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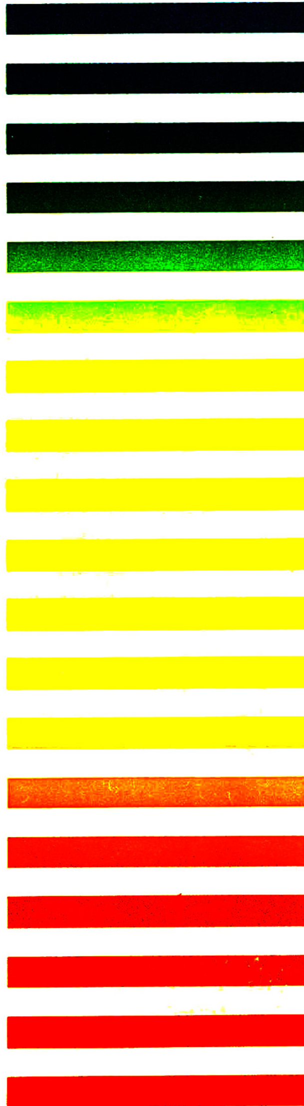

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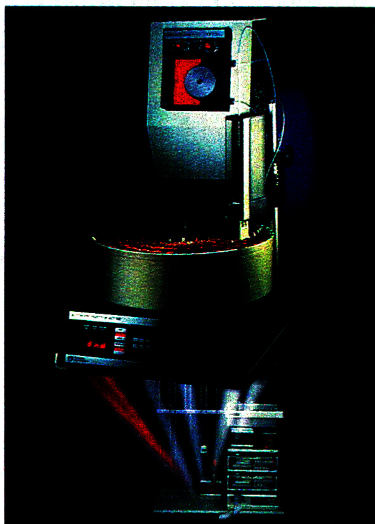

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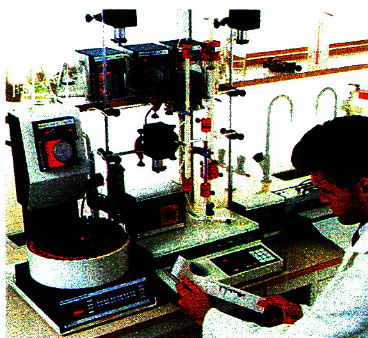

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RAPID AND SELECTIVE DERIVATIZATION METHOD FOR THE NITROGEN-SENSITIVE DETECTION OF CARBOXYLIC ACIDS IN BIOLOGICAL FLUIDS PRIOR TO GAS CHROMATOGRAPHIC ANALYSIS

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(Received May 11th, 1984)

SUMMARY

A rapid and selective derivatization procedure is described for the pre-column labelling of carboxylic acids with a nitrogen-containing label. The carboxylic acid function is activated with 2-bromo-1-methylpyridinium iodide and the activated carboxylic acid function reacts with a primary or a secondary amine to yield an amide. With flurbiprofen as the test compound and dipropylamine as a label the acid was completely converted to the corresponding amide. The method was tested for several aliphatic, aromatic and for phenylacetic or phenylpropionic carboxylic acid derivatives, and was found to result in the complete derivatization of these compounds with a few exceptions only. The derivatization procedure is potentially useful for drug monitoring purposes, as is shown with the analysis of valproic acid and flurbiprofen in plasma.

INTRODUCTION

A number of derivatization methods have been described for derivatization of carboxylic acids, in order to improve the chromatographic behaviour and/or to lower the detection limit in gas chromatographic (GC) systems [1]. Alkylation with different types of alkyl halides such as methyl iodide [2], butyl iodide [3, 4], phenacyl bromide [5] or pentafluorobenzyl bromide [6, 7] as derivatization reagent is the most widely used method for the GC determination of compounds with a carboxylic acid function. In some cases other alkylation reagents such as diazomethane [8] or diazopropane [9], or silylation reactions [10] are used. None of these reagents is selective for the carboxylic acid function. Many carboxylic acids, including therapeutically important compounds, depend for a sensitive GC analysis on detector-orientated derivatization techniques. Methods aimed at improved detection

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with the nitrogen—phosphorus thermionic detector are scarce in the literature.

Schulz and Vîlceanu [11] described the reaction with dimethyl-α-hydroxymethanephosphonate, but this reagent is not easily accessible. In the present study the application of a selective derivatization reaction [12] for the sensitive detection of compounds with a carboxylic acid function is investigated. The method is based on the formation of an amide by coupling the acid with simple aliphatic amines (e.g. dipropylamine or diethylamine).

EXPERIMENTAL

Materials

Butylamine, dibutylamine, dipropylamine, ethylamine, ninhydrin and propylamine, all gold-label quality, were obtained from Janssen Chimica (Beerse, Belgium). Diethylamine, 2-bromopyridine and methyl iodide came also from Janssen Chimica. Benzoic acid, sorbic acid and triethylamine came from E. Merck (Darmstadt, F.R.G.), naproxen from UCB (The Hague, The Netherlands) and flurbiprofen from Boots (Vianen, The Netherlands). Sodium valproate was obtained from Albic (Maassluis, The Netherlands). Acetonitrile, chloroform, dichloromethane, diethyl ether, dimethylformamide, hexane, methanol and toluene, all analytical-reagent grade, were purchased from J.T. Baker (Deventer, The Netherlands) and were distilled from glass before use. The methanol for GLC analysis was obtained from Mallinckrodt (St. Louis, MO, U.S.A.) as nanograde quality. The other compounds used in this study came from various sources or were obtained as gifts from various companies and used as such.

2-Bromo-1-methylpyridinium iodide (BMP) was synthesized following the procedure described by Saigo et al. [13] for the synthesis of 2-chloro-1-methylpyridinium iodide.

The amide of dipropylamine and flurbiprofen (FbDPA) was synthesized on a preparative scale. Purification of the reaction product was performed with thin-layer (TLC) and column chromatography. The resulting product was recrystallized twice from ethanol and stored over phosphorus pentoxide. Its identity was conformed by infrared spectrometry and mass spectrometry.

Gas chromatography

Two gas—liquid chromatographic (GLC) systems were used. System I consisted of an Intersmat IGC 16 (Intersmat Instruments, Pavillons sous Bois, France) equipped with dual flame-ionization detectors. The glass columns (2 m × 1.8 mm I.D.) were packed with 3% OV-17 on 100—120 mesh Chromosorb W HP (Chrompack, Middelburg, The Netherlands). The carrier gas (nitrogen) flow-rate was 20 ml/min, the hydrogen flow-rate 30 ml/min and the air flow-rate 300 ml/min. The injection port and detector temperatures were 290°C and 330°C, respectively.

System II consisted of a Hewlett-Packard HP 5710 A (Hewlett-Packard, Avondale, PA, U.S.A.) equipped with a dual nitrogen—phosphorus flame-ionization detector, Model 18789 A (Hewlett-Packard). The glass column (1.4 m × 1.9 mm I.D.) was packed with 3% SP-1000 on 100—120 mesh Chromosorb W HP (Chrompack). The carrier gas (nitrogen) flow-rate was 52

ml/min, the hydrogen flow-rate 3.8 ml/min and the air flow-rate 50 ml/min. The injection port and detector temperatures were 300°C.

Thin-layer chromatography

TLC plates (E. Merck) of 5 × 10 cm and 20 × 20 cm, precoated with silica gel 60F and a layer thickness of 0.25 mm, were used. Reactions were performed in solutions originally containing 10 μg of carboxylic acid and were analysed by spotting an aliquot of these reaction mixtures on the plate together with solutions of the acid under investigation and the reagent mixtures, respectively. The plates were developed in the ascending mode with eluents consisting of chloroform—methanol or chloroform—hexane mixtures. After evaporation of the eluent the spots were vizualized under ultraviolet radiation of 254 nm or by spraying with a 10% solution of ninhydrin in ethanol.

DERIVATIZATION PROCEDURES

BMP method A

To 10 μl of a solution of 0.01—1.0 μg of carboxylic acid in dichloromethane in a 1.5-ml polypropylene tube, also containing a suitable internal standard, 10 μl of BMP solution (10 μg/μl in acetonitrile), 20 μl of dipropylamine solution (label) (10 μg/μl in dichloromethane), 60 μl of dichloromethane and 10 μl of acetonitrile were added. After vortex mixing for 15 sec the mixture was allowed to stand for 5 min at 5°C. Then 500 μl of dichloromethane were added and the mixture was extracted three times with 500 μl of 2 *M* sulphuric acid. The organic phase was transferred to another polypropylene tube and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 20 μl of methanol; 1-μl portions of the resulting solutions were injected into the chromatograph.

BMP method B

To 10 μl of a solution of 1—10 μg of carboxylic acid in dichloromethane, acetonitrile or dimethylformamide, 30 μl of a BMP solution (10 μg/μl in acetonitrile) and 40 μl of a solution of the label (10 μg/μl in dichloromethane) were added. After vortex mixing for 15 sec the mixture was allowed to stand for 15 min at room temperature. For TLC analysis the mixture was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 50 μl of methanol and 10 μl were spotted on a thin-layer plate. For GLC analysis the mixture was extracted with sulphuric acid and further processed as described under *BMP method A*.

RESULTS AND DISCUSSION

The derivatization procedure

Flurbiprofen, unless mentioned otherwise, was used as a test compound in the derivatization studies with simple aliphatic primary or secondary amines. The derivatization procedure is based on activation of the carboxylic acid function with BMP [12]. Without activating either the carboxylic acid or the amine (reagent) function only minute amounts of the amide are formed under

the mild reaction conditions used in this study. Instead of an amine reagent it is also possible to use a primary alcohol as the reagent [12].

The BMP method as described above was developed by us, based on the work of Saigo et al. [13]. In the BMP method the reaction is base catalysed. However, it was not necessary to add a base (e.g. triethylamine) to the mixture because of the alkaline reaction of the label (e.g. dipropylamine, diethylamine) itself.

In Table I the influence of the solvent on the derivatization yield is summarized. Because BMP is insoluble in many organic solvents but soluble to a concentration of 10 $\mu g/\mu l$ in acetonitrile, this BMP solution was used in combination with the other solvents of Table I. Dipropylamine was used as the label and derivatization method B was used. The derivatization yield was measured by comparison of the peak height ratio of the amide, FbDPA, and the amide of the internal standard (naproxen), which was purified in the same way as described for the purification of FbDPA in this paper, after GLC analysis (system II). Furthermore, the disappearance of flurbiprofen in the derivatization mixture was followed by means of TLC analysis and GLC analysis (system I).

In Table II the influence of the choice of the label on the derivatization yield is summarized. In some cases (e.g. methylamphetamine), it was necessary to add triethylamine, as a base catalyst, to the reaction mixture to achieve 100% conversion to the amide. If simple aliphatic primary or secondary amines

TABLE I

INFLUENCE OF THE SOLVENT ON THE DERIVATIZATION YIELD

Solvent	BMP method A*	Solvent	BMP method A
Acetonitrile	+	Dimethylformamide	+
Butyronitrile	+	Hexane	+/—
Chloroform	+/—	Methanol	—
Dichloromethane	+	Pyridine	—
Diethyl ether	—	Toluene	+/—

*+ = reaction is quantitative, +/— = reaction takes place, but is not quantitative, — = reaction does not take place.

TABLE II

INFLUENCE OF THE LABEL ON THE DERIVATIZATION YIELD

Label	BMP method B*	Label	BMP method B
1,3-Diallyl-6-aminouracil	—	Ethylamine	+
6-Amino-1,3-dimethyluracil	—	Diethylamine	+
Amphetamine	+	Propylamine	+
N-Methylamphetamine	+	Dipropylamine	+
4-Aminoantipyrine	—	Butylamine	+
Guanidine	—	Dibutylamine	+
Guanine	—	Tributylamine	—

*+ = reaction is quantitative, — = reaction does not take place.

(second column of Table II) were used it was never necessary to add base to the mixture. The tertiary amine tributylamine did not react, as expected. The use of a secondary amine in the derivatization reaction is to be preferred to the use of a primary amine, because of the almost two-fold increase in response of the nitrogen—phosphorus detector observed with the former.

A number of other compounds with carboxylic acid functions and some compounds with other acidic functions were tested. The reaction mixtures were investigated by TLC and GLC (system II) analysis with a programmed oven temperature from 100°C to 270°C (10°C/min). The appearance of a new spot in the thin-layer chromatogram and/or a new peak in the gas chromatogram, together with the disappearance of the acid spot in the thin-layer chromatogram were considered as evidence for conversion of the acid under investigation into the corresponding amide. The complete disappearance of the acid spot from the TLC chromatogram and the absence of the acid peak in the GLC chromatogram (system I) indicated that at least 90% of the acid had been converted. In all cases reagent blanks were analysed. Almost all of the carboxylic acids thus tested were completely converted to the corresponding amide. Phthalic acid, a dicarboxylic acid, and gallic acid, a carboxylic acid containing three phenolic functions, did not yield detectable amounts of derivatives. The results are summarized in Table III. The compounds with

TABLE III

DERIVATIZATION OF DIFFERENT ACIDIC FUNCTIONS*

Compound	TLC	GLC	Compound	TLC	GLC
1a. Aliphatic carboxylic acids			1b. Aliphatic carboxylic acids with aromatic groups		
Acetic acid	0	+/—	Diclofenac	+	+
Lauric acid	0	+	Flurbiprofen	+	+
Myristic acid	0	+	Ibuprofen	+	+
Sorbic acid	+	+	Indomethacin	+	0
Stearic acid	0	+	Naproxen	+	+
Valproic acid	0	+	Phenylacetic acid	+	+
2. Aromatic carboxylic acids			3. Carboxylic acids with other acidic or basic functions		
Benzoic acid	+	+	*m*-Aminobenzoic acid	+	0
Nalidixic acid	+	0	ε-Aminocaproic acid	+	0
Nicotinic acid	+	+	Gallic acid	—	—
Phthalic acid	—	—	Salicylic acid	+/—	+/—
Probenecid	+	+	*p*-Sulphamoyl benzoic acid	+	0
4. Compounds with other (than carboxylic) acidic functions					
Barbituric acid	—	—			
Mercaptopurine	—	—			
Phenol	—	—			
Purine	—	—			
Salicylamide	—	—			
Sulphanilic acid	—	—			

*+ = reaction is quantitative, +/— = reaction takes place, but is not quantitative, — = reaction does not take place, 0 = derivative not detectable with the chosen chromatographic system.

acidic functions other than the carboxylic acid function were not derivatized to any detectable degree with this method (Table III).

For subsequent GLC studies a mixture of dichloromethane—acetonitrile was used as the solvent and simple aliphatic secondary amines as labels (BMP method A). The amount of BMP added to the mixture was reduced and the influence of the reaction temperature was investigated. The reaction between flurbiprofen and dipropylamine, performed under the conditions described under *BMP method A*, was completed within 2 min at 5°C. This high reaction rate, even at low temperatures, is an important advantage over many other derivatization reactions, particularly in the case of thermolabile compounds. The reaction could also be performed at room temperature with satisfactory results, but at higher temperatures lower reaction yields were obtained.

GLC analysis

Representative chromatograms from the GLC analysis (system II, column temperature 255°C) of the derivatization mixtures, obtained after reacting flurbiprofen and naproxen (internal standard) with dipropylamine by BMP method A, are shown in Fig. 1. Instead of 3% SP-1000, it was also possible to use 3% OV-17 or 10% Carbowax 20M + 2% potassium hydroxide as the stationary phase. The detection limit of the amide (FbDPA) was about 60 pg with a signal-to-noise ratio of 2. To test the reproducibility of the method nine samples containing 1 μg of flurbiprofen were derivatized following the procedure proposed above. The mean value and the relative standard deviation

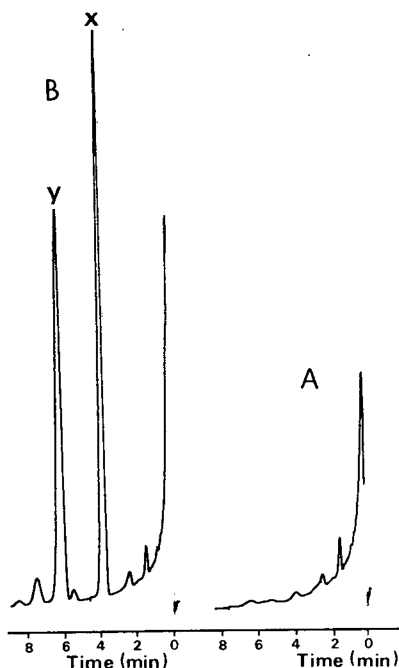

Fig. 1. Chromatograms obtained after derivatization of flurbiprofen with dipropylamine. (A) Reagent blank; (B) chromatogram after derivatization of 0.5 μg of flurbiprofen and 0.5 μg of naproxen following BMP method A. x = FbDPA; y = dipropylaminenaproxate.

of the peak height ratio of FbDPA and the amide of the internal standard were 1.60% and 3.8%, respectively.

The usefulness of the procedure in quantitative analysis was further tested by the analysis of benzoic acid after reaction with diethylamine as label and sorbic acid as the internal standard. GLC system II was used with 10% Carbowax 20M + 2% potassium hydroxide as the stationary phase. After an isothermal period of 6 min at 175°C the oven temperature was programmed to rise by 31°C/min to 240°C. The retention times of the amides of benzoic acid and sorbic acid were 348 and 262 sec, respectively.

The calibration curve showed good linearity; Y = 0.02 (± 0.01) + 1.08 (± 0.02)X (r = 0.997) was the equation for the calibration line after analysing ten samples containing 0.1—1.0 μg of benzoic acid, Y and X being the peak height ratio of the benzoic acid and sorbic acid peaks and the benzoic acid concentration (μg/μl), respectively. The numbers in parentheses are the standard deviations.

Analysis of flurbiprofen and sodium valproate in plasma samples

The potential usefulness of the derivatization with secondary amines for the analysis of carboxylic acids in blood plasma was investigated with flurbiprofen, an analgesic anti-inflammatory drug, and with sodium valproate, an anticonvulsant drug, as test compounds.

Plasma samples of 50 μl, spiked with 0.1—1.0 μg of sodium valproate and 0.5 μg of sorbic acid as the internal standard were acidified with 10 μl of 4 *M* hydrochloric acid and then extracted with 250 μl of chloroform. After 1 min vortex-mixing and 5 min centrifugation (2500 *g*) the aqueous layer was discarded; the chloroform layer was transferred to a clean vial and evaporated to dryness under a stream of nitrogen. The residue was derivatized according to

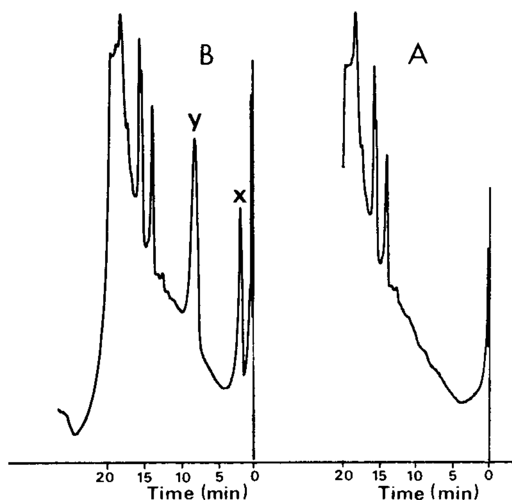

Fig. 2. Chromatograms obtained after extraction of sodium valproate from plasma and derivatization with diethylamine. (A) Plasma blank; (B) chromatogram of a 50-μl plasma sample containing 0.01 μg/μl valproate and 0.01 μg/μl sorbic acid subjected to BMP method A. x = diethylaminevalproate; y = diethylaminesorbate.

BMP method A with diethylamine, and of the final solution 1 μl was subjected to GLC analysis (system II). After an isothermal period of 4 min at 125°C, the oven temperature was programmed to rise by 10°C/min to 270°C. The equation for the calibration line was $Y = -0.08\ (\pm\ 0.04) + 2.04\ (\pm\ 0.07)X$ (r = 0.995, n = 8). A blank plasma sample was treated in the same way. Chromatograms are shown in Fig. 2.

The second application was the analysis of flurbiprofen in plasma. A plasma sample of 50 μl, containing 0.05–0.5 μg of flurbiprofen and 0.5 μg of naproxen as internal standard, was acidified with 10 μl of 4 *M* hydrochloric acid and extracted with 200 μl of dichloromethane. After 1 min vortex-mixing and 5 min centrifugation (2500 *g*) the aqueous layer was discarded and the dichloromethane layer was transferred to a clean vial and evaporated to dryness under a stream of nitrogen. The residue was derivatized with diethylamine according to BMP method A and 1 μl of the final solution was subjected to GLC analysis (system II) with an isothermal oven temperature of 255°C. The equation for the calibration line was $Y = -0.01\ (\pm\ 0.01) + 2.28\ (\pm\ 0.05)X$ (r = 0.998, n = 8).

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DEVELOPMENT AND VALIDATION OF A METHOD FOR MEASURING THE GLYCINE AND TAURINE CONJUGATES OF BILE ACIDS IN BILE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY*

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SUMMARY

We developed and validated a simple method for measuring the individual glycine and taurine conjugates of bile acids in bile by high-performance liquid chromatography with a C_{18} reversed-phase column using an isocratic solvent system of acidified methanol—potassium phosphate. Without preliminary derivatization or purification, complete separation of the ten major conjugated bile acids present in bile could be achieved in 65 min. Total bile acid concentrations were identical when measured enzymatically and by summing the individual bile acids determined by high-performance liquid chromatography. Bile acid composition determined by gas—liquid chromatography correlated with results by high-performance liquid chromatography. Finally, measurements of individual glycine and taurine conjugates in human bile and in mixtures of bile acid standards by high-performance liquid chromatography and thin-layer chromatography gave similar results. This high-performance liquid chromatographic system permits simultaneous quantification of total and individual bile acids and their glycine and taurine conjugates in bile.

INTRODUCTION

A thorough description of bile acid metabolism in man requires measurement of total bile acid concentration, bile acid composition, and the glycine and taurine conjugates of bile acids in bile. These analyses generally require extensive preparative techniques and separate methodologies. For example, total bile acid concentration is usually measured by enzymatic assay while bile acid composition is commonly determined by gas—liquid chromatography

*A part of this work was published in abstract form [1].

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(GLC) after hydrolysis and chemical derivatization. Measurement of the individual glycine and taurine conjugates of bile acids in bile most frequently involves two separate procedures: initial separation by thin-layer chromatography (TLC) and subsequent quantitation by enzymatic assay. These techniques, while well established, require different equipment, utilize separate preparative procedures that can result in artifacts or incomplete recovery, and, taken together, can be quite time-consuming. Therefore, our aim was to develop and validate a single method for separating and quantifying the glycine and taurine conjugates of bile acids in bile using high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Reagents

HPLC-grade organic solvents were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Bile acid standards

Conjugated bile acid standards were purchased from Calbiochem (La Jolla, CA, U.S.A.) and Steraloids (Wilton, NH, U.S.A.). Additional samples of taurocholic acid (TC), taurochenodeoxycholic acid (TCDC), taurodeoxycholic acid (TDC), and tauroursodeoxycholic acid (TU) were kindly provided by Dr. Martin C. Carey and glycoursodeoxycholic acid (GDC) was kindly provided by Dr. Alan F. Hofmann. All standards were $>$ 97% pure by HPLC. [^{14}C]Chenodeoxycholic acid (50 mCi/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.) and was conjugated to glycine as previously described [2]. [^{14}C]Glycochenodeoxycholic acid (GCDC) was greater than 98% pure by TLC in appropriate solvents.

Chromatographic apparatus

We used a Hewlett-Packard 1084 liquid chromatograph (Hewlett-Packard, St. Paul, MN, U.S.A.) equipped with a Waters μBondapak C_{18} (octadecylsilane) column, 30 cm $\times$ 3.9 mm I.D., 10 μm particle size (Waters Assoc., Milford, MA, U.S.A.).

Detection was accomplished with a Schoeffel Spectroflow ultraviolet (UV) detector (Schoeffel Instrument, Westwood, NJ, U.S.A.) at 200 nm. Preliminary experiments by us assessing wavelengths of 195—210 nm had established optimum absorbance of the individual bile acids at 200 nm. Peak area was calculated by slope integration on a programmable Hewlett-Packard 79850B LC terminal.

Preparation of solvent and standards

The mobile phase was methanol—0.1 *M* monobasic potassium phosphate (60:40, v/v), adjusted to pH 4.5 with phosphoric acid. This mixture was then filtered through a 0.45-μm filter (type HA, Millipore, Bedford, MA, U.S.A.) and degassed. The solvent flow-rate was 1.5 ml/min.

To prepare a mixture of the ten conjugated bile acid standards, we dissolved each bile acid individually in methanol at a concentration of 1 mg/ml. A 1-ml

aliquot of each solution was then combined, evaporated under nitrogen, and redissolved in the mobile phase to a volume of 1 ml. A standard elution profile was obtained by injecting 50 μl of the standard mixture representing 50 μg of each individual bile acid.

Sample preparation

Human duodenal bile was obtained from 34 patients undergoing gallstone dissolution therapy [3] and from normal volunteers undergoing bile acid replacement studies [4]. We diluted the bile 1:10 (v/v) in isopropanol, heated it for 10 min at 75°C, centrifuged the sample at 450 *g*, and decanted the supernate. We then filtered the supernate through a Gelman Acrodisc 0.45-μm filter. Of this solution 1 ml was then evaporated under nitrogen and redissolved in the HPLC solvent for injection and subsequent analysis. The injection volume used was generally 100 μl. This volume was sometimes modified to rerun questionable peaks.

Estimation of recovery

[^{14}C]GCDC was used to estimate recovery of the sample [5]. To each ml of sample, we added 10,000 cpm of [^{14}C]GCDC. We collected 2-min fractions (3 ml) of the eluent, added 10 ml of Safety Solve Scintillant (Research Products International, Mount Prospect, IL, U.S.A.), and counted ^{14}C by liquid scintillation spectroscopy with the use of external standardization for quench correction.

Other analytical methods

Total bile acid concentration was measured by an automated modification of the method of Talalay [6]. Bile acid composition was determined by GLC on an AN-600 column (3%), as described elsewhere [7]. Glycine and taurine conjugated bile acids were measured by TLC as previously described [8].

Statistical analysis

Linear regression lines were calculated by the method of least squares and statistical analyses were done by unpaired Student's *t*-test.

RESULTS

Fig. 1A shows the elution profile of the ten major conjugated bile acids prepared as standards representing 50 μg of each individual bile acid. The retention times are given in Table I. The low pH (4.5) contributes to the optimum separation conditions [9], and peak symmetry is maintained by the high salt concentration of the solvent system. Chromatographic runs observed by us as well as other authors [10] show slight asymmetry in the taurolithocholate and glycolithocholate peaks; however, they are integratable by peak area. Free bile acids, cholesterol and phospholipids were not run in this system as their elution patterns were not of specific interest for these analyses.

Fig. 1B shows the separation of the ten major conjugated bile acids in a sample of human duodenal bile with an injection volume of 50 μl.

We established a standard curve for each of the ten conjugated bile acids by

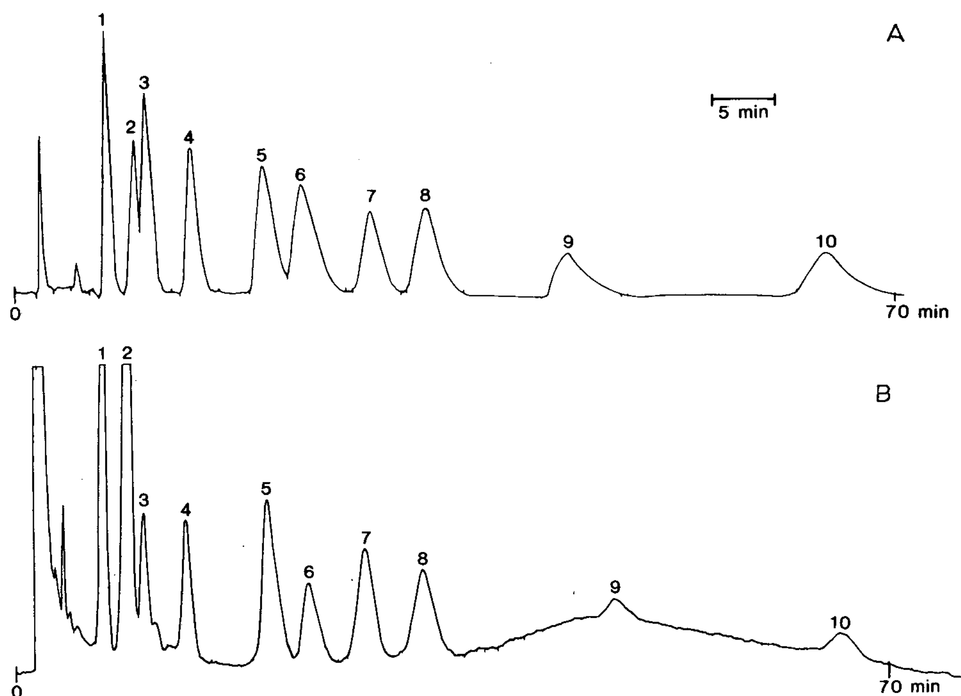

Fig. 1. (A) Elution profile of standard solution of pure bile acids. (B) Elution profile of bile acids of a sample of human duodenal bile. This profile is from bile taken from a patient after twelve months of tauroursodeoxycholic acid treatment resulting in the large percentage of glycoursodeoxycholic and tauroursodeoxycholic acid in the sample. Chromatographic conditions: solvent, methanol—0.1 *M* potassium dihydrogen phosphate, pH 4.5 (60:40); flow-rate, 1.5 ml/min; detector wavelength, 200 nm. For peak identification, see Table I.

TABLE I

RETENTION TIMES AND RELATIVE RETENTION TIMES OF THE TEN MAJOR CONJUGATED BILE ACIDS

Peak number	Bile acid	Retention time (min)	Retention time relative to GCDC
1	Tauroursodeoxycholic	7.4	0.26
2	Glycoursodeoxycholic	9.7	0.34
3	Taurocholic	10.6	0.37
4	Glycocholic	14.3	0.51
5	Taurochenodeoxycholic	19.9	0.71
6	Taurodeoxycholic	22.8	0.81
7	Glycochenodeoxycholic	28.2	1.0
8	Glycodeoxycholic	32.7	1.2
9	Taurolithocholic	44.6	1.6
10	Glycolithocholic	64.6	2.3

plotting concentration injected versus eluted peak area, and obtained linearity from 0.02 to 0.40 μmol. This range compares favorably with the findings of other authors [11]. Correlation coefficients (*r* values) of linear regression for the taurine conjugates ranged from 0.973 to 0.999 ($P < 0.001$) and from 0.997

to 0.999 ($P < 0.001$) for the glycine conjugates. As found by other authors [11], each standard curve was linear, although the slopes differed slightly for each bile acid (Fig. 2).

Total bile acid concentrations in bile (Fig. 3) were virtually identical when measured enzymatically by the 3α-steroid dehydrogenase method [6] and by summing the quantities of individual bile acids determined by eluted peak areas on HPLC ($r = 0.99$; $P < 0.001$). Repeat determination by HPLC of total bile acid concentrations on four separate samples of duodenal bile varied by only 3 ± 1.1% (mean ± S.D.). The four samples included total bile acid concentrations of 44, 70, 106, and 189 μmol/ml showing assay reproducibility within a reasonably broad concentration range.

Bile acid composition of bile determined by GLC (Fig. 4) correlated closely ($r = 0.96$; $P < 0.001$) with results of measurements by HPLC.

A highly significant correlation ($r = 0.96$; $P < 0.001$) existed between measurements by HPLC and TLC for individual glycine and taurine conjugates in human bile (Fig. 5).

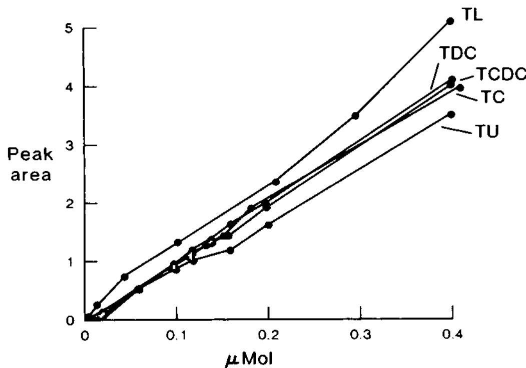

Fig. 2. Relationship of the concentrations of bile acid standards to the peak integrated areas for the taurine bile acid conjugates. Each point represents results for the mean of three determinations; these varied by only 3 ± 1.0% (mean ± S.D.). The same profile and statistics were demonstrated by the glycine bile acid conjugates ($r = 0.973 - 0.999$; $P < 0.001$). For abbreviations of the bile acids, see Table II.

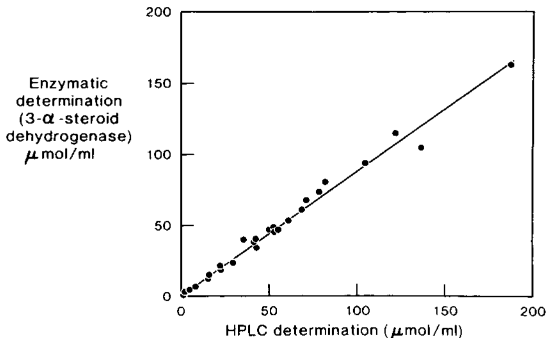

Fig. 3. Concentrations of total bile acids in samples of human duodenal bile obtained from patients with gallstones. The concentration of total bile acid in each of 26 duodenal bile samples was measured enzymatically and by summing the individual bile acid concentrations determined by HPLC ($r = 0.99$; $P < 0.001$).

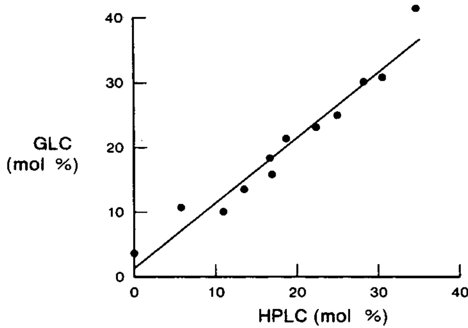

Fig. 4. Bile acid composition of samples of human duodenal bile obtained from normal volunteers undergoing bile acid replacement studies [4]. The bile acid composition in each of ten individual duodenal bile samples was determined separately by HPLC and GLC. This profile shows the correlation between HPLC and GLC for taurodeoxycholic and glycodeoxycholic acid (r = 0.97; P < 0.001). The other four bile acid conjugates also showed excellent correlation (r = 0.96; P < 0.001).

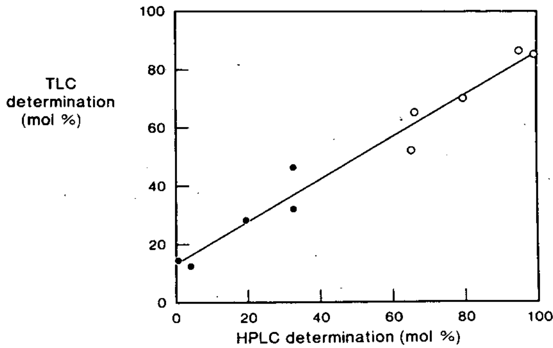

Fig. 5. The amount of individual conjugates of glycine and taurine in samples of human duodenal bile obtained from normal volunteers undergoing bile acid replacement studies [4]. The amount of individual bile acids conjugated to glycine and taurine in each of five samples of duodenal bile was determined by TLC and HPLC. Each point represents the percent of glycine or taurine conjugated bile acids in the bile samples. (○) Glycine conjugates; (●) taurine conjugates (r = 0.96; P < 0.001).

TABLE II

MEAN RECOVERY FOR EACH OF THE MAJOR CONJUGATED BILE ACID

Bile acid	Recovery (%)	Bile acid	Recovery (%)
Tauroursodeoxycholic (TU)	86	Glycoursodeoxycholic (GU)	85
Taurocholic (TC)	85	Glycocholic (GC)	88
Taurochenodeoxycholic (TCDC)	91	Glycochenodeoxycholic (GCDC)	89
Taurodeoxycholic (TDC)	85	Glycodeoxycholic (GDC)	90
Taurolithocholic (TL)	85	Glycolithocholic (GL)	94

Mean ± S.D. = 87.2 ± 2.5%

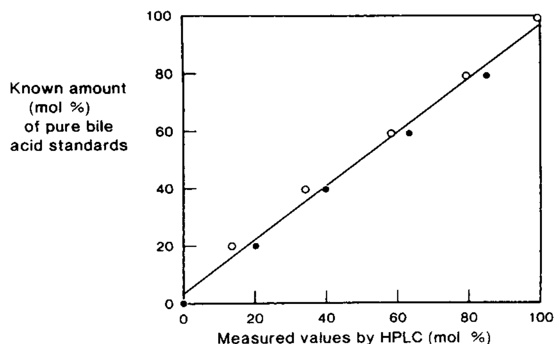

Fig. 6. The amount of individul conjugates of glycine and taurine in each of five mixtures of pure bile acid standards was measured separately and compared to the amount known to be present in each sample. Each mixture was prepared in physiologic proportions of individual bile acids (i.e., 40% cholic, 35% cheno, 15% deoxy, 5% urso, 5% litho). In addition, the mixtures were prepared so that the molar percentages of glycine and taurine, when summed, would equal 100%; for example, 60% glycine plus 40% taurine = 100%. Each point represents the percent of glycine or taurine conjugated bile acids in mixtures of bile and standards. (○) Glycine conjugates; (●) taurine conjugates ($r = 0.99$; $P < 0.001$).

When mixtures of pure bile acid standards enriched to varying degrees with glycine or taurine conjugates were assessed by HPLC, the measured values correlated significantly ($r = 0.99$; $P < 0.001$) with the known values (Fig. 6).

The mean recovery of [^{14}C]GCDC in all of the validation studies was 91 ± 4.7% (mean ± S.D.). Recovery experiments were also carried out by preparing a mixture of known amounts of each bile acid conjugate added in approximate physiologic proportions and running the mixture three times.

The mean recoveries for each of the bile acids are given in Table II. Mean recovery for all the bile acids was 87.2 ± 2.5% (mean ± S.D.).

DISCUSSION

We have developed a rapid, reproducible, accurate and sensitive technique using HPLC which permits the simultaneous determination of total bile acid concentration, bile acid composition and molar percentages of the individual bile acids conjugated to glycine and taurine in bile. Furthermore, using both samples of human bile and mixtures of bile acid standards, we have systematically compared this technique to the enzymatic assay of total bile acids, the GLC measurement of bile acid composition, and the TLC determination of the glycine and taurine conjugates of biliary bile acids.

Others have described HPLC systems for the separation of biliary bile acids. Armstrong and Carey [9] used HPLC to examine quantitatively the hydrophobic/hydrophilic properties of bile salts in monomeric bile salt and mixed bile salt—cholesterol micellar solutions; they did not, however, study samples of human gallbladder or duodenal bile [9]. Nakayama and Nakagaki [12] reported the successful separation of the glycine and taurine conjugates of the commonly occurring bile acids in bile, as did Bloch and Watkins [11]; neither group, however, provided quantitative data on the actual amount of the individual bile acids present in bile. Rubin and Van Berge-Henegouwen [13]

employed HPLC using a radial compression system for separation of biliary bile acids; they provided a partial validation of the method by comparing the quantification of the primary bile acids, chenodeoxycholic and cholic acids, in bile by HPLC and GLC.

Although others have described HPLC systems for the separation and, to a lesser extent, the quantitation of biliary bile acids, we believe our technique is at least as efficient as these published methods and may have several advantages over these techniques. First, methanol—potassium phosphate is superior to acetonitrile as a solvent system because the solubility of bile acids is greater in methanol [9]; this is an advantage which is particularly important for the quantitation of the poorly soluble lithocholic acid conjugates, which have frequently been difficult to clearly separate. We should add that, in our experience, glycolithocholate has a short shelf life when dissolved in methanol and begins to precipitate within one to three weeks. For this reason, it is important that standard solutions be prepared freshly prior to injection.

Second, the isocratic feature of the system enhances the simplicity and reproducibility of the technique, at least in our experience and with the equipment available to us. Indeed, attempts by us at gradient elution were abandoned because of baseline instability. Third, no derivatization or separation of phospholipid and cholesterol is needed with our technique and, except for deproteinization, little preparation of the specimen is necessary. We have not assessed other methods of sample preparation (i.e., Sep-Pak) which might make sample preparation even simpler or faster [14]. With our method, ten samples can easily be run in 12 h with the use of an automatic injector, once the technique has been established. Fourth, our recovery using [^{14}C] GCDC as an internal standard is better than has been reported for estriol [15, 16], testosterone [10], dexamethasone [13], commonly used internal standards which may actually overlap with the bile acids of interest. Finally and perhaps most importantly, others have not validated the accuracy of HPLC for bile acid analyses by comparing it to the conventional methods used to measure total bile acid concentration, bile acid composition, and the glycine and taurine conjugates of bile acids in bile. This latter determination is of increasing importance since the possibility exists that reversing the normal predominance of glycine conjugates by taurine supplementation may enhance the efficacy of gallstone dissolution.

The only limitation of this technique is that it does not measure unconjugated bile acids. Since these rarely occur in human bile, this disadvantage has little practical significance. Our systematic comparison of this HPLC technique to conventional methodology establishes the validity of using HPLC alone for analyses necessary for a complete description of biliary bile acids.

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3-METHOXY-4-HYDROXYPHENYLGLYCOL, 5-HYDROXYINDOLEACETIC ACID, AND HOMOVANILLIC ACID IN HUMAN CEREBROSPINAL FLUID

STORAGE AND MEASUREMENT BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND COULOMETRIC DETECTION USING 3-METHOXY-4-HYDROXYPHENYL-LACTIC ACID AS AN INTERNAL STANDARD

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SUMMARY

To simultaneously measure 3-methoxy-4-hydroxyphenylglycol (MHPG), 5-hydroxyindoleacetic acid (5HIAA), and homovanillic acid (HVA) in human cerebrospinal fluid (CSF), we used an acetonitrile protein precipitation, reversed-phase high-performance liquid chromatography with coulometric detection, and 3-methoxy-4-hydroxyphenyllactic acid (MHPLA) as an internal standard for all three metabolites. MHPG, 5HIAA, HVA, and MHPLA were stable for one month when stored in CSF at —70°C. Three determinations were made in triplicate for each of seven subjects over a 30-day storage period and the coefficients of variation within subject for these determinations ranged from 0.075 to 0.165 for MHPG, 0.045 to 0.148 for 5HIAA and 0.053 to 0.181 for HVA. Means and standard deviations of CSF concentrations were 10.7 ± 3.0 ng/ml for MHPG, 22.4 ± 9.9 ng/ml for 5HIAA, and 39.9 ± 21.4 ng/ml for HVA. This method provides simple sample preparation, sensitivity, and cost advantages, as well as simultaneous extraction and quantitation of MHPG, 5HIAA, and HVA using an internal standard.

INTRODUCTION

3-Methoxy-4-hydroxyphenylglycol (MHPG), 5-hydroxyindoleacetic acid (5HIAA) and homovanillic acid (HVA) are metabolites of the central nervous systems (CNS) monoamine neurotransmitters norepinephrine (NE), dopamine (DA), and serotonin (5HT) [1—6]. The concentrations of these metabolites in cerebrospinal fluid (CSF) may reflect the activity of their relative neutrotransmitter systems in the central nervous system [7, 8]. Levels

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of these amine metabolites have been measured in patients with delirium tremens [9], senile dementia of Alzheimer type [10], sleep apnea syndrome [11], schizophrenia [12], depression and suicidal behavior [13], and Parkinson's disease [14]. The biogenic amine hypothesis of affective disorders posits a functional deficit in CNS monoamine neurotransmitters, the precursors of these metabolites [15–19]. Since intensive study of biological factors in affective disorders is a major part of current research, we wanted to develop a simple and inexpensive, yet sensitive high-performance liquid chromatographic (HPLC) assay to measure the concentrations of MHPG, 5HIAA, and HVA in human CSF.

MHPG, 5HIAA, and HVA have been measured by fluorometry [20–23], radioenzymatic assay [24], radioimmunoassay [25, 26], gas chromatography (GC) with electron-capture detection (ECD) [1, 23, 27–35], GC with mass spectrophotometric (MS) detection [6, 36–49], and by HPLC with amperometric electrochemical detection [50–59]. The earlier-developed methods, fluorometric, radioenzymatic, and GC–ECD, were not as sensitive as more recent methods and required larger amounts of CSF. Radioimmunoassay methods are relatively new for these CNS metabolites. They require derivatization of the compounds and they are sensitive in the picogram range; however, they are not being widely used at this time and have not been applied to CSF measurements. GC–MS is perhaps the most widely used method in recent years. This method is sufficiently sensitive and very specific; however, it requires equipment that is expensive both to purchase and maintain. In addition, the metabolites must be derivatized and all three metabolites cannot be quantitated simultaneously.

Assay methods for MHPG, 5HIAA, and HVA by HPLC utilizing amperometric detection have been used recently by several investigators with excellent results [55–59]. The equipment is less expensive to purchase and less troublesome to operate than that used in previous methods. The HPLC methods have included simple sample preparation and sensitivity in the sub-nanogram range.

The use of the coulometric electrochemical detector with two detector cells in a series provides sensitivity for MHPG, 5HIAA, and HVA in the low picogram range, and has the advantage of measuring compounds at two voltage settings. We here describe an enhancement of HPLC methodology for quantitation of MHPG, 5HIAA, and HVA in human CSF which combines reversed-phase HPLC with coulometric electrochemical detection. This paper is based in part on a presentation at the 5th International Catecholamine Symposium in Goteborg, Sweden [57].

EXPERIMENTAL

Materials

MHPG hemipiperazine, HVA, 5HIAA, and 3-methoxy-4-hydroxyphenyllactic acid (MHPLA) were purchased from Sigma (St. Louis, MO, U.S.A.). Sodium citrate, HPLC-grade phosphoric acid, and HPLC-grade methanol were purchased from Fisher (Pittsburgh, PA, U.S.A.). Nylon-66 filters (0.2 and 0.45 μm) were purchased from EM Science (Gibbstown, NJ, U.S.A.). The Ultrasphere-IP (C_{18}, 5 μm particle size) column was purchased from Beckman (Fullerton, CA, U.S.A.).

Tissue preparation

CSF was collected from the lumbar region of the spinal cord and was immediately placed on ice at 0—4°C [60]. MHPLA, the internal standard (20 ng/ml) was added, then the CSF was stored at —70°C in a capped polypropylene test tube until processing.

On the day of the assay, we thawed the CSF, then added 1 ml of acetonitrile to 200 μl of lumbar CSF in borosilicate test tubes. The samples were vortexed vigorously for 15 sec, then centrifuged at 2700 *g* for 15 min. This step removes approx. 99.8% of the protein in the CSF. Of the supernatant 1 ml was carefully aspirated and dried to residue under nitrogen at 40°C. This sample residue could be stored in the refrigerator at 0—4°C for 24 h, or at —70°C indefinitely, with no degradation of the metabolites.

Next we redissolved the residue in 200 μl of mobile phase, degassed the sample under house vacuum for 5 min, then injected 20 μl into the chromatograph for quantitation of MHPG, 5HIAA, HVA, and MHPLA. In these experiments, each sample was prepared immediately before injection because at pH 5 the 5HIAA is unstable in light. The 5HIAA peak in the external standard solution decreased approx. 25% at pH 5 during an 8-h period in room light. When the standard solution was protected from light, the degradation of 5HIAA did not occur. The MHPG, HVA, and MHPLA were stable at pH 5 in the light over an 8-h period.

High-performance liquid chromatography

The HPLC apparatus consisted of a Beckman Model 110A single-piston pump, a Bio-Rad Labs. Bio-Sil ODS-10 pre-column, an Altex Model 210 injector valve, a Beckman Ultrasphere-IP (250 mm × 4.1 mm I.D.), 5-μm C_{18} column, and an Environmental Systems Associates (ESA) coulometric electrochemical detector.

The isocratic mobile phase usually consisted of 4% (v/v) HPLC-grade methanol and 96% of a solution containing 75 m*M* sodium citrate and 78 m*M* phosphoric acid, pH 5.25 (see legends to figures for individual experiments). The sodium citrate—phosphoric acid solution was prepared with deionized distilled water by dissolving 75 m*M* sodium citrate, then adjusting the pH to 5.25 with phosphoric acid. As recommended by ESA, the citrate—phosphate buffer was filtered consecutively through Rainen Nylon-66 filters of 0.45 μm, then 0.2 μm pore size. The methanol was then added to the citrate—phosphate buffer and the solution was degassed for at least 5 min under house vacuum.

The ESA coulometric detector has two graphite detector cells. The mobile phase and the samples flow through these cells. At the appropriate voltage setting, 100% of an electroactive substance will be reacted as it passes through the cell. The voltage can be different for each cell and, therefore, some selectivity can result. For these experiments, detector 1 (D1) voltage was 0.23 V and detector 2 (D2) voltage was 0.40 V. 5HIAA was oxidized by D1 and MHPG, MHPLA, and HVA were oxidized by D2 (see Fig. 1).

Current—voltage curve

The current—voltage (C—V) curve was obtained by injecting several identical external standard and CSF samples into the chromatograph at different voltage

settings and measuring the current generated, then graphing the voltage (abscissa) in V versus the current (ordinate) as a fraction of the maximum current. The C—V curve is dependent on the mobile phase and provides good qualitative evidence that each peak is the result of one compound since very few compounds have the same C—V curve.

Calculation of CSF concentration

Peak heights were measured directly from a chart recorder. MHPLA in the CSF sample was added exogenously, and the metabolite extraction efficiency was calculated from data in Fig. 2.

$$\frac{\left[\dfrac{\text{metabolite peak height}}{\text{MHPLA peak height}}\right]_{\text{sample}}}{\left[\dfrac{\text{metabolite peak height}}{\text{MHPLA peak height}}\right]_{\substack{\text{external}\\ \text{standard}}}} \times [\text{MHPLA}]_{\text{sample}} \div \begin{array}{c}\text{metabolite}\\ \text{extraction}\\ \text{efficiency}\\ \text{as a fraction}\end{array} = [\text{metabolite}]_{\text{CSF}}$$

RESULTS

Fig. 1A is the chromatogram of a 20-μl injection containing 400 pg each of MHPG, 5HIAA, HVA, and MHPLA as external standards. All four compounds eluted as baseline-to-baseline peaks within 25 min of the injection. At a full-scale detector range of 50 nA, approx. 100 pg of each of these compounds produced at least a 10% of page deflection. The 5HIAA peak (peak 3) appeared on both detectors because 100% of the 5HIAA was not oxidized by D1 at 0.23 V. We used the D1 peak height for calculations because it is directly reflective of the 5HIAA concentration. The ratios of the metabolite peaks to the MHPLA (internal standard) peak were very consistent from injection to injection and from preparation to preparation of external standard. When 400 pg each of MHPG, 5HIAA, HVA, and MHPLA were injected into the chromatograph, the ratios of external standard peak heights for MHPG/MHPLA, 5HIAA/MHPLA, and HVA/MHPLA were 1.37 ± 0.075 (S.D., n = 28), 2.01 ± 0.10 (S.D., n = 11), and 0.868 ± 0.060 (S.D., n = 28), respectively. These values were used in the calculation of the CSF concentrations of MHPG, 5HIAA, and HVA (see Fig. 2).

Fig. 1B is a chromatogram from a pooled CSF sample. Given that this was a biological sample, other peaks than those seen in Fig. 1A are noted; however, the peaks for MHPG, 5HIAA, HVA, and MHPLA are present and well defined based on retention time.

To determine the recovery of MHPG, 5HIAA, HVA, and MHPLA in our sample preparation procedure, we pooled equal amounts of CSF samples from four depressed patients and spiked six aliquots with 20 ng/ml MHPLA and 0—50 ng/ml MHPG, 5HIAA, and HVA. We then processed the samples as explained in Experimental and determined the concentrations of metabolites and internal standard in each of the six aliquots. As seen in Fig. 2, the R value (see legend to Fig. 2) of MHPG, 5HIAA, or HVA was directly proportional to the CSF concentration. Squared correlation coefficients (r^2) for the linear regressions were 0.996 for MHPG ($p < 0.0001$), 0.999 for 5HIAA (p <

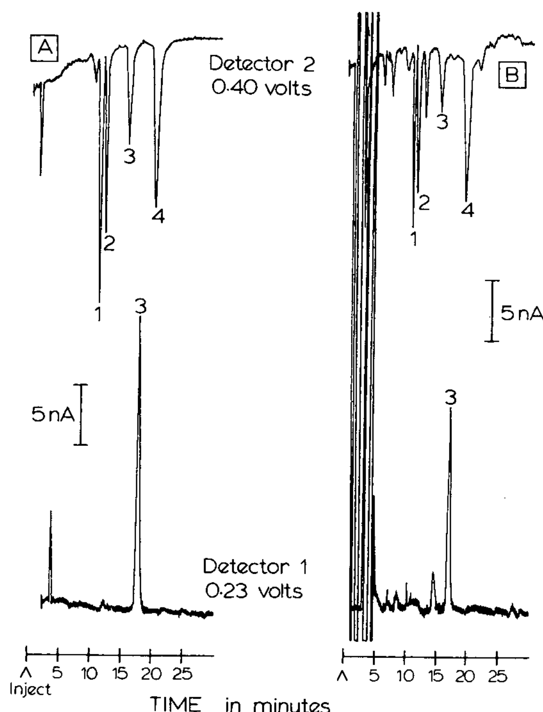

Fig. 1. (A) Chromatogram of MHPG, 5HIAA, HVA, and MHPLA standards. A 20-μl aliquot of sample containing 400 pg each of MHPG, 5HIAA, HVA, and MHPLA dissolved in mobile phase was injected into the HPLC system described in Experimental. Peaks: 1 = MHPG; 2 = MHPLA; 3 = 5HIAA; and 4 = HVA. 5HIAA is not completely oxidized by D1, therefore, a peak is seen on the D2 tracing. MHPG, MHPLA, and HVA are not oxidized by D1 and, therefore, these peaks appear only on the D2 tracing. (B) Chromatogram of a CSF sample. A 20-μl aliquot of a pooled CSF sample processed as explained in Experimental was injected into the chromatograph. The amount of each compound in the peaks is 311 pg for MHPG (1), 349 pg for MHPLA (2), 303 pg for 5HIAA (3), and 382 pg for HVA (4).

0.0001), and 0.994 for HVA ($p < 0.001$). This suggests that MHPLA is a valid internal standard for MHPG, 5HIAA, and HVA. The average recoveries for the four compounds were 94.6 ± 4.0% (S.D.) for MHPG, 76.8 ± 2.0% (S.D.) for 5HIAA, 94.2 ± 4.7% (S.D.) for HVA, and 104 ± 6.4% (S.D.) for MHPLA, the internal standard. The loss of 5HIAA occurs in the deproteinization step, not the evaporation step. These recoveries were used to calculate CSF concentrations of the metabolites.

To verify the metabolite peaks on the chromatogram of the CSF sample, we ran the CSF sample and an external standard sample at several different voltages to obtain the current—voltage curves seen in Fig. 3. The MHPG, 5HIAA, HVA, and MHPLA peak heights in standard and CSF samples increased to a maximum with voltage increases in the range 0.08—0.40 V. The fact that the curves for each compound in CSF and external standard samples were superimposable suggests that each peak was the result of a single compound.

Infrequently, a contaminating peak interfered with the accurate measurement of a metabolite peak and necessitated a minor change in the mobile

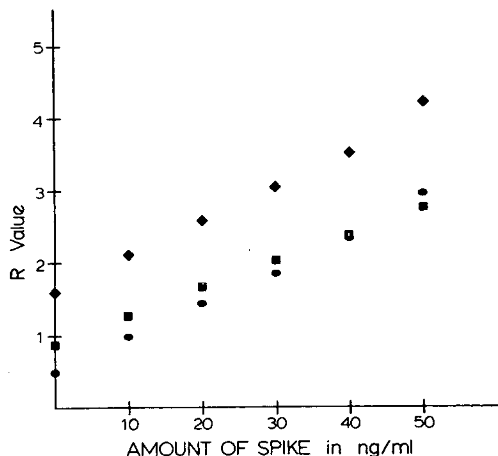

Fig. 2. Graph relating R value of MHPG, 5HIAA, and HVA to amount of spike in CSF. The R value of a compound is the ratio of each metabolite peak height to MHPLA peak height in the CSF sample divided by the same ratio for 400 pg each of the external standards. The R value for a specific compound multiplied by the concentration of the internal standard added to the CSF sample is equal to the concentration of specific compound in CSF. The abscissa is the amount of metabolite added to the CSF sample as a spike. Each of six identical pooled CSF samples was spiked with 20 ng/ml of the internal standard MHPLA and with 0, 10, 20, 30, 40 and 50 ng/ml each of MHPG, 5HIAA, and HVA. These samples were processed as described in Experimental. A 20-µl aliquot of each of the final samples was injected into the chromatograph and R values calculated from the peak height ratios in CSF and external standard injections. ●, MHPG; ■, 5HIAA; ◆, HVA.

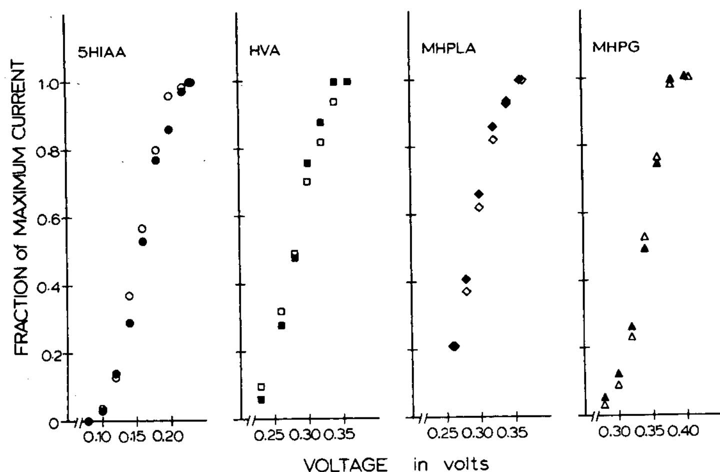

Fig. 3. Current—voltage curves for MHPG, 5HIAA, HVA, and MHPLA in external standard and CSF sample injections. The abscissa is the voltage applied to the detector cell. The ordinate is the ratio of a metabolite peak height at a given voltage to the maximum peak height. The standard solution contained 400 pg each of MHPG, 5HIAA, HVA, and MHPLA. The pooled CSF sample, spiked with only MHPLA, was prepared as explained in Experimental. The same external standard and CSF sample solutions were used throughout the experiment. Open symbols, CSF sample; closed symbols, external standards.

phase. The pH in the range 5.0—6.5 had no effect on the retention time of MHPG, but had a dramatic effect on the retention times of MHPLA, 5HIAA, and HVA. Changes of pH can be used to move the peaks in relationship to each other. Also, organic-strength changes in the mobile phase significantly altered the retention times of the MHPG, 5HIAA, HVA, and MHPLA peaks, but their relative positions on the chromatogram did not change. Ionic strength of the mobile phase had no effect on retention time of the compounds for total mobile phase concentrations of citrate and phosphate up to 200 m*M*.

To establish that the metabolites and the internal standard were stable in the CSF while stored at —70° C, we added 20 ng/ml MHPLA to freshly drawn CSF from seven subjects, then divided each subject's sample into several aliquots. Metabolite measurements were performed in triplicate with one aliquot, then the other aliquots were stored at —70° C. At 14- and 28-day intervals, aliquots were thawed and metabolite measurements were made in triplicate. There were no statistically significant differences within subject for values obtained at days 0, 14, and 28, showing that the compounds are stable when stored in CSF at —70° C. The mean coefficients of variation (standard deviation/mean) for each compound for nine determinations for each of seven subjects ranged from 0.045 to 0.181 (see Table I). These low coefficients of variation for nine determinations also show that the sample preparation was consistent.

Also presented in Table I are the CSF concentrations for MHPG, 5HIAA, and HVA. The means and standard deviations (S.D.) for seven subjects for MHPG, 5HIAA, and HVA were 10.7 ± 3.0, 22.4 ± 9.9, and 39.9 ± 21.4 ng metabolite per ml CSF, respectively. These concentrations compare favorably with the

TABLE I

CSF CONCENTRATIONS (ng/ml) AND COEFFICIENTS OF VARIATION OF MHPG, 5HIAA, and HVA IN STORED CSF SAMPLES

MHPLA, the internal standard (20 ng/ml) was added to freshly drawn CSF from lumbar punctures, then the CSF was divided into 1-ml aliquots and stored in capped polypropylene tubes at —70° C. Measurements of metabolites in triplicate were made the day of CSF drawing, then in approximately two-week intervals for two more measurements. The CSF concentrations expressed as ng/ml (± S.D.) for each metabolite for each subject are shown, and the means for seven subjects are at the bottom of the table. C.V. values are also given for each compound for nine determinations in each of seven subjects.

