 intakes and the following milk B_6 vitamer values: microbiologically derived (M) PL, $r = 0.43$, $p < 0.06$; MPN, $r = 0.57$, $p < 0.008$;

TABLE III

SIGNIFICANT CORRELATIONS BETWEEN MILK B_6 VITAMER VALUES AND STATUS MEASUREMENTS

Individual vitameric values represent the sum of both forms of the vitamer, the unphosphorylated and the phosphorylated.

Parameters*	*r*	*p* values
E-ALAT:MPL	−0.65	<0.002
E-ALAT:MPM	−0.48	<0.03
E-ALAT:MPN	−0.40	<0.08**
E-ALAT:MTL	−0.71	<0.001
E-ALAT:HPL	−0.65	<0.002
E-ALAT:HPM	−0.44	<0.05
E-ALAT:HTL	−0.65	<0.002
RPLP:MPL	0.59	<0.006
RPLP:MTL	0.56	<0.009
RPLP:HPL	0.76	<0.0001
RPLP:HTL	0.74	<0.0001

*Abbreviations used: Coenzyme stimulation of erythrocyte alanine aminotransferase activities, E-ALAT; radioisotopically monitored plasma pyridoxal phosphate levels, RPLP; microbiological (M) and HPLC (H) measurement of pyridoxal (PL), pyridoxamine (PM), pyridoxine (PN), and total B_6 (TL) concentrations in milk.
**Approached significance.

MTL, $r = 0.52$, $p < 0.02$; HPLC-derived (H) PL, $r = 0.74$; $p < 0.0001$; HTL, $r = 0.73$, $p < 0.0002$. Large S.D. values were observed with relation to vitamin B_6 intakes, milk B_6 vitamer, and status parameter values. As reflected by the *r* values, subjects having lower vitamin B_6 intakes usually had higher coenzyme stimulation of E-ALAT activities, lower plasma PLP levels, and lower B_6 vitamer levels in their milk as quantitated by both HPLC and microbiological methods; the reverse was usually true for subjects with higher intakes. Other researchers have also found milk from humans to be quite variable in vitamin B_6 or B_6 vitamer content [1, 16] but they did not relate these findings to biochemical status measurements.

The B_6 vitamer concentration of the milk was affected by the vitamin B_6 status of the women in the current study. The B_6 vitamer concentration of the milk was also affected by supplementation and vitamin B_6 intakes in the current study. Subjects in the current study who had higher intakes of vitamin B_6 also had higher concentrations of B_6 vitamers in their milk. Similar findings obtained by microbiological assay for total vitamin B_6 have been reported by West and Kirksey [1].

Similar B_6 vitamer concentrations were obtained for human milk samples by the newly developed HPLC fluorometric technique as by the AOAC microbiological assay. Both the HPLC method and the microbiological procedures used in the current study seemed to be sensitive and accurate in qualitating and quantitating the B_6 vitamers found in human milk from women who took, as well as who did not take, supplements containing the vitamin. The vitamin B_6 status of lactating women could be determined by quantitating the B_6 vitamer content of their milk.

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DETERMINATION OF INORGANIC SULPHATE IN PLASMA BY REVERSED-PHASE CHROMATOGRAPHY USING ULTRAVIOLET DETECTION AND ITS APPLICATION TO PLASMA SAMPLES OF PATIENTS RECEIVING DIFFERENT TYPES OF HAEMODIALYSIS

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SUMMARY

The determination of sulphate in plasma is described, making use of reversed-phase high-performance liquid chromatography with ultraviolet detection. The concentration of inorganic sulphate determined in plasma of twenty healthy volunteers was 0.307 ± 0.092 mmol/l (mean ± S.D.). In one stable chronic dialysis patient the kinetics of plasma sulphate removal were monitored during and after one single pass dialysis. In addition, plasma sulphate concentrations were determined in three stable chronic dialysis patients during a consecutive scheme of two single pass dialyses, five Redy® dialyses and three single pass dialyses. As expected, plasma sulphate accumulates in plasma to a high steady-state level under Redy dialysis, whereas during single pass dialysis sulphate is efficiently removed.

INTRODUCTION

The inorganic sulphate in the body is formed from sulphur-containing amino acids [1] and derived from resorption of inorganic sulphate from the gastro-

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intestinal tract [2]. The greater part of the inorganic sulphate is excreted by the kidneys, a minor part is used for sulphoconjugation reactions [3]. Mean serum or plasma sulphate values in healthy adults are found in the order of 0.3 mmol/l [4—14].

Up to now sulphate accumulation has only been mentioned in renal failure [1, 4, 15—17]. The exact clinical significance of elevated plasma sulphate concentrations is not known. Blum and Coe [18] mentioned metabolic acidosis in a diabetic patient with an elevated plasma sulphate level (2.6 mmol/l). This observation combined with studies of Hänze [19], who found a close inverse correlation between serum SO_4^{2-} and HCO_3^-, has led to the suggestion that SO_4^{2-} is involved in the development of metabolic acidosis in uraemia [17]. It is also suggested that elevated plasma sulphate levels could be involved in the pathogenesis of renal osteodystrophy [17].

During single pass dialysis many of the accumulated residual products, including sulphate, are efficiently removed [15]. The disadvantage of this dialysis technique, namely the use of ±180 l of dialysis solution, was overcome by the Redy® dialysis system. This system makes use of constantly regenerated dialysis solution (±6 l), which is filtered through a number of filter layers removing heavy metals and oxidizing agents, cations, anions, and organic compounds such as urea, creatinine and uric acid [20]. During this process also calcium, magnesium and potassium are largely removed so that these ions have to be constantly infused into the dialysate. On the other hand, it has been reported that sulphate accumulated in plasma when the original type of Redy cartridges were used [15]. For that reason we decided to undertake a study in order to find out whether such an accumulation also occurs in the more recently developed versions of the Redy system. For sulphate determination in plasma we worked out a reversed-phase high-performance liquid chromatographic (HPLC) method using ultraviolet (UV) detection.

MATERIALS AND METHODS

Materials

The anion-exchange resin AG1-X10 (100—200 mesh) was supplied by Bio-Rad Labs. (Richmond, CA, U.S.A.). All other chemicals were purchased from E. Merck (Darmstadt, F.R.G.) and were of analytical grade. Heparinized tubes (Venoject VT 100H) were from Terumo Europe (Belgium). Glass-distilled water was used throughout. The 10-cm analytical column (I.D. 4.6 mm) and 3-cm guard column RP-18 Spheri-5 (I.D. 4.6 mm) were from Brownlee Labs. (Santa Clara, CA, U.S.A.). For the single pass dialyses the Gambro AK-10 dialysis monitor (Lund, Sweden) was used. For the sorbent cartridge dialyses the Redy system, consisting of Sorbsystem SS-EA with cartridge type D 32/60 (Organon Technika, Turnhout, Belgium), was used.

Patients and samples

A plasma pool was made by mixing plasma samples obtained from healthy persons as well as from patients. Plasma samples of ten healthy males and ten healthy females, aged 20—60 years, were used for the determination of the normal plasma sulphate concentration.

Patient A was a 69-year-old woman, patient B was a 52-year-old woman and patient C was a 44-year-old man, all stable, chronic dialysis patients, on single pass for many years. Patients A and C were dialysed on days 0, 3, 7, 10, 14, 17, 21, 24, 28, 31, zero being the day of the first dialysis. On days 0, 3, 24, 28, 31 they were dialysed by single pass dialysis, on days 10, 14, 17, 21, 24 by the Redy dialysis system. Patient B was dialysed on days 0, 4, 7, 11, 14, 18, 21, 25, 28, 32, zero being the day of the first dialysis. On days 0, 4, 25, 28, 32 she was dialysed by single pass dialysis, on days 7, 11, 14, 18, 21 by the Redy dialysis system. All dialyses lasted 7 h; plasma samples were taken just before the start and immediately after the end of the dialysis. For monitoring the sulphate concentration during and after a single pass dialysis patient A was selected. The plasma samples were taken at 1 min before and 2, 4, 6, 7 h after the start of the dialysis. Seven hours after the start of the dialysis the patient was disconnected and again plasma samples were taken at 8, 9, 10 and 22.5 h (counted from the start of the dialysis).

Determination of inorganic sulphate in plasma

Sample preparation. The anion-exchange material was allowed to stand for 18 h on water before use. Columns with a height of 1 cm and an I.D. of 1 cm were prepared from this material. Before use the columns were washed successively with (a) 10 ml of 100 g/l ammonium acetate, (b) 5 ml of water, (c) 5 ml of 0.1 mol/l hydrochloric acid, and (d) 5 ml of water. This washing procedure was repeated once more. Then 200 μl of plasma or sodium sulphate standard solution were added to the column, which then was rinsed with 3 × 5 ml of water. The sulphate was eluted with 2 × 3 ml of 100 g/l ammonium acetate solution. The eluate was dried at 160°C under a stream of air. After disappearance of the solvent the sample was heated for another 30 min. Then 500 μl of water were added and heated to dryness at 160°C under a stream of air. After cooling to room temperature the samples were redissolved in 1000 μl of the HPLC mobile phase (see below). If necessary the samples were diluted further.

HPLC determinations. Determinations were carried out on a Waters HPLC pump Model M6000A (Waters Assoc., Milford, MA, U.S.A.) equipped with a reversed-phase guard column and a reversed-phase analytical column (see section on materials) and a Pye Unicam (Cambridge, U.K.) Model PU 4020 UV detector. The output filter of the UV detector was set at 2 sec and the range at 0.04 a.u.f.s. The electrical output was fed to a Philips dual-line recorder (Philips, Eindhoven, The Netherlands) Model PM 8252, which was set at 10 mV and 20 mV full scale and coupled to a Spectra Physics computing integrator (San Jose, CA, U.S.A.) Model SP 4100. The mobile phase was a solution of $5 \cdot 10^{-4}$ mol/l potassium hydrogen phthalate and 10^{-3} mol/l tetrabutylammonium bromide. The pH was adjusted to 5.9 with 1 mol/l sodium hydroxide. The flow-rate was 1.5 ml/min. A 100-μl volume of sample was automatically injected on the column with a WISP 710B autosampler (Waters Assoc.). The sulphate concentration was determined by comparing the integrated peak area with those obtained from standard sulphate solutions.

RESULTS AND DISCUSSION

At present, plasma sulphate determinations, in contrast to other anions (i.e. chloride, phosphate and bicarbonate), are not routinely performed in clinical chemical laboratories. Most of the determinations of plasma sulphate reported up to now, are based on the precipitation reaction $Ba^{2+} + SO_4^{2-} \rightarrow BaSO_4$. The $BaSO_4$ formed can be determined by gravimetry [4], nephelometry [5] or by turbidimetry [6]. Sulphate can also be determined indirectly by measuring the remaining Ba^{2+} concentration, after its addition in excess, employing flame photometry [7], radioisotope labelling [8—10] or atomic absorption spectrophotometry [11]. Another indirect method is by colorimetry [12, 21]. Recently, so-called ion chromatography, employing HPLC, has attracted attention as a very elegant method to measure a variety of ions. In general, commercially available instruments, so-called ion chromatographs, using conductimetric detection, are employed. This technique has

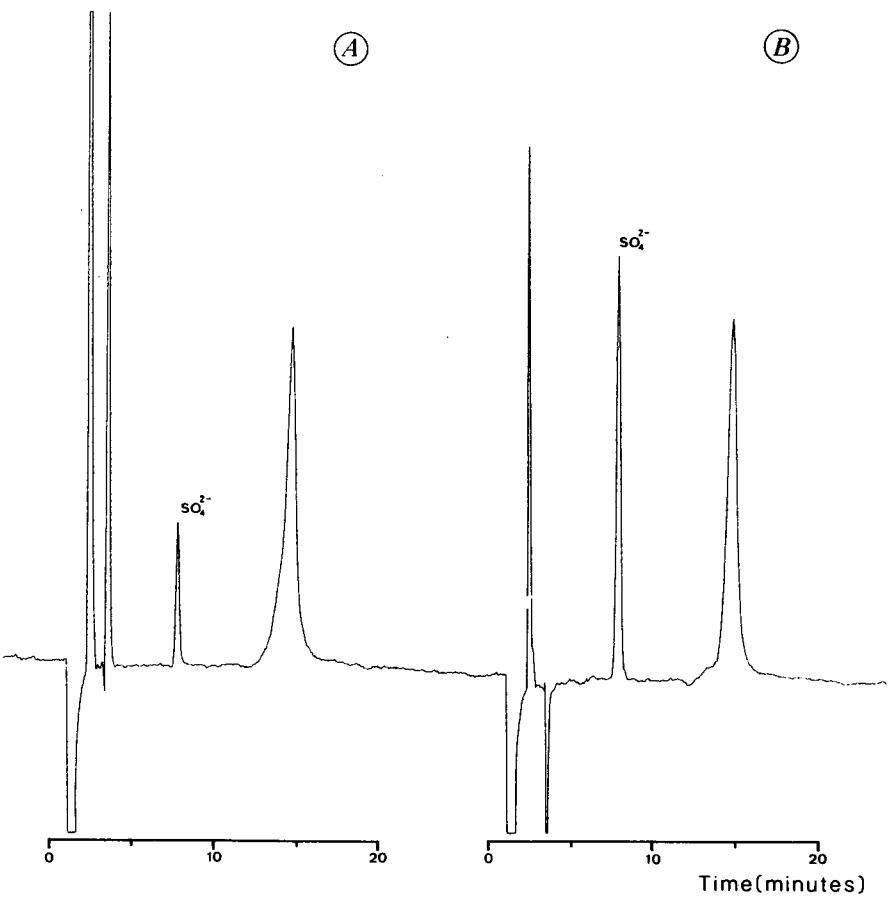

Fig. 1. Examples of chromatographic runs showing sulphate determinations of a normal plasma sample (A, sulphate amount on column 10 nmol) and a 1:1 diluted, elevated (B, sulphate amount on column 48 nmol), cleaned-up plasma sample. Ordinate represents decrease in absorption, a.u.f.s. = 0.04, recorder output 20 mV full scale.

been successfully applied in the determination of sulphate in plasma [13, 14], but most laboratories, including our own, are not in possession of this special instrumentation. However, anions can also be measured by HPLC with UV detection, as has been shown by Cochrane and Hillman [22]. It should be possible to adapt this method for the determination of plasma sulphate. As plasma and serum contain many interfering substances, considerable clean-up is needed before injecting the samples.

An effective clean-up of plasma samples was achieved by passing them through an anion-exchange resin. After thoroughly washing with water, the retained anions, including sulphate, were then eluted as ammonium salts by concentrated ammonium acetate solution. After drying at 160°C, all volatile salts, such as ammonium acetate, ammonium bicarbonate and ammonium nitrate, including the greater part of the ammonium chloride, have disappeared. The "clean" plasma sample is, after redissolution in HPLC eluent, injected into the HPLC system. Examples of a normal and an elevated plasma sulphate level are shown in Fig. 1. Aqueous sodium sulphate standards were similarly worked up. A linear calibration curve was constructed in the range 0—2 mmol/l. Plasma samples with a sulphate concentration above 2 mmol/l were diluted with HPLC mobile phase.

The within-day variation and day-to-day variation in the pooled plasma and the corresponding mean values were determined, being 0.51 mmol/l, C.V. 4.8%, and 0.50 mmol/l, C.V. 5.3%, respectively. In addition, sulphate in varying amounts was added to aliquots of this pooled plasma. After determination of the sulphate concentration, recovery of the added sulphate was calculated. The results are listed in Table I.

After having established that plasma sulphate determinations were reproducible and that the recovery of added sulphate was correct, plasma sulphate levels of twenty healthy individuals were determined. They were normally distributed, the mean value ± S.D. being 0.307 ± 0.092 mmol/l. In Table II normal values of plasma or serum sulphate, as determined using different techniques, are shown and turn out to be in close agreement with those found by the present method.

The plasma sulphate was monitored in patient A during and after one single

TABLE I

RECOVERIES OF SULPHATE ADDED TO POOL PLASMA

Given are the measured amounts (μmol) of sulphate and the recovery percentages (in parentheses).

Amount of SO_4^{2-} added to 1 ml of pooled plasma (μmol)					
0	0.125	0.25	0.50	1.00	2.00
0.55	0.67 (96)	0.79 (96)	0.99 (88)	1.57 (102)	2.53 (99)
0.53			1.03 (100)	1.54 (101)	
0.52			0.98 (92)	1.49 (97)	
0.52			1.06 (108)	1.57 (105)	
0.50			0.98 (96)	1.45 (95)	
0.55			1.01 (92)	1.64 (109)	

TABLE II

NORMAL SERUM AND PLASMA SULPHATE LEVELS IN ADULTS

n = number of samples analysed.

Authors	Method	n	Concentration (mmol/l) (mean ± S.D.)
Kleeman et al. [12]	Colorimetry	15	0.296
Berglund and Sörbo [6]	Turbidimetry	10	0.33
Miller et al. [8]	γ-Spectroscopy	88	0.323 ± 0.085
Leskovar and Weidmann [7]	Flame photometry	50	0.315 ± 0.032
Cole et al. [9]	γ-Spectroscopy	19	0.297 ± 0.038
Cole and Scriver [10]	γ-Spectroscopy	10	0.33 ± 0.05
Michalk and Manz [11]	Atomic absorption spectrometry	12	0.290 ± 0.057
Cole and Scriver [13]	Ion chromatography	16	0.30 ± 0.05
De Jong and Burggraaf [14]	Ion chromatography	40	0.325 ± 0.053
This study	Reversed-phase chromatography	20	0.307 ± 0.092

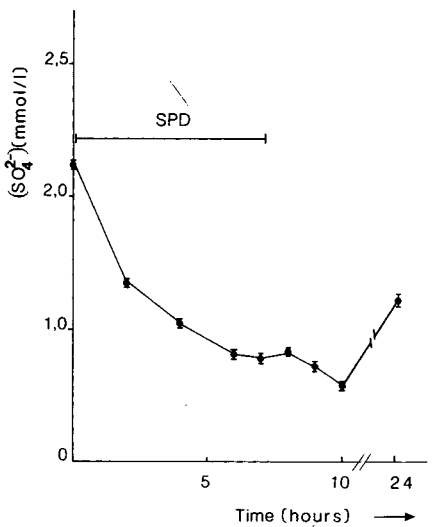

Fig. 2. The plasma sulphate concentration (± S.D.) in patient A during and after one single pass dialysis (SPD).

pass dialysis (see Fig. 2). The results clearly demonstrate a fall in plasma sulphate concentration during dialysis. During the last hour of this dialysis the sulphate seems to reach a steady-state level. After the end of the dialysis the sulphate concentration decreases slightly again, before giving the expected rise.

In the three patients (A, B, C) the plasma sulphate was measured before and after a number of consecutive dialyses: first, two single pass dialyses; secondly, five Redy dialyses, and finally, three single pass dialyses (see Fig. 3). It is clear that in all three patients sulphate accumulation occurs during Redy dialysis, as already found by others using older types of cartridges [15]. The plasma sulphate level finally seems to reach a maximum value during the Redy dialysis;

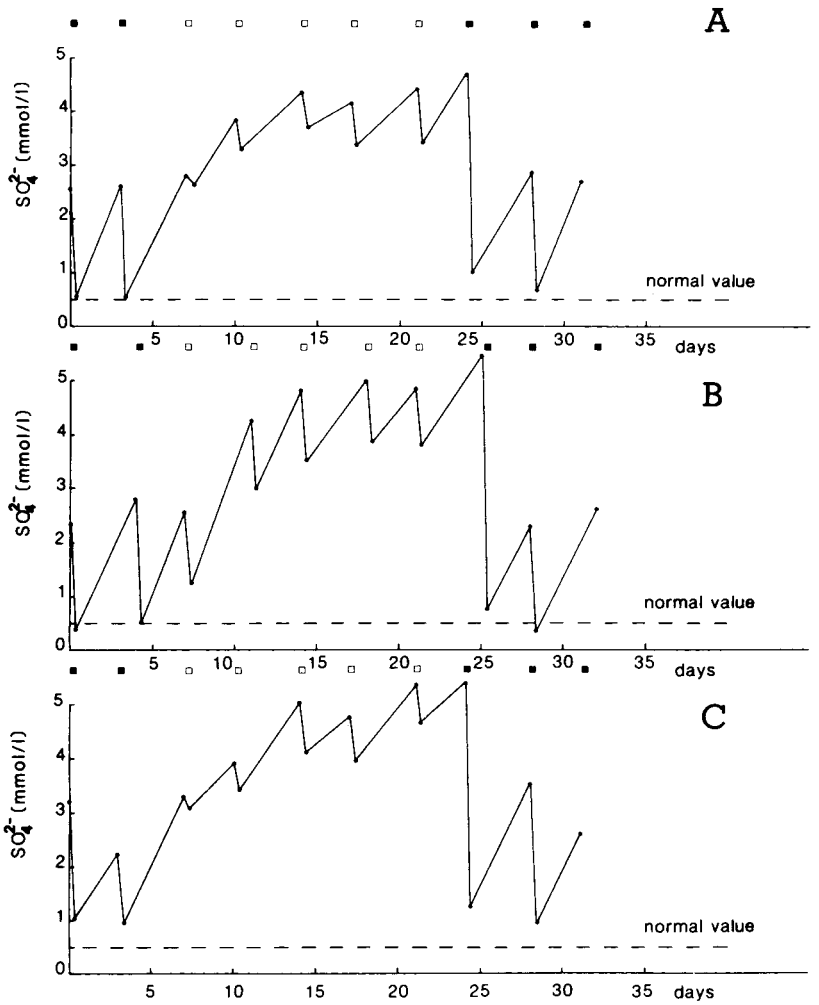

Fig. 3. The plasma sulphate concentration in patients A, B and C during a scheme of consecutive two single pass dialyses, five Redy dialyses and three single pass dialyses. ■, Single pass dialysis; □, Redy dialysis.

after subsequent single pass dialysis the excess sulphate is again efficiently removed.

From these relatively simple experiments it can be concluded, that the present method can be successfully applied in studies concerning the removal of plasma sulphate in patients subjected to different kinds of dialysis.

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DETERMINATION OF INORGANIC SULFATE IN HUMAN SALIVA AND SWEAT BY CONTROLLED-FLOW ANION CHROMATOGRAPHY

NORMAL VALUES IN ADULT HUMANS*

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SUMMARY

Following the previous demonstration that low concentrations of inorganic sulfate (SO_4) in human serum and cerebrospinal fluid can be accurately determined by controlled-flow anion chromatography, the assay has been extended to the quantitation of free SO_4 in saliva and sweat by modification of the established methods of sample collection and preparation. Salivary secretions were ultrafiltered to remove macromolecular polyanions that bind irreversibly to the anion-exchange separator column and reduce resolution. Sweat was collected from 22 fasted adult volunteers using a method which utilizes absorbent filter pads applied to the forearm after secretion had been stimulated by pilocarpine iontophoresis. It was necessary to acid wash the filter pads to reduce sulfate contamination. Saliva ultrafiltrate or sweat was diluted and injected onto a Dionex D-10 Ion Analyzer using the standard anion column system.

The mean inorganic SO_4 concentration in saliva from seventeen adult fasting volunteers was 72 ± 4 μmol/l (± S.E.); the mean SO_4 concentration in sweat was 83 ± 3 μmol/l. Both are significantly less than in matching serum, suggesting that SO_4 is actively removed during formation of these glandular secretions. The ion chromatographic assay is shown to be capable of measuring SO_4 in biological fluids at concentrations that are otherwise undetectable by conventional assay techniques.

INTRODUCTION

The measurement of inorganic sulfate (SO_4) in complex matrices is significantly compromised by the relative insensitivity and non-specificity of

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classical chemical methods [1, 2]. In biological fluids, procedures that depend on selective barium sulfate precipitation suffer from incomplete reaction sequences if the SO_4 concentration is much less than 150 μmol/l [3, 4]. Sensitive precipitations of SO_4 with organic dyes, such as benzidine [5, 6], or precipitations of excess barium with chloranilate [7], or rhodizonate [8], are sufficiently non-specific that the physiological concentrations of co-attendant phosphate and chloride ions render assay results of unmodified fluids uninterpretable [1, 9]. Controlled-flow anion chromatography measures SO_4 by its conductimetric properties after it has been separated from other anions, and is ideally suited to the assay of biological samples [10—13].

Recent experiments have shown that the high sweat chloride, which is a hallmark of cystic fibrosis, arises from a defect in anion transport [14]. With this in mind, we set out to develop methods for measuring trace anions in sweat and saliva, focusing first on SO_4, which may be a useful analogue of chloride in the study of membrane transport [15, 16].

In an effort to extend the method for SO_4 determination to secretions of salivary and sweat glands, it was necessary to modify current procedures for sample collection and preparation before analysis. This report details the assay modifications and estimates of precision. Data are also presented for normal SO_4 concentrations in adult human secretions.

METHODS AND MATERIALS

Subjects

Adult laboratory personnel (mean age 26.2 years, range 18—43 years) gave informed consent to procedures for the collection of saliva, sweat and serum. Subjects were instructed to fast after midnight and to take only small amounts of tap water in the morning. Samples were collected between 09.00 and 12.00 h. If saliva was to be collected, subjects were also asked to forego brushing their teeth since many toothpastes contain sodium dodecyl sulfate. Experimental protocols were approved by the ethics committees of the I.W. Killam Hospital for Children and Dalhousie University Faculty of Medicine.

Sample collection and preparation

Blood

Blood was drawn from the antecubital vein using disposable needles and syringes and transferred to capped 25-ml Corning centrifuge tubes (Corning Glass Works, Corning, NY, U.S.A.). The blood was centrifuged and the serum supernatant stored at −20° C until assayed.

Saliva

Saliva was collected in disposable polystyrene cups, after subjects had thoroughly rinsed their mouths, gargled with distilled, deionized water (ddH_2O), and discarded the first few ml of saliva. Subjects chewed on pieces of Parafilm "M" wax (Marathon, Neenah, WI, U.S.A.) to stimulate flow but no effort was made to standardize or quantitate flow-rate. After 15 min, or when

a volume of 20—30 ml was obtained, the sample was centrifuged for 10 min at 1000 *g* to remove particulates and the supernatant stored at −20° C until assay.

Ultrafiltration of saliva. Injection of unfiltered saliva into the ion chromatograph results in significant loss of resolution. An Amicon Micropartition System (MPS-1, Amicon Corporation, Danvers, MA, U.S.A.) was therefore used to filter saliva samples. The MPS-1 system utilizes YMT cellulosic membrane filters that have a nominal molecular weight cut-off of 25,000, and generates an ultrafiltrate by centrifugation partition [17]. Integrity of the filters was tested by checking the ultrafiltrate for protein with urinary dipsticks (Labstix-R, Ames Company Division, Miles Labs., Rexdale, Canada).

In initial trials, it was noted that ultrafiltered saliva samples had more than twice the SO_4 content of the same samples analysed neat (Table I). The adoption of an acid-washing procedure of the membrane filters prior to processing the saliva was instituted to eliminate contamination. Washing the filters with four successive 1-ml aliquots of 1 m*M* hydrochloric acid reduced the concentration of SO_4 eluted from 57 to 5 μmol/l. (The SO_4 contamination of the hydrochloric acid before filtration was less than 1 μmol/l.) As Table I indicates, samples of saliva that are filtered with membranes washed as described above contain the same amount of SO_4 (71 ± 1 μ*M* vs. 70 ± 1 μ*M*, $n = 3$). This procedure was therefore adopted for all future studies. A saline blank was added in each run to ascertain that any particular batch of washed filters added less than 5 μmol/l SO_4 to the ultrafiltrate.

TABLE I

EFFECT OF ULTRAFILTRATION ON THE SULFATE CONTENT OF HUMAN SALIVA

Results are the mean ± S.E. ($n = 3$).

Sample	SO_4 Concentration (μmol/l)		
	Unfiltered	Filtered	
		Fresh filters	Acid-washed filters
Dilute hydrochloric acid (1 m*M*)	0.37 ± 0.09	58→17→15→5*	5**
Saliva	73 ± 2	171 ± 6***	—
	70 ± 1	—	71 ± 1§

*SO_4 Concentrations of successive 1-ml washes of the filter with 1 m*M* hydrochloric acid.
**If the filters contained more than 5 μmol/l, they were discarded.
***Significantly higher than the same sample before filtration ($p < 0.01$, Student's *t*-test).
§No significant effect if acid-washed filters are used.

Frozen samples of saliva were thawed and recentrifuged at 1000 *g* for 10 min to remove any futher precipitate. Approximately 1 ml of the supernatant was added to the MPS-1 apparatus. A 5-min run at 4° C and 1900 *g*, in an RC2-B Sorvall refrigerated centrifuge equipped with a fixed-angle SS-34 rotor (Sorvall, Newtown, CT, U.S.A.) was used to obtain an initial 100 μl of ultrafiltrate. Because the acid-washed membrane filters could not be adequately dried, the initial ultrafiltrate, diluted somewhat with wash fluid, was discarded. The

sample was then centrifuged for a further 30 min at 1900 *g* and the final 200—300 μl of ultrafiltrate were collected, diluted fifteen-fold with 1 m*M* sodium hydroxide, and analyzed immediately.

Sweat

The collection of sweat on absorbent discs is a standard clinical procedure for measurement of sweat chloride in the diagnosis of cystic fibrosis. A modification of the method of Gibson and Cooke [18, 19] was used, as outlined below.

Preparation of absorbent discs. Cellulose filter discs [25 mm diameter, Type 470 (Lot F25), Schleicher and Schnell, Keene, NH, U.S.A.] also contained significant amounts of SO_4. To remove this contamination, discs were placed in Fisherbrand-R Histological Processing Cassettes (Product 12-653-16A, Fisher Scientific, Dartmouth, Canada), rinsed in three successive solutions of 5 m*M* nitric acid (prepared from reagent-grade nitric acid; J.T. Baker, Phillipsburg, NJ, U.S.A.), and finally in ddH_2O. Drying was facilitated by brief rinses with 95% (v/v) ethanol and acetone. Discs were placed in 25-ml polypropylene weighing bottles (Markson Science, Del Mar, CA, U.S.A.) and stored in a desiccating chamber until used. Under these conditions, the disc dry weight remained constant over 24 h.

Collection. Both ventral forearms were wiped with low-sulfate gauze (Curity Gauze Sponges, 12-ply, 10 × 10 cm; Kendall Canada, Toronto, Canada) that had been dampened with ddH_2O. Pilocarpine hydrochloride (1%, w/v; Sigma, St. Louis, MO, U.S.A.) and sodium nitrate (2%, w/v) were used to soak gauze pads attached to the ventral anodic and dorsal cathodic plates, respectively. The padded plates were applied to each forearm, and the current was maintained at 1.5 μA for 5 min, after which the iontophoresed areas were again wiped clean with dampened gauze, then dried.

Two absorbent filter discs, prepared as described above, were removed from their weighing bottles and placed under a Parafilm covering that was then carefully taped to the arm. After 45 min, the sweat-laden discs were returned to the weighing bottle and the total weight was recorded. Subtraction of the dry weight (in the bottle) yielded the weight of collected sweat and hence its volume. (The density of sweat of any collection in this manner is sufficiently close to that of water that any correction adds less than 1% to the overall accuracy [20].)

If the weight of sweat collected was less than 50 mg, it was discarded; when it was less than 100 mg, it was pooled with the matching sample from the opposite forearm. Exactly 4.00 ml of 1.0 m*M* sodium hydroxide were added to the weighing bottles containing the discs. Sweat was eluted into the sodium hydroxide solution over at least 10 min at room temperature, with occasional agitation of the discs in the solution. The sodium hydroxide eluate was then decanted and spun at 1000 *g* for 5 min to remove any cellulose fibres loosened from the discs. An aliquot of the supernatant was immediately subjected to chromatographic analysis.

Recovery studies

Saliva

Increasing amounts of 50 m*M* sodium sulfate were added to aliquots of a pooled saliva sample, which were then filtered and analyzed by the methods described above.

Sweat

Sweat samples were large enough for analysis, but recovery studies cannot be performed on samples obtained by the iontophoretic method using absorbent discs. Instead, tracer sulfate ($^{35}SO_4$, as carrier-free sulfuric acid; New England Nuclear, Montreal, Canada) was added to prepared dry discs or to discs soaked with varying amounts of ddH_2O or Ringer's lactate. The radioactive sample was then eluted in the standard fashion. The eluate was counted to determine whether any of the added SO_4 remained bound to the disc. Similarly, varying amounts of 100 μM sodium sulfate were added to blank discs and the samples prepared and analyzed in the standard fashion.

Binding studies

Both serum and saliva contain proteins and glycoproteins that are known to bind multivalent ions. To determine whether a significant fraction of sulfate is also significantly bound, small amounts of labelled SO_4 were added to serum, saliva, and saline solutions and subjected to ultrafiltration. The specific activity of the ultrafiltrate was then compared to that of the unfiltered sample.

ANALYTICAL

Controlled-flow anion chromatography

All samples were analyzed on a D-10 ion chromatograph (Dionex, Sunnyvale, CA, U.S.A.). For SO_4 analysis, this instrument was outfitted with two 50 × 4 mm guard columns (containing the same anion-exchange resin as the main separator column), a 250 × 4 mm separator column (AS1, standard anion analysis) and a fixed-bed, 100 × 9 mm S1 suppressor column in series. To reduce the leaching of metal ions from the plumbing into the cation-exchange resin of the anion suppressor column, a cation trap was added to the regenerating system. With biological samples, it was found that the suppressor must be regularly exhausted with 0.1 *M* sodium carbonate, thoroughly rinsed with ddH_2O, and vigorously regenerated with 1 *M* sulfuric acid, if the eluate is to remain free of contaminants and a stable baseline is to be maintained. After approximately 200 samples had been processed, the first pre-column was discarded, the second pre-column moved forward to the position of the first, and the second pre-column replaced with a new one.

Sulfate concentrations were calculated by interpolation using the line of best fit for peak heights of at least four standards. All standard curves used in this study had correlation coefficients greater than 0.99.

Liquid scintillation counting

Aliquots (20 μl) of samples (serum, sweat, saliva, etc.) containing radio-

labelled SO_4 were transferred to mini-vials containing 4 ml of Readi-Solv scintillating fluid (Beckman Instruments, Fullerton, CA, U.S.A.) and counted by liquid scintillation.

Reagents

All chemicals were reagent grade unless otherwise indicated. Solutions were tested by ion chromatography and all contained less than 1 μmol/l SO_4. Because there is a tendency for the sodium hydroxide reagents and solutions to become contaminated with ambient SO_4, fresh 1 m*M* solutions were prepared monthly from individually preweighed vials of pelleted sodium hydroxide (product No. 505-8, Sigma). These solutions had the lowest SO_4 content of the several commercially available preparations assayed. All distilled, deionized water used had a resistance of greater than 10 MΩ and contained no detectable SO_4, as measured by ion chromatography (limit of sensitivity < 0.1 μmol/l).

Statistical analysis

Statistical analyses were performed according to the methods described by Sokal and Rohlf [21]. Like many biological variables, the distribution of SO_4 concentrations often displays upward skewing; where this might distort the statistical interpretation, data were log-transformed prior to analysis.

RESULTS

Chromatographic profiles

Sulfate in saliva emerges as a symmetric peak at 17.0 min (Fig. 1). It is well separated from other anions and readily quantitated by peak height. Sulfate in sweat elutes at 16.3 min as a less symmetric peak that often exhibits tailing (Fig. 1). However, a horizontal baseline can be drawn and the sweat SO_4 measured by peak height as for other samples. Earlier peaks (not labelled) shown in Fig. 1 may be nitrate and bromide [12], but a positive identification was not attempted. Other unidentified substances, indicated by a question mark, were occasionally observed.

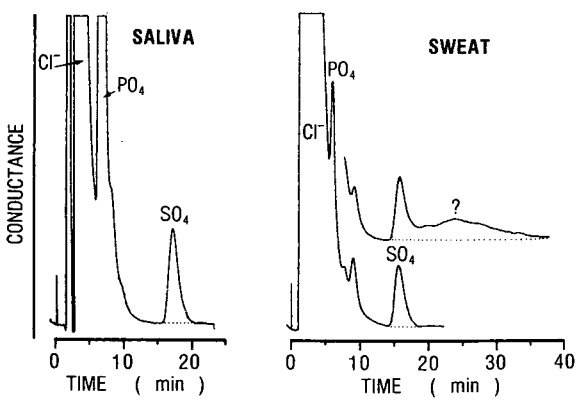

Fig. 1. Chromatographic profiles for saliva and sweat. Scale on the abscissa is arbitrary, based on a 1 μS full-scale deflection and a fixed baseline offset for background conductivity. Baselines for SO_4 determinations are shown as dotted lines.

Recovery studies

As Fig. 2 shows, there is good linear correlation between measured SO_4 and the SO_4 added to saliva prior to ultrafiltration and analysis. The line of best fit ($y = 0.89x + 9.0$; $F_{1,9} = 14.2$, $p < 0.01$) has a slope not significantly different from 1.0 (95% confidence limits: 0.67—1.07). Mean recovery was 96.1 ± 1.4% (mean ± S.E., $n = 10$).

Recovery of radiolabelled SO_4 added to the prepared sweat discs was significantly affected by the ionic strength of solution. Free radiolabelled SO_4 in ddH_2O discs was not completely eluted when fluid volumes of less than 100 μl were added to dry discs; the same radiolabel added to discs soaked in small volumes (< 100 μl) of Ringer's lactate was readily eluted with a recovery of 100 ± 7% (Table II).

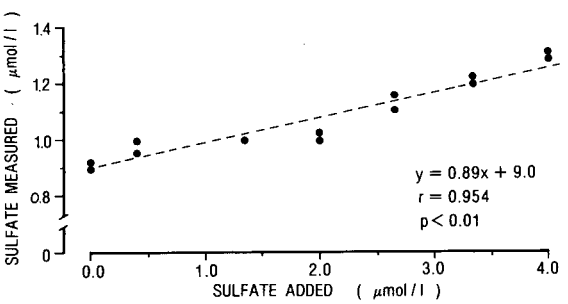

Fig. 2. Recovery of SO_4 added to filtered saliva. Line of best fit, by the least-squares method, is shown; its slope does not differ significantly from 1.00.

TABLE II

RECOVERY OF SO_4 FROM ABSORBENT FILTER DISCS USED IN SWEAT COLLECTION

Addition	*n*	Amount added	Amount recovered	Recovery (%)
Radiolabelled SO_4 (cpm)				
Discs alone	6	671 ± 11*	739 ± 57	110 ± 9
Discs with sweat	2		682 ± 82	102 ± 12
Unlabelled SO_4 (μmol/l)				
Dry discs: < 100 μl	9	100	82 ± 7	82 ± 7
> 100 μl	4	100	105 ± 3	105 ± 3
Discs with Ringer's lactate (50—100 μl)	4	123 ± 4**	123 ± 9	100 ± 7

*Mean ± S.D. of six aliquots.

**Mean ± S.D. of four measurements of Ringer's lactate to which was added a small amount of 50 m*M* sodium sulfate, equivalent to 100 μ*M* in final dilution.

Binding studies

The specific activities of radiolabelled SO_4 were the same in the ultrafiltrates of both serum and saliva as in the original fluids (Table III). Thus, there is no evidence of significant binding of SO_4 to macromolecules, nor to the ultrafiltration membranes themselves.

TABLE III

EFFECT OF MPS-1 ULTRAFILTRATION ON RADIOLABELLED SO_4 IN HYDROCHLORIC ACID, SERUM AND SALIVA

Mean ± S.E. of cpm for three determinations are shown.

Sample	Before ultrafiltration	After ultrafiltration	*p*
1 m*M* Hydrochloric acid	2180 ± 15	2165 ± 20	NS*
Saliva	2080 ± 20	2125 ± 10	NS
Serum	2104 ± 27	2387 ± 16	< 0.05**

*NS, Not significant.

**Specific activity of the ultrafiltrate is marginally but significantly higher than the unfiltered serum. This probably represents the concentrating effect of protein exclusion by ultrafiltration, which amounts to about 7% [32].

Estimates of precision

Within-run coefficient of variance for pooled saliva samples was 4.2% (n = 9). Between-run coefficient of variance was 2.7% (n = 22).

Duplicate samples of sweat from the same individual yielded a maximum estimate for method variability (Fig. 3). Two-way analysis of variance on eighteen duplicates, one from each forearm, yielded a within-patient variability that was 9.9% of the total, of which only a portion can be methodological in origin.

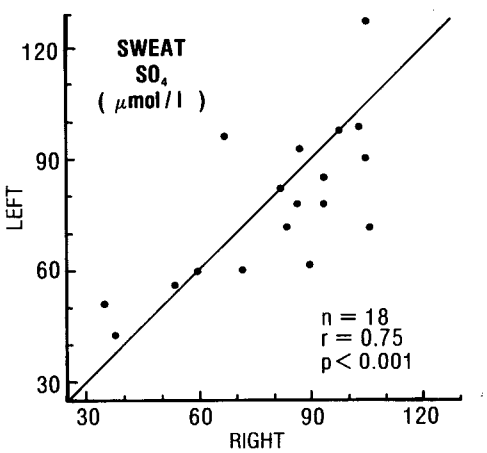

Fig. 3. Correlation between sweat SO_4 concentrations simultaneously obtained from left and right forearms of each subject. The line of identity is shown and with it the correlation coefficient.

SO_4 Concentrations in saliva

The mean concentration of SO_4 in saliva from seventeen adult fasted volunteers was 72 μmol/l (range 52—107 μmol/l) (Fig. 4A). The matching serum concentration was 298 ± 9 μmol/l, which is comparable to values reported previously [11]. The difference between serum and saliva concentra-

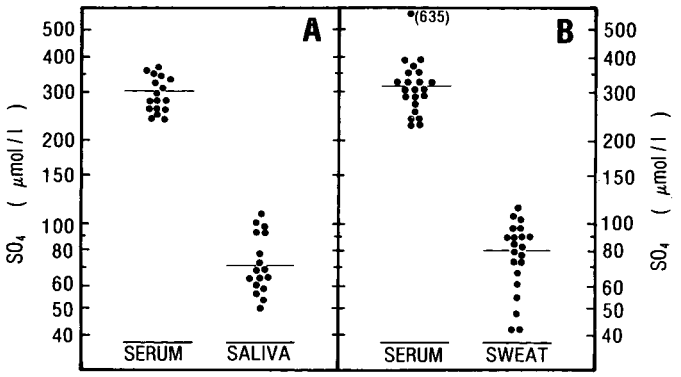

Fig. 4. SO_4 Concentrations in saliva (A) and sweat (B). Note the logarithmic scale of the ordinate. Untransformed means are shown as horizontal lines. Both saliva and sweat SO_4 concentrations are significantly lower ($p < 0.001$) than in matched sera.

tions is highly significant ($p < 0.001$, Student's t-test). There were no significant differences between mean SO_4 concentrations in the ten female (71 ± 6 µmol/l) and seven male (74 ± 7 µmol/l) volunteers. Mean salivary SO_4 concentration was about one-half of that reported previously for cerebrospinal fluid [135 ± 14 µmol/l (mean ± S.E., $n = 25$), $p < 0.001$; Student's t-test] [13].

SO_4 Concentrations in sweat

Mean weight of sweat was 222 ± 16 mg (range 87—431 mg). For sample duplicates ($n = 18$), values for right and left forearms were significantly correlated ($r = 0.75$, $p < 0.01$) (Fig. 3). There was no significant difference in mean sweat weight or SO_4 concentrations between the duplicates (data not shown); therefore, when the sample volume was small, duplicates were pooled for analysis.

Sulfate concentration in sweat from 22 volunteers was 80 ± 3 µmol/l ($n = 39$; range 35—128 µmol/l), while the matching serum concentration was 299 ± 25 µmol/l (Fig. 4B). Serum SO_4 in males and females did not differ significant-

TABLE IV

SULFATE CONCENTRATIONS IN HUMAN SALIVA AND SWEAT

All values given are mean ± S.E. µmol/l.

Group	No. of individuals	Saliva	Serum	No. of individuals	Sweat*		Serum
					Weight (mg)	SO_4**	
Females	10	71 ± 6	287 ± 13	9	221 ± 26	79 ± 5	316 ± 55
Males	7	74 ± 7	306 ± 13	13	531 ± 55	81 ± 7	287 ± 19
Combined	17	72 ± 4	298 ± 9	22	404 ± 47	81 ± 4	299 ± 25

*Total sweat weight from both forearm collections is given.

**SO_4 Concentrations used in this calculation are means of both forearm collections in all but four cases where samples were combined to yield adequate volume for analysis.

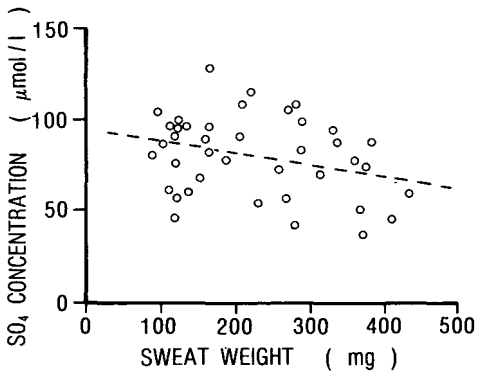

Fig. 5. Dependence of SO_4 concentration on sweat weight. Line of best fit obtained by least-squares regression and analysis of variance is also shown. Note that the influence of sweat weight is modest when compared to the overall variation in SO_4 concentrations.

ly in this study (Table IV) and, although the combined sweat weight in males was more than twice that in females, there was no sex-related difference in sweat SO_4 concentrations.

The possible influence of sweat weight, a measure of secretion rate, on sweat SO_4 concentrations was also examined (Fig. 5). The correlation coefficient was -0.32 ($p = 0.0025$). Analysis of variance for a linear regression model yielded an F statistic ($F_{1,38} = 4.195$) that was just below the 5% level of significance ($p = 0.048$); continued higher-order polynomials did not significantly improve the fit. The slope of the line ($y = -0.067x + 94.8$) is such that sweat weight exerts little influence on normal values.

DISCUSSION

The ultimate composition of the fluids produced by compound exocrine glands is a reflection of two interacting transport processes. The first is initial fluid formation; the second, subsequent modification of fluid by selective reabsorption [22, 23]. Current evidence suggests that the primary secretion is isotonic with extracellular fluid and is equivalent to serum ultrafiltrate [22, 24]. Sweat and salivary glands are no exception, although they differ significantly in fine structure and function. Determination of the solute profiles in human subjects presents a technical challenge for all but the most abundant components. While it is apparent that excretion of electrolytes in sweat or saliva plays only a secondary role in mineral homeostasis [25], fluid composition may provide important clues to the nature of systemic electrolyte transport disorders. Such is the case in cystic fibrosis [26].

Previously, we have shown that controlled-flow anion chromatography is a specific, sensitive and precise method for SO_4 determinations in serum and cerebrospinal fluid [11, 13]. In this report, the same is shown for sweat and saliva. To eliminate the problems of SO_4 contamination, the standard collection procedures had to be modified. In doing so, normal values for saliva and sweat SO_4 in fasting adults have been derived for the first time.

Previous attempts to estimate SO_4 concentrations in human secretions have been made, but total sulfur content [27], or an upper limit [25], are the only values reported. For saliva, the usual procedures which depend on precipitation

of barium or benzidine fail, presumably because these cations bind significantly to strongly charged polyanions (collectively termed "mucus") found in the final salivary product [28]. We found that we could measure SO_4 in the presence of these polyanions, but only at the expense of column degradation and eventual loss of resolution. Ultrafiltered samples, however, were assayed without difficulty. As yet, no attempt has been made to assess the effects of circadian variation [29], gland stimulation, or altered secretion rate on saliva SO_4 concentrations. Moreover, there are significant compositional differences among the fluids of different salivary gland groups which cannot be assessed using whole saliva [30] and would require a more elaborate collection technique.

Reliable collection of human sweat requires attention to technique. This has been amply demonstrated for chloride estimations [20] and proved to be extremely important for SO_4, which is present in a molar abundance that is less than 0.4% of that for chloride. It is fortunate that little manipulation of the sweat sample is required prior to analysis. However, rigorous attention to the elimination of artifacts is still required. As with salivary secretions, we did not attempt to estimate the effects of circadian variation or physiological stimuli on sweat SO_4 concentrations.

Pilocarpine iontophoresis produces a maximum stimulatory response that presumably allows for meaningful comparisons between controls and patient populations [20]. We found a small but significant bias toward lower values at high sweat rates, but the magnitude of the bias was not so large that a corrective factor is required.

It was not surprising to find that SO_4 concentrations are low in both sweat and saliva. The reabsorptive properties of the ductal epithelium are such that the potential difference across the epithelium is highly negative with respect to the lumen. Thus, there is a significant electrical driving force for the transport of anions out of the lumen, across the luminal membrane, and into the epithelial cytosol. From there, the anion diffuses through the cytosol, is transported across the apical membrane, and re-enters the extracellular or intravascular pool [22]. The origin of the potential difference is thought to be the pumping of Na^+ by Na^+—K^+-ATPase across the apical membrane and out of the epithelial cell, followed by influx of cation from the lumen down its chemical gradient into the cell. The passive influx of cation from the lumen thus generates a negative potential in the luminal space.

Chloride reabsorption can thus be viewed as following the pathway of Na^+ absorption into the ductal epithelium, then transcellular diffusion and uphill efflux. Whether the same is true of SO_4 cannot be ascertained from the present data. However, it seems likely that the lower SO_4 concentrations of the final secretion are a reflection of selective ductal reabsorption.

This report illustrates that trace electrolytes can be accurately measured by controlled-flow ion chromatography in very small samples of secreted fluids. It will be of more general interest to determine whether this method can be adapted to generate anion or cation profiles for different secretions. With the appropriate technical modifications, it should be possible to measure chloride, bicarbonate, lactate, phosphate and sulfate simultaneously [10, 31]. Application of such a method might well be very rewarding in the effort to identify the anion transport defect that characterizes the sweat duct in cystic fibrosis [22].

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DETERMINATION OF PHTHALIC ACID, MONO-(2-ETHYLHEXYL) PHTHALATE AND DI-(2-ETHYLHEXYL) PHTHALATE IN HUMAN PLASMA AND IN BLOOD PRODUCTS

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SUMMARY

Pretreatment for the determination of phthalic acid, mono-(2-ethylhexyl) phthalate (MEHP) and di-(2-ethylhexyl) phthalate (DEHP) in human serum or plasma, and the determination of these compounds in blood products by high-performance liquid chromatography was studied. The amount of phthalic acid, MEHP and DEHP, migrated into blood products from a flexible bag, was studied.

About 0.1% of DEHP in a flexible bag was found to have migrated into human platelet plasma. Most of the MEHP and phthalic acid detected in human platelet plasma was not derived from the flexible bag but was produced by enzymatic hydrolysis of the migrated DEHP. The amount of DEHP eluted into blood products from the flexible bag differed, depending upon storage time, storage temperature, etc.

INTRODUCTION

The most common material for medical devices such as flexible bags and tubing is polyvinyl chloride (PVC) with di-(2-ethylhexyl) phthalate (DEHP) as the main plasticizer. Recently the toxicity, mutagenicity and carcinogenicity of DEHP have been questioned [1], and it has been pointed out that DEHP is changed by heat and enzymes into mono-(2-ethylhexyl) phthalate (MEHP) and further to phthalic acid (PA) [2]. Therefore the safety of these compounds has to be considered, particularly as MEHP is considered to be more toxic than DEHP [3].

We have studied the analytical conditions for high-performance liquid chromatography (HPLC) by which these compounds can be determined in human serum or plasma and have established a quantitative method of excellent reproducibility with an internal standard. Poor recovery of these compounds

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with the pretreatment of serum reported in literature [4—6] has, after various attempts, led us to find a method of pretreating serum ensuring nearly 100% recovery. In particular, we have employed, for the first time, deproteinization with an acetonitrile—sodium hydroxide mixture and ultrafiltration for the pretreatment of serum. In order to examine the elution of PA, MEHP and DEHP from a flexible bag into blood products, we determined these compounds in blood products. The interesting results we obtained are reported here.

EXPERIMENTAL

Reagents

Commercially available and chemically pure reagents were used except for MEHP and monomethyl phthalate (MMP), which were synthesized by the method described in the literature [7].

Materials

Materials used were fresh-frozen human plasma (Japan Red Cross, JRC, Tokyo, Japan; this was stored at −30°C for one week in a flexible bag), concentrated human platelet plasma (JRC; this was stored at 22°C for two days in a flexible bag; during one day, centrifugation was carried out), human serum (JRC; this was stored in a glass bottle), human plasma (JRC; this was kept frozen for 21 days in a flexible bag), Plasmanate (Green Cross, Osaka, Japan),

TABLE I

CONDITIONS FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND PRETREATMENT OF SERUM

HPLC			
Compound	PA	MEHP	DEHP
Injection volume (μl)	10	10	10
Column	Senshu-Pak $5C_{18}$ (150 × 4.6 mm)	Senshu-Pak $5C_{18}$ (150 × 4.6 mm)	Senshu-Pak $5C_{18}$ (150 × 4.6 mm)
Temperature	Ambient	Ambient	Ambient
Flow-rate (ml/min)	0.8	0.8	1.2
Detection	UV (254 nm)	UV (254 nm)	UV (254 nm)
Retention time (min)	4.6	10.6	6.0
Mobile phase*	A	B	C
Internal standard	MMP	MBP	DnOP

Pretreatment

Serum PA:	Serum (1 ml), water (3 ml) and 50% phosphoric acid (0.1 ml) were mixed and after ultrafiltration by Centrifree MPS-3, the ultrafiltrate was injected for HPLC.
Serum MEHP:	Serum (1 ml), 50% phosphoric acid (50 μl) and the mixing solution of diethyl ether—methanol, 2:1 (3 ml) were mixed, kept one day at 4°C, again mixed and after centrifugation the diethyl ether layer of the supernatant was injected for HPLC.
Serum DEHP:	Serum (1 ml), 1 *M* sodium hydroxide (1 ml) and acetonitrile (3 ml) were mixed and after centrifugation the upper layer was injected for HPLC.

*A: 5 m*M* Monosodium phosphate aqueous solution—acetonitrile (80:20), pH 2.8 adjusted with phosphoric acid. B: 5 m*M* Monosodium phosphate aqueous solution—acetonitrile (50:50), pH 2.8 adjusted with phosphoric acid. C: Water—acetonitrile (10:90).

dried human plasma (Nippon Pharmaceutical, Tokyo, Japan), human and equine plasma (Japan Biological Materials Center, JBMC, Tokyo, Japan; the human plasma was stored at 22°C for 25 days in a flexible bag and the equine plasma was stored in a glass bottle), equine serum (Flow Labs., VA, U.S.A.; this was kept in a glass bottle), and a flexible bag (Terumo, Tokyo, Japan; this was made of PVC).

Equipment

The high-performance liquid chromatograph (Shimadzu LC-3A) was equipped with an ultraviolet (UV) detector (Shimadzu SPD 2A), data processing equipment (Shimadzu Chromatopac C-RIA) and ultrafilter (Amicon MPS-3).

Procedures

Table I summarizes the HPLC and pretreatment conditions for serum and plasma that we established. Fig. 1 shows representative chromatograms for PA, MEHP and DEHP in human serum. Almost the same chromatograms were obtained for PA, MEHP and DEHP in human plasma.