Subject	MHPG		5HIAA		HVA	
	CSF (± S.D.)	C.V.	CSF (± S.D.)	C.V.	CSF (± S.D.)	C.V.
1	10.4 (0.56)	0.054	9.7 (1.4)	0.148	25.7 (2.6)	0.101
2	11.3 (0.86)	0.076	29.0 (1.3)	0.045	71.8 (3.8)	0.053
3	9.3 (0.59)	0.063	22.6 (1.2)	0.054	54.8 (4.5)	0.082
4	11.8 (0.78)	0.067	19.2 (1.3)	0.068	55.9 (4.3)	0.078
5	7.5 (0.67)	0.090	8.0 (0.63)	0.078	12.4 (2.1)	0.170
6	7.8 (0.53)	0.068	14.4 (1.0)	0.069	35.3 (3.3)	0.093
7	16.5 (1.6)	0.097	14.9 (1.5)	0.104	23.3 (4.2)	0.181
Mean	10.7 (3.0)		22.4 (9.9)		39.9 (21.4)	

TABLE II

CONCENTRATION OF FREE MHPG, 5HIAA, AND HVA IN HUMAN CSF DETERMINED BY VARIOUS METHODS

CSF concentrations and standard deviations for free MHPG, total MHPG, 5HIAA, and HVA were taken from the references listed in the left column of the table. The methods used to determine the concentrations are listed in the right column.

Reference	Ref. No.	MHPG (ng/ml, ± S.D.)		5HIAA (ng/ml, ± S.D.)	HVA (ng/ml, ± S.D.)	Method
		Free	Total			
Gerbode and Bowers (1968)	21			34.0 ± 15	40 ± 29	Fluorometry
Gottfries et al. (1969)	20			40.0 ± 10	60 ± 30	Fluorometry
Wilk et al. (1972)	23			29.0	18.0	Fluorometry
Schanberg et al. (1968)	1	50—200				GC—ECD
Gordon and Oliver (1971)	27		15.1 ± 7.7	29.0	37.0	GC—ECD
Wilk et al. (1971)	28		22.0 ± 7.0			GC—ECD
Bond (1972)	30	18.1 ± 5.2	41.1 ± 18.4			GC—ECD
Sjoquist and Anggard (1972)	32				83.7 ± 5.9	GC—ECD
Wilk et al. (1972)	23		15.0			GC—ECD
Chase et al. (1973)	33	10 ± 1	14.0 ± 1.4	28.0 ± 1.3	30 ± 2.8	GC—ECD
O'Keeffe and Brooksbank (1973)	31		11.0 ± 2.9			GC—ECD
Bertilsson et al. (1972)	39			22.5 ± 9.7		GC—MS
Bertilsson (1973)	40	12.6 ± 0.88				GC—MS
Swahn et al. (1976)	42	6.99		32.5	61.9	GC—MS
Karoum et al. (1977)	45	11.0 ± 1.2	12.9			GC—MS
Jimerson et al. (1981)	38		8.0			GC—MS
Kopin et al. (1983)	49	9.0				GC—MS
Koslow et al. (1983)	36	7.97 ± 1.55		21.2 ± 5.7	41.9 ± 13.7	GC—MS
Sjoquist and Anggard (1972)	32	13.4 ± 1.1				GC—MS
Langlais et al. (1980)	56	8.0				HPLC
Tune et al. (1980)	55	4.9 ± 0.8		22.1 ± 2.1	38.4 ± 3	HPLC
Semerdjian-Rouquier et al. (1981)	58			16.7 ± 2.0	21.0 ± 5	HPLC
Van Woert et al. (1982)	59	8.0				HPLC
Javors et al. (1983)	57	11.3 ± 1.3		17.9 ± 2.5	45.0 ± 6.4	HPLC

determinations in Table II that were made in several other laboratories with various procedures.

DISCUSSION

Our goal was to develop a procedure to collect and store lumbar CSF, then to simultaneously determine MHPG, 5HIAA, and HVA concentrations in these samples. The collection of the CSF was done with a method that has worked well in our studies with infrequent and mild side-effects for the subjects [60].

The internal standard, MHPLA (Fig. 4), has structural characteristics similar to MHPG and HVA. MHPLA also has approximately the same electrochemical sensitivity as all three metabolites. As seen in Fig. 2, MHPLA was extracted from CSF in direct proportion to MHPG and HVA, as well as 5HIAA, and, therefore, was used as an internal standard for all three compounds.

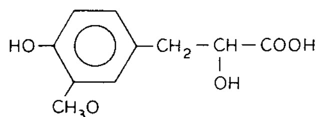

Fig. 4. Chemical structure of MHPLA.

The straightforward sample preparation, which required no derivatization, and the isocratic mobile phase allowed simultaneous extraction and quantitation of the three metabolites of interest. This is a definite advantage in sample determination time.

MHPG, 5HIAA, HVA, and MHPLA were stable for one month when stored at −70°C in capped polypropylene tubes (see Table I). For at least three separate determinations on each of three separate days, two weeks apart, the coefficients of variation for CSF concentrations of MHPG, 5HIAA, and HVA ranged between 0.045 and 0.181. Furthermore, there was no trend for an individual subject's metabolite concentration to increase or decrease during the one-month storage period. The means and standard deviations (S.D.) of the CSF concentrations for the seven subjects in our study were 10.7 ± 3.0 ng/ml for MHPG, 22.4 ± 9.9 ng/ml for 5HIAA, and 39.9 ± 21.4 ng/ml for HVA. As seen in Table II, these values are in the same range as those calculated by HPLC with amperometric detection and GC—MS or GC—ECD. In fact, the values that have been determined over the last fifteen years by various methods are very consistent.

The advantages of the HPLC with coulometric detection are in relative cost, selectivity, and sensitivity. An isocratic HPLC with electrochemical detection is much less expensive to purchase and maintain than either a GC—MS or a GC—ECD system. Reversed-phase HPLC provides exceptional selectivity in the separation of low-molecular-weight compounds. MHPG, 5HIAA, HVA, and the internal standard MHPLA were easily separated by appropriate organic strength and pH of an isocratic mobile phase.

Furthermore, the coulometric detector we used has two detector cells in series which provides further selectivity. Although we have not fully utilized this advantage in this study, it is possible to set each detector at a voltage that

would oxidize a fraction of a specific compound, giving a signal in each channel. The ratio of the signal from D1 to the signal from D2 would be constant for a specific compound, and would confer more selectivity to the system. In addition, in more complex samples, as those from urine or plasma, having two detector cells set at different voltages would allow different compounds to be quantitated on D1 and D2. Finally, HPLC with coulometric detection enabled us to actually measure as little as 20 pg of MHPG, 5HIAA, HVA, and MHPLA. This sensitivity allows for more dilute samples and, therefore greater HPLC column life.

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DETERMINATION OF CARNITINE, BUTYROBETAINE, AND BETAINE AS 4'-BROMOPHENACYL ESTER DERIVATIVES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for determination of carnitine, 4-(N,N,N-trimethylammonio)butanoate (butyrobetaine), and 2-(N,N,N-trimethylammonio)acetate (betaine) is described. These ω-trimethylammonio carboxylates and the chemically analogous internal standards 4-(N,N-dimethyl-N-propylammonio)-3-hydroxybutanoate or 6-(N,N,N-trimethylammonio)hexanoate were derivatized by reaction with 4'-bromophenacyl triflate in the presence of N,N-diisopropylethylamine. The trialkylammonio carboxylate 4'-bromophenacyl ester derivatives were separated from other sample constituents by reversed-phase ion-pair high-performance liquid chromatography with spectrophotometric detection at 254 nm. Standard curves were linear over a sample concentration range of 10—100 nmol/ml. Quantities of 2.5 nmol of ω-trialkylammonio acid derivatives injected into the chromatograph were detected with signal-to-noise ratios greater than 50.

INTRODUCTION

The mitochondrial fatty acid acyltransferase cofactor carnitine [4-(N,N,N-trimethylammonio)-3-hydroxybutanoate] has been the object of various determination schemes. The first reported biological assay procedure related growth of the mealworm *Tenebrio molitor* to growth medium carnitine content [1]. Carnitine also has been determined by various spectrophotometric means based upon complex formation between its quaternary ammonium functionality and chromophoric anions. Periodide [2] and bromophenol blue [3, 4] complexes have been used for this purpose. Visualization of carnitine on thin-layer chromatograms has been accomplished through use of iodine [5], and by complex formation with iodoplatinate [6]. An alternative approach exploited

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enzymatic recognition of carnitine by carnitine acetyltransferase, leading to detection of a chromophoric coupled reaction product [7]. This concept was further modified by use of radioactively acetate-labeled acetyl coenzyme A and measurement of the production of acetate-labeled acetylcarnitine [8—12]. Of related interest is the determination of 4-(N,N,N-trimethylammonio)-butanoate (4-TMA-butanoate, butyrobetaine), which has been established as a biosynthetic precursor of carnitine [13—16]. This compound undergoes complex salt formation with the reagents used for spectrophotometric determination of carnitine [2, 5]. In addition, 4-TMA-butanoate has been determined indirectly by enzymatic conversion to carnitine in the presence of butyrobetaine hydroxylase and subsequent radioenzymatic determination of the carnitine product [16, 17]. 2-(N,N,N-Trimethylammonio)acetate (2-TMA-acetate, betaine) is an intermediate in the metabolism of choline [18, 19], and has been determined spectrophotometrically [2, 5], radioenzymatically [18, 19], and by high-performance liquid chromatography (HPLC) [20].

With the advent of HPLC and its application to analytical problems, we sought to use this new technique for our work related to the biosynthesis of carnitine. The carboxyl group is the most analytically accessible structural feature of carnitine and related ω-trimethylammonio acids (TMA acids). However, carboxyl groups are weakly chromophoric, and are neither fluorophoric nor electrophoric to any useful degree. Therefore, carboxylates with no other functionality permitting sensitive detection are derivatized for their determination at low concentration by HPLC. Among the most useful of reported acid derivatives are 4′-bromophenacyl esters [20—31]. Unfortunately, the four-carbon TMA acids proved to be both unreactive toward common carboxyl-O-alkylation reagents and thermally labile, making the usual conditions of 4′-bromophenacyl ester formation [21, 22, 32] entirely unsatisfactory. To solve this problem, we developed a new derivatizing agent with substantially increased alkylative reactivity, 4′-bromophenacyl triflate [33]. This reagent allows mild, rapid, and quantitative derivatization of carnitine, 4-TMA-butanoate, 2-TMA-acetate, and other trialkylammonio carboxylates. With this reagent, we developed a procedure for the determination of these trimethylammonio acids in standard solutions at concentrations of 10—100 nmol/ml.

This paper presents that method. Sample preparation by ion-exchange chromatography, derivatization by reaction with 4′-bromophenacyl triflate, separation from sample constituents by reversed-phase ion-pair HPLC, and spectrophotometric detection permit quantitative measurements of these compounds within a concentration range potentially useful for biological sample determinations.

EXPERIMENTAL

Equipment

The liquid chromatograph consisted of a Model 6000A pump, a U6K syringe loading sample injection valve, an RCM-100 radial compression module, and a Model 440 fixed-wavelength spectrophotometric detector purchased from Waters Assoc. (Milford, MA, U.S.A.). The chromatographic separation was

accomplished on a 10 × 0.8 cm plastic cartridge containing Radial-Pak C_{18} of 5 μm nominal particle diameter (Waters Assoc.). The chromatographic column was protected against particulate sample contaminants by a 5 × 0.4 cm column constructed from zero-dead-volume chromatographic unions (Crawford Fitting, Solon, OH, U.S.A.) and packed with the pellicular reversed-phase medium Co:Pell ODS (Whatman, Clifton, NJ, U.S.A.). The detector was operated at 254 nm, and its output signal was recorded by a Linear Instruments (Irvine, CA, U.S.A.) Model 291 chart recorder. A Hewlet-Packard (Avondale, PA, U.S.A.) Model 3354C laboratory automation system was used for chromatographic peak area integration, peak height measurement, and calculations derived from those measurements. A Waters Assoc. WISP-710A automatic sampler was used in experiments related to establishment of standard curves. Liquid scintillation counting was performed with a Packard Instruments (Downers Grove, IL, U.S.A.) PRIAS scintillation spectrometer. ^{1}H Magnetic resonance spectra were acquired with a Bruker Instruments (Billerica, MA, U.S.A.) WH-270 pulsed Fourier transform nuclear magnetic resonance (NMR) spectrometer. A Kraft Apparatus (Mineola, NY, U.S.A.) Big Vortex unit was used for continuous mixing of samples during derivatization procedures.

Materials

Acetonitrile (OmniSolv, non-UV grade) was purchased from MCB (Cincinnati, OH, U.S.A.) and filtered through nylon membranes of 0.45 μm nominal pore size before use in chromatographic mobile phases. Acetonitrile intended for use in derivatization reactions was distilled from calcium hydride. Water was prepared for use as a chromatographic mobile phase constituent by passage through a Milli-Q water purification unit (Millipore, Bedford, MA, U.S.A.). Sodium dodecyl sulfate (SDS, electrophoresis grade) was purchased from Gallard-Schlesinger (Carle Place, NY, U.S.A.). Sodium dihydrogen phosphate and phosphoric acid (85%), N,N-dimethylformamide (DMF), and ethyl acetate were purchased from Fisher Scientific (Cleveland, OH, U.S.A.). Triethylamine, N,N-diisopropylethylamine, 3-(N,N-dimethylamino)-1-propanol, and 3-(N,N-dimethylamino)-1,2-propanediol were purchased from Aldrich (Milwaukee, WI, U.S.A.).

(*l*)-Carnitine (chloride) was a generous gift of the Otsuka Pharmaceutical Factory (Naruto, Tokushima, Japan). 2-TMA-acetate and hydroxyacetic (glycolic) acid were purchased from Sigma (St. Louis, MO, U.S.A.). 4-Aminobutanoic acid was purchased from Nutritional Biochemicals (Cleveland, OH, U.S.A.). 6-Aminohexanoic acid was purchased from Chemical Procurement Labs. (College Pt., NJ, U.S.A.). 2,4′-Dibromoacetophenone was purchased from Aldrich. 1-Iodopropane was purchased from Eastman Organic Chemicals (Rochester, NY, U.S.A.). Dowex 1-X8 (200–400 mesh, Cl^- form) anion-exchange resin was purchased from Sigma and converted to the OH^- form according to instructions published by Bio-Rad Labs. (Richmond, CA, U.S.A.).

Synthesis of authentic compounds

4′-Bromophenacyl triflate was synthesized as described previously [33]. Authentic TMA-carboxylates were synthesized from the corresponding ω-amino acids according to modifications [16] of the method of Linstedt and

Lindstedt [14]. 4-(N,N-Dimethyl-N-propylammonio)-3-hydroxybutanoate (N-propylcarnitine) was prepared by N-demethylation of carnitine [34] and alkylation of the resultant 4-(N,N-dimethylamino)-3-hydroxybutanoic acid by 1-iodopropane. Product identity was confirmed by ^1H NMR spectroscopy. [^{14}C-Methyl] trimethylammoniobutanoate was synthesized and purified as described [16], as was [^{14}C-methyl] carnitine [34].

Authentic 4′-bromophenacyl esters of TMA-carboxylates were synthesized by slight modifications of the method of Cooper and Anders [32]. Thus, a TMA acid (Cl^- salt) was applied in aqueous solution to a column of Dowex 1-X8 (OH^- form) anion-exchange resin of excess ion-exchange capacity and eluted with 4 column vols. of deionized water. The effluent was collected, evaporated to dryness, and redissolved in DMF. To this were added 1.1 molar equivalents of both triethylamine and 2,4′-dibromoacetophenone, and the solution was heated to 40°C with stirring. After 3 h, the product was precipitated by addition of ethyl acetate, collected by filtration, and recrystallized from ethanol—acetone. Product identities were confirmed by ^1H NMR spectroscopy, and the products were shown to be of greater than 99% chromatographic purity at 254 nm.

Sample preparation

Standard solutions of carnitine, 4-TMA-butanoate, and 2-TMA-acetate were prepared in water at concentrations of 10—100 nmol/ml. Aqueous working solutions of N-propylcarnitine and 6-TMA-hexanoate were prepared at concentrations of 1 μmol/ml. The former compound was used as a procedural internal standard for carnitine determination, while the latter was used for determinations of 4-TMA-butanoate and 2-TMA-acetate. In a 1.5-ml (13 × 39 mm) polypropylene Eppendorf tube were combined 600 μl aqueous TMA-carboxylate standard solution and 60 μl of the appropriate trialkylammonio carboxylate internal standard working solution. The tube was vortexed, and 550 μl of the contents were applied to a 7 × 0.5 cm column of Dowex 1-X8 (OH^- form) anion-exchange resin contained by a Pasteur pipet. Excluded species were eluted from the column with 2 ml of deionized water. The effluent was collected in a 13 × 100 mm polypropylene test tube and the contents evaporated to dryness by a gentle stream of oil-free compressed air.

Derivatization

The dry residue from the isolation procedure was reconstituted in 100 μl of $2 \cdot 10^{-3}$ *M* N,N-diisopropylethylamine in distilled acetonitrile and vortexed for 2 min. To this were added 100 μl of a $5 \cdot 10^{-3}$ *M* solution of 4′-bromophenacyl triflate in acetonitrile, and the sample was vortexed for 10 min. The excess alkylating agent was destroyed by addition of 100 μl of $1 \cdot 10^{-2}$ *M* hydroxyacetic acid N,N-diisopropylethylammonium salt in acetonitrile and vortexing for 2 min. Sample aliquots of 15 μl were injected directly into the chromatograph.

Chromatography

Two chromatographic eluents were used. The mobile phase used for chro-

matography of carnitine 4′-bromophenacyl ester derivatives was $5.0 \cdot 10^{-4}$ *M* SDS, $2.0 \cdot 10^{-3}$ *M* sodium dihydrogen phosphate, and $5.0 \cdot 10^{-3}$ *M* 3-(N,N-dimethylamino)-1,2-propanediol in acetonitrile—water (75:25, v/v). This was prepared by dissolving 0.070 g ($2.5 \cdot 10^{-4}$ mol) of SDS, 0.140 g ($1 \cdot 10^{-3}$ mol) of sodium dihydrogen phosphate (monohydrate), and 0.30 ml (0.30 g, $2.5 \cdot 10^{-3}$ mol) of 3-(N,N-dimethylamino)-1,2-propanediol in 125 ml water, adjusting the pH to 6.5 with 85% phosphoric acid, and filtering this solution through a 0.2-μm pore diameter cellulose nitrate membrane. This was combined with 375 ml of filtered acetonitrile with thorough magnetic stirring. The mobile phase used for chromatography of 2-TMA-acetate and 4-TMA-butanoate 4′-bromophenacyl esters was $1.0 \cdot 10^{-3}$ *M* SDS, $1.0 \cdot 10^{-3}$ *M* sodium dihydrogen phosphate, and $1.0 \cdot 10^{-2}$ *M* 3-(N,N-dimethylamino)-1-propanol in acetonitrile—water (70:30, v/v). This was prepared analogously. The eluents in both cases were pumped at 5.0 ml/min. Absorbance of the effluent stream was monitored at 254 nm.

Verification of sample recovery through sample preparation columns

The recovery of TMA-carboxylates from the Dowex 1-X8 anion-exchange resin columns was demonstrated by application of aliquots of [^{14}C-methyl]-carnitine to several columns and collection of 0.5-ml eluent fractions directly in 5.5-ml scintillation vials. Scintillation cocktail was added, and the radioactivity of each fraction was determined by liquid scintillation counting. Recovery of applied radioactivity was determined by comparison of eluted radioactivity with the radioactivity found in aliquots of labeled material equal in volume to that applied to the sample preparation column.

Reaction completion verification

The extent of completion of the reaction of 4′-bromophenacyl triflate with the TMA-carboxylates under the recommended conditions was evaluated by subjecting an aliquot of [^{14}C-methyl]carnitine to the entire procedure. The resulting reaction mixture was chromatographed as described, but at an eluent flow-rate of 1.0 ml/min to permit collection of 1.0-ml eluent fractions in 5.5-ml scintillation vials. Scintillation cocktail was added and the contained radioactivity determined by liquid scintillation counting. Recovery of the sample radioactivity from the chromatograph was established by comparison of the radioactivity found within the eluent fractions and that detected in a reaction mixture aliquot equal in volume to that chromatographed.

Quantitation

Standard curves of carnitine:N-propylcarnitine, 2-TMA-acetate:6-TMA-hexanoate, and 4-TMA-butanoate:6-TMA-hexanoate were established over a sample concentration range of 10—100 nmol/ml. All standard solutions were determined in duplicate for standard curve generation. Linearity of detector response over this sample concentration range was established by algebraic least-squares fit of determined chromatographic peak height ratios and their respective sample concentrations to a linear equation.

Time course of the reaction of carnitine and 4-TMA-butanoate with 2,4'-dibromoacetophenone (Fig. 1)

In 15-ml conical screw-capped test-tubes were combined 200 μl of a 10^{-4} *M* solution of carnitine or 4-TMA-butanoate in ethanol, $2 \cdot 10^4$ dpm of the corresponding ^{14}C-labeled substrate, and 3 μl of $6 \cdot 10^{-2}$ *M* N,N-diisopropylethylamine in methanol. The tubes were vortexed, and the solvents were evaporated under a compressed air stream. The reaction was started by addition of 500 μl of $7 \cdot 10^{-3}$ *M* 2,4'-dibromoacetophenone in isopropanol. The tubes were capped, placed in a water bath at 70°C, and shaken continuously during the reaction period. At the indicated time intervals, one tube was cooled to room temperature, opened, and 300 μl of water were added to the reaction mixture. The solution was extracted with 6 ml of *n*-butyl acetate, and a 75 μl-aliquot of the aqueous phase was injected into the liquid chromatograph.

Chromatography was performed with the apparatus described under *Equipment.* A 30 × 0.39 cm steel column packed with μBondapak C_{18} reversed-phase medium (10 μm nominal particle diameter) was used. The mobile phase was $5 \cdot 10^{-3}$ *M* sodium heptanesulfonate in acetonitrile—water (62:38, v/v), and was pumped at 1.5 ml/min. Eluent fractions of 0.5 ml were collected, and the contained radioactivity was determined as described. The extent of reaction was calculated by comparison of the radioactivity which co-chromatographed with the carnitine or 4-TMA-butanoate derivative peak with that contained in a second 75-μl aliquot of the same reaction solution extract. These derivatization and chromatographic conditions were found to be unsuited for determination of TMA-carboxylates at lower sample concentrations.

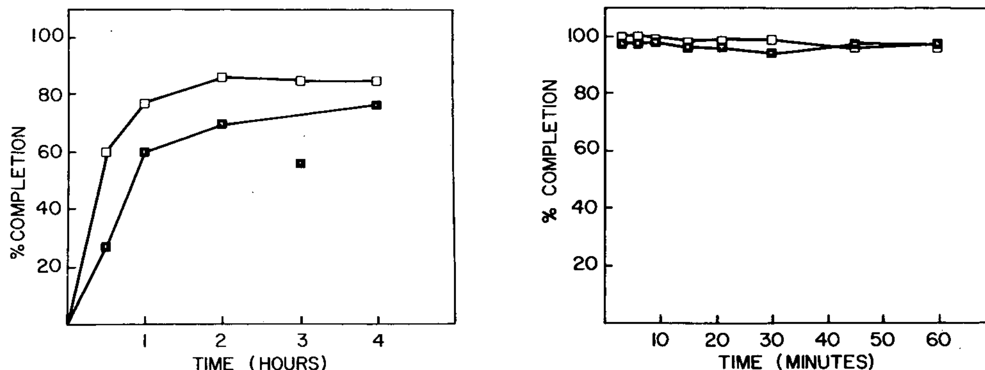

Fig. 1. Extent of reaction of carnitine (■) and 4-TMA-butanoate (□) with 2,4'-dibromoacetophenone versus time. Details are given in the Experimental section. The derivatization and chromatographic conditions were found to be unsuited for determination of TMA-carboxylates at low concentrations.

Fig. 2. Extent of reaction of carnitine (■) and 4-TMA-butanoate (□) with 4'-bromophenacyl triflate versus time. Details are given in the Experimental section. Data points are the average of two determinations.

Time course of the reaction of carnitine and 4-TMA-butanoate with 4'-bromophenacyl triflate (Fig. 2)

In 15-ml conical screw-capped test tubes were combined 2 μl of a 10^{-1} *M* ethanolic solution of carnitine or 4-TMA-butanoate, and 2 μl of a solution of

the corresponding ^{14}C-labeled substrate containing about $8 \cdot 10^4$ dpm of radioactivity. The samples were neutralized by addition of 10 μl of a solution of $8.4 \cdot 10^{-2}$ *M* tri-*n*-butylamine in acetonitrile, and then were evaporated to dryness. The reaction was started by addition of 150 μl of $8.6 \cdot 10^{-3}$ *M* 4'-bromophenacyl triflate in acetonitrile and 50 μl of $2.1 \cdot 10^{-2}$ tri-*n*-butylamine in acetonitrile. The tubes were vortexed, capped, and allowed to stand at room temperature. At the indicated time intervals, a pair of tubes were immersed in a dry-ice—acetone bath, the frozen solvent was removed under vacuum, and the contents reconstituted in 500 μl of 10^{-2} *M* hydrochloric acid. A 50-μl aliquot was injected directly into the chromatograph without extraction. Chromatography, fraction collection, and liquid scintillation counting were performed as described for trials with 2,4'-dibromoacetophenone, although no spectrophotometric detector was used. The extent of reaction was determined by comparison of the radioactivity isolated in fractions containing the carnitine or 4-TMA-butanoate derivative peaks with the sum of the radioactivity isolated in both those fractions and fractions containing unreacted labeled substrate. Overall recovery of radioactivity was calculated by comparison of total radioactivity isolated from the chromatographic column with that contained in a second 50-μl aliquot of the reconstituted residue from the derivatization reaction. These recoveries were 95% (± 7%, $n = 16$) in experiments with carnitine, and 89% (± 7%, $n = 16$) in experiments with 4-TMA-butanoate.

RESULTS AND DISCUSSION

Our work related to the biosynthesis of carnitine necessitated the development of an analytical method for the determination of several TMA-carboxylates with a measurement limit of 10 nmol/ml. These compounds are not amenable to detection by any of the usual methods employed for HPLC without prior derivatization. 4'-Bromophenacyl ester derivatives have proved useful for highly sensitive determinations of carboxylic acids. These derivatives are intensely chromophoric (λ_{max} = 250–260 nm, log ϵ = 3.8–4.5 [21, 22]), the required alkylating agent (2,4'-dibromoacetophenone) is commercially available and inexpensive, and the methodology of the derivatization procedure apparently has been well developed [20—31]. Two important general methods have been employed for preparation of 4'-bromophenacyl esters on the analytical scale. Durst et al. [22] reacted 10^{-3} *M* carboxylate potassium salts with a two-fold excess of 2,4'-dibromoacetophenone and 5—10 mol% of the macrocyclic polyether phase-transfer catalyst 18-crown-6 in acetonitrile at 80°C for periods of 15—30 min. Cooper and Anders [32] prepared the analogous 2-naphthacyl esters by reaction of carboxylate trialkylammonium salts with similar equivalent proportions of 2-bromoacetonaphthone in DMF at 40°C in periods of hours. Variations of both procedures have been used for determination of carboxylates [20—31].

We applied modifications of both procedures to [^{14}C-methyl] carnitine and [^{14}C-methyl] 4-TMA-butanoate with 2,4'-dibromoacetophenone as the alkylating agent. All attempts under reaction conditions similar to the crown-ether phase-transfer catalyzed system failed to produce derivatives, while some suc-

cess was achieved in experiments with variations of the latter mentioned procedure. Eventually, we found that yields of derivatives as great as 90% could be prepared by extension of reaction times and through use of forcing temperature and concentration conditions (Fig. 1). Interestingly, similar yields of homologous two-, five-, and six-carbon TMA-carboxylates could be prepared in shorter reaction times. The resistance of the four-carbon TMA-acids to carboxyl-O-alkylation by 2,4′-dibromoacetophenone may arise from their assumption of a folded conformation permitting strong ionic interaction of the large onium head and carboxylate tail of the molecule, thereby limiting carboxylate approach to electrophiles. Examination of framework models suggests that the folded conformation allowing close proximity of both ends of the molecule are accessible and preferred only in the four-carbon TMA-acid homologues.

The low rate of carboxylic acid O-alkylation by 2,4′-dibromoacetophenone in dilute solution and at elevated temperatures [21, 22, 25, 32] suggested that there is a significant activation barrier to displacement of bromide from the alkylating agent. We reasoned that a more reactive derivatization reagent would provide better derivative yields at lower sample concentrations and at lower temperatures. We therefore designed a synthetic route to 4′-bromophenacyl triflate [33]. This new alkylating agent proved highly reactive toward samples of ^{14}C-labeled carnitine and ^{14}C-labeled 4-TMA-butanoate in acetonitrile solution, with complete reaction occurring within a few minutes at room temperature. Fig. 2 represents a progress curve for the reactions of [^{14}C-methyl]carnitine and [^{14}C-methyl]4-TMA-butanoate with 4′-bromophenacyl triflate in acetonitrile at room temperature.

The derivatization reaction with the new alkylating agent occasionally failed. This problem was solved completely by inclusion of trialkylamines in the reaction solution. Although the TMA-carboxylates probably do not form intimate ion pairs with trialkylammonium ions in solution, the added base does neutralize any residual acid present in the sample. Thus, the reaction succeeds uniformly when the carboxylate conjugate bases are the species actually present in the reaction medium. Among bases tested, the sterically hindered N,N-diisopropylethylamine was alkylated least rapidly by the derivatization reagent [33], and therefore was used for sample neutralization.

The formation of side-reaction products during derivatization of organic acids by 2,4′-dibromoacetophenone has been noted [23]. One of these by-products was identified spectroscopically as 2-chloro-4′-bromoacetophenone by Patience and Thomas [31], who showed that Cl^- ions present in the reaction solution compete successfully with carboxylates for available 2,4′-dibromoacetophenone. Since 4′-bromophenacyl triflate readily underwent the same reaction, a method for removal of Cl^- from samples of TMA-acid (Cl^- salts) was required. The strong anion exchanger Dowex-1 (OH^- form) quantitatively exchanges Cl^- for OH^-, and does not retain TMA-carboxylates as their dipolar ions present in solution at neutral pH. Columns of Dowex-1 were used to prepare samples of the TMA-carboxylates for both semi-preparative and microscale syntheses of their 4′-bromophenacyl ester derivatives. Recovery of carnitine from these columns was investigated by application of [^{14}C-methyl]-carnitine to a 7 × 0.5 cm column of Dowex 1-X8 (200—400 mesh, OH^- form)

anion-exchange resin contained by a Pasteur pipette. Elution of the columns with 2 ml of deionized water allowed the recovery of 95% (± 1%, $n = 4$) of the applied radioactivity in the collected effluent.

To facilitate the development of an HPLC system for separation of the TMA-carboxylate esters from other species in the reaction mixture, authentic 4′-bromophenacyl esters of carnitine, 2-TMA-acetate, and 4-TMA-butanoate were synthesized. These cations are candidates for chromatography by a reversed-phase ion-pair separation mechanism. Preliminary experimentation with the μBondapak C_{18} medium led to the development of chromatographic conditions adequately selective for separation of derivatization reaction products in concentrated samples. However, these conditions proved unsuitable for work with samples of biologically representative concentrations owing to chromatographic interference. We therefore experimented with the considerably more retentive reversed-phase medium Radial-Pak C_{18}. Initially, an eluent system containing $1 \cdot 10^{-2}$ M SDS and $1 \cdot 10^{-2}$ M sodium dihydrogen phosphate in acetonitrile—water (50:50, v/v) was tried. The TMA-carboxylate ester derivatives were retained extensively under these conditions, and the chromatograms exhibited poor peak shapes. These problems were solved by the inclusion of triethylamine in the eluent. It has been proposed that amine modifiers in chromatographic eluents used for reversed-phase chromatography on silica-bonded reversed-phase media masks residual silanol groups which otherwise interact strongly with the basic functional groups of some solutes [35, 36]. This led us to investigate several tertiary amines as possible chromatographic mobile phase constituents. Experimentation with authentic esters and later with micro-scale synthetic mixtures led to the development of the eluents actually used. N-Propylcarnitine and 6-TMA-hexanoate were selected as procedural internal standards on the basis of their retention relative to other sample constituents under these chromatographic conditions. The inversion of the expected reversed-phase ion-pair elution order of 2-TMA-acetate and 4-TMA-butanoate was confirmed by ^{1}H NMR spectroscopy of the authentic compounds.

We found it necessary to remove excess 4′-bromophenacyl triflate from the sample matrix to prevent chromatographic interference by slower forming side-reaction products. This was accomplished by the addition of a molar excess of a carboxylic acid—tertiary amine solution (1:1 molar) to the reaction mixture after allowing a few minutes for TMA acid ester derivative formation. Hydroxyacetic acid—N,N-diisopropylethylamine was suitable for this purpose. The resultant reaction product was nearly unretained under these chromatographic conditions.

To demonstrate the extent of the derivatization reaction, [^{14}C-methyl]-carnitine was subjected to the described reaction conditions and chromatographic system. Of the radioactivity committed to the experiment, 96% was recovered from the chromatographic column, and 98% of the recovered radioactivity appeared in a peak which co-chromatographed with the chromatographic peak of injected carnitine 4′-bromophenacyl ester.

Fig. 3 is a chromatogram of an aqueous blank specimen carried through the analytical scheme and chromatographed with the eluent used for determination of carnitine. A chromatogram of a processed 10 nmol/ml carnitine stan-

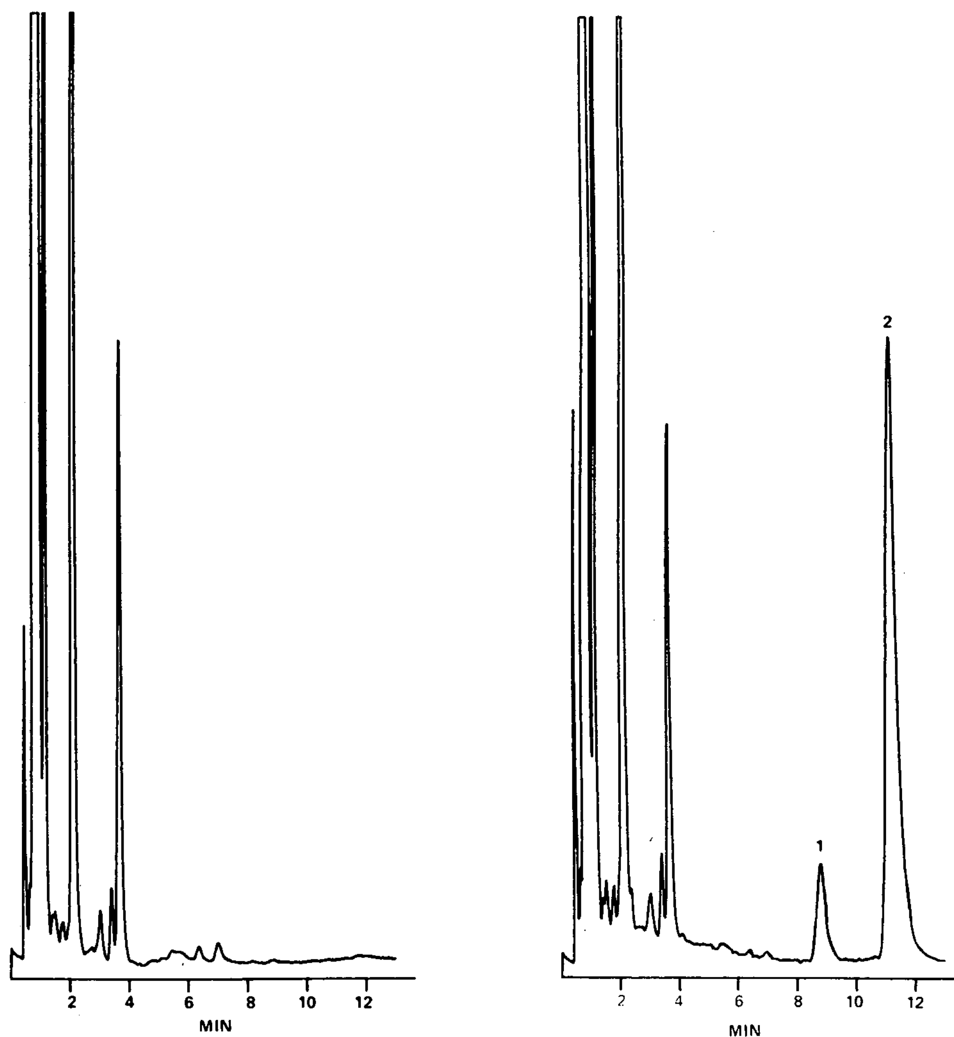

Fig. 3. Chromatogram obtained after isolation and derivatization of an aqueous blank specimen according to the procedure described in the text and chromatography with the eluent used for carnitine determination. The chromatographic column was a 10 × 0.8 cm plastic cartridge containing Radial-Pak C_{18} (5 μm nominal particle diameter). The eluent was $5 \cdot 10^{-4}$ *M* SDS, $2 \cdot 10^{-3}$ *M* sodium dihydrogen phosphate, and $5 \cdot 10^{-3}$ *M* 3-(N,N-dimethylamino)-1,2-propanediol in acetonitrile—water (75:25, v/v). The sample aliquot injected was 15 μl. The pump was operated at a flow-rate of 5 ml/min. The absorbance detector was operated at 254 nm. Full scale of the ordinate is 0.02 absorbance units (a.u.).

Fig. 4. Chromatogram obtained after isolation and derivatization of 5 nmol of carnitine and 50 nmol of N-propylcarnitine. The chromatographic conditions were as described under Fig. 3. A 15-μl aliquot of the reaction mixture was injected into the chromatograph. Peaks: 1 = carnitine 4'-bromophenacyl ester; 2 = N-propylcarnitine 4'-bromophenacyl ester.

dard solution containing N-propylcarnitine internal standard is shown in Fig. 4. Fig. 5 is a chromatogram of a processed aqueous blank carried through the entire analytical scheme and chromatographed with the eluent used for determination of 2-TMA-acetate and 4-TMA-butanoate. Chromatograms of pro-

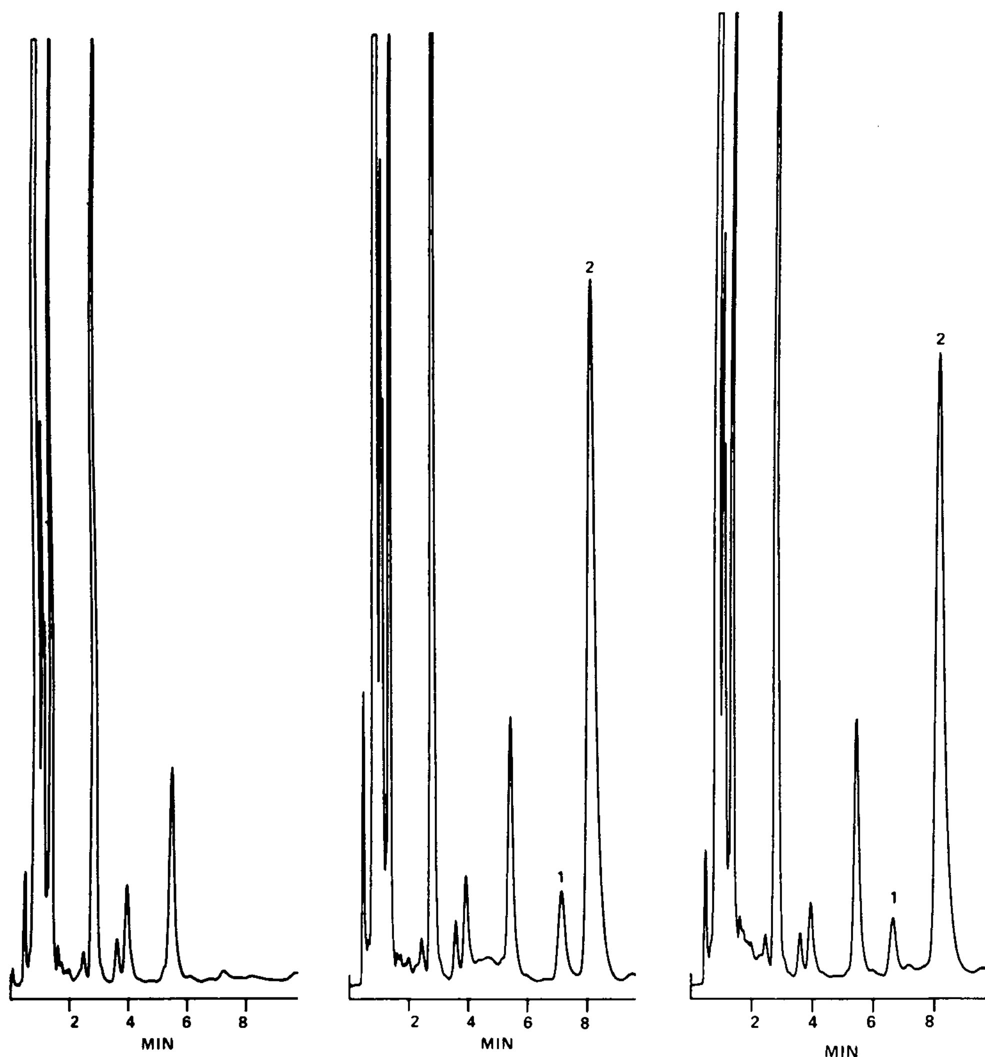

Fig. 5. Chromatogram obtained after derivatization of an aqueous blank specimen and chromatography with the eluent used for 2-TMA-acetate and 4-TMA-butanoate determinations. The apparatus used was that described under Fig. 3. The eluent was $1 \cdot 10^{-3}$ *M* SDS, $1 \cdot 10^{-3}$ *M* sodium dihydrogen phosphate, and $1 \cdot 10^{-2}$ *M* 3-(N,N-dimethylamino)-1-propanol in acetonitrile—water (70:30, v/v). The sample aliquot injected was 15 μl. The pump was operated at a flow-rate of 5 ml/min. The absorbance detector was operated at 254 nm, and the full scale of the ordinate is 0.02 a.u.

Fig. 6. Chromatogram obtained after derivatization of 5 nmol of 2-TMA-acetate and 50 nmol of 6-TMA-hexanoate, internal standard, isolated according to the described procedure. The chromatographic conditions were as described under Fig. 5. Peaks: 1 = 2-TMA-acetate 4'-bromophenacyl ester; 2 = 6-TMA-hexanoate 4'-bromophenacyl ester.

Fig. 7. Chromatogram obtained after derivatization of 5 nmol of isolated 4-TMA-butanoate and 50 nmol of isolated 6-TMA-hexanoate. The chromatographic conditions were as described under Fig. 5. Peaks: 1 = 4-TMA-butanoate 4'-bromophenacyl ester; 2 = 6-TMA-hexanoate 4'-bromophenacyl ester.

cessed aqueous standard solutions containing 10 nmol/ml of 2-TMA-acetate or 4-TMA-butanoate, with included 6-TMA-hexanoate internal standard, are shown in Figs. 6 and 7, respectively.

Standard curves of carnitine:N-propylcarnitine, 2-TMA-acetate:6-TMA-hexanoate, and 4-TMA-butanoate:6-TMA-hexanoate peak height ratios versus sample concentration all were found to be linear over a sample concentration range of 10—100 nmol/ml. The linear regression coefficients were, respectively, 0.9983, 0.9952, and 0.9908; slopes were 0.0152, 0.0115, and 0.0084; *Y*-intercepts were —0.0055, 0.0234, 0.0113. These *Y*-intercept values are less than 18% of the chromatographic peak height ratios obtained at the 10 nmol/ml concentration point.

The method as presented is suitable for determination of the specific radioactivity and radiochemical purity of labeled carnitine and TMA-butanoate. We also have extended the procedure to the determination of carnitine in urine. This application requires complete removal of large accompanying quantities of inorganic salts from the urine sample matrix. These salts otherwise interfere with the derivatization of TMA acids, both by occlusion of the compounds of interest within the salt residue and by the side-reaction of nucleophilic anions with the derivatization reagent [33]. Furthermore, some functionally polar sample constituents not removed by the usual desalting techniques were found to interfere chromatographically, necessitating changes in the composition of the chromatographic eluent. A separate manuscript describing the determination of total carnitine in human urine is in preparation.

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CHROMBIO. 2305

AUTOMATED THEOPHYLLINE ASSAY USING GAS CHROMATOGRAPHY AND A MASS-SELECTIVE DETECTOR

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SUMMARY

An automated gas chromatographic—mass spectrometric assay for theophylline is described. Theophylline is extracted from plasma or urine (50 µl) and transformed into an N-pentyl derivative. The internal standard used for quantitation is [1,3-^{15}N, 2-^{13}C]theophylline. The detection is performed by monitoring the molecular ions 250 for theophylline and 253 for the internal standard with a quadrupole mass specific detector HP 5790 A. The system has been fully automated: injection, calibration, assay, calculation. The method shows excellent analytical parameters: linearity between 2 and 40 µg/ml; day-to-day reproducibility 1.82% for a concentration of 15 µg/ml; repeatability 0.75% (15 µg/ml) and 0.33% (30 µg/ml). Accuracy is also excellent. Due to the use of an internal standard labelled with stable isotopes, the specificity and high analytical quality of the method make it useful as a reference method to compare with routine theophylline assays.

INTRODUCTION

Theophylline (1,3-dimethylxanthine) is widely used to treat asthma, apnea of prematurity and obstructive lung diseases. To be effective, theophylline plasma levels must be within a narrow therapeutic range (7—20 mg/l) [1, 2].

Many factors have been reported to cause variations in the pharmacokinetics of this drug, among them age [3], nutrition [4], diseases [5, 6], administration of other drugs or xenobiotics [7—9]. Because of the narrow therapeutic index

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and numerous sources of kinetic variations, theophylline levels have to be monitored carefully to insure safe and effective use of this drug.

Current methods for measuring theophylline in plasma or serum include spectrophotometry, liquid chromatography and several immunoassay techniques.

We describe here a method using capillary gas chromatographic (GC) separation and detection with a mass specific detector with automatic monitoring of the analytical procedure. This method can deliver one assessment every 16 min using theophylline labelled with stable isotopes (^{15}N, ^{13}C) as an internal standard.

EXPERIMENTAL

Materials

Theophylline (1,3-dimethylxanthine) was purchased from Sigma (St. Louis, MO, U.S.A.). The internal standard, [1,3-^{15}N, 2-^{13}C]theophylline, was synthesized by C.E.A. (Saclay, France). Tetramethylammonium hydroxide, N,N-dimethylacetamide, iodopentane, isopropanol, ethyl acetate and chloroform (analytical-grade reagent) were purchased from E. Merck (Darmstadt, F.R.G.) and used without further purification. In order to check the analytical quality of the method, standard sera containing various known concentrations of theophylline were obtained from Biotrol (Paris, France) and Syva Biomérieux (Lyon, France).

The stock solution of internal standard (100 $\mu g\ ml^{-1}$) was prepared in ethanol. It was stored at 4°C and could be used within a month. The working solution was prepared by a ten-fold dilution in water (10 $\mu g\ ml^{-1}$). The extraction solvent was chloroform—isopropanol (95:5). Before extraction, serum or plasma was buffered with acetate buffer (pH 5.2).

Extraction

To 50 μl of serum or plasma in a 20-ml conical centrifuge tube were added 200 μl of acetate buffer (pH 5.2) and 50 μl of internal standard solution (10 $\mu g\ ml^{-1}$). After mixing and addition of 2 ml extraction solvent, the sample was extracted for 1 min on a vortex mixer. After centrifugation, the organic phase was transferred to another tube and evaporated to dryness under a stream of nitrogen at 40°C.

Derivatization of the NH groups of both theophylline and internal standard was performed according to the alkylation procedure described by Greeley [10] for barbiturates and adapted to theophylline by Johnson et al. [11] for N-butyl derivatives and by Lowry et al. [12], Berthou et al. [13], Joern [14] for N-pentyl derivatives, and for methylxanthines as we described previously [15]. A 50-μl volume of N,N-dimethylacetamide and 25 μl of a 0.1 *M* solution of tetramethylammonium hydroxide were added to the drug residue and thoroughly mixed for 10 sec. Then 25 μl of iodopentane were added to the solution which was shaken and allowed to stand at room temperature for 10 min. The organic phase was transferred to another tube and evaporated to dryness. The sample was then ready for injection.

Chromatographic separation

The gas chromatograph used was a Hewlett-Packard Model 5790 designed for capillary column chromatography. This apparatus was equipped with an automatic injector HP 7672 A. The capillary column used was a cross-linked dimethylsilicone silica column, 12 m × 0.23 mm. Samples were automatically injected into the chromatograph according to the splitless mode. The splitless valve time was 1 min. The injector temperature was set at 250°C. Oven temperature was programmed from 115°C (1 min) to 195°C (5 min) at 15°C/min. The transfer line temperature was 260°C. Helium was used as carrier gas. Before splitless injection, the drug extraction residue was dissolved in 70 μl of a toluene—ethyl acetate mixture (50:20, v/v). The injected volume was 1 μl.

Detection and measurements

A mass-selective detector Hewlett Packard 5970 A was used for detection. The monitored ions were *m/z* 250 for theophylline and *m/z* 253 for the internal standard. The dwell time was 75 msec for each ion. The detector was operating between 7 and 8.5 min, the retention time of theophylline being 7.76 min in the described conditions. Areas were integrated in the horizontal mode with a 5% slope sensitivity and a rejection area of 150.

Analytical-quality parameters measurements

Standardization and linearity were checked from Syva standard sera using the following concentrations: 2.5, 5, 10, 20 and 40 μg ml^{-1}. In order to check reproducibility two samples containing theopylline at 7 and 15 μg ml^{-1} (Biotrol) were extracted and measured once a day for ten days. Precision was measured by the determination of theophylline concentrations ten times a day from standard sera (Biotrol) containing 7.5, 10 or 30 μg ml^{-1} theophylline.

Accuracy was determined by comparison of measured concentrations and true concentrations from fifteen different commercial standard sera ranging between 2.5 and 40 μg ml^{-1}. Each of these sera was extracted twice and extraction residues injected twice.

The method was then validated by the routine determination of 100 samples for therapeutic drug monitoring and the results were compared with those obtained by the enzyme multiplied immunoassay technique (EMIT).

RESULTS

Mass spectra and fragmentograms

Fig. 1 shows the mass spectra of the N7-pentyl derivatives of theophylline (Fig. 1A) and [1,3-^{15}N, 2-^{13}C]theophylline (Fig. 1B) used as internal standard. Molecular ions are *m/z* 250 and 253, respectively, and base peaks *m/z* 180 and 183, respectively. They correspond to the M—pentyl (M-70) fragment ion. Because of slight interference in patient's serum or plasma at *m/z* 180 due to a fatty acid fragment, molecular ions were used for mass fragmentography. The relative abundance of these ions was large enough (45%) to ensure a good analytical quantitation. Typical fragmentograms are shown in Fig. 2a for a standard serum and in Fig. 2b for a patient's serum. They correspond to the monitoring of ions 250 and 253. The retention time of the corresponding chro-

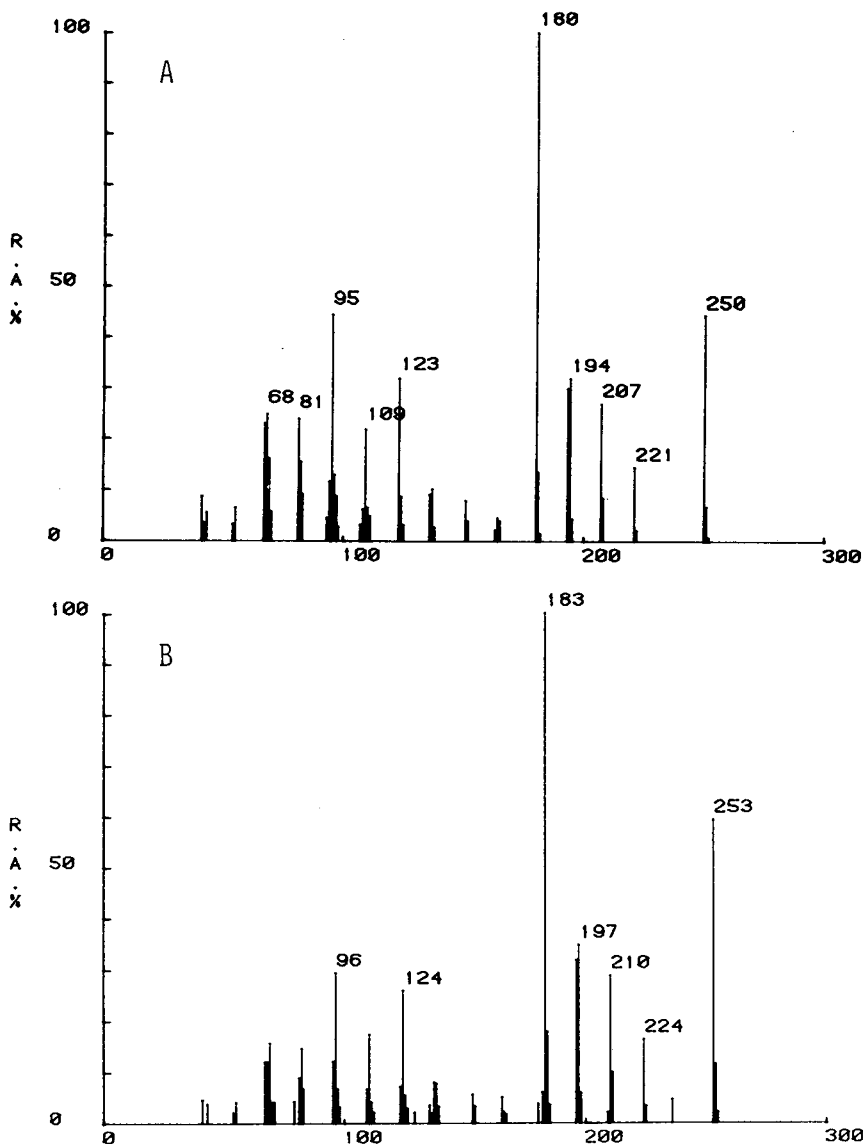

Fig. 1. (A) Mass spectrum of N-7-pentyl derivatives of theophylline. (B) Mass spectrum of N-7-pentyl derivatives of [1,3-^{15}N,2-^{13}C]theophylline.

matographic peak, in the described conditions, is 7.76 min. The fragmentogram of a blank sample does not show any peak at this retention time.

Linearity

A standard curve obtained under the conditions described above is shown with its confidence interval in Fig. 3. Each point is the result of a duplicate injection. The regression analysis gave the following results: slope 0.1108; intercept 0.035, r = 0.997; residual error $1.7 \cdot 10^{-3}$. Samples for another

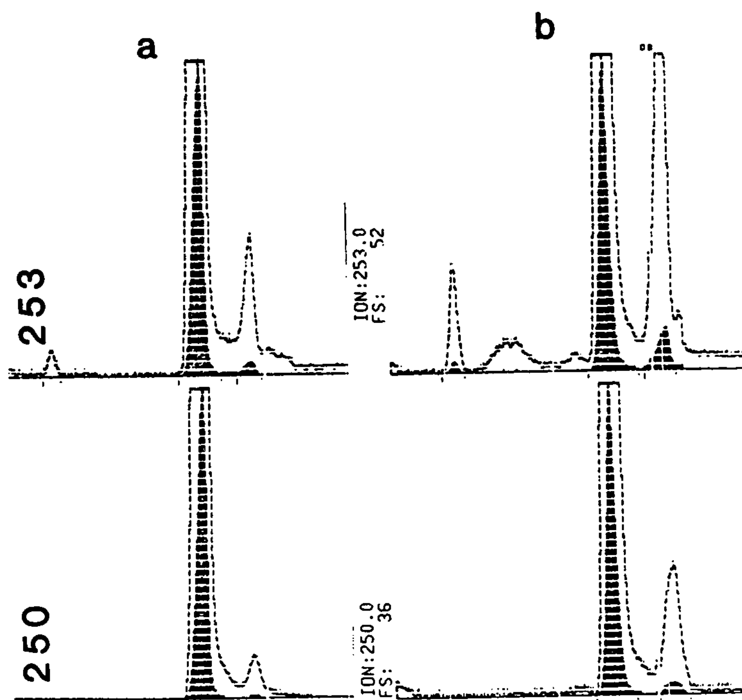

Fig. 2. Typical fragmentograms of ions 250 and 253 (a) from a standard serum, (b) from a patient's serum.

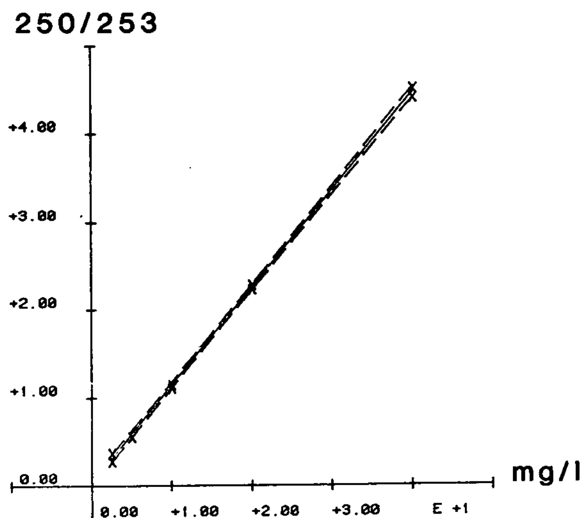

Fig. 3. Calibration curve with confidence interval.

standard curve were extracted and injected only once on day 0 and then on day 6. The results shown in Table I indicate that linearity is very good in the range 2.5—40 μg ml^{-1} and that extracted samples can be stored at 4°C before measurement without any loss.

TABLE I

LINEAR REGRESSION OF A STANDARD CURVE MEASURED ON DAY 0 AND DAY 6

	Day 0	Day 6
Slope	0.1059	0.1056
Intercept	$4.8 \cdot 10^{-2}$	$6.3 \cdot 10^{-2}$
r	0.99993	0.99994
Residual error	$2.34 \cdot 10^{-4}$	$3.25 \cdot 10^{-4}$

Day-to-day reproducibility

The day-to-day reproducibility measured by ten assays corresponding to a ten-day period gave the following results for the coefficient of variation: 1.93% for 7 μg ml^{-1} and 1.82% for 15 μg ml^{-1}.

Repeatability

Ten determinations of the same concentration on the same day gave the following coefficients of variation: 1.87% (7.5 μg ml^{-1}), 0.76% (15 μg ml^{-1}) and 0.33% (30 μg ml^{-1}).

Accuracy

The comparison of measured concentrations against true concentrations in the range 2.5—40 μg ml^{-1} for fifteen different standard sera gave the following results: slope 0.992; intercept 0.035, $r = 0.9999$; residual error $1.7 \cdot 10^{-3}$.

One hundred sera from patients treated with theophylline have been assayed with this method and the results compared with the EMIT. Concentrations ranged between 0 and 25 μg ml^{-1}. The parameters of the correlation (X = EMIT, Y = this method) are: slope 0.951; intercept 0.188; $r = 0.9997$. Results of the two methods are in good agreement.

CONCLUSIONS

This automatic method for theophylline assay exhibits good parameters in terms of analytical quality and practicability. Due to these qualities it can be used as a "reference method" to test other kinds of theophylline assays. Moreover, the automation of the analytical procedure affords a high degree of safety and quality for routine analysis. Finally, a mass-selective detector connected to a capillary gas chromatograph and the use of internal standard labelled with stable isotopes and automatic analytical procedures produce a flexible, practical and versatile tool which can be adapted to many other drug assays as well as therapeutic and biological profiles.

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CHROMBIO. 2303

IMPROVED MICRO-METHOD FOR THE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CAFFEINE AND PARAXANTHINE IN BIOLOGICAL FLUIDS

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SUMMARY

A high-performance liquid chromatographic procedure is reported for reproducibly and sensitively quantitating caffeine and its N-demethylated metabolite paraxanthine in micro-samples. A 5-μm reversed-phase radial compression column and 214-nm fixed wavelength ultraviolet detector were used to attain a sensitivity sufficient to quantitate these compounds at concentrations as low as 80 ng/ml using only 25 μl of sample. The assay is applicable to microliter samples of whole blood, serum, plasma, saliva, amniotic, cerebrospinal and gastric fluids such as might be obtained in studies involving small animals or neonates. The utility of the assay is illustrated with caffeine and paraxanthine levels measured in several maternal and fetal fluids following constant-rate intravenous infusion of caffeine into a rabbit throughout pregnancy.