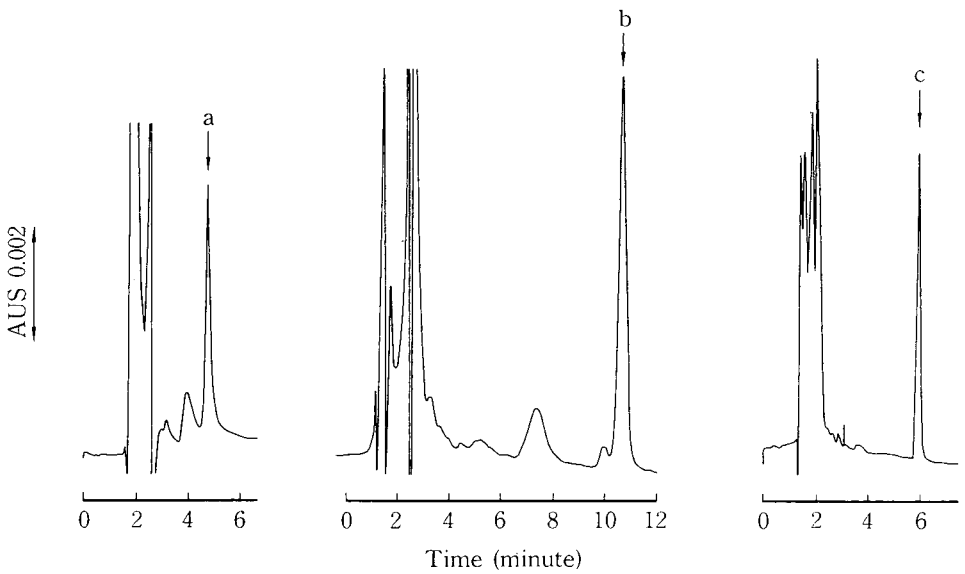

Fig. 1. Typical chromatograms of phthalic acid, mono-(2-ethylhexyl) phthalate and di-(2-ethylhexyl) phthalate in human serum. a, b and c indicate peaks of PA, MEHP and DEHP, respectively. Concentrations of PA, MEHP and DEHP were 6.1, 39.4 and 26.0 μg/ml, respectively; 10 μl were injected. Conditions for serum pretreatment and for HPLC are given in the text. Almost identical chromatograms were obtained for PA, MEHP and DEHP in human plasma.

RESULTS AND DISCUSSION

Identification of synthesized MMP and MEHP

These compounds were identified by elementary analysis, infrared, nuclear magnetic resonance (Fig. 2) and mass spectra (Fig. 3). The values obtained for

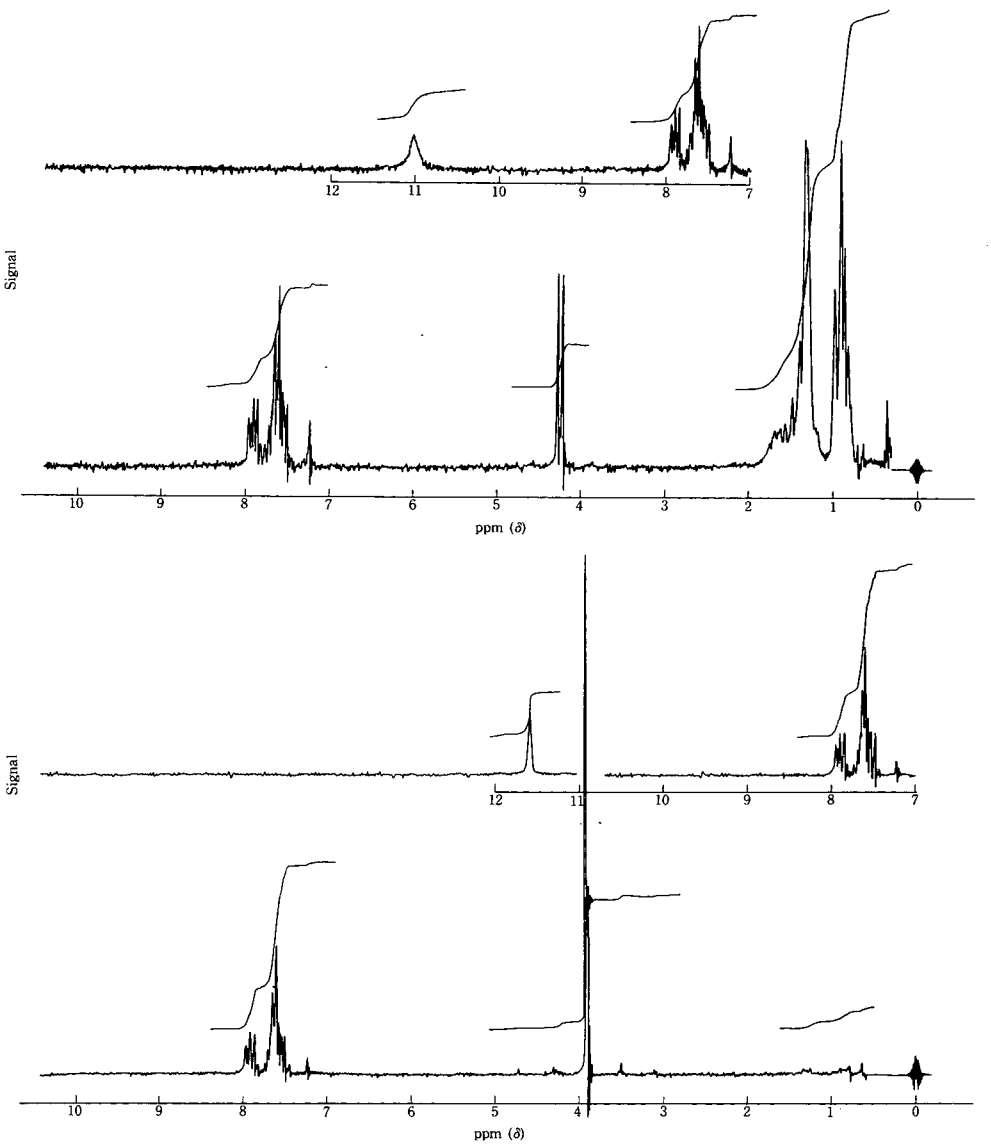

Fig. 2. Nuclear magnetic resonance spectra of mono-(2-ethylhexyl) phthalate (upper two traces) and of monomethyl phthalate (lower two traces). Nucleus ^1H, zero reference trimethylsilane, sample temperature ambient, solvent C^2HCl_3.

elementary analysis of MEHP and MMP were: MEHP, carbon 67.58%, hydrogen 7.45%, m.p. 4°C; and MMP, carbon 60.88%, hydrogen 5.15%, m.p. 82—83.5°C. There was a linear relation between the number of carbons in the alkyl group of the monoalkyl phthalates and the logarithm of the retention time (t_R).

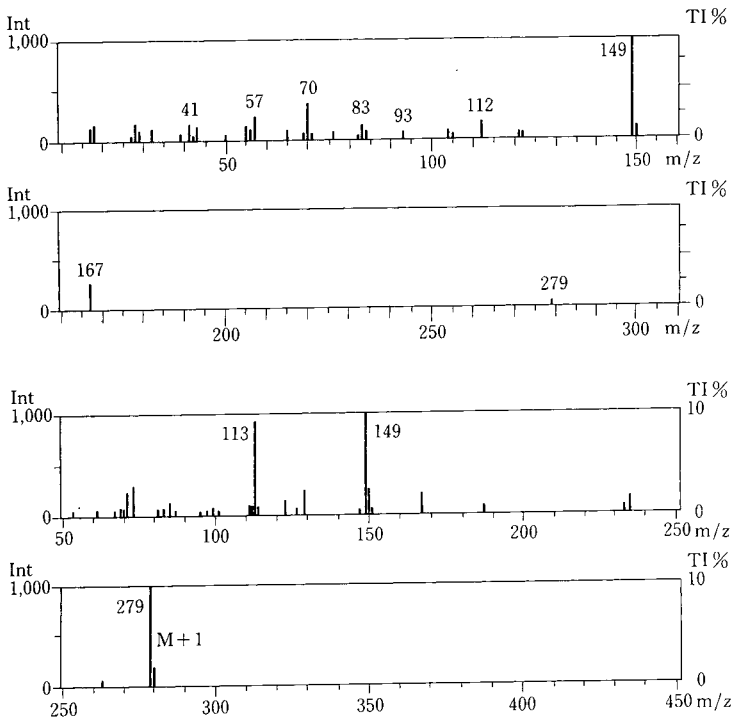

Fig. 3. Electron-impact (EI) mass spectrum (upper two traces) and chemical-ionization (CI) mass spectrum (lower two traces) of mono-(2-ethylhexyl) phthalate. Analytical conditions for EI and CI mass spectra:

Mass spectrum	EI	CI
Equipment	JMA-D 300	JMA-D 300
Data processing	JMA-200 S	JMA-200 S
Ionization voltage	70 V	200 V
Reaction gas	—	Isobutane
Ionization current	300 μA	300 μA
Accelerating voltage	3 kV	3 kV
Ion multiplier voltage	1.25 kV	1.25 kV
Chamber temperature	150°C	130°C

Effect of acetonitrile concentration on the analysis of PA and MEHP in human serum and plasma

Using a mixture of phosphate buffer, which contained 5 m*M* sodium phosphate, and acetonitrile as the mobile phase adjusted with phosphoric acid to pH 2.8, the effect of acetonitrile concentration on the t_R of PA and MEHP was studied (Fig. 4). With 13—20% acetonitrile a good separation of PA from serum admixtures was obtained. The acetonitrile concentration was set at 20% with regard to the t_R of the internal standard, MMP.

With 60% acetonitrile, separation of MEHP from serum admixtures was unsatisfactory, but 50% acetonitrile afforded a good separation of monobutyl phthalate (MBP) as internal standard from MEHP and of these compounds from serum admixtures; 40% acetonitrile failed to elute MEHP from the column.

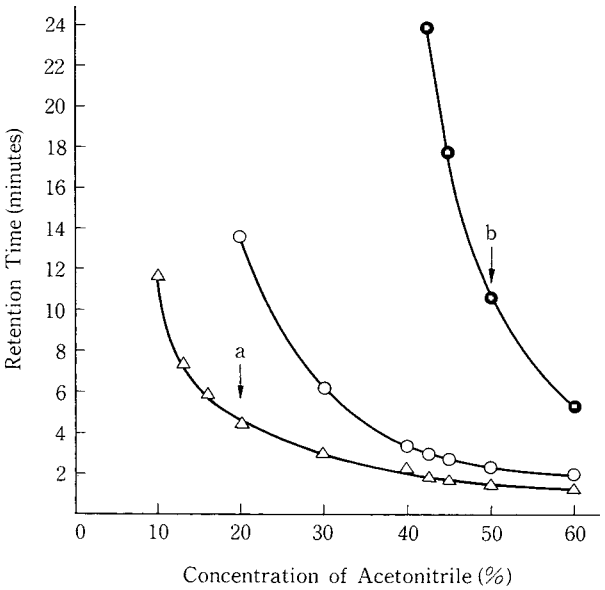

Fig. 4. Relation between retention time and concentration of acetonitrile. a and b indicate the recommended mobile phase for the determination of phthalic acid (△) and mono-(2-ethylhexyl) phthalate (●), respectively, in human serum. With the mobile phase used for the determination of MEHP, PA could not be satisfactorily separated from serum admixtures.

Under the analytical conditions for MEHP, PA was eluted in the vicinity of the void volume, resulting in insufficient separation from serum admixtures. Therefore, the simultaneous determination of PA and MEHP in serum or in plasma under isocratic conditions by the ion-suppression method was impossible. To make it possible to perform the simultaneous determination of PA and MEHP in serum and in plasma under isocratic conditions, paired-ion chromatography was attempted with various mixtures of 1/15 *M* phosphate buffer (pH 7.38) containing 5 m*M* *n*-butyltetraammonium phosphate and acetonitrile (from 50:50 to 80:20) as the mobile phase. However, we failed to find a condition under which satisfactory simultaneous separation of PA from MEHP and of these compounds from serum admixtures is attained.

The gradient elution method, being somewhat unsatisfactory in its reproducibility, was not employed as the quantitative condition in the present study.

The effect of acetonitrile concentration on the analysis of DEHP in human serum and plasma

The use of 100% acetonitrile resulted in poor separation of DEHP and the internal standard di-(*n*-octyl) phthalate (DnOP) from serum admixtures; 80% acetonitrile failed to elute DEHP from the column; at 90% the separation of DEHP from DnOP and of these compounds from serum admixtures was excellent.

Linearity of the calibration curve and the detection limits

Data are presented in Table II. The coefficient of variation (C.V.) of

TABLE II

LINEARITY AND DETECTION LIMIT

Signal-to-noise ratio = 2. Application volume = 10 μl. Sensitivity = 0.02 a.u.f.s. (254 nm).

Compound	Linear range (μg/ml)	Detection limit (ng)
PA	2—250	2.5
MEHP	5—500	10.5
DEHP	5—400	8.8

repeated injections of PA, MEHP and DEHP was within 1% (n = 5) in all cases. Serum that was not stored in a flexible bag and did not contain PA, MEHP or DEHP was treated as blank.

Pretreatment of PA, MEHP and DEHP in human serum and plasma

Deproteinization for the recovery of MEHP and DEHP in human serum and plasma. We failed to find a suitable deproteinization method with satisfactory recovery rates for PA, MEHP and DEHP in the literature [4—6]. Therefore, we tried to find a method of pretreatment which gives a good recovery rate (Table III). To human serum samples of between 5 and 8 ml in which PA, MEHP and DEHP were not detected, were added 20 and 10 μl, respectively, of acetonitrile—water (60:40) solutions of PA and MEHP (10.0 and 39.4 mg/ml) and 10 μl of a hexane solution of DEHP (39.08 mg/ml), shaken well, allowed to stand overnight at 4°C and used for the study of the deproteinization as described below.

At first, the effect of deproteinizing eluate on the elution of DEHP from serum was studied. Although direct injection of a two-fold dilution of serum with water gave good recovery of MEHP and DEHP, deterioration of the column with serum protein occurred (Table III). Addition of 1 *M* sodium hydroxide with acetonitrile to serum for deproteinization resulted in nearly 100% recovery of DEHP. The possibility of DEHP and DnOP being hydrolysed with sodium hydroxide was discounted. Deproteinization with acetonitrile alone did not give a satisfactory recovery of DEHP, and an acid added in place of 1 *M* sodium hydroxide also gave a low recovery.

We then studied the effect of deproteinizing eluents on the elution of MEHP in serum. Although the same pretreatment of serum as with DEHP resulted in about 90% recovery (Table III), a variety of attempts were made for a higher recovery. To suppress ionization of MEHP, phosphoric acid was added to serum and further a mixture of diethyl ether—methanol (2:1) was added followed by stirring, centrifugation and injection of the diethyl ether layer of the supernatant into the HPLC system. The recovery obtained was about 86%, but a recovery of 100% was attained when the mixture was allowed to stand overnight at 4°C after stirring without centrifugation. The mixture was stirred, centrifuged and the diethyl ether layer of the supernatant injected into the HPLC system. Recovery of MEHP and DEHP in human plasma was also about 100% using the above deproteinization method.

An attempt to find an efficient deproteinizing eluent for the recovery of PA

TABLE III

DEPROTEINIZATION PRETREATMENT OF HUMAN SERUM FOR PA, MEHP AND DEHP DETERMINATION

Twenty microlitres of 10 mg/ml PA, 10 μl of 39.4 mg/ml MEHP in acetonitrile—water (3:2) and 10 μl of 39.08 mg/ml DEHP in hexane were added to 5—8 ml of human serum and mixed. It was kept for 24 h at 4°C and was used as the sample named as serum in the column of methods of deproteinization. The recovery ratio in parentheses was that of the sample centrifuged after being stirred overnight.

Method of deproteinization	Recovery ratio (%)		
	PA	MEHP	DEHP
Serum 1 ml + acetonitrile 3 ml	23.0	54.5 (85.6)	72.5 (92.8)
Serum 1 ml + 1 *M* sodium hydroxide 1 ml + acetonitrile 3 ml	15.6	90.4 (87.9)	99.9 (101)
Serum 1 ml + 6 *M* perchloric acid 0.1 ml	76.0	1.3	None
Serum 1 ml + 6 *M* perchloric acid 0.1 ml + acetonitrile 3 ml	45.2	35.0	66.3 (84.8)
Serum 1 ml + 1 *M* sodium hydroxide 1 ml + tetrahydrofuran 3 ml	—	—	99.8
Serum 1 ml + 50% phosphoric acid 50 μl + chloroform 2 ml	4.1	10.1	2.8
Serum 1 ml + 50% phosphoric acid 50 μl + diethyl ether 2 ml	72.2	45.6	9.4
Serum 1 ml + 50% phosphoric acid 50 μl + hexane 2 ml	N.D.	59.0	1.9
Serum 1 ml + 50% phosphoric acid 50 μl + chloroform—methanol (2:1) 3 ml	38.7	10.4	37.7 (37.6)
Serum 1 ml + chloroform—methanol (2:1) 6 ml	—	—	85.7 (82.0)
Serum 1 ml + 50% phosphoric acid 50 μl + diethyl ether—methanol (2:1) 3 ml	78.0 (78.5)	85.6 (100)	75.5 (92.0)
Serum 1 ml + diethyl ether—methanol (2:1) 6 ml	—	—	75.4 (89.4)
Serum 1 ml + 50% phosphoric acid 50 μl + heptane—isopropanol (1:4) 5 ml	58.4	34.6	77.8 (98.0)
Serum 1 ml + heptane—isopropanol (1:4) 6 ml	—	—	78.8 (98.6)
Serum 1 ml + 50% phosphoric acid 50 μl + chloroform—diethyl ether (1:1) 4 ml	66.0	18.6	3.9
Serum 1 ml + water 1 ml	48.0	103.0	101.0
Serum 1 ml + water 3 ml	—	102.0	—

in serum failed within the range of this study (Table III); therefore we investigated the ultrafiltration method described below.

Ultrafiltration method for the recovery of PA in human serum and plasma. With the same materials as used for deproteinization, ultrafiltration was studied as a pretreatment method (Table IV). Of the PA, MEHP and DEHP in human

TABLE IV

ULTRAFILTRATION PRETREATMENT OF HUMAN SERUM FOR PA, MEHP AND DEHP DETERMINATION

The same sample as described in the legend to Table III was used.

Method of ultrafiltration	Recovery ratio (%)		
	PA	MEHP	DEHP
Serum 1 ml	76.8	3.3	None
Serum 1 ml + 1 *M* sodium hydroxide 1 ml	12.6	23.4	None
Serum 1 ml + 50% phosphoric acid 50 μl	80.6	1.8	None
Serum 1 ml + 50% phosphoric acid 200 μl	81.2	1.5	None
Serum 1 ml + water 1 ml + 50% phosphoric acid 100 μl	92.5	1.8	None
Serum 1 ml + water 3 ml + 50% phosphoric acid 100 μl	99.9	6.8	5.6

serum, about 77%, 3% and 0%, respectively, were found to be present in the ultrafiltrate. Addition of acid to the serum increased the amount of PA in the ultrafiltrate to about 80%, but it caused little change in amounts of MEHP and DEHP. An increased amount of acid did not alter the amounts of PA, MEHP and DEHP in the ultrafiltrate, but dilution of serum with water increased the recovery; in particular, PA was found for the most part in the ultrafiltrate after the addition of acid to a four-fold dilution of serum with water. The fact that this method gave 100% recovery for MMP suggests that it is suitable for the pretreatment of hydrophilic compounds. PA in human plasma was about 100% recovered by this ultrafiltration method.

Pretreatment using Sep-Pak C_{18}. To 1 ml of human serum as used for deproteinization, 3 ml of water and 0.1 ml of 50% phosphoric acid were added and shaken vigorously. Then 1 ml of the mixture was applied to Sep-Pak C_{18} and eluted with 8 ml of methanol or acetonitrile. The solvent was evaporated and the residue was dissolved again in 1 ml of the mobile phase for MEHP analysis, with a recovery of 86.6% for PA and 87.0% for MEHP. No difference was found in the recovery ratio between acetonitrile and methanol. Experiments under various conditions revealed that the Sep-Pak C_{18} method for the recovery of PA and MEHP in human serum was inferior to the two pretreatment methods previously described, in terms of the low recovery ratio and the troublesome handling of the evaporation of the solvent.

Determination of PA, MEHP and DEHP in a flexible bag and their migration into blood products

About 1 g of that part of a flexible bag which comes in contact with blood (about 15 g) was cut into fine pieces, to which 100 ml each of diethyl ether and phosphoric acid—diethyl ether (pH 2) were added. After shaking for four days at room temperature, the solvent was evaporated and PA, MEHP and DEHP were determined after re-dissolution in the mobile phase (Table V). The extraction by phosphoric acid—diethyl ether produced a similar result to that obtained with diethyl ether extraction. Table V summarizes total amounts of PA, MEHP and DEHP having migrated into human platelet plasma. About 0.13% of DEHP from the flexible bag migrated into the human platelet plasma.

TABLE V

AMOUNTS OF PA, MEHP AND DEHP EXTRACTED WITH DIETHYL ETHER FROM A FLEXIBLE BAG AND THE AMOUNTS OF THESE COMPOUNDS ELUTED INTO CONCENTRATED HUMAN PLASMA PLATELETS

One gram of flexible bag was cut into small pieces, 100 ml of either diethyl ether or acid—diethyl ether solution were added and the whole was kept constantly agitated for four days at room temperature.

	Amount extracted* (n = 3)	Amount eluted** (n = 3)
DEHP (mg)	3128.1 ± 179.4	4.0 ± 0.5
MEHP (μg)	130.8 ± 14.3	589.5 ± 101.3
PA (μg)	0.0	9.0 ± 1.2

*Amount was that contained in 15 g of flexible bag.
**Amount was that contained in 15 ml of concentrated human plasma platelets.

The fact that PA, which was not detected in the flexible bag, was detected in human platelet plasma suggests that it was produced by enzymatic hydrolysis from the eluted DEHP. It is likely that the speculation will hold true concerning MEHP. This fact will be supported by the findings that in spite of the DEHP/MEHP ratio being about 24,000:1 in the flexible bag, the ratio in human platelet plasma was about 7:1 and that the amount of MEHP in human platelet plasma was greater than in the flexible bag.

MEHP and PA were not detected as impurities in 10,000 ppm of industrial DEHP (Ishikawa Product, Tokyo, Japan) that is added to PVC. This suggests a possibility that MEHP detected in the flexible bag (about $4 \cdot 10^{-3}$% of DEHP) was produced from DEHP by heating during the PVC moulding procedures [8]. During moulding procedures and also during the acid—diethylether extraction procedure PA was not produced from DEHP or from MEHP.

Quantitative determination of PA, MEHP and DEHP in blood products

Using the following blood products — fresh-frozen human plasma, concentrated human platelet plasma, heated human plasma protein of Plasmanate, dried human plasma, human plasma and equine plasma — the amounts of PA, MEHP and DEHP eluted from the flexible bag into these blood products were determined (Table VI). The results indicated that contact time with the flexible bag with or without centrifugation, storage temperature, etc. were responsible for the differences in the amount of DEHP eluted. With high storage temperature (22°C) and vigorous shaking with centrifugation for one day, DEHP was markedly eluted into the concentrated human platelet plasma and MEHP was also present in a large amount. Of importance is the fact that highly toxic MEHP was detected in fresh-frozen human plasma and concentrated human platelet plasma in amounts of about 1/7 to 1/17 that of DEHP. In Plasmanate manufactured after heating for 10 h at 60°C most of the eluted DEHP was found to be hydrolysed to PA by heat and enzymes. PA, MEHP and DEHP were contained in a significantly large amount in human plasma (JBMC), a product that had been imported from the U.S.A. and which had been stored at room temperature of 22°C for the long period of 25 days. Therefore, DEHP was thought to have been eluted in a larger amount during the storage than was

TABLE VI

CONCENTRATION OF PA, MEHP AND DEHP IN SEVERAL BLOOD PRODUCTS AND IN HUMAN PLASMA

Sample	Concentration (μg/ml)		
	PA	MEHP	DEHP
Fresh frozen human plasma (JRC) (n = 3)	0.3 ± 0.1	1.54 ± 0.3	26.7 ± 0.3
Concentrated human plasma platelet (JRC) (n = 3)	0.6 ± 0.1	39.3 ± 6.8	267.0 ± 30.5
Plasmanate (n = 3)	12.0 ± 1.0	0.9 ± 0.1	2.0 ± 0.2
Dried human plasma (n = 3)	0.7 ± 0.1	N.D.	1.0 ± 0.1
Human plasma (JRC) (n = 3)	3.0 ± 0.4	5.6 ± 1.1	72.5 ± 2.9
Human serum (JRC) (n = 3)	N.D.*	N.D.	N.D.
Human plasma (JBMC, imported from U.S.A.) (n = 2)	4.9 ± 0.0	54.4 ± 2.5	172.6 ± 5.6
Horse plasma (n = 1)	0.6	0.4	2.2
Horse serum (n = 3)	N.D.	N.D.	N.D.

*N.D., not detected.

the case for human plasma from JRC. Furthermore, storage at room temperature (22°C) may have caused the eluted DEHP to change into MEHP and PA.

CONCLUSION

For the pretreatment of serum for the determination of PA, serum was diluted four-fold with water followed by the addition of acid and ultrafiltration. The pretreatment of serum for the determination of MEHP was the addition of acid and a mixture of diethyl ether—methanol (2:1), overnight standing and centrifugation to obtain the supernatant. The pretreatment of serum for the determination of DEHP was the addition of sodium hydroxide followed by deproteinization with acetonitrile.

Under the isocratic HPLC conditions it was impossible to separate and determine PA, MEHP and DEHP simultaneously in serum. Therefore, determination of PA and MEHP in serum was carried out by reversed-phase chromatography employing ion suppression, and the determination of DEHP in serum was carried out by reversed-phase chromatography without ion suppression.

The amount of DEHP eluted into blood products from the flexible bag differed, depending upon the period of storage with or without centrifugation, the storage temperature, etc. A large amount of DEHP was detected in human platelet plasma. The amount of MEHP was about 1/7 that of DEHP. Detection of PA and MEHP in addition to DEHP suggests the involvement of hydrolytic enzymes.

About 0.1% of the DEHP in a flexible bag was found to have migrated into concentrated human platelet plasma. Most of the PA and MEHP detected in

human platelet plasma was not derived from the flexible bag but was produced by enzymatic hydrolysis of DEHP, thus causing the migration.

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^{63}Ni ELECTRON-CAPTURE GAS CHROMATOGRAPHIC ASSAY FOR BUPRENORPHINE AND METABOLITES IN HUMAN URINE AND FECES

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SUMMARY

A ^{63}Ni electron-capture gas chromatographic assay is described for buprenorphine, a potent narcotic agonist—antagonist. In addition, the assay is useful for the measurement of the metabolite norbuprenorphine and demethoxybuprenorphine, a rearrangement product resulting when buprenorphine is exposed to acid and heat. An extraction procedure was developed which optimized recovery of buprenorphine from biological samples and produced minimal background interferences and emulsion problems. Extract residues were derivatized with pentafluoropropionic anhydride and assayed by gas chromatography. Samples were analyzed with and without enzyme hydrolysis, thus providing a selective and sensitive assay for both free and conjugated buprenorphine, norbuprenorphine and demethoxybuprenorphine. The lower limits of detection following extraction of a 1-ml sample were ca. 10 ng/ml for buprenorphine and demethoxybuprenorphine and 5 ng/ml for norbuprenorphine. Application of the assay to human samples following a 40-mg oral dose of buprenorphine produced no evidence for the presence of demethoxybuprenorphine in urine or feces. Norbuprenorphine (free and conjugated) was present in urinary and fecal samples; buprenorphine (free and conjugated) was found in high amounts only in feces and in trace amounts in urine as conjugated buprenorphine. The urinary and fecal excretion pattern observed for a human subject following oral dosing of buprenorphine suggests enterohepatic circulation of buprenophrine.

INTRODUCTION

Buprenorphine is a synthetic *endo*-ethanotetrahydrooripavine derivative which has both analgesic and opioid antagonistic properties [1]. As an analgesic it is some 25 to 40 times more potent than morphine, whereas when used as an antagonist it is equivalent in potency to the narcotic antagonist naltrexone. The relatively low toxicity of buprenorphine, minimal abstinence

syndrome on abrupt withdrawal following chronic administration and suppression effects on heroin self-administration [2] led to the proposal that it be used for the chemical maintenance of heroin addicts [2, 3].

Because of the high potency of buprenorphine, assay methods for measurements of drug in plasma and cerebrospinal fluid have been based on sensitive radioimmunoassay [4] and radioreceptor [5] techniques. These methods lack specificity, however, and an assay was needed which would simultaneously measure parent drug, potentially active metabolites and drug artifacts in biofluids. This report describes the development of a ^{63}Ni electron-capture gas chromatographic (GC) assay for buprenorphine, norbuprenorphine and the acid-catalyzed rearrangement product, demethoxybuprenorphine (Fig. 1) in human urine and feces. Biological samples collected from subjects participating in assessment studies of buprenorphine as a treatment drug for narcotic addiction were assayed by these methods.

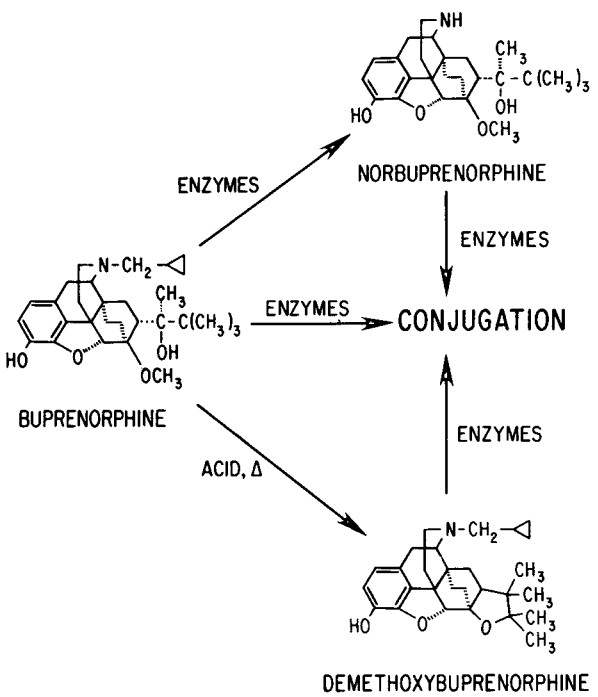

Fig. 1. Buprenorphine biotransformation products and acid-catalyzed rearrangement to demethoxybuprenorphine.

MATERIALS AND METHODS

Standards and reagents

Drug standards were obtained from the following sources: buprenorphine, [15,16-$^3H_2$]buprenorphine (specific activity 38 Ci/mmol, > 95% purity), etorphine (internal standard, Research Technology Branch, Division of Research, National Institute on Drug Abuse, Rockville, MD, U.S.A.); norbuprenorphine (Reckitt and Coleman, Hull, U.K.). The acid-catalyzed

rearrangement product of buprenorphine, demethoxybuprenorphine (N-cyclopropylmethyl-6,14-*endo*-ethano-2′,3′,4′,5′,7,8-hexahydro-4′,4′,5′,5′-tetramethylfurano[2′,3′:6,7]normorphide, DMB) was synthesized from buprenorphine as reported [6]. The identity and purity of these compounds were established by gas chromatography—mass spectrometry and thin-layer chromatography prior to their use. Pentafluoropropionic anhydride (PFPA) was purchased from Pierce (Rockville, IL, U.S.A.). All other chemicals were of reagent grade quality.

Gas chromatography

A Perkin-Elmer gas chromatograph Model Sigma 2 equipped with a ^{63}Ni electron-capture detector was used for the analyses. A glass column (1.8 m × 2 mm I.D.) was packed with 3% OV-17 on Gas-Chrom Q (100—120 mesh). The column was operated isothermally at 260°C. Other conditions were as follows: detector temperature, 320°C; injector temperature, 275°C, carrier gas flow-rate (nitrogen) 20 ml/min : make up gas flow-rate, 40 ml/min.

Subjects, dosing, sample collection

The subjects were healthy, drug-free male volunteers with a history of opiate abuse. Their ages ranged from 21 to 45 years. During the study the subjects were housed in the research ward of the Addiction Research Center (Baltimore, MD, U.S.A.). All gave informed consent to participate in the study, the protocol for which had been approved by the Institutional Review Board of Baltimore City Hospitals. Buprenorphine or placebo was administered orally (10—40 mg) or subcutaneously (1—2 mg) every four days. Urine and feces were collected throughout the study and frozen until time of assay.

Sample preparation and hydrolysis

Frozen fecal samples were weighed and placed in 1.0 l of methanol. The mixture was stirred overnight and decanted into centrifuge tubes. Following centrifugation, aliquots (1.0 ml) were removed and evaporated to dryness under a stream of nitrogen at 40°C.

For enzyme hydrolysis, 1.0 ml of buffer (2 *M* sodium acetate, pH 5.2) was added to the fecal residue or urine sample (1.0 ml) followed by the addition of etorphine, internal standard (I.S., 200—250 ng) and enzyme solution (Glusulase®, Endo Labs., Garden City, NY, U.S.A.) containing 30,000 U of glucuronidase and 6000 U of sulfatase. The samples were mixed and incubated at 37°C for 20 h. Following the incubation period, the reaction was stopped by the addition of 1 ml of 10 *M* phosphate buffer, pH 10.

Extraction and derivatization of buprenorphine and metabolites

Enzyme hydrolyzed or untreated urinary and fecal samples (1.0 ml) were placed in 15-ml centrifuge tubes. An aqueous aliquot of I.S. (100 μl, 200—250 ng) was added to those samples which had not been hydrolyzed. For standard curves, 1.0-ml aqueous aliquots of mixtures of buprenorphine (10—1000 ng/ml), norbuprenorphine (25—2000 ng/ml) and demethoxybuprenorphine (25—2000 ng/ml) were added. The extraction procedure, as outlined in Fig. 2, began with the addition of buffer (1.0 ml of 10 *M* potassium phosphate, pH

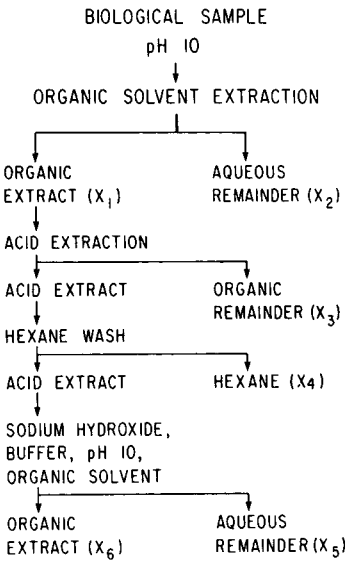

Fig. 2. Extraction procedure for buprenorphine and metabolites from biological samples.

10), and the final pH was adjusted to 10.0 ± 0.1 with 1 *M* sodium hydroxide. Ethyl acetate—heptane (6 ml, 4:1, v/v) was added and the contents were vortexed for 30 sec. For enzyme-hydrolyzed samples, the tubes alternatively were gently shaken on a bench shaker for 30 min in order to prevent emulsion formation. The samples then were centrifuged and the organic layer was transferred to a clean tube. The extraction was repeated and the combined organic extracts were treated with 3 ml of cold 0.1 *M* sulfuric acid. This mixture was vortexed for 30 sec and centrifuged. The organic phase was discarded and the acid extract was treated with 6 ml of hexane. After vortexing, the hexane layer was discarded and the pH adjusted to pH 10 ± 0.1 with 1 *M* sodium hydroxide followed by 1 ml phosphate buffer. Heptane—ethyl acetate (6 ml, 1:5, v/v) was added and the contents were vortexed for 30 sec. After centrifugation the organic layer was transferred to a 13-ml centrifuge tube and evaporated to dryness under a stream of nitrogen.

Derivatization of the residue was accomplished by the addition of toluene (100 μl) and PFPA (20 μl) and incubation at room temperature for 1 h. The excess reagent was removed by evaporation under a stream of nitrogen at room temperature. Ethyl acetate (100 μl) was added and an aliquot (1—2 μl) was withdrawn for GC analysis.

Quantitation of buprenorphine and metabolites

Daily standard curves were constructed for buprenorphine, norbuprenorphine and demethoxybuprenorphine. The curves were prepared from the analyses of standard control solutions containing I.S. (250 ng) and buprenorphine, norbuprenorphine and demethoxybuprenorphine added in equal concentrations in ranges of 10—100, 25—250 or 500—2000 ng/ml. The standard solutions were processed and analyzed in the same manner as drug specimens. Linear responses for peak height ratios versus concentration were

obtained for all concentration ranges with $r \geqslant 0.98$. The lower limits of detection were approx. 10 ng/ml for buprenorphine and demethoxybuprenorphine and 5 ng/ml for norbuprenorphine.

RESULTS AND DISCUSSION

Assay development

During the initial stages of assay development for buprenorphine in biological samples, a variety of experimental conditions were tested and optimized. An extraction scheme (Fig. 2) was developed for the recovery of drug and extractable metabolites from urinary and fecal material. Initial extraction with an organic solvent followed by back-extraction into acid, hexane wash and re-extraction provided a purified drug extract with minimal background interferences when assayed by ^{63}Ni electron-capture GC. The losses and recoveries incurred in the extraction scheme (Fig. 2) with a variety of organic solvents are shown in Table I. The percentages are based on the amount of [^{3}H]buprenorphine added initially and are adjusted for aliquot loss. The percentages X_1 through X_6 reflect the efficiency or loss of each step but also reflect prior losses. Ethyl acetate was slightly superior to methylene chloride in the initial extraction step, X_1, for the recovery of buprenorphine from urine at pH 10. The addition of isopropanol improved initial recoveries (X_1) to about 80%. Other solvent combinations, e.g. pentane—isopropanol (9:1) and ethyl acetate—heptane (4:1) produced moderate (49%) to good (69%) initial recoveries, respectively. Acid extraction of buprenorphine from pentane—isopropanol (9:1) was most efficient (X_3 = 1.6, indicating a small loss), whereas moderate losses occurred with the ethyl acetate based solvents (X_3 ranged from 12.5% to 21.8%) and substantial losses occurred with methylene chloride based solvents (X_3 ranged from 30.1% to 53.4%). Following acid-extraction the hexane wash step (X_4) produced little loss (ca. 1%) in all cases. Recoveries in the final extraction step (X_6) reflect losses in all prior steps as well as losses to the aqueous phase (X_5) and indicate the overall efficiency of the entire

TABLE I

PERCENTAGE RECOVERY AND LOSSES OF [^{3}H]BUPRENORPHINE DURING EXTRACTION

The recoveries reflect the efficiency of each step and that of all prior steps of the extraction scheme (Fig. 2). The percentages ± standard error are based on the amount of [^{3}H]buprenorphine initially added. Corrections were not made for that portion of solvent miscible in the other phase, hence the sum of X_1 and X_2 does not equal 100%.

Solvent (ratio, v/v)	Fraction					
	X_1	X_2	X_3	X_4	X_5	X_6
Ethyl acetate	73.6 ± 0.3	20.7 ± 0.2	16.8 ± 0.1	1.2 ± 0.1	6.7 ± 0.6	47.5 ± 0.6
Ethyl acetate—isopropanol (1:9)	80.2 ± 0.4	15.2 ± 0.4	21.8 ± 0.4	1.2 ± 0.1	3.7 ± 0.1	52.8 ± 0.6
Ethyl acetate—heptane (4:1)	69.2 ± 0.8	23.6 ± 0.5	12.5 ± 0.2	1.2 ± 0.1	6.7 ± 0.6	44.5 ± 1.0
Methylene chloride	56.0 ± 0.5	29.2 ± 0.8	30.1 ± 2.2	1.3 ± 0.2	3.4 ± 0.3	18.9 ± 2.1
Methylene chloride—isopropanol (7:3)	82.3 ± 1.5	12.9 ± 0.2	53.4 ± 1.6	1.3 ± 0.1	2.6 ± 0.1	34.0 ± 0.4
Pentane—isopropanol (9:1)	49.0 ± 3.6	40.7 ± 2.1	1.6 ± 0.1	1.3 ± 0.1	5.6 ± 0.6	28.1 ± 2.4

process. The highest recoveries were obtained with the ethyl acetate based solvents with efficiencies ranging from 44.5% to 52.8%. Ethyl acetate—heptane (4:1) was selected as the solvent of choice based on its overall extraction efficiency of 44.5 ± 1.0%, low background contribution from biological control samples and minimum emulsion problems encountered during extraction.

In addition to solvent selection, a number of other factors were optimized for recovery of buprenorphine from biological samples. During extraction it was found that vortexing samples for 30 sec rather than mechanical shaking for 15 min resulted in slightly higher recoveries. However, severe emulsion problems occurred upon vortexing enzyme-hydrolyzed samples and mechanical shaking for 30 min was substituted in the processing of these samples.

The effect of pH on extraction efficiency was determined across the pH range 7—12. Only small differences in recoveries were found across the entire range and pH 10 was selected as the pH of choice.

Conditions suitable for the complete derivatization of buprenorphine to the mono-PFPA derivative were found to be incubation at room temperature (22—26° C) with 25 μl PFPA in 100 μl of toluene. Heating during derivatization was found to produce extensive decomposition of both buprenorphine and norbuprenorphine and was avoided.

Prior to the completion of the assay development procedure for buprenorphine and metabolites, it was necessary to establish that buprenorphine and metabolites were completely stable under the selected conditions. Buprenorphine has been shown previously to undergo rearrangement in the presence of acid and heat to demethoxybuprenorphine [6]. At pH $\leqslant$ 1 and in the presence of heat, the rearrangement reaction can approach total conversion to demethoxybuprenorphine. However, at pH 1 in the absence of heat, i.e. room temperature, aqueous buprenorphine solutions were shown to be stable up to ten weeks. Thus, in the current extraction procedure, brief exposure of buprenorphine to cold 0.1 *M* sulfuric acid was shown not to produce detectable amounts of demethoxybuprenorphine. Therefore it was possible with this procedure to determine if demethoxybuprenorphine is produced by humans in vivo rather than as an artifact of the assay procedure.

Search for demethoxybuprenorphine in drug samples

Following extraction and derivatization, buprenorphine, norbuprenorphine and demethoxybuprenorphine could be separated, detected and measured with reference to an internal standard by GC (Fig. 3). The stronger response obtained for norbuprenorphine (Fig. 3A) was due to formation of a di-PFPA derivative as opposed to formation of mono-PFPA derivatives for the other compounds. The assay procedure produced no interferences from control samples (Fig. 3D) or other tetrahydrooripavine derivatives and had sufficient sensitivity to measure demethoxybuprenorphine down to ca. 10 ng/ml (Fig. 3C).

Analyses of urine samples following large oral doses of buprenorphine produced no evidence of demethoxybuprenorphine formation and excretion. Fig. 3B is a typical chromatogram of an extract of unhydrolyzed urine obtained from a human subject 8—12 h following a 40-mg oral dose of buprenorphine. The arrows indicate the retention times at which demethoxy-

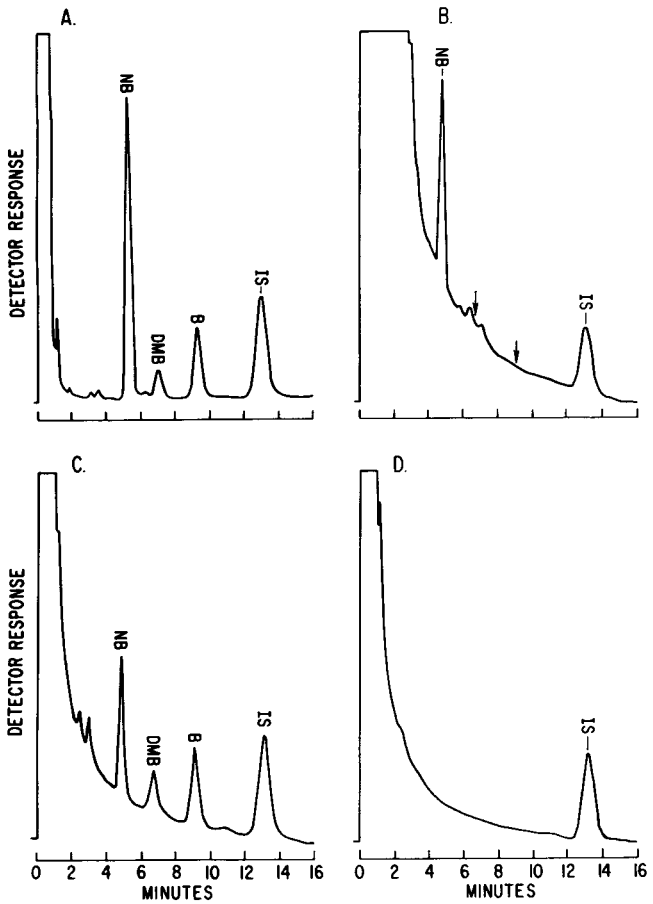

Fig. 3. Gas chromatograms of standards of buprenorphine and metabolites and extracts from human urine. (A) Unextracted standards (1 ng on column); (B) extract of unhydrolyzed urine collected 8—12 h following a 40-mg oral dose of buprenorphine (arrows indicate retention times of demethoxybuprenorphine and buprenorphine); (C) extract of control urine with standards added (NB, DMB and B 100 ng. IS, 200 ng); (D) extract of control urine. All samples were derivatized with PFPA. Peaks: NB = norbuprenorphine; DMB = demethoxybuprenorphine; B = buprenorphine; and IS = etorphine, internal standard.

buprenorphine and buprenorphine would have emerged. Demethoxybuprenorphine was also not present in samples collected at other time periods nor was it detectable following buprenorphine administered by other routes. It should be noted, however, that although demethoxybuprenorphine was not excreted by these subjects, the conversion of buprenorphine to this biologically active compound [7] either by the acidic environs of the stomach or by enzymatic processes cannot be ruled out completely since only a small portion of the administered dose of buprenorphine could be recovered. The possibility exists that demethoxybuprenorphine was biotransformed into other products which were not detectable by this assay.

Excretion of buprenorphine and norbuprenorphine

Human urinary and fecal samples were assayed for buprenorphine and norbuprenorphine by the described assay procedure. Samples were analyzed untreated (free) and following enzyme hydrolysis (total) to provide measures of conjugated metabolites. Fig. 4 illustrates the typical findings for most samples. Fig. 4A is the response for a control sample with 100 ng and 50 ng added, respectively, of norbuprenorphine and buprenorphine. Responses were linear and reproducible down to ca. 10 ng/ml buprenorphine and 5 ng/ml

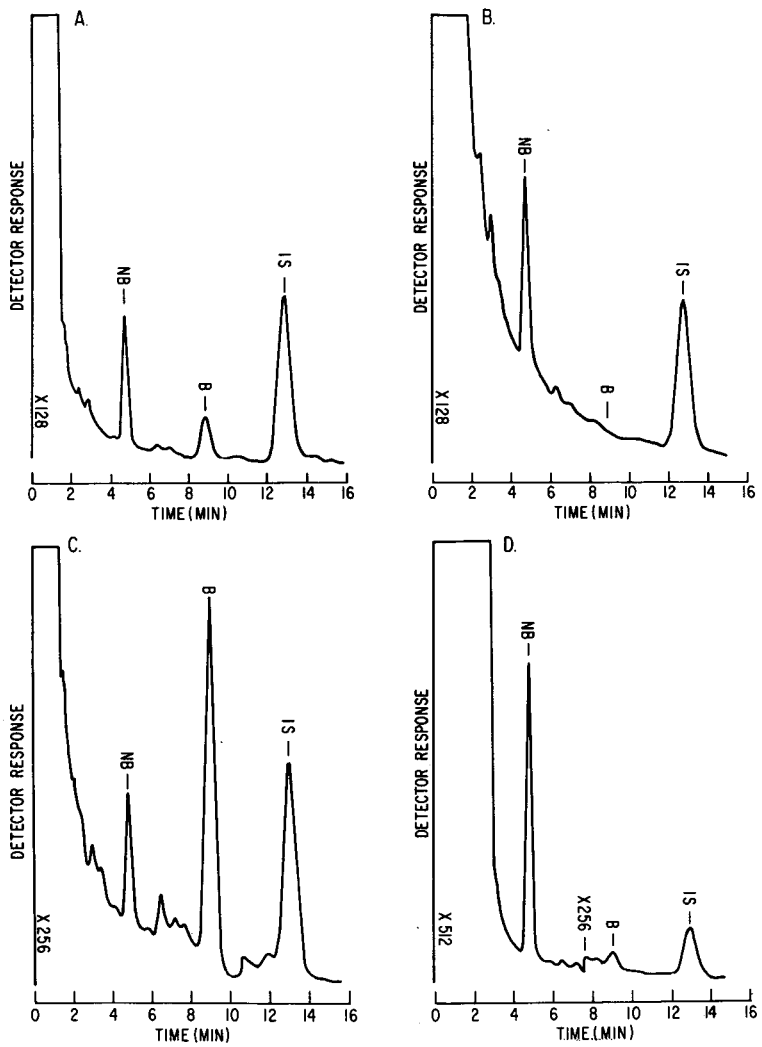

Fig. 4. Gas chromatograms of human biological extracts. (A) Control urine with standards added NB, 100 ng; B, 50 ng; and IS, 250 ng; (B) urine collected 48—60 h following a 40-mg oral dose of buprenorphine; (C) hydrolyzed feces sample collected 73 h following a 20-mg oral dose of buprenorphine; (D) urine, same as (B), hydrolyzed with enzyme. All samples were derivatized with PFPA. Peak: Nb = norbuprenorphine; B = buprenorphine; and IS = etorphine, internal standard.

norbuprenorphine. All of the urinary analyses were consistent in that free buprenorphine was not detectable at any time period (e.g., Fig. 4B) and conjugated buprenorphine was present in very small amounts (e.g., Fig. 4D). Both free and conjugated norbuprenorphine were present at all time periods tested through six days following drug administration. In contrast to the results from urinary analyses, fecal analyses showed larger amounts of free parent buprenorphine present than the metabolite norbuprenorphine (Fig. 4C). It should be noted that this was occurring at time periods during which norbuprenorphine was being excreted in urine in much larger amounts than total buprenorphine. These findings indicate the likelihood of enterohepatic circulation of buprenorphine in human similar to that found for buprenorphine in rat [8].

The overall cumulative urinary excretion of conjugated buprenorphine, conjugated norbuprenorphine and free norbuprenorphine for a human subject following a 40-mg oral dose is shown in Fig. 5. The major metabolite, conjugated norbuprenorphine, accounted for only ca. 4% of the administered dose with free norbuprenorphine and conjugated buprenorphine each accounting for ca. 1%. The remainder of the dose could be partially accounted for by elimination in feces. Other metabolites were not detected but could possibly represent a sizable portion of the administered dose.

Overall, this assay clearly established that buprenorphine is excreted intact in feces over a long time course (at least seven days) and as conjugated norbuprenorphine in urine over an equal time period. Detailed investigations of buprenorphine following other routes of administration are underway.

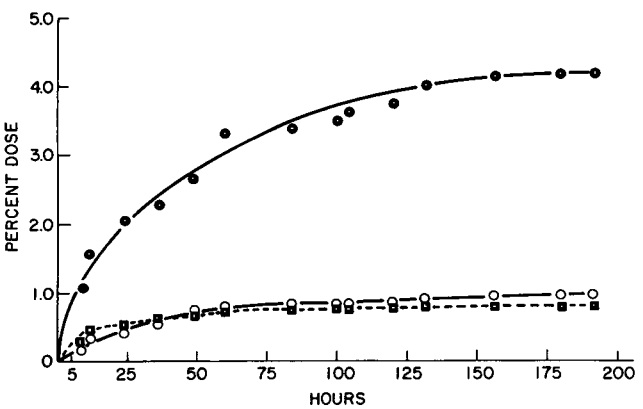

Fig. 5. Cumulative urinary excretion of buprenorphine metabolites following a 40-mg oral dose of buprenorphine to a human subject. ■, Conjugated buprenorphine; ○, free norbuprenorphine; ●, conjugated norbuprenorphine.

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CHROMBIO. 2372

CLAVULANATE-POTENTIATED TICARCILLIN: HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAYS FOR CLAVULANIC ACID AND TICARCILLIN ISOMERS IN SERUM AND URINE

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(Received August 6th, 1984)

SUMMARY

High-performance liquid chromatographic assays for the determination of clavulanic acid and ticarcillin in biological fluids are described. The clavulanic acid assay uses serum ultrafiltrate and direct injection of diluted urine with reversed-phase ion-pair/counter-ion chromatography. The ticarcillin assay allows, for the first time, the separation and quantitation of two isomers of ticarcillin. The performance of these assays has been evaluated and found to be satisfactory for routine clinical use and thus the assays have been applied to the study of the pharmacokinetics of these analytes in a subject with renal failure.

INTRODUCTION

Clavulanate is a potent, irreversible, inhibitor of a number of bacterial β-lactamases [1]. Penicillins susceptible to lactamase attack have their activity enhanced in the presence of clavulanate and its combination with amoxicillin has been shown to be effective both in vitro [2, 3] and in vivo [3, 4].

Ticarcillin has a broader spectrum of activity than amoxicillin but is susceptible to the action of some β-lactamases; however, clavulanate potentiated inhibition has been shown to be effective in vitro and in vivo [2, 5, 6].

Typically, clavulanate and ticarcillin are assayed by microbiological assay [7, 8] which although more suited to large sample numbers may lack precision and also the turn round of such assays tends to be slow. High-performance liquid chromatography (HPLC) has recently been applied to the assay of clavulanate in urine [9]; an assay for serum has been described [10], but its performance characteristics were not validated. This paper describes simple, precise assays for clavulanate and ticarcillin in serum and urine.

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MATERIALS AND METHODS

HPLC equipment

A Gilson 302 B pump with a Series 5 head and an 802 manometric module were used with an Altex 160 fixed-wavelength filter detector (Scotlab Instruments Sales, Bellshill, U.K.). Injections were made with a Waters 710B WISP autosampler (Waters Assoc., Northwick, U.K.). A 25 cm × 4.6 mm I.D. Spherisorb 5-μm ODS column was used for the determination of clavulanate and a 10 cm × 5 mm I.D. Hypersil 3-μm ODS column was used for ticarcillin. Both columns were slurry packed in the laboratory using a Shandon column packer (Shandon Southern Products, Runcorn, U.K.). Results were integrated using a Shimadzu C-R2AX integrator (Dyson Instruments, Houghton-le-Spring, U.K.).

HPLC conditions

Ticarcillin. Methanol—water (30:70) was filtered through an 0.45-μm nylon filter in a glass filtration apparatus under vacuum supplied by a water-driven venturi pump. To the filtered solvent was added concentrated phosphoric acid to give a final aqueous concentration of 0.05 *M*. After gentle mixing the eluent was pumped at a flow-rate of 2 ml/min. A zinc lamp with a 214-nm filter was used to monitor the mobile phase at 0.01 a.u.f.s. Auto-zero was performed by the WISP prior to injection of 50 μl of sample at intervals of 15 min.