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine) is ubiquitous in the American diet. It is also utilized in the therapy of neonatal apnea and produced in clinically significant concentrations as a metabolite of theophylline (1,3-dimethylxanthine) which is similarly administered. Its pharmacological and toxicological effects are not fully understood and are of increasing interest to both the scientific and lay communities. While various high-performance liquid chromatographic (HPLC) assays for caffeine have been published [1—3], they generally (1) suffer from worse reproducibility than determinations of comparable concentrations of theophylline and paraxanthine (1,7-dimethylxanthine), its N-demethylated metabolites, (2) poorly resolve paraxanthine from theophylline and (3) provide inadequate sensitivity to accurately quantitate low levels

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of methylxanthines in microsamples such as might be required in studies involving neonates or small animals. Several published caffeine assays [2, 3] involve sample work-ups that fail to take into account the relative insolubility of caffeine during reconstitution steps, resulting in susceptibility to incomplete and undependable recovery. We report assay modifications that result in the dependable, sensitive and selective quantitation of caffeine and paraxanthine in microliter samples of biological fluids.

EXPERIMENTAL

Chemicals

Paraxanthine, caffeine, theophylline, 3-methylxanthine, 1,3-dimethyluric acid, uric acid and β-hydroxyethyltheophylline (Sigma, St. Louis, MO, U.S.A.), 1-methyluric acid (Adams, Round Lake, IL, U.S.A.), 1-methylxanthine (Vega Biochemicals, Tucson, AR, U.S.A.), theobromine (Merck, Rahway, NJ, U.S.A.) and 1-methyl[^{14}C]caffeine (4 mCi/mmol, ICN, Irvine, CA, U.S.A.) were all used as received. Buffer salts were HPLC grade and water was treated with a Milli-Q water purification system equipped with a 0.45-μm final filter (Millipore). Methylene chloride (Burdick & Jackson Labs., Muskegon, MI, U.S.A.), methanol and tetrahydrofuran (Fisher Scientific) were HPLC grade and used without further purification. Tetrahydrofuran was stored tightly sealed under nitrogen.

Apparatus

The HPLC system consisted of a Varian Model 5020 liquid chromatograph (Varian, Palo Alto, CA, U.S.A.) equipped with a WISP autoinjector, a Model 441 fixed 214-nm ultraviolet (UV) detector (Waters Assoc., Milford, MA, U.S.A.) and a 10-mV Varian Model 9176 recorder. Separations were achieved using a radial compression 5-μm C_{18} reversed-phase column protected with a C_{18} Corasil Bondapak® precolumn (Waters Assoc.).

Standard solutions

Stock solutions of caffeine, paraxanthine, theophylline (1 mg/l) and β-hydroxyethyltheophylline (internal standard, 5 mg/l) were prepared in water and stored refrigerated at 4°C. These solutions were found to be stable for at least one month. Working aqueous solutions were prepared from caffeine, paraxanthine and theophylline stocks at concentrations of 0.025—0.5 mg/l. Volumes of 100 μl of each working solution, when added to 25 μl of plasma, resulted in effective standard concentrations of 0.1—2.0 mg/l of plasma. Results for standards prepared in this manner were identical to those obtained for standards prepared directly in plasma. An aqueous working internal standard solution contained 0.25 mg/l.

Extraction procedure

To 200 μl of 0.2 *M* phosphate buffer (pH 6.0) in a 13 × 100 mm borosilicate glass tube were added 25 μl of plasma and 100 μl of internal standard solution. Methylene chloride (3 ml) was added and the samples shaken for 20 min (120 oscillations per min) and centrifuged (174 *g*). The lower phase was transferred

to a clean 12 × 75 mm silanized borosilicate glass tube and the solvent removed under a stream of nitrogen at 60°C. Water (230 μl) was then added. The sample was held at 90°C in a heating block for 6 min, vortexed and approx. 180 μl were injected onto the column for chromatographic analysis.

Chromatography

The mobile phase consisted of a ternary mixture of tetrahydrofuran—methanol—0.01 *M* potassium dihydrogen phosphate, pH 3.5 (1:9:90). The mobile phase flow-rate was 2.5 ml/min and the effluent was monitored at 214 nm with the recorder set at 0.2 cm/min. Column temperature was ambient (approx. 23°C) since the radial compression system used as not amenable to further control. Peak heights of the methylxanthines were measured and the drug/internal standard ratios compared to those of the calibration standards. Aqueous stock solutions of uric acid, 1-methyluric acid, 1,3-dimethyluric acid, 3-methylxanthine, 1-methylxanthine and 3,7-dimethylxanthine (theobromine) were injected to check for chromatographic interference and worked up according to the above procedure to determine extraction characteristics.

Recovery studies

Drug-free plasma was equilibrated with methylene chloride containing 1-methyl[^{14}C]caffeine (50 ng per 3 ml, 4 mCi/mmol) using the same conditions and phase ratios as described in the extraction procedure above. The organic phase (2.5 ml) was then evaporated to dryness and the tube in which the drying was performed was tared. The residue was redissolved in approx. 200 μl of water and incubated for 6 min at 90°C. The reconstituted sample was weighed at room temperature and the liquid was transferred to a tared scintillation vial. The vial was reweighed to permit correction for losses due to transfer, then the sample was counted in a liquid scintillation counter (Beckman, Model LS-100). The overall recovery of caffeine was calculated as the ratio of the [^{14}C]caffeine present in the entire reconstituted sample to that present in the aliquot of radiolabelled drug which was originally added to the methylene chloride extractant. Recovery from the dry-down procedure was also determined in an attempt to gauge the contribution of irreversible drug loss to glass surfaces. For these experiments the methylene chloride solution of radiolabelled caffeine was dried without extraction and the quantity of reconstituted caffeine compared to that which had originally been placed in the system. In all cases appropriate quench corrections were performed.

Precision

Plasma specimens containing caffeine at concentrations of 0.3 and 1.7 mg/l and paraxanthine at concentrations of 0.3 and 1.5 mg/l were analyzed on six separate occasions to determine the between-day coefficient of variation of the assay. Ten specimens at each of the same concentrations were analyzed on the same day using a common standard curve to elucidate within-day variability. All plasma specimens were stored at −20°C in polystyrene vials between analyses.

Animal study

A refillable infusion pump (Infusaid, Model 300) was implanted in the infrascapular region of the thorax of a female New Zealand white rabbit. The outlet catheter from the pump was passed subcutaneously to the neck region and inserted into the superior vena cava via the jugular vein. A caffeine benzoate solution (80 mg/ml caffeine + 80 mg/ml sodium benzoate in water) was then infused at a nominal caffeine infusion rate of 20 mg/kg/day. The pump had previously been shown to maintain its nominal rate of delivery within a precision of ± 5% over periods in excess of 100 days. Following a seven-day stabilization period the rabbit was bred, then sacrificed at 29 days gestation. Maternal plasma and cerebrospinal fluid were sampled as well as fetal plasma, cerebrospinal, amniotic and gastric fluids. Aliquots of 25 μl of each fluid were analyzed for caffeine and paraxanthine by the present procedure.

RESULTS AND DISCUSSION

The chromatographic resolution of paraxanthine from theophylline using the 5-μm Radial-Pak® C_{18} column with UV detection at 214 nm is shown in Fig. 1. For comparison, a chromatogram run on a 30-cm C_{18} steel column (μBondapak C_{18}, Waters Assoc.) under the same conditions is inset. It can be seen from Fig. 1 that baseline resolution is achieved using the Radial-Pak column. A four-fold increase in signal-to-noise ratio was realized by using a detection wavelength of 214 nm instead of 254 nm. Chromatographic parameters are compared in Table I and retention times of all compounds of interest relative to internal standard for the radial compression system are listed in Table II. None of the uric acid and methylxanthine derivatives tested interfered with peaks of interest and only di- and trimethylxanthines were extracted by our procedure. Peak height ratios of paraxanthine and caffeine to internal

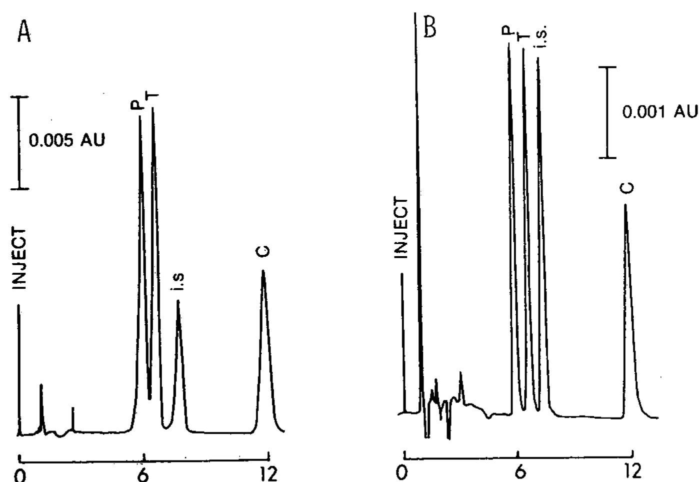

Fig. 1. Comparative resolution of paraxanthine from theophylline. Injected: 35 ng of each component; UV detection at 214 nm. (A) μ-Bondapak C_{18} (10 μm, 30 cm) column. (B) Radial-Pak C_{18} (5 μm) column. Peaks: P = paraxanthine; T = theophylline; C = caffeine; i.s. = internal standard = β-hydroxyethyltheophylline.

TABLE I

CHROMATOGRAPHIC PARAMETERS FOR METHYLXANTHINE SEPARATIONS

Parameter*	μBondapak C_{18}	Radial-Pak
k'_p	4.08	5.78
k'_t	4.50	6.78
k'_β	5.17	7.67
k'_c	8.25	12.9
$R_{p,t}$	0.71	2.21
$\alpha_{p,t}$	1.07	1.68

*p = paraxanthine; t = theophylline; β = β-hydroxyethyltheophylline; c = caffeine; k' = number of column volumes required to elute compound; R = resolution = distance between two band centers/average band width; α = ratio of column volumes required to elute two compounds.

TABLE II

RETENTION CHARACTERISTICS OF VARIOUS XANTHINES USING THE RADIAL-PAK SYSTEM

Compound	Retention time (min)	Relative retention*	Extracted during work-up
Uric acid	1.4	0.197	No
1-Methyluric acid	2.5	0.352	No
3-Methylxanthine	2.7	0.380	No
1-Methylxanthine	3.2	0.451	No
1,3-Dimethyluric acid	3.5	0.493	No
3,7-Dimethylxanthine	3.8	0.535	Yes
1,7-Dimethylxanthine	5.8	0.817	Yes
1,3-Dimethylxanthine	6.4	0.901	Yes
β-Hydroxyethyltheophylline	7.1	1.000	Yes
1,3,7-Trimethylxanthine	11.8	1.660	Yes

*Relative to β-hydroxyethyltheophylline as internal standard.

standard were found to be linear over the concentration range 0.1—2.0 mg/l (r^2 = 0.999).

Examination of several published HPLC assays for caffeine and theophylline [1—3] and our own attempts to develop an assay of sufficient sensitivity to quantify methylxanthines in microsamples from fetal animal studies brought an unsettling characteristic to our attention. While the ionizable dimethylxanthines paraxanthine and theophylline were generally determined with very low coefficients of variation (less than 5%), the variability in caffeine assays was often considerably higher. This was observed even when sample extraction was not part of the assay procedure as in the direct injection procedure of Tse and Szeto [2]. These authors reported a coefficient of variation for caffeine that was 1—2% at concentrations in excess of 10 mg/l and increased systematically to 12% at 0.5 mg/l. Theobromine, theophylline and paraxanthine showed no such effect. We observed similar results and since the total mass of caffeine in our 25-μl samples was 10—100 times lower than that in the 500-μl samples of Tse and Szeto [2], we felt it was necessary to seek a means of improving on

published caffeine assays. We also observed that the caffeine concentrations we obtained using published techniques were not normally distributed. Outliers unpredictably occurred as if some important parameter in the recovery of caffeine were inadequately controlled.

Caffeine has very limited solubility in many solvents, presenting the possibility that the reconstitution of caffeine extracts following dry-down might have been inefficient and undependable. The use of mobile phase as a reconstitution solvent resulted in caffeine recoveries that were both incomplete (77% overall) and highly variable (14.8% coefficient of variation). The use of any of a number of organic solvents dramatically improved the recovery and reduced the variability (Table III). Hot water was also very effective and was selected because of its compatibility with the chromatography. We postulate that it works well because caffeine is about 30 times more soluble in aqueous systems at 90°C than at 25°C. Using this solvent only about 1% of the dried caffeine residue is lost to the silanized tube.

TABLE III

RECOVERY OF CAFFEINE AS A FUNCTION OF RECONSTITUTION SOLVENT

Solvent	Overall recovery (%)	Coefficient of variation (%)
Water (90°C)	97*	2.5
Tetrahydrofuran	93*	2.9
Ethyl acetate	90*	4.9
Mobile phase	77**	14.8

*Measured by liquid scintillation counting.
**Measured by high-performance liquid chromatography.

Adsorptive loss was also a contributing factor and silanization of the final dry-down tube was found to be essential for precise determination of caffeine concentrations. While the slopes and intercepts of calibration curves derived from standards analyzed with and without silanized tubes were not significantly different for either caffeine or paraxanthine, replicate analyses of unknown plasma samples showed significantly greater coefficients of variation for the determination of caffeine concentrations using untreated glassware. The variability of paraxanthine analyses were unaffected by either reconstitution solvent or dry-down surface and under all experimental conditions were 5% or less.

The combined use of silanized tubes, hot water reconstitution and 214-nm detection has resulted in a very reproducible and sensitive assay for caffeine and its major metabolite paraxanthine. A chromatogram from 25-μl sample of plasma containing 1.0 mg/l caffeine, theophylline and paraxanthine which was analyzed using this method is shown in Fig. 2. Standard curves are linear, independent of matrix for plasma, cerebrospinal, amniotic and gastric fluids and the assay can quantitate 2 ng of each compound (80 ng/ml in a 25-μl sample) with a signal-to-noise ratio greater than 4. Table IV lists within- and between-day coefficients of variation for caffeine and paraxanthine at concentrations near the upper and lower limits of the concentration range studied

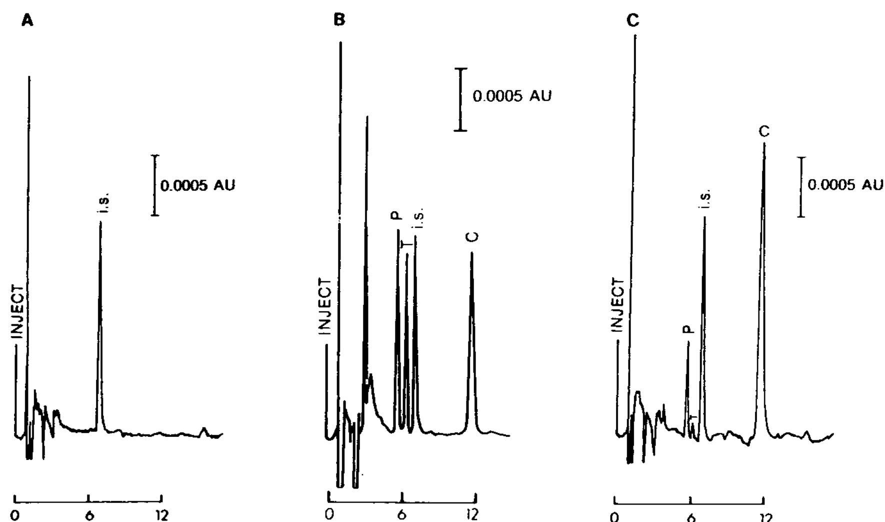

Fig. 2. Chromatograms of extracts of (A) blank rabbit plasma; (B) rabbit plasma spiked with paraxanthine (P), theophylline (T), β-hydroxyethyltheophylline (i.s.) and caffeine (C) all at 1.0 mg/l; (C) plasma from rabbit receiving caffeine (C = 1.72 mg/l, P = 0.48 mg/l).

TABLE IV

INTRA- AND INTER-DAY VARIABILITY IN THE ANALYSIS OF PARAXANTHINE AND CAFFEINE

	Concentration (mg/l)			
	Paraxanthine		Caffeine	
Actual	0.25	1.48	0.30	1.68
Calculated within-day (n = 10)				
Mean	0.25	1.43	0.31	1.65
S.D.*	0.01	0.03	0.02	0.09
C.V.**	5.2%	2.3%	4.8%	5.5%
Dev.***	+1.6%	−3.7%	+5.1%	−1.8%
Calculated between-day (n = 6)				
Mean	0.25	1.47	0.30	1.74
S.D.	0.01	0.03	0.01	0.06
C.V.	3.5%	2.1%	4.3%	3.3%
Dev.	+0.8%	−1.3%	+0.7%	+3.7%

*S.D. = standard deviation.
**C.V. = coefficient of variation (%).
***Dev. = percent deviation from actual.

(0.1—2.0 mg/l). They are about 5% or less in all instances. By comparison, one published assay [1] reports within-day coefficients of nearly 10% for 0.1-ml samples containing 200 and 100 ng of caffeine. We found a within-day coefficient of variation of 4.8% when we quantitated only 7.5 ng in a 0.025-ml sample. We have used a single column for over 1000 injections spanning months while maintaining good resolution and sensitivity.

TABLE V

MEAN FETAL FLUID/MATERNAL PLASMA CONCENTRATION RATIOS AT 29 DAYS GESTATION

Fetal fluid	Concentration ratio (mean ± S.D.)	
	Paraxanthine	Caffeine
Amniotic	0.68 ± 0.05*	0.89 ± 0.09
Gastric	0.70 ± 0.11	0.92 ± 0.11
Plasma	0.68 ± 0.07	0.84 ± 0.06
Cerebrospinal	0.62 ± 0.10	0.94 ± 0.06
	F = 1.06 ns**	F = 1.42 ns

*n = 6.
**ns = not significant.

Table V lists mean fetal fluid/maternal plasma concentration ratios for a variety of fetal fluids taken from a pregnant rabbit following caffeine infusion (20 mg/kg/day) throughout pregnancy. Samples were taken at 29 days gestation which is two days before normal delivery for these animals. There were no significant differences in either paraxanthine or caffeine fetal/maternal ratios among the various fluids although caffeine did exhibit higher ratios than its ionizable (pK_a = 8.7) and more polar metabolite.

The method described herein is well suited to performing drug disposition or pharmacokinetic studies in systems where sample volume is severely restricted. It is also of potential usefulness for the analysis of low levels of the methylxanthines in microliter samples of ultrafiltrate such as those obtained when protein binding is being characterized.

ACKNOWLEDGEMENTS

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DETERMINATION OF THE ANTIALLERGENIC AGENT, N-[4-(1H-IMIDAZOL-1-YL)BUTYL]-2-(1-METHYLETHYL)-11-OXO-11H-PYRIDO[2,1-*b*]QUINAZOLINE-8-CARBOXAMIDE, IN PLASMA BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS USING FLUOROMETRIC DETECTION

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SUMMARY

A rapid, sensitive and selective high-performance liquid chromatographic (HPLC) assay was developed for the determination of the antiallergenic compound N-[4-(1H-imidazol-1-yl)butyl]-2-(1-methylethyl)-11-oxo-11H-pyrido[2,1-*b*]quinazoline-8-carboxamide (I), and its major metabolite, 2-(1-methylethyl)-11-oxo-11H-pyrido[2,1-*b*]quinazoline-8-carboxylic acid (I-A), in plasma. The assay involves precipitation of the plasma proteins with acetonitrile—methanol (9:1), followed by the analysis of an aliquot of the protein-free filtrate by reversed-phase ion-pair HPLC with fluorescence detection for quantitation. The analogous compound, N-[6-(1H-imidazol-1-yl)hexyl]-2-(1-methylethyl)-11-oxo-11H-pyrido[2,1-*b*]-quinazoline-8-carboxamide (II), is used as the internal standard. The overall recovery of compounds I and I-A from plasma is 107.0 ± 8.6% and 107.0 ± 10.0%, respectively. The sensitivity limits of quantitation are 20 ng of I, and 10 ng of I-A per ml of plasma using a 0.5-ml aliquot. The assay was used to monitor the plasma concentrations of I and of I-A in a dog following a 5 mg/kg intravenous infusion of I · 2HCl, a 10 mg/kg oral dose of I · 2HCl and of metabolite I-A.

INTRODUCTION

The compound N-[4-(1H-imidazol-1-yl)butyl]-2-(1-methylethyl)-11-oxo-11H-pyrido[2,1-*b*]quinazoline-8-carboxamide·2HCl, I·2HCl, is a member of a series of amide and ester derivatives of 2-substituted pyrido[2,1-*b*]quinazoline-8-carboxylic acids synthesized by Tilley [1] (Fig. 1), of clinical interest as bronchospasmolytic agents [2]. Studies in the dog have shown that compound I is hydrolyzed at the amide bond to yield 2-(1-methylethyl)-11-oxo-11H-pyrido[2,1-*b*]quinazoline-8-carboxylic acid, compound I-A, as the predomi-

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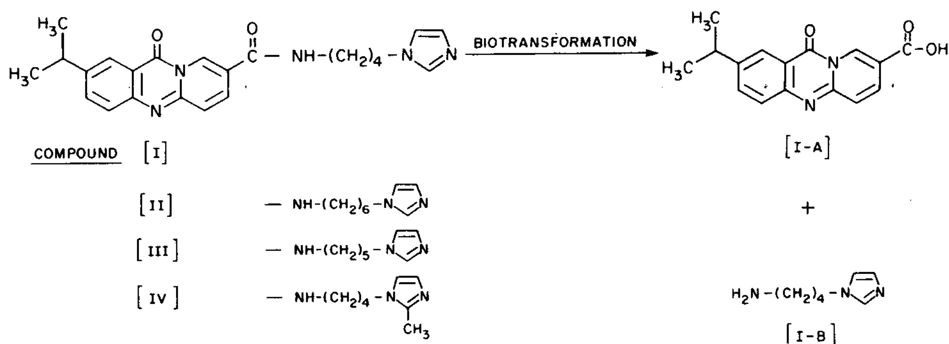


Fig. 1. Chemical structures for the compounds referred to in the text.

nant plasma metabolite. The 2-substituted quinazoline-8-carboxylic acid series of compounds have also been investigated as antiallergenic agents [3—5]. The analogue, 2-methoxy-11-oxo-11H-pyrido[2,1-*b*]quinazoline-8-carboxylic acid, exhibited potent oral activity as an antiallergenic agent [6], and its pharmacokinetics in man have been reported [7]. A high-performance liquid chromatographic (HPLC) assay for this compound was also reported [8].

A rapid, sensitive and selective HPLC assay, with automated injection, was developed for the determination of I and I-A to monitor the biopharmaceutic and pharmacokinetic profile of I in the dog. The parent compound, I, and its major metabolite, I-A, are quantitated in the protein-free filtrate after precipitation of the plasma proteins with acetonitrile—methanol (9:1). An aliquot of the protein-free filtrate is analyzed by reversed-phase ion-pair HPLC with a WISP autoinjector, using fluorescence detection (excitation at 275 nm and emission greater than 418 nm) for quantitation. The assay is selective for the parent drug, I, in the presence of its acid metabolite, I-A. The analogous compound, N-[6-(1H-imidazol-1-yl)hexyl]-2-(1-methylethyl)-11-oxo-11H-pyrido[2,1-*b*]quinazoline-8-carboxamide, II, is used as the internal standard in the assay.

The overall recovery of compounds I and I-A from plasma is 107.0 ± 8.6% and 107.0 ± 10.0%, respectively. The sensitivity limits of quantitation are 20 ng of I, and 10 ng of I-A per ml of plasma using a 0.5-ml aliquot.

The assay was used to monitor the plasma concentrations of I and of I-A in a dog following a 5 mg/kg intravenous infusion of I·2HCl, a 10 mg/kg oral dose of I·2HCl, and of metabolite I-A.

EXPERIMENTAL

Analytical standards

Compound I·2HCl ($C_{23}H_{25}N_5O_2 \cdot 2HCl$, MW = 476.4, m.p. = 261—263°C decomposition), compound I-A ($C_{16}H_{14}N_2O_3$, MW = 282.29, m.p. > 310°C), and compound II [$C_{25}H_{29}N_5O_2$, MW = 431.53, m.p. = 135—136°C, of pharmaceutical grade purity (> 99%)] were used as the analytical standards.

Preparation of standard solution

Standard solutions of I, I-A and II were prepared as follows:

Solution A-1. 118 μg of I·2HCl per ml (equivalent to 100 μg of I per ml) in methanol—water—conc. ammonium hydroxide (75:24:1). Dissolve 1.18 mg of I·2HCl in 10.0 ml methanol—water—conc. ammonium hydroxide (75:24:1) in a 10-ml amberized volumetric flask.

Solution A-2. 11.8 μg of I·2HCl per ml (equivalent to 10 μg of I per ml) (1.0-ml aliquot of solution A-1 diluted to 10.0 ml with methanol).

Solution B-1. 100 μg of I-A per ml in methanol—water—conc. ammonium hydroxide (75:24:1). Dissolve 1.0 mg of I-A in 10 ml of methanol—water—conc. ammonium hydroxide (75:24:1).

Solution B-2. 10 μg of I-A per ml in methanol (1.0-ml aliquot of solution B-1 diluted to 10 ml with methanol).

Solution B-3. 1.0 μg of I-A per ml in methanol (1.0 ml aliquot of solution B-2 diluted to 10 ml with methanol).

Solution C. 100 μg of II per ml in methanol—water—conc. ammonium hydroxide (75:24:1). Dissolve 1.0 mg of II in 10 ml of methanol—water—conc. ammonium hydroxide (75:24:1).

Mixed standard solutions Nos. 1—8 are prepared by diluting aliquots of solution A-1, A-2, B-1, B-2, B-3 and C to 10 ml in methanol given in Table I.

TABLE I

PREPARATION OF MIXED STANDARD SOLUTIONS 1—8

Solution no.	Aliquots (μl) of standard						Final concentration (ng per 100 μl of solution)		
	A-1	A-2	B-1	B-2	B-3	C	I	I-A	II
1	—	100	—	—	500	200	10	5	200
2	—	200	—	—	1000	200	20	10	200
3	—	350	—	300	—	200	35	30	200
4	—	1000	—	500	—	200	100	50	200
5	200	—	—	1000	—	200	200	100	200
6	350	—	300	—	—	200	350	300	200
7	1000	—	500	—	—	200	1000	500	200
8	—	—	—	—	—	200	—	—	200

Solutions of I·2HCl, I-A and II are stable for two months when stored at 5°C in their respective solvents.

Aliquots of 100 μl of solution 1, 2, 3, 4, 5, 6 or 7 were added to separate 0.5-ml specimens of control plasma and processed along with the samples to establish a processed (recovered) standard calibration curve for the direct quantitation of unknowns.

Aliquots of 100 μl of the above solutions were added to 0.5 ml of distilled water plus 0.1 ml of 0.25 *M* potassium phosphate buffer, pH 7.0, and diluted with 1.3 ml acetonitrile—methanol (9:1) to establish an external standard calibration curve to determine the linearity and performance of the HPLC system.

Reagents

All inorganic reagents were analytical reagent grade (ACS). All aqueous solutions were prepared with distilled, carbon-filtered, deionized water, filtered

through a 0.2-μm filter (Type DC System, Hydro-Service and Supplies, Durham, NC, U.S.A.). The inorganic reagents and solutions include: conc. ammonium hydroxide (29.3% ammonia, Baker analyzed reagent grade), 0.25 *M* potassium phosphate buffer, pH 7.0, and 1.0 *M* orthophosphoric acid. The PIC-A (Waters Assoc., Milford, MA, U.S.A.) used for HPLC consists of 0.005 mol of tetrabutylammonium phosphate in phosphate buffer, pH 7.5 (dissolved in approx. 14 ml water). (Note: if the PIC-A reagent is pale yellow to yellow in color and not "water white", it should not be used because it then will contribute significantly to fluorescence background and drastically reduce the sensitivity of the assay.). Acetonitrile and methanol, suitable for spectrophotometry and liquid chromatography, were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Instrumental parameters

Column. The column used for reversed-phase HPLC was a pre-packed 30 cm × 3.9 mm I.D. stainless-steel column containing 10-μm Bondapak C_{18} (Waters Assoc.).

Instrument. The HPLC system consisted of a Model 6000A reciprocating piston pump and a Waters Intelligent Sample Processor (WISP^{TM}) Model 710B (Waters Assoc.) and a Schoeffel Model FS-970 LC fluorometer operated at 275 nm for excitation and emission at wavelengths greater than 418 nm (Kratos, Schoeffel Instruments, Westwood, NJ, U.S.A.). The isocratic mobile phase consisted of two vials of Waters Pic-A reagent, plus 0.5 ml of 1.0 *M* orthophosphoric acid added to 1 l of a mixture of water—methanol—acetonitrile (350:500:150) and was pumped at a pressure of approx. 14 MPa (2000 p.s.i.) and a constant flow-rate of 1.5 ml/min.

The fluorescence detector range was 0.5 μA full scale and the photomultiplier sensitivity was 580 V. The chart speed on the 10-mV recorder, Model 7132A (Hewlett-Packard, Palo Alto, CA, U.S.A.) was 1.27 cm/min. The WISP autoinjector was programmed for a 12-min run time per sample using mobile phase as the rinse solvent. Under these conditions 12.5 ng of I and 3.75 ng of I-A injected gave $>$ 75% of full scale pen response, while 2.5 ng of II injected gave approx. 15—20% of scale. The retention times (t_R) of I, I-A and II were 6.1, 3.4 and 8.8 min, with corresponding capacity factors (k') of 1.6, 0.5 and 2.8, respectively (Fig. 2). The minimum detectable amount of I and I-A was 0.125 ng and 0.063 ng injected, equivalent to 20 ng of I and 10 ng of I-A per ml of plasma, respectively, using a 0.5-ml sample aliquot.

Analytical procedure

Into separate 100 × 13 mm disposable borosilicate culture tubes (Cat. No. 14-962-10C, Fisher Scientific, Pittsburgh, PA, U.S.A.) was added a 0.5-ml aliquot of unknown plasma sample (aliquots of less than 0.5 ml taken of those unknowns with expected concentrations above the highest calibration point were diluted to 0.5 ml with drug-free plasma), a 100-μl aliquot of standard solution 8 (equivalent to 200 ng of II, the internal standard) and 0.1 ml of 0.25 *M* potassium phosphate buffer, pH 7. Each sample was mixed for a few seconds on a Vortex mixer. Then 1.3 ml of acetonitrile—methanol (9:1) were added, each tube was stoppered with polyethylene caps (Plugtite Cat. No. 127-0019-

100, Elkay Products, Shrewsbury, MA, U.S.A.) and mixed again for 10–15 sec at the highest speed setting of the Vortex mixer. The samples were centrifuged at 2100 rpm (1100 *g*) for 10 min at 5°C in a refrigerated centrifuge (Model PR-J with a No. 253 rotor, Damon/IEC, Needham, MA, U.S.A.). The supernatant protein-free fraction was transferred into a standard 4-ml glass vial (Waters part No. 73001). Each vial was sealed with an H-style vial cap (Waters part No. 72711)) fitted with a PTFE septum (Waters part No. 73005). The autoinjector (WISP 710B) was programmed to inject 25 μl out of a total volume of 2.0 ml for HPLC analysis.

Recovered standard curve

Along with the samples, process eight 0.5-ml specimens of control plasma, one to be used as a control blank to which 100 μl of methanol are added and seven to be used for the preparation of the recovered standards to which 100 μl of solutions 1, 2, 3, 4, 5, 6 or 7 equivalent to 0, 10, 20, 35, 100, 200, 350, and 1000 ng of I; 0, 5, 10, 30, 50, 100, 300 and 500 ng of I-A; and 200 ng of II per 0.5 ml of plasma (representing 0, 20, 40, 70, 200, 400, 700 and 2000 ng of I; 0, 10, 20, 60, 100, 200 and 1000 ng of I-A; and 400 ng of II per ml of plasma), respectively, are added. These standards are used to establish the processed (recovered) standard curve for the direct quantitation of the unknowns.

External standard curve

In order to verify the linearity and performance of the HPLC assay, an external standard calibration curve is prepared by adding 100 μl of solutions 1, 2, 3, 4, 5, 6 or 7 to 0.5 ml of distilled water plus 0.1 ml of 0.25 *M* potassium phosphate buffer, pH 7, and 1.3 ml acetonitrile–methanol (9:1) (final volume = 2000 μl) and mixing. Aliquots (25 μl per 2000 μl) are programmed on the WISP for automated injection. Typical chromatograms are shown in Fig. 2.

Calculations

The concentration of I and I-A in the unknowns was determined by interpolation from a least-squares regression equation (weighted linear equation: $Y = a\ X + b$) of the calibration data (processed by a Hewlett-Packard Model 3357B Laboratory Automation System), of the recovered standards processed along with the unknowns using peak height ratios (peak height of compound I or I-A to peak height of internal standard II) versus concentration of I or I-A per ml of plasma.

RESULTS AND DISCUSSION

Compounds I and I-A possess strong ultraviolet (UV) absorption and fluorescence characteristics. The fluorescence excitation maxima of compound I occur at 235, 272, and 360 nm with a broad emission band with peaks at 460 and 480 nm. Compound I-A also has excitation maxima at 235, 272, and 360 nm but with its major emission peak at 450 nm and a smaller one at 475 nm. The Schoeffel Model FS-970 fluorescence detector was used with the excitation set at 275 nm and an emission filter transmitting wavelengths greater than

418 nm and allowed for the quantitation of I and I-A in nanogram concentrations, while avoiding high fluorescence backgrounds from the PIC-A reagent, seen at shorter exciting wavelengths.

Reversed-phase HPLC analysis was the chromatographic method of choice, since it is compatible with an acetonitrile protein precipitation step followed by direct injection of the supernatant. Thus, a rapid sensitive and selective HPLC assay was developed for the determination of compounds I and I-A from plasma using a WISP autoinjector and fluorescence detector for quantitation. The method enabled the accurate quantitation of compounds I and I-A with high sample throughput required for pharmacokinetic and biopharmaceutic studies.

Compound II was chosen as the internal standard in the assay, due to the similarity of its luminescence to that of compound I and its chromatographic resolution from I and I-A.

Chromatographic behavior of I, I-A and II

Compounds I, I-A and II exhibited UV absorbances sufficiently intense for detection at 235 nm. However, the use of PIC-A as an ion-pairing reagent in the mobile phase created a significantly high background UV absorbance, making UV detection impractical. Also, the fact that the UV chromatogram of control plasma (dog and human) contained interfering endogenous peaks made fluorometric detection a necessity for sensitive quantitation.

The HPLC system is flushed initially with methanol, followed by methanol—water (50:50, v/v), to remove deposits from the column accumulated from previous use. The mobile phase is allowed to recycle through the system for at least 2 h at a flow-rate of 1 ml/min to equilibrate the system. Non-equilibration will result in a change in k' values during chromatography. Several μBondapak C_{18} columns used during the course of this project showed variation in equilibration time from column to column with concomitant variation in retention times of $> \pm 0.5$ min for each of the compounds and variation in the separation (α) factors. The preparation of the 100 μg/ml stock solutions in methanol—water—ammonium hydroxide (75:24:1) insures the complete dissolution of the compounds while liberating the free base of I·2HCl, thus the same lots of released material used for dosing were used as the analytical standards in the HPLC analysis of biological samples.

Selectivity of the assay

The known products of either biotransformation and/or instability of I necessitated the use of chromatographic parameters which ensured the stability and selectivity of the assay. Thus the major biotransformation product, I-A, was resolved from I and II. A second breakdown product/metabolite, 1 H-imidazole-1-butanamine, I-B, has very little UV absorption and no significant fluorescence and is therefore not detected even when injected in microgram quantities.

Several analogues were investigated as candidates for an internal standard, Fig. 1. Of these, compound III had a retention time about 1 min longer than I, while compound IV had a retention time similar to III together with a smaller peak (probably an impurity) at the retention time of I. Compound II, with a

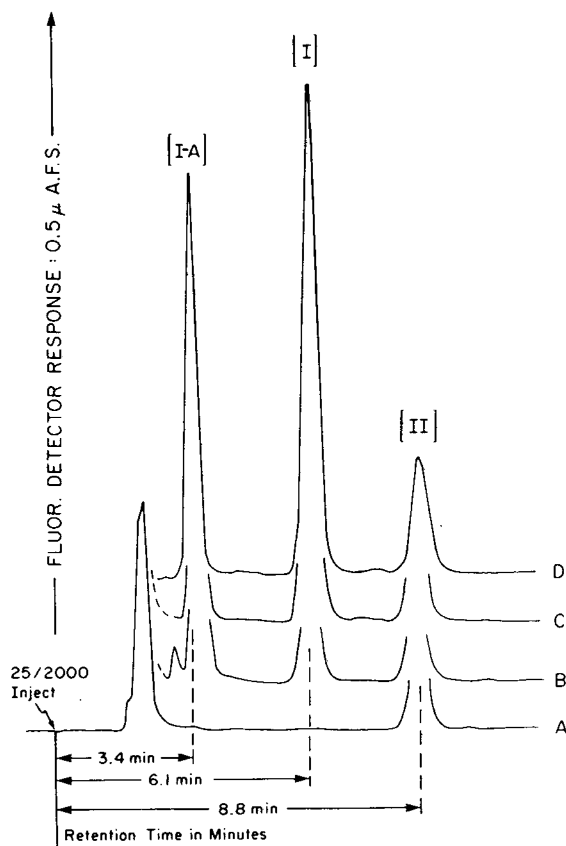


Fig. 2. Chromatograms of the HPLC analysis of the protein-free filtrate of (A) control dog plasma with added internal standard; (B) dog plasma following oral dosing of I· 2HCl at 300 mg/kg; (C) authentic standards recovered from control dog plasma; and (D) authentic standards.

retention time of 8.8 min, was selected as the internal standard since it was completely resolved from I and metabolite I-A, Fig. 2.

Assay validation

Calibration curves for I and I-A ($Y = 0.00239X - 0.00058$ and $Y = 0.00568X + 0.0162$) were linear from 20 to 2000 ng of I and 10 to 1000 ng of I-A per ml of plasma, respectively, using a 0.5-ml specimen. The correlation coefficients (r) were 0.9997 and 0.9988 and the average deviation from the line was 5.0% and 5.4% for I and I-A, respectively. Intra- and inter-assay validation data over the linear concentration range of I and I-A yielded mean coefficients of variation of 3.1% and 5.0%, respectively, for I, and 5.1% and 5.8%, respectively, for I-A, Tables II and III.

Percent recovery and sensitivity limits

The overall recovery of I and of I-A from plasma is 107.0 ± 8.6% and 107.0 ± 10.0%, respectively. The sensitivity limit of the assay is 20 ng of I and 10 ng of I-A per ml of plasma, respectively.

TABLE II

STATISTICAL VALIDATION OF THE HPLC ASSAY FOR COMPOUND I

Amount added (ng/ml)	Amount found (ng/ml)	n	Coefficient of variation (%)
(A) Intra-assay variability			
20	17 ± 0.1	2	0.7
40	43 ± 3.3	3	7.6
70	76 ± 2.5	3	3.3
200	195 ± 4.7	3	2.4
400	391 ± 13.2	3	3.4
700	698 ± 15.0	3	2.2
2000	2010 ± 40.0	3	2.0
		Average ±	3.1
(B) Inter-assay variability			
20	18 ± 1.7	5	9.4
40	41 ± 3.8	9	9.2
70	74 ± 2.8	9	3.8
200	200 ± 10.0	9	5.0
400	401 ± 12.0	9	2.9
700	696 ± 20.0	9	2.9
2000	1990 ± 44.0	8	2.2
		Average ±	5.0

TABLE III

STATISTICAL VALIDATION OF THE HPLC ASSAY FOR COMPOUND I-A (METABOLITE)

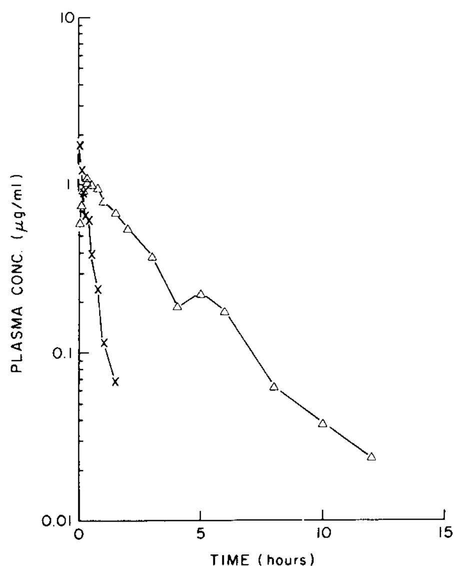
Amount added (ng/ml)	Amount found (ng/ml)	n	Coefficient of variation (%)
(A) Intra-assay variability			
10	10 ± 0.4	3	4.1
20	20 ± 2.5	3	12.5
60	62 ± 0.6	3	1.0
100	95 ± 4.5	3	4.8
200	202 ± 11.0	3	5.5
600	647 ± 22.0	3	3.3
1000	961 ± 41.0	3	4.3
		Average ±	5.1
(B) Inter-assay variability			
10	10 ± 1.2	8	12.1
20	21 ± 1.8	9	8.7
60	61 ± 3.0	9	5.0
100	96 ± 3.6	9	3.7
200	199 ± 9.0	9	4.5
600	624 ± 20.0	9	3.2
1000	972 ± 30.0	9	3.1
		Average ±	5.8

Application of the method to biological specimens: biopharmaceutic/pharmacokinetic studies in the dog

A pilot study was conducted in a dog using the HPLC assay as described, but without the inclusion of II (internal standard) since a pure standard was unavailable at the time. The study involved the intravenous infusion of a single 5 mg/kg dose of I·2HCl and a single oral administration to the same dog of a 10 mg/kg dose of I·2HCl as a solid in a hand-packed gelatin capsule. A 10 mg/kg oral dose of a solution of the metabolite I-A was also administered to the same dog.

Following intravenous (Fig. 3) and oral (Fig. 4) administration, plasma concentrations of I declined rapidly with the major plasma component being the acid metabolite I-A whose concentrations were measurable up to 12 h. These preliminary data, which document the utility of the assay, also suggest first-pass biotransformation of I to yield I-A in the dog, which was substantiated by comparing the plasma concentration versus time profile of I-A following oral administration of I-A.

Plasma concentrations of I and I-A in the above studies were determined using the HPLC assay as described but without the internal standard II. Quantitation was based on absolute peak height versus concentrations of I or I-A. In these experiments the calibration curves for I and I-A ($Y = 6.005\ X + 23.909$ and $Y = 12.549\ X + 7.098$) were linear from 20 to 2000 ng of I and 20 to 600 ng of I-A per ml of plasma, respectively, using a 0.5-ml aliquot. The correlation coefficients (r) were 0.9999 and 0.9987 and the average deviation from the line was 4.0% for I and 5.6% for I-A, respectively. The mean intra-assay ($n = 3$) coefficients of variation were 3.9% and 5.6% for I



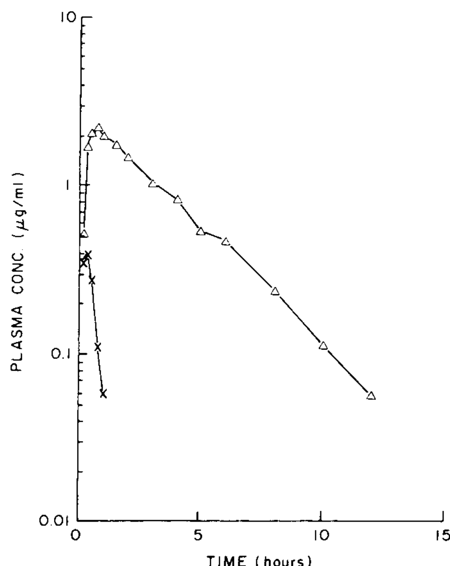


Fig. 3. Plasma concentrations of I (×) and its major metabolite I-A (△) in a dog following a 5 mg/kg intravenous infusion of I·2HCl administered over a 10-min interval.

Fig. 4. Plasma concentrations of I (×) and its major metabolite I-A (△) in a dog following a 10 mg/kg oral dose of I·2HCl (solid) in a hand-packed gelatin capsule.

and I-A, respectively. The mean inter-assay coefficients of variation for eleven experiments were 4.6% for I and 4.7% for I-A, respectively, demonstrating the precision of the assay even without the use of the internal standard.

Stability of I in dog and human plasma on storage at −17°C and −70°C up to 90 days

The stability of I in fresh plasma was evaluated by HPLC analysis at two concentrations (100 and 1000 ng/ml) following storage at −17°C and −70°C for a period of 1, 7, 30, 60 and 90 days. Fresh pools of plasma from the two species were prepared at the respective concentrations by adding 10 μl of solution A-1 and 100 μl of solution A-1 per 10 ml of the respective plasma. Three-ml aliquots of each pool together with a control plasma from each species (total 24) were pipetted into separate 1-dram Wheaton vials. One half of the specimens (12) were stored at −17°C and the other at −70°C.

The stability-indicating plasma samples from day 1, 7, 30, 60 and 90 stored at −17°C and −70°C were analyzed in triplicate as unknowns along with external standards and plasma recovered calibration standards at similar concentrations added to fresh control plasma using the HPLC assay with the internal standard II added. The data tabulated in Table IV were analyzed using a

TABLE IV

STABILITY OF I IN DOG PLASMA AND IN HUMAN PLASMA STORED AT −17°C AND −70°C

Day	Storage temp. (°C)	100 ng/ml added Mean conc. found ± S.D.* (% S.D.)	1000 ng/ml added Mean conc. found ± S.D.* (% S.D.)
(A) Dog plasma			
1	−17	102 ± 2.8 (2.8)	871 ± 11 (1.2)
1	−70	99 ± 3.6 (3.6)	867 ± 47 (5.4)
7	−17	95 ± 2.7 (2.8)	891 ± 16 (1.8)
45	−17	99 ± 3.1 (3.1)	913 ± 24 (2.6)
45	−70	101 ± 3.7 (3.7)	889 ± 20 (2.3)
60	−17	101 ± 2.0 (2.0)	937 ± 117 (12.5)
91	−17	89 ± 3.8 (4.2)	841 ± 42 (5.0)
91	−70	95 ± 1.6 (1.6)	899 ± 10 (1.1)
(B) Human plasma			
1	−17	96 ± 9.7 (10.0)	1143 ± 34 (2.9)
1	−70	101 ± 4.9 (4.9)	1084 ± 57 (5.2)
7	−17	89 ± 1.3 (1.5)	875 ± 32 (3.6)
32	−17	101 ± 2.2 (2.2)	1139 ± 37 (3.2)
32	−70	104 ± 2.6 (2.5)	1108 ± 37 (3.3)
62	−17	97 ± 2.1 (2.1)	1066 ± 49 (4.6)
95	−17	98 ± 3.7 (3.8)	1142 ± 57 (5.0)
95	−70	92 ± 4.6 (5.0)	1086 ± 27 (2.5)

*n = 3 in all cases.

two-tailed *T*-test. The data indicated that I was stable throughout the storage interval at —17°C and —70°C.

The plasma samples collected from dogs in these studies were stored at —70°C prior to analysis.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF SOTALOL IN BIOLOGICAL FLUIDS

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SUMMARY

A sensitive, selective and reproducible reversed-phase high-performance liquid chromatographic method is described for the quantification of sotalol in human serum and urine. Sotalol and the internal standard, atenolol, were extracted from alkalinized serum and urine (pH 9.0) into 1-butanol—chloroform (20:60, v/v). The organic phase was evaporated, and to the residue was added 0.1 *M* sulphuric acid (serum analysis) or mobile phase (urine analysis). The mobile phase consisted of 0.01 *M* phosphate buffer (pH 3.2) and acetonitrile (20:80, v/v) containing 3 m*M* n-octylsodium sulphate. The flow-rate was 1.5 ml/min. The retention times of atenolol and sotalol were 7 and 10 min, respectively. Ultraviolet detection at 226 nm made it possible to achieve a detection limit of 0.03 μmol/l.

INTRODUCTION

The pharmacological properties of sotalol were first described in 1965 by Lish et al. [1]. These and subsequent studies showed that sotalol hydrochloride is a non-selective beta-blocking drug, devoid of intrinsic sympathomimetic and membrane-stabilizing activity [2]. Sotalol seems to differ from other beta-antagonists in general use in that it also exerts amiodarone-like effects of the group III antiarrhythmic drugs [3] and furthermore prolongs, in a concentration-dependent manner, the action potential duration and QTc interval in the electrocardiogram [4]. Although sotalol hydrochloride has been used in the treatment of cardiovascular diseases for about a decade, there are few data about its quantitative measurement in biological fluids. In most of the pharmacokinetic and pharmacodynamic studies where the concentrations of the drug have been measured [5—12], the assay method has been the spectrofluorometric procedure described by Garret and Schnelle [13], or a slight

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modification of it [12]. However, the spectrofluorometric assay needs large volumes of plasma and lacks specificity.

In view of the clinical use of sotalol, a sensitive and reproducible high-performance liquid chromatographic (HPLC) method has been developed for its quantitation in serum and urine. The method involves the structurally related compound atenolol as internal standard, and a relatively simple extraction procedure prior to chromatography.

EXPERIMENTAL

Chemicals and glassware

Sotalol hydrochloride was purchased from Lääke-Farmos (Turku, Finland) and the internal standard, atenolol, from Oy Star Ab (Tampere, Finland). Chloroform, 1-butanol, orthophosphoric acid, potassium dihydrogen phosphate and sulphuric acid were obtained from E. Merck (Darmstadt, F.R.G.) and were of analytical grade. Acetonitrile, HPLC grade, was purchased from Rathburn Chemicals (Walkerburn, U.K.). Only double-glass-distilled water was used. All glassware was silanized before use with a 10% solution of dimethyldichlorosilane in toluene. After 15 min in this solution, the glassware was washed with toluene and methanol.

Apparatus and HPLC procedure

The liquid chromatographic system consisted of an M-45 solvent delivery system, a Model U6K liquid chromatographic injector (Waters Assoc., Milford, MA, U.S.A.), and an LKB 2238 Uvicord SII detector with ultraviolet lamp of wavelength 226 nm (Bromma, Sweden). The absorbance range was 0.05 for serum analysis and 0.1 for urine analysis. The column was a reversed-phase μBondapak(TM) Phenyl column (30 cm × 3.9 mm I.D., 10 μm particles size; Waters Assoc.). Pre-column filter and packing material, Bondapak C_{18}/Corasil, were from Waters Assoc. A one-channel recorder 2210 LKB (Bromma) was used (chart speed 2 mm/min, sensitivity 20 mV).

The mobile phase for the separation of sotalol and internal standard consisted of a mixture of 0.01 *M* phosphate buffer, pH 3.2—acetonitrile (80:20, v/v), containing 3 m*M* *n*-octylsodium sulfate (Merck). The mobile phase was filtered through a Millipore filter unit equipped with Millipore filter, HATF 04700, pore size 0.45 μm (Millipore, Molsheim, France), 0.5 h after *n*-octylsodium sulphate had been added, and then degassed ultrasonically (Sonicor). The flow-rate was 1.5 ml/min.

Calibration graphs

Calibration graphs were prepared by adding sotalol to drug-free serum and urine to provide varying concentrations. These samples were then processed through the analytical procedure described. The internal standard was diluted with 0.5 *M* Tris—HCl buffer (pH 9.0) to give the final concentration of 2.0 μmol/l for serum extractions and 50.0 μmol/l for urine extractions.

The evaluation of the chromatograms was based on the peak height ratios of sotalol and atenolol. The calibration graphs for sotalol in human serum ranged from 0.1 to 4.0 μmol/l, and in urine from 3.25 to 200 μmol/l. The concentration of the stock solution was 1 mmol/l.

Extraction procedure

Serum. A 1-ml volume of the solution of internal standard in 0.5 *M* Tris—HCl buffer (pH 9.0) was aded to 1 ml of sample or standard in a 15-ml PTFE-lined screw-capped tube, and the mixture was gently vortexed. After the addition of 7 ml of chloroform—1-butanol (60:20, v/v), the tubes were shaken in a vertical position for 10 min, then centrifuged for 10 min at 1500 *g*. The organic phase (5 ml) was transferred to a 10-ml conical glass-stoppered test tube and evaporated to dryness under a gentle stream of nitrogen in a water bath (50°C). The residue was redissolved in 50—100 µl of 0.1 *M* sulphuric acid by vortexing for 30 sec; 20—30 µl were injected onto the column.

Urine. The extraction procedure was the same as that for serum except that 0.5 ml of urine was used, and the residue was dissolved in 250—1000 µl of the mobile phase.

Recovery and reproducibility

The absolute analytical recovery of sotalol from human serum and urine was estimated by comparing the peak heights obtained from the injection of known quantities of the compound with those obtained from the injection of extracts of serum and urine samples spiked with sotalol. Within-day reproducibility and accuracy were evaluated by analysis of serum and urine samples (n = 6) containing 4.0 and 50.0 µmol/l sotalol, respectively. Day-to-day reproducibility was determined by assaying serum standards containing 0.1, 0.25, 0.5, 1.0, 2.0 and 4.0 µmol/l on six occasions, and urine standards containing 6.25, 12.5, 25.0, 50.0, 100.0 and 200.0 µmol/l on six occasions. In addition, the influence of injection volume and the initial volume of serum and urine on the reproducibility was evaluated.

Stability and storage of sotalol

To assay the stability of frozen samples, 1-ml aliquots of pooled serum from volunteers were stored frozen at —20°C in polypropylene centrifuge tubes and subsequently assayed by the method described.

RESULTS

Typical chromatograms obtained from serum and urine analyses are shown in Figs. 1 and 2, respectively. The analysis of drug-free serum and urine samples, performed without the addition of internal standard and sotalol, did not reveal the presence of any endogenous interfering compounds. The retention times of atenolol and sotalol were 7 and 10 min, respectively. There was a linear correlation between sotalol serum and urine concentrations and the peak height ratios between the drug and internal standard over the ranges measured: 0.1—4.0 n*M* (serum) and 6.25—200 n*M* (urine). The calibration curve for sotalol in serum had a slope of 0.5539, and an ordinate intercept of —0.0156 with a correlation coefficient (r) of 0.9971 over the range of assay. In urine, the calibration curve had a slope of 0.0260, and an ordinate intercept +0.0014, with r = 0.9987. The amounts of internal standard in the serum and urine analyses were 2 and 50 nmol, respectively.

The absolute recovery of sotalol from serum was determined by comparing

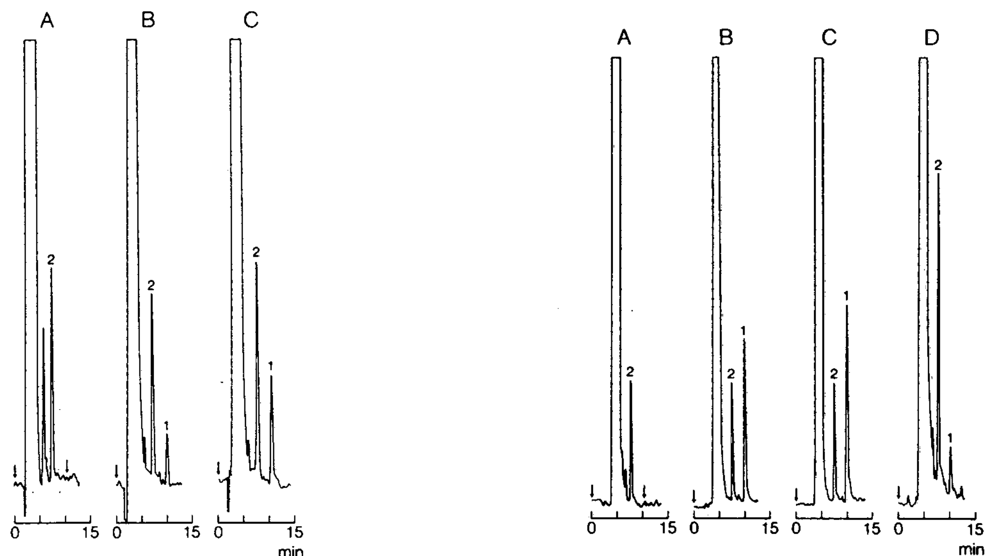

Fig. 1. Chromatograms of human serum: (A) before administration (control); (B) spiked with 0.5 nmol of sotalol; (C) sample obtained from a volunteer 24 h after oral intake of 160 mg of sotalol hydrochloride. Peaks: 1 = sotalol; 2 = internal standard (atenolol). Detector: 226 nm, 0.05 a.u.f.s. Recorder: sensitivity 20 mV, chart speed 2 mm/min.

Fig. 2. Chromatograms of human urine: (A) before administration (control); (B) spiked with 50 nmol of sotalol; (C) sample from 12—24 h collection fraction from a volunteer receiving a 160-mg oral dose of sotalol hydrochloride; (D) sample from 60—72 h collection fraction from a volunteer receiving a 160-mg oral dose of sotalol hydrochloride. Peaks: 1 = sotalol; 2 = internal standard (atenolol). Detector: 226 nm, 0.1 a.u.f.s. Recorder: sensitivity 20 mV, chart speed 2 mm/min.

the peak heights of extracted standards to the peak heights of known concentrations injected. The absolute recoveries ranged from 70% to 75% over the concentration range 0.1—4.0 nmol. Tables I—III show that the recoveries with atenolol as the internal standard varied between 96% and 106% in serum and between 99% and 103% in urine. The sensitivity of the assay was 0.1 μmol/l when 1 ml of the sample was used and 20 μl of the final solution were injected into the column. The detection limit could be lowered by injecting a larger volume into the column and by using a larger volume of the sample in the pharmacokinetic study. Thus the absolute limit of detection was 0.03 μmol/l.

TABLE I

WITHIN-DAY ACCURACY AND PRECISION DATA FOR SOTALOL IN SERUM AND URINE (n = 6)

Sample	Amount added (nmol/ml)	Mean concentration found (nmol/ml)	Standard deviation	Standard error	Coefficient of variation (%)	Recovery (%)
Serum	4.0	4.04	0.02	0.01	0.53	101.0
Urine	50.0	51.36	0.87	0.36	1.70	102.7

TABLE II

DAY-TO-DAY ACCURACY AND PRECISION DATA FOR SOTALOL IN SERUM (n = 6)

Amount added (nmol/ml)	Mean concentration found (nmol/ml)	Standard deviation	Standard error	Coefficient of variation (%)	Recovery (%)
0.1	0.106	0.009	0.004	8.30	106.0
0.5	0.501	0.020	0.009	4.06	100.2
1.0	0.982	0.046	0.019	4.67	98.2
2.0	1.923	0.101	0.041	5.27	96.2
4.0	4.131	0.116	0.047	2.80	103.3

TABLE III

DAY-TO-DAY ACCURACY AND PRECISION DATA FOR SOTALOL IN URINE (n = 6)

Amount added (nmol/ml)	Mean concentration found (nmol/ml)	Standard deviation	Standard error	Coefficient of variation (%)	Recovery (%)
6.25	6.33	0.28	0.11	4.36	101.3
12.5	12.48	0.56	0.23	4.43	99.8
25.0	24.82	1.21	0.49	4.88	99.3
50.0	50.84	1.21	0.49	2.38	101.7
100.0	100.38	2.11	0.86	2.10	100.4

TABLE IV

STABILITY OF SOTALOL IN FROZEN SERUM SAMPLES

Week	Concentration found (nmol/ml)
1	1.88
	2.07
	1.95
2	2.04
	2.19
3	1.98
	2.02
4	1.99
	2.07
Mean ± S.D.	2.02 ± 0.09

The within-day accuracy and precision data for serum and urine are presented in Table I. The corresponding day-to-day data are shown in Tables II and III. These data indicate that the assay used here is sufficiently accurate and precise for pharmacokinetic studies.

No decrease in the measured sotalol concentration was detected when samples were stored for periods of up to four weeks at −20°C (Table IV).

A change in the volume of urine from 100 μl to 2 ml and of serum from 0.5 to 2 ml had no influence on the accuracy and reproducibility studies when the paired urine and serum samples were spiked with sotalol to contain 25.0 and 1.0 nmol, respectively.

DISCUSSION

The extraction of sotalol into the organic phase is pH-dependent. Sotalol has two pK_a values (8.80 and 9.80). It has been shown that the maximum extraction into an organic solvent can be effected at the maximum concentration of the neutral form in equilibrium with the zwitterion, half-way between the two pK_a values, i.e. about pH 9 [13]. When 1.0 ml of Tris—HCl buffer (pH 9.0) was added to 0.1—2 ml of urine (pH varied from 4.8 to 8.2), or to 1—2 ml of serum, the final pH was always 9.0.

Sotalol displays two ultraviolet absorption peaks depending on the pH of the medium [13]. In acidic conditions, the λ_{max} is 227 nm, and above pH 8.5 the λ_{max} is 248 nm. Since the molar absorptivity of sotalol in basic solutions is greater than in acidic conditions, a lower wavelength and an acidic mobile phase were chosen for quantitative measurement to prolong the lifetime of the column. The lower limit of detection in acidic conditions was nevertheless sufficient to be acceptable for pharmacokinetic studies.

The optimal separation of sotalol and the internal standard from endogenous material was obtained when the ratio of phosphate buffer to acetonitrile was 80:20 (v/v). Caffeine interfered with the analysis when the acetonitrile concentration was increased. Bubble formation in the mobile phase was minimized by allowing the solution to stand at room temperature for half an hour before filtration and sonication.

The principal advantages of the present HPLC method, compared to the spectrofluorometric method of Garret and Schnelle [13] and to its modifications [12], are improved sensitivity and specificity. The HPLC method recently described by Lefebvre et al. [14] is also sensitive, but no accuracy and precision studies were provided.

The HPLC method reported here has proved to be particularly useful for pharmacokinetic studies of sotalol in volunteers. The results of those studies will be reported elsewhere.

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CHROMBIO. 2304

DETERMINATION OF (*R*)- AND (*S*)-DISOPYRAMIDE IN HUMAN PLASMA USING A CHIRAL α_1-ACID GLYCOPROTEIN COLUMN

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SUMMARY

The direct resolution and quantitation of (*R*)- and (*S*)-disopyramide, isolated from human plasma, was accomplished using a chiral α_1-acid glycoprotein column. A LiChrosorb RP-2 column (50 × 3.0 mm I.D.) was used as a precolumn. Phosphate buffer, pH 6.20, containing 2-propanol and N,N-dimethyloctylamine was used as mobile phase. The precision of the determination of (*R*)- and (*S*)-disopyramide in human plasma, expressed as the relative standard deviation, was 1.8% and 3.3% for (*R*)- and (*S*)-disopyramide, respectively, at a drug level of 0.5 μg/ml. In two subjects who received a single capsule of racemic disopyramide (150 mg), the plasma levels of the (*R*) isomer were about half those of the (*S*) isomer. The half-lives of (*R*)- and (*S*)-disopyramide were similar.

INTRODUCTION

Disopyramide is an antiarrhythmic agent and is marketed as a racemate, i.e. a mixture of two optical isomers, (*R*)- and (*S*)-disopyramide. It has been demonstrated recently that (*R*)- and (*S*)-disopyramide are equipotent with respect to the antiarrhythmic effect [1]. However, the use of disopyramide is limited because the drug also possesses strong anticholinergic properties, resulting, for example, in severe urine retention [2]. Giacomini et al. [3] reported that (*S*)-disopyramide is about three to four times more potent as an anticholinergic

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agent. These results were obtained using longitudinal muscle strips from guinea pig ileum as test model.

Differences in the disposition of the enantiomers of disopyramide have been observed in dogs [4]. The purpose of the present study was to characterize the in vivo disposition of (*R*)- and (*S*)-disopyramide in man, in an attempt to study the prerequisites for obtaining a reduction of the anticholinergic side-effects by the use of only (*R*)-disopyramide.

Separation and quantitation of the disopyramide enantiomers was accomplished in this study using a chiral α_1-AGP column coupled in series with an RP-2 precolumn. The α_1-AGP column was prepared by immobilization of the plasma protein α_1-acid glycoprotein (orosomucoid) on a solid phase. The preparation of an α_1-AGP column has been described previously [5, 6].

EXPERIMENTAL

Chemicals

Racemic disopyramide was obtained from Roussel Labs. (*R*)- and (*S*)-disopyramide, and (*R*)- and (*S*)-monodesisopropyldisopyramide oxalate were kindly supplied by Professor Wendel L. Nelson (School of Pharmacy, Department of Medicinal Chemistry, Seattle, WA, U.S.A.). N,N-Dimethyloctylamine (DMOA) was obtained from ICN Pharmaceuticals (Plainview, NY, U.S.A.) and LiChrosorb RP-2 with a mean particle diameter of 5 μm from E. Merck (Darmstadt, F.R.G.). The other chemicals used were of analytical or equivalent grade.

Apparatus

The high-performance liquid chromatographic (HPLC) system consisted of a Waters pump Model M6000 A (Waters Assoc., Milford, MA, U.S.A.), a Waters U6K injector and a Shimadzu SPD-2A ultraviolet (UV) detector with variable wavelength, operating at 261 nm.

Column preparation

The columns were made of precision-bore 316 stainless steel and equipped with modified Swagelok connections and Altex 2-μm frits. The dimensions of the RP-2 column were 50 $\times$ 3.0 mm. LiChrosorb RP-2 (0.3 g) was suspended in 3.0 ml of dichloromethane and placed in an ultrasonic bath for 10 sec to be degassed, before being poured into a 10-ml packing column. The packing column was filled with dichloromethane and coupled to a Haskel pump, operating at 300 bars. Acetone (30 ml) was used as the driving liquid. After packing, the column was rinsed with 50 ml methanol and 30 ml of methanol—water (1:1).

The chiral α_1-AGP column (100 $\times$ 3.0 mm) was prepared by immobilization of the plasma protein α_1-acid glycoprotein (orosomucoid) on silica microparticles with a mean particle diameter of 13 μm. The preparation of the chiral phase as well as the packing procedure is described elsewhere [6].

Chromatographic technique

A mobile phase of phosphate buffer, pH 6.20, containing 4.3% (v/v) of

2-propanol and 1.95 mM DMOA was used. The solvent was degassed in an ultrasonic bath before use.

Extraction procedure

A volume of 100 μl of 2 M sodium hydroxide was added to each plasma sample (1.0 ml). Disopyramide was then extracted with 6.0 ml of water-saturated diethyl ether for 15 min. The tubes were centrifuged for 3 min and 5.0 ml of the ether phase were transferred to a conical tube and evaporated to dryness with a stream of dry nitrogen at 40°C. The residue was dissolved in 125 μl of the mobile phase and 50 μl were injected onto the column (the RP-2 column coupled in series with the α_1-AGP column).

Standard curves

Standard curves were prepared by adding 0, 0.3, 1.0, 2.0, 3.0 and 4.0 μg of racemic disopyramide base to 1.0 ml of drug-free human serum. The samples were then handled as described under *Extraction procedure*. Standard curves were constructed by plotting the peak height versus the concentration of (R)- and (S)-disopyramide.

Reproducibility

Reproducibility studies for the analysis of (R)- and (S)-disopyramide were performed at two different concentrations. Six 1.0-ml drug-free serum samples were spiked with racemic disopyramide (1.0 μg or 3.0 μg) and the samples were handled as described under *Extraction procedure*. The peak heights of the enantiomers were measured and the relative standard deviations were calculated.

Subjects

Two male patients, 64 and 79 years' old, were given a commercial Durbis® capsule containing 150 mg of racemic disopyramide. At timed intervals venous blood was drawn into heparinized Venoject tubes. The plasma was separated immediately after collection and frozen until analysed.

RESULTS AND DISCUSSION

The enantiomers of disopyramide are extracted into diethyl ether from the alkalinized plasma samples. The extraction time needed to reach equilibrium was studied using four different extraction times: 15, 30, 45 and 60 min. No significant difference in the peak area ratios (R/S) was obtained when using different extraction times. It was also demonstrated that the peak heights were independent of the extraction time in the above-stated interval and that 15 min is enough to reach equilibrium.

It is demonstrated in this paper and it has also been demonstrated in previous papers from our group [5—7], that (S)-disopyramide is bound with higher affinity than the (R)-enantiomer to orosomucoid. Therefore, it is of vital importance to determine that both enantiomers are extracted to the same extent into diethyl ether. This study was performed using racemic disopyramide concentrations between 0.30 and 2.5 μg/ml and by use of an extrac-

tion time of 15 min. The areas of the peaks and the R/S area ratios were calculated and it was found that the R/S area ratio was independent of the disopyramide concentration. The mean values of the areas of (R)- and (S)-disopyramide as a percentage of the total area (the sum of the areas of (R)- and (S)-disopyramide) are 48.9 and 51.1 with relative standard deviations of 1.83 and 1.95 (n = 14), respectively.

Chromatographic studies

It has been previously demonstrated that the disopyramide enantiomers can be separated using a chiral α_1-AGP silica column [5, 6]. However, the plasma

TABLE I

CHROMATOGRAPHIC DATA OBTAINED ON THE α_1-AGP COLUMN

Conditions: column dimensions, 100 × 3.0 mm I.D.; 183 mg α_1-AGP/g solid phase; mobile phase, phosphate buffer, pH 6.20 containing 1.95 mM DMOA and 4.3% 2-propanol.

R_s = resolution factor = $\frac{(t_{R(S)} - t_{R(R)}) \cdot 2}{W_{t(R)} + W_{t(S)}}$ where W_t is base width.

	$k'_{(R)}$	$k'_{(S)}$	α	R_s
Disopyramide	1.66	5.70	3.43	3.05
Monodesisopropyldisopyramide	0.72	1.74	2.41	2.11

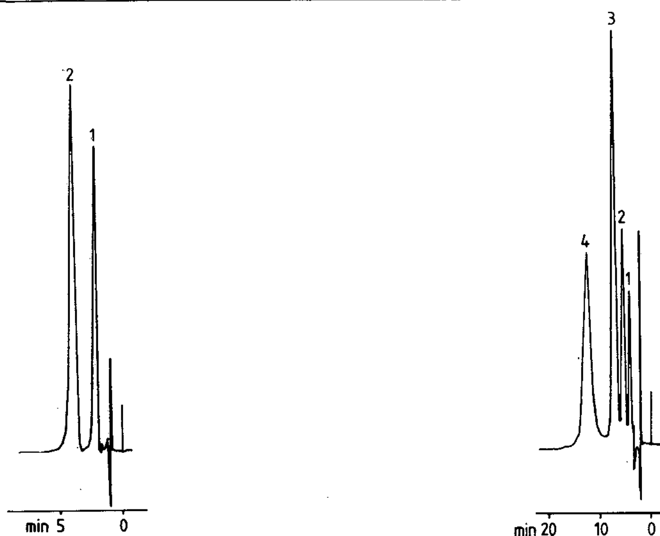

Fig. 1. Separation of disopyramide and monodesisopropyldisopyramide. Column: LiChrosorb RP-2 (50 × 3.0 mm I.D.). Mobile phase: Phosphate buffer pH 6.20 containing 4.3% (v/v) 2-propanol and 1.95 mM N,N-dimethyloctylamine. Flow-rate: 0.5 ml/min. UV detection: 261 nm. a.u.f.s. = 0.01. Samples: 1 = monodesisopropyldisopyramide, 2 = disopyramide.

Fig. 2. Resolution of the enantiomers of disopyramide and the enantiomers of monodesisopropyldisopyramide. Columns: LiChrosorb RP-2 (50 × 3.0 mm I.D.) coupled in series with an α_1-AGP column (100 × 3.0 mm I.D.). Other conditions as in Fig. 1. Peaks: 1 = (R)-monodesisopropyldisopyramide, 2 = (S)-monodesisopropyldisopyramide, 3 = (R)-disopyramide, 4 = (S)-disopyramide. a.u.f.s. = 0.005.

samples also contain the metabolite, monodesisopropyldisopyramide [8, 9] and it was found that (*S*)-monodesisopropyldisopyramide elutes with approximately the same capacity factor as (*R*)-disopyramide when using the α_1-AGP column alone, despite the fact that many different mobile phase compositions were tested. The chromatographic data using a mobile phase composition of 4.3% (v/v) 2-propanol in phosphate buffer, pH 6.2, and with addition of 1.95 m*M* N,N-dimethyloctylamine are summarized in Table I. It is interesting to note the difference in the separation factors obtained for the enantiomers of disopyramide and the enantiomers of its monodesisopropyl metabolite. Monodesisopropylation of disopyramide decreases the separation factor from 3.43 to 2.41 (cf. Table I). Disopyramide and the monodesisopropyl metabolite can be separated by chromatography on a 50-mm long LiChrosorb RP-2 column using the above-stated mobile phase, as demonstrated in Fig. 1. If the RP-2 column is coupled before the α_1-AGP column as a precolumn, a complete separation of (*R*)- and (*S*)-disopyramide and their metabolites can be obtained, as demonstrated in Fig. 2. This separation system was also used for the determination of (*R*)- and (*S*)-disopyramide in human plasma. The resolution between the disopyramide enantiomers decreases when the precolumn is used. This is probably caused by the fact that the sample zone, transferred to the α_1-AGP column, is broadened by passage through the precolumn.

Standard curve, reproducibility and recovery studies

The reproducibility of the method was determined as described under Experimental. The study was performed at two different concentrations (1.0 and 3.0 μg/ml) of racemic disopyramide and the relative standard deviations found are summarized in Table II.

TABLE II

PRECISION OF PLASMA DETERMINATIONS

Concentration of racemic disopyramide (μg/ml)	Relative S.D.* (%)	
	(*R*)	(*S*)
1.0	1.81	3.32
3.0	1.68	1.66

*Calculated for n = 6.

The degree of extraction of (*R*)- and (*S*)-disopyramide into diethyl ether is > 90% with a phase volume ratio, V_{org}/V_{aq}, of 5.45. Standard curves are constructed by plotting the peak height versus the concentration of the disopyramide enantiomers. Linear regression equations of the standard curves were calculated and the equations are as follows: $Y_R = 68.58X - 3.64$, and $Y_S = 32.92X - 1.12$. The standard deviations of the intercept and the slope for (*R*)-disopyramide are 1.52 and 1.24, respectively. The corresponding values for the (*S*) isomer are 0.63 and 0.51. Linear standard curves were obtained in the concentration range studied (0.15—2.0 μg/ml) and the correlation coefficients were in all cases better than 0.999.

Determination of (R)- and (S)-disopyramide in human plasma

The concentrations of (*R*)- and (*S*)-disopyramide in human plasma were determined as described under Experimental. Fig. 3A—C demonstrates chromatograms of blank plasma, of a plasma sample spiked with racemic disopyramide and of a plasma sample obtained from a patient after administration of racemic disopyramide, respectively. The blank plasma chromatogram demonstrates that no interfering peaks are present.

Fig. 4A and B demonstrates the total (free + protein-bound) plasma concentrations of (*R*)- and (*S*)-disopyramide in man in two subjects who received a single oral dose of a 150-mg Durbis® capsule. It can be seen from the plasma concentration—time curves that at all time points there is a higher plasma concentration of (*S*)-disopyramide, i.e. the enantiomer with the strongest anticholinergic potency. It can also be seen that the half-lives of the isomers are similar. The concentration ratio (*S*/*R*) for one subject is about 2 and for the other subject about 1.5.

Kook et al. [10] reported a more than two-fold higher C_{max} and AUC for (*R*)-disopyramide after oral administration of disopyramide to dogs. Obviously, there is a species difference with regard to the disposition of disopyramide between dog and man.

It has been reported that (*S*)- and (*R*)-disopyramide are equipotent with respect to the antiarrhythmic effect [1]. However, it has also been demonstrated that (*S*)-disopyramide is three to four times more potent as an anti-

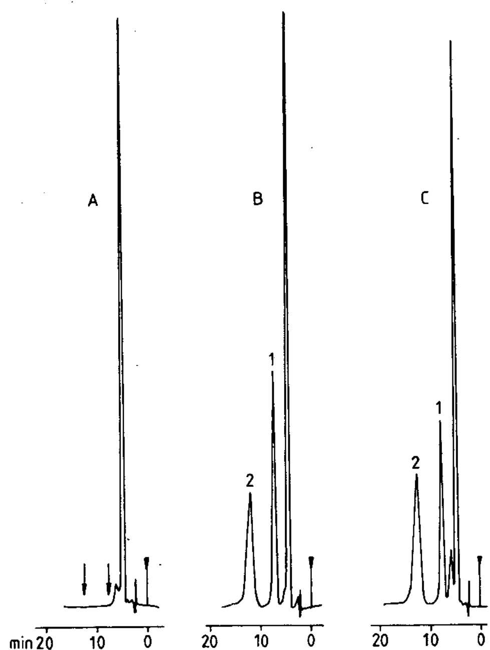

Fig. 3. Separation of (*R*)- and (*S*)-disopyramide isolated from human plasma. (A) Chromatogram of blank plasma. Arrows indicate the retention times of (*R*)- and (*S*)-disopyramide. (B) Blank plasma spiked with racemic disopyramide (1.5 µg/ml). Peaks: 1 = (*R*)-disopyramide, 2 = (*S*)-disopyramide. (C) Plasma sample from a patient obtained 1 h after administration of a 150-mg Durbis capsule. Conditions as in Fig. 2. a.u.f.s. = 0.04.

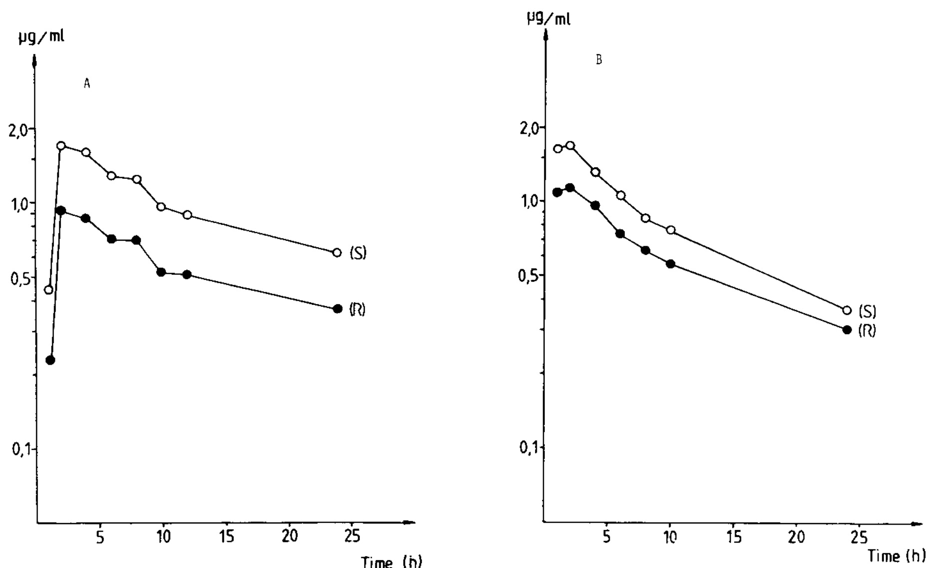

Fig. 4. Plasma concentrations of (*R*)- and (*S*)-disopyramide in two subjects (A and B) each receiving 150-mg commercial capsules containing racemic disopyramide.

cholinergic agent [3]. It was demonstrated in Fig. 4A and B that the concentration of (*S*)-disopyramide was about two times as high as that of the (*R*)-isomer. It is thus reasonable to assume that the anticholinergic side-effects associated with the use of racemic disopyramide can be reduced if only (*R*)-disopyramide is administered. To assess the possibility of a therapeutic improvement by using only the (*R*)-isomer, the free levels of (*R*)- and (*S*)-disopyramide in plasma must be determined. A prerequisite for obtaining this improvement is that the concentration of free (*S*)-disopyramide is high when compared with the free plasma concentration of (*R*)-disoyramide, i.e. a situation comparable with that obtained when using the total (free + protein-bound) plasma concentrations of the enantiomers (Fig. 4A and B).

(*S*)-disopyramide is bound to human α_1-acid glycoprotein (orosomucoid) with higher affinity than the (*R*) form [5—7]. Studies are now in progress to examine the influence of the stereoselective protein binding on the pharmacokinetics and the pharmacodynamics of (*R*)- and (*S*)-disopyramide and to study if any therapeutic benefits can be obtained by using only (*R*)-disopyramide.

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CHROMBIO. 2309

SIMULTANEOUS DETERMINATION OF FLUZINAMIDE AND THREE OF ITS ACTIVE METABOLITES IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A sensitive and selective high-performance liquid chromatographic method has been developed for a new anticonvulsant, fluzinamide, and three of its active metabolites. This method requires only 0.5 ml of plasma, and it involves a single extraction with a mixture of hexane—dichloromethane—butanol (55:40:5). The plasma extract is chromatographed on a 10-μm, C_{18} reversed-phase column and quantitated by ultraviolet absorbance at 220 nm. The concentration—response curves for all four compounds are linear from 0.05 μg/ml to at least 10 μg/ml. The extraction efficiency of this method is greater than 90%. The accuracy and precision of the method were tested by analyzing spiked unknown samples that had been randomly distributed across the concentration range. The mean concentrations found were within ± 9% of the various amounts added with a standard deviation of ± 3.5%. This method has been successfully applied to the analysis of samples obtained from fluzinamide-dosed dogs, healthy unmedicated volunteers, and patients who were at steady state with phenytoin, carbamazepine, and fluzinamide.

INTRODUCTION

Fluzinamide, N-methyl-3-[3-(trifluoromethyl)phenoxy]-1-azetidine carboxamide, is a potential antiepileptic agent. It was shown to be effective in mice and rats in preventing convulsions induced by electrical and chemical stimuli. The profile of activity of fluzinamide most closely resembled those of phenobarbital and valproic acid [1].

Concentrations of anticonvulsants in the plasma of epileptic patients are routinely monitored in order to ensure that drug levels are within the therapeutic ranges. High-performance liquid chromatographic (HPLC) methods with either direct protein precipitation or simple solvent extraction techniques

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[2—7] are preferred because of their selectivity, speed, and sensitivity. Such a method for the determination of fluzinamide is especially important because of its poor solubility in water or aqueous buffer solutions and its potential for poor dosage form performance. During the early drug development process, plasma concentration data were needed to determine the bioavailability of this compound.

An HPLC method was initially developed [8] to monitor only the unchanged drug. This method involved a simple extraction with 40% dichloromethane in hexane, and the extract was chromatographed on a 10-μm, C_{18} reversed-phase column with 35% of acetonitrile in water as the mobile phase.

After the analysis of the first few samples, it was apparent that three metabolites were coextracted from plasma and chromatographed on the system described above. One of the metabolites was not completely resolved from fluzinamide.

In subsequent experiments, the three metabolites that circulated in the plasma along with parent compound were separated, identified, and synthesized [9]. They were all found to be active with structures similar to fluzinamide. The major metabolic pathway of fluzinamide was believed to be the formation, in sequence, of the N-hydroxymethyl derivative (I), followed by the N-formyl derivative (II) and then the 3-[3-(trifluoromethyl)phenoxy]-1-azetidine carboxamide (III), the N-desmethyl metabolite of the unchanged drug. Fig. 1 shows the chemical structures of fluzinamide, its active metabolites in the plasma, and the internal standard.

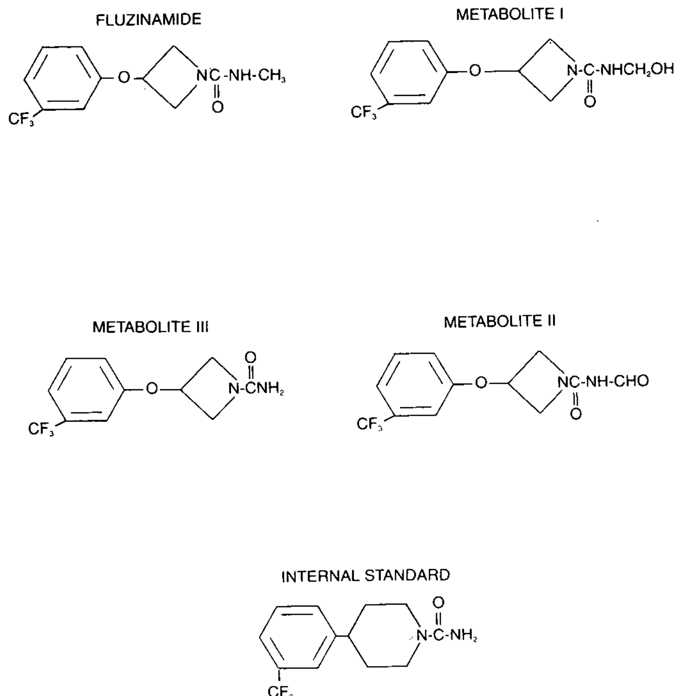

Fig. 1. Chemical structures of fluzinamide, three of its active metabolites, and the internal standard.

This paper is a description of a simple, selective, and sensitive method for the simultaneous quantitation of fluzinamide and its three active metabolites in plasma.

EXPERIMENTAL

Instruments and chromatographic conditions

The HPLC system consisted of a Waters Assoc. M6000A solvent delivery system, an autosampler (WISP Model 710B, Waters Assoc., Milford, MA, U.S.A.) and variable-wavelength detector (Schoeffel FS.770 LCUV, Schoeffel Instrument, Westwood, NJ, U.S.A.).

The chromatographic separation was achieved on a 10-μm, C_{18} reversed-phase Bondapak column. The mobile phase consisted of acetonitrile—tetrahydrofuran—0.025 *M* phosphate buffer, pH = 4.2 (30:5:65). It was delivered at a flow-rate of 1.5 ml/min and the compounds eluted were quantitated by their ultraviolet absorbance at 220 nm.

The output signal generated by the spectrophotometric detector was acquired by a computer-automated laboratory system (Computer Inquiry Systems, Waldwick, NJ, U.S.A.) with a Hewlett-Packard Model 1000 computer (Hewlett-Packard, Palo Alto, CA, U.S.A.). The chromatographic tracings were recorded on a 10-mV chart recorder (Hewlett Packard).

Chemicals and reagents

Acetonitrile, tetrahydrofuran, and monobasic sodium phosphate (analytical grade) were all purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Hexane, butanol, and methylene chloride (analytical grade) were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Fluzinamide, its three metabolites, and the internal standard were synthesized by A.H. Robins (Richmond, VA, U.S.A.).

Standard solutions

Stock solutions, corresponding to 100 μg/ml in acetonitrile—water solution (40:60), were prepared for fluzinamide, the internal standard, and metabolites I, II, and III.

Standard solutions ranging from 0.05 to 10 μg/ml were prepared by spiking drug-free plasma with the appropriate concentrations of all the compounds. To prepare the internal standard for spiking plasma samples, the stock solution was diluted to 50 μg/ml.

Extraction procedure

Samples (0.5 ml) of plasma, either standard or unknown, were transferred to 125 × 16 mm culture tubes. Internal standard (80 μl, 50 μg/ml) and 5 ml of extracting solvent that contained a mixture of hexane—methylene chloride—butanol (55:40:5, v/v/v) were added to each tube. The tube was vortexed for 15 sec and centrifuged for 5 min. The upper organic layer was pipetted to a clean tube and evaporated to dryness under a gentle stream of nitrogen in a water bath at 25°C. The residue was reconstituted in 200 μl of the mobile phase and 100 μl were injected into the HPLC system. All the samples and standards were run in duplicate.

Precision, reproducibility, and accuracy studies

To test the precision and reproducibility of this method, six individual standard curves of all compounds ranging in concentration from 0 to 10 μg/ml were run on consecutive days. The coefficient of variation values were determined for the peak height ratio at each concentration level. The slope, intercept, and correlation coefficient of the daily standard curves were calculated. The accuracy of the method was also determined by assaying thirty randomized samples spiked with various concentrations of the four compounds. The concentrations of these samples were unknown to the analyst at the time of analysis.

RESULTS AND DISCUSSION

Chromatograms of blank plasma spiked with the internal standard and blank plasma spiked with fluzinamide, three of its active metabolites, and the internal standard are shown in Figs. 2 and 3, respectively.

The chromatographic separation of all the compounds was accomplished on the column by the proper adjustment of the mobile phase. Tetrahydrofuran and the ratio of organic to aqueous fractions in the mobile phase appeared to be the two most critical factors. Tetrahydrofuran at 5—10% of the mobile phase composition was optimum for separating all five compounds. If the concentration of tetrahydrofuran exceeded 10%, metabolite II and the internal standard were not resolved; less than 5% tetrahydrofuran resulted in losing the resolution between metabolite II and fluzinamide. Changing the amount of

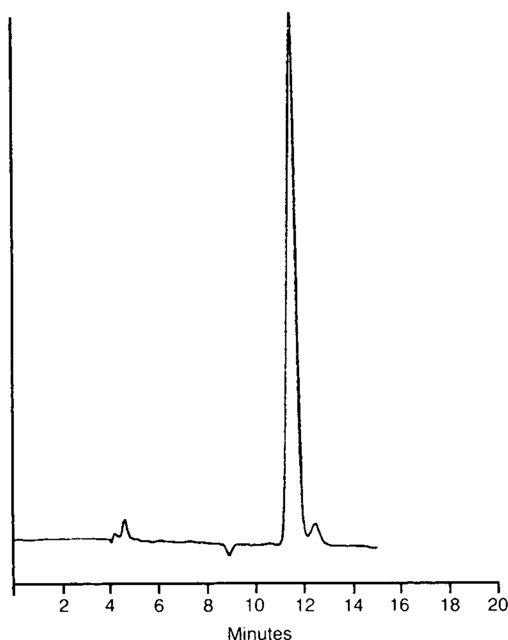

Fig. 2. Chromatogram of extracts from control plasma spiked with 4 μg/ml internal standard.

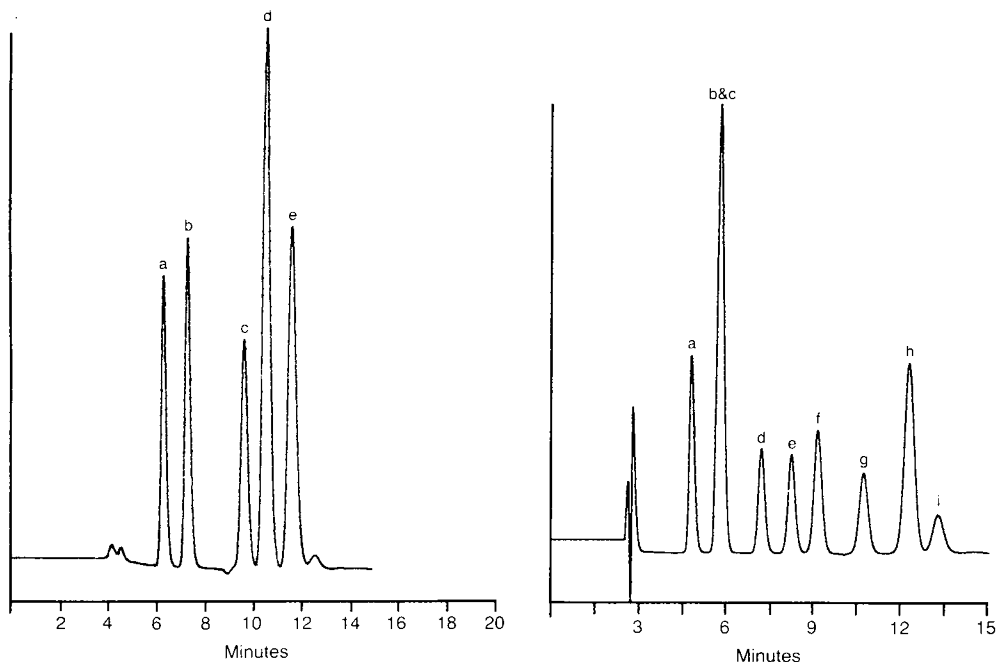

Fig. 3. Chromatogram of extracts from control plasma spiked with 5 μg/ml metabolite I (a), 5 μg/ml metabolite III (b), 5 μg/ml fluzinamide (c), 5 μg/ml metabolite II (d), and 4 μg/ml internal standard (e).

Fig. 4. Chromatogram of extracts from control plasma spiked with 10 μg/ml 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (a), phenobarbital (b), carbamazepine (c), metabolite I (d), metabolite III (e), phenytoin (f), fluzinamide (g), metabolite II (h), and 4 μg/ml internal standard (i).

tetrahydrofuran in the mobile phase was necessary for the lot-to-lot change in column performance and the "aging" of analytical columns.

High plasma concentrations of the commonly used anticonvulsants such as phenytoin and carbamazepine are generally present in epileptic patients. The chromatography described in this method can separate these anticonvulsants from fluzinamide, its active metabolites, and the internal standard. Fig. 4 shows a typical chromatogram of extracts from plasma that had been spiked with 5-(*p*-hydroxyphenyl)-5-phenylhydantoin, phenobarbital, carbamazepine, fluzinamide, three of its active metabolites, phenytoin, and internal standard. The chromatogram shows good resolution for all compounds except between phenobarbital and carbamazepine. Therefore, the commonly used anticonvulsants do not interfere with the analysis of fluzinamide, its active metabolites, or the internal standard by the HPLC conditions described above.

Tetrahydrofuran at 8% appears to be the optimal concentration to be used in separating the large phenytoin peak from that of metabolite III by a 10-μm, C_{18} reversed-phase Bondapak column (Fig. 4). Slight variations in the retention times of all the compounds were observed between different batches of columns, but all the compounds and the internal standard should be eluted within 12 ± 2 min.

During the extraction and evaporation procedures, the temperature was kept

TABLE I

DETERMINATION OF UNKNOWN AMOUNTS OF FLUZINAMIDE AND METABOLITE I ADDED TO PLASMA

Fluzinamide added (μg/ml)	Number of samples	Concentration of fluzinamide found				I added (μg/ml)	Number of samples	Concentration of metabolite I found			
		Mean of set (μg/ml)	S.D. of set	C.V. of set (%)	Found (%)			Mean of set (μg/ml)	S.D. of set	C.V. of set (%)	Found (%)
0.00	4	BQL*	—	—	—	0.00	4	BQL	—	—	—
0.06	4	0.060	0.007	11.79	100	0.05	4	0.045	0.006	12.80	90
0.10	5	0.102	0.008	8.24	102	0.10	5	0.093	0.013	13.60	93
0.41	5	0.406	0.114	2.81	98	0.60	5	0.663	0.061	9.23	111
2.06	5	2.160	0.056	2.56	105	1.60	5	1.640	0.060	3.59	103
6.36	4	6.769	0.166	2.45	106	7.00	4	7.300	0.178	2.45	103
10.60	3	11.570	0.408	3.52	109	9.50	3	9.215	0.163	1.76	97

*BQL = below quantifiable limit, i.e. mean concentration is less than 0.05 μg/ml.

TABLE II

DETERMINATION OF UNKNOWN AMOUNTS OF METABOLITE II AND METABOLITE III ADDED TO PLASMA

II added (μg/ml)	Number of samples	Concentration of metabolite II found				III added (μg/ml)	Number of samples	Concentration of metabolite III found			
		Mean of set (μg/ml)	S.D. of set	C.V. of set (%)	Found (%)			Mean of set (μg/ml)	S.D. of set	C.V. of set (%)	Found (%)
0.00	4	BQL*	—	—	—	0.00	4	BQL	—	—	—
0.05	4	0.050	0.000	—	100	0.05	4	BQL	—	—	—
0.08	5	0.820	0.004	5.33	103	0.10	5	0.118	0.005	4.26	118
0.45	5	0.047	0.006	1.27	105	0.80	5	0.766	0.006	0.72	96
1.50	5	1.610	0.047	2.94	107	1.80	5	1.732	0.044	2.54	96
3.98	4	3.982	0.099	2.46	102	5.00	4	4.550	0.067	2.94	91
8.29	3	8.410	0.445	5.30	101	9.00	3	9.220	0.346	3.72	104

*BQL for metabolite III is 0.1 μg/ml and for metabolite II is 0.05 μg/ml.

at 25°C or below because the N-hydroxymethyl derivative of fluzinamide (metabolite I) is unstable at temperatures higher than 35°C. Preliminary studies on metabolite I showed that a significant amount of the compound was converted to metabolite III at temperatures over 35°C. However, fluzinamide and its active metabolites showed no significant degradation over a period of six months in spiked plasma samples stored at −20°C.

The maximum absorption wavelength for fluzinamide, its three metabolites, and the internal standard was around 220 ± 10 nm and the quantitative determination of all compounds was accomplished at 220 nm. Even though structurally very similar, the molar absorptivity of metabolite II is almost two times higher than fluzinamide or the other two metabolites at 220 nm. Generally, the results of six consecutive standard curves for fluzinamide and its active metabolites showed a linear range between 0 to at least 10 μg/ml. The mean slope values of the regression lines for fluzinamide and its three metabolites were 0.246 ± 0.01, 0.283 ± 0.02, 0.546 ± 0.01, and 0.348 ± 0.01 respectively. Good reproducibility was reflected by the tight correlation coefficients: r = 0.9994 ± 0.001. The intercept values of the regression lines were always negligible. The peak height for all compounds showed a coefficient of variation below 10% in the concentration range of 0.1—10 μg/ml.

Tables I and II show the recovery values of the thirty spiked samples. The percentages found were generally within ± 10% of the theoretical concentrations added. The lower limit of quantitation of fluzinamide, metabolite I, and metabolite II was 0.05 μg/ml; it was 0.1 μg/ml for metabolite III. The recovery by a single extraction step in this method was better than 90% for all the compounds.

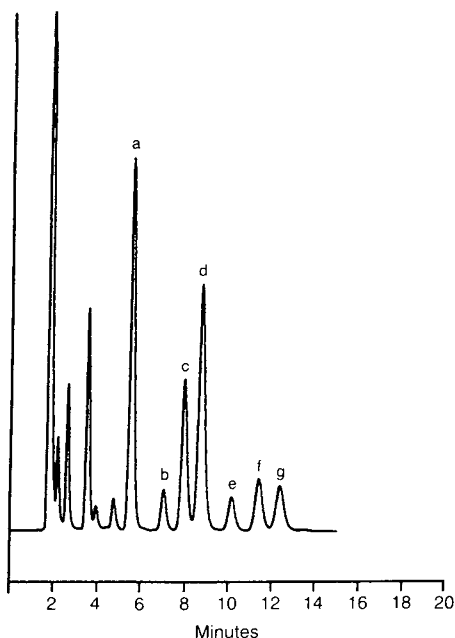

Fig. 5. Chromatogram of extracts from a plasma sample obtained from a patient at steady state of phenytoin, carbamazepine, and fluzinamide. Peaks: a = carbamazepine; b = metabolite I (4.64 μg/ml); c = metabolite III (6.14 μg/ml); d = phenytoin; e = fluzinamide (3.31 μg/ml); f = metabolite II (2.67 μg/ml); and g = internal standard (4 μg/ml).

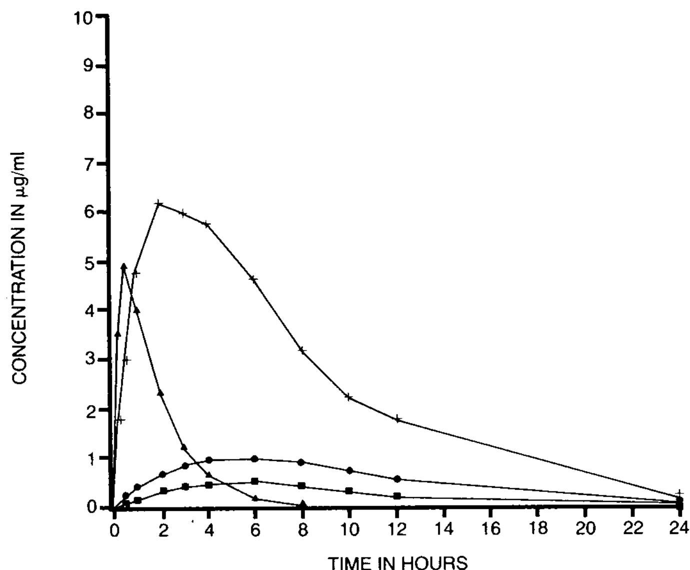

Fig. 6. Plasma concentration—time curve of fluzinamide and three of its active metabolites after a single 10 mg/kg oral dose of fluzinamide in a male dog. ▲, Fluzinamide; +, metabolite I; ■, metabolite II; ●, metabolite III.

This method was utilized in the analysis of plasma samples obtained from patients and dogs dosed with fluzinamide. Fig. 5 shows a chromatogram of extracts of a plasma sample obtained from a patient in which phenytoin, carbamazepine, and fluzinamide were at steady state. Fig. 6 shows the plasma concentration—time profile of fluzinamide and the three metabolites obtained after administration of a single oral dose (10 mg/kg) to a male dog.

CONCLUSION

A rapid, sensitive, and selective method has been developed for simultaneous determination of fluzinamide and those of its active metabolites at low microgram levels, with a linear range of 0.05—10 µg/ml, using 0.5 ml of plasma. The method exhibited a high degree of precision and accuracy, and it is well suited for routine analysis of plasma samples obtained from bioavailability and pharmacokinetic studies.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR PILOCARPINE IN AQUEOUS HUMOR

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SUMMARY

A high-performance liquid chromatographic assay for pilocarpine has been developed for the determination of pilocarpine in aqueous humor. A structurally similar internal standard is used, and pilocarpine is separated from isopilocarpine under the chromatographic conditions used. A 100-μl sample is mixed with an aliquot of internal standard at pH 8.3 and extracted with methylene chloride. The extract is evaporated to dryness and the alkaloids are quaternized with *p*-nitrobenzyl bromide. Following the quaternization, the sample is evaporated to dryness, washed and diluted with a mobile phase—triethylamine mixture and analyzed by high-performance liquid chromatography using a reversed-phase octadecylsilane column with detection at a wavelength of 254 nm. This is a highly sensitive, reproducible and selective assay for measuring pilocarpine at physiological levels in individual aqueous humor samples.

INTRODUCTION

The widespread use of pilocarpine in clinical ophthalmology has stimulated the development of many assay techniques to determine the levels and distribution of the drug after topical application in the eye. Several methodologies have been employed including radioactive isotope techniques, spectrophotometry, polarography, and chromatographic assays [1—5]. Wide disparity in results has occurred with the different techniques. Often sensitive methods have not differentiated between pilocarpine and inactive degradation products [2, 3]. Hydrolysis and epimerization studies of Nunes and Brochmann-Hanssen [6] demonstrated the importance of considering these degradation products. Additionally, most techniques are not sensitive enough to measure concentrations achieved in individual aqueous humor samples.

With the development of high-performance liquid chromatography (HPLC)

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and gas—liquid chromatography it has become possible to separate the epimer of pilocarpine with sufficient sensitivity to measure the drug in small-volume biological samples [4, 5, 7]. The HPLC assay for pilocarpine described by Mitra et al. [7] did not give separation of the epimers in our hands. A serious shortcoming of the procedure is the lack of an internal standard.

The present paper describes an HPLC assay for pilocarpine which has proved reproducible and sufficiently sensitive to measure low concentrations of the drug in individual aqueous humor samples. The technique incorporates the derivatization on the imidazole ring of pilocarpine with *p*-nitrobenzyl bromide to increase sensitivity [7]. Excellent separation of pilocarpine and its epimer is achieved using ion-pair chromatography. Pilocarpic acids are not extracted at the pH used. A structurally similar internal standard is used.

EXPERIMENTAL

Chemicals

Pilocarpine nitrate and triethylamine were obtained from Sigma (St. Louis, MO, U.S.A.). A commercially available pilocarpine solution (Isoptocarpine, Alcon Labs.) was used for administration to laboratory animals. Commercial preparations of therapeutic agents tested for drug interference were obtained from the University Hospital Pharmacy. Isopilocarpine nitrate and *p*-nitrobenzyl bromide were obtained from Aldrich (Milwaukee, WI, U.S.A.). *p*-Nitrobenzyl bromide was recrystallized from ethanol—water before use. Pilosine was obtained from ICN Pharmaceuticals (Plainview, NY, U.S.A.) and recrystallized from ethanol. 1-Octanesulfonic acid, sodium salt, was obtained from Eastman-Kodak (Rochester, NY, U.S.A.). Methylene chloride, cyclohexane, and diethyl ether without preservative, distilled-in-glass grade, were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). All other chemicals were reagent grade. Water was double-distilled. Aqueous humor and all solutions were filtered through a 0.45-μm Millipore filter prior to use. Centrifuge tubes and ampules used in the derivatization procedure were silanized with dimethyldichlorosilane.

Working solutions

An aqueous stock solution containing pilocarpine nitrate at 66 μg/ml was used to prepare a series of standard solutions of the drug in normal rabbit aqueous humor at 0.25, 0.50, 1.0, 2.5, 5.1, 7.6, 8.5, 9.2 and 10.1 μg/ml free base concentrations. Standard solutions of the epimer were prepared from an aqueous stock solution containing 13 μg/ml isopilocarpine nitrate. The working internal standard solution contained pilosine at a concentration of 21 μg/ml in water. Potassium bicarbonate was prepared at a concentration of 0.3 *M*, pH 8.3. The derivatizing solution was *p*-nitrobenzyl bromide, 0.5 mg/ml, in acetonitrile.

Chromatography

The Beckman (Anaheim, CA, U.S.A.) Model 330 isocratic system consisting of a Model 110 A pump, Model 210 sample injection valve and Model 153 detector for ultraviolet (UV) detection at 254 nm was used in conjunction with

a Heathkit strip-chart recorder. An Altex-Beckman Ultrasphere reversed-phase octadecylsilane (RP-ODS) column, particle size 5 μm, 25 cm × 4.5 mm I.D. was used. The mobile phase was pumped at 1.3 ml/min. Column-to-column variation in initial backpressure required occasional adjustment in the flow-rate. The column temperature was maintained at 25—26°C. The mobile phase was prepared by adding 48 ml of 0.25 *M* glycine—hydrochloric acid buffer, pH 2.1, and 20 ml of 0.1 *M* 1-octanesulfonic acid to 1 l of water. The resulting solution was mixed with 440 ml of isopropyl alcohol containing 1.2 ml triethylamine, followed by the addition of water to a final volume of 2 l.

Assay procedure

A 100-μl aliquot of the standard solution or aqueous humor was placed in a 12-ml conical test tube. A 30-μl aliquot of working internal standard solution was added, followed by 600 μl of potassium bicarbonate solution. The mixture was swirl-mixed immediately; 1 ml methylene chloride was added. The sample was mixed for 1 min by swirl-mixing, and centrifuged for 5 min at 300 *g*. The aqueous layer was aspirated and discarded. The organic layer was transferred to an ampule and evaporated to dryness under nitrogen. A 100-μl aliquot of derivatizing solution was added to the dry sample. The ampule was sealed under a nitrogen atmosphere and mixed. The sample was derivatized by placing the ampule in a 40°C oil bath for 40 h. Following derivatization, the ampule was cooled to room temperature and frozen at −40°C until chromatography was performed.

Prior to chromatographic analysis, the ampule was opened and diluted with 500 μl of acetonitrile. The diluted sample was mixed thoroughly and a 200–300 μl aliquot was removed and evaporated to dryness under nitrogen. The dried sample was treated with 0.4 ml of diethyl ether, the tube was swirl-mixed for 30 sec and the diethyl ether was discarded. This washing procedure was performed six times with diethyl ether followed by three times with cyclohexane. The sample was then redissolved in 32 μl of a solution containing mobile phase and 2 m*M* triethylamine (96:4), swirl-mixed, and a 20-μl sample was injected into the HPLC system. Aqueous controls were routinely used.

Milligram quantities of pilocarpine, isopilocarpine and pilosine derivatives were prepared as described by Mitra et al. [7] and used in preliminary work to establish retention times and chromatographic conditions.

Stability of derivatives

Pure derivatized standards of pilocarpine, isopilocarpine and pilosine were dissolved in acetonitrile at a concentration of 6 μg/ml and frozen at −40°C. Ten samples at a concentration of 2.5 μg/ml were carried through the assay procedure and frozen in the unopened ampules. The ampules were opened and the samples assayed as described. Peak height ratios were determined.

Standard curve

Standard curves were constructed by analyzing a series of aqueous humor samples containing known amounts of pilocarpine free base in a concentration range of 0.25 to 10.1 μg/ml.

Precision

Within-day and between-day variability were determined by analyzing ten replicate samples containing pilocarpine at a concentration of 2.5 μg/ml.

Specificity and interference studies

Samples of rabbit aqueous humor, obtained from animals not treated with pilocarpine, were analyzed without the addition of internal standard to identify potential interference by endogenous components.

Interference by therapeutic agents frequently used with pilocarpine or often encountered in our patient population was evaluated. Drugs tested included methazolamide, acetazolamide, diazepam, acetaminophen, caffeine, echothiophate iodide, epinephrine·HCl, ketamine·HCl, pentobarbital, carbachol and timolol maleate. Drugs were tested at a 5 μg/ml concentration except for diazepam, which was studied at a 1 μg/ml concentration.

Animal studies

New Zealand white male rabbits weighing approx. 2 kg were given a 50-μl dose of 4% pilocarpine topically in the right eye. Rabbits were sedated with pentobarbital. At 30 min, aqueous humor was aspirated and frozen at −40°C for subsequent assay.

Column maintenance

The column was washed 30 min with water at a flow-rate of 1.0 ml/min, and 30 min with acetonitrile at a flow-rate of 1.0 ml/min at the end of each day. The column was equilibrated overnight with mobile phase at a flow-rate of 0.5 ml/min. At the end of the week the column was washed with water for 1 h at a flow-rate of 1 ml/min and stored in methanol.

RESULTS

Under the chromatographic conditions used, the retention times of pilocarpine, isopilocarpine and the internal standard were 11.2, 12.2 and 18.4 min, respectively. The resolution factor (R_s) was 1.62 for the two epimers. A separation factor (α) of 1.07 was calculated. Some variability from HPLC column to column was observed, but adequate resolution of pilocarpine and isopilocarpine was always possible. Fig. 1. is a chromatogram of the standards.

Pure standards of derivatized isopilocarpine and pilocarpine, dissolved in acetonitrile at 6 μg/ml concentration, were found to be stable when stored at −40°C for four months. Samples carried through the assay procedure and frozen in unopened ampules were found to be stable for two weeks after derivatization. Ten samples containing pilocarpine at a concentration of 2.5 μg/ml were found to contain 2.8 μg/ml pilocarpine, coefficient of variation (C.V.) 7.3%, after storage for thirteen days at −40°C. Derivatized samples stored for more than two weeks at −40°C had a C.V. of 15.6%. Pilocarpine in aqueous humor samples frozen without sample preparation appeared stable for several months.

Analysis of a series of aqueous humor samples containing known amounts of pilocarpine yielded a standard curve in which the concentration of the drug

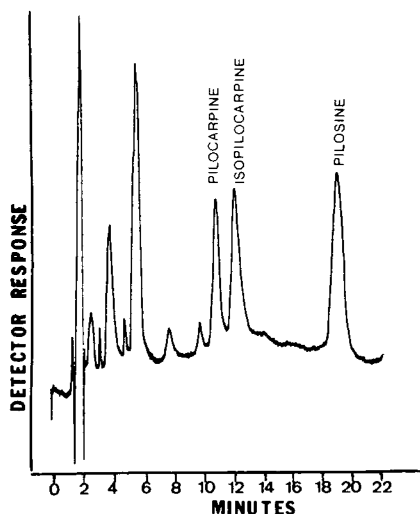

Fig. 1. Chromatogram of a mixture of pilocarpine, isopilocarpine and pilosine standards carried through the assay procedure.

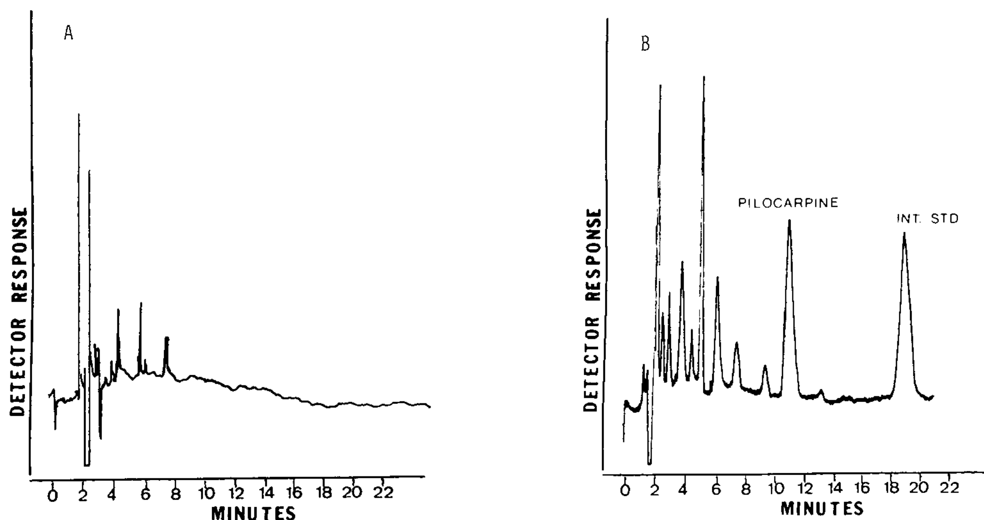

Fig. 2. Chromatograms obtained by analysis of rabbit aqueous humor: (A) obtained from animals not treated with pilocarpine, and without addition of internal standard; (B) 30 min after topical administration of 50 μl of 4% pilocarpine. Concentration: 5.75 μg/ml.

was linearly related to the pilocarpine:internal standard peak height ratios. The data fit the equation of a straight line: peak height ratio = 0.2198[pilocarpine] + 0.0043. Least-square analysis yielded a coefficient of correlation (r) of 0.9984. The assay was developed for a concentration range of 0.25—10.1 μg/ml. The lowest concentration studied was 0.25 μg/ml. Preliminary experiments (data not shown) indicated that the range of linearity may be extended well beyond the 10 μg/ml concentration. Curves constructed from pilocarpine in water were essentially identical to those from aqueous humor. The variability

observed from both sources was no greater than that observed from injection to injection, therefore, aqueous controls were routinely used.

The within-day analysis of ten replicate samples containing 2.5 μg/ml pilocarpine gave a mean drug concentration of 2.48 μg/ml with a C.V. of 7.1%, while between-day results were 2.3 μg/ml, C.V. 7.9%.

No interference by endogenous compounds was found when aqueous humor from normal rabbits was analyzed (Fig. 2A). Methazolamide, acetazolamide, diazepam, acetaminophen, caffeine, echothiophate iodide, epinephrine·HCl, ketamine·HCl and carbachol, did not interfere with the assay. Timolol maleate showed a small peak at the pilocarpine retention time. Pentobarbital, used as the sedating agent, did not interfere.

A 50-μl dose of 4% pilocarpine was given topically to four unsedated young rabbits. At 30 min after administration, the aqueous humor was found to contain pilocarpine in a concentration range of 5—8 μg/ml. Fig. 2B was obtained by analyzing rabbit aqueous humor following topical administration of pilocarpine.

DISCUSSION

Pilocarpine may epimerize to isopilocarpine under certain conditions, and since isopilocarpine is essentially pharmacologically inactive, separation of the two compounds during analysis is important [6, 8, 9]. We were unable to obtain the desired separation using the procedure described by Mitra et al. [7]. One broad peak was obtained with no resolution. This may have been due to the differences in the RP-ODS columns used. We retained the derivatization procedure to obtain the desired sensitivity and turned to other solvents for separation. Acetonitrile—water (75:25) with millimolar ion-pairing agent gave excellent separation of the epimers, but the internal standard was eluted with isopilocarpine. The chromatographic conditions used in the present procedure provide good separation of pilocarpine and isopilocarpine. However, to effect good separation it is necessary to inject the sample in a mobile phase—triethylamine mixture. Triethylamine decreases tailing of the peaks and improves separation of the epimers. Increased concentrations of ion-pairing agent, iso-

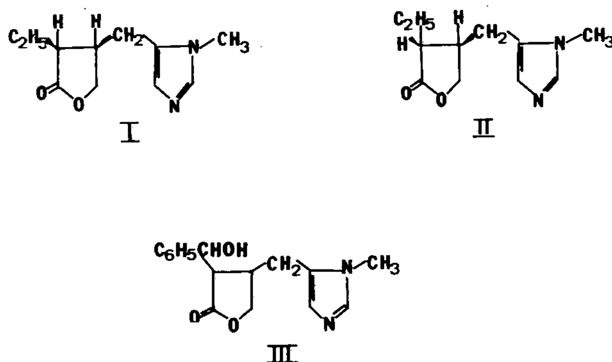

Fig. 3. Structures of the epimers pilocarpine (I) and isopilocarpine (II), and the internal standard, pilosine (III).

propyl alcohol, buffer or triethylamine in the mobile phase decreases the retention times at the expense of resolution.

Pilosine was chosen for the internal standard because it closely resembles pilocarpine in chemical structure (Fig. 3), is extracted and derivatized similarly to the compounds under study and is commercially available. Pilosine is unlikely to epimerize during the procedure [10].

The extraction procedure was also patterned after Mitra et al. [7] but utilized only a single extraction. This was shown to provide significantly cleaner extracts although there was some compromise in extraction efficiency. We found that approx. 85% of the pilocarpine was recovered with one extraction. The derivatization of the compounds of interest with *p*-nitrobenzyl bromide was extended from 24 h [7] to 40 h, since it was found that pilosine required a longer reaction time. The washing procedure with diethyl ether and cyclohexane was included to remove contaminants from the samples.

No isopilocarpine was found in the rabbit aqueous humor after administration of pilocarpine. For this reason a sample of commercial pilocarpine was diluted to 10 μg/ml free base and assayed. The commercial preparation was found to contain 10.9 μg/ml pilocarpine and no detectable isopilocarpine. This finding is similar to those of Drake et al. [11] and Noordam et al. [12].

In the chosen concentration range, one-third to one-half of the derivatized sample was used for each injection. This permitted a repeat injection if necessary. At pilocarpine concentrations of 0.5 μg/ml or less, the entire derivatized sample was required for accurate quantification.

Column temperatures and pressure as well as complete equilibration of the column proved critical in obtaining reproducible retention times. Extensive column washing was necessary to extend column life.

The stability studies indicate that aqueous humor samples may be frozen at −40°C for several months prior to sample preparation. Derivatized samples should be assayed within two weeks after derivatization. The purified standards, made up in acetonitrile, were stable for several months at −40°C. Pure, dried, derivatized standards of pilocarpine and isopilocarpine broke down completely after six months storage in vacuum dessicator at room temperature.

Drugs tested for potential interference were antiglaucoma drugs commonly used with pilocarpine; also diazepam, acetaminophen and caffeine. Only timolol showed a peak which could interfere in the assay. It was not established if this peak were due to the drug itself or a constituent of the commercial solution. Steroids were not tested as they would be removed in the diethyl ether wash. Pilocarpic acids are not extracted into methylene chloride at the pH used. Ketamine and pentobarbital were also tested for interference since they are routinely used in animal work in our laboratory.

The concentration of pilocarpine found in the rabbit aqueous humor was similar to that found by Chrai and Robinson [2] following a dose of 25 μl of 0.1 *M* pilocarpine with an assay using tritium-labeling.

A considerable number of assays for pilocarpine exist for the determination of the drug in commercial preparations. A need existed, however, for a highly sensitive and selective assay for the drug at physiological concentrations. While our assay is elaborate, it is selective, sensitive and reproducible. It is applicable to the study of pilocarpine distribution in the animal eye and to the analysis of pilocarpine in individual human aqueous humor samples.

ACKNOWLEDGEMENT

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Note

Identification of 6-deoxyallitol and 6-deoxygulitol in human urine

Electron-impact mass spectra of eight isomers of 6-deoxyhexitol

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Polyols form a large group of compounds in body fluids, and their study has become important since polyol levels in body fluids change in various diseases. In diabetes mellitus the urinary excretion of mannitol and myoinositol is increased [1], and the concentration of 1-deoxyglucose in plasma [2] and cerebrospinal fluid [3] is low. Increased production of sorbitol in the tissues is considered to be pathogenic in the complications of diabetes mellitus such as cataracts [4] and neuropathy [5]. In uraemia the serum and urinary levels of myoinositol are increased [1, 6], and the accumulation of myoinositol is considered to be a cause of uraemic polyneuropathy [7, 8]. We previously reported [6] that serum and urinary levels of chiroinositol and scylloinositol are increased and the serum level of 1-deoxyglucose is decreased in uraemia and that seven new deoxyalditols including 6-deoxymannitol and 6-deoxy-

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galactitol had been identified. In the present study 6-deoxyallitol and 6-deoxygulitol have been found as normal components of human urine for the first time.

MATERIALS AND METHODS

Chemicals

L-Rhamnose and L-fucose were the products of Tokyo Kasei (Tokyo, Japan). 6-Deoxyglucose was obtained from Sigma (St. Louis, MO, U.S.A.). D-Glucose, D-mannose and ribitol were the products of Yoneyama (Osaka, Japan).