Clavulanate. The filtered, degassed mobile phase was methanol—0.1 *M* potassium dihydrogen phosphate (10:90) containing 0.05 *M* pentane sulphonic acid (HPLC grade, Fisons, Loughborough, U.K.) and 0.1 *M* ethanolamine at a flow-rate of 1.5 ml/min. A mercury lamp with a 313-nm filter was used to monitor the eluent at 0.01 a.u.f.s. Auto-zeroing of baseline prior to injection was again initiated by the WISP autosampler which injected 75-μl samples every 10 min.

Materials

Imidazole (BDH, for penicillin analysis), for the assay of clavulanate was prepared as described by Foulstone and Reading [10]. A 1 mg/l solution of thienylbutyric acid (Sigma, U.K.) in methanol was prepared for use as an internal standard in the ticarcillin assay. All other reagents were BDH AnalaR grade unless specified.

Sample collection

Both ticarcillin and clavulanate are labile, blood samples were allowed to clot for 1 h at room temperature, centrifuged and the serum was stored at −80° C prior to analysis. Urines were volumed and aliquotted prior to dilution 1:10 with 0.1 *M* citrate buffer, pH 6.5, to ensure analyte stability; the diluted urine samples were also stored at −80° C prior to analysis.

Calibration

Ticarcillin. From a freshly prepared stock of 1 g/l ticarcillin sodium (Beecham Pharmaceuticals, Brockham, U.K.) were prepared serum calibrators covering the range 0—500 mg/l using horse serum No. 3 (Wellcome Reagents,

U.K.). Urine calibrators were prepared in fresh human urine diluted 1:10 with 0.1 *M* citrate buffer and covering the range 0—2000 mg/l. The ratio of total peak area of ticarcillin to internal standard peak area was plotted against concentration to provide a calibration line for total ticarcillin. In the determination of ticarcillin isomers, peak height ratio of isomer 1 and isomer 2 to the internal standard was used. The relative contributions of the two isomers were calculated by proportion of peak height to the total ticarcillin concentration, as pure free acid, equal detector response being assumed on a weight for weight basis.

The sum of ticarcillin 1 and 2 compared well with the concentration of total ticarcillin calculated using peak area.

As batch analysis could extend to over 12 h an aqueous calibrator was also prepared in 0.1 *M* phosphate buffer, pH 7.0, with a value of 250 mg/l; during analysis this 'target' calibrator was assayed after every four injections; this allowed assessment and correction of any degradation of samples during sampling.

Clavulanate. In a similar approach to that outlined for ticarcillin, potassium clavulanate (Beecham Pharmaceuticals) 0.5 g/l was used to prepare serum calibrators over the range 0—50 mg/l and urine calibrators again diluted 1:10 with 0.1 *M* citrate buffer, pH 6.5, over the range 0—500 mg/l. The target calibrator was 20 mg/l in phosphate buffer, pH 7.0.

The procedure was externally calibrated, i.e. the peak areas were plotted against concentration and a calibration line produced. The measured product of clavulanate has a half-life in pH 7.0 buffer of around 15 h and the use of the target calibrator is of great importance.

In both the ticarcillin and clavulanate assays the results were subsequently converted to those of the pure free acid. From the material supplied the ticarcillin sodium was equivalent to 80.8% pure free acid and the potassium clavulanate was equivalent to 82.5% pure free acid.

Assay procedures

Ticarcillin. To 1 ml of sample in a Z10 plain tube (Brunswick, U.K.) were added 50 µl of 1 mg/l methanolic thienyl butyric acid and 6 ml of diethyl ether; to each tube were then added 100 µl of 3 *M* hydrochloric acid; the tube was immediately capped and inverted two or three times to ensure complete mixing. It was essential to perform this step immediately as ticarcillin was very labile in acid conditions. Once all the tubes were ready they were shaken for 5 min on a lateral shaker (Griffin and George, London, U.K.). The ether layer was transferred to a second Z10 tube containing 500 µl of 0.1 *M* phosphate buffer, pH 7.0, and the organic layer removed under a stream of air at 37°C. Of the remaining aqueous layer, 200 µl were transferred to a PRO tube (Sarstedt, U.K.) from which the upper 5 mm had been removed; these tubes were placed in the centre of a spring insert in the WISP autosampler vial and the septum screw caps applied; the carousel was then loaded and the samples injected. The procedures for serum and diluted urine were identical.

Clavulanate. Serum (500 µl) in a Z10 tube was diluted with 500 µl of 0.1 *M* phosphate buffer, pH 7.0, after mixing this was transferred to a Syva free drug assembly (Syva, Maidstone, U.K.). (The assembly is a pre-assembled version of

the Amicon MPS-1 ultrafiltration apparatus.) This was centrifuged for 20 min at 4°C at 1500 *g*. Ultrafiltrate (100 μl) was added to 100 μl imidazole reagent in a PRO tube, the tube was capped, mixed by flicking and inversion, the top 5 mm were removed and inserted as described above into the WISP autosampler. For urine the procedure is as described for ultrafiltrate.

Pharmacokinetics

The assays were applied to the study of clavulanate-potentiated ticarcillin in patients with renal failure. Informed consent and ethical permission were obtained and the individuals given an intravenous dose of the combination which comprised of 3 g of ticarcillin and 200 mg of clavulanate. Blood samples were drawn pre-dose and at 0.083, 0.25, 0.33, 1, 1.5, 2, 4, 8, 12 and 24 h post-dose. Urine was collected as passed and diluted with 0.1 *M* citrate buffer; 24-h collections were not made owing to analyte instability. Samples were stored as described above.

RESULTS

Clavulanate

A chromatogram from a pre-dose serum sample is shown in Fig. 1A. A patient's serum with a concentration of 2.5 mg/l clavulanate is illustrated in Fig. 1B. The chromatogram from a urine containing clavulanate is shown in Fig. 2.

Linearity. Linearity was established over the range 0—500 mg/l for serum assay and 0—2500 mg/l for the urine assay. This is equivalent to 0—20 μg on-column weight allowing for the differences in dilution.

Sensitivity. Sensitivity, defined as twice the signal-to-noise ratio, was equivalent to 0.1 mg/l, i.e. 2 ng on-column weight.

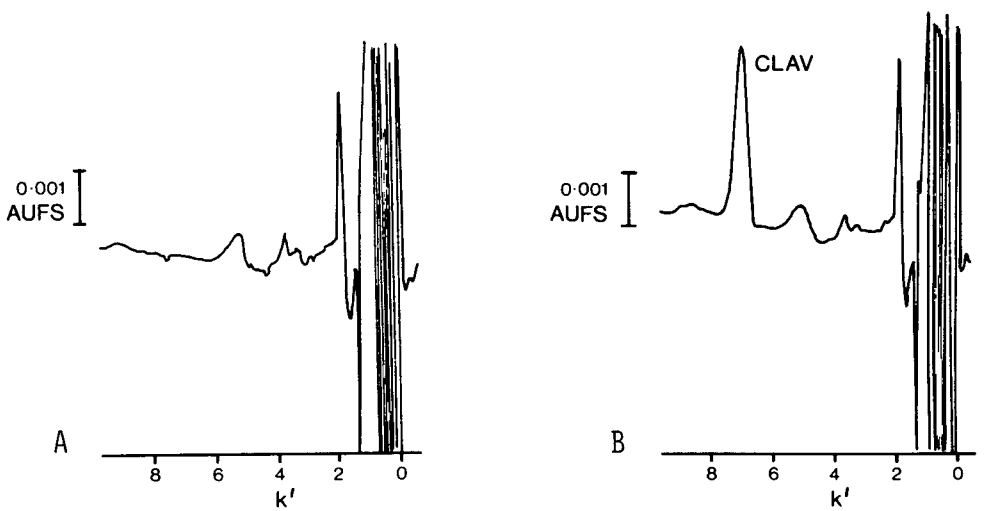

Fig. 1. Chromatograms for serum clavulanate assay. (A) Pre-dose sample; (B) 8-h post-dose serum sample, clavulanate concentration 2.5 mg/l. Chart speed 5 mm/min. Peak: CLAV = clavulanate.

Recovery. By comparison with directly injected calibrators at a concentration of 5 mg/l it was determined that the recovery from an aqueous standard was 93.8 ± 2.9% (n = 5) and for the serum standard was 79.2 ± 4.2% (n = 5). The average loss directly attributable to ultrafiltration was 6.2% and there was a further 12.6% loss associated with the ultrafiltration of serum owing to protein binding.

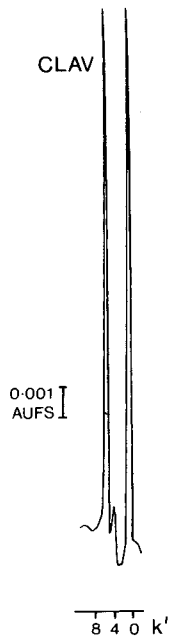

Fig. 2. Chromatogram following assay of clavulanate in urine. Chart speed 2 mm/min. Peak: CLAV = clavulanate.

Precision. The within-batch precision at 1 mg/l was 4.5% (n = 40) and at 8 mg/l 2.95% (n = 40). The between-batch precision at these levels was 6.6% (n = 44) and 4.3% (n = 48), respectively.

Accuracy. Samples from patients on a great variety of co-medication have been assayed with no apparent interference. However, the use of imidazole that is not free from ultraviolet-absorbing impurities results in a number of peaks which were found to interfere with the assay of clavulanate; the use of the recommended grade of reagent avoids this problem.

Ticarcillin

Ticarcillin is an isomeric mixture which chromatographs as two peaks the ratio of which (approx. 45:55) was the same in calibrators and patient samples (Fig. 3B); addition of a penicillinase to the sample resulted in the conversion to the corresponding penicillinoic acid(s). Typically the k' values for the two ticarcillin isomers were 4.5 and 6.1, and 1.6 for both penicillinoic acids. To maintain comparability with earlier work on ticarcillin pharmacokinetics, the total ticarcillin was calculated from the summed areas of the peaks; it was found that by decreasing the methanol content to 25% an endogenous

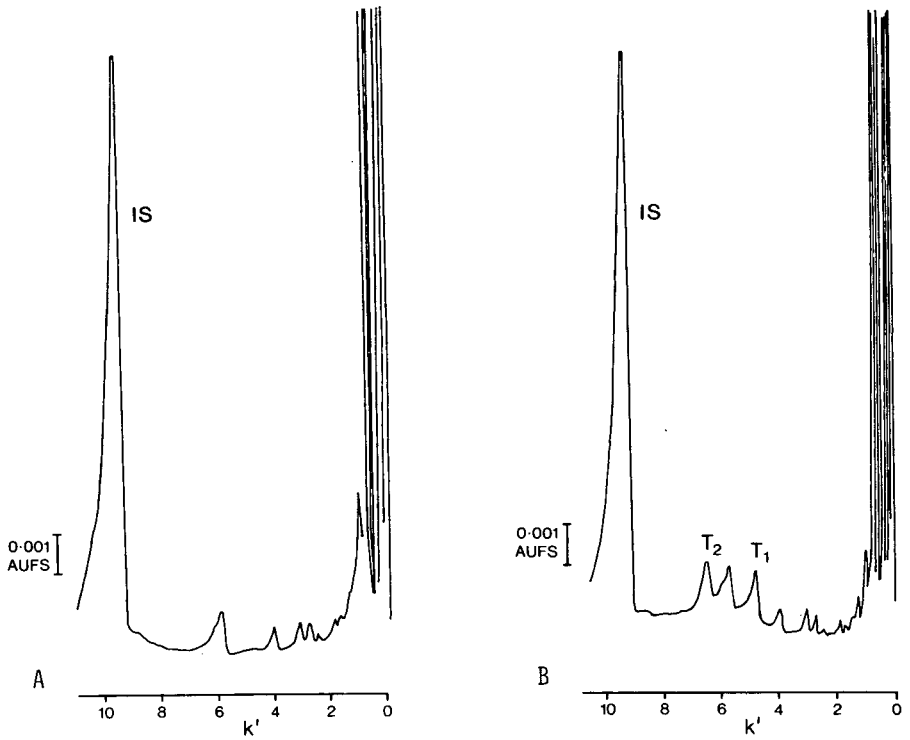

Fig. 3. Chromatgrams of pre-dose (A) and 8-h post-dose (B) sera for ticarcillin. Concentration in B is 10 mg/l. Eluent: methanol—0.05 *M* phosphoric acid (25:75); flow-rate 2 ml/min; chart speed 5 mm/min. Peaks: IS = internal standard, thienylbutyric acid; T_1 = ticarcillin isomer 1; T_2 = ticarcillin isomer 2.

interfering peak eluted between the ticarcillin peaks (Fig. 3A pre-dose, Fig. 3B post-dose total concentration 10 mg/l). In practise it was found that subtraction of the endogenous peak area from ticarcillin areas using the 30% methanol solvent yielded comparable results with a faster assay. The endogenous peak did not interfere with the quantitation of ticarcillin isomer 1 or isomer 2 if peak height measurements were used.

Fig. 4 is a chromatogram obtained from a urine extract from a subject taking the ticarcillin—clavulanate mixture; the 30% methanol solvent was used.

Linearity. The summed area of the ticarcillin peak has been found to be linear over the range 0—600 mg/l for serum and 0—6000 mg/l for urine, this is equivalent to 0—30 μg on-column weight.

Sensitivity. The sensitivity for each ticarcillin isomer is 1 mg/l, i.e. a summed sensitivity of 2 mg/l equivalent to an on-column weight of 200 ng.

Recovery. By comparison with the summed areas from directly injected standards a recovery relative to the internal standard of 98.1 ± 8.3% (n = 20) was found at 80 mg/l.

Precision. The within-batch precision at 5 mg/l was 7.8% (n = 21) and at 80 mg/l was 6.0% (n = 60). The between-batch precision was 12.4% (n = 58) and 8.4% (n = 62), respectively.

Accuracy. The co-eluting endogenous peak had an area equivalent to 3.5

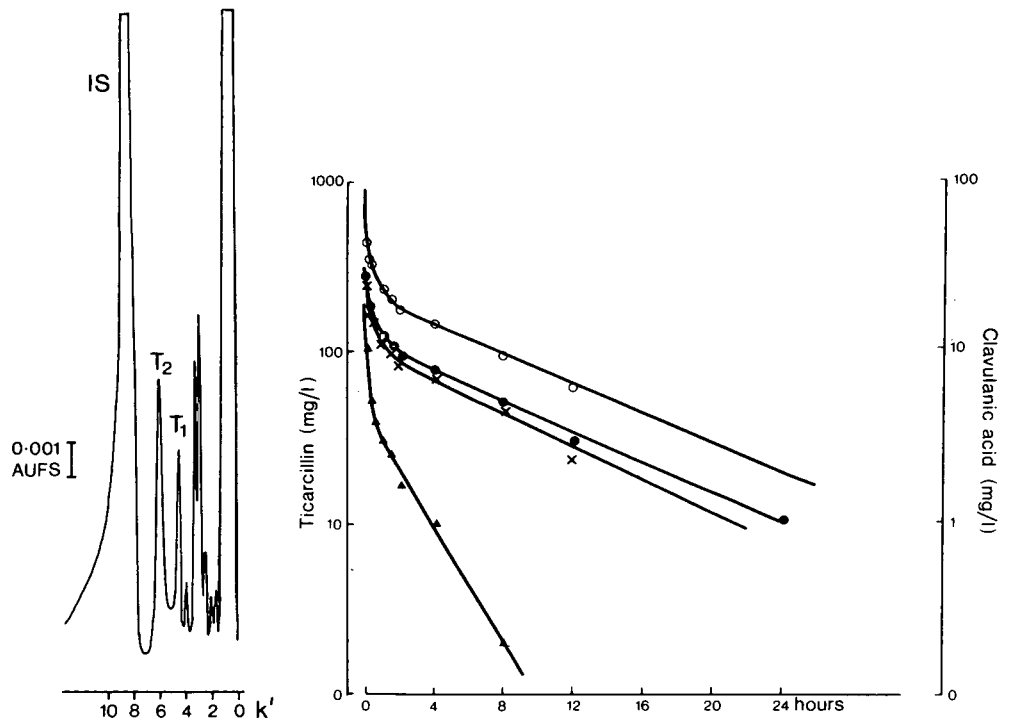

Fig. 4. Chromatogram following assay of ticarcillin in urine. Eluent: methanol—0.05 *M* phosphoric acid (30:70); chart speed 2 mm/min. Peaks: IS = internal standard, thienylbutyric acid; T_1 = ticarcillin isomer 1; T_2 = ticarcillin isomer 2.

Fig. 5. Plasma concentration—time curve for clavulanate and ticarcillin following a 3-g ticarcillin—200-mg clavulanate oral dose in a patient with renal failure. Creatinine clearance 20 ml/min. ○, Total ticarcillin; ×, ticarcillin isomer 1; ●, ticarcillin isomer 2; ▲, clavulanate.

mg/l ticarcillin. No medication yet encountered has given rise to spurious results.

Pharmacokinetics. The plasma drug concentration—time curves for total ticarcillin, ticarcillin isomer 1, ticarcillin isomer 2 and clavulanate from a subject with renal failure (creatinine clearance 20 ml/min), given 3 g ticarcillin—200 mg clavulanate intravenously are shown in Fig. 5. The half-lives of elimination were: total ticarcillin, 7.0 h; ticarcillin isomer 1, 6.5 h; ticarcillin isomer 2, 6.8 h; and clavulanate 1.8 h; the ticarcillin half-lives are comparable within the error of the assay and are excreted in a constant proportion.

DISCUSSION

Clavulanate

The sample preparation procedure used here was closely related to that of Foulstone and Reading [10], but had not been validated by them for a routine procedure. The Syva assemblies used here are very similar to the Amicon MPS-1 system and acceptable results should be obtainable using this. In the sample preparation the buffer was used primarily to stabilise the clavulanate, as

glycerol [10] did not interfere in the chromatography. The pre-column derivatisation step is preferred although it should be possible to develop a satisfactory post-column reaction procedure if desired. The proportions of reagent to sample were satisfactory and were chosen for assay simplicity.

The reasons for the chosen solvent composition are as follows: the potassium phosphate was used to maintain an acid pH at which the clavulanate derivative was relatively stable; pentane sulphonic acid was required to ion-pair the reaction product 1-(8-hydroxy-6-oxo-4-azaoct-2-enoyl)imidazole [10] to improve its retention. No products were found for ticarcillin or its pencillinoic acid when incubated with imidazole reagent.

When well capped reversed-phase material was used some tailing of the peak shape resulted; however, if end-capping was incomplete it was found that peak tailing was excessive; in either case excellent peak shape could be obtained by the addition of a cation which covered the residual silanol groups and also acted as a counter-ion; ethanolamine was found to be satisfactory and adjustment of its concentration could be used to control the retention of the clavulanate—imidazole reaction product, an increase causing a decrease in the k' value and a decrease an increased k' value. The use of longer-chain alkyl cationic surfactants resulted in excessive desorption with a decrease in retention to a k' of < 1. An internal standard was unnecessary as there was little sample preparation for urine samples and a simple preparation for serum with reproducible recovery.

The product stability was such that adjustment for degradation loss within-run was necessary although reproducible within the run; this was well identified with the repeat sampling of the target standard.

The precision of the assay was better than that previously noted with a microbiological assay [8].

Ultrafiltration of serum samples is essential for the maintenance of column selectivity as direct injection of serum, although feasible [10] led to rapid loss of retention; this would be acceptable for small sample numbers only, especially if imidazole, that has not been specially purified, was used owing to the interfering peaks that would be found.

Ticarcillin

Of particular note in the assay described for ticarcillin was the ability to separate and quantitate the two ticarcillin isomers, the pharmacokinetics of which can be studied for the first time. The interference by the small serum peak can be avoided by increasing the retention to allow resolution, by subtraction of the peak area found in a pre-dose sample or by running a penicillinase blank; of the options the second was the most rapid and was routinely adopted.

As would be expected, manual measurement and summation of total ticarcillin was less precise than integrated summation of area, with correction for background especially if faster elution times are used. However, measurement of peak height for each isomer and summation compared well with blank-corrected integrated area; using peak height in this way a negligible contribution from the endogenous peak was noted.

Despite investigating a number of penicillins and their penicillinoic acids,

none had a k' value appropriate for a useful internal standard. The internal standard used was suitable in terms of its chromatographic retention; however, early problems of variable relative recovery proved attributable to variable recovery of thienylbutyric acid, a problem resolved by using a mechanical shaker. There are a number of other areas in the ticarcillin assay which can lead to imprecision; in the extraction step it was found to be essential to add the acid after the diethyl ether and to mix immediately; delay resulted in significant and variable losses of ticarcillin which is acid-labile. Losses, owing to residual acidity, can occur, if the extract is then evaporated to dryness; extracts were therefore concentrated by evaporation into phosphate buffer.

The precision attainable with this assay is superior to that reported for a microbiological assay [8].

CONCLUSION

Since both clavulanic acid and ticarcillin are labile, correct storage and handling of samples are essential for accurate results; the use of HPLC provides an ideal approach for the fast, reliable, precise and accurate assay of both analytes in serum and urine.

The ticarcillin assay is the first to show the existence of the two isomers. The assays developed here are suitable for the estimation of both clavulanic acid and the ticarcillin isomers in clinical studies.

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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND AMPEROMETRIC DETECTION OF 3-O-METHYL ISOPRENALINE SULPHATE: APPLICATION TO STUDIES ON THE PRESYSTEMIC METABOLISM OF *d*-ISOPRENALINE IN MAN*

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SUMMARY

A selective method for the determination of 3-O-methyl isoprenaline sulphate in human urine and blood plasma has been developed using reversed-phase high-performance liquid chromatography with amperometric detection. The sulphoconjugate was subjected to acidic hydrolysis and the liberated 3-O-methyl isoprenaline was isolated by organic extraction and conventional cation exchange. An internal standard of 3-O-methyl isoetharine was synthesized from commercially avilable isoetharine and used to correct for recovery losses. The assay was shown to be linear over the range 5 ng/ml to 20 μg/ml with a limit of detection of 2 ng/ml. The reliability of the analytical method was examined together with its applicability to in-vivo studies in man.

INTRODUCTION

The conjugation of phenolic compounds with sulphate is an important mechanism for the detoxification of xenobiotics and natural compounds [1]. The process is intimately linked to the ingestion of food, which can contain the sulphate precursors cysteine and methionine (either free or in the form of protein) [2] and vitamin A, which is required for the activation of inorganic sulphate to 3-phosphoadenosine-5-phosphosulphate (PAPS) in a two-step reaction catalysed by ATP-sulphurylase (EC 2.7.7.5) and adenosine-5′-phosphosulphatephosphokinase (APS-phosphokinase) (EC 2.7.1.25) [3]. Phenolsulphotransferase (PST, EC 2.8.2.1) catalyses the transfer of sulphate from PAPS to

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an acceptor phenol substrate and has been identified in gut, liver, platelets, lung and kidney [4, 5].

Independent in-vitro evidence for the existence of two functionally distinct forms of human platelet PST has been presented [6, 7]. One form of the enzyme is relatively thermolabile (TL) [8] and catalyses the sulphate conjugation of monoamines (M) [6]. The other is thermostabile (TS) [8], catalyses the sulphoconjugation of micromolar concentrations of phenol and *p*-nitrophenol (P) and is selectively inhibited by 2,6-dichloronitrophenol [9].

It has been suggested that variations in platelet PST activity (measured in a blood sample) might reflect variations in PST activity in other organs such as the gut, which are more directly involved in sulphate conjugation and that such differences in activity might represent one factor contributing to individual variation in the sulphoconjugation of phenolic compounds [10—13].

As a result of in-vivo evidence for the existence of a sulphate conjugation defect [14—17] we selected *d*-isoprenaline (isoproterenol) as a model drug to investigate the possibility of an underlying polymorphism in gut sulphoconjugation [18]. Since isoprenaline is a PST (TL) or (M) specific substrate [19] and is conjugated exclusively with sulphate in man [20], it is likely to provide a clearer insight than that obtained with paracetamol (acetaminophen) [19—21], which is both a mixed TL—TS or M—P substrate and also a candidate for conjugation with glucuronic acid and sulphate in man[22, 23].

The variation observed in the sulphoconjugation of ingested *d*-isoprenaline [18], led us to speculate that low sulphate conjugation might be accompanied by increased O-methylation. In order to investigate this possibility, it was necessary to measure the 3-O-methyl metabolite of isoprenaline in plasma and urine. Previous analytical approaches have mostly used a cation-exchange clean-up step, followed by periodate oxidation and spectrophotometric measurement of the vanillin [24], ferricyanide oxidation and fluorimetric measurement of the trihydroxyindoles [25] or thin-layer chromatography (TLC) and scintillation spectrometry of tritium-labelled metabolites [26]. This paper describes the development and application of a reversed-phase high-performance liquid chromatographic (HPLC) assay with amperometric detection for the determination of 3-O-methyl isoprenaline sulphate in human plasma and urine.

MATERIALS AND METHODS

Apparatus

An Altex 100A HPLC pump provided a pulse-free flow of mobile phase to an Altex 210 injection valve fitted with a 100-μl loop and a 150 × 4.6 mm I.D. stainless-steel analytical column packed with 5-μm diameter Altex Ultrasphere octyl particles (Altex Scientific, Berkeley, CA, U.S.A.). The column was protected by a 50 × 2.1 mm I.D. stainless-steel pre-column hand packed with Whatman Co:Pell ODS (Whatman, Maidstone, U.K.). The electrochemical detection system comprised a Model LC-4 amperometric detector fitted with a TL-5 glassy carbon cell assembly (BioAnalytical Systems, West Lafayette, IN, U.S.A.). The column, detector and cell were enclosed in a Faraday cage of aluminium, itself earthed to the chart recorder, a Servoscribe RE 541.20 (Smiths Industries, London, U.K.).

Reagents

Organic solvents were all AnalaR grade, purchased from May and Baker (Dagenham, U.K.). S-Adenosyl-L-methionine (SAM) and glutathione (GSH) were obtained from Sigma (Poole, U.K.). Bio-Rex 70 cation-exchange resin was purchased from Bio-Rad Labs. (Watford, U.K.). Isoetharine hydrochloride 99% pure by TLC was a gift from Riker Labs. (Loughborough, U.K.). Catechol-O-methyltransferase (COMT, EC 2.1.1.6) was prepared from rat liver according to the method of Axelrod and Tomchick [27]. 3-O-Methyl isoprenaline 97% pure by TLC was supplied by Boehringer (Mannheim, F.R.G.). All other chemicals were of AnalaR grade and obtained from BDH (Chadwell Heath, U.K.).

Standards

A stock solution of 3-O-methyl isoprenaline (6 mg/ml) was prepared in 0.1 *M* hydrochloric acid and stored at 4°C. Subdilutions were made in 0.1 *M* hydrochloric acid and spiking achieved by adding 50 µl of appropriately diluted standard to 5 ml of drug-free urine or plasma.

Preparation of internal standard

No suitable internal standard was available commercially. From a consideration of the structure of 3-O-methyl isoprenaline, 3-O-methyl isoetharine was selected as a likely candidate internal standard (Fig. 1). It was synthesized from isoetharine using a ten-fold excess of SAM, in a reaction catalysed by partially purified COMT. The incubation mixture used was based on the recipe utilised in catecholamine radioenzymatic assays [28]. Initial time course experiments were performed as the yield of 3-O-methyl isoetharine at 1 h was very low. Conversion was found to be quantitative at 15 h incubating at 30°C and using 50 mg COMT preparation, 25 mg GSH, 6 ml Tris—magnesium chloride—EGTA buffer, pH 8.4, 1 ml of 0.5 mg/ml isoetharine and 1 ml of 5.0 mg/ml SAM. After incubation the pH of the solution was adjusted to below 1.0 with concentrated hydrochloric acid, the protein precipitate removed by centrifugation (2050 *g*, 4°C, 5 min) and the supernatant stored at 4°C.

Chromatographic separation

The mobile phase consisted of 0.1 *M* citrate—phosphate (McIlvaine) buffer

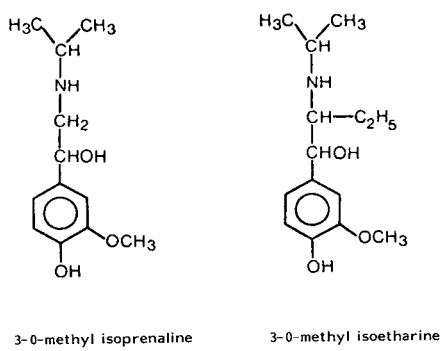

Fig. 1. Structural formulae of analytes.

of pH 3.2, containing 2.5 m*M* disodium EDTA and 11% methanol. This mixture was filtered through a 0.5-μm Gf/f glass microfibre filter (Whatman) and helium-degassed (BOC Special Gases, London, U.K.) prior to pumping at 1.6 ml/min through the HPLC system. Amperometric detection was achieved using a glassy carbon cell held at +0.80 V versus Ag/AgCl reference electrode. Typical sensitivities employed were 10 nA full scale for plasma and 250 nA full scale for urine.

Investigation protocol

Seven healthy volunteers (mean age 31.5 years) were asked to refrain from taking ascorbic acid and paracetamol for 12 h prior to dosing. On the day of the study no breakfast was taken but the subjects were allowed lunch 3 h after taking an oral dose of *d*-isoprenaline-*d*-bitartrate (2 mg/kg) in 100 ml of water. A baseline blood sample (10 ml) was taken into a chilled lithium heparin tube and further samples were taken at 30-min intervals up to 4 h, then hourly up to 8 h or in some cases 10 h. All blood samples were centrifuged at 2050 *g* for 15 min at 4° C and the separated plasma kept at −70° C until assayed. The protocol for this study was approved by the Research Ethics Committee of the Royal Postgraduate Medical School and Hammersmith Hospital and all subjects gave their informed consent. Two 24-h urines were collected.

Hydrolysis of sulphoconjugate

Acid-catalysed hydrolysis of 3-O-methyl isoprenaline sulphate was carried out as previously described [18]. Protein precipitation with perchloric acid was introduced prior to the plasma hydrolysis to avoid the possibility of gel formation on cooling. For plasma the procedure involved adding 250 μl of internal standard and 500 μl of concentrated perchloric acid to 3 ml of plasma in a polystyrene conical tube, standing for 5 min, mixing, centrifuging at 2050 *g* for 5 min at ambient temperature and then transferring the supernatant into heat resistant polyethylene tubes for a 30-min boiling period.

Extraction of 3-O-methyl isoprenaline

A 2-ml volume of toluene—isoamyl alcohol (3:2, v/v) was added to 2 ml of the acidic hydrolysate and the mixture shaken for 10 min (Denley Multivortex), followed by a brief centrifugation at 2050 *g*. The lower aqueous layer was frozen in a mixture of methylated spirit and solid carbon dioxide and the upper organic phase (containing organic acids) poured off. After thawing, the aqueous layer was tipped into a 20-ml plastic container (Sterilin) containing 10 ml of 4% boric acid and 1% disodium EDTA solution. The pH was adjusted to 6.50 with 4 *M* sodium hydroxide and the resultant solution applied to a Bio-Rex 70 cation-exchange column prepared in a Bio-Rad plastic minicolumn. The columns were allowed to drain to waste, then 10 ml of distilled water were used to wash out the Sterilin containers onto the columns, which were again allowed to drain to waste. Large plastic tubes (Sarstedt) containing 2.5 g sodium chloride were placed under the columns and the methoxy compounds eluted with 6 ml of 4 *M* ammonium hydroxide. The tubes were capped and vortex-mixed to ensure saturation with sodium chloride, then 5 ml of ethyl acetate—acetone (2:1, v/v) were added followed by rotary mixing (Matburn) for 20 min.

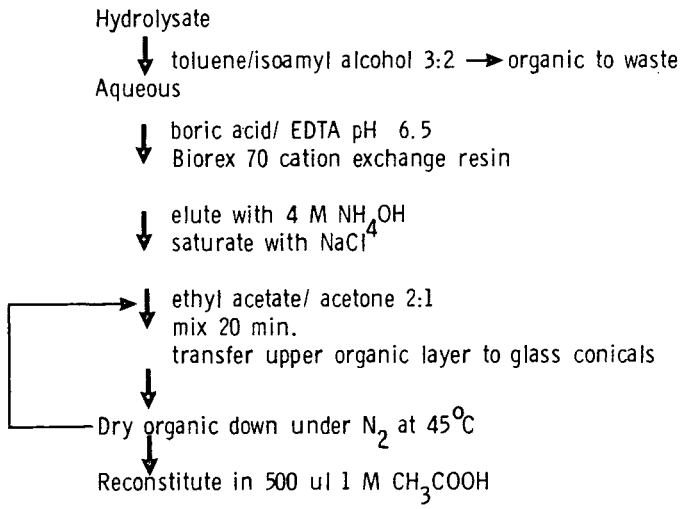

Fig. 2. Flow chart showing the extraction procedure.

The phases were separated by briefly centrifuging at 2050 *g*, the aqueous phase was retained and the upper organic layer was transferred to a conical glass tube. The extract was taken to dryness with nitrogen at 45° C. The aqueous phase was again extracted with 5 ml ethyl acetate—acetone and the extracts pooled for a final drying down, followed by reconstitution in 500 µl of 1 *M* acetic acid. An outline of the extraction procedure is given in Fig. 2.

Calculation

Measurement of 3-O-methyl isoprenaline was carried out by comparison of the peak height ratio of 3-O-methyl isoprenaline to 3-O-methyl isoetharine in the sample, to that obtained from authentic standards prepared in drug free plasma or urine, extracted and chromatographed in the same way. At least four such standards were run with each batch of samples.

RESULTS AND DISCUSSION

Chromatography

Our previous experiences in the separation of catecholamines and related compounds with citrate—phosphate buffers [29] led us to select a similar system, but of higher ionic strength in order to resolve the relatively less polar 3-O-methyl isoprenaline and 3-O-methyl isoetharine from each other. Preliminary experiments with authentic standards gave retention times of 6 and 14 min for 3-O-methyl isoprenaline and 3-O-methyl isoetharine, respectively. Hydrodynamic voltammograms of these two compounds were performed and as a result the optimum applied potential was found to be +0.80 V versus Ag/AgCl. Representative chromatograms of urine samples obtained with the method are shown in Fig. 3. No interfering peaks were observed at the retention time of the two analytes in the chromatograms of urines from *d*-isoprenaline-free subjects. The dopamine metabolite 3-methoxytyramine was present, but sufficiently well resolved from the 3-O-methyl isoprenaline peak to

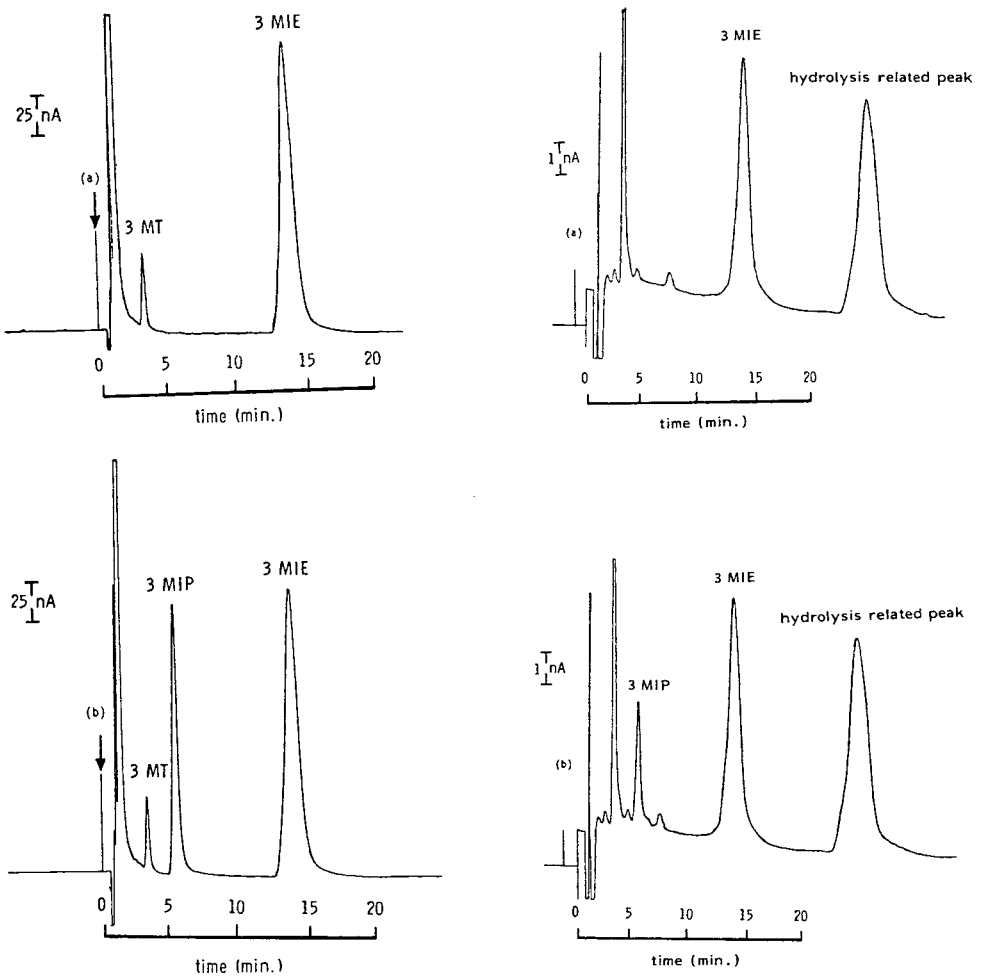

Fig. 3. Chromatograms of (a) 24-h urine from a drug-free subject; (b) 24-h urine from a subject after receiving 2 mg/kg *d*-isoprenaline-*d*-bitartrate. Chromatographic conditions as in text. Peaks: 3MT = 3-methoxytyramine; 3MIP = 3-O-methyl isoprenaline; 3MIE = 3-O-methyl isoetharine (internal standard).

Fig. 4. Plasma chromatograms from (a) drug-free subject; (b) 2.5 h after receiving an oral dose of *d*-isoprenaline (2 mg/kg). Peaks: 3MIE = 3-O-methyl isoetharine (internal standard); 3MIP = 3-O-methyl isoprenaline; hydrolysis related peak.

present no problems. Occasionally, broad, late eluting peaks were seen, towards the end of a batch of urine samples. Typical plasma sample chromatograms are displayed in Fig. 4. Again no interferences were seen in the chromatograms of drug-free plasma, although a large hydrolysis-related peak was present in all samples, with a retention time of 28 min — this extended the analysis time in plasma samples to 35 min.

Hydrolysis

Acidic hydrolysis is a rapid, inexpensive and effective procedure for the

deconjugation of catechol compounds. Being a chemical process it is not subject to the limitations of the enzymic methods using aryl sulphatase (EC 3.1.6.1) [30]. Our finding of quantitative conversion of the 3-O-methyl conjugates after 30 min at 100°C, is in close agreement with other published data [30–32].

Recovery

The absolute analytical recovery of authentic 3-O-methyl isoprenaline spiked into biological samples was 65% in urine (n = 5) and 68% in plasma (n = 5).

Quantitation

The linearity and precision of the measurement of 3-O-methyl isoprenaline by HPLC with amperometric detection, preceded by extraction from plasma or urine, were investigated by analysing samples spiked with known amounts of authentic standard. Calibration curves were constructed from the peak height ratio of 3-O-methyl isoprenaline:3-O-methyl isoetharine versus the concentration of 3-O-methyl isoprenaline, and were shown to be linear over the range 5 ng/ml to 20 μg/ml in plasma or urine. The limit of detection was 2 ng/ml at a signal-to-noise ratio of 2.0. Typical equations for calibration lines were $y = 0.18x + 0.1488$ ($r = 0.9958$) for plasma and $y = 0.0011x + 0.0343$ ($r = 0.9965$) for urine. Within-assay coefficient of variation of the assay was 4.5% (n = 15, $\overline{X}$ = 51.33 ng/ml, S.D. 2.3503) and the between-assay coefficient of variation 9.6% (n = 6, $\overline{X}$ = 51.83 ng/ml, S.D. = 4.9967).

Application to presystemic metabolism

The plasma concentration—time profiles for 3-O-methyl isoprenaline sulphate following the ingestion of 2 mg/kg *d*-isoprenaline-*d*-bitartrate are shown in Fig. 5. There is clearly a considerable inter-individual variation in response to this single oral dose, with peak concentrations of 3-O-methyl isoprenaline varying from only 48 up to 600 ng/ml and with the peak occurring from 2.0 to 5.0 h after the dose. It is possible to classify subjects into low and high areas under the plasma concentration—time curve (AUC). Thus subjects 3, 6 and 7 could be placed into a low AUC group and 1, 2, 4 and 5 into a high AUC group. The urinary excretion of 3-O-methyl isoprenaline sulphate ranged from 6.16 to 20.50 mg per 24 h, this representing between 4.7% and 13.5% of the oral dose given. The urine data are summarised in Table I and are in good agreement with earlier pharmacokinetic studies using tritium-labelled isoprenaline [33]. As expected there is a direct relationship between the plasma 3-O-methyl isoprenaline sulphate AUC and the percentage of the *d*-isoprenaline dose excreted as 3-O-methyl isoprenaline sulphate in the urine, i.e. with subjects in the low AUC group having a lower excretion of 3-O-methyl isoprenaline sulphate than their high AUC counterparts.

The individual variations seen may be due to variable absorption, metabolism or a mixture of the two. By extending the number of subjects and measuring both of the principal metabolites of isoprenaline at the same time, one should be able to investigate the possibility of an inverse relationship between 3-O-methyl isoprenaline sulphate and isoprenaline sulphate.

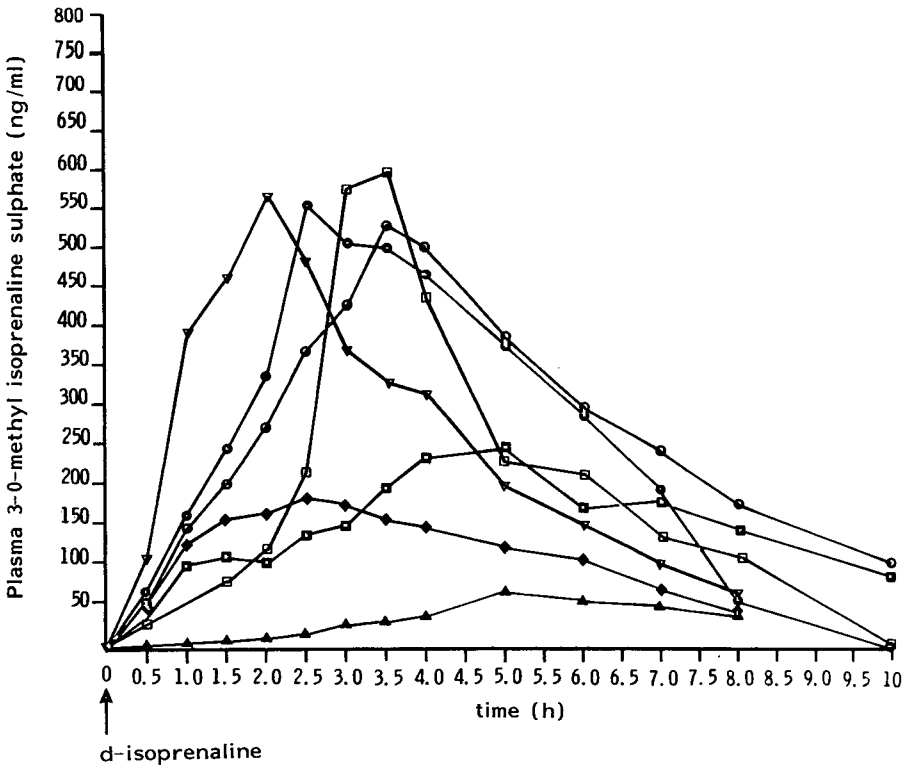

Fig. 5. Plasma concentration—time curves for 3-O-methyl isoprenaline sulphate in seven healthy volunteers. □, Subject 1; ●, subject 2; ■, subject 3; ▽, subject 4; ○, subject 5; ▲, subject 6; ◆, subject 7.

TABLE I

URINARY EXCRETION OF 3-O-METHYL ISOPRENALINE SULPHATE

Subject No.	Urinary excretion of 3-O-Methyl isoprenaline (mg per 24 h)	Percent of oral dose excreted as 3-O-methyl metabolite	Creatinine (g per 24 h)
1	19.80	13.5	1.99
2	20.50	12.3	1.89
3	15.70	9.2	2.17
4	18.00	13.4	1.74
5	16.54	11.2	2.01
6	6.16	4.7	1.54
7	11.80	7.8	1.86

CONCLUSIONS

A selective and sensitive HPLC method for the determination of 3-O-methyl isoprenaline sulphate in human plasma and urine is described. Cation exchange and solvent extraction are combined with reversed-phase ion-pairing HPLC and amperometric detection. It is now possible to study further the role of the gut

in the metabolic inactivation of catecholamines with particular interest in the widely postulated sulphate conjugation defect.

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CHROMBIO. 2389

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF 6-METHYLTHIOGUANINE, A MAJOR METABOLITE OF 6-THIOGUANINE, IN URINE

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SUMMARY

A flow-fluorimetric high-performance liquid chromatographic assay for 6-methylthioguanine in urine has been developed. This compound is a major catabolite of 6-thioguanine, an important drug in cancer chemotherapy. The metabolite was extracted from alkaline urine with ethyl acetate which was injected onto a reversed-phase high-performance liquid chromatographic system for separation and detection. The method is simple, rapid and sensitive to below 500 ng ml^{-1} which is below the levels encountered following a therapeutic dose of 6-thioguanine. Another metabolite was chromatographically separated from 6-methylthioguanine and partially characterised.

INTRODUCTION

Thioguanine (TG) is widely used in the treatment of acute myeloid leukaemia, yet there is little known of its metabolism in patients. After an oral dose of [^{35}S]TG up to 40% is excreted in urine in 24 h [1] largely as inorganic sulphate with 6-methylthioguanine (MTG) and 6-thiouric acid (TU). The structures of TG and some of its metabolites are given in Fig. 1. There is a large variation between patients in the fraction of the dose excreted, possibly due to metabolic differences [1, 2]. Moreover, there have been reports of quantitative differences in excreted metabolites according to the disease being treated. Patients with leukaemia excreted much more MTG than TU [1] whereas in those with solid tumours the reverse was true [3]. Further, malignant disease associated alterations in purine metabolism (ring methylation) have been reported [4, 5].

It is possible, therefore, that increased methylation of TG might be a useful

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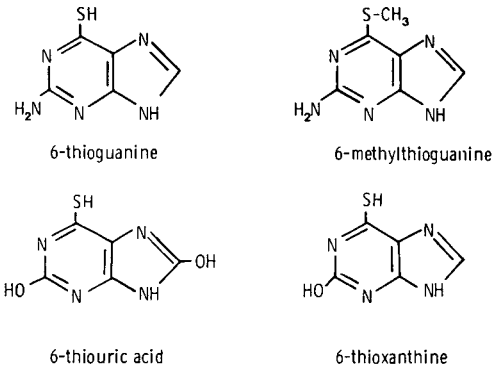

Fig. 1. Structures of 6-thioguanine and some of its metabolites.

marker for leukaemic cells. To investigate this we have developed a new assay for urinary MTG, suitable for clinical studies. The method involves solvent extraction and injection of the extract onto a reversed-phase high-performance liquid chromatographic (HPLC) system.

Another urinary metabolite of TG was separated from MTG and partially characterised through its resemblance to a product from the methylation of 6-thioxanthine (TX).

Chromatography

MTG was separated from urine constituents and other metabolites by extraction into ethyl acetate followed by HPLC using an ODS (C_{18}) reversed-phase on Spherisorb (particle size 5 μm) column, dimensions 250 mm × 4.8 mm, with a solvent system of 5% glacial acetic acid in methanol—water, pH 2.8 (60:40) at a flow-rate of 1 ml min^{-1} using an Altex Model 110A pump.

The separated MTG was detected using a Shoeffel FS-970 flow-spectrophotofluorimeter, exciting at 330 nm and measuring emission through a 389-nm cut-off filter. No other filters were used. The photomultiplier output was measured on a Servoscribe 1S flat-bed recorder. The sample injector was a Waters Model U6K. All liquid volumes were delivered using Gilson automatic pipettes P20 to P5000.

As standard practice in our laboratories, all glass tubes used in fluorimetric analyses were decontaminated in 30% nitric acid for over 15 h and rinsed in glass-distilled water.

Chemicals and reagents

6-Thiouric acid (TU) was obtained from Calbiochem (Hertfordshire, U.K.). 6-Thioguanine (TG), 6-mercaptopurine (6MP), 6-methylmercaptopurine (6MMP), 6-methylthioguanine (MTG) and 6-thioxanthine (TX) were obtained from Sigma (London, U.K.).

Methanol and ethyl acetate were obtained from Rathburn Chemicals (Peebleshire, U.K.). Trimethylanilinium hydroxide (MethElute, Pierce and Warriner, Cheshire, U.K.) was used as supplied (0.2 *M* in methanol). All water used in solution preparation was glass-distilled and deionised; all other reagents were standard analytical grade. Thin-layer chromatography (TLC) was carried out using Eastman-Kodak 13255 cellulose plates (without fluorescent indicator) and 13179 silica plates (without fluorescent indicator).

Determination of urinary MTG concentrations

Standard solutions of MTG in urine were prepared in the range 0.5—10 μg ml^{-1} by the addition of a fixed volume (300 μl) of aqueous standard in the range 5—100 μg ml^{-1} to urine (2.7 ml). These analytical standards were alkalinated by the addition of 1 *M* sodium hydroxide (500 μl) to each sample, and extracted with ethyl acetate (3 ml) by shaking on a linear agitator at 280 rpm for 10 min. After centrifugation at 800 *g* for 2—3 min the ethyl acetate layer was analysed by injection of an aliquot (100 μl) into the HPLC system described above (see *Chromatography*). MTG showed a retention time of approximately 8 min.

Methylation of TG metabolites

A sample of each of TU, TX TG, 6MP, and 6MMP (3—4 mg each, approximately 20 μmol) was dissolved in methanol (5 ml). Trimethylanilinium hydroxide (MethElute), 0.2 *M* in methanol (100 μl), was added and the mixture heated at 35°C for 40 min. Excess MethElute was destroyed by the addition of water (5 ml). For the methylation of TX in later experiments, the reactants were heated at 50°C for 1 h after a method described for the methylation of xanthine [6].

RESULTS

A linear relationship between peak height and urine MTG concentration was seen. The mean inter-assay variation over all points was 3.8% with intra-assay variation for a single point at 3.0%.

As observed with guanine [7] the fluorescence of MTG in methanol—water mixtures was greater than in aqueous media. It was thought that the addition of methanol to urine might impart sufficient fluorescence to any MTG present

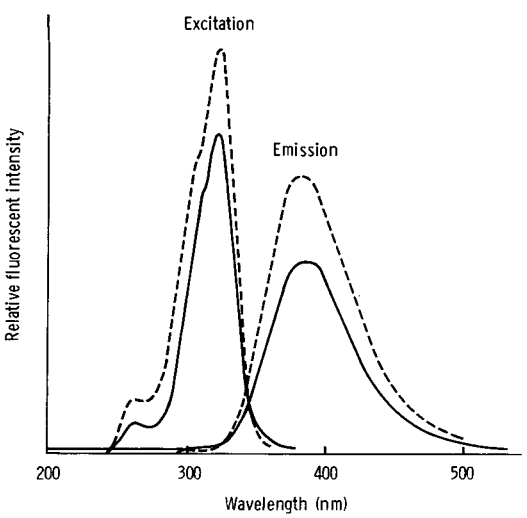

Fig. 2. Excitation and emission maxima of 6-methylthioguanine and a metabolite extracted from the urine of a patient following an oral dose of 6-thioguanine (160 mg). The spectra designated by a broken line correspond to 6-methylthioguanine.

to permit its fluorimetric detection. However, the urine constituents also showed quite intense fluorescence on excitation following the addition of methanol which prevented the detection of MTG. On extraction with ethyl acetate, MTG was found to be fluorescent in this solvent also and could be assayed by direct fluorimetry of the extract. Extraction was most efficient from alkaline urine (83%) and no detectable interference was encountered from TU and TG.

On extracting the urine from a patient receiving orally TG, however, an interfering metabolite was observed with similar fluorescent characteristics to MTG (Fig. 2). This was uncovered by cellulose thin-layer chromatographic (TLC) analysis with low-temperature luminescent detection [8]. MTG gave a higher R_F when applied in dilute sodium hydroxide over that when applied in dilute hydrochloric acid, ethyl acetate or methanol, water being the eluent in each case (Table I). No such variation was observed with the urinary metabolite.

TABLE I

CELLULOSE TLC OF 6-METHYLTHIOGUANINE (MTG) AND A URINARY CATABOLITE OF 6-THIOGUANINE

All chromatograms were developed in water.

Application medium	R_F MTG	R_F Metabolite
0.1 *M* Hydrochloric acid	0.16	—
0.1 *M* Sodium hydroxide	0.38	—
Ethyl acetate	0.16	0.44
0.1 *M* Hydrochloric acid in urine	0.20	0.44
0.1 *M* Sodium hydroxide in urine	0.38	0.44

Using the HPLC solvent system methanol—water (60:40) MTG was separated from urine constituents following ethyl acetate extraction; however, the metabolite gave a very similar retention time with this solvent system (Fig. 3). In order to separate the metabolite from MTG, the pH of the solvent system was reduced by the addition of glacial acetic acid (5%). This increased the retention of MTG to approximately 18 min leaving the metabolite retention unchanged (Fig. 4). Increasing the flow-rate gave a retention time of 8 min for MTG (Fig. 5) and this was the system finally used for the assay of MTG.

Identification of the urinary metabolite

A number of thiopurines were methylated separately and the products from each reaction were analysed separately by HPLC. The chromatograms are described in Table II. The retention times for the parent thiopurines were also obtained individually with no attempt made to separate them chromatographically. A product from the methylation of TX had a similar retention time to that of the urinary metabolite in the mobile phase methanol—water (60:40), with and without the addition of 5% glacial acetic acid. A 6MP methylation product also had a similar retention time but unlike the metabolite and methylated TX this could not be detected fluorimetrically.