6-Deoxymannitol was synthesized by the reduction of L-rhamnose with sodium borohydride, 6-deoxysorbitol by the reduction of 6-deoxyglucose with sodium borohydride, and 6-deoxygalactitol by the reduction of L-fucose with sodium borohydride. 6-Deoxyallitol was synthesized by the sodium borohydride reduction of 6-deoxy-D-allose [9], which was prepared by chemical conversion of D-glucose. 6-Deoxygulitol was synthesized by the sodium borohydride reduction of 6-deoxy-L-gulose [9], which was prepared by chemical conversion of D-mannose. 6-Deoxyiditol was synthesized by the sodium borohydride reduction of 6-deoxy-L-idose, which was obtained by the acid hydrolysis of methyl β-L-idopyranoside prepared from D-glucose according to the method of Ikeda et al. [10]. 6-Deoxyaltritol was synthesized by the sodium borohydride reduction of 6-deoxy-D-altrose, which was obtained by the acid hydrolysis followed by deacylation of methyl 2,3-di-O-acetyl-4-O-benzoyl-6-deoxy-α-D-altropyranoside prepared from D-glucose according to the method of Chiba and Tejima [11]. 6-Deoxytalitol was synthesized by the sodium borohydride reduction of 6-deoxy-L-talose [9], which was prepared by chemical conversion of D-glucose.

Samples

24-h Urine samples were obtained from five healthy adults and ten patients with chronic renal failure. Four of ten uraemic patients were on 5-h haemodialysis three times a week.

The urine samples were kept at −20°C prior to analysis.

Sample preparation

A volume of urine equivalent to 1 mg of creatinine was applied to a Dowex 50W-X8 column (H^+, 5 cm × 0.8 cm I.D.) after the addition of 50 μg of ribitol as internal standard. Polyols were eluted with 30 ml of distilled water. The eluate was applied to an Amberlite IRA 400 column ($HCOO^-$, 5 cm × 0.8 cm I.D.). Polyols were eluted with 30 ml of distilled water. After lyophilization, the polyols were redissolved with 9 ml of hot methanol, then transferred to a sample vial and dried with a nitrogen stream. The polyols were trimethylsilylated with 90 μl of N,O-bis(trimethylsilyl)trifluoroacetamide and 10 μl of trimethylchlorosilane at 60°C for 20 min. Of the sample 2 μl were subjected to gas chromatography—mass spectrometry.

Instrumentation

A Hewlett-Packard 5710A gas chromatograph was directly coupled to the

source of a JMS-D300 mass spectrometer (JEOL, Tokyo, Japan). The gas chromatograph was equipped with a 30 m × 0.25 mm I.D. OV-101 open tubular glass capillary column and a splitless injector. The column temperature was programmed from 120°C to 260°C at 3°C/min. Electron-impact ionization (EI) mass spectra were recorded at an ionizing energy of 70 eV, an ionization current of 300 μA and an accelerating voltage of 3 kV.

RESULTS AND DISCUSSION

Fig. 1 shows the gas chromatogram of polyols in the urine of a patient with chronic renal failure (upper chromatogram). The identification of the peaks

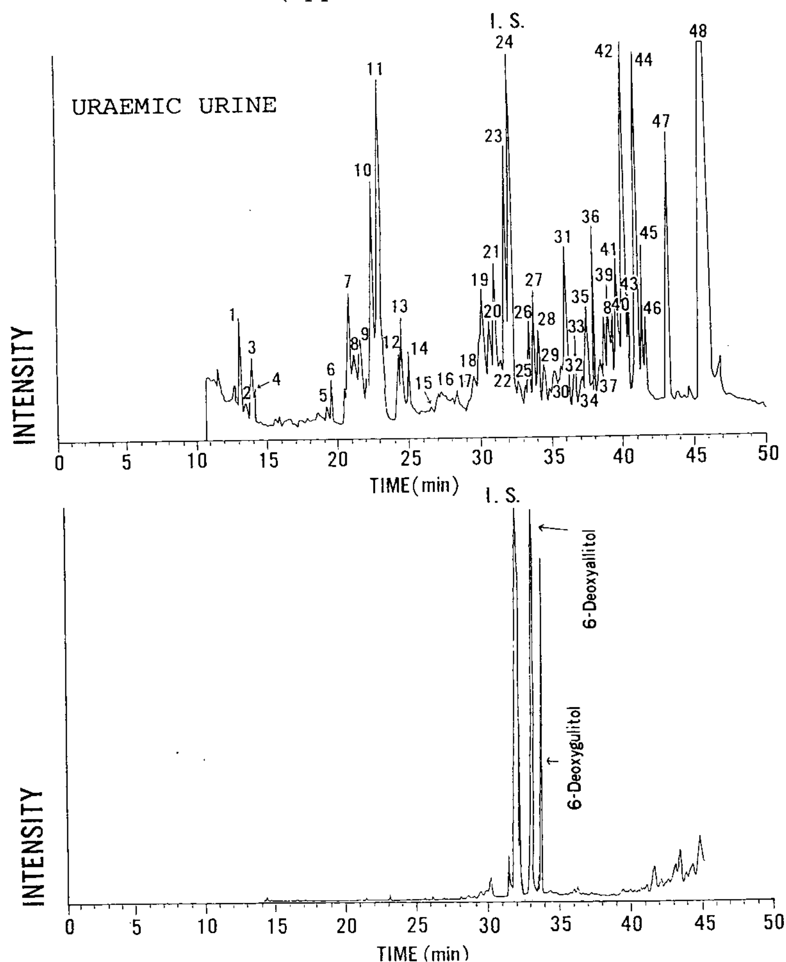

Fig. 1. Gas chromatograms of polyols in urine of a patient with chronic renal failure (upper chromatogram), and of synthesized deoxyhexitols (lower chromatogram). Peak identifications: 1 = glycerol, 3 = 4-deoxythreitol, 4 = 4-deoxyerythritol, 10 = threitol, 11 = erythritol, 12 = 5-deoxyxylitol, 13 = 5-deoxyarabitol, 15 = 2-deoxyribitol, 19 = xylulose, 22 = xylitol, 23 = arabitol, 24 = ribitol (internal standard), 26 = 6-deoxyallitol, 27 = 6-deoxymannitol, 28 = 6-deoxygulitol, 29 = 6-deoxygalactitol, 30 = fructose, 31 = 1-deoxyglucose, 36 = α-glucose, 37 = β-galactose, 39 = neoinositol, 42 = mannitol, 43 = sorbitol, 44 = chiro-inositol, 45 = β-glucose, 46 = epi- or *cis*-inositol, 47 = scylloinositol, 48 = myoinositol.

was based on the fact that the EI mass spectra and the relative retention times of the peaks were the same as those of the trimethylsilylated authentic compounds or that the EI mass spectra of the peaks were the same as those reported in the literature.

The EI mass spectrum of peak 26 is shown in Fig. 2 (lower spectrum). The molecular ion was found to be 526 by chemical ionization. The base peak at *m/z* 117 and the intense peak at *m/z* 219 in addition to the peaks at *m/z* 103, *m/z* 205, and *m/z* 307 suggested the structure of 6-deoxyhexitol. Eight isomers of 6-deoxyhexitol were synthesized, and the relative retention times and the EI mass spectra of the trimethylsilylated isomers were measured, and listed in Table I. Peak 26 was identified as 6-deoxyallitol, since only the trimethylsilyl (TMS) derivative of 6-deoxyallitol showed the same retention time (Fig. 1) and the same EI mass spectrum (Fig. 2) as those of peak 26.

The EI mass spectrum of peak 28 is shown in Fig. 3 (lower spectrum). The molecular ion of peak 28 was 526. Peak 28 and peak 26 showed similar mass spectra, suggesting an isomeric relationship. Although the TMS derivatives of 6-deoxygulitol, 6-deoxyiditol and 6-deoxyaltritol showed almost the same retention times as peak 28, only the TMS derivative of 6-deoxygulitol showed an EI mass spectrum identical with that of peak 28 (Fig. 3). The TMS derivative of 6-deoxyiditol was excluded because the relative intensity of *m/z* 217

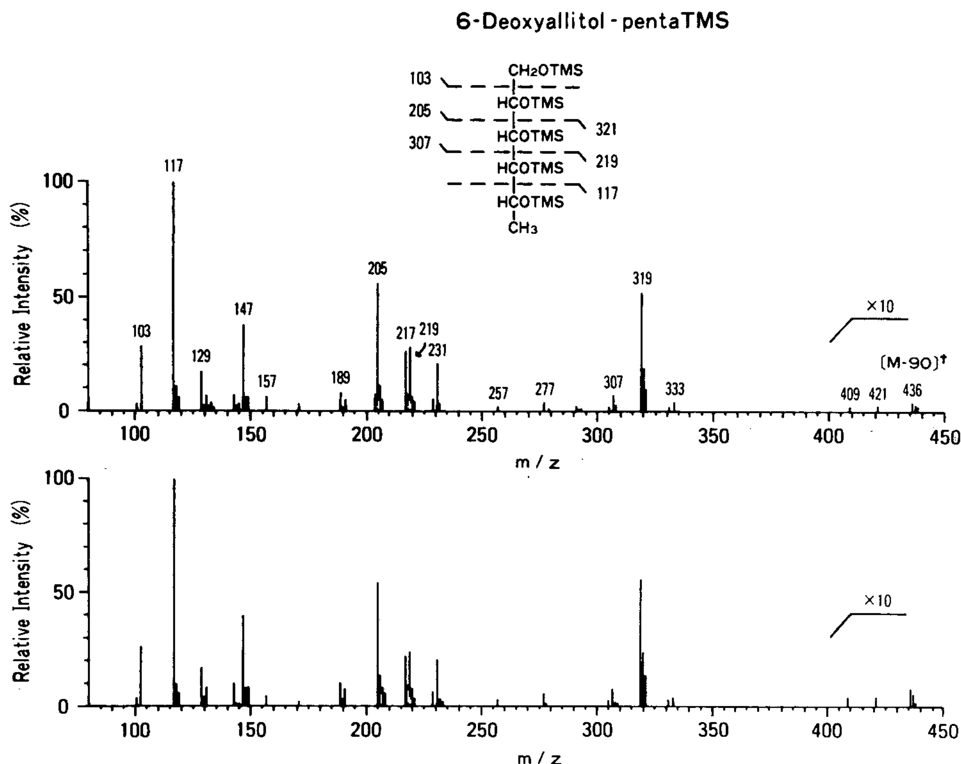

Fig. 2. EI mass spectra of TMS derivative of 6-deoxyallitol (upper spectrum) and of peak 26 (lower spectrum) in the gas chromatogram of Fig. 1.

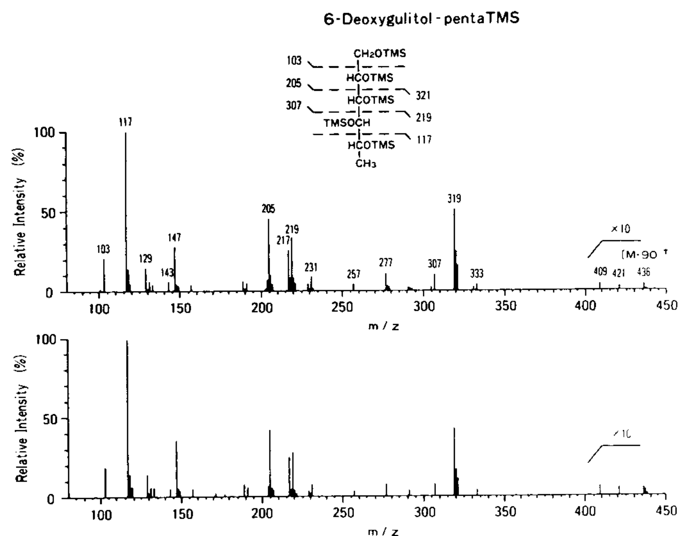

Fig. 3. EI mass spectra of TMS derivative of 6-deoxygulitol (upper spectrum) and of peak 28 (lower spectrum) in the gas chromatogram of Fig. 1.

TABLE I

RELATIVE RETENTION TIMES AND EI MASS SPECTRA OF EIGHT TRIMETHYLSILYLATED ISOMERS OF 6-DEOXYHEXITOL

m/z	Relative abundance (%)							
	6-Deoxy-allitol	6-Deoxy-mannitol	6-Deoxy-sorbitol	6-Deoxy-gulitol	6-Deoxy-iditol	6-Deoxy-altritol	6-Deoxy-galactitol	6-Deoxy-talitol
103	29	26	12	21	19	26	34	26
117	100	100	84	100	100	100	100	100
129	17	17	12	14	9	11	18	16
131	7	11	6	6	7	8	15	8
133	4	10	6	4	7	7	12	6
143	7	11	9	6	6	8	11	10
147	38	56	42	28	36	39	63	36
157	6	6	6	4	2	1	4	5
189	8	7	5	6	3	4	7	6
191	5	6	4	5	4	6	8	8
204	7	10	10	7	7	9	14	7
205	56	56	51	45	40	47	35	54
217	26	30	38	25	26	31	48	32
219	28	29	26	33	22	38	35	37
229	5	5	6	4	2	2	4	2
231	21	10	22	9	9	23	14	10
257	2	5	6	4	2	2	4	2
277	4	6	6	11	6	6	5	3
291	2	4	6	2	2	2	4	1
293	1	2	0.5	1	1	1	1	1
305	2	3	4	2	2	2	4	2
307	7	10	16	10	11	20	18	12
319	52	63	100	51	31	42	31	52
331	2	2	3	2	4	2	1	2
333	4	3	5	4	2	4	3	3
409	0.2	0.3	6	0.4	0.6	0.9	0.8	0.2
421	0.2	0.2	0.5	0.3	0.1	0.8	0.3	0.3
436	0.4	0.7	1	0.4	0.1	0.7	0.6	0.7
RRT*	1.04	1.05	1.05	1.06	1.06	1.06	1.07	1.08

*Relative retention time. Ribitol was used as standard (RRT = 1.0). The retention time of ribitol was 32.2 min on a 30 m × 0.25 mm I.D. OV-101 glass capillary column. The column temperature was programmed from 120°C to 260°C at 3°C/min.

(26%) was higher than that of *m/z* 219 (22%). The TMS derivative of 6-deoxy-altritol was excluded because the relative intensity of *m/z* 231 was as high as 23%. Peak 28 was then identified as 6-deoxygulitol.

Peak 27 and peak 29 were identified as 6-deoxymannitol and 6-deoxy-galactitol, respectively, as reported in the literature [6].

6-Deoxyallitol and 6-deoxygulitol were newly detected in normal urine as well as in uraemic urine. The metabolic origin and the physiological significance of these new deoxyhexitols are obscure at present, and should be studied.

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CHROMBIO. 2333

Note

Elevation of certain polyols in the cerebrospinal fluid of patients with multiple sclerosis

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Multiple sclerosis (MS), a severely disabling disease of the central nervous system (CNS), may be caused by vascular factors, disturbed immune mechanisms, viral infections, or metabolic alterations. While a combination of various factors [1] is believed to be ultimately responsible for the demyelination process in MS, much remains to be learned about the disease etiology. Metabolic alterations of either genetic, dietary, or immune origin could modify certain CNS structures and prepare them for attack by a pathogenic agent (e.g., a virus).

Since lipids are major constituents of the myelin sheath, several studies have been concerned with this class of compounds or related factors [1—7]. Other biochemical studies of this disease are currently rare. This work suggests that an additional biochemical factor, related to the metabolism of carbohydrates, may be implicated. The enhanced concentrations of fructose and sorbitol observed in this study with MS patients suggest the existence of the "sorbitol pathway." This pathway is also believed to be implicated in diabetic neuropathies [8—12].

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EXPERIMENTAL

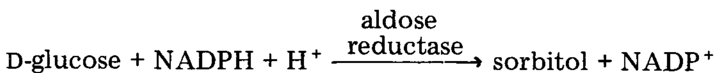
During the development of multicomponent analytical methodology for polyols [13] and other constituents [14] of human cerebrospinal fluid (CSF), a variety of pathological samples were screened together with normal specimens (i.e., from patients with slipped or cracked disks, but no other known pathological problems). CSF samples (obtained by lumbar puncture) from seven patients diagnosed as having definite symptoms of MS were used to record polyol metabolic profiles [13]; all of these samples were also found positive for oligoclonal bands [15, 16] in the electrophoresis of CSF proteins. Samples of 0.5 ml CSF were first subjected to chromatography on a 4 cm × 0.5 cm DEAE Sephadex column to remove interfering compound classes [14]. The fraction eluted with 5.0 ml deionized water was collected and lyophilized to dryness. Dodecanol was added as an internal standard [13], and 100 μl TSIM (trimethylsilylimidazole, from Pierce, Rockford, IL, U.S.A.) was used to derivatize the samples at 70°C for 5 h. Removal of excess reagent on a Lipidex-5000 column, sample preconcentration and chromatography on a glass capillary column were carried out as described in a previous publication [13].

RESULTS AND DISCUSSION

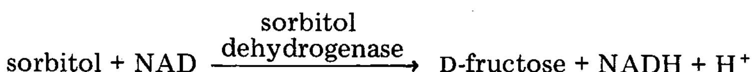
While analyzing the data obtained from the seven MS patients versus six normal samples, increases in fructose and sorbitol (glucitol) were consistently observed in the former sample type. Fig. 1 shows a typical example of such elevations, while no substantial variations are seen in the remaining identified polyols [13].

A summary of the concentration of polyols, including the standard deviations, is given in Table I. Because of the widely varying concentrations of the different polyols in CSF, normal levels have been scaled to 1.0 to permit inclusion of all data points on the same graph (Fig. 2), indicating also the range of normal values. The points plotted for the MS patients represent average values. A significant difference in the means (normal versus MS) for fructose is placed at better than 99% confidence level, while it is somewhat less for sorbitol (more than 95% confidence).

While there is little doubt about a characteristic elevation of fructose and sorbitol in the MS patients as indicated above, a possible biochemical explanation is also readily offered. The following pathway has been postulated [9] to result in accumulation of fructose and sorbitol:



$$\text{D-glucose} + \text{NADPH} + \text{H}^+ \xrightarrow[\text{reductase}]{\text{aldose}} \text{sorbitol} + \text{NADP}^+$$



$$\text{sorbitol} + \text{NAD} \xrightarrow[\text{dehydrogenase}]{\text{sorbitol}} \text{D-fructose} + \text{NADH} + \text{H}^+$$

It is of interest that this polyol pathway is suspected of having implications in neurological complications of diabetes mellitus [8, 11, 12] in humans and the CNS damage in experimental diabetic animals [9, 10]. Suggestions have been made that accumulation of these polyols may result in osmotic

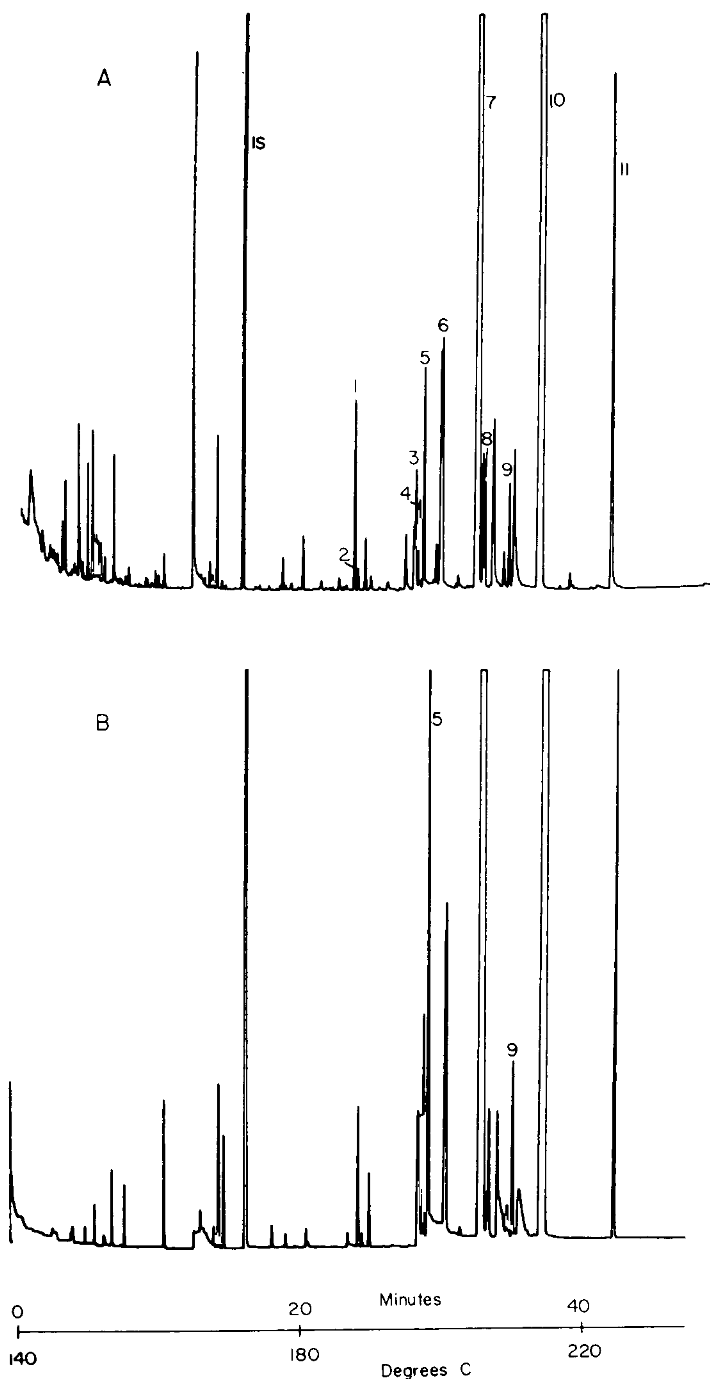


Fig. 1. Chromatograms of the polyols in CSF. (A) Normal sample; (B) sample from patient with multiple sclerosis. Peaks: IS = dodecanol (internal standard); 1 = arabinitol; 2 = ribitol; 3 and 5 fructose; 4 and 8 = mannose; 6 = 1,5-anhydroglucitol; 7 and 10 = glucose; 9 = glucitol (sorbitol); 11 = myo-inositol. Conditions: 50 m × 0.25 mm I.D., glass capillary column, temperature programmed from 140°C to 240°C at 2°C/min; flame-ionization detector.

TABLE I

CONCENTRATION OF POLAR NEUTRAL COMPOUNDS IN THE CEREBROSPINAL FLUID OF NORMAL AND MULTIPLE SCLEROSIS PATIENTS

Concentrations (mg/l ± S.D.) are based on six normal and seven multiple sclerosis samples.

Compound	Normal	Multiple sclerosis
Ribitol	3.4 ± 2.0	2.6 ± 3.8
Fructose	7.6 ± 3.6	31.2 ± 11.5
Mannose	8.5 ± 5.5	12 ± 8
1,5-Anhydroglucitol	37.1 ± 16.5	44 ± 12
Glucose	789 ± 275	838 ± 204
Glucitol (sorbitol)	4.8 ± 3.0	9.4 ± 3.2
Inositol	54 ± 12	49 ± 11

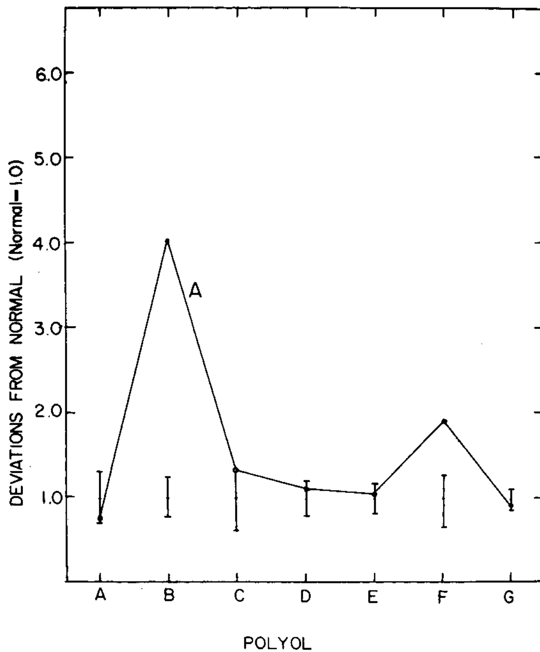


Fig. 2. Normal values for six CSF polar neutral compounds scaled to 1.0 (range of standard deviations indicated). Ordinates: A = ribitol; B = fructose; C = mannose; D = 1,5-anhydroglucitol; E = glucose; F = glucitol (sorbitol); G = myo-inositol. A = plot of average values for polyols in CSF from seven multiple sclerosis patients.

dysequilibrium [12] and subsequent cellular damage, induced abnormalities in lipid synthesis by Schwann cells [17], and the demyelination process itself [18]. If the above were consequences of the polyol accumulation in diabetic neuropathies, the elevated CSF levels of fructose and sorbitol reported in this work would suggest a similar mechanism to be a contributory factor to MS conditions.

While remaining polyols (as seen in Figs. 1 and 2) may have clinical significance in various other CNS diseases [13, 19, 20], we found their levels in MS

within the normal range. This includes inositol in spite of its obvious importance for the cerebral tissue. Only slight elevations of this substance have been found in the CSF of patients with diabetic neuropathies [12].

ACKNOWLEDGEMENT

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CHROMBIO. 2324

Note

Gas chromatographic quantitative determination of 1- and 3-methylhistidine in urine and muscles: comparison with glass capillary determination

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(First received May 15th, 1984; revised manuscript received June 20th, 1984)

Interest in the determination of the methylhistidine isomers, 1-methylhistidine (1-MH) and 3-methylhistidine (3-MH), has been increasing recently. 3-MH is present as a single residue in the peptide chains of actin and myosin [1, 2]. It is thus particularly abundant in muscle. 1-MH has been isolated in a peptide of the neuromuscular junction, whose function is still unknown. Upon breakdown of actin and myosin, 3-MH is not re-utilized, but is rapidly and quantitatively excreted in the urine [3, 4]. It is therefore a useful urinary marker of muscular turnover. The usual analysis techniques for these two MH isomers are based on ion-exchange chromatography with either the amino acid analyser [5] or a colorimetric method using ninhydrin and *o*-phthalaldehyde [6, 7]. High-performance liquid chromatography [8] or gas chromatography [9] can also be used.

Recently, we have developed a method for the isolation of the two MH isomers from biological specimens and their quantitative determination by glass capillary gas chromatography (GC) [10]. This method has been applied for the determination of 1- and 3-MH in various animal and human studies [11—13]. However, the glass capillary GC method requires sophisticated equipment and experienced maintenance staff, so it is not practical for routine clinical analysis.

The aim of the present study was to develop a simpler GC method using a normal GC apparatus and a packed column. The method was applied for the determination of 1- and 3-MH in human urine and rat muscles, and its capabilities were compared with the glass capillary GC method.

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EXPERIMENTAL

Isolation of 1- and 3-MH by charcoal column chromatography

Column adsorption chromatography was performed as previously described [10] using columns (1.5 × 1.0 cm I.D.) packed with charcoal—Celite (1:1, w/w) and buffered at pH 5 with 0.33 *M* acetate buffer. Dried hydrolysates of biological samples were dissolved in 0.33 *M* acetate buffer (pH 5) and an amount corresponding to 0.25 ml of urine and 15 mg of wet muscle was applied to the charcoal—Celite column which was first washed with 20 ml of water, then with 5 ml of 80% acetone; 1- and 3-MH were eluted with 30 ml of dichloromethane—methanol—33% ammonium hydroxide (70:25:5). The eluates were evaporated to dryness under vacuum.

Derivatization of 1- and 3-MH for gas chromatography

The carboxy group of 1- and 3-MH was esterified with 5 ml of a mixture of 5% dry acetyl chloride in *n*-propanol. Each tube was sealed, mixed and left to react overnight at 90°C in a Reacti-Therm heating module (Pierce, Rockford, IL, U.S.A.). Samples were evaporated to dryness under vacuum and then N-acetylated with 150 μl of trifluoroacetic anhydride and 200 μl of dichloromethane for 30 min at room temperature. The two derivatives were evaporated to dryness under vacuum and redissolved in ethyl acetate solution containing as reference standard 2-N-methylamino-5-chlorobenzophenone (MACB, 25 μg/ml) before GC analysis.

GC analytical conditions

A Fractovap 2150 gas chromatograph (Carlo Erba, Milan, Italy) equipped with a flame-ionization detector was used. The glass column (2 m × 4 mm I.D.) was silanized and packed with 1.5% SP-2250 and 1.95% SP-2401 on 100—120 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). The temperatures of the injection port, column and detector were kept at 275°C, 210°C and 275°C, respectively. Nitrogen was used as the carrier gas at a flow-rate of 33 ml/min. The practical sensitivity limit was about 4 μg/ml, compared to 2 μg/ml with the glass capillary GC method.

The use of an electron-capture detector increased the sensitivity about 100-fold but this detector was not routinely used because human urine contains large amounts of 1- and 3-MH. This detector should be very useful for measuring low concentrations of the two isomers as, for example, in muscle proteins.

Mass spectra of the two MH isomers have already been reported [10].

Quantitation

Quantitation was by the internal standardization method with MACB. The calibration curves for 1- and 3-MH derivative concentrations from 12.5 to 100 ng/μl showed a linear response within this range. Urinary creatinine was determined on urine samples using a modification of the alkaline picrate technique [14]. Proteins were determined by the method of Lowry et al. [15].

RESULTS AND DISCUSSION

A comparison of the resolution capacity of the packed column and the glass capillary column in the separation of a sample of human urine is shown in Fig. 1. The 1- and 3-MH derivatives were satisfactorily separated, with short retention times and symmetrical peaks in the packed GC analytical conditions described. No interfering peaks were detected in biological samples.

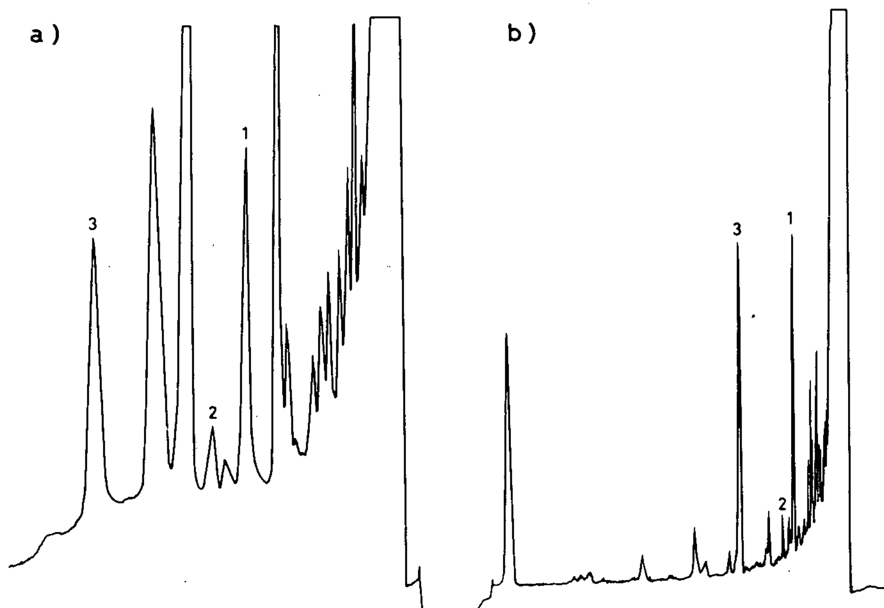

Fig. 1. Comparison of urine assays of methylhistidine isomers by packed (a) and capillary (b) chromatographic systems. In both analyses the amount injected corresponded to 1 µl of human urine. Peaks: 1 = 3-MH, 2 = 1-MH, 3 = internal standard.
Packed column GC conditions: a Fractovap 2150 gas chromatograph (Carlo Erba) equipped with a flame-ionization detector. The glass column (2 m × 4 mm I.D.) was packed with 1.5% SP-2250 and 1.95% SP-2401 on 100—120 mesh Supelcoport. Temperatures: oven 210°C, detector and injector 275°C. Carrier gas: nitrogen, flow-rate 33 ml/min.
Glass capillary GC conditions: gas chromatograph 3900-B (DANI, Monza, Italy) equipped with a flame-ionization detector. Capillary column (20 m × 0.85 mm O.D., 0.30 mm I.D.; Duran 50 with a 0.15 µm thick Pluronic F-68 coat). The split injection mode was used (flow 15 ml/min). Temperatures: oven 200°C, detector 280°C, injector 300°C. Carrier gas: hydrogen, flow-rate 0.7 ml/min.

TABLE I

PRECISION OF 1-MH AND 3-MH ASSAY

	Concentration (µg/ml)	n	C.V. (%)	
			1-MH	3-MH
Within-day	5.0	10	0.6	0.5
	50.0	10	0.7	0.5
Between-day	5.0	10	0.7	0.6
	50.0	10	0.8	0.7

TABLE II

COMPARISON OF 1- AND 3-MH LEVELS IN HUMAN URINE AND RAT MUSCLE ASSAYED BY GLASS CAPILLARY GC AND BY PACKED COLUMN GC

Sample	Creatinine excretion (mmol per 24 h)*	Methylhistidine level (μmol per 24 h)			
		Glass capillary GC		Packed column GC	
		1-MH	3-MH	1-MH	3-MH
Human urine*					
P.A.	7.31	58.30	184.80	50.00	185.20
M.R.	5.34	41.90	151.10	42.10	152.00
P.M.	3.50	61.08	112.29	61.94	112.83
S.A.	5.82	80.40	189.17	80.65	190.00
Mean ± S.E.	5.49 ± 0.95	60.33 ± 9.62	159.34 ± 19.72	60.92 ± 9.63	160.00 ± 19.29
	Protein in wet muscle (mg/g)				
Rat muscles**					
Palmaris longus	74.00 ± 2	1429 ± 7	888 ± 8	1430 ± 8	889 ± 7
Pectoralis	87.04 ± 2	2760 ± 5	664 ± 7	2759 ± 7	663 ± 5
Gastrocnemius	99.12 ± 3	1733 ± 8	774 ± 8	1730 ± 8	775 ± 4
Heart	105.12 ± 4	205 ± 4	196 ± 6	206 ± 3	197 ± 6

*Healthy youths (18—20 years), weight 60—70 kg, fed a diet containing no meat.
**Male CD-COBS rats (Charles River, Calco, Italy), body weight 250 g, eating a diet containing no meat. Muscles from two animals were pooled for each determination. Values are expressed as mean ± S.E.M. ($n = 4$).

The efficiency of the method (recovery) was tested for 1- and 3-MH derivatives from four spiked human urines and the same urines unspiked. Average recovery was 97 ± 2%. No significant differences were found between recoveries for different concentrations of either of the MH isomers (10, 25, 50, 75 ng). The reproducibility of the present method, summarized in Table I, was fairly good compared with that of GC using the glass capillary column [10]. Before developing the glass capillary GC method for 1- and 3-MH derivatives, we tried unsuccessfully to work with packed columns (SE-30, OV-1, OV-17, OV-101, 2% OV-17 + 1% OV-210), but there was no resolution between 1- and 3-MH peaks. The mixed stationary phase 1.5% SP-2250 and 1.95% SP-2401 on 100—120 mesh Supelcoport, however, shows excellent resolution properties as regards the two MH isomers.

Table II reports assays of 1- and 3-MH in human urine and rat muscles and compares the results with those obtained by the glass capillary GC method. The results agree well, confirming the reliability of the proposed method, associated with its greater simplicity, and suggest possible biomedical application in studies on muscle-wasting diseases. The stationary phase used has a long life and is therefore economical for routine use.

In conclusion, the newly developed GC method is satisfactory with respect to sensitivity, accuracy, precision and economy. The assay procedure is simple and convenient, and therefore clinically applicable to the routine analysis of 1- and 3-MH in biological samples.

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CHROMBIO. 2314

Note

Reversed-phase high-performance liquid chromatography of C_{21} metabolites of progesterone

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Preliminary results of studies of the metabolism of progesterone in a perfused canine isolated gravid uterine preparation established that the preparation produced a number of metabolites that were poorly separated by the use of gas—liquid chromatography (GLC). The methods described here are the results of efforts to establish means for separating and identifying many of the C_{21} compounds that may be metabolites of progesterone. Lin et al. [1] have reported separation of the reduction products of progesterone by employing a combinaton of adsorption and reversed-phase high-performance liquid chromatography (HPLC). The methods described here are simple isocratic reversed-phase HPLC systems that separate C_{21} ketonic metabolites of progesterone, the stereoisomers of pregnanediol, and eight isomers of pregnane-3,6,20-triol. Separations have been achieved using a single commercially available column and three mobile phases.

EXPERIMENTAL

Materials

The solvents were HPLC-grade methanol and acetonitrile (Fisher Scientific, Fair Lawn, NJ, U.S.A.). Distilled water was passed through a Milli-Q water purification system (Millipore, Bedford, MA, U.S.A.). Solutions were filtered through 0.5-μm Millipore FHUP-047-00 filters and then sparged with helium for 30 min just before chromatography. Isomers of pregnane-3,6,20-triol were synthesized in our laboratory according to the method published by Allen

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and Knights [2]. The other steroids were obtained from commercial sources. Their purity was evaluated by paper chromatography, thin-layer chromatography, determination of melting point, and GLC [3], as well as by HPLC. Analysis by the latter two methods indicated trace impurities in some of the steroids.

Chromatography

Reversed-phase HPLC was performed using a DuPont system (DuPont, Wilmington, DE, U.S.A.). The system consisted of an 870 pump module equipped with a universal septumless injector with a 50-μl sample loop, an ultraviolet (UV) spectrophotometer detector with a flow cell with a pathlength of 10 mm and a capacity of 8 μl, and a refractive index (RI) detector. A dual-channel Liniar Model 285 recorder (Liniar Instruments, Irvine, CA, U.S.A.) was connected to the two detectors.

A 250 × 4.6 mm I.D. commercially packed column of Zorbax ODS (5—6 μm particle size) (DuPont) was employed for all separations. The mobile phases, flow-rates, and column pressures were as follows: acetonitrile—water (60:40, v/v), 1 ml/min, 44 bars for ketones, 48 bars for diols; methanol—water (70:30, v/v), 2 ml/min, 220 bars for ketones, 237 bars for diols; methanol—water (11:9, v/v), 2 ml/min, 252 bars for triols, 290 bars for 17-hydroxypregn-4-ene-3,20-dione. Chromatography was carried out at room temperature.

RESULTS AND DISCUSSION

The steroids chromatographed and their retention times (t_R) using methanol—water and acetonitrile—water as mobile phases are listed in Table I. The steroids within each one of the three groups of ketonic metabolites, diols, and triols are listed in order of increasing t_R in the system methanol—water (70:30) and have each been given an identification number.

An example of the separation of a mixture of some ketonic C_{21} steroids using the mobile phase methanol—water (70:30) is illustrated in Fig. 1 and the separation of the same mixture using acetonitrile—water (60:40) is shown in Fig. 2. From Fig. 1 it can be seen that 5α-pregnane-3,20-dione (9) and 3α-hydroxy-5β-pregnan-20-one (10) did not separate using methanol—water as the mobile phase. However, these steroids were well separated using acetonitrile—water (Fig. 2). In the chromatography employing acetonitrile—water as the mobile phase (Fig. 2), 3β-hydroxy-5α-pregnan-20-one (11) and 5β-pregnane-3,20-dione (6) did not separate and the separation of 20β-hydroxy-pregn-4-en-3-one (4) and progesterone (3) was not optimal. These two pairs of steroids were separated using the methanol—water system (Fig. 1). Mixtures of the four 3-hydroxypregnan-20-one epimers were separated using either mobile phase. Acetonitrile—water is the mobile phase of choice for the separation of the four 20-hydroxypregnan-3-ones, since 20β-hydroxy-5α-pregnan-3-one (13) and 20β-hydroxy-5β-pregnan-3-one (14) were not well separated using methanol—water. Other pairs of steroids not optimally resolved or unresolved using one mobile phase can be well separated in the alternative system, e.g., methanol—water is the better mobile phase system for the pairs progesterone (3) and 20α-hydroxy-5β-pregnan-3-one (7); 20α-hydroxy-5α-pregnan-3-one

TABLE I

RETENTION TIMES OF SOME POSSIBLE C_{21} METABOLITES OF PROGESTERONE

The experimental conditions employed are described in the section *Chromatography* in the text and in the legends of the figures. The retention times are those obtained by refractive index detection.

No.	Steroid	Retention time (min)		
		Acetonitrile—water (60:40, v/v)	Methanol—water	
			70:30, v/v	11:9, v/v
Ketones				
1	17-Hydroxypregn-4-ene-3,20-dione	9.6	7.2	30.2
2	20α-Hydroxypregn-4-en-3-one	16.0	14.2	
3	Pregn-4-ene-3,20-dione (progesterone)	22.1	15.4	
4	20β-Hydroxypregn-4-en-3-one	21.3	20.2	
5	20α-Hydroxy-5α-pregnan-3-one	25.2	21.8	
6	5β-Pregnane-3,20-dione	30.2	22.8	
7	20α-Hydroxy-5β-pregnan-3-one	22.9	24.3	
8	3β-Hydroxy-5β-pregnan-20-one	27.4	24.8	
9	5α-Pregnane-3,20-dione	35.8	25.8	
10	3α-Hydroxy-5β-pregnan-20-one	25.0	26.3	
11	3β-Hydroxy-5α-pregnan-20-one	29.9	27.7	
12	3α-Hydroxy-5α-pregnan-20-one	31.8	31.3	
13	20β-Hydroxy-5α-pregnan-3-one	33.8	32.8	
14	20β-Hydroxy-5β-pregnan-3-one	31.7	33.5	
Diols				
15	5β-Pregnane-3β,20α-diol	18.5	21.4	
16	5α-Pregnane-3β,20α-diol	20.8	23.1	
17	5β-Pregnane-3β,20β-diol	24.9	31.7	
18	5α-Pregnane-3β,20β-diol	25.9	32.8	
19	5β-Pregnane-3α,20α-diol	19.5	33.4	
20	5α-Pregnane-3α,20α-diol	23.4	35.9	
21	5β-Pregnane-3α,20β-diol	25.5	42.2	
22	5α-Pregnane-3α,20β-diol	29.9	45.6	
Triols				
23	5β-Pregnane-3α,6β,20β-triol		3.0	9.2
24	5β-Pregnane-3α,6β,20α-triol		4.0	15.4
25	5β-Pregnane-3α,6α,20β-triol		4.3	18.0
26	5α-Pregnane-3β,6β,20β-triol		5.0	21.4
27	5α-Pregnane-3β,6β,20α-triol		5.6	25.7
28	5α-Pregnane-3α,6α,20β-triol		5.7	28.1
29	5β-Pregnane-3α,6α,20α-triol		5.8	30.5
30	5α-Pregnane-3α,6α,20α-triol		7.9	44.7

(5) and 3α-hydroxy-5β-pregnan-20-one (10), and 3α-hydroxy-5α-pregnan-20-one (12) and 20β-hydroxy-5β-pregnan-3-one (14).

The results of chromatography of a mixture of the eight pregnanediol stereoisomers using the mobile phase methanol—water (70:30) are shown in Fig. 3A. The chromatogram of the same mixture obtained using acetonitrile—water (60:40) as the mobile phase is shown in Fig. 3B. Again, the problems

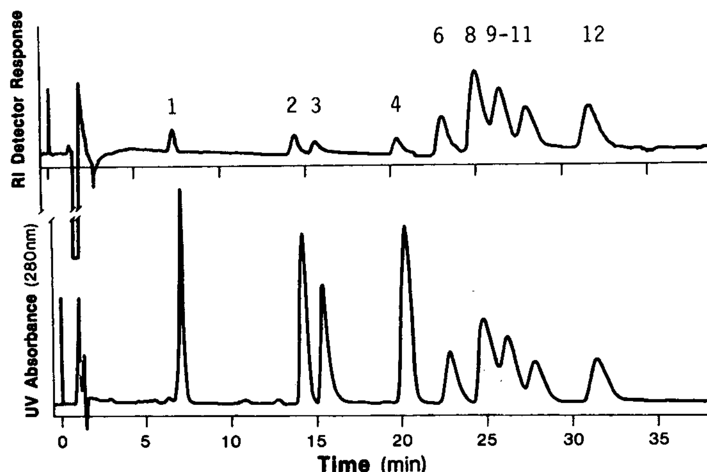

Fig. 1. Chromatogram of ketonic C_{21} steroids using the mobile phase methanol—water (70:30, v/v). A mixture of 10 μg each of 17-hydroxypregn-4-ene-3,20-dione, 20-hydroxy-pregn-4-en-3-ones, and progesterone, 40 μg each of pregnane-3,20-diones, and 75 μg each of pregnanolones dissolved in methanol was chromatographed on a Zorbax ODS column (250 mm × 4.6 mm I.D., 5—6 μm particle size). The flow-rate was 2 ml/min and the pressure was 220 bars. The effluent passed through two detectors in series, the first a UV spectrophotometer set at 280 nm, range 0.04, and the second a refractive index (RI) detector with the attenuator set at $0.05 \cdot 10^{-3}$ RI units. The dual-channel recorder was set at a span of 1 mV. The chart speed was 1 cm/min. For peak identification, see Table I.

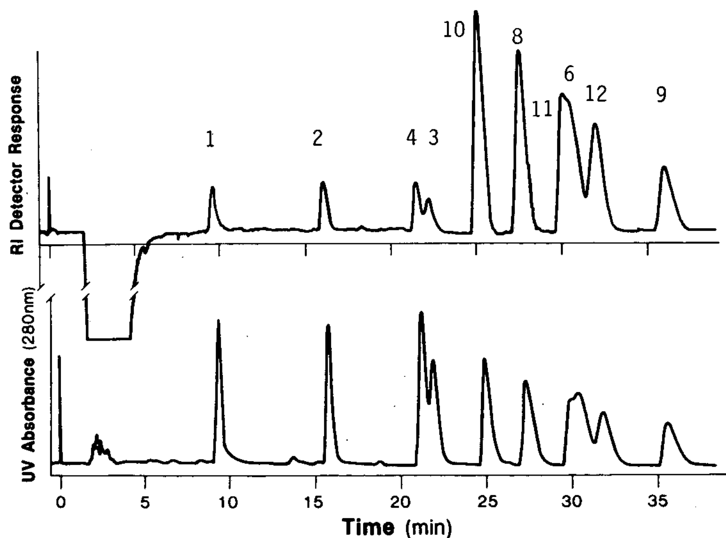

Fig. 2. Chromatogram of ketonic C_{21} steroids using the mobile phase acetonitrile—water (60:40, v/v). The flow-rate was 1 ml/min and the pressure was 44 bars. The UV spectrophotometer range was set at 0.08. The other parameters are as described in Fig. 1. For peak identification, see Table I.

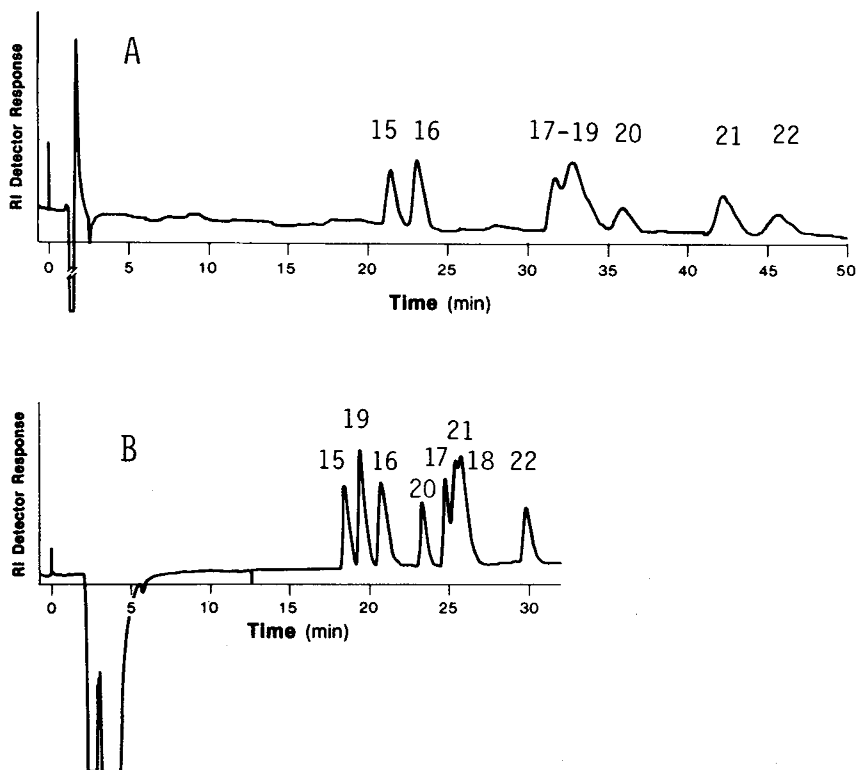

Fig. 3. Chromatograms of pregnanediols. A mixture of 70 μg of 5α-pregnane-3α,20β-diol and 85 μg each of the other pregnane-3,20-diols dissolved in methanol was chromatographed using (A) the mobile phase methanol—water (70:30, v/v), flow-rate 2 ml/min, pressure 237 bars, and refractive index detector attenuator at $0.05 \cdot 10^{-3}$ RI units, or (B) the mobile phase acetonitrile—water (60:40, v/v), flow-rate 1 ml/min, pressure 48 bars, and refractive index detector attenuator at $0.1 \cdot 10^{-3}$ RI units. The parameters for the column and recorder are as described in Fig. 1. For peak identification, see Table I.

encountered with one mobile phase system are solved by substituting the other system. Using the mobile phase methanol—water (Fig. 3A), 5α-pregnane-3β,20β-diol (18) and 5β-pregnane-3α,20α-diol (19) were not separated. 5α-Pregnane-3β,20β-diol (18) was only partially separated from 5β-pregnane-3β,20β-diol (17). However, a mixture of 5β-pregnane-3β,20β-diol (17) and 5β-pregnane-3α,20α-diol (19) will be resolved. The group of three steroids was separated from one another by employing acetonitrile—water as the mobile phase (Fig. 3B). Upon chromatography of the pregnanediols using this latter mobile phase, there was little separation of 5β-pregnane-3α,20β-diol (21) and 5α-pregnane-3β,20β-diol (18), and incomplete separation of 5β-pregnane-3α,20β-diol (21) and 5β-pregnane-3β,20β-diol (17). The two pairs of steroids have sufficiently different t_R values to allow separation using methanol—water as the mobile phase.

The components of a mixture of eight pregnane-3,6,20-triols were well separated using the Zorbax-ODS column and the mobile phase methanol—water

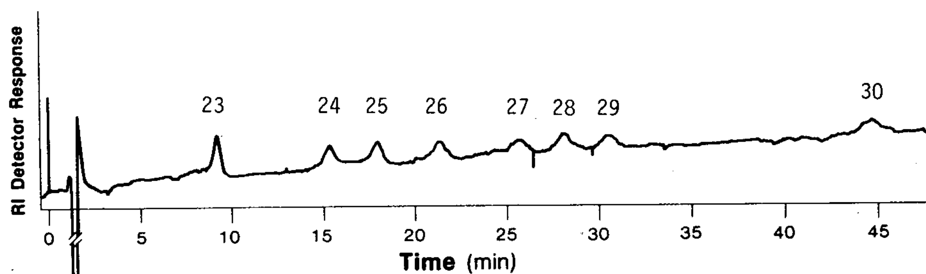

Fig. 4. Chromatogram of pregnane-3,6,20-triols. A mixture of between 37 and 45 μg of the pregnanetriols dissolved in methanol was chromatographed using the mobile phase methanol—water (11:9, v/v), flow-rate 2 ml/min, and pressure 252 bars. The parameters for the column, refractive index detector, and recorder are as described in Fig. 1. For peak identification, see Table I.

(11:9) (Fig. 4). This system was selected because of published results employing it for the HPLC of $C_{19}O_3$ steroids [4]. The attenuator setting on the refractive index detector was chosen as that optimal for securing both sufficient sensitivity with the limited amounts of these steroids available as reference standards and a satisfactory baseline. As can be seen from Table I, the employment of the mobile phase methanol—water (70:30) resulted in the separation of only certain of the pregnanetriols from one another. However, it would offer a further method of characterization by means of t_R for some of the previously separated pregnane-3,6,20-triols.

Lin et al. [1] have achieved separation of ketonic steroids and the pregnanediols using a combination of adsorption and reversed-phase partition HPLC. The adsorption systems consisted of two chromatographic tubes, each 300 × 2 mm I.D., packed in their laboratory with Partisil 5 (5 μm particle size, Whatman, Clifton, NJ, U.S.A.) connnected in series, and the mobile phases 0.25% ethanol in dichloromethane, hexane—isopropanol (97:3), and *n*-hexane—methanol—ethanol (96:3:1). The reversed-phase system consisted of two tubes, each 250 × 4 mm I.D., packed in their laboratory with Zorbax BP-ODS (7—8 μm particle size) and a mobile phase of 60% aqueous acetonitrile. The order of elution of the steroids obtained by us with the single-tube Zorbax-ODS column and acetonitrile—water (60:40) is similar to that obtained by Lin et al. [1] with their reversed-phase system with the exception that progesterone (3) preceeds 20α-hydroxy-5β-pregnan-3-one (7) in our system. Therefore, our results conform to their generalizations concerning the stereochemistry of isomers. For some ketonic steroids the use of the longer reversed-phase column appears to offer an advantage, since the greater differences in t_R between some of the pairs of steroids result in separations not achieved on the single-tube column with the mobile phase acetonitrile—water (60:40).

There are obvious benefits to be derived from prior fractionation of organic residues by Girard's *T* and digitonin partitions and various methods of chromatography [3]. However, the reversed-phase HPLC systems using a single column and easily exchangeable mobile phases employed in this investigation can be used in tandem with them or alone, depending on the complexity of the mixture and the purpose of the analysis, to achieve resolution of C_{21} metabolites of progesterone. The biological studies using the perfused canine

isolated gravid uterine preparation for which these methods were developed employed steroids labeled with ^{3}H and ^{14}C. Fractions of the elutriates from the HPLC column were collected and then assayed for radioactivity in a scintillation counter. Labeled metabolites of the progesterone injected were successfully separated and identified by these methods [5].

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CHROMBIO. 2310

Note

Ion-exchange chromatography of fluorogenic derivatives of maltooligosaccharides for preparation of α-amylase substrates

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Because human pancreatic α-amylase in serum increases with pancreatic lesions, and salivary α-amylase increases during mumps, the assay of α-amylase activity is very effective in clinical diagnoses. We have proposed a new α-amylase assay in which fluorogenic derivatives of maltooligosaccharides were used as the substrates, the products being separated from the digests and quantitated by high-performance liquid chromatography (HPLC) [1, 2]. The assay is unique in that it is not subjected to interference from endogenous glucose, maltose, or α-glucosidase in the sample. The fluorogenic substrates, O-6-deoxy-6-[(2-pyridyl)amino]-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-D-glucose (FG5) and O-6-deoxy-6-[(2-pyridyl)amino]-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-D-glucitol (FG6R), can be synthesized via many steps from maltopentaose and maltohexaose, but this requires skilful technique and a long time. We have prepared them from dextrin using a relatively simple method including (1) partial introduction of pyridylamino groups into the glucose residues of dextrin, (2) digestion of the modified dextrin to fluorogenic maltooligosaccharides and glucose by α-amylase and glucoamylase, and (3) fractionation of the digest to each oligosaccharide by gel filtration and HPLC. Gel chromatography on Bio-Gel P-4 was not so effective for fractionating the fluorogenic maltooligosaccharides [1]. We tried to separate them by ion-exchange chromatography, because the pyridylamino group has a positive charge at acidic pH and because the hydrophilic properties of the oligomers must vary with the degree of polymerization. This

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paper reports the effective fractionation of pyridylamino derivatives of malto-oligosaccharides and their sugar alcohols by ion-exchange chromatography.

MATERIALS AND METHODS

Materials

Liquefying α-amylase from *Bacillus subtilis* (EC 3.2.1.1) and glucoamylase from *Rhizopus niveus* (EC 3.2.1.3) were purchased from Seikagaku Kogyo (Tokyo, Japan). Amylose EX-1 with an average of seventeen glucose residues was from Hayashibara Biochemical Labs. (Okayama, Japan). Bio-Gel P-4 was purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). Dowex 50W-X2 was from Dow Chem. (Midland, MI, U.S.A.). All chemicals were of the highest grade available.

Preparation of F-amylose

The partial introduction of pyridylamino groups into some of the C-6 positions of the glucose residues of amylose was carried out in a manner basically similar to that for dextrin [1]. A mixture of 0.4 ml of dichloroacetic acid and 4 ml of dimethylsulphoxide was added to a solution of 2 g of amylose and 3 g of N,N′-dicyclohexylcarbodiimide in 38 ml of dimethylsulphoxide. The mixture was stirred at room temperature for 50 min, and then 1.2 g of oxalic acid in 5 ml of methanol were added. To this reaction mixture, a mixture of 8.5 g of 2-aminopyridine, 3 ml of acetic acid, 3.2 g of sodium cyanoborohydride, and 12 ml of water was added, and the mixture was heated at 90°C for 30 min. Water (300 ml) was added to the reaction mixture, and the precipitate was removed by filtration. The pH of the filtrate was adjusted to 1.0 with 1 *M* hydrochloric acid to decompose excess sodium cyanoborohydride. After the pH was adjusted to 7.0 with 1 *M* sodium hydroxide, the solution was concentrated in vacuo. The residue was dissolved in water and applied to a Bio-Gel P-4 column (90 × 4.5 cm) equilibrated with 0.01 *M* ammonium bicarbonate. Elution was monitored by measuring the absorbance at 320 nm due to pyridylamino groups. The fist peak (F-amylose) was collected and lyophilized. The yield of F-amylose was 1.6 g, and 7.4% of the glucose residues were modified, judging from the absorbance due to pyridylamino groups.

Digestion of F-amylose by glucoamylase and α-amylase

F-Amylose (1.5 g) was dissolved in 160 ml of water and the pH was adjusted to 4.8 with 1 *M* hydrochloric acid. The solution was incubated with *Rhizopus niveus* glucoamylase (10 mg) at 40°C for 5 h. After the pH of the digest was adjusted to 6.0 with 1 *M* sodium hydroxide, 1.6 ml of 0.013% *Bacillus subtilis* liquefying α-amylase in 0.1 *M* calcium acetate buffer of pH 6.0 was added to the digest, and the mixture was incubated at 40°C for 1 h. The digestion was stopped by heating the reaction mixture at 100°C for 10 min. The pH of the digest was adjusted to 4.8 with 1 *M* hydrochloric acid, and then the digest was further incubated with 5 mg of glucoamylase at 40°C for 5 h.

Sodium borohydride reduction

To one-third of the above digest, 200 mg of sodium borohydride were added, and the mixture was kept at room temperature for 3 h. By adding 1 *M* hydrochloric acid to the solution, the remaining sodium borohydride was decomposed, and the pH of the solution was adjusted to 4.0 with 1 *M* sodium hydroxide.

Ion-exchange chromatography on Dowex 50W-X2

The sample was applied on a column of Dowex 50W-X2 (124 × 1.5 cm) equilibrated with 0.1 *M* pyridine—acetic acid buffer of pH 5.6. After washing with 350 ml of the same buffer, the column was developed by a linear gradient generated by mixing 1 l of this buffer with 1 l of 0.35 *M* pyridine—acetic acid buffer, also of pH 5.6. The elution was monitored by measuring absorbance at 310 nm due to pyridylamino group.

High-performance liquid chromatography

The HPLC apparatus used was a Waters Assoc. Model M-45. A column (150 × 4.6 mm) packed with Cosmosil $5C_{18}$ (Nakarai Chemicals, Kyoto, Japan) was used. The elution was carried out with 0.1 *M* ammonium acetate buffer of pH 4.0 containing 0.05% 1-butanol at a flow-rate of 1.8 ml/min. The detector was a Hitachi fluorescence spectrophotometer, Model 650-10M. The wavelength of excitation was 320 nm and that of emission was 400 nm. O-6-Deoxy-6-[(2-pyridyl)amino]-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-D-glucose (FG4), FG5, and O-6-deoxy-6-[(2-pyridyl)amino]-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-O-α-D-glucopyranosyl-(1→4)-D-glucose (FG6) and their sugar alcohols (FG4R, FG5R, and FG6R), of which the structures had been confirmed [1, 2], were used as the standard compounds for HPLC analysis in this study.

RESULTS AND DISCUSSION

Pyridylamino derivatives of maltooligosaccharides were prepared from amylose as summarized in Fig. 1. Glucoamylase was used to liberate non-reducing-end glucose from F-amylose one by one, and to produce the limit-dextrins of which the non-reducing-end glucose residues are modified. The digest of F-amylose or its reduction product with sodium borohydride was applied on the Dowex 50W-X2 column and the elution was carried out as described under Materials and methods. The elution patterns are shown in Figs. 2 and 3. The peaks in the figures were identified by comparing their elution positions on HPLC with the standards as illustrated in Fig. 4A—D. Peaks X and Y seemed to be heptamers from their elution positions, but they are not, because they were eluted faster than the corresponding hexamers on HPLC (Fig. 4E and F). They may be products formed by a side-reaction on the introduction of pyridylamino group into amylose. It was estimated that a higher oligomer would be eluted faster than a lower one on ion-exchange chromatography from analogy with the high-mannose-type glycopeptides obtained from Taka-amylase A or ovalbumin [3, 4]. The fractionations of the fluorogenic

```
--------G-G-G-G-G-G-G-G-G--------- (Amylose)
                  ↓ DCC-DMSO
                  ↓ 2-Aminopyridine,NaCNBH3
                  F
--------G-G-G-G-G-G-G-G-G--------- (F-amylose)
                  ↓ Glucoamylase
                  F
        G<  +  G-G-G-G-G---------
                  ↓ Liquefying α-amylase
                  ↓ Glucoamylase
                  F
        G<  +  G-G-G-G< (FG4)          F
                  F                    G- :
               G-G-G-G-G< (FG5)
                  F
               G-G-G-G-G-G< (FG6)
```

Fig. 1. Preparation of pyridylamino derivatives of maltooligosaccharides. G, Glucose residue; F/G, 6-deoxy-6-[(2-pyridyl)amino]-D-glucose residue; G< , reducing-end glucose residue; —, α-1,4-glucosidic bond.

Fig. 2. Ion-exchange chromatography of the digest of F-amylose on Dowex 50W-X2. A digest of F-amylose (500 mg) was diluted with water to 100 ml, and then applied on the column. The elution was carried out as described in Materials and methods.

derivatives of the maltooligomers were satisfactorily performed, taking advantage of their ionic features. By this method, it will be easy to scale up the production of the substrates for α-amylase assay.

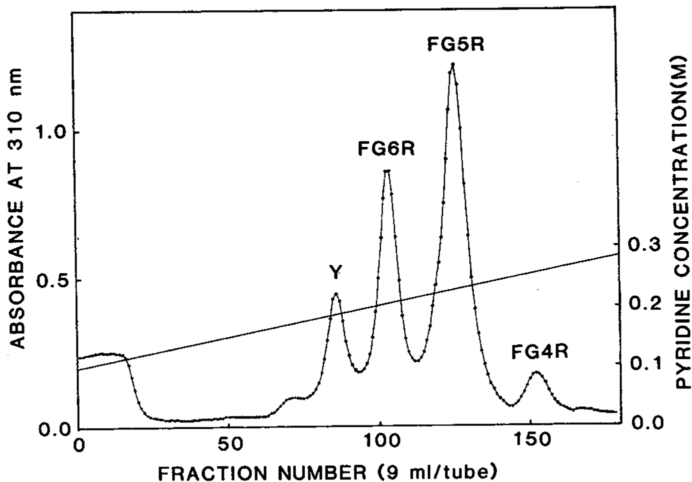

Fig. 3. Ion-exchange chromatography of the reduction product of the digest of F-amylose on Dowex 50W-X2. The reduction product of the digest of F-amylose (500 mg) was diluted with water to 250 ml, and then applied on the column. The elution was carried out as described in Materials and methods.

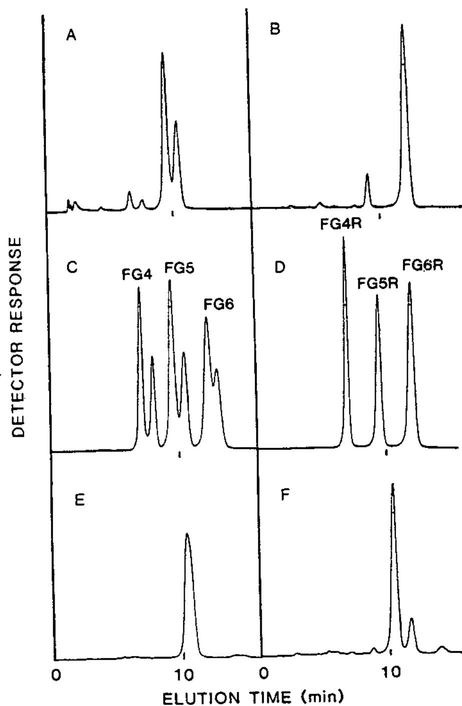

Fig. 4. HPLC analyses of the peaks isolated by ion-exchange chromatography. Analyses were carried out as described in Materials and methods. (A) FG5 in Fig. 2; (B) FG6R in Fig. 3; (C) standard mixtures (FG4, FG5, and FG6); (D) standard mixtures (FG4R, FG5R, and FG6R); (E) peak X in Fig. 2; (F) peak Y in Fig. 3. FG4, FG5, and FG6 were detected as the two peaks of their anomeric forms (α, β) on HPLC analysis as reported previously [1].

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CHROMBIO. 2321

Note

Direct clean-up and analysis of urinary catecholamines

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Catecholamines play an important role as neurotransmitters in the central nervous system, in the regulation of blood pressure or for the detection of catecholamine-secreting tumours, e.g. phaeochromocytoma [1, 2].

The development of high-performance liquid chromatographic (HPLC) techniques during the last decade has shown that this method is particularly useful for the determination of catecholamines in physiological fluids. With respect to the chemical properties of the catecholamines and their metabolites different modes of HPLC such as reversed-phase, reversed-phase ion-pair, ion-exchange including either electrochemical detection or fluorescence detection after pre- or postcolumn derivatization have been exploited [3, 4].

For the quantitation of catecholamines in physiological fluids preliminary sample clean-up steps still present a crucial factor for routine clinical analysis. Liquid—solid extraction methods, e.g. the use of cation exchangers, acid-washed aluminium oxide or the use of boric acid affinity gels either in the column under low-pressure conditions or in the batch mode are in common use [3, 4].

Recently Neidhart et al. [5] introduced a column-switching technique consisting of an aluminium oxide precolumn and a cation-exchange analytical column which allows the direct analysis of epinephrine and norepinephrine in urine with fluorimetric detection after postcolumn derivatization. Hansson et al. [6] developed a column-switching technique with a boronic acid—silica supported precolumn and a reversed-phase C_{18} analytical column allowing the electrochemical detection of 3,4-dihydroxyphenylacetic acid.

By use of a column-switching technique we introduced a combined boric acid high-performance liquid affinity chromatography (HPLAC)—RP-C_{18} HPLC method for the on-line clean-up and analysis of ribonucleosides in

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physiological fluids [7, 8]. First results in catecholamine research with on-line ion-pair reversed-phase chromatography of spiked urines by ultraviolet detection showed that the column-switching technique is applicable for analysis of these biogenic amines [9]. Because, under these conditions no reproducible retention times were achieved, we have now developed a method for the on-line clean-up and analysis of the principal urinary catecholamines epinephrine (E), norepinephrine (NE) and dopamine (DA) including a boric acid HPLAC—cation-exchange HPLC column-switching technique with electrochemical detection. This provides a powerful improvement compared to the system introduced by Hansson et al. [6] for the analysis of just one of the dopamine catabolites.

EXPERIMENTAL

Chemicals

Norepinephrine (NE), epinephrine (E) and dopamine (DA) were purchased from Aldrich (Steinheim, F.R.G.). In all buffer preparations double-distilled water and salts from E. Merck (Darmstadt, F.R.G.) of the purest grade available were used.

Urine treatment

Fresh human urine (500 μl) was membrane-filtered (0.2 μm; Schleicher & Schüll, Dassel, F.R.G.) and an aliquot of 100 μl was applied to the high-performance liquid affinity chromatography column (HPLAC column 1). To check for possible interfering compounds a blank urine was prepared from another 500-μl aliquot by adjusting the pH to 10 with concentrated ammonia to destroy endogenous free catecholamines. After standing for 5 h at 25°C an aliquot of this pretreated urine was analysed.

Chromatography

HPLAC column 1 was filled with laboratory-prepared phenylboronic acid-substituted silica [10] in a stainless-steel column (50 × 4 mm I.D.). Column 2 was filled with a cation-exchange material (Nucleosil 10 SA, 10 μm; 250 × 4 mm I.D.; Macherey & Nagel, Düren, F.R.G.).

Column 2 was isocratically eluted with 0.45 mol/l formic acid adjusted to pH 3.0; flow was 2.0 ml/min. The elution of column 1 is described in the results section. Under these chromatographic conditions the following retention times (min) were found: NE (6.71), E (10.16) and DA (12.59). Electrochemical detection was carried out with an electric potential set at +0.75 V.

HPLC apparatus

The HPLC equipment consisted of two Altex Model 110 A pumps (Altex, U.S.A.) controlled by a Model 420 microprocessor and a Rheodyne Model 7125 loop injector for sample introduction. Detection was performed with an electrochemical detector (ELCD 656 and 641 VA detector; Metrohm, Switzerland) with a glassy carbon electrode and Ag/AgCl reference electrode. Areas under the peaks were integrated with a Hewlett-Packard Model 3390 integrator (Hewlett-Packard, Frankfurt, F.R.G.).

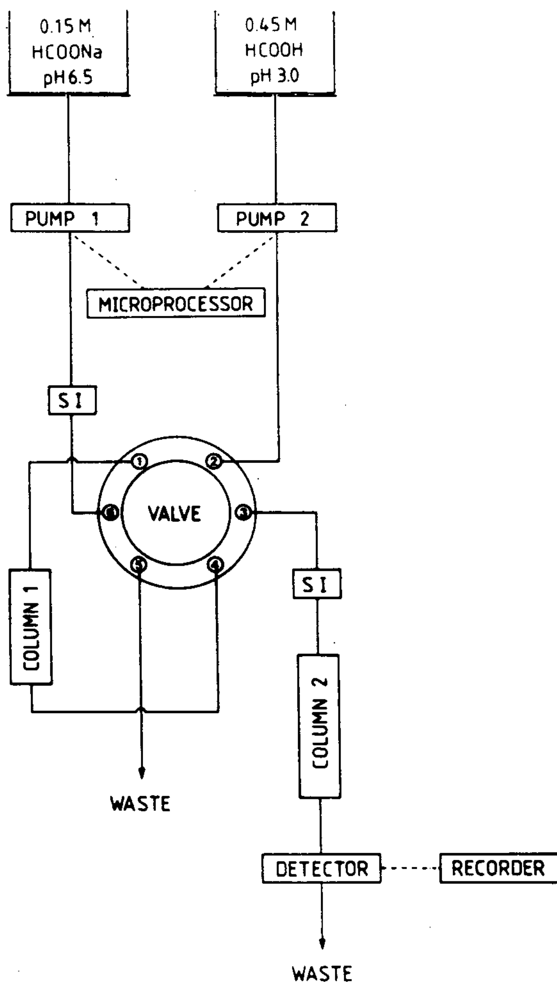

Fig. 1. On-line system set-up.

For the direct on-line analysis the gradient system was additionally equipped with a second Rheodyne 7125 loop injector (SI) and a Rheodyne Model 7010 six-port valve which were incorporated as shown schematically in Fig. 1.

RESULTS AND DISCUSSION

For the direct clean-up and analysis of catecholamines in urine the HPLAC column 1 was equilibrated for 2 min in valve position "Load" (Fig. 2) with 0.15 mol/l sodium formate pH 6.5. After sample injection (synthetic mixture or urine) column 1 was washed with the same buffer for 3.30 min at 1.0 ml/min. During that time catecholamines were selectively retarded on the HPLAC column whereas the sample matrix was discharged. After this clean-up step the valve was switched to "Inject" and thereby connected in series in front of column 2 (Fig. 2). The group-specifically bound catecholamines on column 1 were then eluted under acidic conditions (0.45 mol/l formic acid

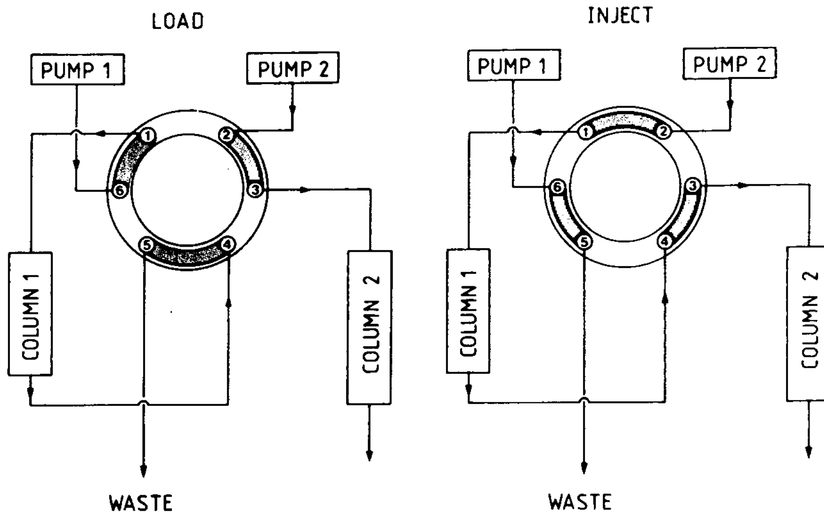

Fig. 2. Switching-valve positions.

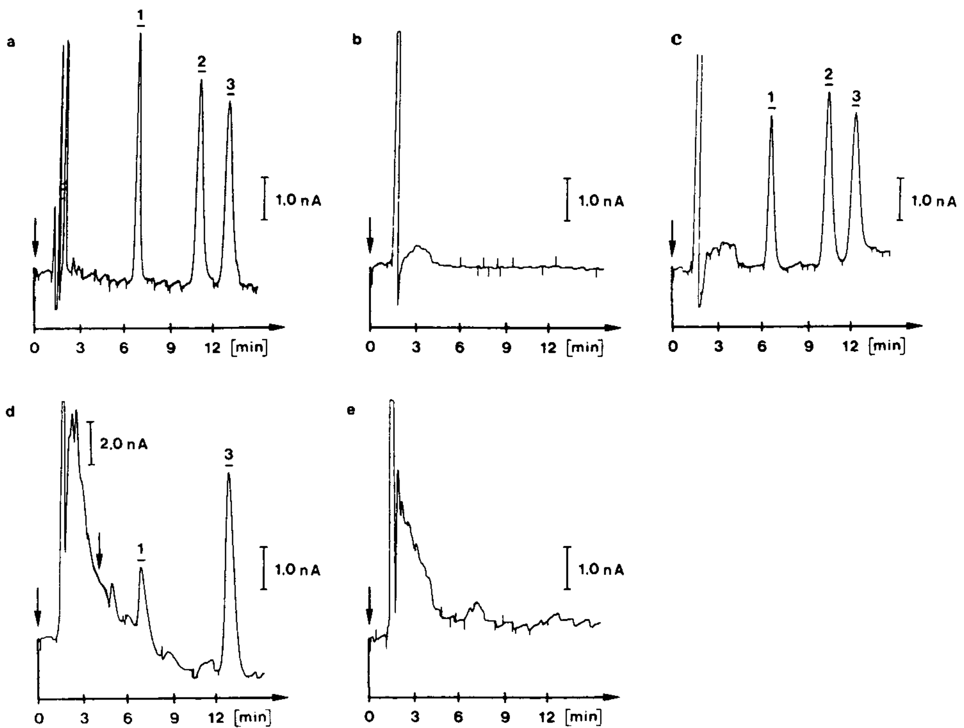

Fig. 3. (a) Off-line cation-exchange HPLC analysis of a synthetic mixture of 50 pmol each of the catecholamines NE (1), E (2) and DA (3). (b) On-line blank run. (c) On-line HPLAC—cation-exchange HPLC of a sample identical to that in (a). (d) On-line analysis of 100 μl of membrane-filtered native human urine. The arrow indicates change in sensitivity. (e) On-line analysis of 50 μl of urine as in (d) after destruction of endogenous catecholamines.

pH 3.0) in a small volume through positions 2—1—4—3 of the valve and transferred to the top of column 2 over a period of 1.10 min at 2.0 ml/min. This elution buffer was also used for the subsequent isocratic analytical separation. The valve was then switched back into position "Load" and elution of column 2 could be carried out.

A comparison of the analysis of E, NE and DA on the cation-exchange column (Fig. 3a) with the on-line procedure (Fig. 3c) shows that the latter one does not essentially affect band broadening and resolution of the catecholamines investigated. The column-switching technique also does not influence the electrochemical detection under the conditions applied (Fig. 3b). The method described allows the direct analysis of the principal catecholamines in native urine in a quarter of an hour (Fig. 3d). As shown in Fig. 3e for the analysis of a urine blank, possible interfering peaks are not detected. This allows the quantitative determination of the compounds of interest.

To monitor the accuracy of the overall chromatographic system, the matrix-dependent and -independent recovery of catecholamines was determined. For matrix-independent recovery synthetic mixtures of catecholamines were applied directly to the cation-exchange column 2 and identical mixtures subsequently analysed by the on-line HPLAC—cation-exchange HPLC system. For matrix-dependent recovery the amount of catecholamines present in a control urine was determined by the external standard method. The control urine was then spiked with defined amounts of catecholamines and analysed anew. Results are summarized in Tables I and II.

TABLE I

MATRIX-INDEPENDENT RECOVERY OF CATECHOLAMINES

Catecholamine	Recovery* (%)	R.S.D.** (%)
Norepinephrine	98.9	0.6
Epinephrine	101.4	2.3
Dopamine	97.8	1.0

*Each value is an average of three runs.
**Relative standard deviation.

TABLE II

MATRIX-DEPENDENT RECOVERY OF CATECHOLAMINES ADDED TO CONTROL URINE

Catecholamine	Catecholamine amount* (ng)			Average recovery (%)
	Urine	Spike	Urine + spike (found)	
Norepinephrine	2.5 ± 0.1	8.2 ± 0.2	10.1 ± 0.8	94.7
Epinephrine	—	10.0 ± 0.3	9.9 ± 0.4	99.0
Dopamine	7.7 ± 0.9	8.1 ± 0.5	15.5 ± 0.1	98.1

*Each value is an average of three runs ± standard deviations.

The recovery of catecholamines is very high, due to the fact that sample clean-up and analysis can be carried out under acidic conditions and error-prone evaporation and redissolution steps are avoided.

The method described consists of only a few working steps and allows the direct and quantitative determination of the principal free urinary catecholamines NE, E and DA in a quarter of an hour.

This on-line HPLAC—HPLC system should represent the method of choice, as it is particularly suitable for automation and applicable for routine clinical analysis.

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CHROMBIO. 2329

Note

Simultaneous determination of L-dopa and 3-O-methyldopa in human serum by high-performance liquid chromatography

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Levodopa (3,4-dihydroxyphenylalanine), in combination with carbidopa (L-α-hydrazino-3,4-dihydroxy-L-α-methylcinnamic acid), a peripheral decarboxylase inhibitor, has been shown to improve motor function in the majority of patients with Parkinson's disease [1]. However, long-term treatment with levodopa/carbidopa is associated with a variety of untoward effects, including unpredictable patterns of motor fluctuation ("on-off phenomenon"), abnormal involuntary movements, and end-of-dose akinesia ("wearing off") [2]. Elevated blood levels of 3-O-methyl dopa (3-methoxy-tyrosine, OMD), a major metabolite of L-dopa, have been associated with the

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occurrence of L-dopa-induced dyskinesias in Parkinsonian patients; furthermore, several groups have suggested that plasma OMD levels and/or OMD/L-dopa ratios may be predictive indicators of the long term response to L-dopa therapy [3, 4]. This hypothesis has not been experimentally verified, mostly because of the lack of simple, rapid, and reliable quantitation of blood OMD/L-dopa levels in human plasma.

Heretofore, plasma levodopa and OMD have been determined separately by the time-consuming and tedious method of fluorescent analysis [5]. Although high-performance liquid chromatography (HPLC) with electrochemical detection (ED) has been used successfully for determination of catecholamines in most tissues [6], simultaneous determinations of L-dopa and metabolites by HPLC and ED so far have not included measurements of OMD [7, 8], or sample preparations and elution patterns have been too long (40 min to 20 h per sample) to realistically permit multiple sample analysis [9, 10].

We report here a rapid, sensitive, and specific method for the simultaneous determinaton of these compounds in deproteinized plasma through separation by reversed-phase HPLC and ED.

MATERIALS AND METHODS

Standards of L-dopa and its metabolites were purchased from Sigma (St. Louis, MO, U.S.A.). Carbidopa was the generous gift of Merck Sharp & Dohme (West Point, PA, U.S.A.). Ammonium phosphate was HPLC grade and was purchased from A.H. Thomas (Philadelphia, PA, U.S.A.). All other chemicals were of reagent-grade quality. All solvents used in the HPLC system were HPLC grade, were solubilized with doubly distilled water further treated with a Millipore Milli-Q system (Milford, MA, U.S.A.), and were degassed and filtered under vacuum with a 0.45-μm Millipore HAWP-type filter before use.

High-performance liquid chromatography

The HPLC system from Waters Assoc. included a Model 6000A solvent delivery system, a Model U6K loop injector, and a Model RCM100 radial compression module. The column, also from Waters, was a Radial-Pak reversed-phase Partisil column (10 cm $\times$ 8 mm μBondapak C_{18}, 10 μm particle size). The system was also fitted with a 30 $\times$ 2.9 mm guard column composed of Waters C_{18} Corasil, 37—50 μm particle size range.

The electrochemical detector consisted of a TL5 glassy carbon electrode and an LC4A potentiostat (BioAnalytical Systems, West Lafayette, IN, U.S.A.). The working electrode was set at +660 mV relative to an Ag/AgCl reference electrode. Potentiostat sensitivity ranged from 5 to 100 nA. Elution profiles were continuously recorded on a Pedersen 37 MR strip-chart recorder at 1 V full scale. A good quantitative correlation was observed between peak heights and concentration of various standards injected. The elution of compounds from the column was carried out at ambient temperature in an isocratic mode. The mobile phase consisted of 100 m*M* ammonium phosphate, pH 4.3, and was pumped across the column at a flow-rate of 3.0 ml/min.

Sample preparation

Blood samples (5—10 ml) were drawn by venipuncture, transferred to agar separation tubes, and centrifuged at 1000 *g* for 5 min at 4°C. Serum (1 ml) was withdrawn and deproteinized by addition of 50 μl of 72% perchloric acid (PCA) and mixing on ice for 10 min. PCA was then removed by precipitation with 500 μl of 1 *M* dipotassium hydrogen phosphate, pH 11, with 5 m*M* NaEDTA (ethylenediamine tetraacetic acid), and final sample pH was adjusted to 8 with 1 *M* potassium hydroxide (typically 700 μl), resulting in formation of insoluble K^+ perchlorate. Samples were spun at 11,000 *g* for 4 min at 4°C. The supernatant was removed, reacidified to pH < 5 with 5 *M* hydrochloric acid or 2.5 *M* sulfuric acid, and aliquots of 20 μl were injected for HPLC determination.

RESULTS AND DISCUSSION

A representative chromatogram illustrating resolution of standard mixtures of L-dopa, 3-O-methyl dopa, and carbidopa, as well as norepinephrine and epinephrine, is shown in Fig. 1A. The complete separation of these compounds occurs within 10 min. Other major metabolites of L-dopa metabolism: 3,4-dihydroxyphenylacetic acid, vanilmandelic acid, homovanillic acid, as well as α-methyl dopa (a commonly used antihypertensive medication) do not interfere with the resolution of L-dopa or OMD. Dopamine, the carboxylated

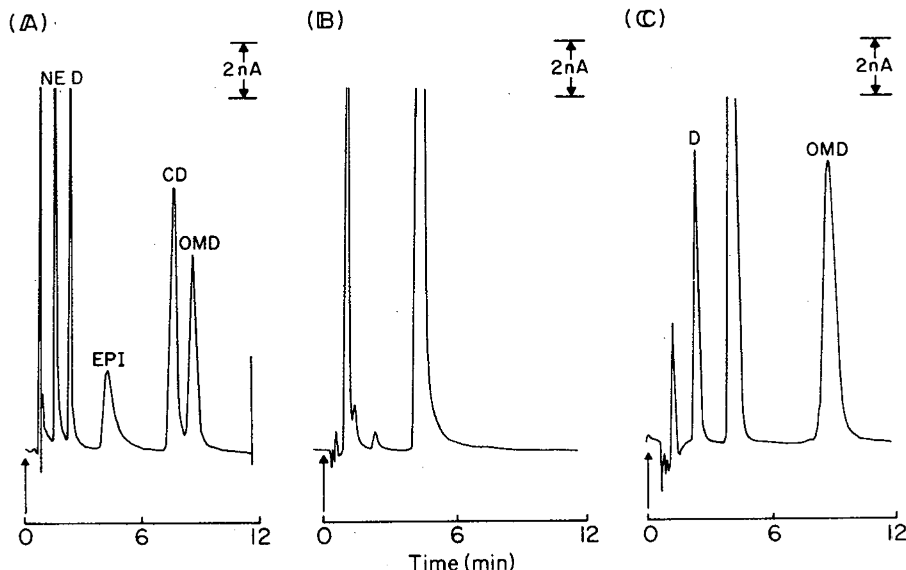

Fig. 1. HPLC elution profiles of: (A) standard mixture of L-dopa metabolites after single injection. The 10-μl injection consisted of aqueous mixture containing 100 pmol each of L-dopa (D), 3-O-methyl dopa (OMD), epinephrine (EPI), norepinephrine (NE), and carbidopa (CD); (B) 20 μl control serum obtained from healthy male volunteers prepared as described in the text; (C) 20 μl identically prepared serum obtained from a Parkinsonian patient 2 h after administration of 200 mg of Sinemet. See text for details on the mobile and stationary phases, flow-rates, and detector settings.

product of L-dopa conversion by dopa decarboxylase, has a retention time closest to that of OMD, but does not interfere with quantitation of OMD peak heights. In addition, dopamine is usually present at concentrations 10^4 times less than that of typical OMD blood levels found in Sinemet (levodopa/carbidopa) treated Parkinsonian patients and was not detectable in any of our plasma chromatograms. Carbidopa, the dopa decarboxylase inhibitor, has a retention time similar to dopamine, but likewise was found not to inhibit quantitation of OMD peak heights.

A chromatogram of control serum (Fig. 1B) obtained from healthy male volunteers demonstrates that no contaminating peaks are introduced by our serum preparation process. Additionally, plasma obtained by separation of blood containing the anti-coagulant EDTA can also be utilized for analysis without alteration of resolution of the L-dopa/OMD peaks (not shown). However, EDTA (which elutes immediately after the dead volume) does overlap with the peak of norepinephrine, and therefore, under these particular chromatographic conditions, quantitation of norepinephrine would be difficult. Fig. 1C is a chromatogram of a serum sample obtained from a patient with advanced Parkinson's disease 2 h after administration of 200 mg of Sinemet. This patient, who has severe involuntary movements and random fluctuations in motor performance, can be seen to have clearly detectable levels of L-dopa and OMD in this chromatographic profile.

The absolute recovery of L-dopa and OMD from human serum was estimated by comparing peak heights obtained from the injection of known quantities of the compounds with peak heights obtained from injection of extracts prepared from control serum spiked with the compounds. This gave values of 98% for L-dopa and 102% for OMD (n = 4). Since alumina extraction of samples is not utilized and analytical recovery is close to 100% for both compounds, the use of extracted, spiked serum standards in quantitations was found to be unnecessary.

Repeated determinations (n = 5) of standard preparations at a concentration of 1 μg/ml gave coefficients of variation of 2.6% for L-dopa and 2.4% for OMD. Although all of the measurements were carried out in frozen samples within three days of deproteination, standards frozen under identical conditions were found to be relatively stable over much longer periods of storage. A decay in activity of 8.8% and 8.0% was observed for a month's storage at −18°C for L-dopa and OMD, respectively.

Standard curves for L-dopa and OMD were prepared from injecton of pure stock solutions over a wide range of concentrations. Linearity of our chromatographic technique and detector was routinely observed for both compounds over a concentration range of nearly three orders of magnitude (2—1000 pmol injected compounds). Measurement of L-dopa and OMD was achieved at a sensitivity of 10—100 nA/V full scale at an applied electrode potential of +660 mV. Under these conditions, the limits of detection were 10 ng/ml (1 pmol injected compound) with a signal-to-noise ratio greater than 10 for both L-dopa and OMD. Additional improvements on these lower limits of detection could be achieved simply by making the applied voltage across the detector more positive (e.g. +720 mV), or by increasing the amount of sample injected. Since serum levels of L-dopa and OMD typically range from 0.10 to 10 μg/ml, our

described settings were clearly adequate for detection of L-dopa/OMD in all samples assayed from our patient population.

CONCLUSIONS

We have presented here a rapid, reliable, and sensitive assay for simultaneous detection of L-dopa and its metabolite 3-O-methyl dopa in human sera using reversed-phase HPLC and amperometric detection without interference from other known L-dopa metabolites. Sample preparation has been simplified, requiring only precipitation of serum proteins, and does not require more time-consuming steps such as pH-dependent alumina absorption/elution. The described technique therefore seems ideally suited for the study of L-dopa metabolism requiring multiple sample analyses in Parkinsonian patients currently on levodopa/carbidopa combination therapy.

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CHROMBIO. 2326

Note

Reversed-phase high-performance liquid chromatographic method for determination of brain glutamate decarboxylase suitable for use in kinetic studies

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γ-Aminobutyric acid (GABA) is well established as a major inhibitory neurotransmitter in the central nervous system [1], thus any disturbance of GABA metabolism which lead to altered levels of GABA may result in neurological dysfunction. The major synthetic pathway for GABA is by decarboxylation of L-glutamate catalyzed by the pyridoxal 5′-phosphate-dependent enzyme, L-glutamic acid-1-carboxylyase (GAD; EC 4.1.1.15) and it has been shown that inhibition of this enzyme can result in the appearance of convulsive episodes [2]. The interest in our laboratory involves a study of the mechanism of homocysteine-induced seizures which has application both in the study of the genetic metabolic disorder, homocystinuria and in human epilepsy.

Homocysteine has been shown to inhibit a number of pyridoxal 5′-phosphate-dependent enzymes [3, 4] and preliminary studies indicate that it may interfere with GAD activity. In order to investigate this, a sensitive and specific assay, suitable for the determination of initial velocities is required so that inhibition of the enzyme can be characterised kinetically. A number of methods for assaying GAD activity have been reported (for reviews, see refs. 5 and 6) but many of these have the disadvantage of indirectly measuring the product and so may be subject to artefacts. Methods have been described where the product, GABA, is isolated by liquid chromatography [6] although none of the reports demonstrate their suitability for use in kinetic analysis.

We have therefore developed a method based on the reversed-phase high-performance liquid chromatographic (HPLC) separation of the *o*-phthalaldehyde (OPA) derivative of GABA and its subsequent fluorimetric detection,

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which optmizes both the sensitivity and rate of separation while being eminently suitable for use in kinetic analysis.

EXPERIMENTAL

Chemicals

HPLC-grade methanol was obtained from Fisons (Loughborough, U.K.). Potassium acetate, glacial acetic acid and boric acid of AnalaR grade, and potassium dihydrogen orthophosphate of GPR grade were obtained from BDH (Dorset, U.K.). GABA was obtained from Koch-Light (Berkshire, U.K.). Amino acid standards, OPA, L-glutamic acid, triethanolamine, pyridoxal 5′-phosphate and aminoethylisothiouronium bromide (AET) were purchased from Sigma (Dorset, U.K.). Reagent-grade water was prepared by running tap water through a Milli R/Q water purification system (Millipore, U.K.) followed by filtration through a 0.2-μm membrane filter.

Enzyme source

Crude enzyme preparations were obtained by homogenisation of whole mouse brains in 10 vols. of 0.3 *M* triethanolamine buffer (pH 6.8), 1 m*M* AET and 2 m*M* pyridoxal 5′-phosphate. The homogenate was exposed to 30-sec periods of ultrasonication four times followed by centrifugation at 15,000 *g* for 20 min. The resulting supernatant was used as a source of GAD.

Enzyme assays

The assay was carried out in a total volume of 100 μl (pH 6.8) containing: 200 m*M* potassium dihydrogen orthophosphate, 5 m*M* L-glutamic acid and 0.2 m*M* pyridoxal 5′-phosphate. Each assay was started by the addition of enzyme in a 30-μl aliquot (typically 5 mg/ml protein) and incubation was carried out at 37°C. The reaction was terminated by the addition of 1 ml of ice-cold ethanol followed by centrifugation in an Eppendorf 5413 micro-centrifuge at 8800 *g* for 5 min. A 5-μl aliquot of the supernatant was derivatized as described.

Preparation of amino acid standards

Individual amino acids were prepared in aqueous solutions at concentrations of 100 μM and filtered through a 0.45-μm membrane filter.

Derivatization

OPA (10 mg) was dissolved in 500 μl absolute ethanol, then 500 μl of 2-mercaptoethanol were added followed by dilution to 10 ml with 0.4 *M* boric acid (adjusted to pH 10.4). To maintain the reagent strength, 50 μl of 2-mercaptoethanol were added every two days while the solution was stored at 4°C. An aliquot (5 μl) of amino acid standard or reaction supernatant was mixed with 10 μl of OPA in a 1.4-ml Eppendorf tube. The contents were mixed and after 90 sec at room temperature a 5-μl aliquot was used for analysis.

Apparatus

A high-performance liquid chromatograph (Gilson International) consisting

of two Model 303 pumps with a Model 802 manometric module and a Model 702 gradient manager was used. Samples were loaded via a Rheodyne Model 7125 syringe loading sample injector fitted with a 20-μl sample loop. The detection system consisted of a Gilson International Model 121 fluorescence detector equipped with a Corning 7-60 excitation filter and a Corning 3-73 emission filter utilizing a flow-cell of 9 μl volume. Peak areas were quantified by the method of area normalization using a Shimadzu Chromatopac C-RIB data processor. A μBondapak C_{18} (10 μm particle diameter) reversed-phase column, 30 cm × 3.9 mm I.D. (Waters Assoc.) was used in conjunction with a CSK guard column, 6 cm × 2.5 mm I.D., packed with ODS Co:Pell (Whatman).

Chromatography

Anhydrous potassium acetate (0.98 g) was dissolved in 900 ml of reagent-grade water and the pH adjusted to 5.6 with glacial acetic acid. The resulting solution was diluted to a total volume of 1 l to make a 0.1 *M* potassium acetate solution. The potassium acetate buffer and methanol were filtered through a 0.2-μm membrane filter (Millipore) before use. Each of the mobile phases was degassed daily by ultrasonic treatment and under vacuum. The mobile phase gradient was run from 20% to 70% methanol in a single 20-min step at a flow-rate of 1.5 ml/min. The elution programme was followed by a 10-min isocratic washing step prior to equilibration of the column with 20% methanol.

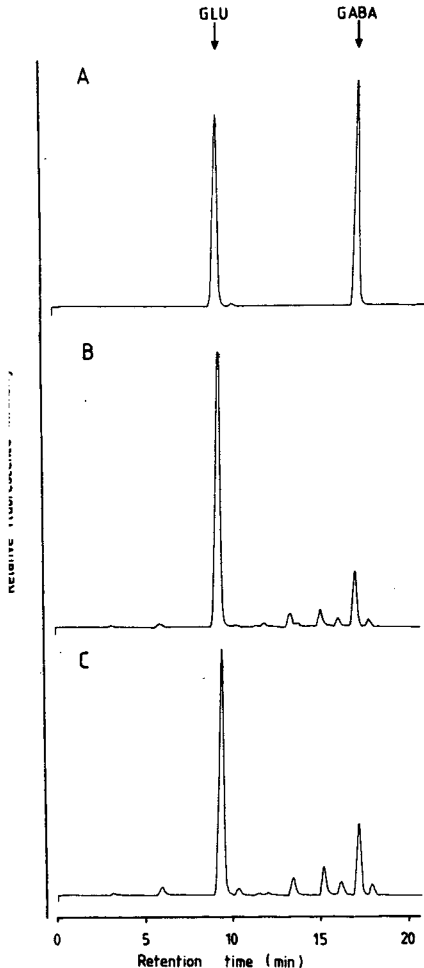
Protein determination

Protein determination was carried out using the dye-binding method of Bradford [7] utilizing bovine serum albumin as a standard.

RESULTS

The results shown in Fig. 1A illustrate a typical chromatogram of OPA-derivatized GABA and L-glutamic acid standards. It is clear that separation of these compounds was achieved and the calculated elution times were within ± 1—2%. L-Glutamic acid (GLU) elutes at 9.83 min with GABA eluting at 17.64 min. Using this method we have been able to detect less than 1.0 pmol of GABA. In our hands, when analysed a variety of commercially available GABA standards, described as approx. 98—99% pure, were separated into a number of peaks, the largest representing no more than 75% of the total peak area. Such an extent of impurity was unsatisfactory for calibration purposes. However, the source of GABA employed in this study gave a major peak representing 99% of the total peak area. Construction of a calibration curve showed that the peak areas were directly proportional to the concentration of the OPA-derivatized GABA in the range 0—100 μ*M*, the concentration of GABA (μ*M*) being equal to $9.2 \cdot 10^{-4}$ times the relative peak area.

Endogenous levels of GABA in the brain extract were obtained by OPA derivatization of a replicate incubate in which the reaction was terminated at time zero. Fig. 1B shows the chromatographic separation of substrate, L-glutamic acid (retention time 9.46 min) from endogenous GABA (retention time 17.51 min). Endogenous GABA concentrations were routinely subtracted from the total amount of GABA formed under assay. In Fig. 1C, a chromato-



'ig. 1. (A) Chromatogram illustrating the separation of OPA-derivatized authentic L-lutamic acid (GLU) and GABA (160 pmol of each). The standards were separated on a μBondapak C_{18} reversed-phase column, elution being effected with a mobile phase of 20—70% methanol—potassium acetate, pH 5.6, in a single 20-min run at a flow-rate of 1.5 ml/min. The retention times of L-glutamic acid and GABA were 9.83 min and 17.64 min, respectively. (B and C) Chromatographic analysis of reaction mixtures, under GAD assay conditions, as a function of time. (B) Time zero showing substrate peak, L-glutamic acid (retention time 9.46 min) and the second major peak, endogenous GABA (retention time 17.51 min). (C) Time 1 min, showing separation of GABA (endogenous + enzymically formed) with a retention time of 17.67 min from L-glutamic substrate (retention time 9.58 min). Assays were carried out using 150 μg rat brain enzyme protein extract plus assay mixture which included 5 m*M* L-glutamic acid. For full details of assay see Experimental.

graphic separation of enzymically formed GABA from L-glutamic acid is shown, following incubation with brain extract for 1 min. Even after this short incubation, the proportion of the peak area representative of enzymically formed GABA was 4.5% greater than that of the endogenous level, in spite of the relatively low V_{max} (92 pmol GABA formed per mg per min) of the enzyme. It should be noted that the change in the peak size of L-glutamic acid

will be negligible due to the high initial substrate concentration required in the kinetic assay. The smaller peaks observed in the chromatogram of Fig. 1C are representative of endogenous metabolites in the brain extract, which do not interfere with quantitation of GABA. Under the assay conditions described, the separation remained highly reproducible with insignificant fluctuation in peak elution time.

In any kinetic study employing a discontinuous assay system, it is essential to establish that the procedure for termination of the reaction is effective. When zero-time controls were compared with controls in which the brain extract had been heat-inactivated, no difference in product (GABA) levels were observed, indicating that the addition of ice-cold ethanol terminates the reaction instantaneously.

The data shown in Fig. 2 illustrate a progress curve of GAD activity for assays carried out in triplicate. The reaction is linear for at least a 15-min incubation period during which time easy measurements of initial velocity for use in kinetic studies can be made. Experiments to establish true initial velocity are frequently neglected so that published data may represent only an extent of reaction which is erroneously utilized for determination of kinetic parameters. Bearing this in mind, assays were performed at a range of L-glutamic acid concentrations and initial rates of GABA formation determined for each. The maximum velocity (V_{max}) of 92 pmol GABA formed per mg per min and the Michaelis constant (K_M) for L-glutamic acid of 1.35 mM were determined by regression analysis of the data; moreover the KM value for L-glutamic acid was in excellent agreement with values determined by other workers, for example Wu and Roberts [8] and Taberner et al. [9] who report K_M values of 0.7 mM and 1.8 mM, respectively.

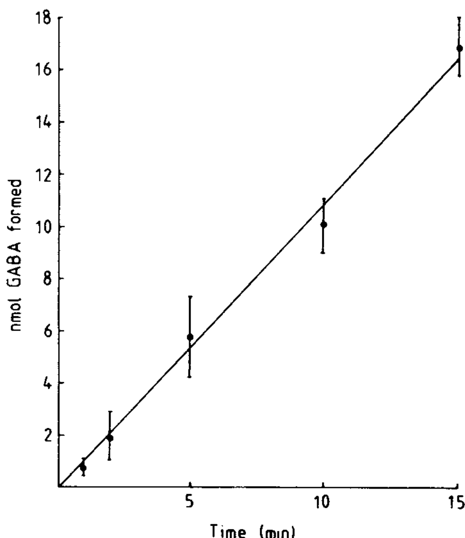


Fig. 2. Progress curve of GAD reaction plotted against time. Rat brain extract was prepared as described in the text. GAD assays were carried out for varying periods of time, at a fixed L-glutamic acid concentration, and GABA formation was measured following chromatographic separation as described in Experimental. Assays were carried out in triplicate and the data presented as the means ± S.D.

DISCUSSION

Although a number of non-chromatographic procedures are available for analysis of GAD activity [10—12], chromatographic methods are preferable owing to their specificity of analysis. The use of HPLC has the added advantages of increased resolution and sensitivity as well as being more rapid. A number of HPLC methods for determination of GABA have been reported [6] although very few were designed to specifically measure GAD activity [13—15]. Pahuja et al. [14] have applied reversed-phase HPLC to the separation of dansylated GABA from retinal cells. However, in this method, GAD activity was quantified by liquid scintillation spectrometry after separation of the dansylated derivatives by HPLC. Furthermore, the use of incubation times of at least 1 h is not commensurate with kinetic analysis, since the rate of the reaction is non-linear at this stage. A further limitation of this method is the level of detection which the authors state to be 1 nmol of GABA.

More recently, Holdiness [15] reported a fluorimetric procedure for analysis of GAD activity in sub-regions of rat brain using cation-exchange HPLC, which attained a lower limit of detection at 0.1 nmol of GABA. Although appearing more sensitive than the method of Pahuja et al. [14], supporting evidence for use in kinetic analysis was not available. To our knowledge, none of the previously reported methods of assay, which employ the advantages of HPLC, have been demonstrated as being suitable for kinetic studies. We have therefore developed a cost-effective and robust assay which specifically measures GABA formed during the reaction. Using this method, it is possible to detect < 1 pmol of enzymically formed GABA utilizing periods of incubation well within the linear portion of the progress time course.

The method for determination of GAD activity, reported herein, is an improvement over other methods and is being presently utilized in our studies on human homocystinuria and generalised epilepsy in which a possible defect in GABA metabolism may result in the appearance of seizures. In the course of our present research, we have found this assay suitable for determination of GAD activity in crude brain extracts, specific brain regions and subcellular organelles and in cultured cells.

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Note

Separation of biologically important angiotensin peptides by high-performance liquid chromatography on a weak cationic exchange bonded phase

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Members of the angiotensin peptide group have a variety of biological actions including pressor responses involving vascular receptors producing systemic vasoconstriction [1], pressor responses mediated by angiotensin receptors in the central nervous system (CNS) [2], influences on water and salt intake [3] as well as the control of adrenal steroid secretion [4]. Biological production of active angiotensin involves cleavage of a circulating precursor glycoprotein, angiotensinogen, by the enzyme, renin. The decapeptide product, angiotensin I, is cleaved at the C-terminal to produce the octapeptide, angiotensin II, the most significant biologically active member of the group [5]. Further metabolism of both angiotensin I and angiotensin II may yield peptides with additional biological function [5]. Production of angiotensin in the periphery is an extracellular process occurring in the circulating blood. Blood plasma or serum has been analyzed for angiotensin immunoreactivity by radioimmunoassay (RIA); typical normal ranges of human arterial plasma angiotensin II immunoreactivity are 10—50 fmol/ml [6].

Angiotensin may also be produced in the CNS [7]. Production of the peptide here may be an intracellular process [8], with active peptide released in a neuromodulatory role [2]. Angiotensin has been detected extracellularly in the CNS by both RIA and high-performance liquid chromatography (HPLC) [9, 10] and cell surface receptors have been characterized in the CNS [11]. Estimates of normal and abnormal levels of angiotensin II in cerebrospinal fluid have relied on RIA estimates and have produced disparate results [9].

Clinical measurements of the activity of the renin—angiotensin system have relied predominantly on estimates of plasma renin activity (PRA) by RIA quantification of angiotensin I production in plasma samples incubated with

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excess renin substrate. Although angiotensin II levels would be the most relevant for clinical diagnosis of angiotensin effects on cardiovascular function, and the correlation between PRA and angiotensin II levels is weak [6], RIA of angiotensin II has not been widely used because of the influence of cross-reactive precursors or metabolites, particularly in venous blood samples. Further investigation into the production, function and degradation of angiotensin peptides requires techniques which are capable not only of quantifying angiotensins in the femtomolar range, but also of discriminating between the structurally closely related precursors, active components and metabolites. For this reason a system has been established to accomplish rapid and efficient separation of immunoreactive angiotensins by HPLC. Furthermore, since present HPLC detection methods do not permit accurate quantification of peptides at biological levels in femtomolar concentrations, the HPLC system was developed so that peptides in samples fractionated by HPLC could subsequently be quantified by RIA.

MATERIALS AND METHODS

Peptides used were synthetic and had a specified purity in excess of 95%. Angiotensin I, angiotensin III, angiotensin C-terminal hexapeptide and C-terminal peptapeptide were prepared by Cambridge Research Biochemicals (Atlantic Beach, NY, U.S.A.). Des. Asp′ angiotensin I, angiotensin II and angiotensin C-terminal tetrapeptide were obtained from Bachem (Torrance, CA, U.S.A.). Amino acid composition of the peptides are shown in Table I. Solvents used in the gradient elution system were: solvent A, 10 mmol/l aqueous ammonium formate—acetonitrile (90:10, v/v) pH 4.2 with formic acid; and solvent B, 50 mmol/l aqueous ammonium formate—acetonitrile (80:20, v/v), pH 4.2 with formic acid. Ammonium formate and formic acid were reagent grade from Sigma (St. Louis, MO, U.S.A.), acetonitrile was HPLC grade from Fisher Scientific (Plano, TX, U.S.A.) and water was either HPLC grade from J.T. Baker (Phillipsburg, NJ, U.S.A.) or double-glass-distilled water.

Chromatography was performed using a Waters HPLC system comprising microprocessor-based gradient controller, twin pumps (Model 6000A), sample injector (Model U6K) and a fixed-wavelength ultraviolet absorbance detector

TABLE I

AMINO ACID SEQUENCE OF ANGIOTENSIN PEPTIDES

N-Terminal 1	2	3	4	5	6	7	8	9	C-Terminal 10	Angiotensin peptide
H-Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu-OH	Angiotensin I
H	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu-OH	des. Asp′ Angiotensin I
H-Asp	Arg	Val	Tyr	Ile	His	Pro	Phe-OH			Angiotensin II
H	Arg	Val	Tyr	Ile	His	Pro	Phe-OH			Angiotensin III
	H	Val	Tyr	Ile	His	Pro	Phe-OH			C-Terminal hexapeptide
		H	Tyr	Ile	His	Pro	Phe-OH			C-Terminal pentapeptide
			H	Ile	His	Pro	Phe-OH			C-Terminal tetrapeptide

at 254 nm (Model 440). The column was a 25 cm × 4.6 mm weak cation exchanger comprised of carboxylic acid functional sites fixed on 5-μm spherical silica particles (CBA, Analytichem International, Harbor City, CA, U.S.A.). Solvent flow-rate was 2 ml/min. The column was equilibrated with solvent A prior to sample injection. Samples were 25 μl of aqueous solutions of the peptides. Following sample injection, solvent A was run isocratically for 5 min. A linear gradient from 100% solvent A to 100% solvent B was performed between 5 and 6 min after injection. The remainder of the elution was performed under isocratic conditions using 100% solvent B. Ultraviolet absorbance at 254 nm was continuously monitored and output recorded on a Texas Instruments strip-chart recorder.

RESULTS

The separation of a multicomponent mixture of angiotensin peptides on a carboxylic acid weak cation-exchange column is illustrated in Fig. 1. All peaks were satisfactorily separated from adjacent peaks and demonstrate good symmetry. At pH 4.2, amino acid residues in angiotensins are predominantly protonated enhancing functional interaction with the carboxylic acid groups of the ion exchanger. Chromatographic separation involved modification of this interaction over a gradient of salt strength. However, satisfactory separation of peptides could not be accomplished without the presence of a gradient of the polar organic solvent, acetonitrile. This latter observation suggests the possibility of both ion-exchange and hydrophobic interactions (perhaps with exposed silica subsurface sites not blocked by carboxylic acid groups) between the analyte and the stationary phase.

The contribution of functional groups associated with amino acid residues in each of the peptides to the interaction between the peptide and the stationary

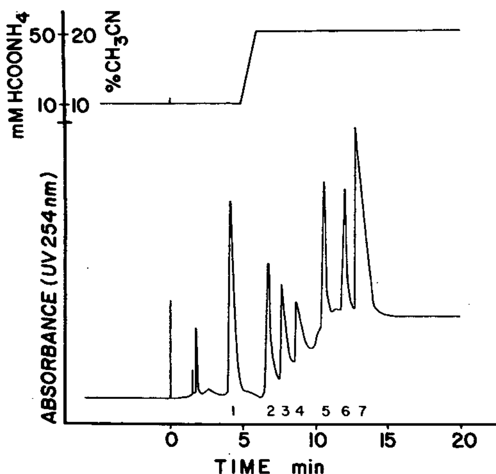

Fig. 1. Separation of seven angiotensin group peptides (25 pmol each) using gradients of salt strength and acetonitrile concentration over a weak cation exchanger (carboxylic acid). Peaks: 1 = angiotensin C-terminal tetrapeptide; 2 = angiotensin C-terminal pentapeptide; 3 = angiotensin II; 4 = angiotensin C-terminal hexapeptide; 5 = angiotensin III; 6 = angiotensin I; and 7 = des. Asp′ angiotensin I.

phase is revealed to a certain extent by the elution sequence of the peptides (see Table I for peptide composition). Presence of the basic residue of arginine at the exposed N-terminal such as in des. Asp′ angiotensin I and angiotensin III was associated with increased retention compared to the retention of angiotensin I and II, respectively, where the N-terminal residue is the less positively charged aspartate. However, the complexities of concurrent hydrophobic and ion-exchange interactions make further analysis uncertain.

The compatibility of the present separation system with RIA of angiotensin II has been evaluated by comparison of the assay standard curve of angiotensin II obtained in the presence and absence of the non-volatile HPLC solvent residue. The only non-volatile component of the solvent system was ammonium formate. The effect of 1 ml of dried solvent from a variety of solvent mixtures on the RIA estimation of angiotensin II was assessed. Fig. 2 shows the displacement of ^{125}I-labelled angiotensin II in the normal standard curve range, and the displacement of ^{125}I-labelled angiotensin assayed in tubes containing 1 ml of dried solvent B. The superimposition of the curves indicates clearly that accurate estimates of angiotensin II can be made by RIA of peptide in samples fractionated in the present chromatographic system.

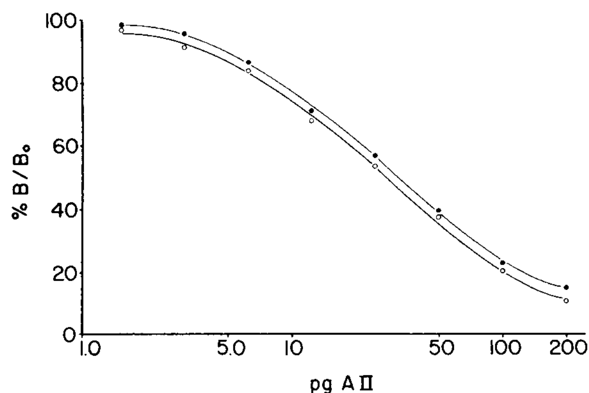

Fig. 2. RIA standard curve of angiotensin II (AII) in absence (•) and presence (○) of dried residue of 1 ml of HPLC solvent B.

Recovery tests have been performed to evaluate the recovery of angiotensin II standards fractionated in the HPLC system. Mean (± S.E.M.) recovery of 50-fmol samples of angiotensin II fractionated by HPLC and subsequently quantified by RIA was 89.6 ± 17.5% (n = 8).

DISCUSSION

Several methods have been previously described for the chromatographic separation of some angiotensin peptides [12—16]. Methods used have included paper chromatography [12], thin-layer chromatography on silica gels [13], and HPLC [14—16]. While some of these methods have been potentially useful in permitting both separation and subsequent quantification of some angiotensins, none has permitted the rapid and routine analysis of the principal immunoreactive precursors and metabolites of angiotensin II. The present

method systematically separates each of seven peptides all of which possess a greater or lesser cross-reactivity in RIA measurement of angiotensin II.

Other authors [15, 16] report the use of both reversed-phase C_{18} and weak anion exchangers in HPLC analysis of angiotensins. While these methods document satisfactory separation of some of the peptides used in the present study, the peptides do not cover the entirety of the closely related, broad group of peptides used here. Early attempts in this laboratory using ion-pairing techniques with halogenated organic acids in C_{18} systems yielded only partial separations of the peptides examined here. The results of the present study indicate that weak cation exchangers may yield a more suitable substrate for the separation of these and possibly other peptides and their metabolites. Suitability for application to quantitative assessments has been further demonstrated by the finding that HPLC solvent components do not interfere with RIA measurements. At present, studies are underway to further evaluate the application of this system to quantification of angiotensins in biological fluids under a variety of physiological conditions.

In conclusion, the HPLC separation of major immunoreactive angiotensin peptides has been satisfactorily accomplished. The separation method provides a means of rapidly separating peptides of biological origin and subsequently quantifying peptides by RIA. Many potential applications of the method can be envisaged ranging from an assessment of the metabolism of angiotensins in the blood to studies examining the role of angiotensin in the central nervous system.

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Note

Anomalous behavior of lectins in size-exclusion high-performance liquid chromatography and gel electrophoresis

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In characterizing the molecular structure of oligomeric proteins such as lectins, it is common practice to estimate the molecular weight of subunits by sodium dodecyl sulphate (SDS) gel electrophoresis and the native molecular weight by gel filtration. The oligomeric form of the protein can then be obtained by comparing these two molecular weights. Media such as Sephadex G-200 have sufficient resolving power that dimers can be readily distinguished from tetramers though in the fortunately rare case of trimers mistakes could be made. The advent of size-exclusion chromatography media such as the TSK SW series [1] has made it possible to rapidly obtain molecular weight estimates for proteins on high-performance liquid chromatography (HPLC) equipment. These media also have greater resolving power than the traditional gels. However in experiments with a group of lectins from leguminous plants reported here, we have found that results obtained by HPLC experiments were less reliable than those previously obtained by gel filtration.

MATERIALS AND METHODS

Published affinity chromatographic procedures were used to purify concanavalin A [2], pea (*Pisum sativum*) lectin [3], peanut (*Arachis hypogaea*) agglutinin [4], *Griffonia simplicifolia* GSI lectin [5] and *Phaseolus vulgaris* hemagglutinin [6]. The N-acetyl-D-galactosamine specific lectins from *Sophora japonica*, *Wisteria floribunda*, *Caragana arborescens* and *Glycine max* (soybean agglutinin) were purified on the affinity medium described for the *Cytisus scoparius* lectins [7]. *Ph. coccineus* and *Ph. lunatus* lectins were purified by chromatographic procedures [8, 9]. The *Dolichos biflorus* lectin was purchased

from Sigma (St. Louis, MO, U.S.A.), and the *Bauhinia purpurea*, *Vicia villosa* and *Cy. sessilifolius* lectins were purchased from E-Y Labs. (San Mateo, CA, U.S.A.).

SDS slab-gel electrophoresis was carried out by the method of Weber and Osborn [10] with a 10% gel and by the method of Laemmli [11] with a 12% gel. The standard proteins used were phosphorylase b (96,000 molecular weight, M_r) bovine serum albumin (68,000), ovalbumin (45,000), carbonic anhydrase (30,000), chymotrypsinogen (25,600), β-lactoglobulin (18,400), myoglobin (17,200) and lysozyme (14,300). For size-exclusion HPLC, a TSK 3000-SW column (60 × 0.7 cm) and TSK-SW precolumn (10 × 0.7 cm) from Beckman Instruments were used. The column was eluted at 0.5 ml/min, using a Pharmacia P500 pump and the effluent was monitored at 280 nm with a Spectraphysics Model SP 8440 detector. Samples of approx. 100 μg of protein were injected in 20—50 μl vols., using a Pharmacia FPLC injector. Some preliminary experiments were carried out on a Beckman Model 330 HPLC system.

RESULTS

The subunit molecular weights (mol.wt.) previously reported for the lectins (Table I) were checked in experiments using two SDS gel electrophoresis methods. In both systems, the protein standards gave linear calibration plots and the lectins migrated in a narrow range of mobilities, close to the position of carbonic anhydrase (Fig. 1). The literature data of Table I were obtained mainly by the method of Weber and Osborn [10].

TABLE I

LECTIN MOLECULAR WEIGHTS FROM SIZE-EXCLUSION HPLC AND LITERATURE SUBUNIT MOLECULAR WEIGHTS

Lectin	Apparent mol.wt.	Subunit mol.wt.	Reference
Gr. simplicifolia	120,000	32,500	14
Ph. lunatus	120,000	31,000	15
B. purpurea	120,000	(195,000)*	16
W. floribunda	105,000	28,000	17
Ph. coccineus	100,000	34,000	8
Ph. vulgaris	96,000	34,000	18
S. japonica	96,000	32,500	19
V. villosa	96,000	30,000	20
Ca. arborescens	82,000	30,000	21
D. biflorus	78,000	27,500	22
Cy. scoparius	70,000	30,000	7
G. max	68,000	27,600**	13
Cy. sessilifolius	64,000	(110,000)*	23
A. hypogaea	48,000	27,500	24
Concanavalin A	29,000	25,600**	12
P. Sativum	37,000	7000 17,000	3

*Oligomer molecular weight.
**Calculated from amino acid sequence.

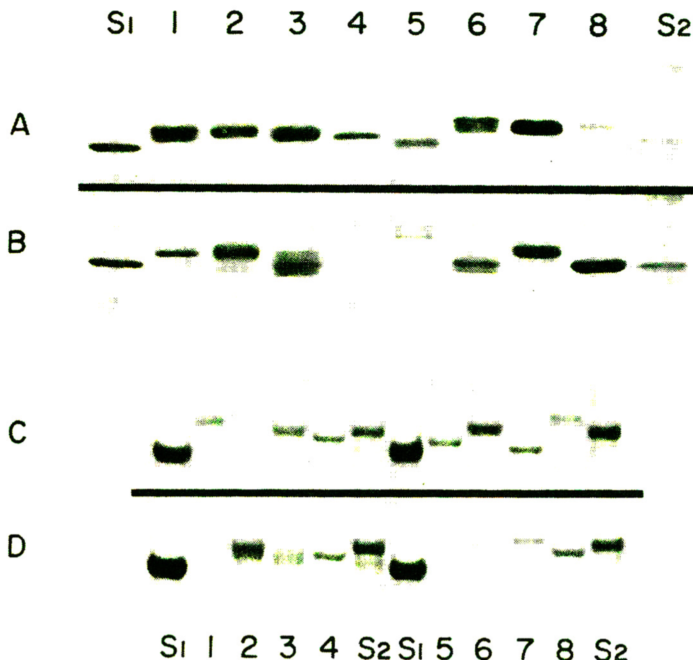

Fig. 1. SDS gel electrophoresis of lectins. The gel sections shown in A and B were obtained by the method of Laemmli [11] and those in C and D by the method of Weber and Osborn [10]. The standard proteins shown are carbonic anhydrase, mol.wt. 30,000 (AS_1, BS_1, CS_2, DS_2) and chymotrypsinogen, mol.wt. 25,600 (AS_2, BS_2, CS_1, DS_1). The lectins are *Ph. vulgaris* (A1, C1); *Ph. coccineus* (A2, C2); *Ph. lunatus* (A3, C3); concanavalin A (A4, C4, D4). *D. biflorus* (A5, C5); *G. max* (A6, C6); *A. hypogaea* (A7, C7); *Gr. simplicifolius* GSI (A8, C8); *S. japonica* (B1, D1); *Cy. scoparius* (B2, D2); *Cy. sessilifolius* CSIa (B3, D3); *B. purpurea* (B5, D5); *Ca. arborescens* (B6, D6); *V. villosa* (B7, D7) and *W. floribuda* (B8, D8).