Although methylation might be expected to derivatise a number of other

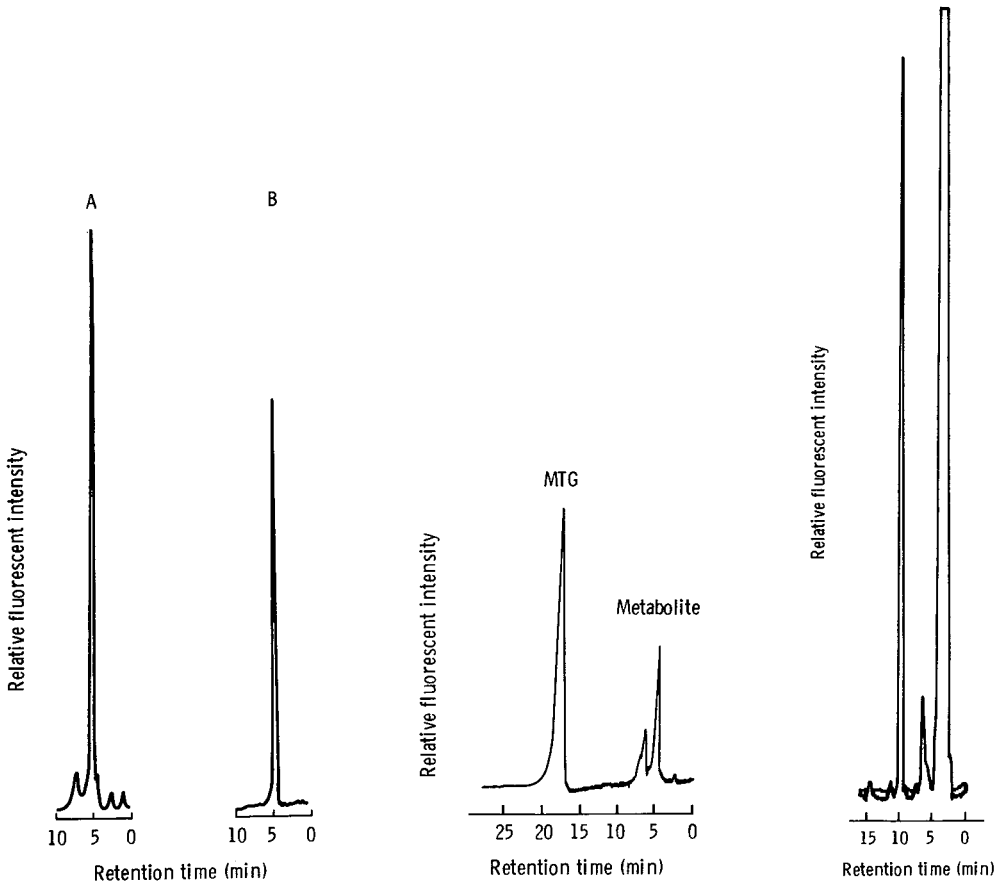

Fig. 3. Chromatograms of (A) 6-methylthioguanine extracted from urine, and (B) an extract from the urine of a patient treated orally with 6-thioguanine (160 mg). Chromatographic conditions: Whatman Sperhisorb 5 ODS (particle size 5 μm) reversed-phase (C_{18}) column (250 × 4.8 mm) with a methanol—water (60:40,v/v) mobile phase at a flow-rate of 0.4 ml min^{-1}.

Fig. 4. A chromatogram of 6-methylthioguanine (MTG) standard and a metabolite extracted from a patient's urine following an oral dose of 6-thioguanine (100 mg). Chromatographic conditions: Whatman Spherisorb 5 ODS (particle size 5 μm) reversed-phase (C_{18}) column (250 × 4.8 mm) with a solvent system of methanol—water (60:40, v/v) containing 5% glacial acetic acid at a flow-rate of 0.4 ml min^{-1}.

Fig. 5. A typical chromatogram of an extract from urine containing 6-methylthioguanine. Chromatographic conditions: Whatman Spherisorb 5 ODS (particle size 5 μm) reversed-phase (C_{18}) column (250 × 4.8 mm) with a solvent system of methanol—water (60:40, v/v) containing 5% glacial acetic acid at a flow-rate of 1 ml min^{-1}.

groups in the molecule [9] the fluorescent characteristics of these species are likely to differ from those of the S-methyl derivative [10]; hence any S-methyl TX may be selectively detected fluorimetrically in the presence of other methylation products. Further, on methylating TG in this way, a product showing similar fluorimetric and chromatographic properties to MTG was observed.

TABLE II

RETENTION TIMES OF SOME THIOPURINES AND THEIR METHYLATION PRODUCTS

Methylation conditions are described in *Methylation of TG metabolites*. Chromatographic conditions: Whatman Spherisorb 5 ODS (particle size 5 μm) reversed-phase (C_{18}) column (250 × 4.8 mm) with a solvent system methanol—water (3:2, v/v) at a flow-rate of 0.4 ml min^{-1}. Column effluent was analysed for UV absorbance using a Pye-Unicam LC 3 UV detector.

Parent compound	Retention time (min)	Retention times (min) of methylation products
6-Thioguanine	4.0	4.2
6-Methylthioguanine	4.2	—
6-Thiouric acid	2.0	2.0 + late, broad peaks
6-Thioxanthine	2.5	3.8
6-Mercaptopurine	2.5	3.8 + late, broad peaks
6-Methylmercaptopurine	3.0	4.3 + late, broad peaks

DISCUSSION

This assay for MTG is suitable for both routine clinical analysis and metabolic studies. To the knowledge of the authors, no such method has been described to date.

The potential pharmacokinetic importance of MTG urine concentrations has been indicated by its occurrence as a major catabolite of TG together with evidence showing that it may be the precursor for inorganic sulphate formation, the overall major urinary metabolite of TG [11, 12].

Following induction chemotherapy for leukaemia, the alteration in TG metabolism resulting in decreased urinary MTG concentrations could provide a marker for the achievement of remission thus avoiding unnecessary prolongation of the use of cytotoxic drugs. After remission, a state of relapse can only be ascertained clinically through the current methods of blood and bone marrow evaluation which require that the disease be relatively advanced [13]. This delay in diagnosis could be avoided if the increased methylation of TG associated with malignancy proves to be a sensitive indicator of the early stages of proliferation of the disease.

A body of information is emerging, pointing to a number of specific changes in purine and nucleic acid metabolism which occur at the onset of neoplastic disease [4, 5, 14]. Assay systems will be required if these metabolic changes are to be monitored and observations so obtained may provide powerful diagnostic tools for the management of malignant disease.

ACKNOWLEDGEMENT

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CHROMBIO. 2394

LIQUID CHROMATOGRAPHIC DETERMINATION OF INDECAINIDE, A NEW ANTIARRHYTHMIC DRUG, AND ITS MAJOR METABOLITE, DESISOPROPYL INDECAINIDE, IN BIOLOGICAL SAMPLES

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SUMMARY

A simple, sensitive, and selective method for the determination of indecainide and its metabolite, desisopropyl indecainide, in human plasma (or serum) and urine is described. The compounds are extracted from alkalinized plasma or urine samples with ethyl acetate—hexane (9:1); the solvent is evaporated under nitrogen and the residue is reconstituted in the mobile phase. The compounds are chromatographed on a Zorbax C_8 column, using 0.25 *M* ammonium acetate—acetonitrile—methanol—tetrahydrofuran (60:20:16:4) as the eluent at 1.5 ml/min. The effluent is monitored at 270 nm or by using a fluorescence detector (λ_{exc} 270 nm, λ_{em} 315 nm).

INTRODUCTION

Indecainide, 9-[3-(isopropylamino)]propyl-9H-fluorene-9-carboxamide (I, Fig. 1), is a potent new antiarrhythmic drug that is currently undergoing clinical trials [1]. Indecainide was found to be effective when administered orally or intravenously in dogs and humans [2—4]. In laboratory animals, I was more potent than reference drugs, such as aprindine and disopyramide, in converting experimentally induced arrhythmias and was free of central nervous system (CNS) and anticholinergic side-effects at therapeutically effective doses [5].

During dose-ranging studies in patients with ventricular arrhythmias, a direct relationship between the concentration of I in plasma and the percentage suppression of the ventricular ectopic activity was observed. Therefore, a sensitive and specific method for the determinaton of I in biological samples was needed for the routine monitoring of the drug's concentration in biological samples

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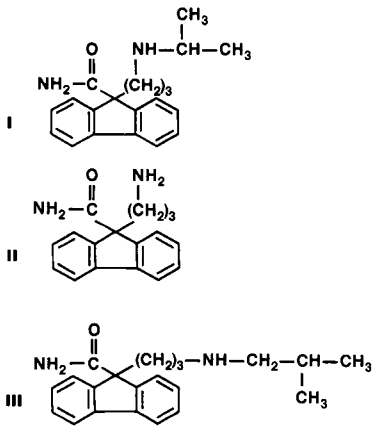

Fig. 1. Structures of indecainide (I), its metabolite (II), and the internal standard (III). I = 9-[3-(isopropylamino)]propyl-9H-fluorene-9-carboxamide; II = desisopropyl indecainide, 9-(3-aminopropyl)-9H-fluorene-9-carboxamide; III = 9-[3-(isobutylamino)]propyl-9H-fluorene-9-carboxamide.

and to study its pharmacokinetics. This report describes a rapid, sensitive, and selective liquid chromatographic (LC) method for the determination of I and its desisopropyl metabolite (II, Fig. 1), in plasma (or serum) and urine samples, using either ultraviolet (UV) or fluorescence detection, as well as the factors affecting the chromatographic separation and the selectivity of detection.

EXPERIMENTAL

Chemicals and reagents

Indecainide hydrochloride, desisopropyl indecainide hydrochloride, and the internal standard, 9-[3-(isobutylamino)]propyl-9H-fluorene-9-carboxamide hydrochloride (III, Fig. 1), were obtained from Eli Lilly and Company (Indianapolis, IN, U.S.A.). All solvents used were distilled in glass. All other reagents were of analytical reagent grade.

Liquid chromatography

The liquid chromatograph consisted of a Hewlett-Packard Model 1081B pump system equipped with a Model 79841A autoinjector (Hewlett-Packard, Avondale, PA, U.S.A.), a Perkin-Elmer Model 650-10S fluorescence detector, and a Model LC-75 variable-wavelength detector (Perkin-Elmer, Norwalk, CT, U.S.A.). The column was stainless steel, 25 cm × 4.6 mm I.D., packed with 6-μm Zorbax C_8 particles (DuPont, Wilmington, DE, U.S.A.), protected by a guard column (Waters Assoc., Milford, MA, U.S.A.), and packed with Co:Pell ODS (Whatman, Clifton, NJ, U.S.A.). The analytical column temperature was maintained at 35°C. A laboratory computer, Hewlett-Packard Model 1000, was used for peak integration and data calculations.

The eluent was prepared by mixing 600 ml of 0.25 *M* ammonium acetate solution, adjusted to pH 6.0 with acetic acid, with 200 ml of acetonitrile, 160 ml of methanol, and 40 ml of tetrahydrofuran. The eluent flow-rate was 1.5 ml/min. For UV detection, the wavelength was 270 nm, with the sensitivity

set at 0.01 a.u.f.s. For fluorescence detection, the excitation wavelength was 270 nm (slit 10 nm), the emission wavelength was 315 nm (slit 5 nm), and the range was set at 0.3.

Standard solutions

Standards in plasma were prepared to contain 100, 200, 500, and 1000 ng/ml I, and 50, 100, 250, and 500 ng/ml II. The urine standards contained 10, 20, 50, and 100 μg/ml I, and 1, 2, 5, and 10 μg/ml II. The internal standard solution was prepared by dissolving 5 mg of III (hydrochloride) in 1 l of water.

Plasma extraction procedure

To 0.5 ml of plasma sample or standard, placed in a disposable centrifuge tube with a PTFE-lined screw cap, were added 100 μl of the internal standard solution, 1 ml of 0.5 *M* sodium carbonate, and 6 ml of ethyl acetate—hexane (9:1, v/v). The tubes were capped, shaken for 3 min, and then centrifuged (800 *g*) for 5 min. Of the organic phase 5 ml were transferred to a clean tube, 0.1 ml of 1% hydrochloric acid in methanol was added, and the solution was evaporated to dryness under nitrogen. The residue was dissolved in 200 μl of the mobile phase, and 50 μl were injected onto the LC column. The concentrations of the compounds in the samples were determined from their peak height ratios relative to the internal standard and the corresponding least-squares line of the calibration standards.

Procedure for urine samples

To 0.1 ml of urine sample or standard was added 1 ml of the internal standard solution. The sample was vortexed, and 100 μl were injected onto the LC column. If the urine sample contained less than 1 μg/ml I, the extraction procedure detailed for the plasma samples was used.

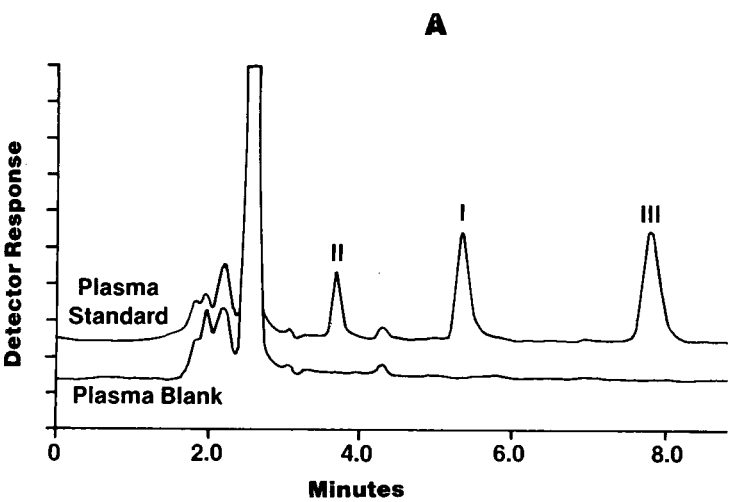

Fig. 2.

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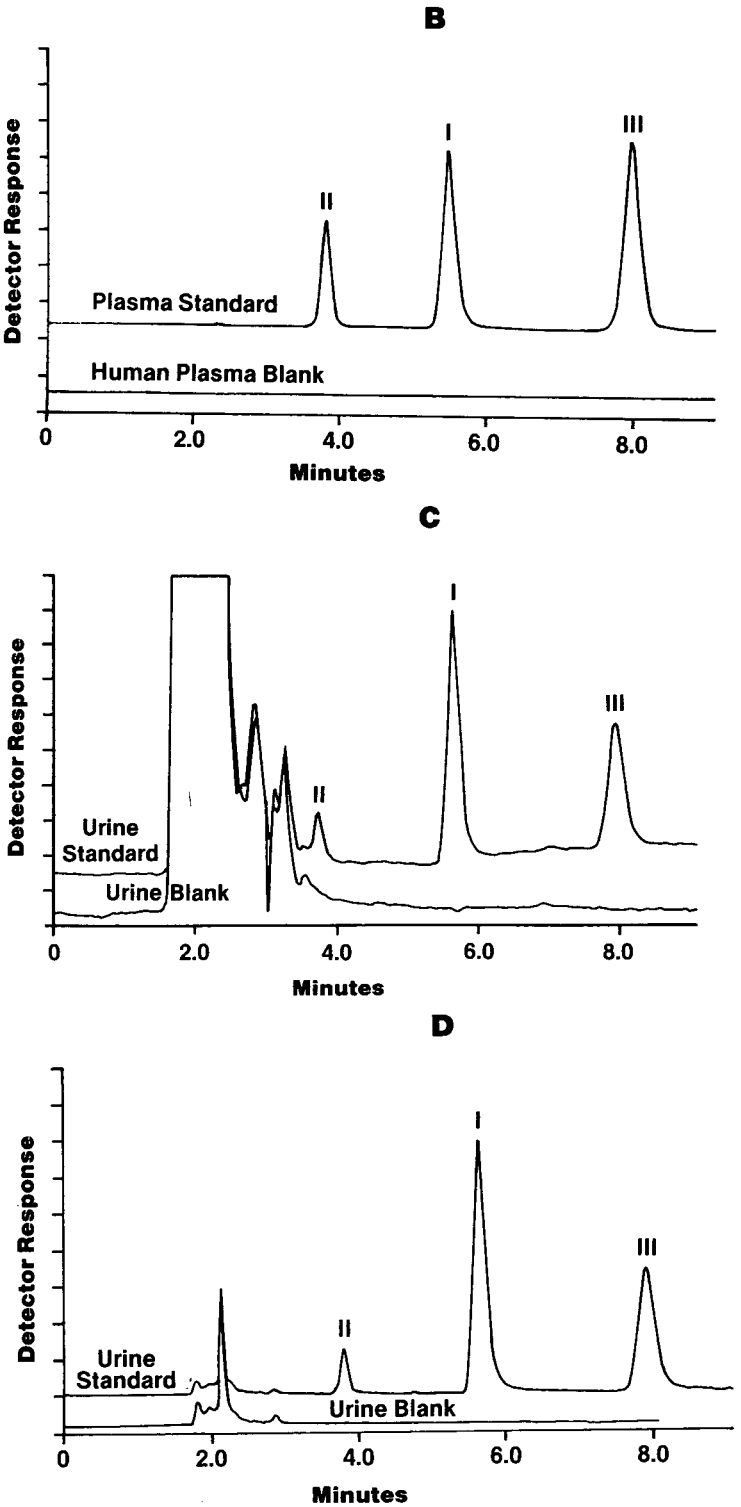
B
Detector Response
II
I
III
Plasma Standard
Human Plasma Blank
0
2.0
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Fig. 2.

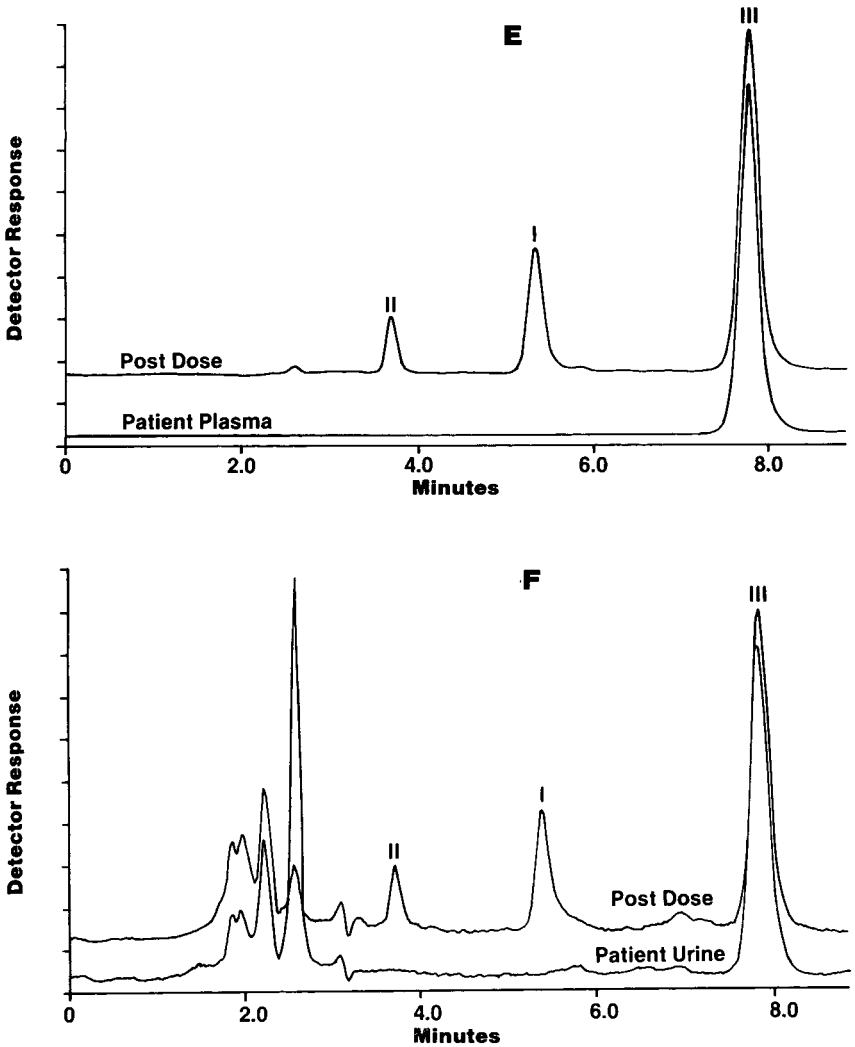

Fig. 2. Chromatograms of plasma and urine samples. (A) Human plasma blank and a standard plasma sample containing 500 ng/ml indecainide (I), 250 ng/ml desisopropyl indecainide (II) and the internal standard (III), using UV detection. (B) The same as A, using spectrofluorescence detection. (C) Human urine blank and a standard sample containing 100 μg/ml I, 10 μg/ml II and the internal standard (III), using UV detection. (D) The same as C, using spectrofluorescence detection. (E) Patients' plasma before dose and following a 50-mg dose of indecainide (spectrofluorescence detection). (F) Patients' urine before dose and following administration of indecainide, 50 mg (spectrofluorescence detection). Chromatographic conditions: column: Zorbax C_8, 25 cm × 4.6 mm I.D.; flow-rate: 1.5 ml/min; eluent: 0.25 *M* ammonium acetate, pH 6.0—acetonitrile—methanol—tetrahydrofuran (60:20:16:4).

RESULTS AND DISCUSSION

Chromatography

Typical chromatograms of plasma and urine samples obtained using the absorbance and fluorescence detectors are shown in Fig. 2A—F. Under the conditions detailed above, the retention times for I, II, and III were 5.3, 3.8, and 7.8 min, respectively. Several factors influencing the elution profile were studied. The combination of methanol, acetonitrile, and tetrahydrofuran was critical, as it has a profound effect on peak symmetry and the overall resolution of the three compounds. The effect of the pH of the aqueous portion of the mobile phase on the retention of the compounds of interest and their resolution from an unknown contaminant found in some patients' plasma is shown in Fig. 3. As the pH was lowered from 7 to 4, the retention time of all three compounds decreased, while the retention time of the unknown contaminant (which absorbed in the UV range but did not fluoresce) was affected to a lesser extent. This resulted in better separation of the

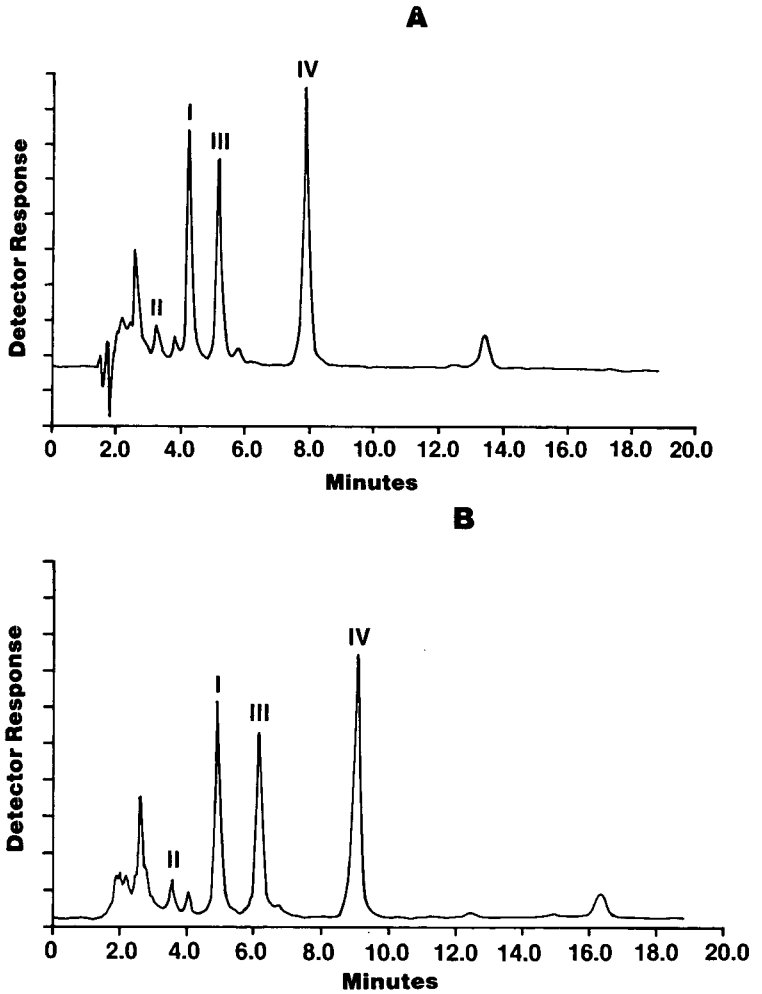

Fig. 3.

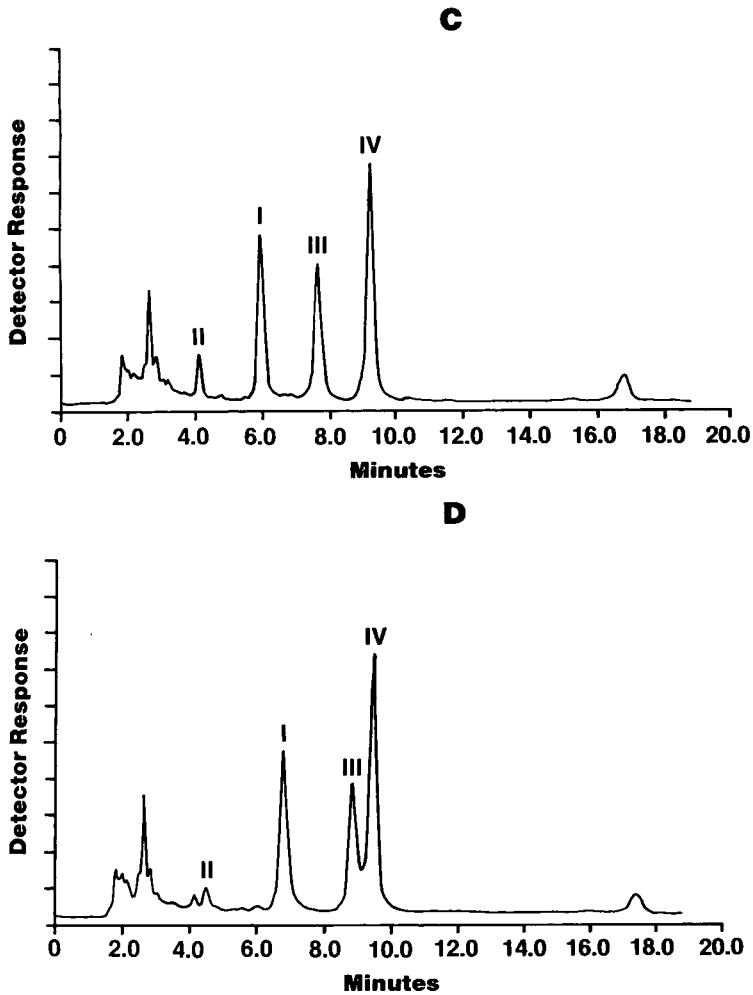

Fig. 3. Effect of mobile phase pH on the chromatographic separation. (A) pH 4; (B) pH 5; (C) pH 6; (D) pH 7. Peaks: I = indecainide; II = desisopropyl indecainide; III = internal standard; IV = unknown contaminant from patients' plasma.

contaminant and III at lower pH values. The resolution was inadequate at pH 7.0. Hence, pH 6.0 was selected since good resolution of I, II, and III as well as endogenous interferences was achieved.

Extraction recovery

Several organic solvents such as hexane, toluene, diethyl ether, butyl chloride, and ethyl acetate were tried for the extraction of I, II, and III from plasma samples. Ethyl acetate gave the best recovery for all three compounds; however, background interference from the plasma was considerably greater, particularly when using UV detection. The addition of hexane (10%) to the ethyl acetate reduced the background from plasma without greatly affecting the recovery of the compounds. The addition of a small amount of methanolic hydrochloride in the evaporation step improved the recovery of the three

TABLE I

SUMMARY OF PRECISION AND ACCURACY DATA OF ASSAY FOR INDECAINIDE AND DESISOPROPYL INDECAINIDE IN PLASMA BY UV DETECTION

		Added (ng/ml)							
		10		60		100		300	
		II	I	II	I	II	I	II	I
Day 1	$\bar{X}$	12.52	11.82	63.38	63.0	106.5	105.5	324.6	320.8
	R.S.D.	5.19	7.58	4.8	3.9	4.0	2.8	0.33	2.5
	n	5	5	5	5	4	4	3	4
Day 2	$\bar{X}$	12.37	12.9	64.2	62.7	105.6	106.4	313.0	311.0
	R.S.D.	4.72	4.1	1.4	1.2	1.5	7.5	1.5	2.2
	n	5	5	5	5	5	5	5	5
Day 3	$\bar{X}$	13.9	12.5	64.7	61.5	104.6	100.0	307.7	298.8
	R.S.D.	5.5	8.9	5.7	3.0	3.0	2.9	2.2	1.8
	n	5	5	5	5	5	5	5	5
Overall precision									
	$\bar{X}$	12.9	12.4	64.1	62.4	105.5	103.9	313.6	309.4
	R.S.D.	7.3	7.6	4.2	2.9	2.8	5.6	2.6	3.6
	n	15	15	15	15	14	14	13	14
Overall accuracy (relative error, %)		29	24	6.8	4	5.5	3.9	4.5	3.1

TABLE II

SUMMARY OF PRECISION AND ACCURACY DATA OF ASSAY FOR INDECAINIDE AND DESISOPROPYL INDECAINIDE IN PLASMA BY FLUORESCENCE DETECTION

		Added							
		10 ng/ml II	20 ng/ml I	60 ng/ml II	120 ng/ml I	200 ng/ml II	400 ng/ml I	600 ng/ml II	1200 ng/ml I
Day 1	$\bar{X}$	10.6	19.9	61.85	120.9	195.8	389.2	599.9	1144.4
	R.S.D.	6.84	4.77	1.35	1.14	2.03	0.73	2.5	0.75
	n	5	5	4	4	5	5	5	5
Day 2	$\bar{X}$	9.52	17.7	60.48	118.2	191.4	389.0	600.7	1162.6
	R.S.D.	7.55	6.65	3.54	2.29	2.7	1.31	2.69	2.27
	n	5	5	5	5	5	5	5	5
Day 3	$\bar{X}$	10.38	18.1	54.6	103.5	194.9	397.6	590.9	1150.0
	R.S.D.	1.58	3.18	4.68	3.91	2.97	2.98	6.86	5.37
	n	5	5	5	5	5	5	5	5
Overall precision									
	$\bar{X}$	10.17	18.59	58.8	113.7	194.0	391.9	597.2	1152.3
	R.S.D.	7.2	7.1	6.4	7.4	2.6	2.1	4.2	3.2
	n	15	15	14	14	15	15	15	15
Overall accuracy (relative error, %)		1.7	−7.05	−2.0	−5.25	−3.0	−2.0	−0.47	−3.97

compounds, particularly of II. This is possibly owing to the reduction of adsorption of the amines to the glass surface. The recovery of known concentrations of I and II from plasma samples containing 100—1000 ng/ml I and 50—500 ng/ml II was determined by comparing the peak heights of the extracted compounds to those obtained from aqueous standard solutions injected directly. The recoveries for the concentration ranges specified were 97.6 ± 3.8% and 83.6 ± 3.8% for I and II, respectively (n = 16). The recovery of III was 97.1 ± 2.5% (n = 16).

Precision and accuracy

The precision and accuracy of the method was determined by spiking blank plasma with I and II at four different concentrations. Five replicates of each plasma sample were assayed on three different days. The concentrations ranged from 10 to 300 ng/ml for UV detection, and from 10 to 1200 ng/ml for fluorescence detection. The results are shown in Tables I and II. The between-

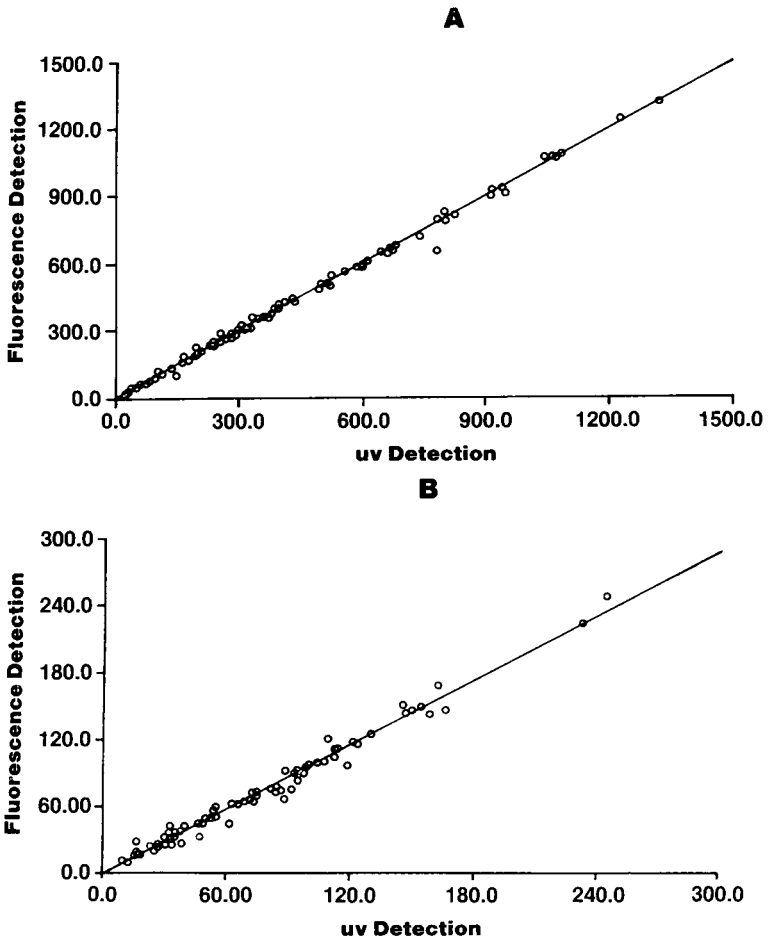

Fig. 4. Correlation between the UV detection and fluorescence detection. (A) Indecainide (ng/ml) in plasma, (B) desisopropyl indecainide (ng/ml) in plasma.

day and within-day precisions were good for both detection methods, ranging from 3.0 to 10.3% using the UV detector, and from 2.0 to 7.2% using fluorescence detection. The overall accuracy, expressed as relative error, ranged from 1.6 to 7.5% using fluorescence detection, and from 3.1 to 6.8% using UV detection, except for the plasma containing 10 ng/ml in which the latter case yielded a relative error of 29%. This indicated that UV detection was subject to marked interferences at lower concentrations.

Comparison between the UV and fluorescence detection

To compare the UV and fluorescence detection of I and II in plasma, the two detectors were connected in series to monitor the effluent from the chromatographic column. About 300 plasma samples from clinical studies were analyzed. The assay results, using the two detectors, were obtained and plotted (Fig. 4). Excellent correlation was obtained for I (r = 0.999). The correlation was equally good for II, with the exception of certain patients' samples where results based on UV detection were significantly higher, showing the susceptibility of the UV detection to interferences. Upon further investigation, the interference was found to be due to the presence of triamterene in these plasma samples. Triamterene co-eluted with II under the chromatographic conditions

TABLE III

RETENTION DATA OF DRUGS TESTED FOR INTERFERENCE IN THE ASSAY OF INDECAINIDE AND DESISOPROPYL INDECAINIDE

Compound	Amount injected (μg)	Retention times* (min)
Acetaminophen	1	2.2
Aprindine	2	ND**
Caffeine	1	2.2
Captopril	0.1	ND
Chlorpheniramine	1	10.1 (tailing)
Clonidine	0.05	ND
Diazepam	0.05	ND
Digitoxin	1	ND
Digoxin	1	ND
Disopyramide	1	ND
Furosemide	2	3.25
Hydralazine	0.2	3.0
Hydrochlorothiazide	0.5	2.75
Lidocaine	2	ND
Methyldopa	0.5	ND
Phenobarbital	5	ND
Procainamide	1	2.2
Propranolol	0.2	12.5***
Quinidine	2	12.8
Secobarbital	1	ND
Theophylline	1	2.2
Triamterene	0.2	4.0

*Detected by UV except where noted.
**ND, not detected.
***Also detected by fluorescence.

discussed, and absorbs at 270 nm, but was not detectable using the spectrofluorometer at the excitation and emission wavelengths specified. However, it was found that if a filter fluorometer was used with the emission cut-off filter of 295 nm, triamterene was also detectable. This resulted in falsely high values of II since the emission wavelength of triamterene is 470 nm. Because triamterene, a diuretic, may be co-administered with indecainide, the use of a spectrofluorometer having excitation- and emission-grating monochromators is the detector recommended when assaying for I and II.

Other drugs that may be administered concomitantly with indecainide were tested for potential interference in the assay. These compounds included analgesics, CNS stimulants, β-blockers, diuretics, cardiac glycosides, and other antiarrhythmic drugs. Solutions of these compounds were injected directly onto the chromatographic column and monitored by both the UV and fluorescence detectors. Table III lists the compounds tested and their retention times. With the exception of triamterene, discussed above, none of the drugs tested interfered in the assay using either detector.

Linearity and sensitivity

The relationship between the concentrations of I and II and their peak height ratios relative to the internal standard, using either detection system, was linear over a wide concentration range (25—1000 ng/ml), with correlation coefficients of 0.998 or better for both compounds. The detection limits varied, depending on the detection mode used. Using the UV detector (270 nm), the limit of detection was 10 ng/ml for I and II; whereas using the spectrofluorometer, the sensitivity of the assay for both compounds was 0.5 ng/ml.

Application of the method in pharmacokinetics studies

Three patients with ventricular arrhythmias were given oral doses of I, ranging from 75 to 125 mg every 8 h over nineteen days in a single blind study

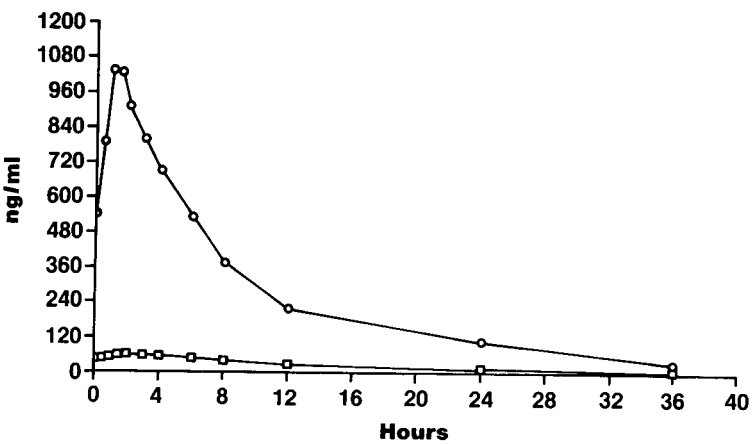

Fig. 5. Mean plasma concentration of indecainide and desisopropyl indecainide following the last 100-mg dose of indecainide in a multiple-dose study. ○, Indecainide; □, desisopropyl indecainide.

with a placebo lead-in period. Blood samples were collected periodically before the morning dose and at pre-determined time intervals up to 36 h following the last dose. Urine was collected, starting on day 19, every 6 h for up to 36 h. The concentrations of I and II in plasma and urine samples were determined by the described method. The plasma profile following the last dose is illustrated in Fig. 5. The results indicated that there was no accumulation of I or II following multiple doses of indecainide hydrochloride. The results also demonstrate the suitability of the method for pharmacokinetic studies.

ACKNOWLEDGEMENTS

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF BACLOFEN IN PLASMA AND URINE OF MAN AFTER PRECOLUMN EXTRACTION AND DERIVATIZATION WITH *o*-PHTHALDIALDEHYDE

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SUMMARY

A reversed-phase high-performance liquid chromatographic method for the determination of the skeletal muscle relaxant baclofen in human plasma and urine is described. Cation-exchange extraction, precolumn derivatization with *o*-phthaldialdehyde, and on-column concentration precede fluorimetric detection (excitation at 340 nm, emission at 460 nm). The precision of the assay was always better than 6%. Recoveries of standards added to plasma and urine were 92% and 93%, respectively. With a sample size of 0.5 ml, a detection limit of a few nanograms, and the possibility of analysing up to four samples per hour, this method is suitable for pharmacokinetic studies. An example is presented.

INTRODUCTION

Baclofen, 4-amino-3-*p*-chlorophenylbutyric acid, is a skeletal muscle relaxant, which has been used in spastic disorders since its introduction for therapy in 1967 [1]. Several symposia have been dedicated to its pharmacological actions and clinical applications, but no conclusive evidence has yet been acquired for its mode of action [2—4]. Besides motor disorders other indications have also been proposed, such as schizophrenia [5—7], tardive dyskinesia [8, 9], and trigeminal neuralgia, but only the latter shows promising results [10, 11].

Baclofen is a *p*-chlorophenyl analogue of γ-aminobutyric acid (GABA), with the substituent rendering it a centre of asymmetry (Fig. 1). The discussion about its mode of action has been complicated by the different effects of the two enantiomers. The efficacy in spasticity has been attributed to (—)-baclofen, a substance with $GABA_B$-mimetic properties [12, 13]. The commercially available drug (Lioresal®) is the racemic mixture.

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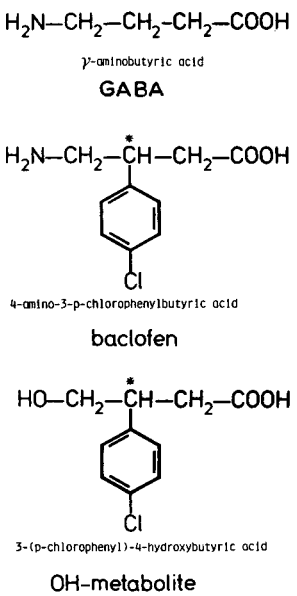

Fig. 1. Structural formulae of γ-aminobutyric acid (GABA), baclofen and its hydroxy metabolite.

Baclofen is metabolized to only a minor extent. Deamination yields 3-(*p*-chlorophenyl)-4-hydroxybutyric acid (Fig. 1), a metabolite which has been identified in the urine of rat, dog, and man. In man about 85% of a ^{14}C-labelled oral dose was found to be excreted unchanged, primarily in the urine. Most of the remaining radioactivity was accounted for by the deaminated metabolite, which was inactive in animals [14, 15].

The development of a procedure to determine baclofen in body fluids encountered several analytical problems due to its amino acid structure. For the measurement of concentrations in the nanogram range, as needed for pharmacokinetic studies, it has to be separated from the endogenous amino acids. Degen and Riess [16] developed a gas—liquid chromatographic method with electron-capture detection, requiring lengthy derivatization reactions and resulting in only a 50% recovery. Gas chromatography in combination with mass spectrometry (GC—MS) has been described by Swahn et al. [17].

No high-performance liquid chromatographic (HPLC) method for the determination of baclofen has yet been published. One problem is that baclofen itself, like other amino acids, cannot be detected in low concentrations in biological material with ultraviolet spectrometry or fluorimetry. However, a considerable number of methods to assay endogenous amino acids, using reversed-phase HPLC in combination with fluorophore formation, have recently been published. Commonly used derivatization agents for pre- or postcolumn fluorescence detection of amino acids are Dns chloride, fluorescamine, and *o*-phthaldialdehyde (OPA) [18]. Derivatization with OPA is relatively simple and rapid. In the presence of alkylmercapto compounds highly fluorescent isoindoles are formed [19—21]. We developed an analytical assay for baclofen based upon precolumn derivatization with OPA. Separation

from the endogenous amino acids was achieved by cation-exchange extraction prior to the derivatization. High sensitivty was obtained with an on-column concentration and cleaning procedure which allows for injection of large volumes [22].

MATERIALS AND METHODS

Reagents and chemicals

Solutions were made in distilled water. All glassware was rinsed with distilled water prior to use. Chemicals were of analytical grade and were used without further purification. A stock solution of baclofen (a gift from Ciba-Geigy, Arnhem, The Netherlands) containing 100 mg/l was diluted with water, urine, or plasma to produce concentrations in the range 0.02—2 mg/l. For the extraction procedure the following solvents and solutions were used: hexane, methanol, saturated sodium chloride solution, citrate buffer pH 2.6 (0.1 *M* citric acid—0.2 *M* dibasic sodium phosphate, 89.1:10.9, v/v), and borate buffer pH 10.4 (0.1 *M* borax adjusted to pH 10.4 with sodium hydroxide). The derivatization reagent consisted of 250 mg of *o*-phthaldialdehyde dissolved in 1.5 ml of methanol, 23 ml of borate buffer pH 10.4 (0.4 *M* boric acid adjusted to pH 10.4 with potassium hydroxide), and 0.5 ml of thioglycolic acid. The pH was adjusted to 10.4 after mixing [23]. Two mobile phases were used: eluent A, 0.9% (w/v) sodium chloride solution; eluent B, methanol—tetrahydrofuran—phosphate buffer pH 8.5 (0.067 *M* dibasic sodium phosphate adjusted to pH 8.5 with monobasic potassium phosphate) (40:2:58, v/v/v).

Apparatus

Extraction was performed with the Baker-10 extraction system (Baker Chemicals, Cat. No. 70180, Deventer, The Netherlands), fitted with 3-ml disposable extraction columns packed with aromatic sulphonic acid bonded to silica gel (Cat. No. 70903).

The chromatographic system consisted of a double-head solvent pump (Orlita, DHP-1515, Bakker, Zwijndrecht, The Netherlands), two sampling valves (Valco, Houston, TX, U.S.A.), and a sampling loop of 1.0 ml. Complete pulse quenching was achieved with a pulsation dampener (Orlita, PDM 3.350 M, Bakker) between pump and injection valve. The analytical column (25 cm × 4.6 mm I.D.) was packed with reversed-phase material Cp-Spher C_8, particle size 8 μm (Chrompack, Cat. No. 28502, Middelburg, The Netherlands). The concentration column (5 cm × 3.0 mm I.D.) was filled with LiChrosorb RP-8, 10 μm (Chrompack). A fluorescence detector (Perkin-Elmer, Model 3000, Delft, The Netherlands), equipped with a red sensitive photomultiplier and a doubly mirrored flow cuvette, was used. The detector was connected to a 10-mV recorder (Kipp & Zonen, BD 40, Delft, The Netherlands).

Extraction and derivatization

The extraction column was conditioned with two column volumes of hexane, two column volumes of methanol, two column volumes of water, and three column volumes of saturated sodium chloride solution. To 0.5 ml of plasma (low concentrations 1.0 ml) or 0.5 ml of urine (high concentrations

were diluted prior to use) an equal volume of citrate buffer pH 2.6 was added. This mixture was loaded onto the column. After five subsequent column washings, four with water and the last with saturated sodium chloride solution, the sample was eluted with four 0.5-ml aliquots of borate buffer pH 10.4. To the collected eluent 0.4 ml of the derivatization reagent was added. After mixing on a vortex mixer and subsequent centrifugation at 2000 *g*, a 1.0-ml sample was taken to be used for HPLC. The time elapsed between the addition of the reagent until injection into the HPLC system was standardized at 150 sec.

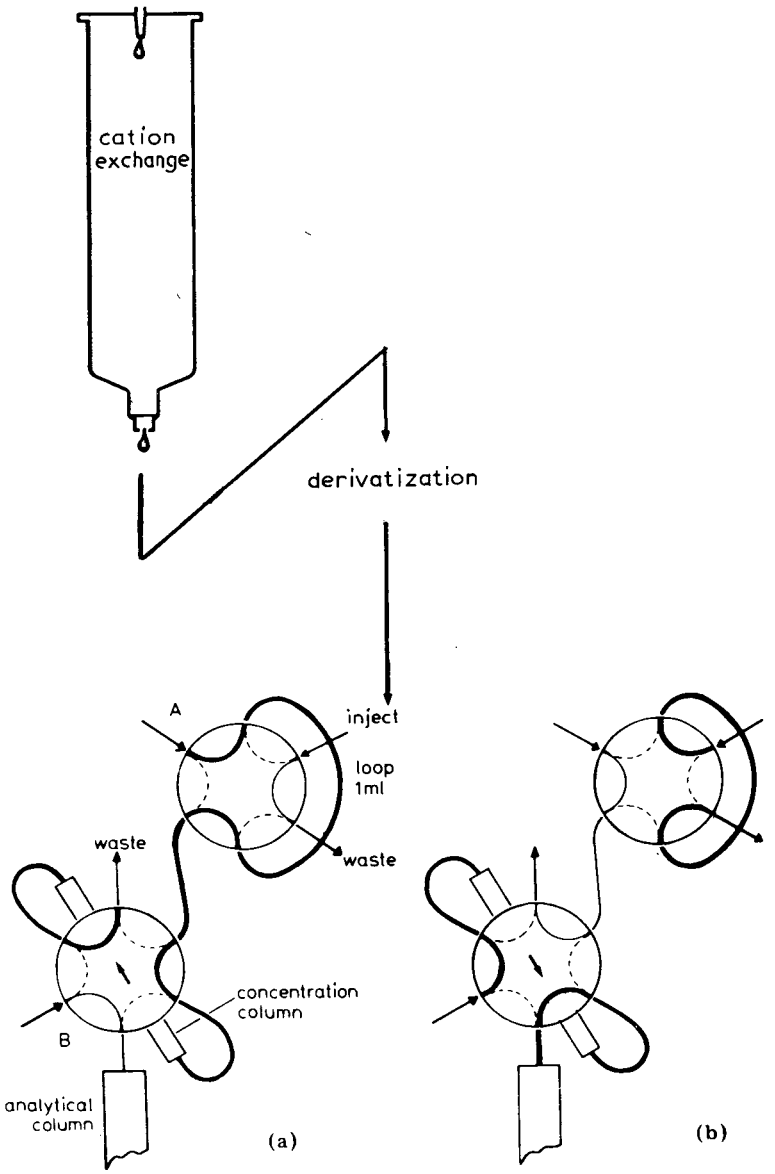

Fig. 2. Schematic diagram of the assay of baclofen. For further explanation, see text.

HPLC and detection

The sample loop was filled as shown in Fig. 2b. By turning the upper valve, solvent A (flow-rate 1.5 ml/min, pressure approx. 5 MPa) transported the sample onto the concentration column (Fig. 2a). After 5 ml of solvent A had been used, the lower valve was turned and with 1.5 ml of solvent B (flow-rate 1.0 ml/min, pressure approx. 10 MPa) the concentrated sample could be flushed onto the analytical column (Fig. 2b). With an excitation wavelength of 340 nm and emission at 460 nm the fluorophore was detected quantitatively by measuring the peak height. The experiments were carried out at room temperature. Between samples the concentration column was flushed with several 1-ml methanol washings.

Recovery

The recoveries of the standards that had been added to water, plasma, and urine were measured in triplicate for three different concentrations in the range 0.10—2.1 mg/l and compared to a direct (i.e. no extraction) assay in water.

Experiment in a healthy volunteer

A 35-year-old Caucasian woman (67 kg) was given a single oral dose of 20 mg baclofen (Lioresal®, two tablets of 10 mg each) 2 h after breakfast. Blood samples of 1—2 ml were drawn at predetermined intervals by fingertip puncture for a total period of 14 h. Samples from spontaneously voided urine were collected for 50 h. All blood specimens were collected in heparinized tubes. Plasma and urine were stored at −20° C until analysis.

RESULTS AND DISCUSSION

Chromatograms of baclofen in plasma and urine are shown in Figs. 3 and 4. Blanks (Fig. 3A and 4A) did not show interfering substances. In Fig. 3B a plasma sample obtained from a patient treated with a daily oral dose of 45 mg is shown. The detection limit in plasma at a signal-to-noise ratio of 3 was approx. 1.5 ng (Fig. 3C). An example of baclofen measured in the urine of a volunteer is given in Fig. 4B. The detection limit in urine was approx. 5 ng (Fig. 4C). The capacity ratio (k') was 3. Calibration curves showed good linearity between peak heights and concentrations (r^2 always > 0.99). The precision of the determination in water, plasma, and urine was measured for three different concentrations in the range 0.10—2.1 mg/l ($n = 4$). Coefficients of variation were always less than 6%. Recoveries in a similar concentration range were 97% for extraction from water, 92% and 93% for extraction from plasma and urine, respectively.

With the cation-exchange extraction procedure interfering endogenous amino acids could be effectively removed, owing to their low $pK_{a,1}$ values [24] as compared to baclofen ($pK_{a,1} = 3.87$, $pK_{a,2} = 9.62$) [15]. Thus, based upon the pK_a values of baclofen and the pH needed for the subsequent derivatization reaction, the different buffers were chosen. When the conditioning with sodium chloride was omitted, baclofen was not reproducibly held on the column. Prior to elution, another wash with sodium chloride was necessary for complete recovery. Derivatization of baclofen with OPA was as simple and rapid as for

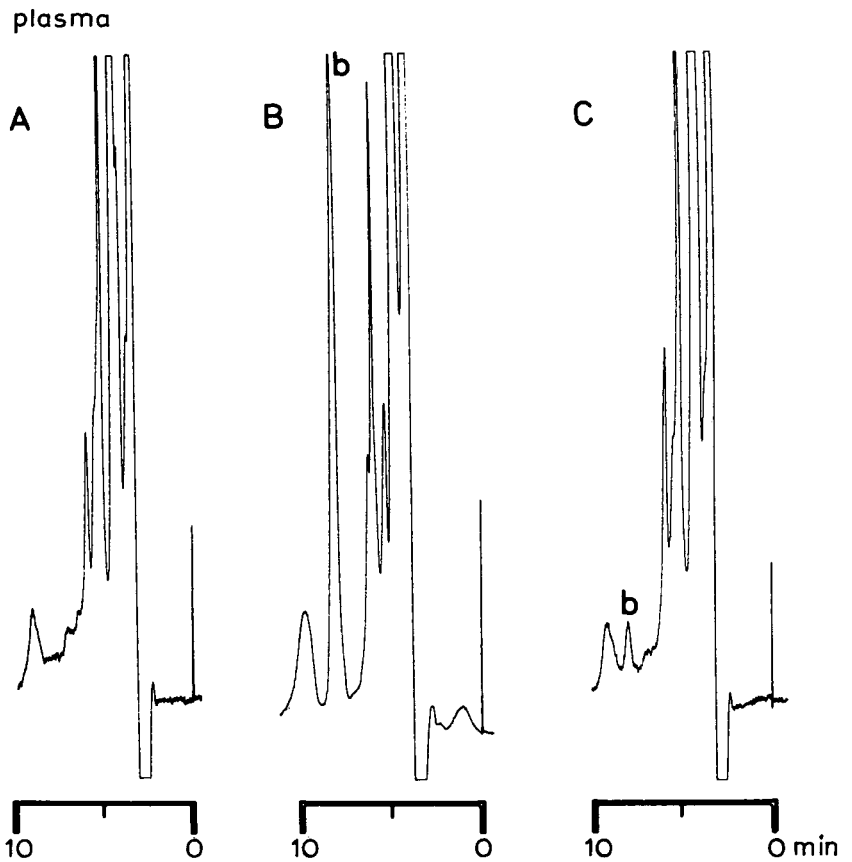

Fig. 3. Chromatograms of plasma samples: (A) plasma blank, (B) plasma of a patient on chronic oral therapy with 0.603 mg/l baclofen, (C) plasma spiked with 0.021 mg/l baclofen. b = baclofen.

endogenous amino acids. The structure of the proposed reaction product is given in Fig. 5. As the stability of the fluorescent derivatives of OPA with amino acids can vary with time [19—21], the baclofen fluorophore was always measured at a fixed time, in this case 150 sec after starting the reaction.

Although baclofen has been in clinical use for over fifteen years, hardly any pharmacokinetic data are available. Only a few studies have been published mentioning pharmacokinetic parameters, in volunteers [14, 15] and in patients [25, 26]. This seems to be mainly due to the lack of analytical procedures suitable for routine measurements.