Molecular weights were estimated for the same lectins by size-exclusion chromatography on a TSK 3000-SW column, run in 0.2 *M* potassium phosphate buffer, pH 7.0 (Table I). Eight of the lectins had mol.wt. between 95,000 and 120,000, consistent with tetrameric forms. Six others, including the well characterized soybean and peanut agglutinins had mol.wt. in the range 50,000—82,000. Concanavalin A should have been tetrameric under these buffer conditions [25] but eluted later than the position expected for the dimer species. The pea lectin also eluted a little later than expected for its $\alpha_2\beta_2$ structure. When the specific sugar inhibitors, D-galactose for peanut agglutinin, N-acetyl-α-galactosamine for soybean agglutinin and methyl-α-D-glucoside for concanavalin A, were added to the buffer, each lectin eluted slightly earlier.

The behavior of the soybean and peanut agglutinins when chromatographed with buffers of different pH, ionic strength and buffer ion is shown in Fig. 2. The apparent molecular weights were little changed by running in 0.1 *M* acetate buffer, pH 5.5. At lower ionic strengths, the column calibration changed considerably, but the apparent molecular weights of the lectins were still below those expected for tetrameric lectins. Similar results to those with the 20 m*M* sodium acetate and 20 m*M* potassium phosphate buffers shown in

Fig. 2, were obtained with 20 mM sodium acetate buffer, pH 5.5. One characteristic of the agglutinin peaks was that they were often significantly broader than those of the standard proteins used to calibrate the system. The column efficiencies calculated for bovine serum albumin, ovalbumin and the two agglutinins in the different buffers are given in Table II, and are calculated from the formula 5.54 $(t_R/W_0)^2$, where t_R is the retention, and W_0 the width at half-height.

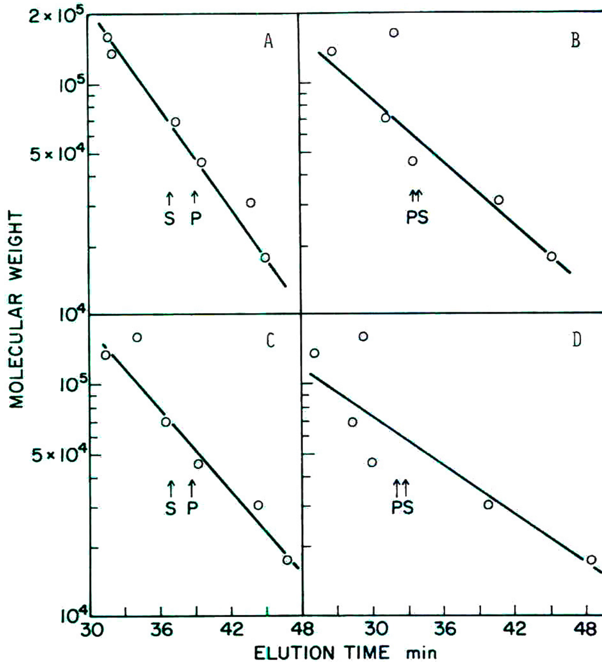

Fig. 2. Size-exclusion chromatography on TSK 3000-SW in various buffers. The standard proteins (○) from top to bottom in each calibration are IgG (160,000), bovine serum albumin dimer (136,000), bovine serum albumin (68,000), ovalbumin (45,000), carbonic anhydrase (30,000) and myoglobin (17,000). The elution positions of soybean (S) and peanut (P) agglutinins are indicated by arrows. The buffers were (A) 0.2 M potassium phosphate, pH 7.0; (B) 0.02 M potassium phosphate, pH 7.0; (C) 0.1 M sodium acetate, pH 5.6; (D) 0.02 M sodium acetate, pH 6.5.

TABLE II

EFFICIENCIES OF THE TSK 3000-SW COLUMN MEASURED WITH VARIOUS PROTEINS AND BUFFERS

Protein	Column efficiency (theoretical plates)				
	0.2 M phosphate, pH 7.0	0.02 M phosphate, pH 7.0	0.02 M acetate, pH 6.5	0.1 M acetate, pH 5	0.02 M acetate, pH 5
Bovin serum albumin	7900	4700	4600	9200	5500
Ovalbumin	6100	4300	5800	7800	3700
Peanut agglutinin	4800	1900	600	4200	800
Soybean agglutinin	6200	2800	1200	6200	2300

DISCUSSION

The gel electrophoresis experiments showed that (a) the range of molecular weights for lectin subunits is much narrower than current literature values (Table I) suggest, and (b) the subunit weights can be significantly overestimated by these methods, when standard proteins such as carbonic anhydrase are used rather than lectins such as concanavalin A. With the exception of the *B. purpurea* lectin, all the lectins ran close to the positions of soybean agglutinin and concanavalin A, whose amino acid sequences [12, 13] give mol.wt. of 27,600 and 25,600, respectively. Thus estimates of 30,000 or above for some of these lectins (Table I) are probably 10—20% too high. Values for the subunit molecular weights of the *B. purpurea* and *Cy. sessilifolius* lectins have not been previously reported; they are 32,000 and 28,000, respectively. The oligomeric form of *B. purpurea* lectin may therefore be different from that of other lectins since the mol.wt. of 195,000 obtained by ultracentrifugation [16] would be consistent with a hexameric structure, though the HPLC value is consistent with a tetramer.

The average mol.wt. for the various lectins is approx. 27,000, giving a tetramer weight of 108,000. Several lectins, including the well characterized soybean and peanut agglutinins, had substantially lower apparent molecular weights. Therefore if TSK 3000-SW is used to determine lectin molecular form, an apparent mol.wt. of 100,000 or more may reliably indicate a tetrameric structure, but an apparent mol.wt. of 50,000—60,000 cannot be taken as evidence for a dimeric structure. The peak width may also be an indicator of aberrant behavior, as the data of Table II show.

Experiments conducted with various buffers for peanut and soybean agglutinins did not suggest a mechanism for their late elution. These two proteins have been well characterized by ultracentrifuge experiments and they are clearly tetramers [24,26,27]. Also, their behavior on Sephadex G-200 was consistent with their molecular weight [24, 26]. Their behavior on TSK 3000-SW was not improved by changing to a pH further from their isoelectric points, nor by lowering the ionic strength to reduce hydrophobic interactions. Adding the specific sugar inhibitors had little effect, hence interactions of their combining-sites with hydroxyl groups on the packing is not significant. Dissociation of the lectin tetramers into dimers might be occurring but the elution position of soybean agglutinin was not changed over a 40-fold range of sample load, including amounts equivalent to those used in the Sephadex G-200 experiments of Lotan et al. [26].

Since even members of one protein family behaved differently in these experiments, it is evident that molecular weight estimates obtained by size-exclusion HPLC must be viewed with caution, particularly when dealing with oligomeric proteins, and preferably be confirmed by gel chromatography on Sephadex or similar media.

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Note

Quantitation of cibenzoline in human plasma by gas chromatography—negative-ion chemical-ionization mass spectrometry

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Cibenzoline (I, Ciprolan®, Fig. 1), is currently under clinical investigation for use as a cardiac antiarrhythmic agent [1, 2]. Recently, gas chromatography with electron-capture detection [3] and high-performance liquid chromatography (HPLC) [4] methods for I have been reported. This paper reports a gas chromatographic—mass spectrometric (GC—MS) assay for I which uses a simple sample work-up, and which is more sensitive than existing assays for I. The method features the use of a $^{15}N_2$-stable isotope analogue of I, compound II (Fig. 1), as the internal standard, and detection in the GC effluent by selected-ion monitoring of the negative-ion chemical-ionization (NICI) generated $(M - 2HF)^{-\cdot}$ ions of the pentafluoropropionyl (PFP) derivatives of I and II.

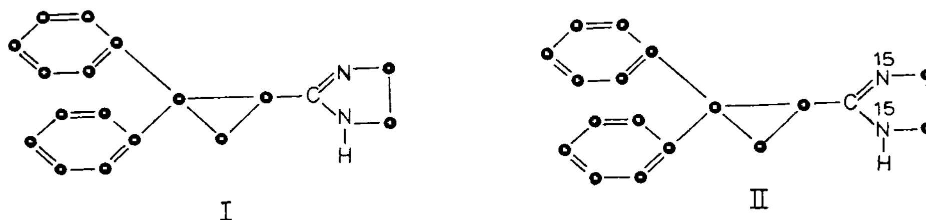

Fig. 1. Chemical structures of cibenzoline (I) and its $^{15}N_2$-stable isotope analogue (II).

EXPERIMENTAL

Chemicals

Compound I was supplied as the free base by Dr. P. Sorter (Chemical Research Department, Hoffmann-La Roche, Nutley, NJ, U.S.A.). Compound II

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was supplied as the butanedioate salt by Dr. C. Perrry (Isotope Synthesis Group, Hoffmann-La Roche). Nanograde ethyl acetate, methylene chloride, acetonitrile, methanol and benzene were obained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Pentafluoropropionic anhydride (PFPA) was purchased from Pierce (Rockford, IL, U.S.A.). The anhydride was stored under nitrogen at −4°C to prevent decomposition. Stock solutions (μg/ml) of I and II were prepared in acetonitrile. Aliquots of the stock solutions were diluted with acetonitrile to give a series of working solutions containing 100 ng/ml II and either 0 ng/ml I (solution A), 10 ng/ml I (solution B), 50 ng/ml I (solution C), 100 ng/ml I (solution D), 250 ng/ml I (solution E) or 500 ng/ml I (solution F). All stock solutions were stored at −4°C when not in use.

Equipment

All test tubes and pipettes were washed with detergent, rinsed with distilled water, dried and siliconized by immersion for 15 min in a 1% aqueous solution of Prosil 28® (PCR Research Chemicals, Gainesville, FL, U.S.A.) prior to rinsing with distilled water and sonication with dichloromethane and methanol.

A Finnigan 3200 mass spectrometer was used with a Finnigan 9500 gas chromatograph. A glass GC column (120 cm × 2 mm I.D.) was packed with 3% SP-2250 on 80–100 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). Methane at a pressure of 1.2 kg/m^2 was used as GC carrier gas (ion source pressure of 67 Pa). The temperature of the injection block, column and GC–MS transfer line were 275°C, 265°C and 210°C, respectively. The continuous dynode electron multiplier was operated at −1.2 kV, and the conversion dynode was operated at +2.5 kV. Modifications to the mass spectrometer to permit the detection of negative ions have been described [5].

Selected-ion monitoring measurements at *m/z* 368 and *m/z* 370 were made using a Finnigan PROMIM® peak monitor. The responses were recorded on a multichannel chart recorder (Rikadenki KA-41). Both channels were operated at gain of 10^{-8} A/V, 100 msec dwell time and a filter setting of 0.5 Hz. The recorder was operated at a chart speed of 2 cm/min.

Procedure

Calibration curve samples were prepared in duplicate by fortifying 1-ml aliquots of control plasma with 100 μl of either solution A, B, C, D, E or F to give final concentrations of 10 ng/ml II and either 0, 1, 5, 10, 25 or 50 ng/ml I, respectively. All experimental plasma samples were fortified with 100 μl of solution A (10 ng of II).

Extractions were performed with C_{18} bonded-phase disposable columns (Cat. No. 607101) using a vacuum manifold from Analytichem (Harbor City, CA, U.S.A.). Column washings and eluents were drawn through the columns by a vacuum of approx. 400 Pa. The columns were washed with methanol (2 × 1 ml), distilled water (2 × 1 ml), and 0.02 *M* phosphate buffer, pH 11 (2 × 1 ml) before the plasma samples (1 ml) were applied to the columns. The columns were washed with 0.02 *M* phosphate buffer, pH 11 (2 × 1 ml) and acetonitrile (1 ml). Compounds I and II were then eluted with methanol (2 × 1 ml). The methanol was transferred to 75 × 10 mm disposable culture tubes, and was evaporated to dryness at 40°C under a stream of nitrogen.

The residues were reconstituted with 50 μl of ethyl acetate followed by 10 μl of 10% PFPA in ethyl acetate. The tubes were tightly capped and allowed to stand for 60 min at room temperature. Following evaporation of the derivatizing agent under a stream of nitrogen, the residues were reconstituted in 25—50 μl of ethyl acetate just prior to analysis of 1 to 5 μl of this solution by GC—NICI-MS.

The slope (m) and intercept (b) values from a linear least-squares regression analysis of the observed m/z 368 to m/z 370 ion ratio (R) versus amount (ng) added data from the analysis of the calibration curve samples were used to calculate the amount (x) of I in an experimental sample from the measured R using the equation $x = (R - b)/m$. The concentration of I in an experimental sample was calculated by dividing the amount found by the volume of plasma analyzed.

Determination of extraction recoveries

In order to determine the extraction efficiency, pooled human plasma was fortified with [^{14}C]cibenzoline (10 ng, $5.8 \cdot 10^5$ dpm/ml). Aliquots of 1 ml were applied directly to the C_{18} disposable columns, which were washed first with 2 × 1 ml of 0.02 *M* phosphate buffer, pH 11 and finally with 1 ml of acetonitrile. The [^{14}C]cibenzoline was eluted with 2 × 1-ml aliquots of methanol. The samples were collected and counted for radioactivity.

RESULTS AND DISCUSSION

The electron-capture NICI mass spectra of the PFP derivatives of I and II are shown in Fig. 2. The spectra consist principally of $(M - 2HF)^{\cdot-}$ ions. The loss of 2HF has been previously observed in the NICI mass spectra of PFP derivatives of other amines [6, 7].

Typical selected-ion current profiles from the assay are shown in Fig. 3. Calibration curves were linear for concentrations between 1 and 50 ng/ml (correlation coefficients greater than 0.99). Mean intra- and inter-assay precisions were 2.1% and 8.1%, respectively (Table I).

The concentrations of cibenzoline in plasma samples from four subjects each given a 50-mg oral dose of cibenzoline are given in Table II. These samples were also analyzed using the published HPLC method [4]. Plasma concentration—time curves based on the mean concentration data reported in Table II for both methods are shown in Fig. 4. Data from the two methods are linearly related (correlation coefficient = 0.96, HPLC concentration = 1.105 · GC—MS concentration − 1.44 ng/ml). A two-tailed paired Student's t-test of the two sets of concentration data suggests that they are not statistically different at the $p = 0.05$ level of significance [8]. The same result is also obtained if natural logarithms of the concentrations are calculated prior to the paired Student's t-test in order to normalize the relatively wide range of concentrations compared [8].

In summary, a GC—MS assay for cibenzoline was developed which is approximately 2.5 times more sensitive than a published HPLC assay [4] for the compound. Plasma concentration data determined by the GC—MS and HPLC assays [4] are not statistically different.

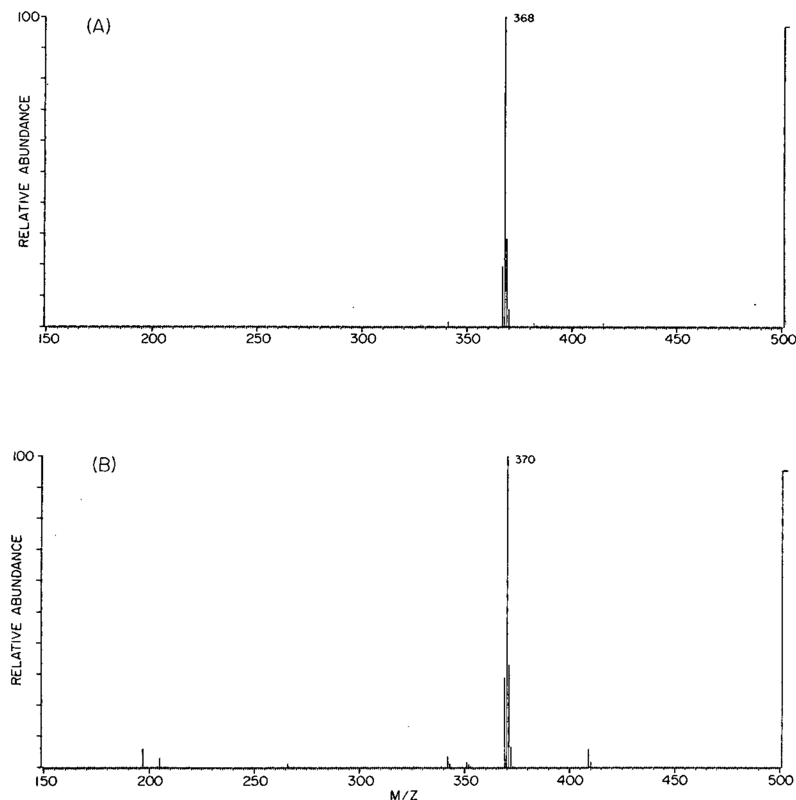

Fig. 2. NICI mass spectra of the PFP derivatives of (A) cibenzoline (MW = 408) and (B) [$^{15}N_2$]-cibenzoline (MW = 410). Methane was the CI reagent gas.

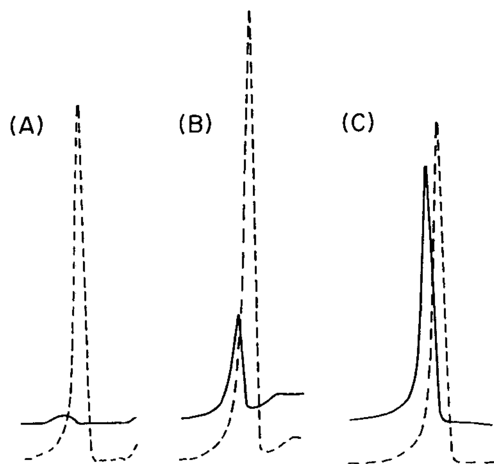

Fig. 3. Selected-ion current profiles from the analysis of 1-ml plasma samples each containing 10 ng of [$^{15}N_2$]-cibenzoline (- - -). Profiles A and B are from plasma samples also fortified with 0 and 1 ng of cibenzoline, respectively. Profile C is from plasma from a subject given cibenzoline. The concentration of cibenzoline in this sample is 5.0 ng/ml.

TABLE I

STATISTICAL VALIDATION OF THE GC—NICI-MS ASSAY FOR CIBENZOLINE IN PLASMA

Amount added (ng/ml)	Intra-assay*		Inter-assay**	
	Amount found ± S.D. (ng/ml)	R.S.D.*** (%)	Amount found ± S.D. (ng/ml)	R.S.D. (%)
1.00	1.06 ± 0.04	3.4	0.81 ± 0.15	18.6
5.00	4.59 ± 0.08	1.7	5.25 ± 0.48	9.1
10.00	10.17 ± 0.01	0.1	10.64 ± 0.72	6.7
25.00	26.20 ± 1.06	4.0	26.60 ± 0.94	3.5
50.00	49.12 ± 0.72	1.5	48.05 ± 1.21	2.5
	Average = 2.1%		Average = 8.1%	

*Intra-assay precision from the analysis of the data from three separate calibration curves run on the same day.
**Inter-assay precision from the analysis of the data from four separate calibration curves run over a 12-day period.
***Relative standard deviation.

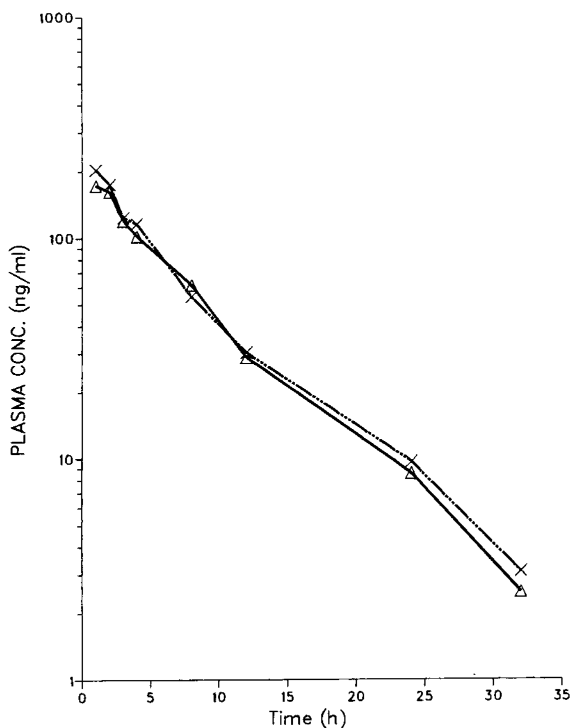

Fig. 4. Plasma concentration—time curves based on the mean concentration data reported in Table II for both the GC—MS (△) and HPLC (×) methods.

TABLE II

CONCENTRATIONS (ng/ml) OF CIBENZOLINE IN PLASMA FOLLOWING A SINGLE 50-mg ORAL ADMINISTRATION OF CIBENZOLINE TO FOUR MALE VOLUNTEERS

Time (h)	Concentration (ng/ml)									
	Subject 1		Subject 2		Subject 3		Subject 4		Mean ± S.D.	
	GC–MS*	HPLC**	GC–MS	HPLC	GC–MS	HPLC	GC–MS	HPLC	GC–MS	HPLC
0.00	NM***	NM	NM	NM	NM	NM	NM	NM	NM	NM
1.00	183.00	200.00	195.00	299.00	104.00	109.00	209.00	206.00	172.8 ± 47.0	203.5 ± 77.6
2.00	152.00	170.00	198.00	230.00	101.00	111.00	198.00	191.00	162.3 ± 96.2	175.5 ± 49.7
3.00	95.60	124.00	154.00	140.00	86.40	84.70	144.00	147.00	120.0 ± 33.9	123.9 ± 27.9
4.00	76.50	118.00	147.00	148.00	62.10	72.80	122.00	125.00	101.9 ± 39.4	116.0 ± 31.5
8.00	54.80	51.70	99.90	74.90	32.20	36.70	59.10	53.90	61.5 ± 28.1	54.3 ± 15.7
12.00	29.70	32.60	34.70	45.30	22.30	14.60	28.50	28.70	28.8 ± 5.1	30.3 ± 12.6
24.00	8.12	9.75	14.50	16.20	3.20	4.66	8.47	8.25	8.6 ± 4.6	9.7 ± 4.8
32.00	4.23	3.60	3.18	5.84	NM	NM	2.66	2.84	2.5 ± 1.8	3.1 ± 2.4
48.00	1.68	NM	NM	NM	NM	NM	NM	NM	NM	NM
72.00	NM	NM	NM	NM	NM	NM	NM	NM	NM	NM

*Concentrations obtained using the assay reported in this paper.
**Concentrations obtained using the assay reported in ref. 4.
***NM = non-measurable.

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Note

High-performance liquid chromatographic determination of imipramine and desipramine in human serum

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(First received April 5th, 1984; revised manuscript received July 31st, 1984)

Quantitative determination of antidepressants in blood from depressive patients has been suggested to be essential for pharmacotherapeutic and clinical investigations of depression [1—3]. The present studies were also attempted to establish a routine laboratory method capable of assaying simultaneously imipramine and its active metabolite, desipramine, in serum from the patients. Reversed-phase high-performance liquid chromatographic (HPLC) methods have now been evaluated to be time saving for routine monitoring of drugs as compared with methods such as normal-phase HPLC and gas chromatography. Since several reversed-phase HPLC methods for imipramine have already been presented from other laboratories [3—7], we re-examined such procedures. In our hands, the application of these methods produced chromatograms that did not allow quantitation of imipramine and desipramine, because of interference from unidentified peaks.

In the present methods following precipitation of proteins in serum with methanol, a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.) was used for purification of the HPLC sample. Our chromatographic quantitative analyses of imipramine and desipramine were simple and reproducible, demonstrating each single peak on the chromatogram with a peak of clomipramine as internal standard. Practically, the concentrations of both drugs were measured versus time in serum from patients and volunteers who were orally administered imipramine for two weeks.

EXPERIMENTAL

HPLC conditions

The equipment used was a Waters Model 6000A solvent delivery system,

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Model U6K universal injector, Data Module, Model 440 ultraviolet detector fixed at 254 nm. A reversed-phase column (15 cm × 4.6 mm I.D.; particle size 5 μm), Cosmosil $5C_{18}$ (Nakarai Chemicals, Kyoto, Japan) was employed. The operating conditions were as follows: mobile phase, acetonitrile—1% triethylamine (pH 6.0, adjusted with phosphoric acid) (38:62) at a flow-rate of 1 ml/min; injection volume, 50 μl; detector sensitivity, 0.005 a.u.f.s.

Sample preparation

Human serum was conventionally prepared by centrifugation and kept frozen at −20°C until the following procedures. Serum (1 ml) was transferred into a polypropylene centrifuge tube, and then 200 ng of clomipramine were added as internal standard, followed by addition of methanol (4 ml). The mixture was shaken for 1 min and centrifuged for 3 min at 11,000 *g*. An aliquot (4.3 ml) of the resultant supernatant was applied on a Sep-Pak C_{18} cartridge. After the cartridge was washed by 4 ml of 50% methanol, imipramine, desipramine and clomipramine were eluted with 2 ml of acetonitrile—1% triethylamine · HCl (9:1). When 200 ng of each drug were added to serum, approx. 65% of the amount was recovered in the eluate. The eluate was collected in a glass tube and evaporated under a stream of nitrogen gas. The residue was dissolved with 100 μl of the mobile phase and an aliquot (50 μl) was injected into the HPLC system.

Materials

Imipramine and clomipramine were donated by Dainihon Pharmaceutical (Osaka, Japan). Desipramine was a gift from Ciba-Geigy (Basel, Switzerland). Acetonitrile, methanol and triethylamine, which were purified for HPLC, were obtained from Nakarai Chemicals (Kyoto, Japan). The conventional distilled water was further purified by Milli-Q Reagent Water System.

RESULTS AND DISCUSSION

When a solution containing imipramine (25 ng), desipramine (25 ng) and clomipramine (100 ng) was subjected to HPLC, a chromatogram as shown in Fig. 1 was obtained with retention times of 7.6, 5.4, and 14.1 min, respectively, and each peak was sharp and symmetrical. Serum was obtained from a healthy volunteer receiving no drugs. To a portion of the serum were added three kinds of antidepressant as described above.. An HPLC sample was prepared from the serum with or without the drugs and injected for HPLC. As shown in Fig. 2, the peaks of desipramine, imipramine and clomipramine in human serum were quite distinguishable in the chromatogram and each retention time was in good agreement with that in Fig. 1. However, a small unidentified peak (peak a in Fig. 2) was observed near the peak of imipramine, whether the serum contained drugs or not.

Calibration curves were produced as follows. Working solutions were prepared by dissolving 10, 20, 30, 50, 100, 200, 300, and 500 ng each of imipramine and desipramine in 1 ml of human serum, which contained 200 ng of clomipramine. These solutions were processed, using the HPLC conditions as described in the experimental section. The ratios of the peak area of either

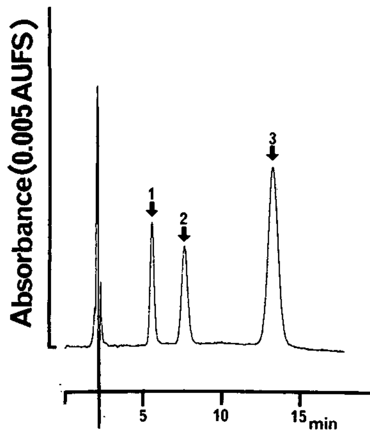

Fig. 1. Chromatogram of imipramine and desipramine with clomipramine. The standard HPLC conditions were used except that the mixture containing imipramine (25 ng), desipramine (25 ng) and clomipramine (100 ng) was directly injected into the HPLC system. The arrows at 1, 2 and 3 indicate the peaks of desipramine, imipramine and clomipramine, respectively. Each retention time is described in the text.

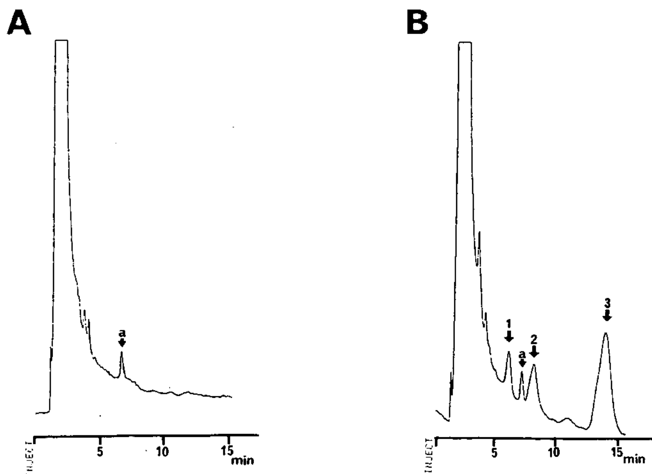

Fig. 2. Chromatograms of human serum in the absence (A) and presence (B) of antidepressants. The serum was obtained from a healthy volunteer with no medication. A 1-ml portion of the serum was spiked with desipramine (50 ng) and imipramine (50 ng) with clomipramine (200 ng). The sera with and without the drugs were used as HPLC samples. The other experimental conditions are described in the text. The arrows at 1, 2 and 3 are defined as described in Fig. 1. The arrow at "a" is an unidentified peak, of which the retention time was 6.8 min.

imipramine or desipramine to the area of clomipramine on the chromatogram were plotted versus the concentrations of imipramine or desipramine in the working solution. The curves exhibited linear relationships over concentrations of both drugs between 10 and 500 ng/ml, with a detection limit each of approx. 10 ng/ml. The reproducibilities of the calibration curves were determined by performing eight replicate analyses on aliquots of human serum, to which the tricyclic drugs were added. In four different concentrations (50, 100,

200, and 500 ng/ml) of the drugs in serum and the coefficients of variation were less than 7%, except that the variation was 11% for the lowest concentration of desipramine. For the quantitative determination of desipramine with better reproducibility than in the present methods, the use of two internal standards might be needed.

Two healthy volunteers and two depressive patients were orally administered imipramine for two weeks. The dose was 75 mg per day, divided into three equal doses at intervals of 8 h. Blood was sampled between 9.00 and 10.00

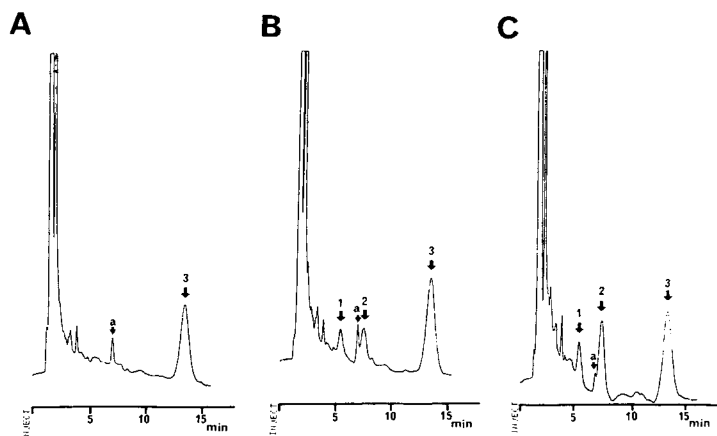

Fig. 3. Chromatograms of the serum from a healthy volunteer with no medication (A), and during the first (B) and second (C) weeks of continuous dosing with imipramine. Detailed experimental conditions are described in the text. The arrows in the chromatograms are as defined in Fig. 2.

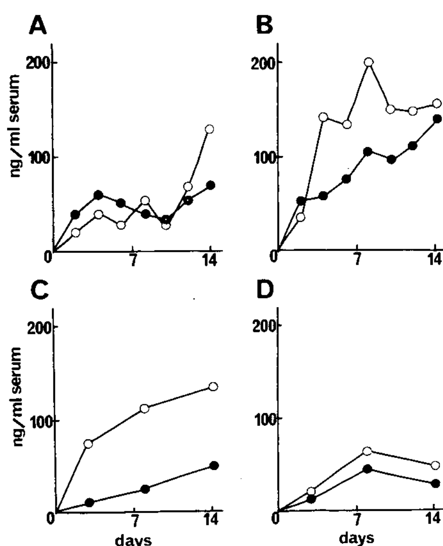

Fig. 4. Serum levels of imipramine (○) and desipramine (●) during the continuous dosing of imipramine. The abscissa shows the days after starting the administration. Two volunteers (A and B) and two depressive patients (C and D) were orally given the drug as described in the text. The other experimental conditions are described in the Experimental section.

a.m. Fig. 3 demonstrates chromatograms of the serum from one of the volunteers during the first and second weeks after the start of imipramine administration. The concentrations of imipramine and desipramine in the serum were calculated to be 53 and 40 ng/ml, respectively, in the first week. In the second week the values increased to 130 for imipramine and 70 for desipramine. The concentrations of both drugs in serum from two volunteers and two patients were quantitatively determined at various times during continuous dosing. The results are shown in Fig. 4, suggesting interindividual variation of the ratio of desipramine to imipramine and on the rates of increases in their serum levels.

Using a Sep-Pak C_{18} cartridge and a reversed-phase column, the present procedures were simple and reproducible with fewer unidentified peaks in the chromatogram. The usefulness of C_{18} bonded-phase disposable columns such as Sep-Pak C_{18} cartridges has been demonstrated for gas chromatographic analyses of tricyclic antidepressants [8, 9]. Also in the present methods the Sep-Pak C_{18} procedure was essential for the purification of the HPLC sample from serum. But a small unidentified peak (peak a) was always present near the peak of imipramine in the chromatogram and was suggested to originate from human serum itself. In the present methods the hydroxylated derivatives of imipramine and various kinds of minor tranquillizers such as benzodiazepines were washed out by 50% methanol from the cartridge and no peaks attributable to them were observed in the chromatogram.

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Note

Determination of clenbuterol in the high nanogram range in plasma of mice by high-performance liquid chromatography with amperometric detection

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Clenbuterol is a selective beta-2-adrenoceptor agonist in animal and man and has been suggested for the treatment of asthma [1]. Recently, an anti-depressive-like activity related to beta-stimulation has been demonstrated in animals [2]. Thus, clenbuterol has been suggested for human endogenous depression [3].

Various methods for the evaluation of beta-2-agonists, such as salbutamol, terbutaline and fenoterol [4—7] have been reported. No method for the measurement of clenbuterol in biological samples has yet been described. This is probably because of the low plasma levels (< 1 ng/ml) obtained after an oral dose in humans. Experimentally the efficacy of clenbuterol leads to dosing schedules in the order of 10—40 μg for asthma and about 100 μg for depression.

The recent observation of its antidepressive-like activity in animals necessitated the determination of plasma clenbuterol concentrations for a better understanding of its effect. This has led us to develop a method of measuring clenbuterol in biological fluids. This paper proposes a method sufficiently sensitive for the analysis of clenbuterol in plasma of mice using reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection (ED).

EXPERIMENTAL

Animals and blood-sampling procedure

Male Swiss NMRI mice weighing 20—25 g were used. Blood was drawn from

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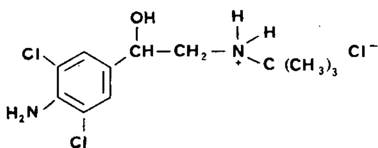
the orbital sinus and collected in heparinized tubes. After centrifugation plasma was frozen in polypropylene tubes and kept at −20° C until assay.

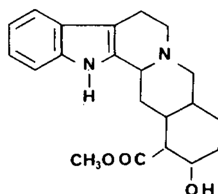
Chemicals and drugs

Clenbuterol hydrochloride (NAB 365) (Fig. 1) (Boehringer, Ingelheim, France) and the internal standard, yohimbine hydrochloride (Fig. 1), (Sigma, St. Louis, MO, U.S.A.) were used.

Standard solutions of clenbuterol and internal standard were prepared by dissolution in methanol at concentrations of 1 and 10 μg/ml.

All reagents used were of analytical grade: methanol (Prolabo, France), chloroform (U.C.B., Belgique), monobasic ammonium phosphate (Sigma), sodium hydroxide (Prolabo) and orthophosphoric acid (Prolabo).





CLENBUTEROL INTERNAL STANDARD

Fig. 1. Chemical structures of clenbuterol and internal standard.

Apparatus

The chromatographic system consisted of a Model A 802 solvent delivery pump (Lirec, France), and a Rheodyne sample valve fitted with a 50-μl loop. The column was a Resolve RP18, 5 μm particle size (15 cm × 3.9 mm I.D.) (Waters Assoc., Milford, MA, U.S.A.). A guard column packed with C_{18} material was used to protect the analytical column.

A Metrohm ED system composed of a 641 VA detector, a 656 electrochemical detector equipped with a glassy carbon electrode and an Ag/AgCl reference electrode was used to oxidize the compounds at a potential of 1.15 V. The sensitivity was set at 10 nA full scale. All chromatograms were recorded on a Servotrace recorder (Sefram, France) at a chart speed of 5 mm/min.

Mobile phase

The mobile phase consisted of 1 m*M* ammonium phosphate—methanol (10:90, v/v). The pH was adjusted to 5.0 by adding orthophosphoric acid. The flow-rate was kept constant at 1 ml/min. The mobile phase was thoroughly degassed and filtered through a 0.2-μm filter disc (Millipore, Bedford, MA, U.S.A.).

Extraction

To 500 μl of plasma of mice were added 80 μl of a solution of 1 μg/ml of internal standard, 0.5 ml of 0.1 *M* sodium hydroxide and 6 ml of chloroform. The mixture was shaken for 20 min using an alternating agitator (Realis type

44-40, France) or for 1.5 min using a Vortex mixer (Bioblock, France). The solution was then centrifuged for 10 min at 900 *g* at −2°C and the supernatant discarded. The lower organic phase was transferred to a clean tube and then evaporated to dryness using a Vortex evaporator (Buchler, NJ, U.S.A.). The residue was dissolved in 100 µl of the mobile phase; a 25-µl aliquot was injected into the chromatograph.

Calibration curves

The calibration curves were obtained by adding clenbuterol to mouse control plasma to obtain concentrations of 10, 20, 40, 80, 120, 160 and 200 ng/ml. These standards were extracted under the experimental conditions as described above.

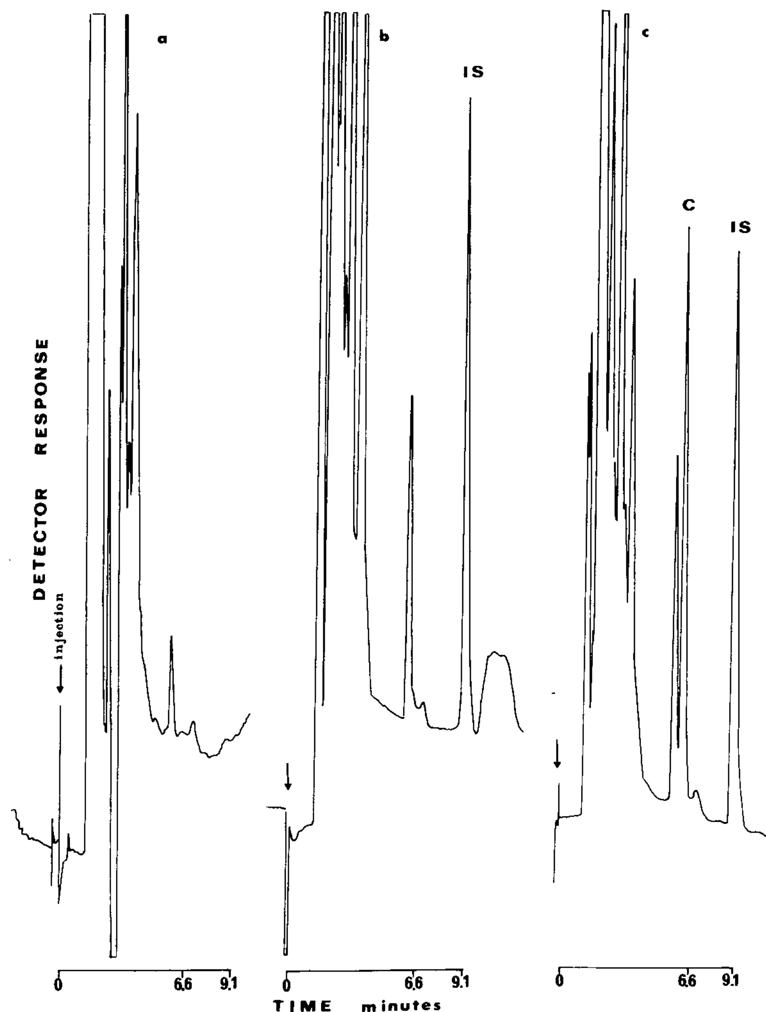


Fig. 2. Typical chromatograms obtained from mouse plasma after injection of (a) blank plasma control, (b) plasma control spiked with internal standard (IS), and (c) plasma control spiked with internal standard and 140 ng/ml clenbuterol (C).

The peak heights were measured and the peak height ratios of clenbuterol over internal standard were plotted against concentration.

RESULTS

Fig. 2 illustrates typical chromatograms obtained after extraction of blank and spiked plasma of mice. The retention times for clenbuterol and internal standard are 6.6 and 9.1 min, respectively (capacity coefficients $k' = 4.0$ and 6.0, respectively). Fig. 3 illustrates chromatograms obtained from plasma of mice collected 30 min after intraperitoneal administration of 0.5 mg kg^{-1} clenbuterol.

Calibration curve shows good linearity (correlation coefficient 0.9969 ± 0.0026) in the range 0—200 ng/ml. The equation of the curve is $Y = 0.013072 (\pm 0.0007)X - 0.0122255 (\pm 0.0212)$.

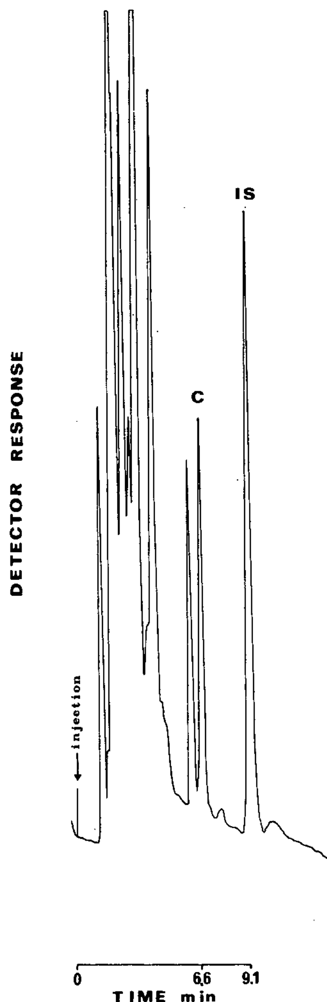


Fig. 3. Chromatogram obtained from mouse plasma 30 min after a 0.5 mg kg^{-1} intraperitoneal administration of clenbuterol.

Precision

The reproducibility of the method was checked for three plasma concentrations: 40, 120, and 200 ng/ml. Ten determinations were made on the same day.

The coefficients of variation are shown in Table I. The day-to-day reproducibility was assessed at 40, 120 and 200 ng/ml over a period of five days. The coefficients of variation are shown in Table I.

TABLE I

REPRODUCIBILITY AND ACCURACY OF HPLC—ED ASSAY FOR CLENBUTEROL

Clenbuterol (ng/ml)	Coefficient of variation (%)	
	Reproducibility	Accuracy (day-to-day)
40	4.41	5.96
120	8.24	7.56
200	3.57	6.69

Recovery

Recovery of clenbuterol was assessed by comparing the peak height after an injection of a pure solution of clenbuterol with that obtained after an injection of extracted plasma containing the same amount of clenbuterol. The percentage recovery of the extraction procedure is shown in Table II.

TABLE II

RECOVERY OF CLENBUTEROL FROM MOUSE PLASMA

Clenbuterol (ng/ml)	Recovery (%)
40	46.25 ± 0.85
120	46.51 ± 1.20
200	43.6 ± 0.99
$\overline{X}$	45.45 ± 1.13

Sensitivity

Under the experimental conditions described above the minimum detectable concentration is 3 ng/ml. But either by increasing the volume injected into the chromatograph or by setting the sensitivity of the detector at 5 nA full scale the limit of detection is 1 ng/ml.

DISCUSSION

This method, with a limit of detection of 3 ng/ml, is sufficiently sensitive for the determination of clenbuterol levels in plasma of mice. It requires a small volume of sample and so allows measurement in an individual mouse, avoiding the need to pool several plasmas. In our laboratory the method has been applied

to study the pharmacokinetics of clenbuterol after intraperitoneal administration in mice (0.5 mg kg^{-1}).

A good resolution of the chromatogram is obtained with a 5-μm Resolve™ column. Compared to a standard one, such a column gives a better sensitivity. However, it is tremendously sensitive to very small modifications in the mobile phase (which then involves significant changes in resolution and retention times).

This procedure shows a good reproducibility; however, before obtaining reproducible results some care must be taken. For example, the sensitivity of the working electrode gradually declines and cleaning of this electrode is necessary to restore it. If a rapid decrease in the response occurs immediately after cleaning, it is essential to stabilize the electrode until the response of the detector is constant. For better results, a new column must be conditioned with the mobile phase before use for 24 h; if this is not done, modifications in the retention times occur.

Many chromatographic problems arose from the extraction and purification of mouse samples. Clenbuterol is not extracted by non-polar solvents but its extraction by polar solvents leads to chromatograms containing endogenous contaminants. The solvent chosen (chloroform) yielded chromatograms containing fewer contaminants and was used for extraction although the percentage recovery of clenbuterol was low. However, the recovery of clenbuterol proved to be constant and unaffected by the concentration of the drug.

The choice of yohimbine as internal standard seems unusual at first sight since its chemical structure is different from that of clenbuterol and more related compounds can be obtained. But according to the chromatographic conditions which were chosen to obtain the best response of clenbuterol (consistent with a sufficiently short retention time), yohimbine has the advantage of being well separated from clenbuterol and from other peaks. Other beta-agonists, such as salbutamol and isoproterenol, tested as internal standard cannot be separated from endogenous peaks. Terbutaline showed a retention time consistent with a good separation, but it was not well extracted in chloroform whatever the pH. Ethyl acetate, which can be used for the extraction of terbutaline [5], was also tried. But such a solvent cannot be conveniently used with electrochemical detection; many chromatographic problems occur, endogenous plasma components appearing in the chromatogram and interfering with terbutaline and clenbuterol.

Thus, our procedure performed as described above can only be used for the determination of clenbuterol. With a few modifications to the mobile phase, it can be applied for determinations of other beta-2-agonists. Thus, this method does not allow the simultaneous measurement of different beta agonists.

In summary, the technique developed using HPLC—ED is reproducible, and is sensitive enough for the determination of clenbuterol in mouse plasma. It is easy to perform and is inexpensive.

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CHROMBIO. 2319

Note

Sensitive high-performance liquid chromatographic determination of 6-mercaptopurine, 6-thioguanine, 6-mercaptopurine riboside and 6-thioguanosine in biological fluids

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For parallel studies on 6-mercaptopurine (6MP) and 6-thioguanine (6TG), a system is desirable to measure both drugs, as well as their metabolites 6-mercaptopurine riboside (6 MPR) and 6-thioguanosine (6TGR). The method we described previously [1] is not suitable for measuring 6TG and 6TGR, because 6TG was not separated from 6MP, and because the peak of 6TGR was very broad. Other methods for the determination of 6-thiopurines have been reported, but are less suitable for our purposes either because the lower limit of detectability is not low enough [2—6] or because extraction of the samples is too time-consuming [7—10]. In the present paper we describe a high-performance liquid chromatographic (HPLC) method for the identification and quantitation of 6MP, 6TG, 6MPR and 6TGR in plasma, cerebrospinal fluid (CSF) and urine.

EXPERIMENTAL

Chemicals

6MP and 6TG were products of Fluka (Hicol, Rotterdam, The Netherlands); 6MPR and 6TGR were from Sigma (St. Louis, MO, U.S.A.); xanthine oxidase and dithiothreitol (DTT) were from Boehringer (Mannheim, F.R.G.); all other chemicals were from E. Merck (Darmstadt, F.R.G.); helium was from Hoekloos (Amsterdam, The Netherlands). Water used for all solutions was purified through a Milli-Q-System (Millipore, Bedford, MA, U.S.A.).

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HPLC procedure

Experiments were performed on a Spectra Physics HPLC SP 8000B (Spectra Physics, Santa Clara, CA, U.S.A.), connected to an automatic sampler MSI 660 (Kontron, Electrolab, London, U.K.). Column effluents were monitored with a fixed-wavelength ultraviolet (UV) detector SP 8210 (Spectra Physics) at 312 nm and a variable-wavelength UV/VIS detector Model 770 (Spectra Physics) set at 342 nm. Detector signals were plotted on a two-channel printer—plotter of the HPLC apparatus. Peak areas were correlated to concentrations, as will be discussed later.

The columns were packed in our laboratory with Nucleosil 10 C_{18}, particle size 10 μm (Chrompack, Middelburg, The Netherlands). During packing a reservoir was connected directly on top of the column tube, and a slurry of Nucleosil (3 g of Nucleosil in 10 ml of methanol) was pumped into the column (250 mm × 4.6 mm I.D.) by means of the HPLC pump. Methanol 50% (v/v) was used as eluent and the pressure was kept at about 21 MPa by controlling the flow-rate.

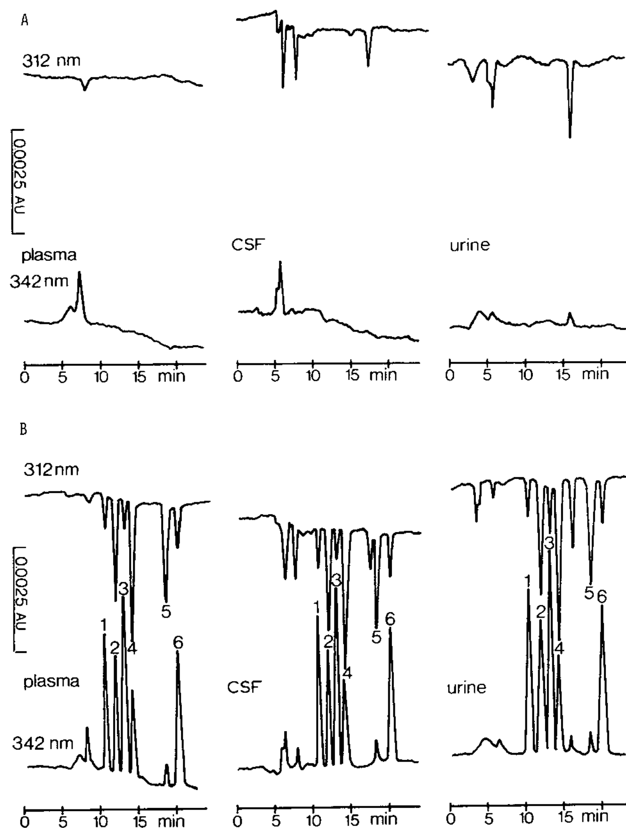

Fig. 1. Scan patterns of separations of 6-thiopurines, following the mobile phase sequence of Table I (injected volume 195 μl). (A) Blanks of plasma, CSF and urine samples; (B) plasma, CSF and urine samples, spiked with 6TU (1), 6TX (2), 0.7 μM 6TG (3), 0.4 μM 6MP (4), 0.4 μM 6MPR (5) and 0.7 μM 6TGR (6). 6TX and 6TU are products of xanthine oxidation reaction on 6MP. The absorption is expressed in absorbance units (AU). The scale is indicated in the figures.

TABLE I

MOBILE PHASE SEQUENCE USED FOR SEPARATION OF 6-THIOPURINES

A = 0.025 *M* phosphoric acid, pH 2.75; B = methanol 50% (v/v); and C = 0.10 *M* potassium dihydrogen phosphate, pH 6.6.

Time (min)	A (%, v/v)	B (%, v/v)	C (%, v/v)
0.0	100.0	0.0	0.0
5.0	98.0	2.0	0.0
10.0	30.0	3.5	66.5
20.0	30.0	3.5	66.5

Chromatography was carried out on two columns in series, at a constant flow-rate of 1.7 ml/min and at a temperature of 33°C (in a water bath).

To achieve a separation such as seen in Fig. 1, we eluted with 0.025 *M* phosphoric acid (pH 2.75), methanol 50% (v/v) and 0.10 *M* potassium dihydrogen phosphate (pH 6.6) following the mobile phase sequence of Table I. The eluents were degassed before and during HPLC runs by continuous helium purging. Before use, solutions were filtered through a Millipore filter (type HA, pore size 0.45 μm). Total run time (including equilibration time for the next run) was 40 min. It is advisable to wash columns with methanol 50% (v/v) after several runs. Depending on the injected volume, 195 μl or 500 μl, we wash the columns after every twenty or ten runs, respectively.

Stock solutions

The 6-thiopurines were dissolved in 0.025 *M* potassium dihydrogen phosphate and the solutions were adjusted to a pH of about 11 with 4.0 *M* potassium hydroxide. After the 6-thiopurines had dissolved completely, the solutions were re-adjusted to a pH between 6.5 and 7.0 with 4.0 *M* phosphoric acid and diluted to a thiopurine concentration of 0.1 mg/ml. Before addition of DTT (final concentration 60 mg/l), 20 μl of each solution were diluted to 500 μl with 0.05 *M* potassium dihydrogen phosphate pH 4.6, and the exact thiopurine concentrations were determined spectrophotometrically.

Parameters on which the exact concentrations were calculated are [11]: 6MP: λ_{max} = 322 nm, ϵ_{max} = 21.5 mM^{-1} cm^{-1}. 6TG: λ_{max} = 342 nm, ϵ_{max} = 25.6 mM^{-1} cm^{-1}. 6MPR: λ_{max} = 322 nm, ϵ_{max} = 27.6 mM^{-1} cm^{-1}. 6TGR: λ_{max} = 342 nm, ϵ_{max} = 26.7 mM^{-1} cm^{-1}.

Stock solutions kept at a temperature of 4°C were stable for at least two weeks.

Enzymatic preparation of 6-thioxanthine (6TX) and 6-thiouric acid (6TU)

6-Mercaptopurine was catabolized enzymatically by xanthine oxidase (EC 1.2.3.2) into 6TX and 6TU according to the procedure described by the manufacturer of the enzyme.

Sample preparation

Venous blood samples of 2 ml were collected in tubes containing heparin

plus 120 μg of DTT. After mixing, the blood samples were centrifuged (5 min, 2000 *g*) and plasma was pipetted into micro test-tubes (type 3810, Eppendorf, Hamburg, F.R.G.). CSF samples of 0.5 ml were collected in micro test-tubes containing 30 μg of DTT. Plasma and CSF samples were put on ice and deproteinized by adding one-tenth of the sample volume of freshly prepared ice-cold 50% (w/v) trichloroacetic acid (TCA).

From the urine samples, 2 ml were taken and pipetted into tubes containing 120 μg of DTT. The urine samples were filtered through a Millipore filter (pore size 0.22 μm) before measurement.

DTT was added to increase the stability and the recovery of the assay [1, 8]. If not measured immediately, extracts of all samples were stored refrigerated and analysed within two weeks, since with older solutions additional absorption peaks were observed.

Thiopurine solutions for intravenous injection

Solutions for intravenous injections were freshly prepared; 6MP or 6TG was dissolved in 0.178 *M* sodium bicarbonate to which sodium hydroxide was added until the thiopurine had dissolved completely. The pH of the final solution was about 10. Intravenous injection was performed through a sterile Millipore membrane filter, type Millex GS (pore size 0.22 μm).

RESULTS AND DISCUSSION

Stability and recovery

As discussed previously [1] thiopurines are poorly soluble at low pH. At basic pH solubility is much better, but oxidation of the SH group has to be prevented by addition of, for example, DTT [1, 8]. With the extraction procedure used, the recovery of thiopurines added to plasma is 94% [1].

Quantitation and calibration

Calibration curves were made using standard solutions of known concentrations. Peak areas of 6MP and 6MPR were integrated at 312 nm, peak areas of 6TG and 6TGR were integrated at 342 nm. Peak areas were calculated in mm^2 : peak area = peak height × peak width at half height. The relationships we found between concentrations and integrated areas and the corresponding

TABLE II

CALCULATION FACTORS FOR CALIBRATION CURVES OF 6-THIOPURINES

Concentrations are given in μ*M*, areas in mm^2, optical scale = 0.01 absorbance units at 10 mV, and injected volume is 195 μl.

Thiopurine	Concentration factor	Correlation coefficient
6MP	Conc. = (area × 0.006938) + 0.002873	0.9999
6MPR	Conc. = (area × 0.009792) − 0.028761	0.9989
6TG	Conc. = (area × 0.008236) − 0.001824	0.9997
6TGR	Conc. = (area × 0.009714) + 0.021745	0.9995

correlation coefficients are given in Table II. With an injected volume of 500 μl, we found a lower limit of detection for 6MP, 6TG, 6MPR and 6TGR of 20 n*M*, 25 n*M*, 65 n*M* and 60 n*M*, respectively.

For 6TX and 6TU, no calibration curves have been made because of the lack of purified 6TX and 6TU.

Accuracy, precision and reproducibility

The accuracy of the method was evaluated by analysing plasma samples containing known amounts of 6MP, 6MPR, 6TG and 6TGR in a concentration range of 100—5000 n*M*. The 95% confidence intervals for single determinations of the thiopurines in plasma were calculated, using the *t*-value from a one-tailed Student's *t*-distribution table and the variance of absolute differences between the actual concentrations (100, 250, 500, 1000, 2000 and 5000 n*M*, respectively) and the concentrations found. The results indicate that any found value would fall within approx. 17.3%, 18.6%, 21.4% and 25.9% of its true value in experiments with 6MP, 6TG, 6MPR and 6TGR, respectively. The validation of

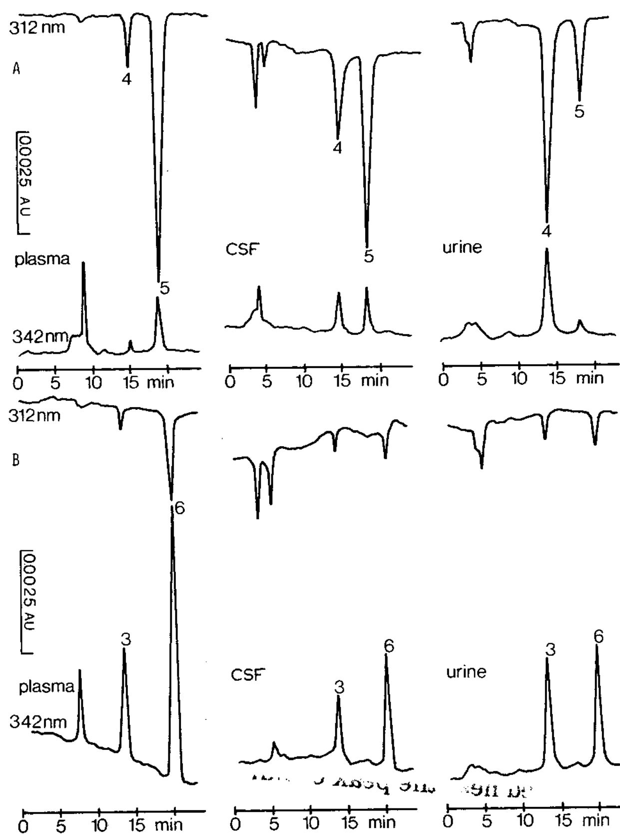

Fig. 2. Scan patterns of plasma, CSF and urine samples (injected volume 195 μl) of a goat, 4 h after an intravenous injection. (A) 6MP (20 mg/kg body weight), urine sample is diluted 5000 times before injection; (B) 6TG (5 mg/kg body weight), urine sample is diluted 25 times before injection. For absorbance units and for identification of the peaks, see legend of Fig. 1.

the method was applied to plasma at the concentrations mentioned above, by determination of the precision (within-day variability) and of the reproducibility (day-to-day variability).

The coefficient of variation for the within-day variation (n = 5) at any concentration of 6MP, 6TG, 6MPR and 6TGR was in the range 1.4—1.7%, 2.1—8.7%, 2.8—10.6% and 3.2—12.5%, respectively. The day-to-day variation (days 1—3) for the same set of data was in the range 2.3—6.0%, 2.8—7.3%, 4.5—9.4% and 4.9—11.8% for 6MP, 6TG, 6MPR and 6TGR, respectively.

Analysis in biological fluids

The method has been applied to the measurement of concentrations of thiopurines in several body fluids after administration of 6MP or 6TG to a goat by an intravenous bolus injection. Scan patterns of analysis in plasma, CSF and urine show the presence of 6MP and 6MPR (Fig. 2A). Chromatographic plots of samples taken after administration of 6TG are given in Fig. 2B. In Fig. 3A and B, time-dependent concentration curves can be seen after push injection of 6MP and 6TG, respectively.

The concentrations of the ribosides of both 6MP and 6TG are higher than the concentrations of the parent compounds. The concentration—time curves of the parent drugs only are far short of representing the total biologically active compounds, at least in goats. In man, it was demonstrated that 6MP and 6MPR are equitoxic at equimolar doses, when given intravenously [12].