To test the applicability of the presented HPLC method in pharmacokinetic studies a pilot experiment was done in a healthy volunteer. Fig. 6 shows the plasma concentration—time and renal excretion rate—time profiles of baclofen after a single oral dose of 20 mg. Some pharmacokinetic parameters are listed in Table I, calculated according to standard methods [27, 29]. The values of t_{max} and of C_{max} are similar to those reported by Swahn et al. [17] with the GC—MS method. After 50 h, 85% of the dose administered was recovered as unchanged drug in the urine. This is in agreement with data from experiments

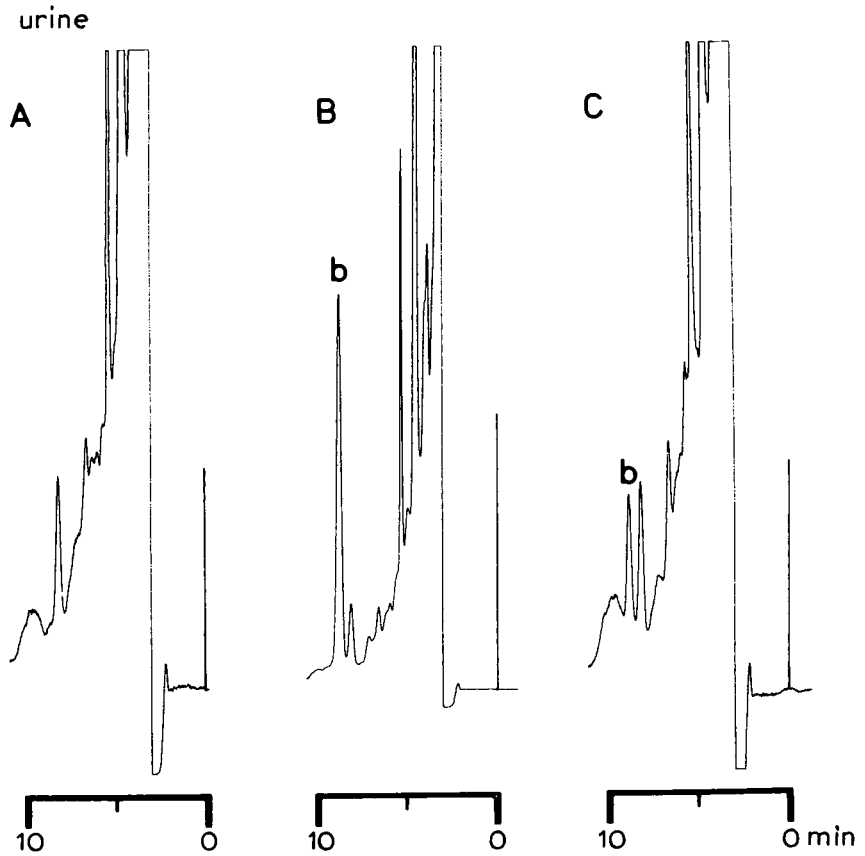

Fig. 4. Chromatograms of urine samples: (A) urine blank, (B) urine sample of a volunteer after a single oral dose of 2.51 mg/l baclofen, (C) urine spiked with 0.205 mg/l baclofen. b = baclofen.

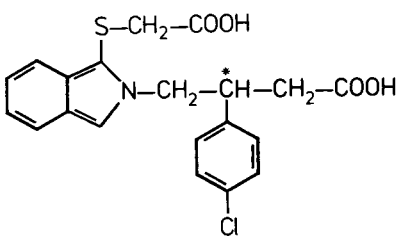

Fig. 5. Proposed reaction product of *o*-phthaldialdehyde and thioglycolic acid with baclofen.

with radioactively labelled baclofen [14, 15]. Clearance values have not been reported earlier. A total plasma clearance (Cl) of 0.16 l h^{-1} kg^{-1} was calculated. In this volunteer the renal clearance of baclofen (Cl_R) was equal to the creatinine clearance. Half-lives from plasma data have been reported to range from 2.5 to 6 h [14, 15]. After massive overdoses, however, half-lives of more than 30 h have been observed [30, 31]. We found a half-life of 5.4 h from terminal plasma data. When the combined plasma and urine data were fitted to a two-compartment open model using NONLIN [27], a higher terminal

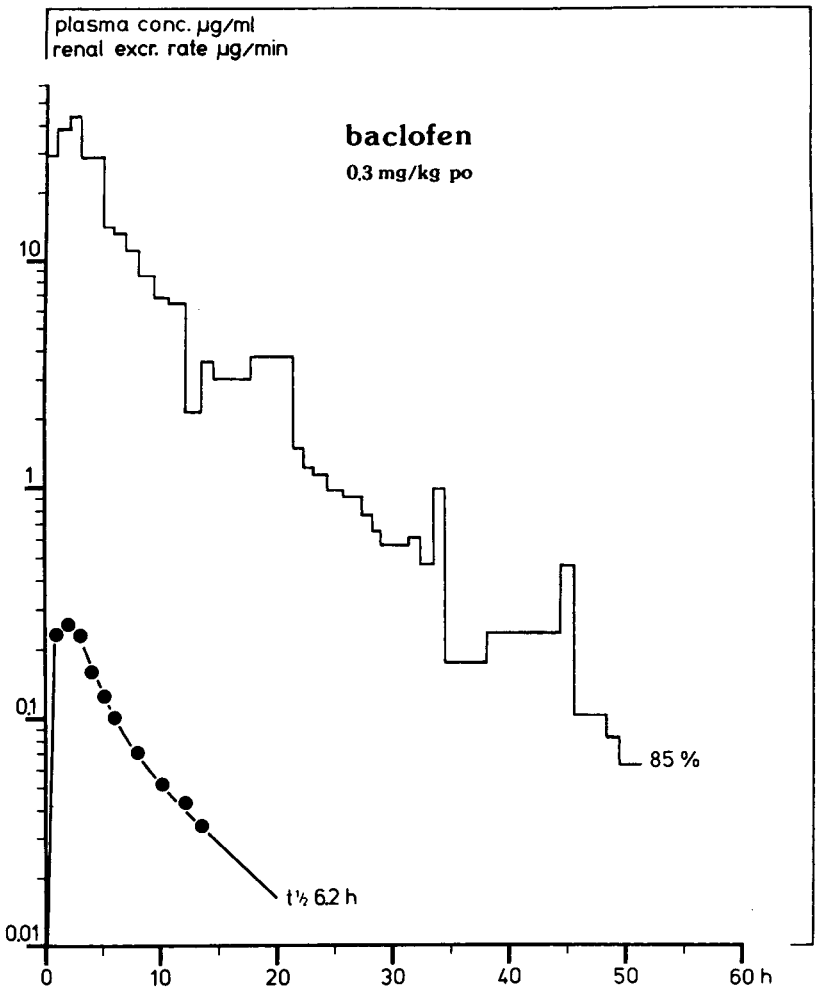

Fig. 6. Plasma concentration—time and renal excretion rate—time profiles of baclofen in a human volunteer following a single oral dose of 20 mg. % = percentage of the dose excreted unchanged in the urine.

TABLE I

SOME PHARMACOKINETIC PARAMETERS OF BACLOFEN IN MAN

Single oral dose of 20 mg (n = 1). Abbreviations according to ref. 28, calculations according to refs. 27 and 29.

C_{max}	0.24 mg/l
t_{max}	2.0 h
$t_{½z}$	6.2 h
Cl^{*}	0.16 l h^{-1} kg^{-1}
Cl_R**	0.12 l h^{-1} kg^{-1}
$A_e(\infty)$***	86% (of dose in urine)

*Assumption F = 1.
**Calculated from total plasma concentration data.
***Extrapolated to infinity.

half-life of 6.2 h was found. In a preliminary study in eighteen patients on chronic oral therapy with daily baclofen doses ranging from 0.26 to 1.2 mg/kg, we measured plasma concentrations varying from 0.078 to 0.60 mg/l, with a mean total plasma clearance of 0.21 ± 0.11 l h^{-1} kg^{-1}. Samples were drawn 3 h after the morning dose.

With the described reversed-phase HPLC method, only the parent drug is measured. The hydroxymetabolite lacks the NH_2 group, which reacts with OPA. Also no separation of the enantiomers is obtained. These disadvantages also apply to the gas—liquid chromatographic methods. From the results presented, the possibility of measuring up to four samples (plasma or urine) per hour, with a sample size of only 0.5 ml, and a detection limit of a few nanograms, it appears that baclofen can be measured with adequate sensitivity and selectivity for pharmacokinetic purposes.

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DETERMINATION OF CHLORPROTHIXENE AND ITS SULFOXIDE METABOLITE IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ULTRAVIOLET AND AMPEROMETRIC DETECTION

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SUMMARY

This communication describes a rapid, sensitive and selective method for the assay of chlorprothixene and its sulfoxide metabolite in human plasma, using reversed-phase high-performance liquid chromatography. Alkalinized plasma was extracted with heptane—isoamyl alcohol (99:1), after addition of thioridazine as the internal standard. The residue obtained after evaporation of this extract was chromatographed on a cyano column, using acetonitrile—0.02 *M* potassium dihydrogen phosphate pH 4.5 (60:40) as the mobile phase with ultraviolet (229 nm) detection. Quantitation was based on peak height ratios over the concentration range of 5.0—50.0 ng/ml for both compounds with 85% and 90% recovery for chlorprothixene and its sulfoxide metabolite, respectively, using a 1.0-ml plasma sample. The assay chromatographically resolves chlorprothixene and the sulfoxide metabolite from the N-desmethyl metabolite, which can only be semi-quantitated owing to low and variable recoveries.

The method was used to obtain plasma concentration versus time profiles in two subjects after oral administration of 100 mg of chlorprothixene suspension and in two additional subjects following overdosages of chlorprothixene estimated to exceed several hundred milligrams. These analyses demonstrated that the sulfoxide metabolite is the predominant plasma component following therapeutic administration and overdosages.

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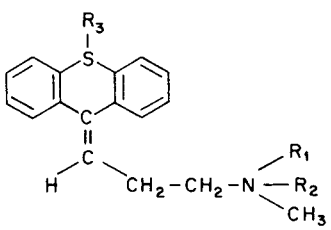
High-performance liquid chromatography with oxidative amperometric detection with the glassy carbon electrode was also evaluated. Although this procedure demonstrated comparable sensitivity and precision to ultraviolet detection for the analysis of chlorprothixene and N-desmethyl chlorprothixene, the sulfoxide metabolite could not be measured with high sensitivity (< 100 ng/ml) owing to endogenous interferences. Hence the utility of this alternative assay technique is limited.

INTRODUCTION

Chlorprothixene (I, 2-chloro-N,N-dimethylthioxanthene-$\Delta^{9,\gamma}$-propylamine) (Fig. 1) is presently marketed as the antipsychotic agent Taractan®. The biotransformation of I in man via oxidation and N-demethylation to chlorprothixene sulfoxide (II) [1—3], N-desmethyl chlorprothixene (III) [3, 4], N-desmethylchlorprothixene sulfoxide (IV) [2, 3] and chlorprothixene sulfoxide-N-oxide (V) [2] has been reported (Fig. 1).

Specificity for the determination of I in the presence of its metabolites requires either selective extraction [1, 5—8] or thin-layer chromatographic (TLC) separation [4, 8]. Selective extraction procedures have been described for the simultaneous determination of I and its major metabolite (II) in blood plasma and urine. These assays require extraction of I and II into heptane or heptane—isoamyl alcohol mixtures from the biological sample adjusted to an alkaline pH. Selectivity against II is assured by back-extraction of II from the organic phase into a pH 5.6 buffer [9]. Intact I is then extracted into dilute acid from the organic phase. Compound I is determined by spectrofluorometry in strong acid as either the intact drug (I) [1, 5, 7, 8] or after potassium permaganate oxidation to 2-chlorothioxanthen-9-one [6, 10]. The S-oxide metabolite (II) is reduced back to I and then determined by spectrofluorometry.



Compound	R_1	R_2	R_3
Chlorprothixene [I]	$-CH_3$	—	—
Chlorprothixene Sulfoxide [II]	$-CH_3$	—	→O
N-Desmethyl Chlorprothixene [III]	—H	—	—
N-Desmethyl Chlorprothixene Sulfoxide [IV]	—H	—	→O
Chlorprothixene Sulfoxide-N-Oxide [V]	$-CH_3$	→O	→O

Fig. 1. Biotransformation of chlorprothixene [1—4].

Toxicological investigations using spectrofluorometry with TLC separation to determine I—IV [4] or selective extraction to determine I and II [8] in post-mortem samples have been reported. In addition, the presence of 2-chlorothioxanthen-9-one was recently identified in the gastric aspirate following a severe chlorprothixene poisoning [11]. Only limited therapeutic drug monitoring data for I are reported [7, 12, 13] which are difficult to correlate owing to the lack of information pertaining to the actual dosage form ingested. Following a single oral dose of 200 mg of I in adults, blood concentrations of I and II ranging from non-measureable (< 10 ng/ml) to 60 ng/ml and from 30 to 140 ng/ml, respectively, were determined 1—8 h post administration [12]. Following single oral 30-mg doses of chlorprothixene peak blood concentrations of I of 12—17 ng/ml were determined 3—4 h post administration [7, 13]. The half-life of the drug following intravenous administration was reported to be in the order of 8—12 h [7].

Separation of tricyclic antidepressants and their metabolites by high-performance liquid chromatography (HPLC) allows for their specific determination in biological fluids without the time-consuming selective extraction or TLC procedures [14]. HPLC procedures have been described for the separation of the *cis*- and *trans*-isomers of I [15] and for the determination of the stability of I under a variety of conditions of photo-irradiation [16]. Amperometric detection coupled with the HPLC separation further increases the specificity of the determination as has been reported for the analysis of phenothiazines, thioxanthene and butyrophenones [17—23] in biological fluids.

The present work describes a reversed-phase HPLC assay with ultraviolet (UV) (cadmium source; 229 nm) detection for the determination of chlorprothixene and its principal plasma metabolite, chlorprothixene sulfoxide (II) using thioridazine (VI) as the internal standard. The assay chromatographically resolves I and II from the N-desmethyl metabolite (III), which can be only semi-quantitated owing to poor and variable recovery. The HPLC—UV assay was utilized to measure concentrations of I, II and III in man following single oral (100 mg) and overdosages exceeding several hundred milligrams of chlorprothixene.

HPLC with oxidative amperometric detection with the glassy carbon electrode was also evaluated and yielded comparable sensitivity and precision for the analysis of I and the semi-quantitative analysis of III. The sulfoxide metabolite (II) could not be measured with high sensitivity (< 100 ng/ml) due to endogenous interferences which limited the utility of this assay technique.

EXPERIMENTAL

Column

The column used was a 25 cm × 4.6 mm I.D. stainless-steel column containing 5-μm LC-PCN (cyano) packing (Supelco, Bellefonte, PA, U.S.A.).

Instrumental parameters

The HPLC system consisted of a Model 6000A pumping system, a Model U6K loop injector and a Model 440 UV detector (with an extended wavelength module and a 229-nm kit) (Waters Assoc., Milford, MA, U.S.A.). The mobile phase for isocratic reversed-phase HPLC was composed of acetonitrile—0.02 *M*

potassium dihydrogen phosphate pH 4.5 (60:40), prepared by mixing 16.0 ml of 1 *M* potassium dihydrogen phosphate pH 4.5 with 784.0 ml distilled water and diluting to 2 l with acetonitrile. The mobile phase was degassed prior to use and kept under constant sparging with helium (99.995%). The column was maintained at a temperature of 40°C (Goldenfoil™ Column Temperature control system; Systec, Minneapolis, MN, U.S.A.), with a flow-rate of 2.0 ml/min and a pressure of 9.7 MPa (1400 p.s.i.). Under the above conditions, the retention times of compounds I, II, III, and VI were 7.6 min (k' = 9.55), 4.8 min (k' = 5.66), 6.9 min (k' = 8.55), and 8.5 min (k' = 10.82), respectively (Fig. 2A).

Amounts of 50 ng of I, II, and III and 150 ng of VI, yielded peaks of approximately full scale deflection at a UV detection sensitivity setting of $5 \cdot 10^{-3}$ a.u.f.s.

The chart speed on a 10-mV recorder was 15 in./h (0.25 in./min) (Hewlett-Packard, Model 713A, Avondale, PA, U.S.A.). Upon completion of a day's analysis, the phosphate buffer and any endogenous materials were flushed from the column with acetonitrile—water (60:40).

Standard solutions

Weigh out 10.0 mg of chlorprothixene (I, $C_{18}H_{18}ClNS$, mol. wt. 315.85,

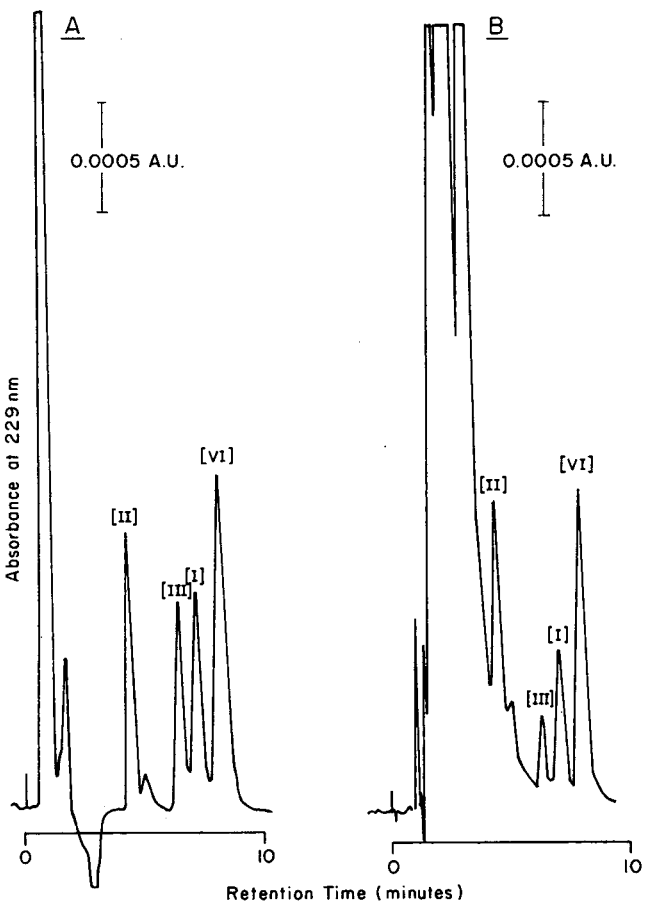


Fig. 2.

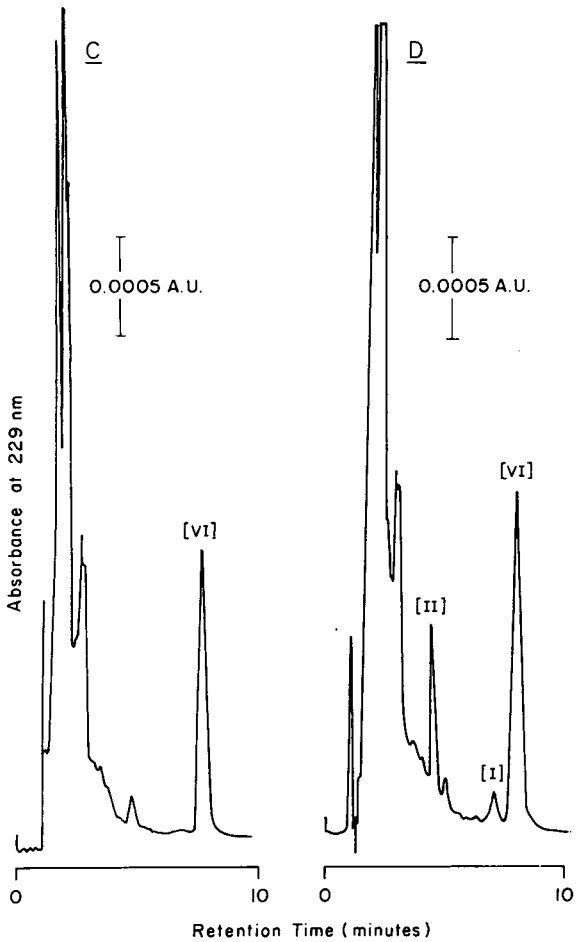


Fig.2. HPLC—UV Chromatograms of (A) external standard injection of 10.0 ng I—III and 50.0 ng of VI; (B) plasma recovered standard supplemented with 40 ng I—III and 250 ng VI; (C) patient plasma control supplemented with 250 ng VI per ml; (D) patient plasma sample (4 h post administration) concentration of I and II were 8.8 and 29.8 ng/ml, respectively.

m.p. = 97—98°C) into a 100-ml volumetric flask, dilute to volume with methanol. This stock solution (A) contains 100 μg/ml I.

In a similar manner, prepare stock solution B of chlorprothixene sulfoxide (II, $C_{18}H_{18}ClNOS$, mol. wt. 331.365, m.p. = 94—95°C), stock solution C of N-desmethylchlorprothixene (III, $C_{17}H_{16}ClNS \cdot HCl$, mol.wt. 338.294, m.p. = 202—204°C), and stock solution D of thioridazine (VI, $C_{21}H_{26}N_2S_2 \cdot HCl$, mol. wt. 407.01, m.p. = 158—160°C).

A series of mixed working standard solutions of I, II and III are prepared by transferring aliquots of 0.010, 0.020, 0.040, 0.060, 0.080 and 0.100 ml of A, B, and C each into 10-ml volumetric flasks and diluting to volume with methanol. These solutions 1—6 contain 5, 10, 20, 30, 40 and 50 ng per 50 μl of solution, respectively, I, II and III.

Aliquots (10 μl) of solutions 1—6 are injected to verify the performance of the HPLC system and to calculate percent recovery from plasma.

A working standard solution of VI, the internal standard, containing 250 ng per 50 μl (solution 7) is prepared by transferring 0.5 ml of D into a 10-ml volumetric flask and diluting to volume.

Reagents

Reagent-grade chemicals were used to prepare 1 *M* potassium dihydrogen phosphate and 2 *M* sodium hydroxide in deionized distilled water.

Other reagents include methanol, acetonitrile (UV) and heptane (Burdick & Jackson Labs., Muskegon, MI, U.S.A.), isoamyl alcohol (Mallinckrodt, St. Louis, MO, U.S.A.) and thioridazine, the internal standard (Sandoz Pharmaceuticals, Hanover, NJ, U.S.A.).

Assay in plasma

Into a 10-ml borosilicate disposable centrifuge tube add 1.0 ml of unknown plasma, 0.050 ml of internal standard (solution 7), 2.0 ml of distilled water and 2.0 ml of 2 *M* sodium hydroxide. Mix well and extract with 10.0 ml of 1% isoamyl alcohol in heptane by slowly shaking on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) for 15 min.

Centrifuge the sample in a refrigerated centrifuge (Model PRJ with a No. 253 rotor, Damon, IEC Corporation, Needham, MA, U.S.A.) at 5—10°C for 5 min at 2200 rpm (1207 *g*). Transfer 8.5 ml of supernatant into a 10-ml borosilicate disposable centrifuge tube. Evaporate to dryness under a stream of nitrogen in a water bath at 60°C using a N-EVAP evaporator (Organomation Assoc., Worcester, MA, U.S.A.). Dissolve the residue in 250 μl of acetonitrile—water (60:40). Inject a 50-μl aliquot for HPLC analysis.

A specimen of 1.0 ml of control plasma and six 1.0-ml aliquots of control plasma containing 0.050 ml of solutions 1—6 (equivalent to 5, 10, 20, 30, 40 and 50 ng of I, II and III per ml plasma) and 0.050 ml of internal standard (solution 7) are processed with the samples as the recovered standards. The peak height ratios of I, II and III to VI versus concentration of the respective compound are used to perform a weighted linear regression analysis to establish the calibration curves for the determination of drug and metabolites concentrations in the unknown samples.

RESULTS

Analytical parameters

Inter-assay linearity and precision of the HPLC—UV method were determined over the plasma concentration range of 5.0—50.0 ng/ml for I and II (Table I). The data were accumulated from five separate analytical experiments, in which two runs included triplicate analysis at each concentration. The average coefficients of variation for I and II over this concentration range were 6.4% and 10.0%, respectively. The recoveries of I and II were approximately 85% and 90%, respectively. The sensitivity limit for III was 10 ng/ml with a recovery of 25% and an average coefficient of variation of 20%. Due to the poor and variable recovery of III and the resultant lack of precision, the assay can only be considered semi-quantitative for III.

TABLE I

INTER-ASSAY STATISTICAL EVALUATION (HPLC—UV METHOD)

Concentration added (ng/ml)	n^*	Mean concentration found (ng/ml)	Coefficient of variation (%)
Chlorprothixene (I)			
5.0	9	4.9 ± 0.4	7.3
10.0	9	10.1 ± 0.7	6.5
20.0	9	20.5 ± 1.7	8.1
30.0	9	29.8 ± 1.8	6.1
40.0	9	39.9 ± 2.2	5.5
50.0	9	50.2 ± 2.6	5.1
		Average =	6.4
Chlorprothixene sulfoxide (II)			
5.0	8	5.0 ± 0.6	11.5
10.0	8	10.0 ± 0.8	8.0
20.0	9	21.3 ± 2.4	11.2
30.0	9	31.6 ± 3.3	10.6
40.0	8	40.1 ± 4.3	10.7
50.0	9	49.8 ± 3.9	7.7
		Average =	10.0

*Data accumulated in five separate analytical experiments; two runs included triplicate analysis of each concentration.

The selectivity of the HPLC—UV assay for the N-desmethylchlorprothixene sulfoxide (IV) and chlorprothixene sulfoxide-N-oxide (V) was not evaluated due to the unavailability of authentic standards. However, the two compounds are expected to be more polar than II and should therefore elute in the solvent front in the chromatograms. Significant interferences by other neuroleptics in the retention areas of I—IV were noted under the specified HPLC conditions. Thus, in toxicological analysis the presence of I—IV will require confirmation with a second technique, i.e. gas chromatography—mass spectrophotometry.

The stability of I upon collection and handling was determined in that no loss of compound was detected for a period of 4 h at both 25°C and 37°C. The stability of I under extended periods of storage was not evaluated. However, the clinical samples from patients receiving 100 mg Taractan® (described below) were stored at —70°C prior to analysis.

Studies in man

Clinical study. The HPLC—UV analysis of I, II and III was applied to the measurement of chlorprothixene and its metabolite in two healthy subjects following a single oral administration of 100 mg. Serial blood pressure recordings were taken with the subject tilted in a 60° upright position to study the pharmacodynamic effects of the drug.

After fasting overnight two male volunteers received 5 ml of Taractan concentrate (containing 100 mg chlorprothixene per 5 ml) with 250 ml of water. Blood samples (12 ml) were drawn by glass syringe at 0, 1, 2, 3, 4, 5, 6,

8, 12, 16, 24, 32 and 48 h post dosing and immediately transferred into oxalated tubes. The samples were immediately spun down to separate the plasma, which was transferred to another tube and stored at −70°C until analysis. The subjects received water ad libitum until 4 h after drug administration when lunch was served. Standard meals were served for 24 h while the volunteers were confined to the study site. The volunteers reported back to the study site for the 32- and 48-h blood collection. Plasma concentrations of I were measurable in subject 1 at 5 h (5.3 ng/ml) and in subject 2 from 1 to 6 h. A maximum plasma concentration of I of 8.8 ng/ml was measured at 4 h for subject 2. These measurable concentrations of I correlated well with clinical response in that subject 1 displayed an orthostatic response at 6 h, and that subject 2 could not be tilted passively from 2 to 7 h because of frank fainting. Concentrations of II were measurable from 1 to 32 h and 1 to 48 h, in subjects 1 and 2, respectively. Maximum concentrations of II at 4 h of 39.9 and 29.8 ng/ml were measured, which declined to 9.8 ng/ml at 32 h and 7.7 ng/ml at 48 h in subjects 1 and 2, respectively (Table II, Fig. 3). Concentrations of III were non-measurable (< 10 ng/ml) throughout the entire collection period.

Overdose study. The assay was also applied to the analysis of blood samples from two patients suspected of ingesting overdosages of chlorprothixene exceeding several hundred milligrams. Subject 3 was a 2.5-year-old female suspected of ingestion of an unknown quantity of 50-mg Taractan tablets. The blood sample was drawn approximately 24 h following the suspected ingestion. Subject 4 was a 25-year-old female suspected of ingestion of an unknown amount of 25-mg Taractan tablets. The blood sample was drawn

TABLE II

CONCENTRATION OF I AND II IN TWO SUBJECTS FOLLOWING A SINGLE ORAL DOSE OF 100 mg CHLORPROTHIXENE (SUSPENSION OF 100 mg/5 ml OF TARACTAN)

Time post administration (h)	Concentration (ng/ml)			
	Subject 1		Subject 2	
	I	II	I	II
0	n.d.*	n.d.	n.d.	n.d.
1	n.d.	8.4	4.9	6.5
2	n.d.	22.4	7.7	16.7
3	n.d.	28.9	6.1	22.0
4	n.d.	39.9	8.8	29.8
5	5.3	34.6	8.1	22.3
6	n.d.	33.4	6.9	26.0
8	n.d.	23.7	n.d.	21.5
12	n.d.	15.3	n.d.	20.0
16	n.d.	14.9	n.d.	12.5
24	n.d.	11.1	n.d.	11.5
32	n.d.	9.8	n.d.	n.s.**
48	n.d.	n.d.	n.d.	7.7

*n.d., Non-detectable (≤ 5.0 ng/ml).
**n.s., No sample.

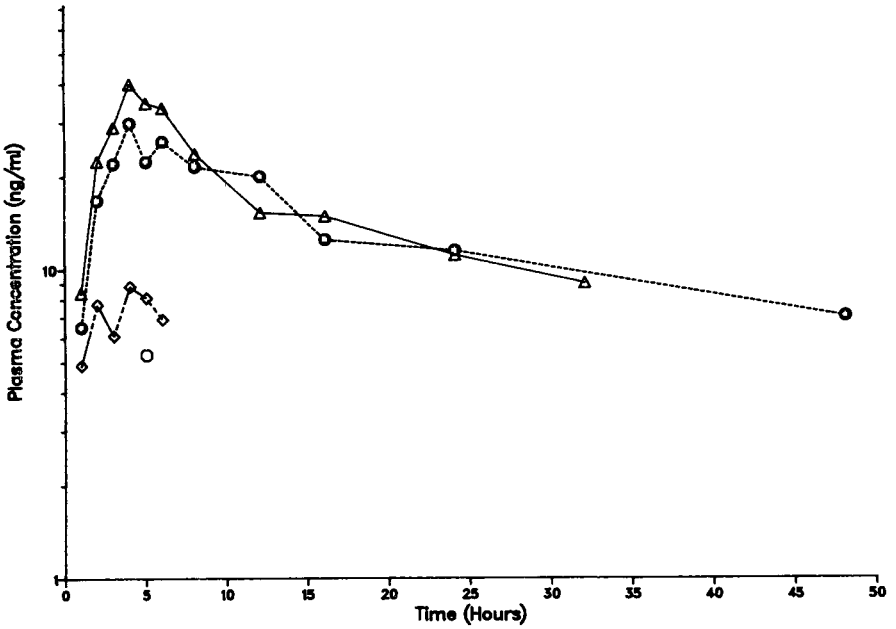


Fig. 3. Plasma concentration versus time profit for chlorprothixene (I) and chlorprothixene sulfoxide (II) in two healthy human subjects following single oral 100-mg doses of Taractan concentrate. (○) Chlorprothixene, subject 1; (△) chlorprothixene sulfoxide, subject 1; (◇) chlorprothixene, subject 2; (●) chlorprothixene sulfoxide, subject 2.

approximately 30 h following the suspected ingestion. Blood samples collected at the respective hospitals were frozen (—17°C) and sent to Hoffmann-La Roche for analysis. Plasma could not be separated from blood for these samples due to freezing, and hence whole blood was used for analysis.

The assay was performed as described in the experimental section with the exception that a CN-AQ cyano column (Varian Assoc., Palo Alto, CA, U.S.A.) was used and maintained at room temperature. A Spectromonitor III detector (LDC Corporation, Riviera Beach, FL, U.S.A.) monitored the column effluent at 230 nm. Dilution of the blood sample or final injection solution was made to bring the concentration of the unknowns within the calibration range. The data (Table III, Fig. 4A) demonstrate that the sulfoxide metabolite (II) was the major blood component and that much lower concentrations of I and III were present.

TABLE III

ANALYSIS OF I—III IN BLOOD FOLLOWING OVERDOSAGE OF TARACTAN

Subject	Concentration (μg/ml)					
	I		II		III	
	UV	Amperometric	UV	Amperometric	UV	Amperometric
3	0.13	0.13	1.0	0.92	0.08	0.06
4	0.25	0.24	0.72	0.61	0.26	0.22

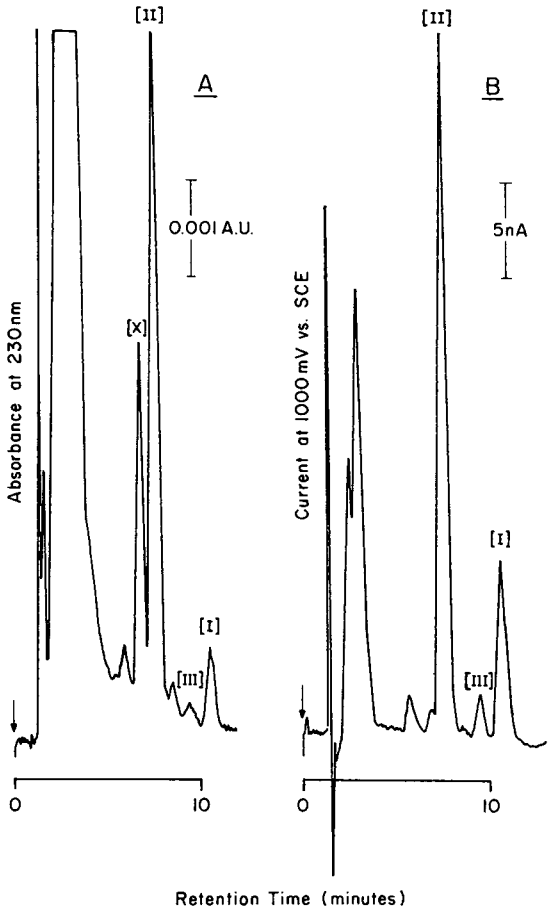


Fig. 4. HPLC analysis with (A) UV and (B) amperometric detection of a blood sample taken from subject No. 3 (see Table III) following an overdosage of Taractan.

Amperometric detection

The goal of the present study was to develop an HPLC assay with simultaneous UV and amperometric detection for I, II and III in both blood and plasma samples following therapeutic drug administration and for toxicological investigations.

Initial investigations with amperometric detection (Model 230; IBM Instruments, Danbury, CT, U.S.A.) monitored changes in oxidation current (50 nA full scale) at the glassy carbon electrode (Model 3892, Kel-F; IBM) with repeated injections and successive increases of 50 mV applied potential (Fig. 5). The data indicated that for injections of 10 ng per 10 μl the sulfoxide metabolite (II) gave only 30—40% of the response of I and III over the potential range examined. This suggested that the product of oxidation of the three compounds may be the sulfone and that the lower current associated with the sulfoxide being converted to the sulfone is due to the lesser number of electrons involved in that oxidation process. The choice of 1000 mV versus saturated calomel electrode (SCE) as the applied potential for the analytical

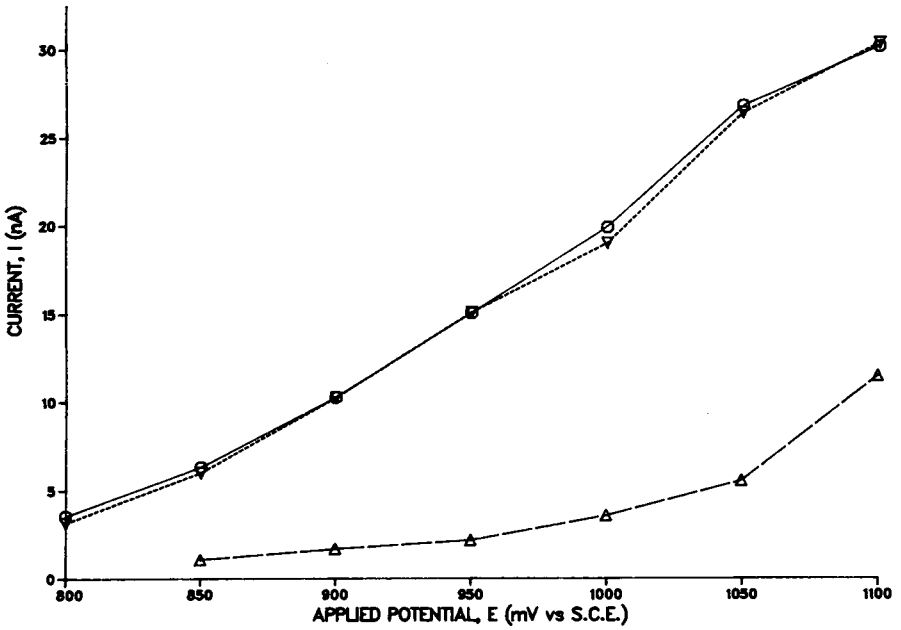


Fig. 5. Hydrodynamic voltammograms for the HPLC analysis of compounds I—III. (○—○) Chlorprothixene; (△— —△) chlorprothixene sulfoxide; (▽- - - - -▽) N-desmethylchlorprothixene.

determination was a compromise between the need for high sensitivity with a reasonably stable baseline. A concentration of 20 ng each of I, II and III yielded peaks of approximately 50%, 20% and 50% of full scale deflection, respectively, at an amperometric detection sensitivity setting of 100 nA with an applied potential of 1000 mV versus SCE.

Statistical validation for the HPLC—amperometric method for I over the concentration range of 10.0—50.0 ng/ml showed approximately equal precision and linearity to the HPLC—UV method. The decreased oxidation current for II along with an insufficiently clean extract for amperometric detection limited the sensitivity of the assay for this compound to < 100 ng/ml. These detection limits are insufficient to determine I or II following the administration of a single therapeutic dose, [7, 12, 13]; hence amperometric detection is recommended only for monitoring the administration of multiple therapeutic doses and for toxicological investigations (see below).

The HPLC—amperometric method was also utilized in the analysis of the samples taken from the two overdosage patients. The UV and amperometric detectors were placed in series with the electrochemical detector downstream of the UV detector. Good agreement was obtained between the two modes of detection (Table III). A typical chromatogram for the toxicological investigations is shown in Fig. 4. A large unknown peak (X) which eluted just prior to II was noted in the UV, but not in the amperometric tracing; hence it does not appear to be related to the ingestion of chlorprothixene.

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ASSAY METHODOLOGY FOR QUANTIFICATION OF THE ESTER AND ETHER GLUCURONIDE CONJUGATES OF DIFLUNISAL IN HUMAN URINE

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SUMMARY

Diflunisal is a salicylate derivative with analgesic and anti-inflammatory properties. It is excreted in the urine as an ether glucuronide, a 1-O-acyl glucuronide and as unchanged drug. The 1-O-acyl glucuronide rearranges to isomeric esters of glucuronic acid under neutral to alkaline pH conditions. The development of a urine assay for the conjugates enables the elucidation of diflunisal non-linear pharmacokinetics. The assay quantitates the ether and ester glucuronides and free diflunisal in urine at 0.5—1.0 μg/ml. Analysis of the glucuronides does not require authentic standards.

INTRODUCTION

Diflunisal (2′,4′-difluoro-4-hydroxy-3-biphenylcarboxylic acid) is a salicylic acid derivative with analgesic and anti-inflammatory activity [1]. The elimination of diflunisal from the human circulatory system is concentration-dependent. Its major route of metabolism is glucuronidation to ether and ester conjugates (Fig. 1) [2—4]. A reliable assay method for the glucuronides is necessary to study the pharmacokinetics of the drug. Several assay methods are available for determining total and free levels of diflunisal in biological fluids [2, 5—10]. The assay method reported earlier [3] for the glucuronides did not achieve sufficient selectivity and specificity since it was based on solvent extraction and cross-contamination could not be avoided. A recently reported assay [11] using the isolated ether and ester glucuronides to calibrate concentrations did not consider the instability of the ester glucuronide and the unavailability of the standards.

The development of the assay methodology for the ether and ester glucuronides of diflunisal has resulted in different approaches of analysis due to

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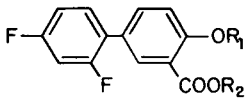


Diflunisal: $R_1 = H, R_2 = H$

Ether Conjugate: $R_1 = Glu, R_2 = H$

Ester Conjugate: $R_1 = H, R_2 = Glu$

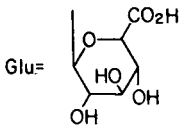


Fig. 1. Structures of diflunisal and its ether and ester glucuronide conjugates.

the lack of availability and the instability of the standards, and the acyl rearrangement of the ester glucuronide. Similar rearrangements have been reported for other drugs [12, 13]. The assay described herein requires no glucuronide standards. The concentration of the ether glucuronide conjugate (EtG) is extrapolated from a diflunisal calibration curve and a molecular weight conversion factor. The ester glucuronide (EsG) is initially hydrolyzed to diflunisal, its value is corrected for free diflunisal and the EsG concentration is determined from a diflunisal curve and an appropriate conversion factor. The EtG and EsG have been isolated for validation and stability data.

MATERIALS AND METHODS

Chemicals

Diflunisal and 5-(4′-fluorophenyl)salicylic acid were synthesized at Merck Sharp & Dohme Research Labs. [14]. Radioactive diflunisal* was synthesized at Merck Sharp & Dohme with ^{14}C in the carboxyl group [2]. Bovine liver β-glucuronidase, Type B-10, was obtained from Sigma.

Isolation of diflunisal glucuronides from human urine

A flow diagram in Fig. 2 depicting the isolation procedure indicates (a) an initial extraction of the urine with diethyl ether to remove diflunisal and waste, (b) urine pH adjustment to 1.0 with 1 *M* hydrochloric acid and (c) a second solvent extraction removing the EtG and EsG, plus some diflunisal. This latter organic extract (organic phase B) was further purified by extracting it with a sodium phosphate buffer (0.1 *M*) at pH 6.0 (2 vols.), readjusting the extract pH value to 1.0 with 1 *M* hydrochloric acid and finally back-extracting the analytes into diethyl ether (2 vols). The ether extracts (organic phase B′)* were concentrated to a thin oil-film and kept in the freezer.

The extracted glucuronide conjugates of diflunisal and diflunisal in the

*The glucuronide metabolites of diflunisal were not successfully synthesized at Merck Sharp & Dohme. The glucuronides were isolated from the urine of several healthy male subjects who received [^{14}COOH]diflunisal in 250-mg capsules in an earlier clinical study. Urine samples were collected in 6- or 12-h intervals for a maximum of fourteen days.

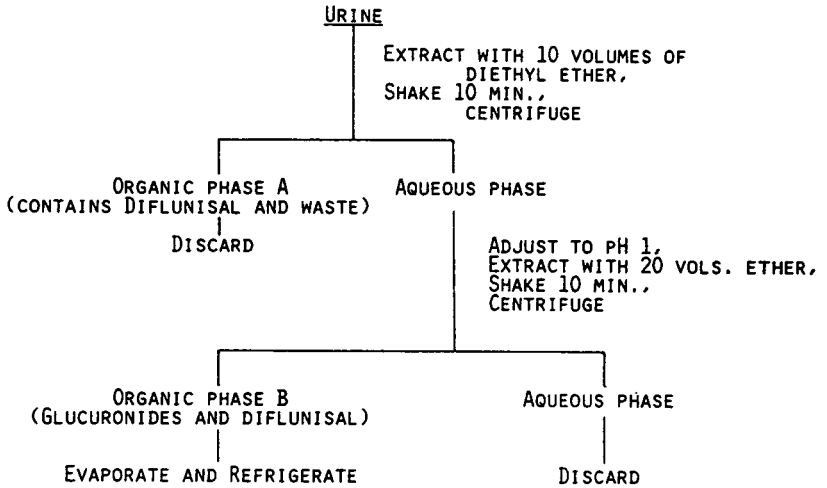


Fig. 2. Flow diagram for the procedure to isolate the two conjugates from urine.

organic phase B′ were further separated on thin-layer chromatography (TLC) or high-performance liquid chromatography (HPLC) (see *HPLC conditions*). On TLC silica gel (250 μm) the components were resolved from each other by the solvent mixture acetone—toluene—*n*-butanol—glacial acetic acid—water (6:6:5:4:4). The relative retentions of the glucuronides on TLC were determined by radioautography: for the EtG, R_F = 0.43; EsG, R_F = 0.57; diflunisal, R_F = 0.94. Authenticity of the EtG and EsG was determined by mass spectrometry (MS) and co-chromatography (TLC and HPLC) with previously reported standards [2].

The radioactive glucuronides of diflunisal used in this investigation as analytical standards were isolated by extraction and TLC from clinical human urine samples*. The isolated standards were not purified further. The amount of glucuronide present in the isolates was determined from their radioactivity (^{14}C) and the specific activity of diflunisal (14.8 μCi per 250 mg). A typical TLC isolate (organic phase B′) was reconstituted in 1.0 ml of acetonitrile, and an aliquot (100 μl) was mixed with 10 ml of scintillation cocktail Aquassure from New England Nuclear and counted for 4.0 min on a Packard Tricarb liquid scintillation spectrometer.

HPLC conditions

Separation and quantification of analytes was performed on a reversed-phase Hypersil ODS column (30 cm × 5 mm I.D.) from Shandon Southern and a guard column Co:Pell ODS from Whatman. Two pumps Model 6000A and a system controller Model 720 from Waters Assoc. performed a linear gradient elution at 2 ml/min. Solvent A consisted of 30% methanol and 70% of dilute aqueous acetic acid (2%). Solvent B consisted of 65% methanol and 35% of dilute acetic acid (2%). The composition of the mobile phase changed from 60% solvent A and 40% B initially to 0% A and 100% B in 25 min. It flowed

*See footnote on p. 364.

at 100% B for an additional 6 min. Re-equilibration of the mobile phase back to 40% of B took 3 min.

Detection and integration involved an ultraviolet (UV) detector Model 441 (fixed-wavelength filter at 254 nm a.u.f.s.) and an integrator Model 730 (0.5 cm/min) from Waters Assoc. For comparison detection by fluorometry using a Schoeffel GM 970 (Fig. 7) was operated at λ_{ex} 215 and λ_{em} 440 nm (range 1.0 μA).

Procedure for quantification of EsG, EtG and free diflunisal in urine with an EtG standard and with the hydrolysis of EsG to diflunisal

Sample preparation. (1) Incubate a 1.0-ml urine sample with 1.0 ml of 0.25 *M* sodium hydroxide at ambient temperature for 1.5 h; (2) a second 1.0-ml aliquot of the above sample is mixed with 1.0 ml of water and 3 drops of glacial acetic acid from a pasteur pipet; (3) after 1.5 h of incubation 3 drops of glacial acetic acid are added to the above sample in step No. 1; (4) the internal standard 5-(4'-fluorophenyl)salicylic acid (30 μg/ml of urine) is added to both above samples in steps Nos. 2 and 3; (5) the above samples are centrifuged (657 *g*) and analyzed by HPLC.

Standards for calibration curves. A 1.0-ml aliquot of control urine is mixed with an appropriate volume of the EtG stock solution and of the diflunisal stock solution. The standard is prepared for analysis as discussed in the above steps Nos. 2—5.

Calculations for the ether, ester and free acid concentrations. Concentrations of the ether conjugate and the free acid of diflunisal are calculated from the chromatogram generated from the pre-hydrolyzed urine aliquot (in step No. 2). Peak area ratios are measured and the concentrations are extrapolated from the above standard curves. Concentration of the ester conjugate is the difference of the diflunisal concentration measured from the hydrolyzed urine aliquot (step No. 1 above) and the prehydrolyzed urine (No. 2). The difference is multiplied by 1.79.

Procedure for quantification of EsG, EtG and free diflunisal in urine without an EtG standard and without EsG hydrolysis

Sample preparation. (1) A 1.0-ml aliquot of a urine sample is mixed with 1.0 ml of water and 3 drops of glacial acetic acid; (2) the internal standard is added, and the sample is mixed, centrifuged and analyzed, as discussed above.

Standards for calibration curves. A 1.0-ml sample of control urine is mixed with an appropriate volume of the diflunisal stock solution. The standard is prepared for analysis as discussed above.

Calculations. The concentration of EtG is calculated from its peak area ratio (EtG/internal standard), a diflunisal standard curve and the molecular weight factor 1.79. The free diflunisal concentration is determined from its peak area ratio and the diflunisal standard curve. The EsG concentration is determined from the sum of the peak areas of EsG and rearrangement products divided by the peak area of the internal standard, a diflunisal curve and 1.79.

Chemical rearrangement

An aliquot (1 ml) from human clinical urine sample* was mixed 1:1 with

*Human clinical urine sample, 6—12 h collection interval, 500-mg dose of unlabeled diflunisal, p.o.

0.2 *M* potassium phosphate buffer, pH 7.5 giving a final pH of 7.4. The sample was placed in an autosampler vial and chromatographed repetitively over time at ambient temperature.

Similarly, an aliquot from the above urine sample was mixed 1:1 with 0.17 *M* potassium acetate buffer, pH 4.0, final pH 4.3. The sample was monitored as above over time.

Enzymatic hydrolysis

An aliquot from a clinical urine sample* was mixed with 0.17 *M* sodium acetate buffer, pH 4.0 (1:1, v/v). An aliquot from the same sample was mixed with the buffer and β-glucuronidase (2 mg/ml). The two samples were incubated at 37°C over 12 h.

Stability studies

(A) An aliquot from a clinical urine sample* was mixed with 1.0 ml of 0.25 *M* sodium hydroxide. An aliquot from the same sample was mixed with 0.17 *M* sodium acetate buffer, pH 4. The two samples were allowed to stand at room temperature over 4.28 h.

(B) The TLC-isolated EtG and EsG of diflunisal were incubated (70 μg/ml and 90 μg/ml, respectively) at ambient temperature in acetonitrile, methanol, ethanol, mobile phase (solvent B) and aqueous buffers at pH 1.19 (hydrochloric acid—potassium chloride buffer, 0.2 *M*), pH 2.77 (citrate—phosphate buffer, 0.1 *M* and 0.2 *M*), pH 4.88 and pH 6.93. The incubates were monitored by HPLC at 3, 6, 9, and 12 h.

(C) Aliquots (1 ml) from a clinical urine sample* were adjusted to pH 4.0, 5.8, 6.9 and 7.6 with a sodium acetate buffer (pH 4.0, 0.17 *M*) and potassium phosphate buffers (pH 5.9, 7.0 and 8.0, 0.2 *M*) (1:1, v/v). The 1-O-acyl glucuronide (89.3 μg/ml) was monitored by HPLC over time at room temperature.

Comparison of ultraviolet and radioactivity chromatograms

A human urine collection sample (6—12 h)** was extracted (organic phase B′, discussed earlier) concentrated and reconstituted in mobile phase. A 30-μl aliquot was injected into the HPLC system and fractions were collected every 15 sec up to 30 min. The 15-sec fractions were mixed with 10 ml of scintillation cocktail and counted for 4.0 min. The UV chromatogram was monitored at 254 nm.

RESULTS AND DISCUSSIONS

The development of an assay for the EtG and EsG glucuronides of diflunisal in urine required their initial isolation [2], characterization and stability studies. Characterization involved co-chromatography by TLC and HPLC of the glucuronide standards isolated by Tocco et al. [2]. Additional mass spectral (MS) data were obtained to substantiate previous results and identify rearrange-

*See footnote on p. 366.
**See footnote on p. 364.

ment products of the EsG. Stability data dictated the direction of the assay development for the EsG. Poor stability of the conjugates suggested the development of an assay without standards.

Stability studies

Room temperature (22—24°C) stability studies of the isolated EtG reveal less than 5% loss over 12 h in all aqueous buffers and organic phases tested, except in neat methanol and ethanol (Tables I and II). The EsG shows similar results except in a pH 6.93 phosphate buffer. The degradation of glucuronide conjugates in neat alcohols has been reported for other drugs [12, 13, 15]. For the ester conjugate, losses may result from nucleophilic substitution during acyl

TABLE I

SOLVENT STABILITY OF THE EtG AND EsG AT ROOM TEMPERATURE

Time (h)	Percent remaining			
	Aceto-nitrile	Mobile phase*	Methanol	Ethanol
EtG				
3	100.0	100.0	99.4	97.2
6	97.6	97.6	94.1	95.0
9	95.2	96.4	96.0	94.1
12	96.4	95.7	92.0	93.0
EsG				
3	98.3	98.3	97.3	94.6
6	98.1	100.0	96.5	93.4
9	97.6	100.1	94.7	91.4
12	93.0	100.1	93.0	91.3

*Solvent B, see Materials and methods.

TABLE II

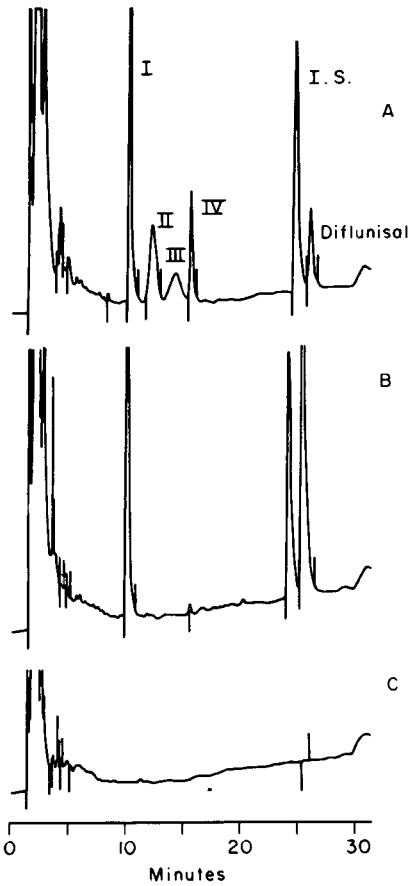
pH STABILITY OF THE EtG AND EsG AT ROOM TEMPERATURE

Time (h)	Percent remaining			
	pH* 1.19	pH 2.77	pH 4.88	pH 6.93
EtG				
3	97.0	98.1	100.0	100.0
6	97.0	100.0	100.0	96.8
9	96.0	100.0	98.6	97.6
12	98.2	100.0	96.4	95.6
EsG				
3	99.0	97.3	100.0	74.0
6	97.2	97.6	100.0	48.0
9	97.2	100.0	98.0	39.0
12	97.5	97.8	95.0	24.0

*pH of aqueous buffers; see Materials and methods.

rearrangement. In the mobile phase, however, which contains methanol, acetic acid and water (solvent B, pH 3.4) observed losses are less than 5%.

In pH 6.93 phosphate buffer only 24% of the EsG remains after 12 h. In urine with pH adjustments, the half-life of the EsG changes dramatically from 300 h at pH 4.0 to 39.5 min at pH 7.6, respectively, at room temperature. At pH 5.8 the half-life is 37.5 h, and at pH 6.9 it is 2.42 h. Without pH adjustment of the above urine matrix (pH 5.2—5.3), 23% of the EsG is lost over 43 h (89.3 μg/ml). The extrapolated half-life is 135 h. Over the same period with addition of citric acid (50 mg/ml) or glacial acetic acid (3 drops/ml), less than 5% is lost.



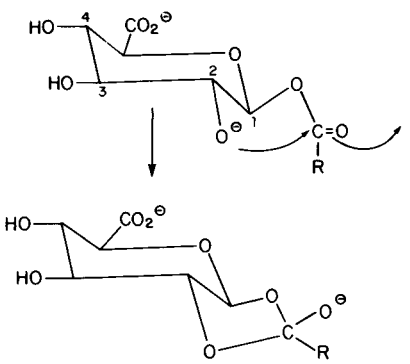


Fig. 3. (A) Chromatogram (before hydrolysis) of a urinary clinical sample containing the EtG of diflunisal (I), EsG rearrangement products (II and III), 1-O-acyl-glucuronide (IV), internal standard and diflunisal. The ether conjugate and free diflunisal are quantitated from this sample. (B) Chromatogram of the above urine sample subjected to alkaline hydrolysis. It contains the ether conjugate (I), internal standard and diflunisal. The ester conjugate is quantitated from this diflunisal peak minus the free diflunisal concentration (from A). (C) Chromatogram of a urine blank before hydrolysis.

Fig. 4. 1,2-*Ortho* acid ester intermediate, proposed mechanism of ester rearrangement [16].

Characterization of the conjugates

The EtG and EsG of diflunisal have been previously isolated from human urine by TLC and characterized by MS [2]. The isolation techniques have been slightly modified (see flow diagram, Fig. 2) resulting in a procedure that clearly separates the EtG and EsG and free diflunisal from urine and each other (TLC: R_F = 0.43, 0.57 and 0.94, respectively). HPLC methodology has been developed that similarly separates the above components (t_R = 10.1, 15.4 and 25.8 min, respectively). Co-chromatography of the standards isolated by Tocco et al. [2] in the above systems supports our assignment of the TLC spots and HPLC peaks. In addition, there exist in the chromatograms (Fig. 3A) of human urine samples from volunteers administered diflunisal other HPLC peaks between the ether and ester conjugates that did not exist in the predose samples (Fig. 3C). Reports from the literature of EsG rearrangements occurring

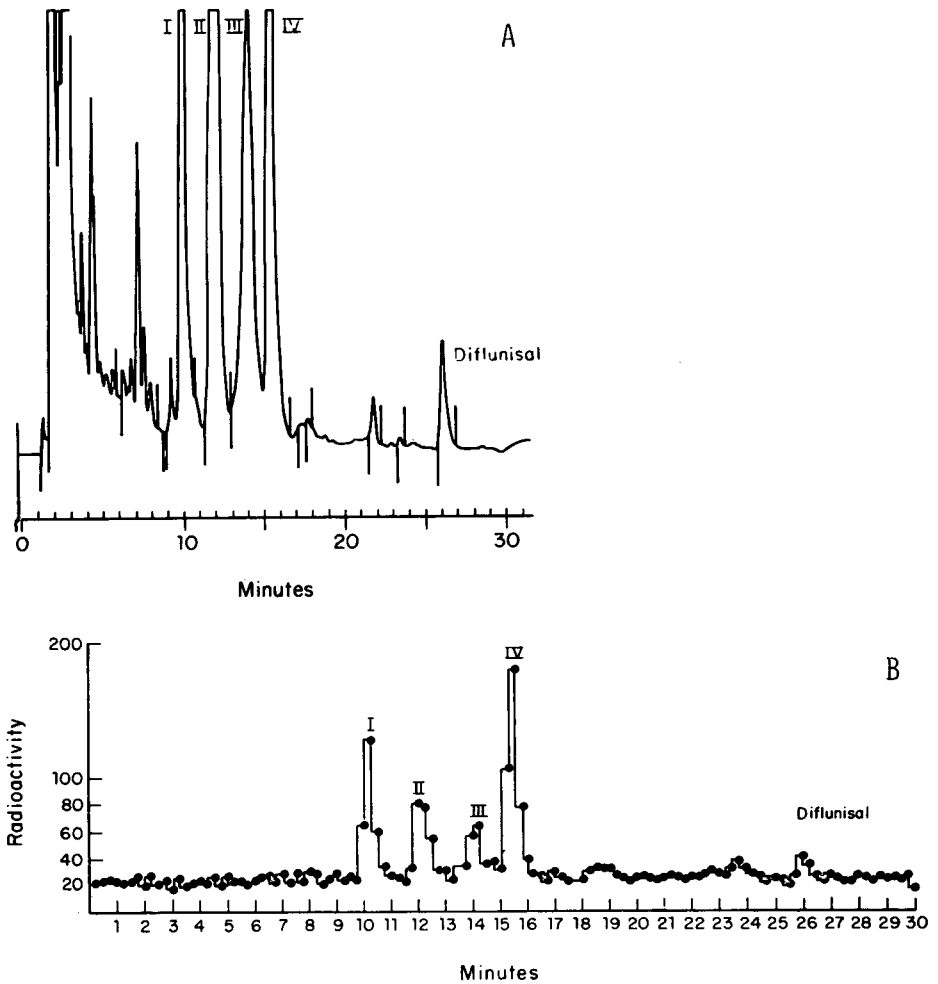


Fig. 5. UV chromatogram (A) versus a radioactivity chromatogram (B) of a human urine collection (6—12 h) post dose (a 250-mg, oral administration of radiolabeled diflunisal). Peaks: 1 = ether conjugate, IV = ester, II & III = ester rearrangements.

with other drug conjugates [12, 13] suggest a similar phenomenon may be happening for the ester conjugate of diflunisal. The 1-O-acyl conjugate would pass through a 1,2-*ortho* acid ester intermediate [13, 16] going to 2-, 3- and 4-O-esters of glucuronic acid (Fig. 4).

Confirmation of the rearrangement products of diflunisal in the urine of volunteers given radiolabeled diflunisal is depicted in a close comparison of a UV chromatogram and a radioactivity chromatogram (Fig. 5). The residue of a human urine extract (organic phase B′) is chromatographed with 15-sec eluent fractions collected and counted. The chromatographic peaks in Fig. 5 are denoted (henceforth) as I, II, III, and IV. I represents the EtG and IV represents the EsG, isolated and identified by Tocco et al. [2]. II and III represent the acyl rearrangements of IV.

The isomerization is further supported by the observation that the loss of the ester peak (IV) corresponds to the appearance of two broad peaks (II and III) overtime (Fig. 6) in a human urine sample. The loss of IV seems to be irreversible to II and III with slow hydrolysis to diflunisal.

Additionally, the literature [12, 13, 17] reports that only the 1-O-acyl

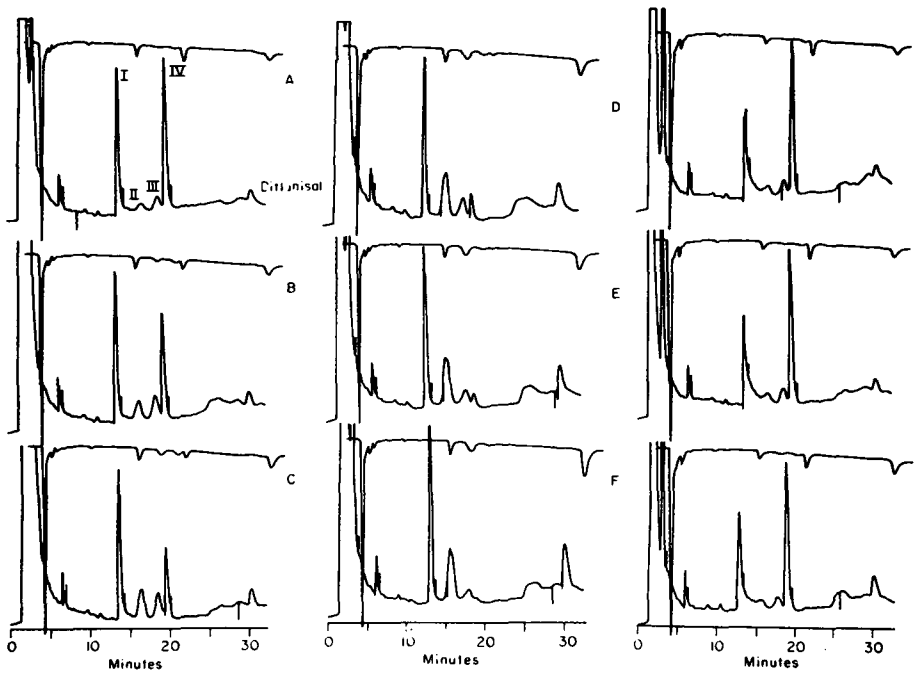


Fig. 6. Chemical rearrangement and hydrolysis of the EsG. (A) Chromatogram of a clinical human urine sample at pH 7.4 (0.2 *M* potassium phosphate buffer at pH 7.5, and urine, 1:1, v/v) at T = 0 min at room temperature; (B) chromatogram of the same sample at T = 77 min; (C) T = 154 min; (D) T = 353 min; (E) T = 505 min; (F) T = 1360 min; (G) chromatogram of the above sample at T = 0 min, pH 4.3 (0.17 *M* sodium acetate buffer at pH 4.0, and urine, 1:1, v/v) at room temperature; (H) T = 78 min; (I) T = 1323 min. The 1-O-acyl glucuronide (IV) rearranges to peaks II and III. The ester isomers slowly hydrolyze to diflunisal. The top tracing accompanying each UV chromatogram is the fluorometer response. The comparison shows similar detector response for diflunisal, but much less for the glucuronide conjugates.

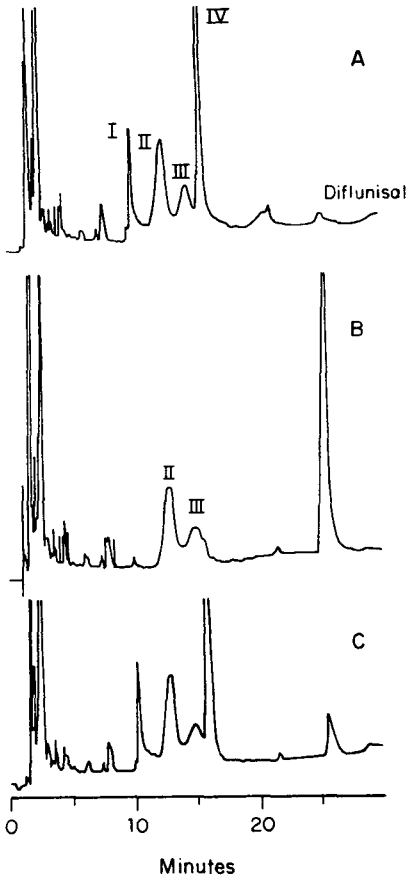


Fig. 7. Enzymatic hydrolysis of the EsG and EtG by β-glucuronidase. (A) Chromatogram of a clinical human urine sample, at pH 4.3 (0.17 *M* sodium acetate buffer at pH 4.0, and urine, 1:1, v/v); (B) chromatogram of the above sample incubated over 12 h at 37°C with β-glucuronidase; (C) chromatogram of the above sample incubated without β-glucuronidase over 12 h at 37°C. The study suggests (supports previous findings [17]) that peaks I and IV are naturally forming glucuronides of diflunisal. Peaks II and III (may be peak clusters) are rearrangements of the ester conjugate (migration of diflunisal from C_1 to C_2, C_3, C_4 on the sugar moiety).

glucuronides can be enzymatically hydrolyzed. The enzymatic hydrolysis was performed on a clinical sample at pH 4.0 containing 1—IV (Fig. 7). Only peaks I and IV completely disappear within an hour. A control at pH 4.0 incubated without β-glucuronidase is not affected. The results suggest peaks I and IV are C_1-O-conjugates and II and III are rearrangements of IV. Since the samples are incubated for 24 h at 37°C and there is no loss of II and III, the migration of diflunisal from C_1 to C_2, C_3 and C_4 appears to be irreversible. Peaks II and III are broader than I and IV and suggest the presence of multiple isomers ($\geqslant 2$) of IV (2-, 3- and 4-O-esters of glucuronic acid).

Final evidence for peaks II and III involved MS analysis following their isolation by HPLC from organic phase B′ and concentration by high vacuum to residues. Electron-impact mass spectra of underivatized II, III and IV gave

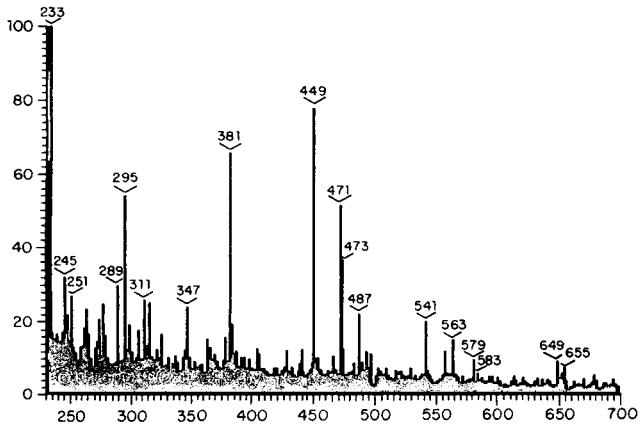


Fig. 8. Fast atom bombardment mass spectrum of IV. HPLC peaks II—IV were collected individually and concentrated under vacuum to a residue. The residue was analyzed by electron-impact and FAB MS.

identical mass fragmentation ions. Fast atom bombardment (FAB) MS (MS analysis was performed on a Varian Mat 731 mass spectrometer with a FAB ion source using a glycerol matrix) gave mass ions at *m/z* 427, 449, 471 and 487 which correspond to the molecular weight plus one (M + H), molecular weight plus sodium (M + Na), M + Na_2 and M + Na + K for II, III and IV, respectively (Fig. 8).

Assay methodology

During the development of an assay for the glucuronides three approaches were considered: (a) the use of the isolated EtG and EsG (1-O-acyl) as standards (as described in the Materials and methods); (b) the use of the isolated EtG only as a standard and hydrolyzing the EsG to diflunisal; and (c) calibrating the ether and ester peak areas (ratios) against a diflunisal standard curve. The first approach was abandoned because of the ester's vulnerability to hydrolysis and rearrangement (human clinical samples contained II and III).

TABLE III

STABILITY OF DIFLUNISAL'S CONJUGATES IN DILUTE BASE

Human clinical sample (6—12 h after dose) is mixed with 0.25 *M* sodium hydroxide (1:1).

Time (h)	Percent remaining				
	I	II	III	IV	Diflunisal
0*	100	100	100	100	100
0.12	98.4	106.8	112.5	26.4	152.7
0.73	96.2	36.0	25.0	7.1	409.1
1.35	98.4	18.0	0	0	490.9
2.08	100.7	0	0	0	518.2
2.82	96.2	0	0	0	463.6
4.28	96.2	0	0	0	477.3

*Clinical sample (above sample) mixed with 0.17 *M* sodium acetate buffer, pH 4.0.

The second was developed and applied to samples. The third was compared to the second.

The second approach involves the hydrolysis of the ester IV and the rearrangement products to diflunisal without affecting the EtG. The effects of 0.125 *M* sodium hydroxide on the five components in urine is summarized in Table III. By 1.5 h the ether conjugate has not hydrolyzed and peaks II—IV are completely gone. Also, the area ratio of the summation of peaks II—IV to I before hydrolysis and the area ratio of diflunisal with I after hydrolysis of the same samples are 1.177 ± 0.117 (n = 3) and 1.179 ± 0.036 (n = 3), respectively. A ratio of the above two numbers 0.9983 points to equal molar absorptivity for the EsGs (peaks II, III and IV) and diflunisal. Since the assay for the EsG involves quantitating diflunisal before and after hydrolysis, the integrity of diflunisal in 0.125 *M* sodium hydroxide was tested further in urine. Less than 3% degradation was observed over 9.6 h.

A typical urine sample collection would be split: one aliquot would be treated with 0.25 *M* sodium hydroxide (Fig. 3B); the second with glacial acetic acid or citric acid (Fig. 3A). After 1.5 h the alkaline aliquots would be treated with acid. The internal standard would be added at this stage and particulates centrifuged. A chromatogram of the second aliquot would yield data on the EtG and free diflunisal. The first would give a diflunisal concentration consisting of the ester equivalent and free diflunisal. The EsG concentration is the difference times a molecular weight factor. A standard calibration curve would consist of the EtG, diflunisal and internal standard. Sensitivity, precision and linearity are presented in Table IV. Urines from two subjects who received 500 mg of diflunisal orally were analyzed by the second method. The EtG and EsG concentrations and free diflunisal are listed in Table V. Accumulative

TABLE IV

ASSAY CHARACTERIZATION

Analyte	Minimum detection limit (μg/ml)	Precision		Linear regression analysis
		Concentration (μg/ml)	Coefficient of variation (%)	
EtG*	0.5	1.88	6.0	r = 0.9978
		3.77	6.8	$y = 0.218x - 0.010$
		18.9	5.2	
		70.3	3.7	
EsG**	1.0	3.2	8.0	r = 0.9994
		6.4	8.1	$y = 0.0324x - 0.010$
		32.0	3.14	
Diflunisal	0.5	1.7	5.8	r = 0.9966
		8.5	7.4	$y = 0.0512x - 0.0645$
		34.0	4.9	

*Based on standards containing the isolated ether conjugate. The concentration of the ether is calculated from the specific activity of the labeled conjugate.

**Based on standards containing the hydrolyzed ester conjugate. The concentration of the ester is caluclated from the specific activity of the labeled conjugate.

TABLE V

REPRESENTATIVE HUMAN CONCENTRATION LEVELS (μg/ml) OF THE EsG AND EtG OF DIFLUNISAL AND FREE DIFLUNISAL

Subjects received a single oral dose of 500 mg of diflunisal.

Time interval (h)	EtG based on standards	EtG based on diflunisal*	EsG based on diflunisal after hydrolysis	EsG based on diflunisal before hydrolysis**	Free diflunisal
Subject 1					
0	0	0	0	0	0
0—3	200.7	200.1	446.8	388.3	32.3
3—12	458.2	459.4	560.1	547.6	62.6
12—24	199.4	199.3	144.1	144.6	19.7
24—36	81.0	82.1	119.6	121.2	14.2
36—48	10.2	12.1	15.9	19.8	0
48—72	6.15	8.0	13.8	11.49	0
72—96	0	0	0	0	0
Subject 2					
0	0	0	0	0	0
0—3	137.3	146.1	243.9	251.9	13.2
3—6	78.7	83.4	149.8	121.7	7.11
6—12	53.5	56.6	89.2	70.9	7.68
12—14	124.6	132.5	146.1	111.70	12.2
24—36	19.6	20.4	29.5	28.3	3.19
36—48	30.7	32.2	51.0	49.1	0
48—72	7.83	7.74	12.1	11.8	0
72—96	0	0	0	0	0

*Concentrations are determined from peak area ratios of the ether, a diflunisal calibration curve and a conversion factor (1.79).

**Concentrations are determined from the peak area ratios of the ester peaks (includes rearrangements), a diflunisal curve and a conversion factor.

urinary levels of subject 2 are plotted in Fig. 9. Dose recovery is 95.7% for subject 1 and 77.7% for subject 2.

The third approach for the assay methodology involves extrapolating a conjugate concentration (with an appropriate peak area ratio) from a diflunisal curve and a molecular weight factor. From the area of peak I, the EtG concentration is determined; from the sum of the areas of peaks II—IV, the ester is determined. The approach requires the chromatography of only one stabilized urine sample. No standards are needed. The methodology (e.g. relative response factors, linearity, etc.) has been evaluated with the EtG initially by comparing concentrations of prepared standards (see Materials and methods) with respective concentrations calculated from the peak area ratios of the EsG standards, a diflunisal standard curve and 1.79 (Table VI). The maximum difference over the linear concentration range 1.88—70.3 μg/ml is 27.7% at 1.88 μg/ml. The average ratio of standard concentrations (based on radioactivity) to extrapolated over the concentration range is 1.005 ± 0.156. Table V compares clinical sample concentrations of subjects 1 and 2 (discussed

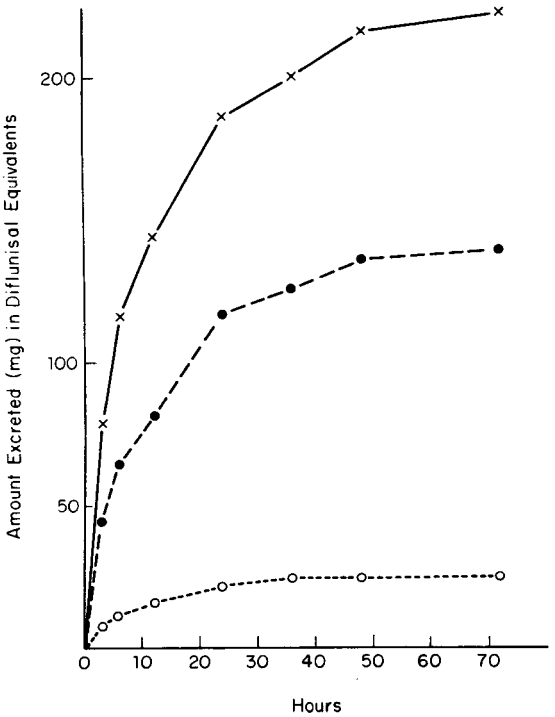


Fig. 9. Accumulative urinary levels of the ether and ester conjugate and free diflunisal of a single subject given a 500-mg oral dose of diflunisal. (•) EtG; (×) EsG; (○) free diflunisal.

TABLE VI

EtG: A COMPARISON CONCENTRATIONS BASED ON RADIOACTIVITY AND ON DIFLUNISAL

x: Concentration based on radioactivity* (μg/ml)	y: Concentration based on diflunisal** (μg/ml)	y/x
1.88	2.4	1.28
3.77	3.86	1.02
9.43	7.75	0.82
18.9	17.6	0.93
37.7	35.4	0.94
70.3	73.4	1.04
		Mean ± S.D.: 1.005 ± 0.156

$r^2 = 0.9978$
$y = 0.0218x—0.010$

*Standards were prepared with the isolated, ^{14}C-labeled ether conjugate.
**The concentrations were extrapolated from the peak areas of the ether standards, a diflunisal calibration curve and the molecular weight factor 1.79.

earlier) based on an EtG standard and on a diflunisal calibration curve. The average concentration difference of both approaches over all time intervals for both subjects is 6.6 ± 8.6%.

A similar evaluation has been made of the EsG assay over the concentration range 2.4—96.2 μg/ml. The average ratio of standard concentrations (based on radioactivity) to extrapolated before hydrolysis (Table VII) is 1.15 ± 0.4. Concentrations extrapolated after hydrolysis have an average ratio of 0.99 ± 0.09.

TABLE VII

EsG: A COMPARISON OF CONCENTRATIONS BASED ON RADIOACTIVITY AND ON DIFLUNISAL

x: Concentration based on radioactivity*	y: Concentration based on diflunisal before hydrolysis**	y/x	z: Concentration based on diflunisal after hydrolysis***	z/x
2.4	4.7	1.96	2.41	1.0
4.81	5.8	1.21	5.44	1.13
12.0	11.4	0.95	11.9	0.99
24.0	22.6	0.94	21.0	0.87
48.0	45.2	0.94	44.8	0.93
96.2	84.2	0.88	95.5	0.99
Mean ± S.D.		1.15 ± 0.40		0.99 ± 0.09

*Standards were prepared with the isolated, labeled EsG.
**The concentrations were extrapolated from the sum of the areas of peaks II—IV, a diflunisal curve and the conversion factor 1.79.
***The concentrations were extrapolated from the areas of diflunisal peaks after hydrolysis, a diflunisal curve conversion and the factor 1.79.

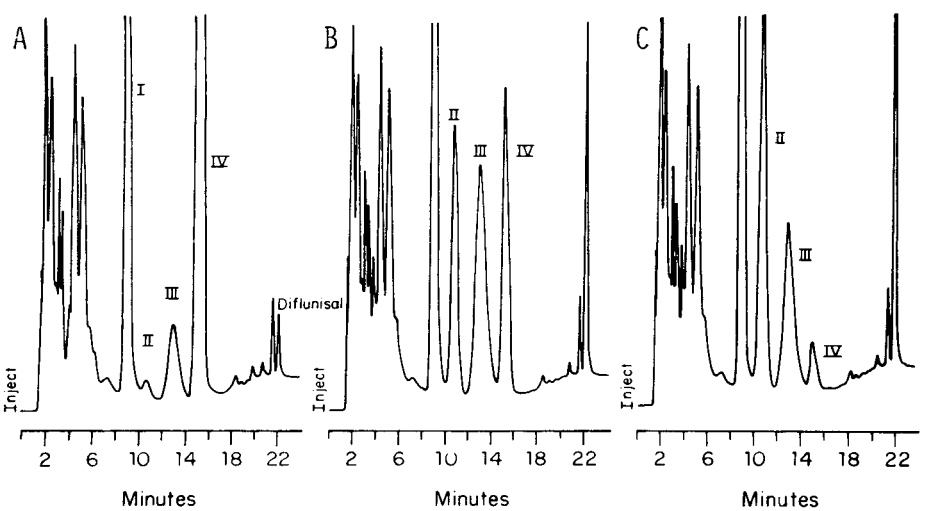


Fig. 10. Rearrangement of the 1-O-acyl glucuronide of diflunisal in rat bile. Chromatograms of (A) a rat bile sample, injected into the HPLC 5 min after collection; (B) the same sample injected 1 h later; (C) the same sample injected 2 h later. The pH of this sample is 6.8.

Table V compares clinical sample concentrations of the EsG based on diflunisal after hydrolysis and on diflunisal before hydrolysis. The average concentration difference for both subjects is 10.3 ± 9.3%.

The preferred assay should be the latter discussed above. However, if the rearrangement products of the EsG exist in the urine samples, summing the areas of peaks II—IV for the EsG may compromise the assays minimum sensitivity and its accuracy at the lower concentrations. A combination of parts of the second and third approach might be more appropriate. The EtG concentration would be extrapolated from a diflunisal curve and the EsG would be hydrolyzed to diflunisal for quantification. The rearrangement of the 1-O-acyl glucuronide conjugate in general and of diflunisal in particular has not been determined to be exclusively a chemical isomerization (versus enzymatic). Although stabilization of diflunisal's 1-O-acyl glucuronide in urine after collection can be accomplished with acid treatment, stabilization of the biological samples before (in vivo) acyl rearrangement would not be possible. The rearrangement products are observed in the urine and bile of rats administered diflunisal (30 mg/kg intravenously) collected in the presence of acetic acid. The acyl rearrangement occurs quickly in rat bile (pH 6.8) not treated with acid (Fig. 10). With the acid, there is no change over the same time period.

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Note

Total oestriol in maternal serum or plasma as measured by liquid chromatography

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Although several authors [1—3] consider that the level of unconjugated oestriol in serum is the most reliable biochemical indicator of foetal well-being, the assay of total oestriol in serum is still the parameter most widely used. Moreover, the assay of total oestriol can be more useful than that of unconjugated oestriol in the management of some pathological pregnancies [4].

Recently, we reported a procedure involving high-performance liquid chromatography (HPLC) with fluorimetric detection for determining unconjugated oestriol in pregnancy serum or plasma [5].

We describe here a rapid procedure for the assay of total oestriol in serum or plasma from pregnant women, based on enzymatic hydrolysis with purified *Helix pomatia* juice, extraction of oestriol with Carbopack B and quantification by HPLC with fluorimetric detection. Our method is as sensitive and accurate as the radioimmunoassay (RIA) method, and has the advantages of being more economical and avoiding manipulation of hazardous materials.

MATERIALS AND METHODS

Instrumentation

A Series 3B liquid chromatograph equipped with a Model 650 S LC

0378-4347/85/$03.30

fluorescence detector having a 20-μl flow cell, and a Rheodyne Model 7125 injector with a 20-μl loop (all from Perkin-Elmer, Norwalk, CT, U.S.A.) was used. The column was 25 cm × 4.6 mm, filled with 5-μm (average particle size) C_{18} reversed-phase packing and protected by a Pelliguard guard column (all from Supelco, Bellefonte, PA, U.S.A.). A mixture of acetonitrile—phosphate buffer, pH 6.2 (24:76, v/v) at a flow-rate of 1.5 ml/min was used as mobile phase. Fluorescence detection was used with excitation at 280 nm, emission at 308 nm, and slit widths of 12 nm.

Reagents

Stock oestriol, oestriol 16-glucuronide ($E_3$16-G) and oestriol 3-sulphate ($E_3$3-S) standards (all from Sigma) were dissolved in methanol to give a concentration of 1 g/l, expressed as oestriol. These standards were diluted with methanol to give 20, 10, 5, and 1 mg/l working standards. To prepare the working plasma or serum standards, 100 μl of either $E_3$16-G or $E_3$3-S working standards were evaporated and the residue was reconstituted in 10 ml of oestriol-free fresh serum or plasma. These standards were stable for at least fifteen days if kept at 4°C.

Working phosphate buffer, pH 6.2, was prepared by diluting a stock solution ten fold, which was prepared by dissolving 27.6 g of $NaH_2PO_4 \cdot H_2O$ in 1 l of freshly distilled water and adjusting the pH to 6.2 with 1 mol/l potassium hydroxide. The stock solution was stable at 4°C for at least one month.

Unpurified *Helix pomatia* juice containing (per ml) 120,000 Fishman units of β-glucuronidase (EC 3.2.1.31) and 3,430,000 Roy units of arylsulphatase (EC 3.1.6.1) was from Merck (Darmstadt, F.R.G.).

Acetonitrile, chromatography grade, was obtained from Carlo Erba. All other solvents (Carlo Erba) were of analytical grade and were distilled in a glass system before use. Carbopack B (80—120 mesh) was kindly supplied by Supelco.

Procedure

Enzyme purification. The Carbopack B column was prepared by pouring 0.25 g of the adsorbent into water and then introducing the suspension into a 15 × 0.6 cm glass column with a small pledget of glass wool in the bottom. One ampoule (2 ml) of crude *Helix pomatia* extract was diluted with 5 ml of 0.5 mol/l acetate buffer, pH 4.6, and passed through the column followed by 3 ml of acetate buffer. The effluent was collected and stored at 4°C. This enzyme preparation is stable at least for fifteen days.

We determined the activities of the purified enzymes relative to those of the untreated ones. Both untreated and treated enzyme preparations were diluted 1000-fold with acetate buffer and 50 μl of these solutions were used to partially hydrolyse 0.5 μg of $E_3$16-G at 37°C for 30 min. The same procedure was used for 1 μg of $E_3$3-S. After that, 4 ml of the mobile phase were added and 20 μl were injected into the chromatograph. In all cases, the efficiency of hydrolysis was assessed by measurement of the peak height of the free oestriol.

Hydrolysis and sample purification. A 600-μl volume of 0.5 mol/l acetate buffer, pH 4.6, and 200 μl of the buffered and purified enzyme preparation were added to 100 μl of serum or plasma. After mixing gently, the mixture was

incubated at 60°C for 30 min. The hydrolysate was diluted with 9 ml of 10 mmol/l hydrochloric acid and passed through a Carbopack column prepared in the same way as described for the enzyme purification. The vial was rinsed successively with two 2.5-ml portions of aqueous 3 mmol/l hydrochloric acid and the rinsings passed through the column. The column was washed with 10 ml of 0.3 mol/l formic acid in methanol, followed by 3 ml of chloroform—methanol (15:85, v/v). The oestriol was eluted with chloroform—methanol (60:40, v/v) and 3 ml of the eluate were collected, starting from the moment the eluting solution was applied to the column. The solution was evaporated under a stream of nitrogen at 60°C. The residue was reconstituted in 60 μl of the solution used as mobile phase and 20 μl were injected into the chromatograph. The total oestriol concentration of standard and patient samples was calculated by comparing the height of the peak produced by oestriol in the sample with that of an authentic oestriol standard. The latter is prepared for chromatography by evaporating 10 μl of one of the unconjugated oestriol working standard solutions and reconstituting with 60 μl of the mobile phase. The response of the fluorimetric detector is linearly related to amount of injected oestriol within the range 0.08—30 ng.

RESULTS AND DISCUSSION

Enzyme purification

The hydrolysis of the oestriol conjugates is the critical point in this assay. The crude solution from *Helix pomatia* contains a lot of organic impurities which are not completely eliminated by the isolation procedure of the oestriol freed by hydrolysis. These compounds produce a number of chromatographic peaks, some obscuring that of the oestriol. Neither the use of purified *Helix pomatia* extracts commercially available nor a purification procedure involving the use of XAD-2 [6] gave satisfactory results.

The procedure elaborated by us, which exploits removal of organic impurities from the extract by adsorption onto the Carbopack B surface, completely eliminates any positive bias in the analysis of total oestriol. Under the experimental conditions selected, repeated (n = 6 in each case) activity tests showed no loss of the β-glucuronidase activity, while only a 6% loss of arylsulphatase activity occurred owing to the purification procedure. Fig. 1 shows typical chromatograms of serum samples hydrolysed with the purified enzyme preparation and carried through the procedure.

The amount of Carbopack B and the preliminary dilution ratio of the *Helix pomatia* juice with acetate buffer are both critical parameters in the purification procedure. We observed a significant loss of enzymic activity by increasing the amount of the adsorbing material or the dilution ratio. This loss can be explained by assuming that the adsorption rate of complex large molecules such as proteins is much lower than that of simpler molecules. Therefore, the enzyme passes unretained through the column provided that the percolation time is made short enough. On the other hand, insufficient enzyme purification was noted when the enzyme preparation was passed undiluted through the Carbopack column, probably because impurities bound to enzymes via non-specific interactions are not retained by the Carbopack column. Dilution of the enzyme preparation has the effect of weakening these interactions.

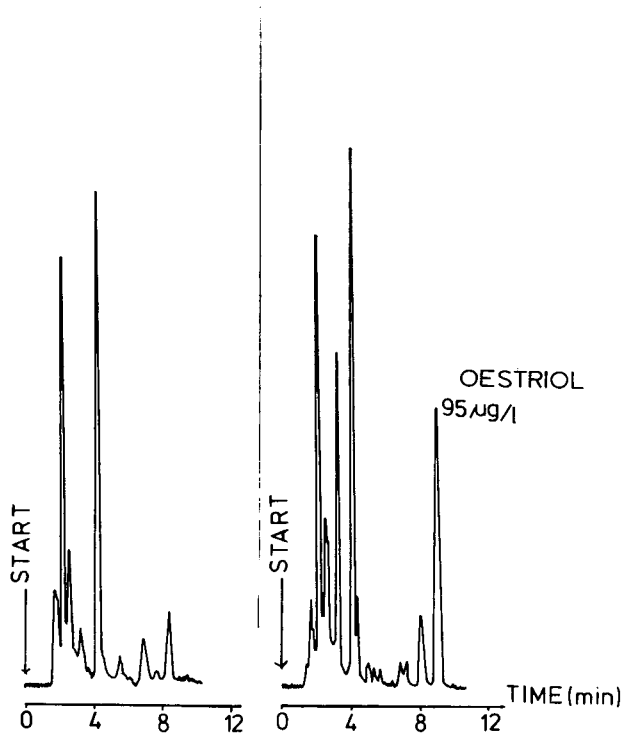

Fig. 1. Chromatograms of purified plasma from a non-pregnant woman (left) and from a pregnant woman at 34 weeks of gestation (right).

Recovery and precision

We assessed the analytical recovery of oestriol and the within-run precision of the method by adding increasing amounts of authentic $E_3$16-G and $E_3$3-S to oestriol-free pooled serum (Table I). The average recovery in the concentration we considered was 92.9%. Each serum sample was assayed eight times during a month. The day-to-day C.V. ranged from 4.0% at 20 µg/l to 2.3% at 400 µg/l.

Sensitivity

The limit of sensitivity (signal-to-noise ratio = 3) was set at 5 µg/l of serum. At this concentration the C.V. was 7.9%.

Specificity

The washing of the Carbopack B column with 10 ml of acidified methanol followed by 3 ml of chloroform—methanol (15:85, v/v) removed residual impurities in the enzyme preparation. The analytical procedure adopted has the same effectiveness in eliminating interfering compounds which may be present in a serum sample as that reported for unconjugated oestriol [5]. Moreover, we observed that either plasma or serum can be used for this assay. EDTA was used as anticoagulant.

TABLE I

RESULTS OF NINE REPLICATE ANALYSES OF OESTRIOL CONJUGATES ADDED TO POOLED SERUM

Added* (μg/l)	Found (μg/l, mean ± S.D.)	C.V. (%)	Recovery (%)	
			Mean	Range
20	18.6 ± 0.73	3.9	93.0	89.1—96.6
100	92.1 ± 2.7	2.9	92.1	89.6—95.6
200	185 ± 5	2.7	92.5	90.5—95.7
400	375 ± 9	2.3	93.8	91.3—96.1

*Data are expressed as oestriol.

Method comparison

Serum samples from 40 patients in their last trimester of pregnancy were analysed by our method and by a commercially available total oestriol RIA kit [Amerlex oestriol (total) RIA kit, Amersham International, U.K.]. Linear regression analysis of data obtained by our HPLC procedure (y) and by the RIA method (x) gave the following results: slope = 0.942, intercept = —0.318 μg/l, r = 0.966. The mean of the HPLC values was 102 (S.D. 74.2) μg/l; the mean of the RIA values was 108 (S.D. 76.1) μg/l.

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Note

Simple and fast analysis of adrenaline and noradrenaline in plasma on microbore high-performance liquid chromatography columns using fluorimetric detection

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The concentration of catecholamines in body fluids can provide valuable information about certain metabolic disorders in man associated with hypertension [1], psychiatric syndromes [2] and adrenal or neuronal tumours [3]. Therefore there is a need for a fast and sensitive method to analyse these compounds in body fluids, preferably in plasma.

Among available techniques such as fluorimetry [4], gas chromatography [5, 6] and radioimmunoassay [7], high-performance liquid chromatography (HPLC) combined with electrochemical [8, 9] or fluorimetric detection [10] has shown to be eminently suited for this analytical problem. However, in our hands, with HPLC until now reliable results could be only obtained with samples containing relatively large catecholamine concentrations as found in urine or tissues. With plasma samples, in which the common catecholamine concentrations are very low and moreover the available sample quantity is limited, a large scattering in the results is found.

The analysis in plasma has to be performed close to the detection limit (i.e. small signal-to-noise ratio). This is a typical case of limitation in sample amount and in combination with a concentration sensitive detection for which small-bore columns offer improved detectability:

$$c_i^{max} = \frac{Q_i}{\sqrt{2\pi}\,\epsilon_m\, A_s\,(1 + k'_i)\,\sqrt{LH_i}} \qquad (1)$$

where c_i^{max} = the maximal concentration (the peak height) of a solute at the end of the column,

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Q_i = amount of solute injected (injection volume × concentration of the solute in the sample),
ϵ_m = mobile phase porosity,
A_s = cross-sectional area of the column,
L = length of the column,
H_i = theoretical plate height.

According to eqn. 1, the detectability will be improved, at constant injected amount of solute, by decreasing the cross-sectional area of the column, the length of the column and the theoretical plate height (the capacity ratio k' has usually already been optimized). This points to short small-bore columns filled with small particles, providing the contribution of the external peak broadening caused by the injection and detection system can be kept small.

In the present study we report the results of an investigation into the applicability of 200 × 1.2 mm I.D. columns for the analysis of catecholamines in 0.5—1 ml of plasma, using on-column concentration and fluorimetric detection. Fluorimetric detection was chosen instead of electrochemical detection, which is quite suitable to combine with microbore columns, because of its greater selectivity which simplifies the sample pretreatment.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a high-pressure pump (Eldex Type B 100-5-2, Menlo Park, CA, U.S.A.), a flow-through manometer acting as a pulse dampener, a high-pressure sampling valve (Rheodyne 7125, Berkeley, CA, U.S.A.), and a fluorimeter (Perkin Elmer, LS-4, U.S.A.) equipped with a 3-μl cell. The wavelengths were set at 278 nm (excitation) and 317 nm (emission). The columns were of 316 stainless steel with dimensions of 200 × 1.2 mm I.D. The inner surface of the tubes was extensively polished before packing [11]. The columns were directly fitted into the injection valve in order to minimize extra peak broadening.

Materials

All solvents were of analytical grade and used without any pretreatment. For the liquid—liquid extraction of the catecholamines (see ref. 9) the quality of *n*-octanol was found to be of crucial importance in order to obtain high recoveries and clean extracts. In this respect, excellent results were obtained with *n*-octanol p.a. from Riedel-De Hean (F.R.G.).

The reversed-phase support was Hypersil ODS 5 μm (Shandon, U.K.).

Procedures

Chromatography. The microbore columns were packed by a slurry technique [11], at constant flow-rate (1.2 ml/min) and a maximum pressure of 600 bars. The slurry liquid consisted of tetrachloromethane + 5% (v/v) methanol and the displacer liquid was pure methanol. The columns were washed with 10 ml of methanol and then equilibrated with the mobile phase. The mobile phase consisted of water—methanol (3:1, v/v) containing 0.15 *M* acetic acid buffer (pH 4.7) 0.04—0.06% (w/v) sodium dodecyl sulphate, 0.01% (w/v) EDTA and 0.01% (w/v) sodium chloride.

Extraction. For the isolation of adrenaline and noradrenaline from plasma, use was made of a selective liquid—liquid extraction system [9]: 0.5—1 ml of plasma was mixed with 0.5 ml of a 2 *M* NH_4OH—NH_4Cl buffer of pH 8 containing 0.2% (w/v) diphenylborate—ethanolamine (DPBEA) and 0.5% (w/v) EDTA. To this mixture 2.5 ml of the extraction solvent, consisting of *n*-heptane containing 1% (w/v) *n*-octanol and 0.25% (w/v) tetraoctylammonium bromide, were added, intensively mixed for 2 min and centrifuged for 5 min at 1200 *g*. Then 2 ml of the organic phase were transferred into a conically shaped glass tube, and 1 ml of *n*-octanol and 0.125 ml of 0.08 *M* acetic acid added. After intensively mixing for 2 min and centrifugation for 5 min at 1200 *g*, 5—100 µl of the aqueous layer were injected into the HLPC system.

It must be noted that the pH of the NH_4OH—NH_4Cl buffer should not exceed a value of 8, as at higher pH the methylated catecholamines, metanephrine and normetanephrine, present in plasma can be partly converted into adrenaline and noradrenaline and thus give rise to misleading results.

RESULTS AND DISCUSSION

Chromatography

The efficiency of the microbore columns was tested with a chromatographic set-up with negligible external peak broadening [11] using methanol—water (8:2, v/v) as the mobile phase and toluene ($k' = 2$) as the solute. Under these experimental conditions the plate number varied between 15,000 and 17,000, at a linear velocity of 1 mm/sec. This corresponds to a reduced plate height of about 2.5. When installing the microbore column in the chromatographic set-up for the catecholamines, the observed plate number dropped to about 8000. This loss can be attributed to the significantly greater external peak broadening

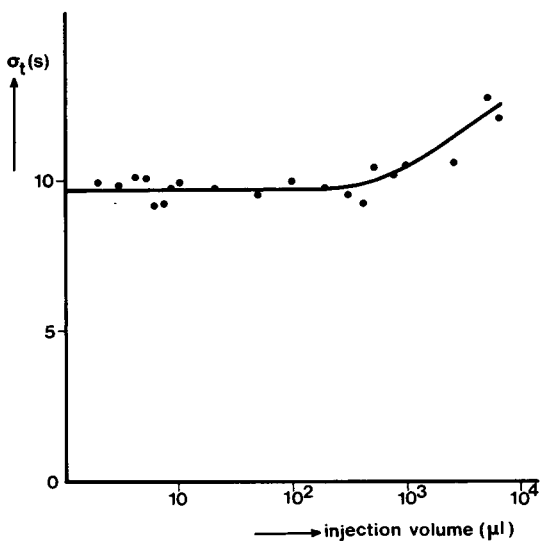

Fig. 1. The standard deviation of the elution peak of noradrenaline (σ_t) as a function of the injection volume of an aqueous test solution on a microbore column. For conditions, see experimental section.

caused by the injection and detection system and by the type of phase system and solutes used. Despite this loss in efficiency, the microbore columns still show an efficiency comparable to a 150 × 4.6 mm I.D. column. Thus the profit of less dilution in the detection of catecholamines can be still exploited, providing the same sample quantity can be injected as on the usual sized columns. Unfortunately the sample volume that can be injected on the microbore columns without losing efficiency is very small (< 1 μl) when the sample is dissolved in the mobile phase. Under these conditions the application of microbore columns is less favourable. However, it is possible to inject much larger sample volumes, without influencing the efficiency, when the sample can be concentrated at the top of the column. This occurs when the sample is dissolved in a non-eluting liquid [12]. On reversed-phase columns, water behaves as a non-eluting liquid and thus is very suitable as a sample liquid.

In order to test water as non-eluting sample liquid and to determine the sample volume which can be injected under these conditions, the standard deviation of noradrenaline was measured as function of the injection volume of the aqueous sample. The results of these measurements are shown in Fig. 1. The standard deviation was chosen for monitoring the efficiency rather than the theoretical plate height because of the uncertainty in measuring the retention time when injecting samples in non-eluting liquids. From Fig. 1 it can be seen that the standard deviation of the peak is virtually constant up to an injection volume of 1 ml and then slowly increases at larger injection volumes. These results show that, when injecting the catecholamines in an aqueous

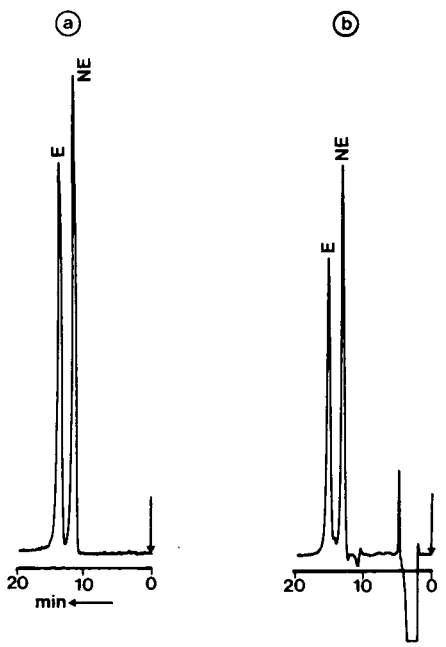

Fig. 2. Chromatograms of a test mixture of adrenaline (E) and noradrenaline (NE) on a microbore column: (a) 0.5-μl injection of the catecholamines dissolved in the mobile phase; (b) 100-μl injection of the catecholamines dissolved in water. For conditions, see experimental section. Sodium dodecyl sulphate concentration = 0.04% (w/v).

solution, similar injection volumes can be applied as on normal sized columns. This is demonstrated in Fig. 2, which shows chromatograms of a 0.5-μl injection of a solution of catecholamines in the mobile phase and of a 100-μl injection of an aqueous solution of the catecholamines.

The catecholamines are isolated from 0.5—1 ml of plasma by liquid—liquid extraction and then back-extracted into 125 μl of aqueous phase. From Fig. 1 it can be seen that, if necessary, the whole 125-μl extract (corresponding to 0.5—1 ml of plasma) can be injected onto the microbore column without influencing the performance.

Analysis of plasma samples

The developed method was applied to plasma samples of essentially healthy persons and of patients. Fig. 3a shows a chromatogram of a 50-μl injection of the extract from plasma of a healthy person. The catecholamine levels were found to be 120 pg/ml adrenaline and 200 pg/ml noradrenaline. Fig. 3b shows a chromatogram of a 20-μl injection of the plasma extract of a patient suffering from hypoglycaemia. The catecholamine levels are significantly higher (2.1 ng/ml adrenaline and 2.3 ng/ml noradrenaline) which simplifies the analysis. Fig. 3a and b shows that owing to the selective liquid—liquid extraction system and the selective measurement via native fluorescence a very clean chromato-

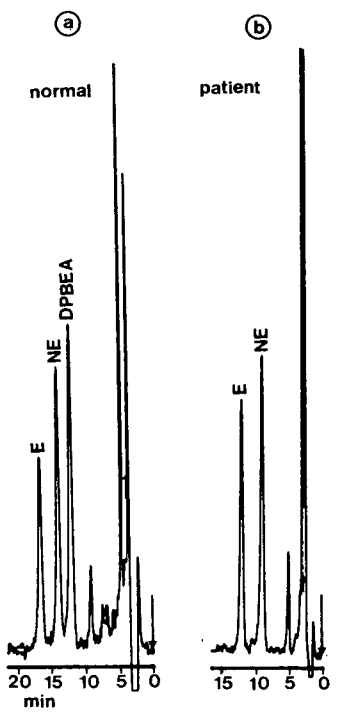

Fig. 3. Chromatograms of extracted plasma samples on the microbore HPLC system: (a) 50-μl injection of an extract obtained from 1 ml plasma of a healthy person; (b) 20-μl injection of an extract obtained from 1 ml plasma of a patient suffering from hypoglycaemia. For conditions, see experimental section. Sodium-dodecyl sulphate concentration = 0.06% (w/v). Peaks: E = adrenaline; NE = noradrenaline; DPBEA = diphenylborate—ethanolamine.

gram is obtained, which allows detection of adrenaline and noradrenaline at levels of about 10 pg/ml of plasma. The relatively large peak eluting in front of noradrenaline in Fig. 3a is diphenyl borate used in the extraction procedure. This peak appears only at very sensitive detector setting and shifts under the noradrenaline peak when the sodium dodecyl sulphate concentration in the mobile phase is $< 0.06\%$ (w/v).

Quantitative aspects

The precision and linearity of the chromatographic method were determined by injecting 50 μl of aqueous solutions of adrenaline and noradrenaline (20—16,000 pg/ml) and measuring the peak area or peak height. The calibration curves were linear over the investigated concentration range, with regression coefficients of 0.9998. The precision of the chromatographic method was determined from replicate analyses of a test mixture of the catecholamines. The relative standard deviation was found to be $\pm$ 0.5% (n = 25) at 500 pg injected amount. This is comparable to the results commonly obtained on normal sized columns.

The detection limit, defined as three times the standard deviation of the noise, was determined to be 5 pg for both catecholamines.

The recovery and reproducibility of the extraction were determined with aqueous test samples, containing 1 ng/ml of both adrenaline and noradrenaline. For both solutes the recovery was found to be 98.5% $\pm$ 0.5 (n = 6), in agreement with earlier reports [9]. The reproducibility of the whole analytical method was determined from replicate extractions of a pooled plasma sample and analysis of the extract by microbore HPLC. For adrenaline a mean value of 430 $\pm$ 8 pg/ml (n = 9) and for noradrenaline 706 $\pm$ 10 pg/ml (n = 9) was found.

CONCLUSIONS

Microbore columns, combined with on-column sample concentration and fluorimetric detection, are eminently suited for the analysis of adrenaline and noradrenaline in 0.5—1 ml of plasma down to levels of 10 pg/ml. The precision and reproducibility of microbore columns are comparable to those obtained on normal sized columns. In our experience microbore columns show a better column stability than normal sized columns. More than 800 plasma extracts could be analysed without any column deterioration.

Analysis of adrenaline and noradrenaline and possibly dopamine at the fg/ml level on microbore columns and fluorimetric detection looks feasible by applying a laser excitation source. This is now the subject of investigation in our laboratory.

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Note

Determination of neurotransmitter amino acids by high-performance liquid chromatography with fluorescence detection

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The analysis of amino acids in brain tissue has been performed by several chromatographic methods. Some amino acids are contained in the central nervous system at very low concentrations (< 0.1 μmol/g); therefore pre- and postcolumn derivatization procedures have been developed. Ion-exchange liquid chromatography has been applied for this purpose by using postcolumn derivatization and detection as fluorescent compounds. With this method all amino acids could be separated by using an anion-exchange column for acid and neutral amino acids and a cation-exchange column for basic amino acids [1]. The determination of some amino acids has been described using separation on a cation-exchange resin and postcolumn fluorescence detection of the *o*-phthaldialdehyde (OPT) derivatives [2].

Thin-layer electrophoretic separation has been shown to be useful for the quantitation of some amino acids in brain tissue [3]. Other authors [4] determined amino acids in several brain areas by converting them to their dinitrophenyl (DNP) derivatives, which were then separated by two-dimensional thin-layer chromatography. These procedures are time-consuming [1, 2, 4], suitable only for a few amino acids [2, 3], require complex sample preparation [4], or employ expensive equipment [1].

Korf and Venema [5] applied the high-performance liquid chromatographic (HPLC) method of Jones et al. [6] for the quantitation of twelve amino acids by OPT precolumn derivatization and fluorescence detection, but in their paper several column, instrumental and derivatization conditions are not given.

The need for a simple, rapid, accurate and sensitive method for the determination of amino acids in brain tissue has led us to develop and to describe here an HPLC analysis with fluorescence detection of Dns derivatives, using the

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precolumn derivatization method mentioned in our previous work [7] for the determination of γ-aminobutyric acid. This procedure has been applied to the determination of amino acid concentrations in some brain areas under physiological conditions.

MATERIALS AND METHODS

Reagents and solutions

Amino acids were obtained from Sigma (St. Louis, MO, U.S.A.). All other solvents and chemical reagents were of liquid chromatography and analytical grade (Carlo Erba, Milan, Italy).

Stock solutions of amino acids (10 μmol/ml in 0.1 *M* hydrochloric acid) were stored at 5°C.

A 0.1 *M* sodium bicarbonate solution in water was employed. Dns chloride stock solution (100 mg/ml in anhydrous acetone) was stored at 5°C protected from light. The working solution was 100 μl of stock solution diluted in 2 ml of acetone.

Liquid chromatography

A Series 2/2 Perkin-Elmer (Norwalk, CT, U.S.A.) liquid chromatograph with a variable-wavelength spectrofluorimeter 650 (Perkin-Elmer) and a variable-wavelength ultraviolet detector LC-75 (Perkin-Elmer) were employed. The injection valve was a Model 7125 (Rheodyne, Berkeley, CA, U.S.A.). The system was connected to a Hitachi—Perkin-Elmer Model 56 recorder. Two RP-8 columns (10 and 5 μm particle size, 25 and 12.5 cm long) (Perkin-Elmer) were operated at room temperature.

Gradient elution was performed by using mixture A (acetonitrile—isopropanol, 90:10) and mixture B (0.011 *M* sodium acetate—0.008 *M* acetic acid). The pH of mixture B was adjusted to 3.00 with phosphoric acid. The flow-rate was 0.8 ml/min. The column effluent was monitored with the fluorescence detector at 345 nm for excitation and 545 nm for emission. The bandwidths of both monochromators were selected at 10 nm. Mixtures A and B were degassed in an ultrasonic bath for 5 min. The multi-step gradient elution of the mobile phase was carried out as described in Table I. The column was initially equilibrated with the starting mobile phase for 15 min. At the end of the separation the column was washed with mixture A for 7 min.

TABLE I

MOBILE PHASE COMPOSITION DURING GRADIENT ELUTION

Time (min)	Mixture A (%)
0	10
15	25
18	34
24	34
39	100
43	100

Animals and analysis

Male Sprague—Dawley rats (Charles River, 180—200 g) were killed by exposing their heads to microwave radiation. Brains were immediately removed, then striata were taken frozen on dry ice and stored at −30° C until assayed. 5-Aminovaleric acid (5-AVA) as internal marker (600 μg/g of sample) was added to the tissues which were homogenized with 15—20 vols. of 85% methanol.

After centrifugation at 1500 *g* for 10 min a 100-μl volume of supernatant was introduced into a screw-capped tube and dried under a nitrogen flow at 50° C. Derivatization was carried out by addition of 50 μl of bicarbonate solution and 100 μl of working Dns chloride solution followed by heating for 15 min at 90° C. The resulting solution (3—5 μl) was injected into the chromatograph. Calibration curves were constructed for each amino acid by adding to tissue extracts increasing amounts of all amino acids and the internal marker. Recovery was evaluated by using striatum homogenates, which were divided into two aliquots. One aliquot was analysed directly while the other was analysed after addition of amino acids in amounts similar to the expected tissue content. The results were then compared with those obtained by analysis of pure amino acids.

RESULTS

Recovery of the amino acids tested was found to be in the range 93 ± 7%. The sensitivity of the method varied for each amino acid, depending on the structure, ranging from 0.1 pmol for γ-aminobutyric acid to 4 pmol for glutamine. Amino acid concentrations in samples were determined by using calibration curves for each amino acid. Peak height ratios using 5-AVA as a marker were plotted against the amount of amino acid added to tissue extracts and the relationship was found to be linear over the ranges tested.

When the same tissue extract was independently analysed at three different times, the mean percentage standard deviation between trials was 9%.

The chromatogram of blank reagent (Fig. 1A) does not show interfering peaks with added amino acids.

Fig. 1B shows a chromatogram for a standard pool with a concentration of 0.1 μmol/ml for each amino acid except taurine (0.4 μmol/ml) and glutamine (0.8 μmol/ml). The separation is quite satisfactory even though the peak of aspartic acid overlaps that of histidine. The latter is present at very low levels in brain tissue and therefore does not significantly affect the height of the aspartic acid peak. Lysine shows an unusually long retention time which may be the result of lysine binding to two Dns groups.

Fig. 1C shows the chromatogram of a striatum extract processed according to the method described above. It is seen that besides the reagent peaks there are unidentified peaks. These could be attributed to other amine compounds present in brain tissue, because at the pH of reaction only these may react. Table II gives the concentrations of amino acids (μmol/g wet tissue) found in striatum; these are in a good agreement with data previously published [1, 3, 5]. Although the use of a 10-μm (25 cm) column resulted in an unsatisfactory separation between aspartic acid and glutamine, the use of a 5-μm (12.5 cm)

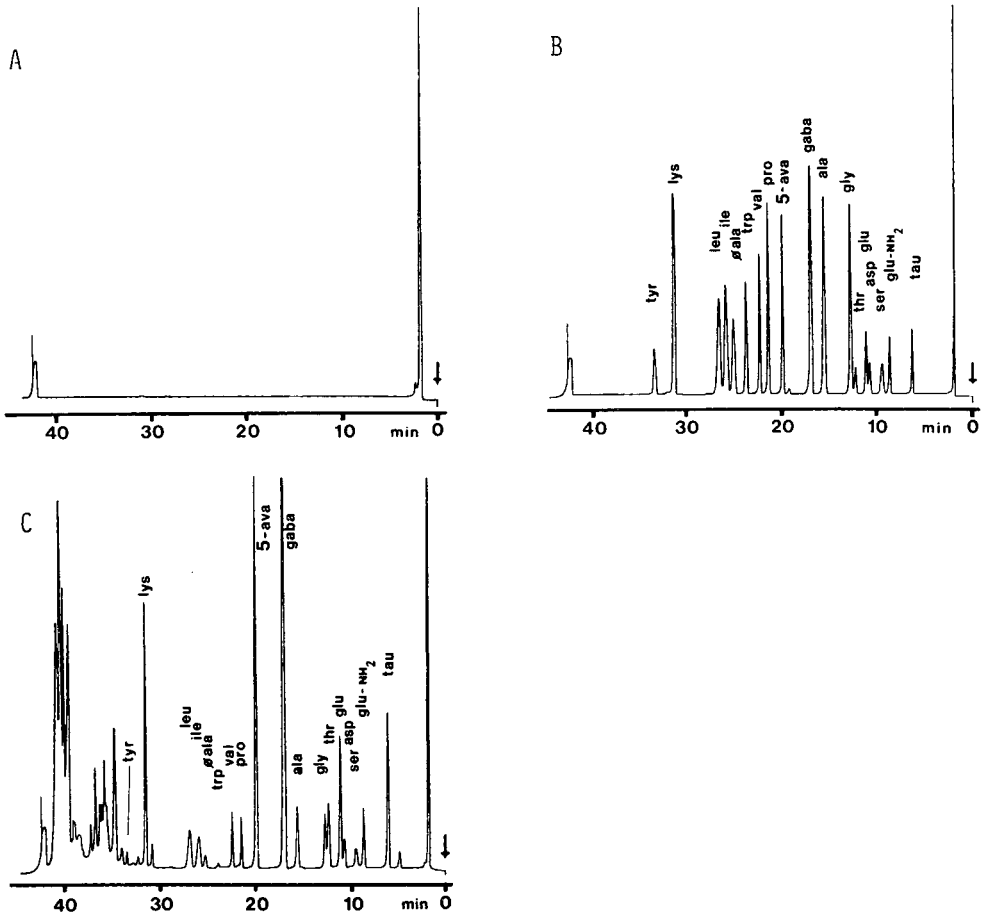

Fig. 1. Chromatograms of (A) blank reagent; (B) standard pool of seventeen derivatized amino acids; (C) derivatized striatum extract with 600 μg/g 5-AVA added. For abbreviations see Table II.

TABLE II

AMINO ACID CONCENTRATIONS IN RAT STRIATUM

Values are given as nmol amino acid per mg tissue ± S.E.M.

Amino acid	Concentration (nmol/mg)	Amino acid	Concentration (nmol/mg)
Taurine (tau)	7.81 ± 0.51	Proline (pro)	0.08 ± 0.01
Glutamine (glu-NH_2)	3.83 ± 0.47	Valine (val)	0.15 ± 0.03
Serine (ser)	1.54 ± 0.09	Tryptophan (trp)	0.09 ± 0.01
Aspartic acid (asp)	1.62 ± 0.14	Phenylalanine (ϕala)	0.03 ± 0.01
Glutamic acid (glu)	5.53 ± 0.32	Isoleucine (ile)	0.06 ± 0.01
Threonine (thr)	0.48 ± 0.04	Leucine (leu)	0.06 ± 0.01
Glycine (gly)	1.12 ± 0.07	Lysine (lys)	0.75 ± 0.06
Alanine (ala)	1.08 ± 0.06	Tyrosine (tyr)	0.15 ± 0.01
γ-Aminobutyric acid (gaba)	2.21 ± 0.36		

column gave the desired separation and had the additional advantages of lower void volume, shorter analysis time and lower solvent consumption. Some amino acids tested with this method (methionine, histidine) are present at very low levels in brain tissue and their peaks are not seen in the chromatogram of striatum (Fig. 1C). When a comparative analysis of striatum extract was made using this method and the amino acid analyser, good agreement was obtained. It is advisable to inject into the chromatograph volumes less than 10 μl, to avoid alteration in the pH of the mobile phase which results in variability of peak retention times.

This assay is highly sensitive and was applied with satisfactory results to several brain nuclei (suprachiasmatic, habenular region, dorsal and median raphe regions, periaqueductal region) with dry weights ranging from 0.5 to 2 mg. However, even smaller amounts of tissue could be analysed.

DISCUSSION

The HPLC determination of amino acids as their Dns derivatives in biological samples other than brain tissue is well known [8—13]. However, their analysis in brain tissue involves specific problems such as the presence of typical amino acids (γ-aminobutyric acid and taurine), a suitable internal standard, sensitivity, interfering compounds, etc. The use of an internal standard such as 5-AVA was found to be very important, since it is a non-endogenous amino acid and elutes well without interference in the chromatogram.

In this work the column used was selected carefully by testing different columns purchased from several manufacturers. Packing materials with nominally the same characteristics showed very different separations, by comparing plate number, peak symmetry, retention times and resolution. Such tests were not performed in other reports on the HPLC analysis of brain amino acids [5—7]. The different results observed in these tests could be explained by the lack of such packing parameters as the carbon loading, area bonded, pore diameters and other important characteristics. Unfortunately, such data are not given by the manufacturers.

The difference in response of amino acids is due to the different reactivity to Dns chloride and to the number of amino groups which undergo the reaction. The detector response was maximum for γ-aminobutyric acid, as its amino group is less subjected to inductive and steric hindrance than α-amino groups. Lysine shows a higher fluorescence response than other amino acids; this may result from two Dns-bonded groups.

The sensitivity of the method was sufficiently high to enable the analysis of the smallest brain areas; this possibility was not reported in previous studies [1—5].

In comparison to the method of Korf and Venema [5] it should be pointed out that the Dns derivatives used here are stable for many hours, whereas some OPT derivatives are extensively decomposed after 5—10 min. Moreover, the present procedure allows the analysis of seventeen amino acids, employing an appropriate internal standard.

Our method is the most rapid which allows the determination of seventeen amino acids in brain tissue. Indeed it takes only 35 min for the separation and

one sample can be injected every 55 min. The preparation of the sample is fast, simple and does not exceed 90 min. The precision of the method was also good as demonstrated by the low standard deviation.

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CHROMBIO. 2379

Note

High-performance liquid chromatography assays of phenylpyruvate and phenylpyruvate oxidase

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Phenylketonuria (PKU) is an inborn error of metabolism affecting phenylalanine metabolism. The normal catabolic path of phenylalanine to tyrosine is blocked owing to the deficiency of the enzyme phenylalanine hydroxylase. The lack of phenylalanine hydroxylase activity in phenylketonurics causes dramatically elevated levels of blood phenylalanine. Much of the accumulated phenylalanine is shunted to make phenylpyruvate, also causing a significant rise in blood levels of this metabolite. Phenylpyruvate has profound inhibitory effects on many enzyme systems and, therefore, may create severe metabolic problems for phenylketonurics. A recent review is provided by Scriver and Clow [1]. Mammalian liver contains the enzyme, phenylpyruvate oxidase (EC 1.14.2.2), which converts phenylpyruvate to the non-toxic *o*-hydroxyphenylacetate. It is clear, however, from plasma analyses, that the activity of mammalian phenylpyruvate oxidase is not sufficient to cope with the conversion of the grossly elevated levels of phenylpyruvate found in PKU patients. As shown by previous studies [2], assistance in such conversion could be provided by an immobilized phenylpyruvate oxidase hollow fiber reactor.

Studies on phenylpyruvate oxidase have been hampered by difficult, tedious and often inaccurate assay methods. Manometric [3] and colorimetric [4] methods were among the first to be used but these were replaced by spectrophotometric [5] and radiochemical procedures [6] in the 1970s. The radiochemical assay, based on a procedure originally developed by Kobayashi [7] for histidine decarboxylase, is the most commonly used today. While the method is relatively sensitive, it is hampered by the fact that it is both time-consuming and expensive. Radiolabelled phenylpyruvate is not available commercially and is typically prepared by reacting ^{14}C-labelled phenylalanine

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with amino acid oxidase (EC 1.4.3.2) [2]. Conversion is incomplete and the amount of product formed has to be checked by an enol—borate assay [8]. Owing to the relative instability of the substrate and the fact that small amounts of radioactive impurities could interfere with the assay, the radiolabelled substrate is prepared immediately before use. The preparation involves a 1-h incubation followed by a chromatographic step.

This paper describes a new high-performance liquid chromatographic (HPLC) procedure that is sensitive, rapid, uses readily available substrate and avoids the requirement of radioactive materials. The method can be used for the assay of blood phenylpyruvate and *o*-hydroxyphenylacetate levels as well as for determining the enzymatic activity of phenylpyruvate oxidase preparations.

EXPERIMENTAL

Phenylpyruvate, *o*-hydroxyphenylacetate, dithiothreitol, catalase, 2,6-dichlorophenol-indophenol and sulfosalicylic acid were purchased from Sigma (St. Louis, MO, U.S.A.); ultrapure, enzyme-grade ammonium sulfate was from Schwartz-Mann (Spring Valley, NJ, U.S.A.); HPLC-grade methanol was from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.); HPLC-grade phosphoric acid was purchased from Spectrum Chemical Manufacturing (Redondo Beach, CA, U.S.A.) and sucrose was from Mallinckrodt (Paris, KY, U.S.A.). Deionized and then glass-distilled water was used for chromatography and for the preparation of all reagents.

HPLC was performed with a Waters Assoc. (Milford, MA, U.S.A.) system consisting of a U6K injector, Model 6000A pump, Model 45 pump, data module M730 and system controller M720 together with an LDC (Riviera Beach, FL, U.S.A.) Spectro Monitor III variable-wavelength detector. The column used was a Waters 10-μm μBondapak C_{18} (30 cm × 3.9 mm) reversed-phase unit.

Phenylpyruvate and *o*-hydroxyphenylacetate were separated by HPLC using a linear gradient beginning with 1% phosphoric acid in water and going to 100% methanol in 1% phosphoric acid in 20 min at a flow-rate of 1.5 ml/min.

A crude preparation of phenylpyruvate oxidase was made by dicing fresh rabbit liver (150 g) and homogenizing it in ice-cold 0.25 *M* sucrose (400 ml) for 2 min in a blender. The suspension was centrifuged at 35,000 *g* for 30 min in a Sorvall RC2B refrigerated centrifuge. The resulting supernatant fraction was filtered through cheese cloth and glass wool and the filtrate brought to 35% ammonium sulfate saturation by addition of the solid salt. The pH was maintained at 7 by the addition of 10% ammonium hydroxide. When the ammonium sulfate was completely dissolved the mixture was kept on ice for 30 min. It was then centrifuged at 35,000 *g* for 1 h in a Sorvall RC2B centrifuge. The supernatant fraction from this step was used as a source of phenylpyruvate oxidase.

A phenylpyruvate oxidase assay reagent was prepared weekly. It consisted of 0.82 g of phenylpyruvic acid, 2.25 mg of 2,6-dichlorophenol-indophenol, 60 mg of dithiothreitol and 500 μl of catalase in 50 ml of 0.1 *M* imidazole—HCl buffer, pH 7.0. For the assay of phenylpyruvate oxidase activity 0.5-ml samples of the liver extract were preincubated for 5 min in a shaking waterbath at

37° C. The reaction was initiated by adding the extract to 1.0 ml of the assay reagent in the waterbath. The samples were removed at various intervals and the reaction was stopped by the addition of 2.5 ml of ice-cold 6% sulfosalicylic acid and the samples were held on ice for 30 min with periodic shaking. The samples were then centrifuged at 12,000 *g* for 10 min and the supernatant fractions were held at −80° C until immediately prior to HPLC analysis.

RESULTS AND DISCUSSION

Separation of phenylpyruvate and *o*-hydroxyphenylacetate on a reversed-phase C_{18} column is dependent upon these compounds being in their neutral form. This was accomplished by using a solvent phase brought to approximately pH 2 by the addition of phosphoric acid. Using the methanol gradient described, *o*-hydroxyphenylacetate is eluted with a retention time of 8.4 min at a methanol concentration of 40% (Fig. 1) while phenylpyruvate is eluted with a retention time of 13.7 min with 68% methanol (Fig. 2). Excellent linearity was observed between peak heights and metabolite concentrations up to 1000 nmol. Phenylpyruvate oxidase assays were carried out as described in the experimental section and the rate of phenylpyruvate conversion was monitored by HPLC. The sulfosalicylic acid is eluted at the void volume of the column. The peak heights for phenylpyruvate were converted to nanomoles of phenylpyruvate using a standard curve. A typical assay is shown in Fig. 3. The rate of phenylpyruvate conversion is linear up to approximately 10 min but the reaction then slows down, probably because of product inhibition [5]. The

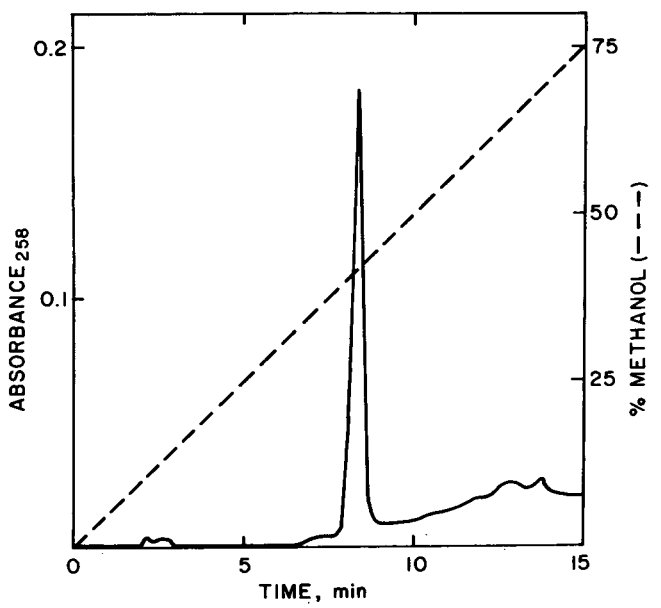

Fig. 1. Elution profile of *o*-hydroxyphenylacetate on reversed-phase HPLC. *o*-Hydroxyphenylacetate was eluted by 40% methanol with a retention time of 8.4 min from a 5-μm C_{18} column (30 cm × 3.9 mm). The column was pre-equilibrated with 1% phosphoric acid in water and a linear gradient from 0% to 100% methanol in 1% phosphoric acid was run in 20 min at a flow-rate of 1.5 ml/min. The column was monitored at 258 nm.

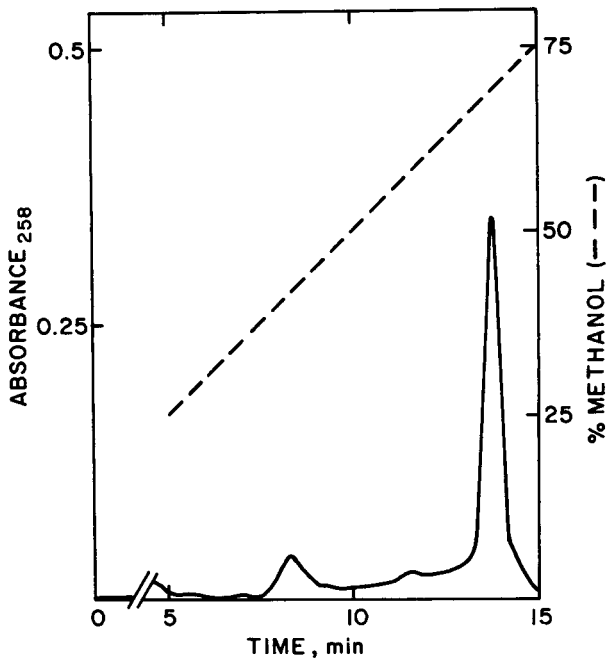

Fig. 2. Elution profile of phenylpyruvate on reversed-phase HPLC. Conditions were as described in Fig. 1. Phenylpyruvate was eluted by 68% methanol with a retention time of 13.7 min from a 5-μm C_{18} column (30 cm × 3.9 mm).

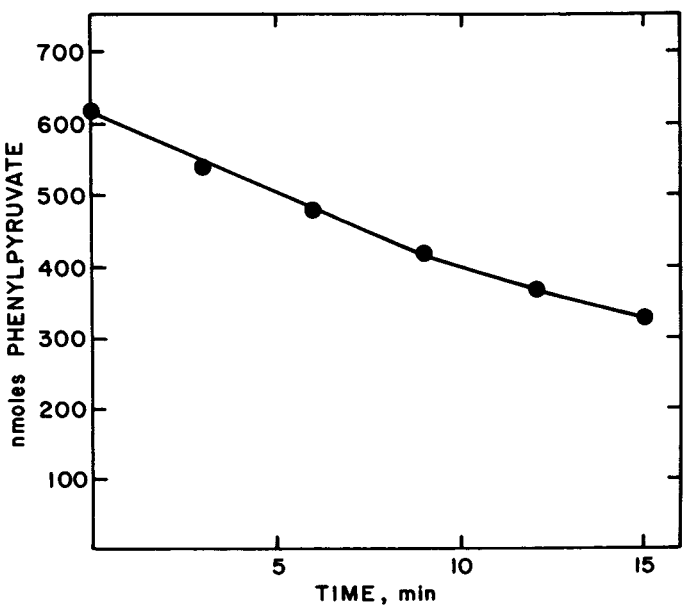

Fig. 3. HPLC assay of a crude phenylpyruvate oxidase preparation. Samples (0.5 ml) of the crude phenylpyruvate oxidase were preincubated for 5 min at 37°C. The reaction was initiated by addition of 1.0 ml of the substrate containing assay reagent, at 37°C, as described in the experimental section. At the indicated time intervals samples were taken from the water bath and the reaction stopped by addition of 2.5 ml of cold 6% sulfosalicylic acid. After 30 min on ice, the samples were centrifuged and the phenylpyruvate concentrations determined by HPLC.

activity of phenylpyruvate oxidase can also be measured in terms of appearance of the reaction product, *o*-hydroxyphenylacetate. In practical terms, it has proven more convenient to measure the decrease in phenylpyruvate concentration with time, to obtain initial rates, rather than attempting to measure very low concentrations of *o*-hydroxyphenylacetate.

The peak heights for phenylpyruvate, obtained from control assay samples lacking enzyme, provide a means for monitoring spontaneous breakdown of phenylpyruvate during the assay period. Little degradation of phenylpyruvate was observed up to 30 min at 37°C. The HPLC assay procedure also provides an internal check on the actual phenylpyruvate content of the assay reagent, thus avoiding one of the difficulties associated with the radiometric assay procedure. The HPLC assay reagent proved stable up to one week when stored at 4°C, but beyond that period increasing breakdown of phenylpyruvate was observed. It is therefore recommended that the reagent be prepared fresh weekly.

The phenylpyruvate oxidase assay by HPLC offers many advantages over the radiochemical procedure. The substrate, phenylpyruvate, is commercially available at much lower cost than the radiolabelled compound required to synthesize [^{14}C]phenylpyruvate, and the fact that the substrate does not have to be synthesized reduces subtantially the time for setting up the assay. The high sensitivity of HPLC detectors makes both the enzymes and metabolite assays viable for low concentration levels.

During the course of the work detailed here, Lefeng et al. [9] reported a similar HPLC procedure for the separation and quantitation of serum phenylalanine. The availability of rapid, accurate assays for the simultaneous monitoring of these important metabolites of phenylketonuria should facilitate future studies of this genetic disease.

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Note

Quantification of nitrendipine in plasma by a capillary column gas chromatographic—mass spectrometric method

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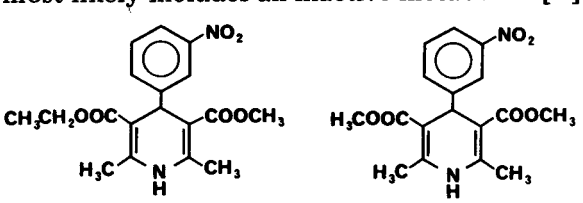
Calcium-blockers represent a group of drugs originally used for the treatment of angina pectoris, but in recent years also for hypertension [1, 2]. Nifedipine (Adalat®) was the first calcium-blocker of the dihydropyridine type. Although nifedipine has been found to effectively reduce the blood pressure of hypertensive patients [3], it has been difficult to find a significant correlation between the plasma concentration and the drop in blood pressure. This has been suggested to be due to the activation of compensatory mechanisms which may mask the "pure" effects of the drug [4]. Another reason may be that the analytical method predominantly used to determine nifedipine in plasma most likely includes an inactive metabolite [5].



I II

Fig. 1. Structures of nitrendipine (I) and the internal standard (II).

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Nitrendipine (I, Fig. 1) is a new calcium-blocker, structurally related to nifedipine. Pharmacological studies indicate that nitrendipine is a potent calcium-blocker, probably slower in onset of action, but more potent and with longer antihypertensive action than nifedipine [6, 7]. Recent studies indicate that nitrendipine may not trigger the compensatory mechanisms (causing, for example, elevated heart rate) to the same extent as nifedipine [8]. This increases the possibilities of finding a correlation between the blood pressure reducing effect and plasma concentration.

Prior to performing a pharmacokinetic study of nitrendipine, the following capillary column gas chromatographic—mass spectrometric (GC—MS) method was developed to ensure high sensitivity and specificity of the assays.

EXPERIMENTAL

Chemicals

Nitrendipine (I) and the structural analogue used as internal standard, 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine dicarboxylic acid dimethyl ester (II, Fig. 1), were obtained from Bayer (Wuppertal, F.R.G.). Stock solutions were prepared (approx. 1 mg/ml) in 20% methanol and stored in the dark at 4°C. Other chemicals used were of analytical purity and were obtained from commercial sources.

Plasma samples

The plasma samples were obtained from subjects suffering from essential hypertension of mild to moderate severity. The subjects received a single oral dose of 20 or 40 mg of nitrendipine, after which the plasma samples were drawn. A blood sample 24 h after administration was also obtained after three weeks' treatment. The plasma samples were protected from light and stored at −70°C prior to analysis.

Sample preparation

Aliquots of plasma (1.0 ml) were pipetted into 15-ml glass-stoppered test-tubes containing 79.2 pmol of the internal standard (II). Following the addition of 1.0 ml of 0.1 *M* sodium hydroxide and 2.5 ml of diethyl ether, the samples were shaken and centrifuged at 1000 *g* for 5 min. The diethyl ether layers were transferred to new test-tubes and evaporated to dryness under a stream of nitrogen. The residues were redissolved in 50 μl of ethyl acetate.

Gas chromatography—mass spectrometry

An LKB 2091 gas chromatograph—mass spectrometer was used for the analyses of plasma extracts and recording of mass spectra.

The GC separations were achieved using a 10 m × 0.32 mm I.D. WCOT SE-52 glass capillary column. Helium was used as a carrier and make-up gas. Splitless injections were carried out using a "moving needle" device. The GC conditions were: injector heater 300°C, column temperature 225°C, column flow-rate approx. 2 ml/min, and make-up gas flow-rate approx. 12 ml/min. Aliquots of 1 μl of the plasma extracts were injected and an initial delay of 0.5 min in opening the separator valve was used to avoid contamination of

the ion source. Under these conditions the retention time of nitrendipine and the internal standard was about 2 min. The MS conditions were: separator temperature 275°C, ion source temperature 240°C, electron energy 70 eV, and trap current 50 μA.

Quantification

Standard samples were prepared by spiking blank serum with nitrendipine (0—72.8 ng/ml). Calibration curves were constructed by plotting the peak height ratio nitrendipine/internal standard obtained in the GC—MS analyses against the concentration of nitrendipine. The nitrendipine levels were then

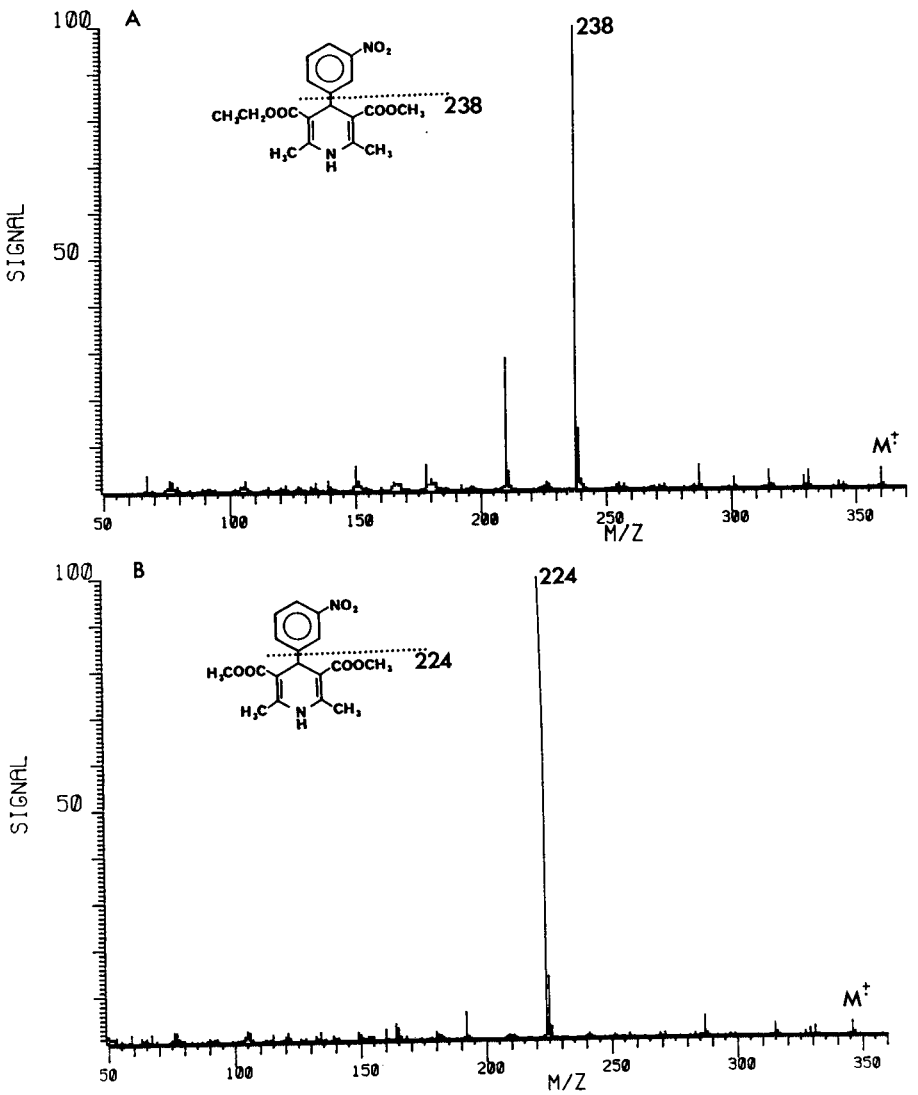

Fig. 2. Electron-impact mass spectra of (A) nitrendipine (I), and (B) the internal standard (II).

determined from the peak height ratio of each plasma extract by reference to the calibration curve.

RESULTS AND DISCUSSION

The electron-impact mass spectra of nitrendipine and the structure analogue (II) chosen as internal standard revealed molecular ions at *m/z* 360 and 346, respectively, which were of low intensity (Fig. 2). The base peaks at *m/z* 238 and 224 were formed by elimination of the 3-nitrophenyl moieties. These mass numbers were monitored in the ion-specific analysis of the plasma extracts.

A representative ion trace chromatogram obtained from the analysis of a plasma sample is shown in Fig. 3. The precision of the quantitative determinations was estimated by repeated analysis of two pools of plasma which had been spiked with nitrendipine (3.64 and 36.4 ng/ml), and was found to be better than 5%. The peak height ratios nitrendipine/internal standard were found to possess a linear relationship to the concentration of nitrendipine in the standard samples in the range of 0—72.8 ng/ml. The yield of nitrendipine and the internal standard through the sample preparation procedure was estimated to be greater than 90%. No evidence for a conversion of the internal standard to nitrendipine or vice versa was recorded under the conditions employed in the analytical procedure. The method was capable of determining nitrendepine levels below 0.5 ng/ml of plasma.

The method was used to determine the plasma concentrations after oral intake of 20 mg or 40 mg of nitrendipine (Fig. 4). The plasma concentration of nitrendipine peaked approximately 90 min after the administration of both 20

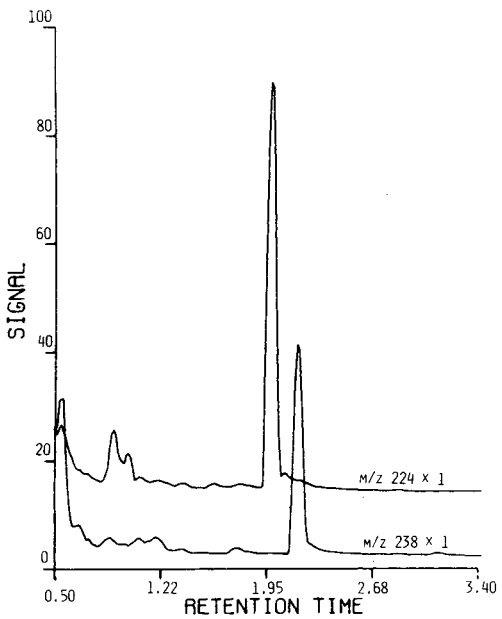

Fig. 3. Chromatogram obtained from the analysis of a plasma sample. The mass numbers (*m/z*) and relative amplification factors are indicated. The retention time is expressed in min.

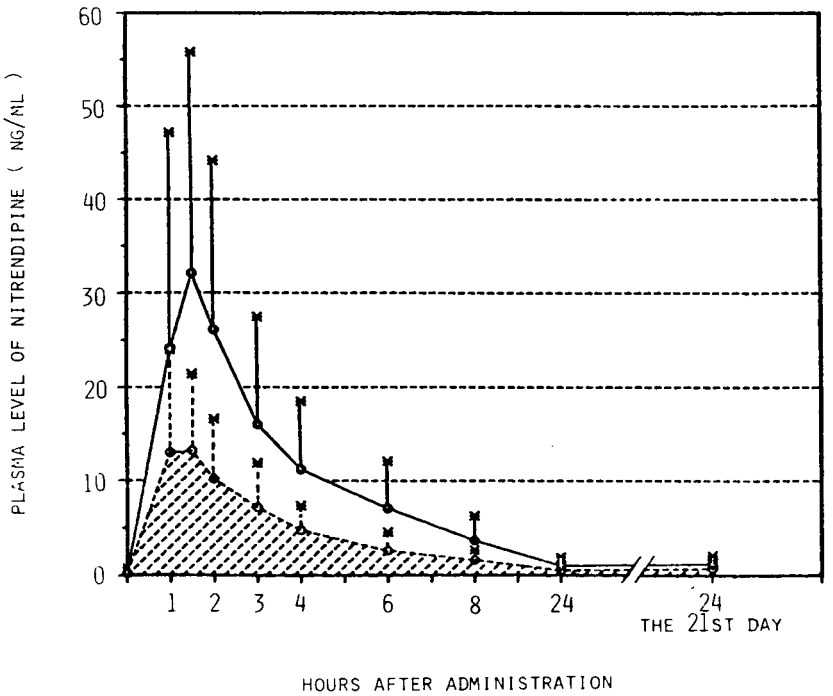

Fig. 4. Time course of nitrendipine levels in human plasma after oral intake or 20 (○) or 40 (●) mg.

and 40 mg. Despite the fact that the area under the curve was nearly twice as big on 40 mg [144.2 (ng/ml) × h] as on 20 mg [69.6 (ng/ml) × h], no additional blood pressure reduction was obtained, indicating that the maximum effect can be reached with 20 mg.

The results of the pharmacokinetic and pharmacodynamic analysis also revealed that the β-elimination half-life, i.e. the half-life of nitrendipine in plasma during the elimination phase, was 11.4 h on 20 mg and 8.1 h on 40 mg, and furthermore that there was a significant correlation between the plasma concentration of nitrendipine and blood pressure lowering effect (20 mg: $r = 0.72, p < 0.05$; 40 mg: $r = 0.84, p < 0.001$) [8].

In conclusion, a highly sensitive and specific method for the analysis of nitrendipine in plasma was developed. The precision and specificity of the analysis have made it possible to demonstrate a correlation between plasma levels and decrease in blood pressure for these types of drugs. This fact emphasizes the importance of using reliable analytical methods when performing pharmacokinetic research.

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Note

Rapid gas chromatographic assay for monitoring valnoctamide in plasma

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(First received June 25th, 1984; revised manuscript received September 19th, 1984)

Valnoctamide (valmethamide, or 2-ethyl-3-methylpentamide) has been used as a tranquillizer and in the treatment of anxiety and tension [1—3], and as an antiepileptic drug [4]. In the current literature, there is not a single report on an assay for monitoring plasma levels of valnoctamide.

The aim of this work was to develop a rapid and sensitive gas chromatographic method for routine assay of valnoctamide in plasma. Pharmacokinetic application of the new gas—liquid chromatographic (GLC) method is presented by plasma monitoring of valnoctamide after oral administration of a commercially available tablet of the drug (2 × 200 mg of Nirvanil®; Clin Midy, France) to a dog.

EXPERIMENTAL

Reagents and standards

Valnoctamide was obtained from Clin Midy (Paris, France), *p-tert.*-butylphenol was obtained from Fluka (Switzerland), chloroform (AnalaR) from BDH (U.K.) and hydrochloric acid from Frutarom (Israel). An organic stock solution of valnoctamide was prepared by dissolving the drug in chloroform. An aqueous stock solution was prepared by dissolving valnoctamide in water. The concentration of each stock solution was 0.5 mg/ml. *p-tert.*-Butylphenol was used as an internal standard and was dissolved in chloroform at a concentration of 0.25 mg/ml. Stock solutions were stored at −20°C.

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Apparatus and conditions

A Packard Model 437 gas chromatograph equipped with a flame-ionization detector and a recorder (Unicorder 225; Kyoto, Japan) was used. The glass column was 180 cm × 2 mm I.D. and was packed with 5% free fatty acid phase (FFAP; Applied Science Labs., State College, PA, U.S.A.) on 80—100 mesh Gas-Chrom Q. Flow-rates were as follows: hydrogen 25 ml/min; air 250 ml/min; carrier gas (nitrogen) 28 ml/min. The temperatures were: column 185°C; injector 205°C; detector 220°C. The same conditions were used in the work with the gas chromatograph—mass spectrometer (LKB Model 2091) which was operated with an ionization electron beam energy of 70 eV.

Extraction procedure

To 0.5 ml of plasma spiked with an appropriate aliquot of valnoctamide (or taken from the dog), were added 0.2 ml of chloroform, 50 μl of internal standard solution and 0.25 ml of 1 *M* hydrochloric acid. The sample was vortexed for 15 min, shaken for 30 min and centrifuged at 3000 *g* for 15 min. Of the organic phase, 3 μl were injected into the gas chromatograph.

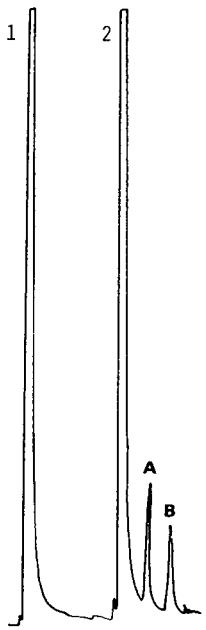
In order to determine the precision of the assay, 5 ml of human plasma were spiked with appropriate aliquots of the aqueous stock solution of valnoctamide and were stored at —20°C during the two months of the precision study. On different days, 0.5 ml was taken from the various stored samples and analysed against a fresh calibration curve made according to the extraction procedure on the same day.

RESULTS AND DISCUSSION

Typical chromatograms of a plasma extract and drug-free plasma are presented in Fig. 1. Under the assay conditions, the following retention times were obtained: valnoctamide 3.0 min; internal standard 3.6 min. The identification of peak A (Fig. 1) as valnoctamide was confirmed by GLC—mass spectrometric (MS) analysis. There was no interference from endogenous plasma components.

Calibration curves from plasma extracts showed a linear correlation between peak height ratio (y) (valnoctamide against internal standard) and plasma concentration of the drug (x). The linear calibration equation was $y = 0.138x + 0.0015$. To calculate this curve, a least-squares linear regression method was used. The minimal detectable concentration was 0.3 μg/ml of plasma. The linearity range of the assay was between 0.3 and 200 μg/ml.

Analytical recoveries of the drug were established as follows. Various amounts (10, 20, 30, 40, 50, 60, 80 and 100 μg) were taken from the aqueous stock solution of valnoctamide, and the volume was made up to 1 ml with drug-free plasma. After acidification, plasma samples were extracted into 0.20 ml of chloroform, which contained 50 μl of internal standard solution. A series of external standards were prepared by adding 50 μl of internal standard solution to 0.2 ml of chloroform containing the various amounts taken from the organic stock solution (10, 20, 30, 40, 50, 60, 70, 80 μg) of valnoctamide. Analytical recoveries were calculated by comparing peak height ratios of the extracted standard to the external standards (Table I). The standard deviation



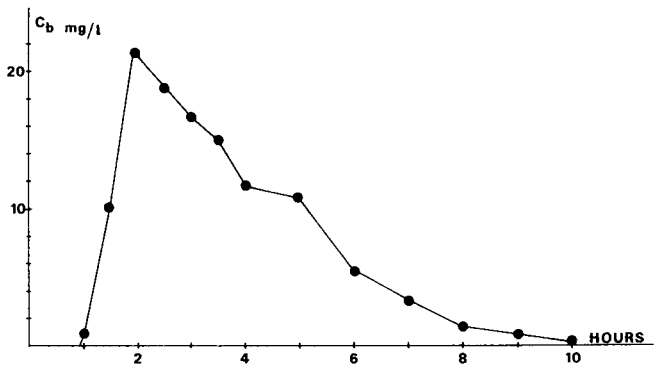


Fig. 1. Examples of chromatograms: (1) human plasma blank; (2) valnoctamide (A) 10 μg/ml (t_R 3.0 min), and internal standard (B) 50 μg/ml (t_R 3.6 min) in human plasma.

Fig. 2. Plasma levels of valnoctamide (Nirvanil) to a dog.

TABLE I

RECOVERY AND REPRODUCIBILITY OF VALNOCTAMIDE IN HUMAN PLASMA

Concentration (μg/ml)	Recovery* (%)	S.D.	C.V. (%)
10	90.6	5.5	6.1
20	89.0	4.3	4.8
30	87.4	3.7	4.2
40	87.6	3.2	3.6
50	89.6	2.3	2.6
60	88.1	2.2	2.5
80	87.6	2.7	3.1
100	90.9	5.8	6.4
Mean	88.9	1.4	1.6

*Mean of eight determinations.

of the analytical recoveries can serve as a good estimate of reproducibility (Table I).

Precision or accuracy of the assay was determined by performing eight replicate analyses of five control samples containing 10, 20, 30, 50 and 80 μg/ml of the drug on different days over a two-month period. The results are shown in Table II. The mean (± S.D.) percentage recovery of valnoctamide, as presented in Table I, is 88.9% ± 1.4 (n = 8). The observed values of the

TABLE II

PRECISION OF THE ASSAY FOR VALNOCTAMIDE IN HUMAN PLASMA

Concentration (μg/ml)	Conc. found* (μg/ml)	S.D.	C.V. (%)
10	10.47	0.41	3.92
20	20.43	0.81	3.96
30	29.35	1.22	4.16
50	48.61	1.88	3.87
80	78.84	2.41	3.06

*Mean of eight determinations.

various valnoctamide concentrations (Table II) were not different statistically from the added concentrations ($P > 0.05$) [5].

A biomedical application of the new GLC method is presented in a preliminary pharmacokinetic study. In this study, valnoctamide (400 mg) was administered orally to a dog (mongrel, 20 kg). The plasma levels of valnoctamide obtained are presented in Fig. 2 (mean of three replicates). The coefficient of variation among the three replicates at each data point was 5%. GLC—MS analysis of peak A (Fig. 1) in various samples obtained from the dog after oral administration of valnoctamide gave an identical MS fragmentation to that of a valnoctamide standard.

Although the retention time of valnoctamide in this assay was significantly different from those of related drugs such as valpromide and valproic acid, in humans there is a possibility of interference by other endogenous substances normally present in plasma or by other prescribed drugs which might be co-administered with valnoctamide.

The proposed method describes for the first time a very rapid, convenient and specific assay for valnoctamide. This assay is very useful in any pharmacokinetic study or therapeutic plasma monitoring of valnoctamide.

ACKNOWLEDGEMENTS

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Note

High-performance liquid chromatographic measurement of verapamil in plasma using a diol column

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Verapamil is a calcium antagonist drug used in the treatment of cardiac arrhythmias, angina and hypertension [1]. Several high-performance liquid chromatographic (HPLC) methods for assay of verapamil in plasma, using C_8 or C_{18} reversed-phase columns and fluorimetric detection, have been reported [2—7]. However, in some of these methods, norverapamil, a major metabolite of verapamil with some pharmacological activity, has not been considered [2, 3, 7]. This metabolite has been reported to accumulate in man during repeated dosing with verapamil, and plasma levels of norverapamil comparable to those of the parent drug have been observed [8, 9]. Other published HPLC procedures [4—6] have described incomplete chromatographic resolution of verapamil and norverapamil and this problem has been addressed by introducing an acetylation step into the sample preparation [4, 5].

The use of fluorimetric detection limits the choice of compounds that can be used as internal standards to those with fluorescence spectra similar to those of verapamil. Analogues of verapamil such as D600 and D517, which do not form part of the metabolic pathway for the drug, have been used but complete chromatographic separation of these compounds from verapamil or norverapamil using reversed-phase columns has not yet been achieved in a run time acceptable for routine analysis [4—6]. Satisfactory resolution, however, has been obtained through the use of silica [10] or cation-exchange columns [11].

The present study describes an HPLC assay of verapamil in plasma using a

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diol column and fluorimetric detection. The method is both sensitive and selective in that complete chromatographic resolution of verapamil, norverapamil and internal standard is readily obtainable.

MATERIALS AND METHODS

Chemicals and solutions

Verapamil {α-isopropyl-α-[(N-methyl-N-homoveratryl)-γ-aminopropyl]-3,4-dimethoxyphenylacetonitrile}, the N-desmethyl metabolite, norverapamil, and the internal standard α-isopropyl-α-[(N-methyl-N-homoveratryl)-β-aminoethyl]-3,4-dimethoxyphenylacetonitrile (D517, I.S.) were obtained as hydrochloride salts from Knoll. Stock solutions of each compound were prepared in water at a concentration of 5.0 μg/ml base (Ajax Chemicals, Melbourne, Australia). All other chemicals were of analytical quality. Water was distilled. Glassware was silanized using Aqua Sil (Pierce, IL, U.S.A.).

Instrumentation

A Constametric IIG high-pressure pump (Labortory Data Control, Riviera Beach, FL, U.S.A.), a Model 7120 sample injection valve with a 20-μl loop (Rheodyne, Berkeley, CA, U.S.A.) and a Model MPF 3L fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT, U.S.A.) fitted with a 20-μl flow cell formed the basis of the liquid chromatograph. Sample fluorescence was monitored using an excitation wavelength of 280 nm and an emission wavelength of 314 nm. A slit width of 7 nm was used for both excitation and emission.

Chromatography was performed on a 250 mm × 4 mm, 5 μm particle size LiChrosorb Diol column (E. Merck, Darmstadt, F.R.G.) at ambient temperature. The mobile phase consisted of methanol—acetonitrile—0.001 mol/l aqueous diammonium hydrogen phosphate (70:10:30). The pH of the phosphate buffer was adjusted to 6.6 with 0.2 mol/l orthophosphoric acid prior to mixing of the mobile phase. The flow-rate was constant at 1.0 ml/min. Column back-pressure was approximately 17 MPa.

Sample preparation

To 1-ml plasma samples in PTFE-lined screw-cap glass tubes were added 30 μl (150 ng) of the internal standard stock solution, 200 μl of saturated aqueous sodium carbonate (pH 11.5) and 3 ml of 20% *tert.*-amylalcohol in hexane. After vortex-mixing for 3 min and centrifugation for 5 min at 1500 *g*, the organic layer was transferred to a clean glass tube, acidified with 200 μl of 1 mol/l hydrochloric acid and vortex-mixed for 2 min. After centrifugation, the organic layer was discarded. The aqueous phase was made alkaline by addition of 200 μl of saturated aqueous sodium carbonate (pH 11.5) and extracted with 2 ml of 20% *tert.*-amylalcohol in hexane as described above. The organic layer was separated by centrifugation, transferred to a silanized tapered tube and evaporated to dryness at room temperature under a gentle stream of nitrogen. The extract was reconstituted with 50 μl of the mobile phase of which 20 μl were injected into the chromatograph.

The assay was calibrated by addition of verapamil and the internal standard

to pooled blood bank plasma. Calibration curves were established for concentrations of verapamil up to 400 ng/ml, and for concentrations of norverapamil up to 200 ng/ml. Peak height ratios were used in quantitation of the assay.

RESULTS AND DISCUSSION

Typical chromatograms of extracted plasma specimens are shown in Fig. 1. The retention times for verapamil, norverapamil and the internal standard (I.S.) were 6.5, 9.0 and 3.6 min, respectively. Chromatograms of blank plasma extracts were free from any interfering peaks and no endogenous substances with prolonged retention were detected. Chromatograms of plasma extracts from patients who had been taking verapamil consistently contained an additional substance which eluted immediately after norverapamil (Fig. 1c). This was assumed to be one of the minor metabolites of verapamil. Monitoring of the chromatography up to 15 min did not reveal any additional peaks.

Calibration curves for verapamil passed through the origin and were linear up to 400 ng/ml, the maximum concentration used. The limit of sensitivity for the assay was approximately 1 ng/ml. The recovery of verapamil from plasma, following extraction, was assessed by comparison of the peak heights from plasma extracts with those obtained from standard solutions of the drug in water. At a concentration of 40 ng/ml verapamil, mean recovery was 78.5% (S.D. 5.3; n = 4). The intra-assay coefficient of variation for plasma extracts at a concentration of 40 ng/ml was 2.3% (n = 7). For plasma concentrations of 20 ng/ml and 80 ng/ml, the respective inter-assay coefficients of variation, assessed over a period of four weeks, were 2.1% (n = 7) and 4.3% (n = 7).

For norverapamil, mean recovery from plasma at a concentration of 20

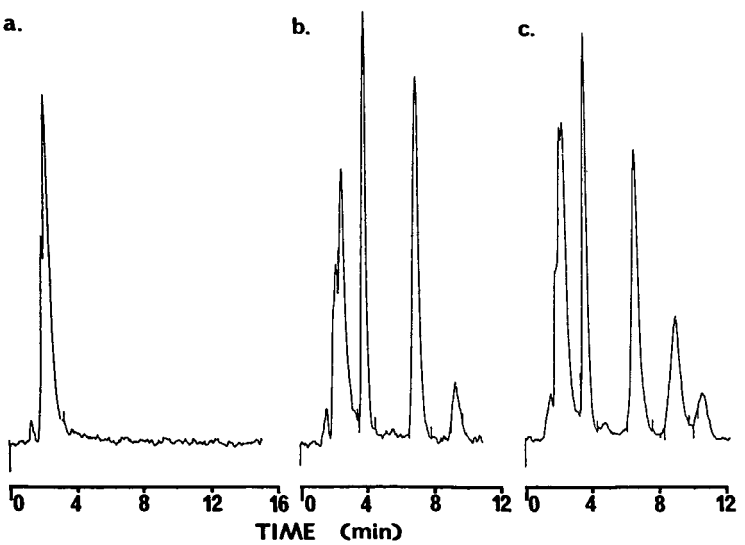

Fig. 1. Chromatograms of human plasma extracts: (a) blank plasma, (b) plasma containing 150 ng/ml I.S., 200 ng/ml verapamil and 100 ng/ml norverapamil, (c) plasma from a patient taking verapamil 80 mg t.d.s. Retention times of I.S., verapamil and norverapamil were 3.6 min, 6.5 min and 9.0 min, respectively.

ng/ml was 54.5% (S.D. 9.7; $n = 4$). The minimum detectable concentration was 3 ng/ml. The calibration curve was linear up to 200 ng/ml, the maximum concentration used, and at a concentration of 40 ng/ml, the intra-assay coefficient of variation was 2.3% ($n = 7$).

The assay has been evaluated using plasma samples from patients receiving verapamil for treatment of heart disease. Despite concurrent medication with a number of drugs including prazosin, theophylline, frusemide, chlorothiazide, disopyramide, propranolol, quinidine, hydrallazine and procainamide, no interference with the chromatographic measurement of verapamil or norverapamil has been detected. However, the potassium-sparing diuretic, amiloride, elutes in 11.0 min and may be detected after the elution of norverapamil.

The use of a diol column has resulted in improved separation of verapamil, norverapamil and I.S. in comparison to earlier methods which employed alkyl-chain reversed-phase columns [2—7]. Using the diol column, norverapamil elutes after verapamil, probably as a result of a greater degree of hydrogen bonding of the more polar norverapamil with —OH groups of the diol-bonded stationary phase. Chromatography on the diol columns has proven stable and reproducible. Unlike silica columns, the diol column undergoes rapid equilibration and is not susceptible to deterioration in performance through contamination of the chromatographic system with water. As a result, the method represents a reliable alternative to normal-phase chromatography of verapamil [10], and is of sufficient sensitivity and selectivity to be used in pharmacokinetic studies.

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Note

Simplified liquid chromatographic analysis for cyclosporin A in blood and plasma with use of rapid extraction

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Cyclosporin A is a new fungal cyclic undecapeptide with a potent immunosuppressive activity. It is notably effective in the prevention of graft-versus-host disease in allogenic bone marrow transplantation [1, 2] and in the prevention of graft rejection after kidney [3, 4], heart [5] and liver transplants [6]. The clinical success of cyclosporin and the utility of measuring blood or plasma concentrations of the drug have been well documented [4, 7].

Several methods have been developed for the analysis of cyclosporin A in plasma and/or in blood; these are generally time-consuming or need a high technology [7—15] (Table I).

TABLE I

PUBLISHED METHODS OF CYCLOSPORIN A ANALYSIS

Authors	Method	Sample	Extraction
Donatsch et al. [8]	RIA	Blood/plasma	—
Niederberger et al. [9]	HPLC, gradient	Plasma	Liquid—liquid
Lawrence and Allwood [10]	HPLC, isocratic	Serum	Column
Sawchuk and Cartier [16]	HPLC, isocratic	Blood/plasma	Liquid—liquid
Nussbaumer et al. [11]	HPLC, column switching	Blood/plasma	Protein precipitation
Leyland-Jones et al. [12]	HPLC, isocratic	Plasma	Column
Kahan et al. [7]	HPLC, isocratic	Blood/plasma	Liquid—liquid
Yee et al. [13]	HPLC, gradient	Serum	Column
Carruthers et al. [17]	HPLC, isocratic	Plasma	Liquid—liquid
Smith and Robinson [14]	HPLC, column switching	Blood/plasma	—

The following study reports the development of a rapid and selective high-performance liquid chromatographic (HPLC) method for the routine analysis of cyclosporin A in blood and plasma. Cyclosporin D was used as the internal standard. This method is suitable for pharmacokinetic studies.

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MATERIALS AND METHODS

Reagents

Chemicals used were cyclosporin (cyclosporin A and D) (Sandoz, Rueil Malmaison, France), diethyl ether Spectrosol (S.D.S., Peypin, France), *n*-hexane Spectrosol (S.D.S.), acetonitrile for chromatography LiChrosolv (E. Merck, Darmstadt, F.R.G.), trifluoroacetic acid (TFA) Uvasol (Merck), methanol Normapur (Prolabo, Paris, France), hydrochloric acid Normapur (Prolabo), buffer pH 10 (Merck).

Water was demineralized and filtered on a Millipore filter Type HA 0.4 μm.

A stock solution of cyclosporin was prepared by dissolving 10 mg of cyclosporin A (or D) in 100 ml of methanol. Further dilutions of this stock solution were made with methanol prior to preparing plasma and blood standards.

Chromatographic conditions

A Waters 6000A pump including a U6K injector was used for the chromatographic analysis (Waters Assoc., Milford, MA, U.S.A.). The analytical column was a RP-18 Hibar LiChrocart 4 μm (25 × 4.6 cm) (Merck). The ultraviolet (UV) detector was a UVIKON 722 LC. A 4 × 0.4 cm precolumn dry-packed with μBondapak C_{18} reversed-phase packing served to protect the analytical column from the adsorption of sample constituents.

The precolumn and analytical column were maintained at 75°C with a column heater block (built by Y. Clavel, Biophysics Department, Nice). This temperature was reached from room temperature in 30 min in order to minimize damages.

To separate cyclosporin A and D from other co-extracted serum or blood constituents, the following mobile phase was used: acetonitrile—1 ml/l trifluoroacetic acid in water (70:30) with a flow-rate of 1.2 ml/min and column pressure of 143 bar, with the detector set at 210 nm. The mobile phase was continuously degassed by helium flow. Under these conditions, the retention times were 6 min for cyclosporin A and 7.2 min for cyclosporin D (see Figs. 1 and 2).

Sample preparation

Blood samples were drawn into heparinized tubes from patients having received oral or intravenous cyclosporin A. If the determinations are performed in the whole blood, the sample may be immediately extracted, whereas when the determinations are performed on plasma, the samples must remain for 2 h at room temperature before centrifugation in order to stabilize exchanges between blood cells and plasma.

Conical glass centrifuge tubes (25 ml) were cleaned with diethyl ether and then dried. Different volumes of the standard solution of cyclosporin A were added to standard tubes. The same volume of the internal standard solution was added to all standard curve tubes and to each sample tube. All tubes were dried with a nitrogen flow. Then 0.5—1 ml of drug-free human blood or plasma was added to each standard tube and 0.5—1 ml of patient's blood or plasma to the appropriate sample tubes. The blood samples were lysed by rapid deep freezing and then thawed.

Hereafter all tubes were treated identically; 0.5—1 ml of pH 10 buffer was added to each tube, mixed for 5 sec with a vortex mixer and then 7 of ether were dispensed with an automatic pipette. The tubes were shaken mechanically for 5 min on a horizontal shaker and centrifuged for 5 min at 800 *g*. A 6-ml aliquot of the separated diethyl ether layer was then transferred to another conical centrifuge tube, which had been previously rinsed with diethyl ether. Another extraction was performed with 7 ml of diethyl ether and then the two ether extracts were mixed and evaporated to dryness under vacuum and nitrogen flow for 10 min at 40°C.

The plasma or blood residue concentrated at the bottom of the conical tube and was then dissolved in 200 μl of methanol—0.1 *M* hydrochloric acid (1:2); 1 ml of *n*-hexane was added and then mixed for 20 sec with the vortex mixer.

An aliquot of 30 μl was injected into the chromatograph.

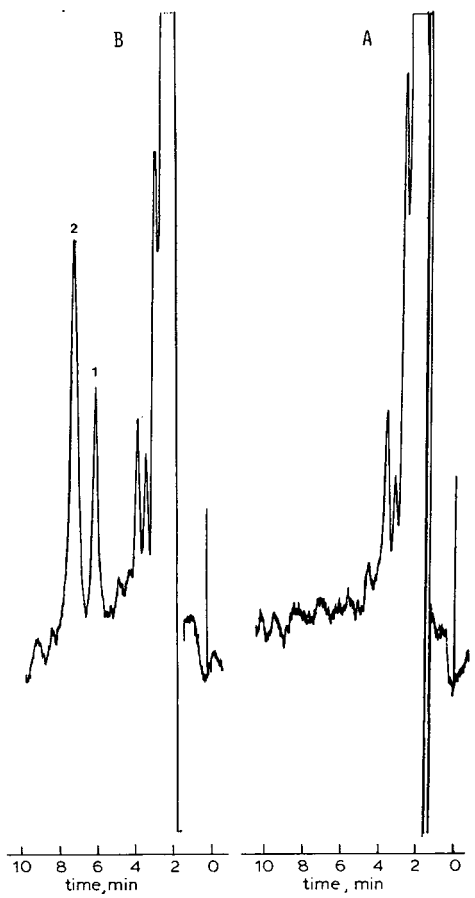

Fig. 1. (A) Chromatogram obtained from analysis of an extract of cyclosporin-A-free human plasma without addition of internal standard. (B) Chromatogram obtained from analysis of an extract of a plasma sample containing 250 μg/l cyclosporin A (1) and internal standard (cyclosporin D) (2).

RESULTS

Chromatograms

No interfering peak was detected in the plasma or blood used for blank standards or from subjects who had received cyclosporin A.

The chromatograms obtained in the analysis of 1 ml of blank plasma or blood and 1 ml of patient's plasma or blood sample containing 250 μg/l (plasma sample) and 150 μg/l (blood sample) are shown in Figs. 1 and 2.

Concentrations of cyclosporin A in plasma or blood were determined from calibration curves of peak height ratios of cyclosporin A to cyclosporin D. The use of the internal standard cyclosporin D was essential for a good precision and linearity of the method.

Linearity

The relationship between the peak height ratio of cyclosporin A to the internal standard (cyclosporin D) and the cyclosporin A concentration in blood

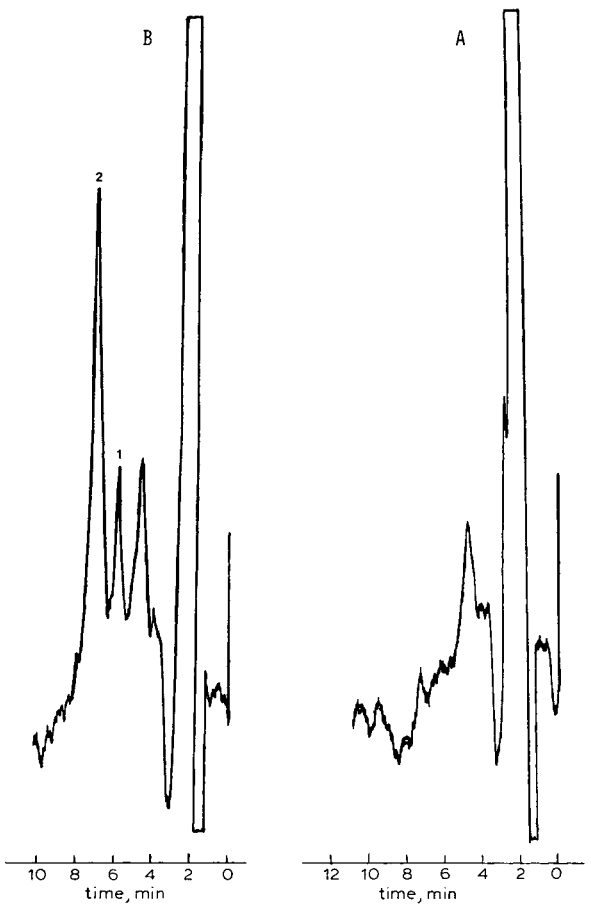

Fig. 2. (A) Chromatogram obtained from analysis of an extract of cyclosporin-A-free human blood without addition of internal standard. (B) Chromatogram obtained from analysis of an extract of a blood sample containing 150 μg/l cyclosporin A (1) and internal standard (2).

or plasma was linear over the range 0—1000 μg/l (plasma, r = 0.980) and 0—2000 μg/l (blood, r = 0.988).

Analytical recovery, sensitivity and reproducibility

Recovery of cyclosporin A was determined by comparing the peak height ratios after extraction of plasma and blood samples containing known amounts of cyclosporin A with those of unextracted samples supplemented with the same amounts of cyclosporin A. For the purpose of this calculation, the internal standard was added to the samples just before injection into the chromatograph.

Cyclosporin A recoveries of 85% and 74% from plasma and blood, respectively, were calculated at a concentration of 300 μg/l.

The sensitivity of this method was evaluated by analysing plasma and blood samples after addition of cyclosporin A in concentrations approaching the sensitivity limit. Method parameters remained consistent to 25 μg/l for plasma and 35 μg/l for blood.

The reproducibility was evaluated after extraction of several plasma and blood samples with given concentrations of cyclosporin (inter-assay) or after repeated injections of the same extract (intra-assay).

The results are given in Table II.

TABLE II

INTRA- AND INTER-ASSAY COEFFICIENTS OF VARIATION (C.V.) FOR CYCLOSPORIN A IN PLASMA AND BLOOD

	Concentration (μg/l)	n	C.V. (%)
Intra-assay			
Plasma	150	8	5
Blood	300	8	4.5
Inter-assay			
Plasma	100	5	7
	400	5	5
Blood	150	5	4
	1000	5	7

APPLICATION OF THE METHOD

Approximatively 600 plasma and blood samples from ten patients have been analysed by this method, with dosage just before treatment and sometimes 2 or 4 h after to assess the serum or blood peak. Fig. 3 shows a typical blood concentration curve after a single dose of cyclosporin A in a treated patient (2 × 200 mg per 24 h per os).

DISCUSSION

The first reported method employed for cyclosporin A determination was radioimmunoassay (RIA) [8] which was initially developed for plasma and, as shown later, was also applicable to blood samples, Its main drawback has been

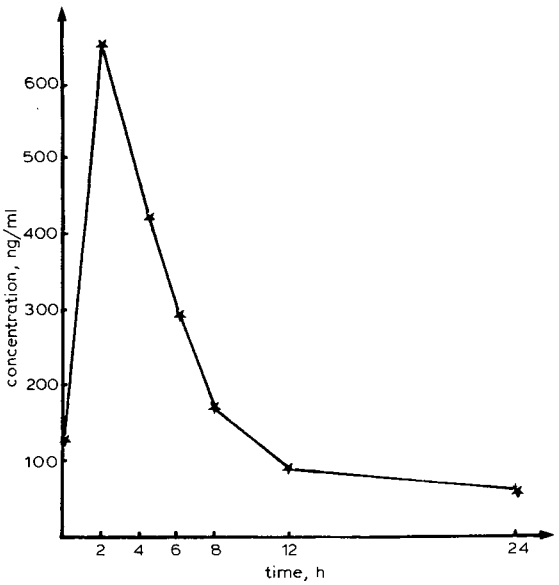

Fig. 3. Blood concentration curve of cyclosporin A from a patient who received an oral dose of 200 mg (chronic treatment).

the cross-reactivity of the antisera with some of the circulating metabolites of cyclosporin A.

The chemical nature of cyclosporin A has delayed the development of analytical techniques, but in the last three years high-performance liquid chromatography (HPLC) has proved to be a valuable technique for the analysis of this drug. Compared to RIA, HPLC methods have a greater specificity. But most of these HPLC methods have disadvantages, especially the long duration of sample preparation and/or chromatography times which make them unsuitable for routine determinations [16, 17]. Other methods have a shorter clean-up procedure, but require a higher technical complexity based on gradient elution or column switching [7, 9, 11, 13, 14].

We describe a method that is now routinely used in our laboratory to quantitate cyclosporin A in concentrations as low as 35 μg/l in whole blood or 25 μg/l in plasma. Compared to other methods, this method provides a combination of rapidity of extraction and simplicity of chromatographic analysis.

The use of an alkaline extraction allows us to reduce the number of steps in the clean-up procedure. Thus, the protein precipitation and the double extraction (acid then alkaline) described by Carruthers et al. [17] can be prevented. Moreover, the double wash with *n*-hexane introduced by Sawchuk and Cartier [16] is not useful; a similar result is obtained without delay by adding *n*-hexane just before injection, together with methanol—0.1 *M* hydrochloric acid. Furthermore, a good recovery is obtained with this extraction procedure.

The use of the internal standard (cyclosporin D) which differs only slightly from cyclosporin A in structure and chromatographic properties, confers to the analytical method a good precision and linearity.

The analytical column used, RP-18 4 μm, gives a good selectivity, sufficient

resolution and allows for isocratic conditions with the mobile phase 0.1% TFA—acetonitrile (30:70). We preferred 0.1% TFA to water, because its use results in sharper peaks.

The chromatograms of blanks from plasma and blood show fluctuations of the baseline, which cannot be considered as interfering peaks, and are due to the very high sensitivity of the detection (0.005 a.u.f.s.) and the short wavelength (210 nm).

The time required for a sample analysis (extraction and chromatographic analysis) is less than 45 min. The sensitivity limit appears adequate for the monitoring of plasma or blood cyclosporin A concentrations in patients receiving regular treatment with this drug.

The possibility of blood determination minimizes the problems of the kinetics of cyclosporin A exchange between red cells and plasma. Moreover, as the concentrations in whole blood are always significantly higher than in plasma, we need in many cases a lower sensitivity.

CONCLUSION

The method reported is rapid and simple, and suitable for routine analysis and therapeutic monitoring in most pharmacology laboratories.

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Note

Reproducible measurement of amphotericin B in serum by high-performance liquid chromatography in alkaline buffer

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Amphotericin B has been used as a chemotherapeutic agent for nearly 25 years and remains today the drug of choice for most systemic fungal infections [1—4]. The major limitation to the use of amphotericin B is the need for parenteral administration and the induction of serious side-effects. The renal side-effects are the most serious with renal vasoconstriction and tubular degeneration [1, 3].

The antimycotic properties of amphotericin are related to its ability to bind sterols, especially ergosterol, in the membranes of fungal cells [5, 6]. Membrane sterols are constituents of eukaryotic cells and this binding may contribute to the prolonged tissue retention of the drug. During intravenous therapy, 90% of amphotericin B disappears from the blood and only low levels are found in biological fluids other than serum. Moreover, the amphotericin B portion remaining in the blood is strongly bound to serum lipoproteins. But investigations providing a rational pharmacokinetic basis of amphotericin B treatment are scarce and several important phenomena are poorly understood [7, 8].

Monitoring the concentration in biological fluids and in tissues may, in theory, assist the management of an amphotericin B regimen. However, the assay of this product is difficult [9]. Microbiological assays are time-consuming, not sensitive and imprecise. Among several different physicochemical assays recently developed [10—14], high-performance liquid chromatography (HPLC) is rapid, specific and sensitive, and looks promising for the assay of amphotericin B in biological fluids as well as in tissue homogenates [3, 5, 15]. However, great variations can appear during the methanolic extraction, and the

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introduction of an internal standard recently proposed has not fully solved the problem [5, 15, 16]. We report here a sensitive, accurate and reproducible HPLC assay for amphotericin B using an original extraction procedure in an alkaline buffered medium.

MATERIALS AND METHODS

Reagents and standard

Methanol, dimethylsulphoxide (DMSO) and hydrochloric acid were purchased from E. Merck (Darmstadt, F.R.G.), ethylenediaminetetraacetic acid (EDTA) was obtained from Riedel-Dahaen (Hannover, F.R.G.). Amphotericin B was a gift of Squibb Labs. (Neuilly-sur-Seine, France).

A stock standard solution of amphotericin B (1 g/l) was prepared in DMSO while a working standard solution (10 mg/l) was prepared by dilution of the stock solution with the mobile phase. The stock solution was stored at −20°C; the working standard was stored at 4°C and its stability was verified by absorbance at 405 nm (E_m = 106,457).

Chromatography and extraction procedure

The analyses were carried out using a Waters high-performance liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with a 6000A Waters pump and a U6K injector. The detector was a Waters Model 440 spectrophotometer with 405- and 340-nm filters, connected to an Omniscribe stripchart recorder (Houston Instruments, Austin, TX, U.S.A.). The column was a reversed-phase Waters Rad-Pak C_{18} (10 cm × 8 mm; 10 μm average particle size). The mobile phase was a mixture of methanol—5 m*M* EDTA (80:20), adjusted to pH 7.8 with dilute hydrochloric acid.

The mobile phase was added to serum in the proportion 3:1, vortexed for 2 min, kept at ambient temperature for 10 min and centrifuged at 2000 *g*. Then, 100 μl of supernatant were injected into the chromatograph.

Microbiological assays

The bioassays were performed according to a modification of the method of Shadomy et al. [17] using *Paecilomyces variotii* as test organism [18].

Human samples

Physicochemical (HPLC) and microbiological assays were simultaneously performed on 60 human sera from patients treated for deep mycosis with amphotericin B associated or not with 5-fluorocytosine.

RESULTS

Chromatography

Fig. 1 shows chromatograms of the working standard solution and of a human serum extracted as described in Materials and methods. Absorbance was simultaneously read at 405 and 340 nm and interfering substances were detected by measuring the ratio of absorbance at 405 nm to that at 340 nm.

Linearity and recovery

Different concentrations of amphotericin B in the range of concentrations of

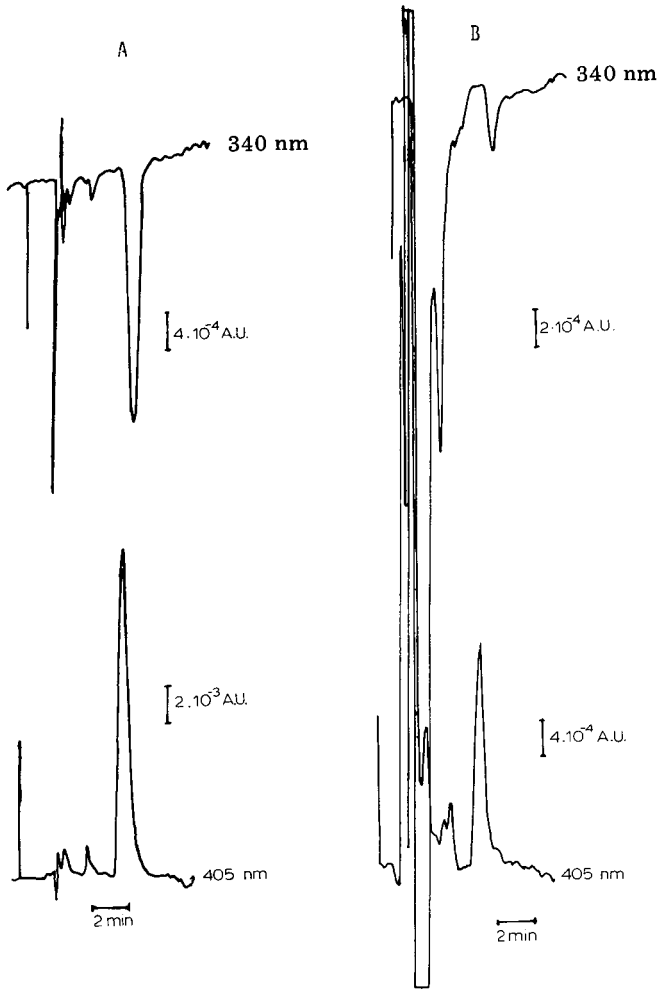

Fig. 1. Typical chromatograms of (A) extract of a standard solution containing 1.25 mg/l amphotericin B and (B) extract of serum sample containing 1.67 mg/l. A.U. = absorbance units.

TABLE I

LINEARITY AND RECOVERY STUDIES

Concentration added (mg/l)	Concentration recovered (mg/l)	Yield (%)
0.1	0.075	75
0.2	0.149	75
0.4	0.307	77
0.6	0.48	80
0.8	0.67	83
1.0	0.97	93
1.2	0.95	79
1.4	1.16	83

clinical interest were added to the same drug-free human serum. The concentration recovered and the yield of the assay are reported Table I. The mean yield of the assay was 81 ± 6%. The regression equation was $y = 0.838x \pm 0.001$ with a correlation coefficient of 0.993. The sensitivity of the assay, i.e. peak height corresponding to three times the baseline noise, was found to be 0.05 mg/l.

Precision

The internal coefficient of variation, by analysing the same serum ten times, was ± 3% (average concentration 1.55 mg/l).

Selectivity

Since amphotericin B is frequently used in association with 5-fluorocytosine

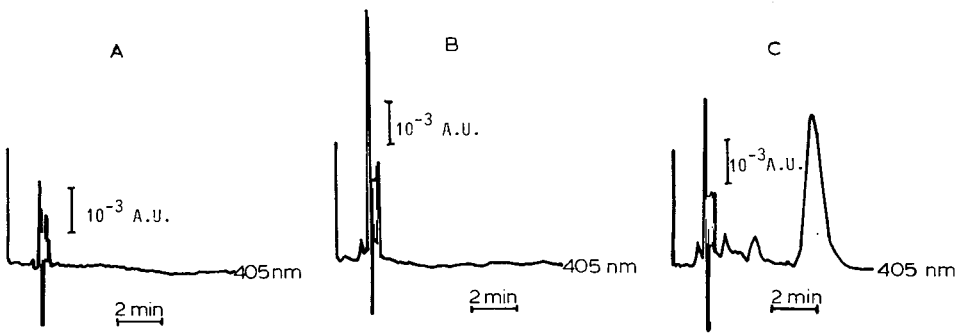

Fig. 2. Chromatograms of extracts of a normal serum sample with (A) no drug, (B) 10 mg/l 5-fluorocytosine, and (C) amphotericin B. A.U. = absorbance units.

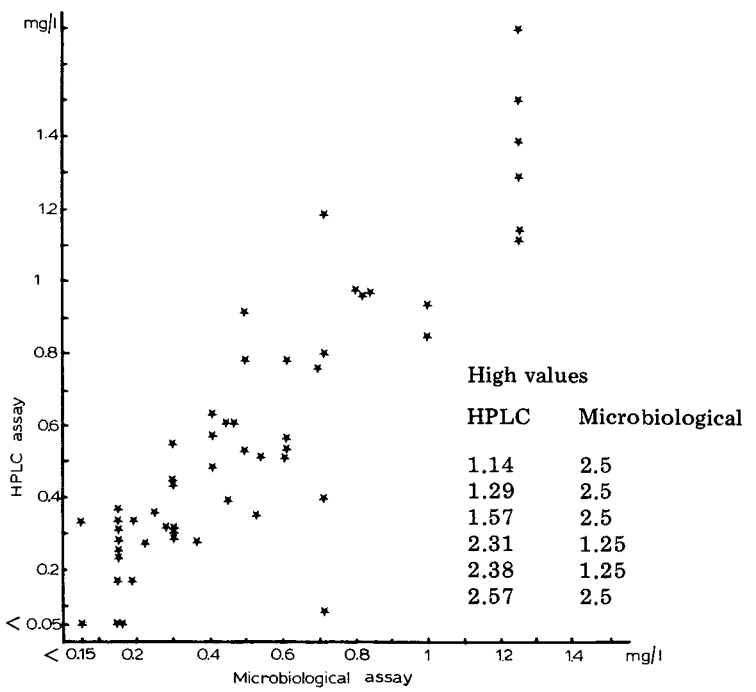

Fig. 3. Comparison of results of the microbiological assay and those of HPLC assay for determination of amphotericin B levels in 60 serum samples.

in the treatment of deep mycosis, we have verifyied that 5-fluorocytosine did not interfere with the amphotericin B assay. Fig. 2 shows a chromatogram of an extracted serum without any drug, and of the same serum with either 5-fluorocytosine or amphotericin B added. It is clear that 5-fluorocytosine does not interfere in the method.

Correlation of the physicochemical assay with the microbiological assays

The microbiological assays and the HPLC method were used simultaneously for 60 serum samples. The correlation coefficient calculated from the values represented in Fig. 3 was 0.7851 (regression equation $y = 0.2234 \pm 0.7008x$).

DISCUSSION

Nilsson-Ehle et al. [15] proposed a rapid and selective HPLC assay for amphotericin requiring many steps (deproteinization, centrifugation, filtration). However, Mayhew et al. [5], using identical experimental conditions, reported a fairly large coefficient of variation of 18% and an extraction yield varying between 53% and 71% within a concentration range of 0.08—10.0 mg/l.

Our previous results using this procedure gave an extraction yield from a standard solution of about 50% (Fig. 4). Furthermore, great variations were

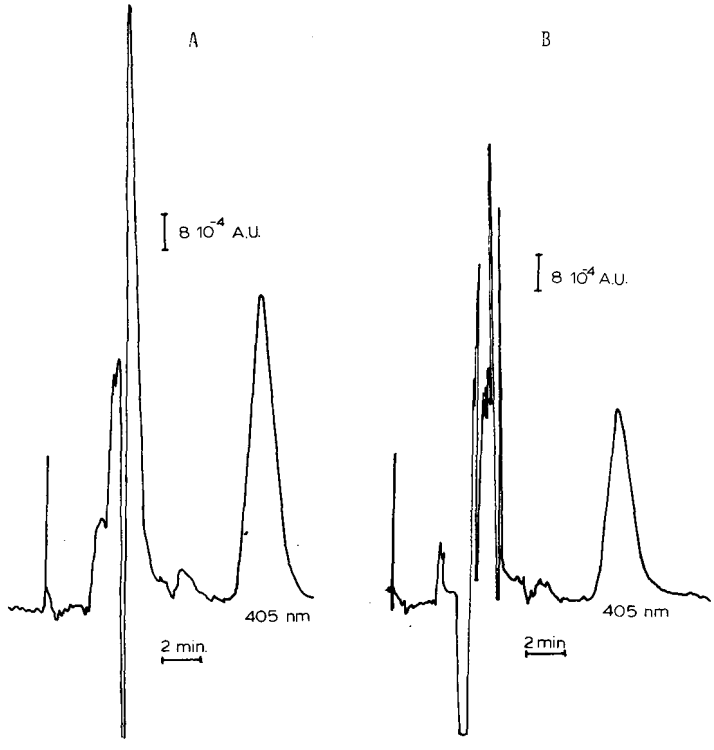

Fig. 4. Influence of pH of mobile phase and extraction buffer on the recovery. (A) Extract of 0.20 μg of standard solution with a mixture (80:20) of methanol—EDTA pH 7.8 as extraction buffer and mobile phase. (B) Extract of 0.20 μg of standard solution with a mixture (80:20) of methanol—water as extraction medium and mobile phase.

observed with patients, the HPLC results being in agreement with those of the bioassay only for some patients. It is well known (Merck Index) that the solubility of amphotericin B in water varies considerably with pH, being insoluble at pH 6—7 and soluble at 0.1 g/l at pH 2 and 12. We considered it important to use extraction and chromatographic conditions of pH compatible with amphotericin B solubility. We have retained pH 7.8 since a more alkaline pH could damage the column. Moreover, the procedure was simplified since we used the same alkaline buffer as mobile phase and for protein precipitation and amphotericin B extraction. Using the experimental conditions, the amphotericin B extraction step was satisfactory and reproducible (Fig. 4) and, since the recovery reached 80% (Table I), our assay was equivalent to previous results [15, 16]. Moreover, in contrast with Golas et al. [16], our HPLC method and the microbiological assays were simultaneously used for 60 patients' serum samples and a good correlation coefficient was obtained. Monitoring the concentration of amphotericin B in biological fluids is the usual way to adjust the drug regimen and it has been considered that we should produce, 1 h after infusion, a serum concentration of about twice the minimal inhibitory concentration of the fungus. However, the clinical usefulness of this approach has been discussed since increasing amphotericin posology was not always accompanied with increased serum concentrations. Most of the administered dose is bound to sterol-containing membranes in different tissues [4, 6, 8]. This rapid, sensitive and reproducible HPLC assay for amphotericin will allow us to determine tissue levels which may be more relevant for the clinical management of a patient [4, 8].

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Note

New sensitive method to determine noscapine in serum by reversed-phase liquid chromatography

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Noscapine is widely used in pharmaceutical preparations for its antitussive effect. Several methods have been described for the analysis of this isoquinoline alkaloid in biological fluids, including fluorometric [1, 2], gas and thin-layer chromatographic [3, 4] and liquid chromatographic [5, 6] techniques. The liquid chromatographic method of Johansson et al. [5] which has a detection limit of 5 ng/ml of plasma is a straight-phase system combined with ultraviolet (UV) detection at 313 nm. It involves several extraction and purification steps and is therefore time-consuming. The reversed-phase system of Jensen [6] is highly dependent on the chromatographic conditions because no internal standard is used. The detection limit at 230 nm is 10 ng/ml of serum.

This paper presents a new, simple and sensitive reversed-phase high-performance liquid chromatographic (HPLC) method for pharmacokinetic study of noscapine in serum with a detection limit of 1 ng/ml. The method is also suitable for analysis of noscapine in serum after oral administration of noscapine embonate.

EXPERIMENTAL

Chemicals

Noscapine hydrochloride and papaverine hydrochloride were of Ph. Eur. grade. Both compounds were dried at 105°C before use. Methanol was of HPLC grade (Rathburn Chemicals). Noscapine embonate (1:1) was synthesized at the analytical laboratory of the University Pharmacy, and had a noscapine content of 45.1% and a particle size of 250 μm. All the other chemicals were of analytical grade.

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Extraction

The extractions were performed on glass columns (20 mm I.D.) packed with Extrelut® (E. Merck).

Chromatographic procedure

The HPLC system consisted of a Pye Unicam PU 4010 pump, LC-XP gradient programmer, PU 4020 UV detector and a Rheodyne Model 7125 injection valve with a sample loop of 50 μl. The chromatograms were recorded on a PU 8251 single-pen recorder. The column was a Hibar LiChrosorb CN (12.5 cm × 4 mm), particle size 5 μm.

The UV detector was set at 220 nm wavelength and 0.005—0.02 a.u.f.s. detector sensitivity range. The flow-rate was 1 ml/min. Peak heights were measured manually.

The mobile phase consisted of 25% methanol and 75% 0.02 *M* disodium hydrogen phosphate and 0.1 *M* sodium perchlorate adjusted to pH 3.8 with 2 *M* phosphoric acid. The time needed to stabilize the column was about 2 h.

Sample preparation

To the serum sample (2.00 ml) were added 400 ng of papaverine hydrochloride (internal standard) dissolved in 1.00 ml of water and 5.0 ml of phosphate buffer pH 4.0 (I = 1.0). The mixture was poured into the column packed with 4.0 g of Extrelut and allowed to adsorb over the supporting matrix for 10—15 min. Noscapine and papaverine were eluted with 25 ml of dichloromethane—butanol (19:1). The eluate was collected in 15-ml conical tubes siliconized before use with Aquasil® and then evaporated to about 2 ml under a stream of warm air (30—40°C), the tubes being in a water bath maintained at 38°C. The residue was evaporated to about 200 μl in the same way but with the bath at 50°C. Then 200 μl of *n*-heptane and 200 μl of 0.33 *M* sodium hydrogen phosphate solution adjusted to pH 2.0 with phosphoric acid were added and the tubes shaken gently by hand for 1 min. After centrifugation for 5 min at 2000 *g* an aliquot of 40—50 μl of the lower phase was injected into the chromatographic system.

The standard curve was prepared by analysis of standard samples containing 2.00 ml of blank serum, 400 ng of internal standard and spiked with noscapine hydrochloride in a concentration range corresponding to 2.9—230 ng of noscapine per ml of serum. The curve was constructed by plotting the peak height ratio of noscapine to the internal standard.

RESULTS AND DISCUSSION

Extraction and purification

Column extraction by dichloromethane—butanol has earlier been used for opiate analyses in biological samples [5, 7]. Jõhansson et al. [5] reported that the absolute recovery from plasma samples spiked with 92 ng of noscapine per ml was 81%. The method includes a complicated purification step from the crude extract.

In the present method noscapine and the internal standard are back-extracted after partial evaporation of the organic phase used in the column

extraction into an aqueous phase which is then directly chromatographed without any further purification. The absolute recovery of noscapine is about 83% and a little more for papaverine.

Chromatography

Typical HPLC chromatograms obtained from blank (A), spiked (B) and volunteers' (C, D) serum are shown in Fig. 1. The eluted peaks are clean, sharp and symmetrical and the blank serum does not show any interfering peaks at all near the elution volumes for drug or internal standard.

The pH and the electrolyte of the mobile phase (sodium perchlorate) have a significant effect on the retention times and symmetry of the peaks. With the conditions used, noscapine and papaverine are almost completely in their ionized forms, and ion-pair formation probably occurs during elution.

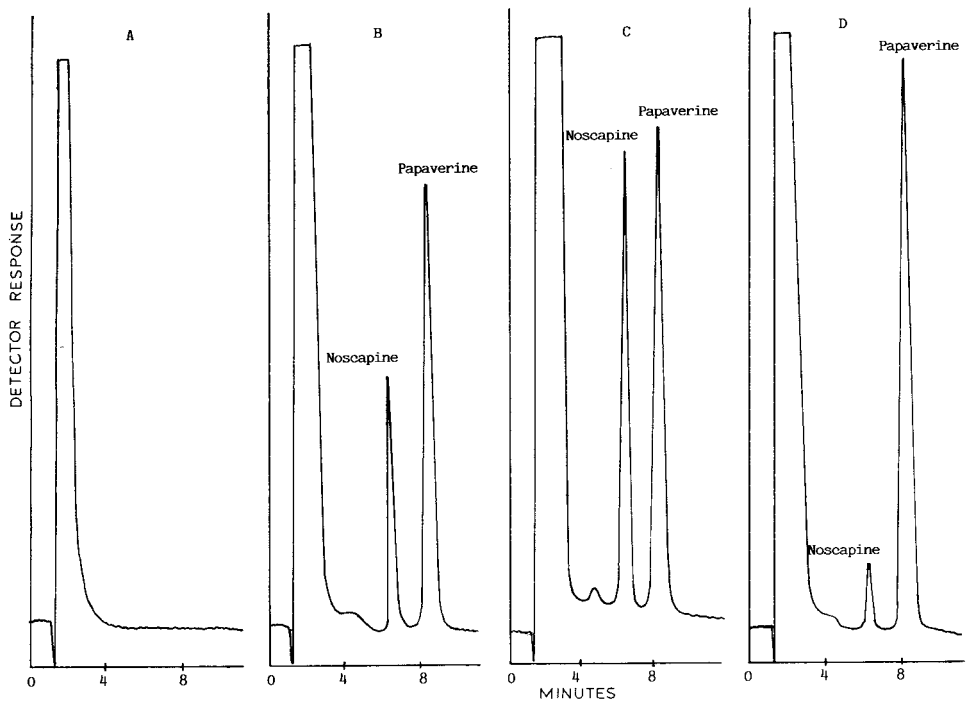

Fig. 1. Chromatograms of noscapine in human serum. (A) Control serum; (B) serum, spiked with 48 ng/ml noscapine with internal standard added; (C) serum, 90 min after an oral dose of 196 mg of noscapine embonate with internal standard added; (D) serum, 360 min after an oral dose of 196 mg of noscapine embonate with internal standard added. Sample size 40—50 µl. Detector sensitivity 0.01 a.u.f.s. See text for other conditions.

Linearity

A straight line was obtained by plotting ng/ml noscapine in serum against the peak height ratio of noscapine to internal standard. The relationship was linear in the concentration range 2.9—230 ng/ml of serum (higher concentrations were not tested). The standard curve had an equation of $y = (1.160 \cdot 10^{-2}x) + (9.85 \cdot 10^{-3})$ and a correlation coefficient of 0.999 ($n = 52$).

Precision

The precision of the method is represented by the relative standard deviation of the mean of replicate assays of the same sample. Because of the simple method used for sample preparation and the high selectivity and sensitivity, precision is good even at low concentrations, as shown in Table I.

TABLE I

PRECISION OF THE METHOD AT DIFFERENT SERUM LEVELS

Drug level (ng/ml)	Number of determinations (n)	Relative standard deviation (%)
184	7	2.2
46	7	3.1
11.5	7	3.3
5.7	5	5.7
2.9	4	9.3

Sensitivity

Using mobile phase as the solvent, noscapine has two absorption maxima, one at 310 nm and the other at 213 nm. The latter is a very strong one with a molar absorptivity of about $6 \cdot 10^4$. To obtain greater baseline stability, detection was performed at 220 nm with a sensitivity 24% below that for the maximum at 213 nm. Based on a signal-to-noise ratio of 3:1, 1 ng/ml was detected with an injection volume of 50 μl and the highest detector sensitivity (X 0.005 a.u.f.s.).

Stability of noscapine in serum

According to earlier reports [5, 6] no transformation of noscapine to noscapic acid occurs within 24 h at 37°C in blood or plasma samples.

In order to investigate the stability of noscapine in frozen serum, five serum samples with different noscapine concentrations (110—223 ng/ml) were stored at −20°C for two weeks. No deterioration of the samples occurred during this time. The average finding was 99.9%.

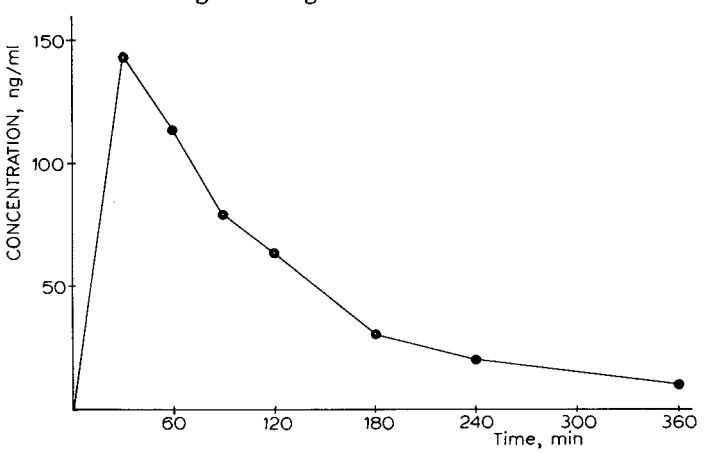

Fig. 2. Serum concentrations of noscapine following an oral dose of 196 mg of noscapine embonate corresponding to 88 mg of noscapine.

Application to biological samples

Serum concentrations of noscapine following an oral dose of 196 mg of nocapine embonate corresponding to 88 mg of nosapine (suspended in 75 ml of water) were monitored in a young healthy volunteer over a 6-h period (Fig. 2). The maximum concentration of noscapine in serum was reached within 30 min.

CONCLUSION

The procedure described in this paper provides a sensitive and reliable method for the determination of noscapine in serum. The preparation of the sample is easy and the rapid chromatographic analysis allows for determination of at least 35 samples in two normal working days.

ACKNOWLEDGEMENTS

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Note

Quantitation of iothalamate in serum and urine by high-performance liquid chromatography

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Assessment of the glomerular filtration rate (GFR) is useful in managing certain critically ill patients, in evaluation of renal function in patients with suspected kidney disease, and to define the role of renal function on drug disposition. The standard method of evaluating GFR is by measuring the renal clearance of endogenous creatinine [1—3]. This process is subject of permutation by ingested creatinine, has a diurnal variation, and varies with the degree of muscular exertion. Renal clearance of creatinine, usually in 24 h, depends on the quantitation of the metabolite by a relatively specific, colorimetric assay [4].

Certain xenobiotics are not metabolized and are cleared exclusively by renal excretion. Iodinated aromatic compounds used as contrast reagents for intravenous angiography and pyelography are in this class. Clearance of these compounds has been used to assess GFR, and the estimate of this function correlates well with the standard creatinine clearance. Radionuclide-labelled compounds ([^{125}I]iothalamate, for example) were used [5, 6] with the concentration of the drug in serum determined from the contained radioactivity. This method has several disadvantages: repeated administration exposes the patient to radiation, the specific activity changes over short intervals, and special precautions are required to dispose of serum, urine, and equipment containing the radionuclide.

Two high-performance liquid chromatographic (HPLC) assays for

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iothalamate have been developed [7, 8]. One requires large sample volume ($\geqslant$ 0.5 ml) and involves a tedious extraction procedure. The other, recently published, is similar to this method but does not utilize dual-wavelength monitoring.

We developed a rapid and sensitive HPLC assay for iothalamate. This procedure utilizes dual-wavelength monitoring to ensure specificity which uses the common 254 nm detector and more sensitive 229 nm detector. This method also uses Hypaque (iodinated aromatic contrast reagent) as the internal standard.

MATERIALS AND METHODS

Separation was achieved by reversed-phase HPLC. A dual-piston Constametric pump (Laboratory Data Control/Milton Roy, Riviera Beach, FL, U.S.A.) delivered the mobile phase to a 5-μm Radial-Pak C_{18} cartridge (10 cm $\times$ 8 mm I.D.) held under radial compression by an RCM-100 radial compression module (both from Waters Assoc., Milford, MA, U.S.A.). Two fixed-wavelength UV III monitors connected in series measured absorbance of the eluate at 229 nm and 254 nm. A separate Spectral Supply high-voltage power source is required for the 229-nm Cd lamp (all Laboratory Data Control/Milton Roy). The 10-mV signals generated by the detectors were delivered to an Omniscribe B-5217-5 dual-pen strip chart recorder (Houston Instruments, Austin, TX, U.S.A.). Samples were either injected with a Model 7010 sample injection valve fitted with a 20-μl sample loop and Model 7011 loop filler port (Rheodyne, Berkeley, CA, U.S.A.) or 20 μl were injected with a WISP (Waters intelligent sample processor, Waters Assoc.).

Sodium iothalamate comes in the form of a U.S.P. 66.8% (w/v) solution for injection labelled as Conray-400 (Mallinckrodt, St. Louis, MO, U.S.A.). The concentration in each lot of the formulation was determined from its molar extinction coefficient ($E = 3.3 \cdot 10^4$ l/mol/cm at pH 6 at 240 nm.).

Analytical reagent grade Type I water produced by the Milli-Q system (Millipore, Bedford, MA, U.S.A.) was used to prepare all aqueous solutions unless otherwise indicated in the text.

Ultrapure 70% perchloric acid (Alpha Products, Danvers, MA, U.S.A.) was diluted with Type I water to give a 6% (w/v) solution and was used as a serum protein precipitant. The internal standard solution consisted of sodium diatrizoate in a U.S.P. 50% injection (Hypaque from Winthop Labs., New York, NY, U.S.A.) which was diluted with 6% perchloric acid to yield a final concentration of 12.5 μg/ml and was stored at 4°C.

A 4% (w/v) solution of detergent to decrease protein binding of the compound was made by dissolving electrophoresis-pure sodium dodecyl sulfate (BioRad Labs., Richmond, CA, U.S.A.) in Type I water. This was stored in an amber bottle at room temperature.

A 40 mmol/l potassium phosphate buffer, pH 6.0, was prepared from analytical reagent grade monobasic and dibasic potassium phosphate salts (Mallinckrodt) and Type I water. Phosphoric acid and/or aqueous potassium hydroxide were added, if necessary, to achieve pH 6.0. It was filtered using a

0.22-μm MF-Millipore Type GS filter membrane (Millipore) immediately prior to its use in the assay. The buffer was stored in a tightly capped amber bottle at room temperature. This buffer was used within one week.

Iothalamate standards

Fresh standards were prepared daily or aliquots were stored at −70°C. An iothalamate solution of 113.6 μg/ml was prepared in distilled water. A 1:1 dilution of this working standard and distilled water yielded three standards used for this assay with the following concentrations: 113.6, 56.8 and 0 μg/ml. The absorbance of 1:50 dilutions of each standard was measured at 240 nm with an Acta III spectrophotometer (Beckman Instruments, Fullerton, CA, U.S.A.). This step was used to verify the accuracy of the dilutions.

Sample preparation

Serum (25 μl) was added to 25 μl of 4% SDS solution (25 μl of distilled water were added to aqueous samples). This was mixed and diluted with 50 μl of internal standard solution. This mixture was mixed vigorously for 10 sec and then centrifuged (10,000 *g*) with an Eppendorf centrifuge (Brinkman Instruments, Westbury, NY, U.S.A.) for 3 min. If the supernatant was cloudy, the centrifugation was repeated.

Assay

A 25-μl aliquot of the supernatant was injected into the chromatograph and eluted with the mobile phase at a flow-rate of 3 ml/min at room temperature. Approximate column back-pressure was 124—138 bars. Detector sensitivity was set at 0.016 absorbance units full scale (a.u.f.s.) with a chart speed of 0.5 cm/min. We found that recycling the mobile phase during the assay did not affect resolution or retention times but considerably saved buffer usage. Fresh buffer was used daily. Precolumns with 30—50 μm C_{18} Corasil packing material (Waters Assoc.) protected the analytical column. These precolumns were routinely changed after approx. 100 injections. Columns were washed with filtered Type I water and a mixture of glass-distilled acetonitrile—Type I water (50:50, v/v). When the WISP was utilized, 200 μl of dimethyl sulfoxide were injected when the water wash was employed. This reduced back-pressure and maintained column's resolution. The duration of reliable use of the analytical column varied from 300 to 500 samples.

Calculations

Retention times of iothalamates and internal standard were 10 and 8.25 min, respectively. Chromatograms of an aqueous and a serum sample are depicted in Fig. 1. A standard curve from aqueous standards was determined daily from the ratio of iothalamate:internal standard peak heights and known concentrations. Iothalamate concentrations of unknown specimens were determined from the above standard curve. Specificity was insured by dual-wavelength monitoring. Interferences by other compounds with iothalamate can be detected by a deviation that is twice the standard deviation of the 229/254 nm peak height ratio. The 229/254 nm peak height ratio of iothalamate is 1.11 ± 0.03.

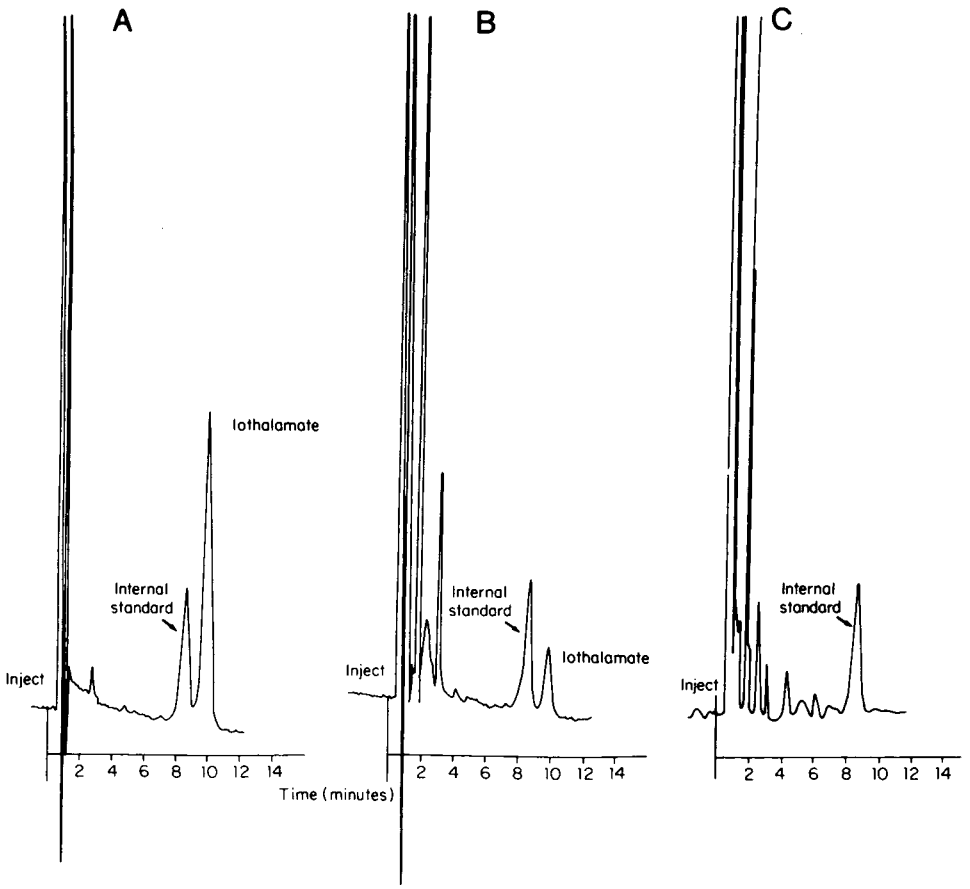

Fig. 1. Chromatogram of (A) an aqueous iothalamate standard, 113.6 μg/ml; (B) a serum sample with calculated iothalamate concentration of 12.5 μg/ml; (C) a serum sample without iothalamate. In all chromatograms, absorbance is at 229 nm with 0.02 a.u.f.s. All samples were prepared as described in Materials and methods.

Linearity

Serial dilutions of iothalamate working standards were made in distilled water to concentrations of 113.6 (no dilution), 56.8, 28.4, 7.1, 0.89, and 0.44 μg/ml. Iothalamate:internal standard peak height ratios were calculated and linear regression was done to calculate the slope, y-intercept and correlation coefficient (r^2).

Precision

Iothalamate was diluted in pooled human serum to three concentrations: 27.5, 50, and 15 μg/ml. Two concentrations, 15 μg/ml (low) and 50 μg/ml (high) were aliquoted and frozen at −70° C. An aliquot for both concentrations was assayed daily which determined run-to-run precision. With statistical analysis, a coefficient of variation was calculated. The 27.5 μg/ml serum solution was assayed ten times on the same day which determined within-run precision. Again, with statistical analysis, a coefficient of variation was calculated.

Accuracy

Recoveries from serum were determined by comparing the peak height ratios of aqueous solutions and serum solutions at the same concentration. Recovery from urine was also determined.

Sensitivity

An aqueous standard was serially diluted with water and assayed until a signal was produced that was twice the background noise. This concentration was defined as lowest sensitivity level of the assay.

Stability

Serum aliquots that were used for precision analysis were stored under four temperature conditions: room temperature (approx. 21°C), 4°C, −20°C, and −70°C. One aliquot was removed from the storage conditions and assayed for iothalamate at zero time, 2, 4, 8, 24, 48, and 72 h, and 1, 2, 4, and 8 weeks.

RESULTS AND DISCUSSION

Linearity

For the concentrations given in Materials and methods (n = 6), a linear regression analysis described the line $y = 0.0319x + 0.0255$ with a correlation coefficient (r^2) of 0.9996. For samples with concentrations greater than 125 μg/ml, dilution to a concentration less than 125 μg/ml was performed.

Precision

Mean values of aliquoted serum samples described in Materials and methods with standard deviations (S.D.) are shown in Table I. Within-run coefficient of variation (C.V.) was 1.58%. The run-to-run coefficients of variation for the high and low concentrations were 3.6% and 5.3%, respectively (n = 25).

TABLE I

PRECISION

Concentrations are given in μg/ml.

	Within-run	Run-to-run	
		High	Low
n	10	25	25
Mean	27.75	50.54	16.19
S.D.	0.43	1.86	0.86
C.V.* (%)	1.58	3.60	5.31

*Coefficient of variation expressed as a percentage of the mean.

Accuracy

Initial mean recoveries of serum were 92% or less. We enhanced recovery by adding 4% (w/v) solution of sodium dodecyl sulfate. The mean recovery improved to 99.8% for serum, while the mean recovery for urine was 99.5%. Measured versus assayed concentrations and recoveries are depicted in Table II.

TABLE II

ACCURACY

Concentrations are given in μg/ml.

Serum			Urine		
Measured concentration	Assayed concentration	Percentage recovered	Measured concentration	Assayed concentration	Percentage recovered
113.6	111.2	97.9	56.8	60.6	106.7
56.8	57.1	100.5	45.4	46.3	102.0
28.4	29.4	103.5	34.1	34.4	100.9
7.1	6.9	97.2	22.7	22.3	98.2
			11.4	12.0	105.3
	Mean =	99.8	56.8	58.4	102.8
			45.4	45.9	100.9
			34.1	32.5	95.3
			22.7	21.3	93.8
			11.4	10.2	89.5
				Mean =	99.5

Sensitivity

The assay was sensitive to 0.3 μg/ml as defined in Materials and methods.

Stability

The stability study at the four different temperatures was conducted for two months. We found no significant difference in the peak height ratios or calculated concentration at any of the temperature conditions after two months of storage as noted. In addition, iothalamate was stable in serum at −70° C for at least twelve months.

A non-radioactive assay of iothalamate is extremely beneficial for the determination of GFR and renal function. The method described here is simple and rapid, yielding results with minimal sample preparation. The small volume required (25 μl) lends itself to pediatric populations. Except for the initial investment of capital equipment, the assay is relatively inexpensive. The assay is well characterized and consistent over time. Interferences from co-eluting compounds have not been encountered even with dual-wavelength monitoring. Since the eluate is totally aqueous, most drugs tend to be retained by the C_{18} column. One would assume that chromatographic peaks from these compounds would only temporarily offset the baseline and not affect the integrity of the iothalamate or internal standard peak. Loss of resolution between iothalamate and the internal standard peaks or high back-pressure indicated a new column was needed.

This method precludes the necessity of using radionuclide-labelled compounds in the assessment of GFR, and thus eliminates the problems inherent with using them. Urine collection, a potential problem in many cases, is circumvented by utilizing the single-injection method of assessing GFR. This allows for easily repeated evaluations when necessary.

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CHROMBIO. 2374

Letter to the Editor

Quantitative analysis of (3-methoxy-4-sulphooxyphenyl)ethylene glycol (MHPG sulphate) in human urine

Sir,

We have read with interest the communication by Eichholtz et al. [1] which appeared recently in this journal. The authors describe a method for measuring (3-methoxy-4-sulphooxyphenyl)ethylene glycol (MHPG sulphate) in urine using gas—liquid chromatography (GLC) and state that this method is an improvement on that developed several years previously by ourselves [2]. We feel that the criticisms of the earlier work given by Eichholtz et al. [1] in justifying this statement are not valid.

Firstly, our procedure uses combined gas chromatography—mass spectrometry (GC—MS) with selected-ion monitoring, a very sensitive technique which is much more specific than GLC alone. Secondly, we synthesised a deuterated analogue of MHPG sulphate for use as an internal standard in the GC—MS assay [2]. This is an ideal internal standard in that it parallels as closely as is possible losses of MHPG sulphate through the analytical procedure. Thus, overall recovery, as long as it is not so low as to limit the sensitivity of the assay, becomes irrelevant. The method of Eichholtz et al. [1] does not incorporate an internal standard and a standard curve is not constructed. Instead, exogenous MHPG sulphate is added to duplicate urine samples as a recovery standard. Thirdly, we have made a detailed study of the reaction of a range of sulphate esters with trifluoroacetic anhydride, pentafluoropropionic anhydride and heptafluorobutyric anhydride and have found that aromatic sulphates readily form perfluoroacyl derivatives of the parent alcohol in quantitative yield [3]. Thus, MHPG sulphate can be readily converted to a perfluoroacyl derivative of MHPG without the need for an enzymatic hydrolysis step, which can often be inefficient and introduce impurities.

We feel that the superiority of the earlier method is demonstrated by the fact that while the procedure of Eichholtz et al. [1] has a standard deviation of ± 20%, our method [2] gives a standard deviation of ± 2%. The time taken for each of the analyses would appear to be about the same (2—3 days) and the only obvious advantage of the method of Eichholtz et al. [1] is that less expensive analytical equipment is required.

Eichholtz et al. [1] report that they developed a new assay for MHPG

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sulphate in urine because they believe that this conjugate is formed predominantly in the central nervous system and may therefore be used as a measure of central catecholamine turnover. We [4] and other groups have shown this theory to be incorrect.

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CHROMBO. 2395

Letter to the Editor

Quantitative analysis of (3-methoxy-4-sulphooxyphenyl)ethylene glycol (MHPG sulphate) in human urine

Reply to Murray and Davies

Sir,

We thank Drs. Murray and Davies for their reaction to our communication on the direct determination of MHPG sulphate [1]. There is no doubt about the sensitivity of the method used by Murray et al. [2].

In fact the method needs to be very sensitive because of the low recovery of the extraction procedure and the perhaps not complete fluoridation of MHPG sulphate, at least in our hands. Therefore, after hydrolysis of the sulphate conjugate we fluoridated MHPG itself and used dissolved MHPG piperazine (Sigma) as a reference standard.

Besides this, our method does not require very complex and expensive equipment, nor a laboratory licensed for radioactive activities. The overall standard deviation of about 20% is high, but results in volunteers do not seem to differ much from that found by other authors, Murray et al. included [1]. This should, however, not be compared with a simple test—retest reliability.

Although our method has some disadvantages we think it can be used in clinical research.

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