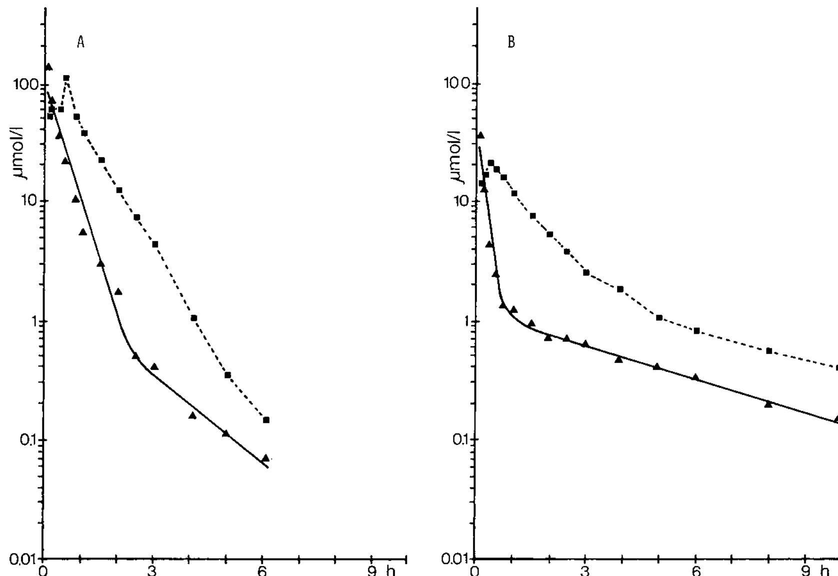

Fig. 3. Concentration—time curves of plasma levels in a goat after an intravenous injection. (A) 6MP (20 mg/kg body weight); (▲) 6MP, (■) 6MPR. (B) 6TG (5 mg/kg body weight); (▲), 6TG; (■), 6TGR.

CONCLUSIONS

In this paper we describe an HPLC procedure to measure 6MP, 6TG, 6MPR

and 6TGR in one single run. The sensitivity of the method enables the pharmacokinetic behaviour of 6-thiopurines in plasma, CSF and urine to be followed for several hours. Since the introduction of HPLC many applications for thiopurines have been reported [2—10]. Several extraction methods do not seem optimal since low recoveries are found [8, 9]. To prevent oxidation of the mercapto (SH—) group of the thiopurines, DTT is added to standards and samples [1, 8]. Other measures have to be taken against oxidation of the thiopurines. Therefore the extraction procedure should be kept short and simple, and should be performed in the cold. The stabilizing effect of DTT increases recovery to 94% [1], and together with all other optimized conditions of sample preparation and chromatography a sensitive and reliable method has been created to measure 6-thiopurines. From our study it may be concluded that all studies on pharmacokinetics of 6MP and 6TG in all kinds of species, including man, should be completed by involving all biologically active derivatives of 6-thiopurines.

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CHROMBIO. 2320

Note

Modified extraction and chromatography for the measurement of plasma melphalan by ion-pair high-performance liquid chromatography

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Melphalan (Alkeran®) is currently used in the treatment of several malignant disorders, including multiple myeloma and carcinoma of the breast, ovary and testis [1]. Although the effectiveness of the drug in the therapeutic management of these patients is unquestionable, several potentially serious side effects, primarily related to bone marrow toxicity, may occur [2]. Consequently, the optimisation of melphalan therapy must take into account the predisposition of the patient towards side-effects [3]. Despite awareness of this problem for over 20 years, very little pharamacokinetic data are available on melphalan, primarily due to difficulties in measuring the low plasma concentrations of the drug following oral therapeutic doses (5—15 mg daily).

Recently, several methods have been described for the measurement of melphalan which provide acceptable chromatography and sensitivity at therapeutic levels [4—6]. Based on these reports we describe a modified assay which allows ultraviolet (UV) determination of the drug at concentrations (5 ng/ml) previously requiring fluorescence detection [4]. This procedure gives an acceptable resolution value with a relatively short retention time (9.5 min). Sample clean-up involves precipitation of plasma macromolecular components with concentrated perchloric acid [5] and extraction of drug from the supernatant by C_{18} Sep-Pak [4] which are straightforward in preparation and use.

MATERIALS AND METHODS

Instrumentation

High-performance liquid chromatography (HPLC) equipment from the Pye Unicam PU 4000 system (Cambridge, U.K.) incorporated a dual reciprocating pulseless pump and a variable-wavelength UV detector set to 260 nm (the mea-

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sured λ_{max} of melphalan in methanol). Detector sensitivity was set at 0.08 a.u.f.s. Chromatograms were recorded on a Pye CDP4 computing integrator with 100-mV input f.s.d. The column was Spherisorb ODS 5 μm particle size, 250 × 4.6 mm I.D., from Phase Separations (Queensferry, U.K.). Both column and Rheodyne 7125 injection valve (Cotati, CA, U.S.A.) with a 200-μl loop were mounted in a block heater (Jones Chromatography, Cardiff, U.K.) and maintained at 40°C. The guard column (3 cm) packed with LiChrosorb ODS (10 μm particle size) was also enclosed in the block heater.

Mobile phase

The mobile phase was a mixture of 80% methanol (Fisons, Loughborough, U.K.), 20% water and 0.0135% (w/v) sodium dodecyl sulphate (BDH, Poole, U.K.) as counter ion [6]. Mobile phase pH was adjusted to 3.11 using sulphuric acid. All materials and reagents were of HPLC grade and were filtered, when appropriate, with a 0.5-μm Millipore filter (Millipore, Bedford, MA, U.S.A.) prior to use. The flow-rate of the mobile phase was 1.3 ml/min.

Sample preparation and extraction

Whole blood (6 ml) was taken from a patient with an indwelling venous catheter and stored in blood collection tubes with lithium heparin anticoagulant. After centrifugation (1500 *g*; 10 min), 3-ml aliquots of plasma were removed and the macromolecular components precipitated with 132 μl [5] of cold concentrated perchloric acid (−20°C). The mixture was vortex-mixed for 5 min prior to centrifugation (−6°C; 1300 *g*; 15 min) in an MSE Coolspin (Fisons). The clear supernatant was removed and passed through a C_{18} reversed-phase Sep-Pak (Waters Assoc., Taunton, MA, U.S.A.) [4] which was subsequently washed with 10 ml of 15% methanol in water (4°C). The melphalan was eluted from the Sep-Pak with 2 ml of cold methanol (−20°C) [4]. The eluate was stored at −20°C prior to injection of 200-μl aliquots onto the column.

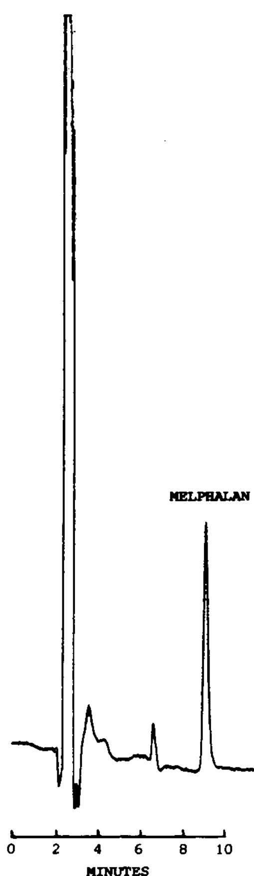
RESULTS AND DISCUSSION

Methanol has been used previously to precipitate the macromolecular components of plasma in an HPLC assay for melphalan, utilising fluorescence detection [4]. However, when this procedure was used in conjunction with UV detection, the method was unsatisfactory, due to superimposition of interfering peaks arising from plasma components onto the peak of interest. Precipitation with cold concentrated perchloric acid overcame this problem, in that most extraneous UV-absorbing material from the plasma was removed and resolution improved.

It was found that the packing material of the guard column had a significant effect on the separation of melphalan. A 5-cm Co:Pell ODS (30—38 μm particle size) guard column resulted in poor resolution. Increasing the pH of the mobile phase above 3.10 improved resolution somewhat but beyond pH 3.20 the retention time of the plasma components also lengthened. The best results were obtained using a 3-cm Pye LiChrosorb RP-18 (10 μm particle size) guard cartridge or a 5-cm Spherisorb ODS (10 μm particle size) guard column. Optimum retention times were in the region of 9.5 min. The measured resolution for

melphalan from the nearest plasma component was 2.15 (Figs. 1—4). Extraction of the drug with Sep-Pak cartridges [4] dispensed with the necessity to ultracentrifuge in order to remove solid material suspended in the eluate. The particulate material was either retained on the inlet plug of the Sep-Pak or removed by washing. This sample clean-up procedure is sufficiently consistent to allow the determination of sample concentrations from a standard addition calibration graph, using plasma spiked with 50, 100 and 150 ng/ml, each chromatographed in duplicate.

The coefficient of variation for ten concentration duplicates (10—2000 ng/ml) extracted from plasma was 2.67%. The correlation coefficient for the curve was 0.99. Within-batch variability for standard solutions of 20, 200 and 2000 ng/ml, each chromatographed ten times was 6.7%, 1.5% and 0.5%, respectively. Recovery of drug from plasma was in the order of 60% with a limit of detection of 5 ng/ml.



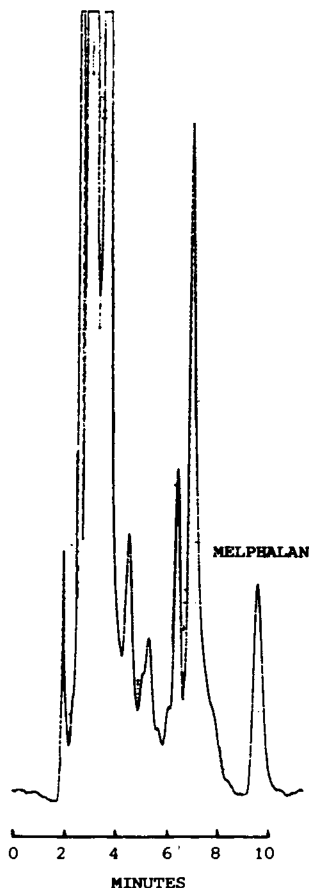


Fig. 1. Chromatography of 100 ng/ml melphalan in methanol.

Fig. 2. Extract of plasma spiked with 100 ng/ml melphalan. The anticoagulant citrate phosphate dextrose resulted in poorer resolution than lithium heparin, which was used in subsequent patient studies.

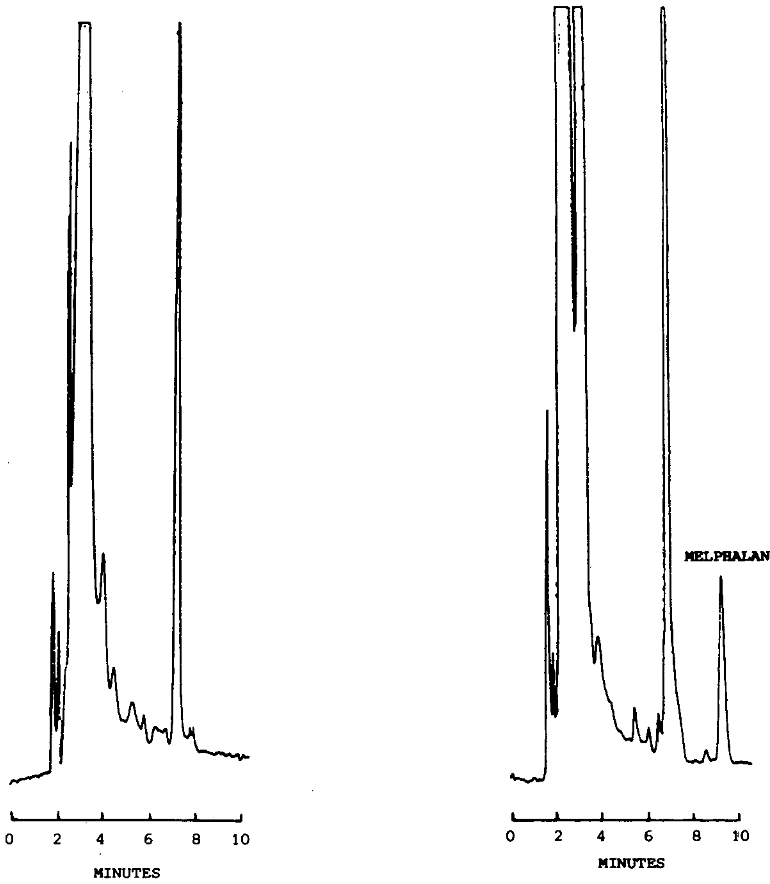


Fig. 3. Extract of a patient plasma. Sample before melphalan administration.

Fig. 4. Extract of a patient plasma sample taken 90 min after oral administration of 12 mg melphalan.

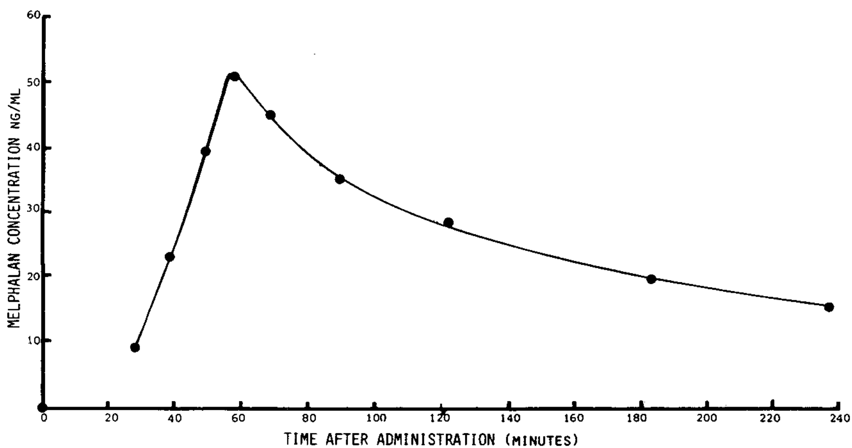


Fig. 5. Plasma levels of melphalan in a patient given 12 mg of oral melphalan. Patient samples were taken at intervals over a period of 4 h.

Using this method we have determined plasma levels of melphalan in six patients with multiple myeloma receiving 10 mg of oral melphalan daily for four days. Samples of peripheral blood taken at intervals over 4 h were stored in an ice bath to minimise drug degradation prior to analysis. Peak plasma concentrations of melphalan ranging from 34 to 190 ng/ml occurred 34—120 min after administration. Five patients exhibited normal time—concentration drug profiles similar to that shown in Fig. 5. Melphalan could not be detected in the plasma from one patient. Chromatography of methanolic solutions of prednisone and prednisolone confirmed that concurrent medication did not interfere with the assay for melphalan.

CONCLUSION

In this paper we have described a simple and rapid technique for the determination of melphalan in plasma. The use of UV spectrophotometry and a modified extraction technique enables a limit of detection (5 ng/ml based on four times baseline noise) which previously required fluorescence detection. We are currently using this assay to monitor patients with multiple myeloma receiving melphalan chemotherapy and for elucidating the pharmacokinetics of melphalan.

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CHROMBIO. 2308

Note

High-performance liquid chromatographic determination of etoposide in plasma using electrochemical detection

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Etoposide [4′-demethylepipodophyllotoxin 9-(4,6-O-ethylidene-β-D-glucopyranoside), I] is a semisynthetic derivative of podophyllotoxin and is increasingly used in the treatment of a variety of malignant conditions [1]. Etoposide inhibits nucleoside transport within cells and thereby interferes with DNA and RNA synthesis. In addition breaks in DNA strands and inhibition of protein synthesis have been demonstrated [2].

The pharmacokinetics of I were demonstrated initially by Allen and Creaven [3] using the tritium-labelled drug, and subsequently by a number of investigators using high-performance liquid chromatography (HPLC) [4—6]. These chromatographic methods have included ultraviolet absorption and spectrofluorometry which provide detection limits from 0.03 to 0.5 μg/ml etoposide in plasma. Electrochemical detection, however, enables these limits to be decreased at least ten-fold [6]. A simple, rapid assay for I with electrochemical detection using teniposide [4′-demethylepipodophyllotoxin 9-(4,6-O-thenylidene-β-D-glucopyranoside), II] as internal standard in plasma is described which significantly decreases the retention times for I and II previously described using electrochemical detection [6, 7].

EXPERIMENTAL

The system comprised a Constametric Model III pump (Laboratory Data Control), an ESA Model 5100A electrochemical detector, a Model 5010 analytical cell and a Model 5020 guard cell. A Waters Assoc. 30 cm × 4.9 mm I.D. stainless-steel column containing 5-μm Bondapak phenyl was used.

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Reagents

Methanol was liquid chromatographic grade (Fisons) and 0.05 *M* phosphate buffer (pH 7.0) was prepared using glass-distilled water. The mobile phase was filtered through a 0.22-μm Millipore membrane immediately before use. 1,2-Dichloroethane (AnalaR) was supplied by BDH and pure I and II were kindly provided by Bristol Myers (U.K.).

High-performance liquid chromatography

The isocratic mobile phase was methanol—phosphate buffer (60:40) at a flow-rate of 1 ml/min. The potential difference across the analytical and guard cells was set at 800 mV.

Extraction procedure

Plasma (1 ml) containing 10 μg each of I and II was placed in a 100 mm × 18 mm "Quickfit" glass tube and 4 ml of 1,2-dichloroethane were added. The solution was mixed for 10 min using an Eschmann Rotamix and then centrifuged at 650 *g* for 5 min. The organic phase was removed and evaporated to dryness in a water bath at 90°C. The residue was reconstituted with 0.4 ml of the mobile phase and 20-μl aliquots were applied to the liquid chromatograph.

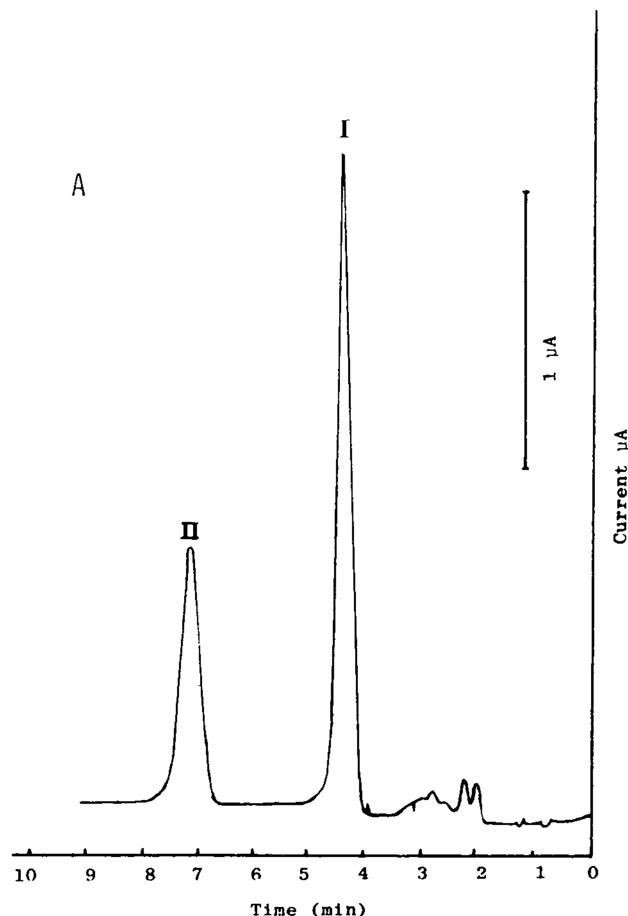

Fig. 1.

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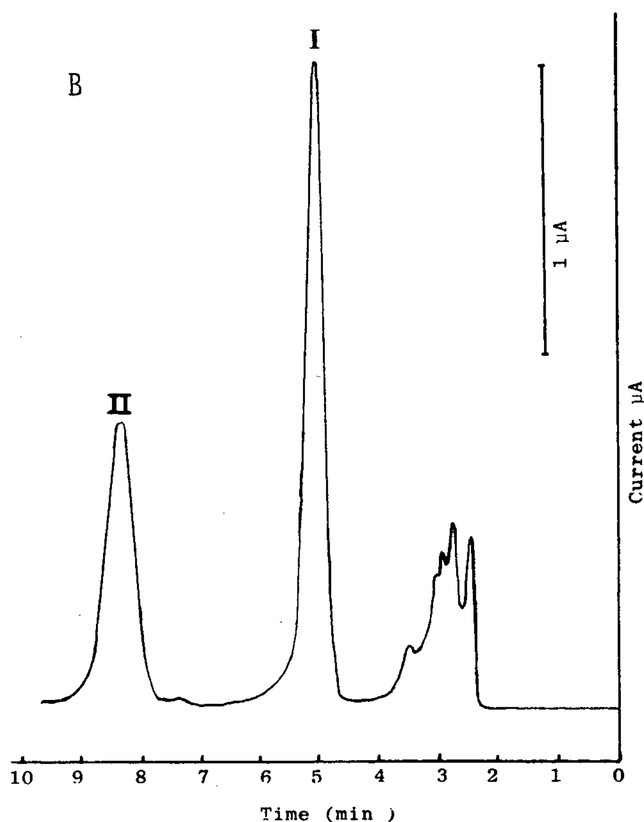

Fig. 1. (A) High-performance liquid chromatogram of human plasma spiked with 10 μg each of etoposide and teniposide. (B) High-performance liquid chromatogram of plasma after the administration of etoposide to the patient.

RESULTS AND DISCUSSION

Optimum conditions for chromatography were found to be different from those cited by Evans et al. [7]. A mobile phase of water—acetonitrile—acetic acid (74:25:1) at a flow-rate of 1 ml/min produced retention times for I and II of 13 and 20 min, respectively, whereas using a mobile phase of methanol—phosphate buffer (60:40) retention times of 5 and 9 min were obtained. Differing proportions of methanol relative to the phosphate buffer were investigated. A ratio of 60:40 produced optimum chromatography with short retention times and good resolution between I and II (Fig. 1).

A number of organic solvents including 1,2-dichloroethane, dichloromethane, chloroform and ethyl acetate were investigated for the extraction of I and II from plasma. The extraction percentage [8] was greater than 90% for each solvent (Table I) but 1,2-dichloroethane produced the fewest interfering peaks on the chromatogram. The oxidative potential of 800 mV was determined from analysis of the voltametric curves of I and II which demonstrate that oxidation for both these compounds is a two-electron process with the second electron released at 650 mV and a plateau reached at 800 mV.

TABLE I

EXTRACTION PERCENTAGES OF I AND II FOR DIFFERENT SOLVENTS

Organic solvent	Percentage extraction	
	I	II
1,2-Dichloroethane	93	94
Dichloromethane	92	93
Chloroform	93	92
Ethyl acetate	92	93

Pools of plasma containing 1, 5, 10, 20, 30 and 50 μg/ml of I were prepared and 1-ml aliquots of each pool were assayed as described above after addition of a similar concentration of II. Ten samples from each pool were assayed in duplicate and the results obtained from the peak height ratios of I and II produced a linear calibration curve (correlation coefficient r = 0.9987) for 2—500 ng of I applied to the column.

The lower limit of detection of I was 0.005 μg/ml. The coefficients of variation were found to be 6.93%, 4.37%, 1.84%, 4.68%, 1.60% and 1.49% for concentrations of I of 1, 5, 10, 20, 30 and 50 μg/ml, respectively.

In order to estimate the recovery the peak height ratio of I and II following extraction from plasma was compared to the ratio obtained following extraction from water. Recoveries were 110%, 106%, 104%, 102% and 101% for concentrations of 1, 10, 20, 30 and 50 μg/ml, respectively.

Using this simple, reproducible assay complete resolution of I and II was achieved in 9 min which offers an improvement over the retention times for I of 12 min previously described using electrochemical detection [7] and in addition detection limits were nearly ten-fold less then those described using ultraviolet absorption.

ACKNOWLEDGEMENT

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CHROMBIO. 2313

Note

Micromethod for the determination of cefotaxime and desacetylcefotaxime in plasma and urine by high-performance liquid chromatography

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(First received April 3rd, 1984; revised manuscript received July 23rd, 1984)

Cefotaxime (CTX), a third generation cephalosporin, has a broad antibacterial spectrum and resistance to β-lactamases. Its main metabolite, desacetylcefotaxime (dCTX), is a microbiologically active product and its pharmacokinetics are different from CTX [1]. Therefore, it is of particular interest to determine CTX and dCTX concentrations in biological fluids. High-performance liquid chromatography (HPLC) presents an advantage in comparison with microbiological assays, which have poor specificity when CTX is determined in the presence of dCTX or other antibiotics.

Several HPLC methods have been described [2—8]. Among these techniques, only two require a small volume of biological sample (100—300 μl) [6, 7]. Kees et al. [6] determined the concentrations of CTX and dCTX using an external standard and no results on accuracy and precision were reported. In the technique described by Lecaillon et al. [7], CTX and its metabolite were not analysed under the same conditions.

We report a micromethod allowing the simultaneous determination of CTX and dCTX in plasma and urine in the presence of an internal standard, cephaloridine. Since the aqueous solubility of cephalosporins is too high to allow their extraction by organic solvents, we have chosen a sample pretreatment which obviates the need for an extraction stage. This two-step method comprises first protein precipitation by propanol-2, then removal of remaining endogenous compounds by an organic solvent (chloroform), leaving the drugs in an aqueous phase of suitable pH to prevent degradation of cephalosporins. This sample pretreatment is short (6 min for each sample). Separation is carried out by ion-pair chromatography on a Radial-Pak C_{18} column within 7 min.

The precision and accuracy of this method were studied, and the coefficients

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of variation were lower than 7% over all concentrations investigated. This technique has been used to analyse samples obtained following pharmacokinetic studies on patients with renal failure.

MATERIAL AND METHODS

Reagents

Cefotaxime and desacetylcefotaxime were obtained from Roussel Uclaf Labs. (France); cephaloridine was from Eli Lilly (France). Reagent-grade propanol-2, chloroform, isoamyl alcohol, ammonium acetate, acetic acid, acetonitrile (E. Merck, Darmstadt, F.R.G.) were used without further purification. Pic-A (tetrabutylammonium hydroxide in phosphate buffer) was obtained from Waters Assoc. (Milford, MA, U.S.A.). Water was glass-distilled deionized.

Apparatus and chromatographic conditions

A Waters Assoc. liquid chromatograph was used. The instrument was equipped with a Model M 45 pump, a Lambda-Max Model 480 ultraviolet detector at 270 nm, a Wisp Model 710 B automatic injector, and a radial compression Z module. The column was a Radial-Pak C_{18} (Waters Assoc.) (100 mm × 8 mm I.D.; 10 μm particle size). Detector output was recorded on a Model 730 data module. The mobile phase was prepared by dissolving one vial of Pic-A in 1 l of acetonitrile—water (17:83, v/v). The working pressure of the pump was 35 bars at a flow-rate of 4 ml/min.

Standard solutions

Stock solutions of CTX, dCTX and cephaloridine were prepared in bidistilled water and stored at −20°C.

Plasma standards. Blank human plasma was spiked daily with CTX and dCTX stock solutions to yield concentrations ranging from 2 to 50 μg/ml. An appropriate dilution of cephaloridine stock solution was prepared in 0.1 *M* acetate buffer (pH 5) to provide a concentration of 50 μg/ml.

Urine standards. From stock solutions of CTX and dCTX, appropriate dilutions were prepared in blank urine to yield concentrations ranging from 5 to 100 μg/ml. The internal standard was at a concentration of 100 μg/ml.

Calibration curves. The calibration curves were constructed by plotting peak height ratios of CTX or dCTX to cephaloridine against concentration of CTX and dCTX in plasma or urine. Standard solutions were prepared daily.

Sample preparation

Plasma and urine samples are stored at −20°C until analysis.

In a 1.5-ml conical centrifuge tube, 100 μl of plasma or urine and 50 μl of 0.1 *M* ammonium acetate buffer (pH 5) containing cephaloridine were mixed with 500 μl of propanol-2; the mixture was vortexed for 30 sec and centrifuged for 2 min at 8700 *g* (Beckman Microfuge). The clear supernatant was transferred into another conical tube (1.5 ml) and 500 μl of chloroform—isoamyl alcohol (100:4, v/v) were added. The tube was vortexed for 30 sec and centrifuged for 2 min at 8700 *g*. A 5—20-μl portion of the upper aqueous phase was injected into the chromatograph.

RESULTS

Under the described chromatographic conditions, CTX, dCTX and cephaloridine were well resolved. Endogenous plasma or urine components did not give any interfering peaks (Fig. 1). Potential interference of some other drugs was investigated (Table I). None of these substances interfered with the quantitation of CTX and dCTX.

The reproducibility (intra-day assay, $n = 8$) and the repeatability (inter-day assay, $n = 8$) of the HPLC procedure were tested with plasma and urine of subjects who intravenously received 1 g of CTX. For reproducibility, the coefficients of variation (C.V., %) were: in plasma, 2.2% for CTX and 3.0% for dCTX; in urine, 2.4% for CTX and 4.0% for dCTX. The study of repeatability has led to the following results (C.V., %): in plasma, 2.4% for CTX and 3.7% for dCTX; in urine, 2.4% for CTX and 5.6% for dCTX.

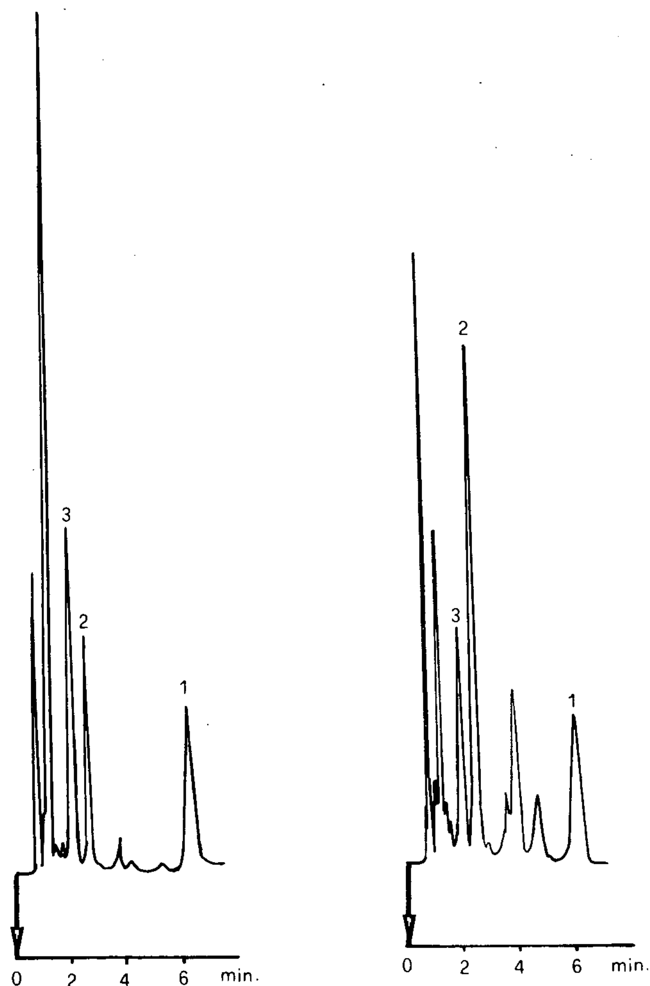

Fig. 1. Chromatograms of a plasma sample (left) and of a urine sample (right) from a patient after a single intravenous dose of CTX. Peaks: 1 = cefotaxime; 2 = desacetylcefotaxime; 3 = internal standard, cephaloridine.

TABLE I

RETENTION TIMES OF SOME DRUGS (1 μg INJECTED)

Drug	Retention time (min)
Cefotaxime	6.03
Desacetylcefotaxime	2.50
Desacetylcefotaxime lactone	2.61
Cephaloridine	1.95
Cephalexin	6.73
Cefazolin	7.24
Cephalothin	26.92
Moxalactam	Two peaks: 10.12 and 10.85
Theophylline	1.45
Caffeine	1.64
Acetaminophen	1.50
Salicylic acid	14.0
Phenobarbital	8.21
Phenacetin	8.04
Amobarbital	No response
Indomethacin	No response
Flufenamic acid	No response
Mefenamic acid	No response
Niflumic acid	No response
Ketoprofen	No response

The accuracy and precision were evaluated by adding known amounts of CTX and dCTX to blank serum and urine. For plasma, the precision of the method was determined by eight replicate assays at concentrations of 7.5 μg/ml and 25 μg/ml for CTX and dCTX, respectively. The coefficients of variation were, respectively: for CTX, 1.9% and 1.0% (intra-day assay) and 2.6% and 1.5% (inter-day assay); for dCTX, 2.3% and 2.2% (intra-day assay) and 2.9% and 6.8% (inter-day assay). In urine, a similar study was performed from two spiked concentrations: 12.5 μg/ml and 25 μg/ml, respectively. The coefficients of variation were, respectively: for CTX, 1.9% and 2.2% (intra-day assay, $n = 8$) and 2.6% and 3.0% (inter-day assay, $n = 8$); for dCTX, 4.5% and 3.1% (intra-day assay, $n = 8$) and 4.6% and 5.8% (inter-day assay, $n = 8$).

In plasma the calibrations curves were linear within the range of 2—50 μg/ml [$Y = 0.034X + 0.013$ ($r = 0.995$) for CTX ($n = 8$); $Y = 0.069X + 0.006$ ($r = 0.998$) for dCTX ($n = 8$)]. In urine, the relationship between concentration and peak height ratio was linear in the calibration range 5—100 μg/ml [$Y = 0.017X + 0.011$ ($r = 0.998$) for CTX, ($n = 8$); $Y = 0.039X - 0.023$ ($r = 0.995$) for dCTX ($n = 8$)].

Under these conditions the detection limit was 0.5 μg/ml for CTX and 0.25 μg/ml for dCTX (signal-to-noise ratio $\geqslant 2$).

DISCUSSION

In this study, potential degradation of cephalosporins during sample conservation, extraction procedure or automatic injection has been carefully taken into consideration, leading us to find analysis conditions to avoid this degradation.

After centrifugation, plasma and urine were stored at −20°C over a short period not exceeding ten days, and thawed just prior to analysis. A plasma sample was kept during 21 days at −20°C: no degradation of CTX and dCTX was noted (Table II). For the storage of samples over several months, a temperature of −70°C was necessary [6]. Blood samples must not be haemolysed. Indeed, blood esterases rapidly hydrolyse CTX and dCTX and this may explain the unexpectedly high levels of metabolite [9].

For sample preparation, a single protein precipitation by methanol or acetonitrile was not satisfactory: endogenous compounds prevented the determination of CTX and dCTX. However, a double purification of sample first by protein precipitation with propanol-2 then by extraction, which removes the

TABLE II

STABILITY OF CTX AND dCTX IN PLASMA FROZEN AT −20°C

Day	Concentration of CTX (μg/ml)	Concentration of dCTX (μg/ml)
0	7.5	7.5
1	7.5	7
2	7.0	7.5
3	7.0	7.5
8	7.5	7.0
21	7.5	7.5

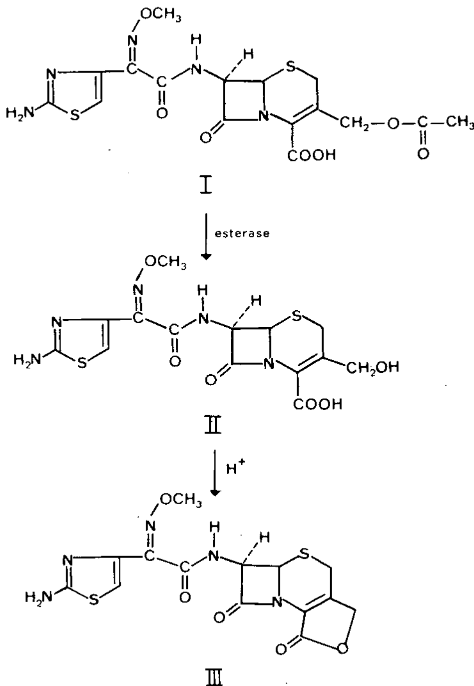

Fig. 2. Transformation of cefotaxime (I) into desacetylcefotaxime (II) and into desacetylcefotaxime lactone (III).

remaining endogenous compounds and the initial solvent, provided a good sample for HPLC. Using the indicated proportions of propanol-2—chloroform (1:1, v/v), the upper aqueous phase containing the drugs was clear.

The recovery of drugs in the upper aqueous phase was assessed by comparing the peak heights of CTX and dCTX obtained after processing plasma samples containing known concentrations of drugs (1, 5, 10, 25, 50 μg/ml for CTX and dCTX). The results obtained were: 92.0 ± 5.2% for CTX and 100.5 ± 4.7% for dCTX.

Under the described conditions, automatic injection of 50 samples required only 7 h. We have studied a possible degradation of CTX into dCTX then into its lactone (Fig. 2) in the final aqueous layer. No degradation was observed (Fig. 3). Several authors have studied the stability of 3-acetoxymethylcephalosporins in aqueous solution [10—12]. They have shown that at 25°C, in the pH range 4.5—6.5, the stability of CTX was maximal and desacetylcefotaxime lactone only appeared below pH 4.

This method allowed us to determine serum concentrations of cefotaxime

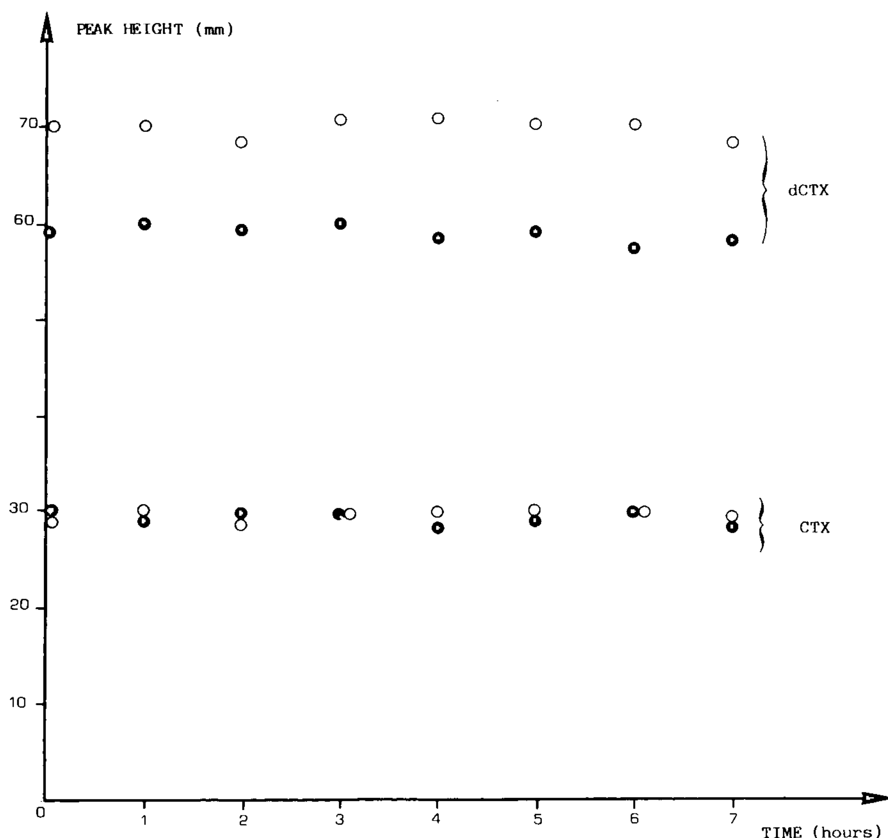

Fig. 3. Stability of CTX and dCTX during 7 h at 25°C in the final aqueous phase. (•), Blank plasma spiked with 12.5 μg/ml each of CTX and dCTX; (○), blank urine spiked with 75 μg/ml each of CTX and dCTX.

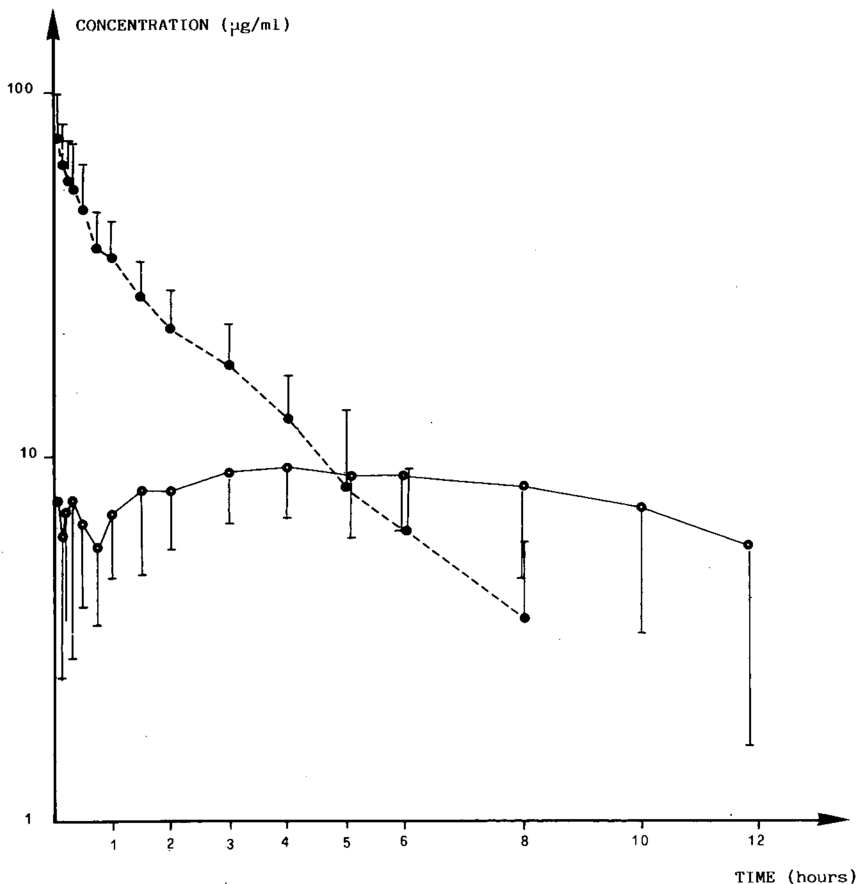

Fig. 4. Mean plasma concentrations of cefotaxime (•- - -•) and desacetylcefotaxime (○—○) in eight patients following administration of 1 g of cefotaxime intravenously.

and desacetylcefotaxime in eight patients undergoing peritoneal dialysis with creatinine clearance below 5 ml/min. These subjects received a slow intravenous injection (3 min) of 1 g of cefotaxime (Fig. 4).

CONCLUSIONS

The present paper describes a rapid and sensitive quantitative micromethod for CTX and dCTX determination in plasma and urine. The presence of an internal standard is not absolutely necessary since there is no extraction of drugs from biological fluids. However, as the chromatographic or detection conditions may undergo fluctuations, the use of an internal standard seems important to enhance the assay precision and reproducibility.

This micromethod is particularly useful in paediatrics and in patients with renal failure where sample size is of major concern.

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Note

Determination of 4,5-dihydro-6-[4-(1H-imidazol-1-yl)phenyl]-3(2H)-pyridazinone hydrochloride, a new cardiotonic, in plasma and urine by reversed-phase high-performance liquid chromatography

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4,5-Dihydro-6-[4-(1H-imidazol-1-yl)phenyl]-3(2H)-pyridazinone hydrochloride (I, CI-914 hydrochloride), is a new cardiotonic [1] currently under phase 1 clinical investigation. This work describes reversed-phase high-performance liquid chromatographic (HPLC) methods for the determination of I in plasma and urine. Optimal conditions for drug extraction and chromatography were selected in order to achieve a satisfactory recovery and adequate sensitivity. The methods have been applied to pharmacokinetic and pharmacodynamic studies in laboratory animals.

EXPERIMENTAL

Chemicals

I and the internal standard, PD 111,908 (Fig. 1), were synthesized at Warner-Lambert/Parke-Davis Research Labs. ^{14}C-Labeled I was custom synthesized by Amersham (Arlington Heights, IL, U.S.A.). Dichloromethane, distilled in glass, was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.); acetonitrile, distilled in glass, from Matheson, Coleman, Bell

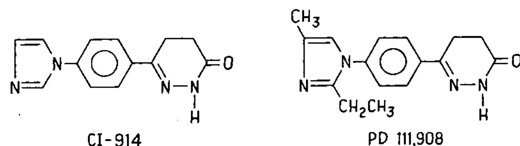

Fig. 1. Chemical structures of I (CI-914) and internal standard (PD 111,908).

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(Cincinnati, OH, U.S.A.); Ready-Solv MP® from Beckman Instruments (Fullerton, CA, U.S.A.); drug-free human plasma from Plasma Alliance (Knoxville, TN, U.S.A.). All other chemicals were of analytical grade. Water used in reagent preparation was purified using the Water-I system from Gelman Sciences (Ann Arbor, MI, U.S.A.).

Reagents

Stock solutions of I and the internal standard (1 mg/ml as the free base) were prepared in 0.1 mol/l hydrochloric acid. Appropriate aliquots of I stock solution were added to drug-free human plasma and dog urine to yield 200 and 1000 ng/ml working standards. A working solution of the internal standard was prepared weekly by diluting (1:400) the stock solution with 0.1 mol/l hydrochloric acid.

Preparation of plasma samples

Aliquots of plasma (0.1—0.5 ml) were pipetted into disposable glass tubes and drug-free human plasma was added to provide a final volume of 0.5 ml. A 0.2-ml aliquot of the internal standard solution (500 ng) was added to each tube. The samples were deproteinized with 2 ml of 10% trichloroacetic acid (TCA), vortexed for 30 sec, and allowed to stand 15 min at room temperature. After centrifugation, the supernatant was decanted into 16-ml glass-stoppered extraction tubes containing 0.7 ml of 2 mol/l sodium hydroxide and 6 ml of dichloromethane. The tubes were shaken for 5 min and centrifuged. The organic layer was transferred into disposable tubes and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 0.2 ml of 30% acetonitrile in water and transferred to autosampler vials containing limited volume inserts; 25 μl of this solution were injected for chromatography.

Preparation of urine samples

Aliquots of urine (0.1—1.0 ml) were pipetted into 16-ml glass-stoppered extraction tubes and drug-free dog urine was added to provide a final volume of 1.0 ml. A 0.4-ml aliquot of the internal standard (1 μg), 0.2 ml of 2 mol/l sodium hydroxide, and 6 ml dichloromethane were added to each tube. The tubes were shaken for 5 min and centrifuged. A 5-ml aliquot of the organic phase was back-extracted with 3.5 ml of 0.1 mol/l hydrochloric acid in a second set of tubes for 5 min. Following centrifugation, 3 ml of the aqueous phase was made alkaline with 0.2 ml of 2 mol/l sodium hydroxide and the mixture was extracted with 5 ml dichloromethane as described above. The organic layer was transferred into disposable tubes, evaporated to dryness under a stream of nitrogen, and analyzed as described for plasma samples.

Instrumentation

An automated HPLC system comprised a Model M-45 pump, a Model 441 ultraviolet (UV) detector operated at 280 nm, a Model 710B WISP sample processor (all from Waters Assoc., Milford, MA, U.S.A.), and a Model 3390A integrator (Hewlett-Packard, Avondale, PA, U.S.A.). The 5-μm LiChrosorb RP-2 column (stainless steel, 125 mm × 4 mm I.D.) was purchased from E. Merck (Gibbstown, NJ, U.S.A.).

Samples were eluted isocratically at a flow-rate of 0.8 ml/min with a pressure of 6.89 MPa. The mobile phase used consisted of a mixture of 0.005 *M* octane-sulfonic acid in 0.1% acetic acid—acetonitrile (70:30).

A Hewlett-Packard Model 1040A high-speed scanning diode array UV spectrophotometric detector was used to determine the specificity of the plasma assay under identical chromatographic conditions described above.

Radioactivity determinations were performed using a Model 3255 liquid scintillation spectrometer (Packard, Downers Grove, IL, U.S.A.).

Calibration and precision

Since preliminary experiments showed that drug-free animal and human plasma yielded equivalent results, human plasma was used for all subsequent plasma validation studies and calibration standard preparations.

Six calibration standards containing 100, 200, 400, 600, 800, and 1000 ng I per ml drug-free human plasma or dog urine were processed daily with each set of unknowns. Calibration curves were constructed by plotting peak height ratios of I to the internal standard as a function of the concentration of I. The best-fit straight line was determined using reciprocal concentration weighted least-squares linear regression. To determine the precision of the assay procedures, triplicate plasma and urine standards were analyzed on three separate days to yield nine replicate values for each of the six concentrations.

Selectivity

Selectivity of the plasma method was determined by examining the homogeneity of the eluted I peak in the extract of a 4-h postdose dog plasma sample. Absorbance of the peak was measured as a function of wavelength and time simultaneously.

Recovery determination of plasma

^{14}C-Labeled drug was used to assess the recovery of I for both the protein precipitation step and the extraction of drug into dichloromethane. The minimum volume of 10% TCA required to provide an optimum recovery of I from plasma was determined by treating 0.5-ml aliquots of a 2 μg/ml plasma standard with 0.5—2.5 ml of 10% TCA. In a separate study, the extraction efficiency of [^{14}C]I from alkalinized TCA supernatant (pH 14) into dichloromethane was also determined. Radioactivity was measured by liquid scintillation counting using Ready-Solv MP® as the scintillation cocktail and external standardization for quench correction.

Recovery determination of urine

Recovery of I from urine was determined by extraction of triplicate 500 ng/ml urine standard solutions and comparison to aqueous standards injected directly.

Quality control and stability studies

Quality control samples in the concentrations of 300, 500, and 700 ng/ml were prepared in drug-free human plasma or dog urine, and aliquots were stored frozen in disposable glass vials. Individual samples at the three concen-

trations were then analyzed daily with each set of unknowns to determine the reliability of the day's analyses and the stability of I in frozen plasma and urine.

RESULTS AND DISCUSSION

Representative chromatograms obtained from plasma and urine analyses are depicted in Figs. 2 and 3, respectively. Retention times for I and the internal standard were 3.0 and 4.9 min, respectively, and no interfering peaks were detected at either retention time.

Homogeneity of the I peak in a dog plasma extract was determined by multiple UV scans at the upslope, apex, downslope, and baseline over the range 211—400 nm. The resulting concentration normalized and background-cor-

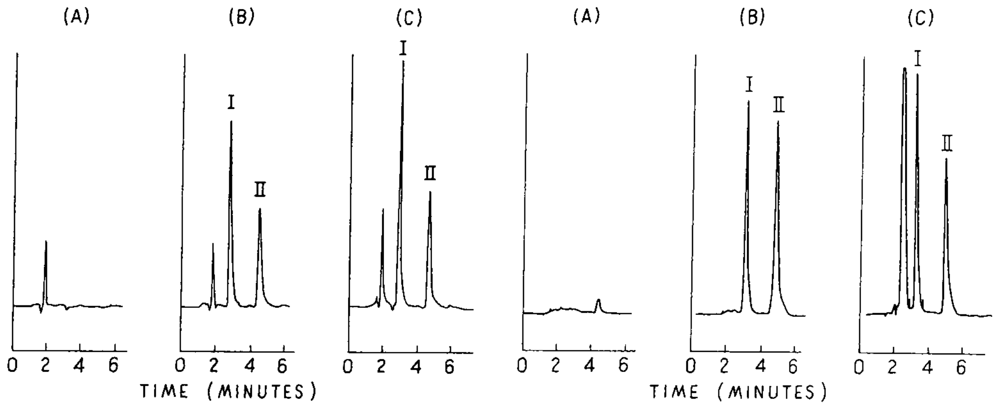

Fig. 2. Chromatograms of an extract of (A) drug-free human plasma; (B) 600 ng/ml plasma calibration standard; (C) 3-h postdose dog plasma sample (1 mg/kg oral dose) containing 690 ng I per ml. Peaks: I = CI-914; II = internal standard.

Fig. 3. Chromatograms of an extract of (A) drug-free dog urine; (B) 600 ng/ml urine calibration standard; (C) 8—12 h postdose dog urine sample (0.5 mg/kg oral dose) containing 770 ng/ml I. Peaks: I = CI-914; II = internal standard.

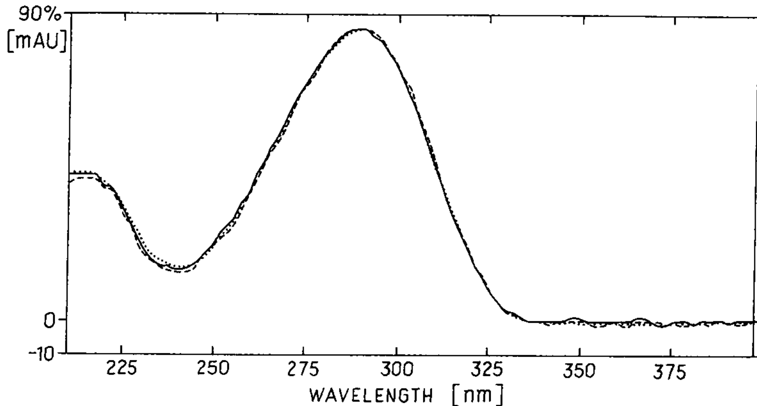

Fig. 4. Spectra of I peak in a postdose dog plasma extract. —, Upslope; • • • • •, apex; - - - -, downslope.

rected spectra (Fig. 4) were superimposable with that of a standard I solution, indicating that the I peak was homogeneous and that the plasma assay procedure is specific for unchanged drug.

Based on peak height ratios of six replicate sequential injections of 100 and 1000 ng/ml standards, the HPLC system reproducibility had a relative standard deviation (R.S.D.) of 0.7% and 0.5%, respectively.

The relationship between peak height ratio (I/internal standard) and I concentration was linear over the concentration range studied (100–1000 ng/ml) and yielded a correlation coefficient of 0.999 or greater for both the plasma and urine assays.

The assay precision and accuracy varied from 1.8% to 4.9% for plasma analyses and from 0.8% to 5.4% for urine analyses (Table I).

For the plasma assay, recovery of I for the protein precipitation step ranged from 54%, when equal volumes of plasma and TCA were used, to 86% when the ratio of plasma to TCA was 1:4. Further increases in TCA volume failed to improve the recovery. The extraction efficiency into dichloromethane was 90%, giving a total recovery of 77% for the entire plasma assay procedure. The urine assay showed 97% recovery of I.

Plasma and urine quality control samples in the concentrations of 300, 500, and 700 ng/ml I were frozen and assayed over a three-month period (Table II).

TABLE I

PRECISION AND ACCURACY OF THE METHODS APPLIED TO I CALIBRATION STANDARDS ($n = 9$)

I added (ng/ml)	I found			
	Plasma		Urine	
	ng/ml	R.S.D. (%)	ng/ml	R.S.D. (%)
100	101.4	3.0	99.5	5.4
200	194.7	1.9	200.6	3.0
400	403.2	4.9	399.4	1.7
600	607.5	3.5	602.4	0.8
800	796.5	2.1	797.4	2.8
1000	996.7	1.8	1000.0	2.7

TABLE II

STABILITY OF I IN PLASMA AND URINE QUALITY CONTROL SAMPLES

I added (ng/ml)	I found					
	Plasma ($n = 14$)			Urine ($n = 3$)		
	ng/ml	Percent theoretical	R.S.D. (%)	ng/ml	Percent theoretical	R.S.D. (%)
300	302.7	100.9	2.5	293.6	97.9	8.0
500	502.0	100.4	2.1	517.8	103.6	2.6
700	702.0	100.3	1.3	719.7	102.8	2.5

For the plasma samples, mean values obtained were within 1% of theoretical values with an R.S.D. less than 3%. The urine quality control samples were within 4% of the theoretical values with an R.S.D. less than 8%. These results indicated excellent drug stability in frozen plasma and urine.

The limit of assay quantitation was defined as the concentration of I per ml of plasma or urine at which replicate analyses have an R.S.D. of 15%. Analysis of 0.5-ml aliquots of 40 and 80 ng/ml spiked plasma samples (n = 6) yielded an R.S.D. of 15.5% and 9.0%, respectively. The reported limit of quantitation was 40 ng/ml. Variability at the lower concentrations was largely derived from plasma preparation since HPLC system reproducibility was essentially the same for replicate injections of 40 and 400 ng/ml standards (1.6% and 1.2% R.S.D. respectively, n = 6). The limit of quantitation can be extended to 20 ng/ml by analyzing 1.0-ml aliquots of plasma, deproteinizing with 4 ml of 10% TCA, and alkalinizing with 1.4 ml of 2 mol/l sodium hydroxide (R.S.D. = 6.0%, n = 6). For urine samples, the reported limit of quantitation was 100 ng/ml.

The described methods have been successfully applied to pharmacokinetic and dose ranging studies in laboratory animals following single 0.5 mg/kg intravenous and oral doses [2, 3]. The resulting plasma concentrations ranged from 40 to 500 ng/ml and showed that I was readily absorbed. Urinary excretion of unchanged I accounted for less than 1% of the dose.

In summary, the described methods are selective, reproducible, and sufficiently sensitive for single-dose pharmacokinetic studies.

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Note

Determination of diastereoisomeric pairs of thioridazine 5-sulfoxide by high-performance liquid chromatography

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(First received April 12th, 1984; revised manuscript received August 7th, 1984)

Thioridazine 5-sulfoxide (thioridazine ring sulfoxide) is a major metabolite of thioridazine, a phenothiazine neuroleptic drug used in the management of psychotic disorders. Thioridazine possesses an asymmetric carbon and has been separated into enantiomers [1]. Oxidation of the ring sulfur atom of thioridazine gives rise to an additional chiral center; therefore, thioridazine 5-sulfoxide exists as two diastereoisomeric pairs of enantiomers. Juenge et al. [2] recently reported the chemical synthesis, isolation, and structural analysis of these two stereoisomer pairs.

Wells et al. [3] developed a high-performance liquid chromatographic (HPLC) method utilizing post-column oxidation and fluorometric detection for the determination of thioridazine and its major metabolites in plasma, including the pairs of ring sulfoxides. Applying this method, Poklis et al. [4] demonstrated the presence of the thioridazine 5-sulfoxide stereoisomeric pairs in blood from patients receiving thioridazine. Previous metabolic studies of thioridazine had failed to recognize the presence of the two ring sulfoxide pairs. The pharmacokinetics and pharmacodynamics of these stereoisomeric pairs have not been studied.

This communication presents a normal-phase HPLC procedure for the rapid determination of the two diastereoisomeric pairs of enantiomers of thioridazine 5-sulfoxide in serum. The ring sulfoxide pairs are tentatively identified as thioridazine ring sulfoxide fast eluter (RSF) and slow eluter (RSS). The analysis is performed in a short silica column with an ultraviolet detector. The method is particularly suited for pharmacokinetic studies in animals; studies that require the rapid analysis of a large number of serum or blood samples.

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EXPERIMENTAL

Chemicals and reagents

Thioridazine and mesoridazine were obtained from Sandoz Pharmaceuticals (Hanover, NJ, U.S.A.). 2-Propanol and *n*-butyl chloride (HPLC grade) were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) and diethylamine from Mallinckrodt (St. Louis, MO, U.S.A.). Distilled water was purified for HPLC use with a Millipore filtration unit. All glassware was silanized with 2% dimethyldichlorosilane in benzene (Sigma, St. Louis, MO, U.S.A.).

Standards

Standards of the diastereoisomeric pairs of thioridazine ring sulfoxide were prepared by the method of Juenge et al. [2]. A stock standard solution of the racemic ring sulfoxides (100 μg/ml) was prepared by dissolving 10 mg of the drug in 100 ml of ethanol. An intermediate standard of 10 μg/ml was prepared by dilution of 0.10 ml of the stock standard with 0.90 ml of ethanol. A second intermediate standard of 1 μg/ml was prepared by dilution of 0.10 ml of the 10 μg/ml standard with 0.90 ml of ethanol. Mixed standards in serum were prepared daily by adding the following amounts of intermediate standards to 15-ml reusable culture tubes and diluting to 2.0 ml with serum: 0.10, 0.20, and 0.50 ml of the 1 μg/ml standard and 0.10 ml of the 10 μg/ml standard. The resultant serum standards contained the following concentrations of RSF and RSS (ng/ml serum): 25, 50, 125, and 250.

The internal standard was prepared by dissolivng 300 μg of mesoridazine in 100 ml of ethanol. A 0.40-ml aliquot of this standard was added to each sample before extraction. Although mesoridazine is also an oxidative metabolite of thioridazine, data from additional experiments indicate that after administration of the ring sulfoxides to rats, the compounds are not reduced and then oxidized again on the side-chain sulfur to form mesoridazine. Instead the ring sulfoxides are oxidized further to the disulfoxide and sulfone.

Chromatography

Analyses were performed with a Waters Assoc. (Milford, MA, U.S.A.) HPLC system consisting of a Model M6000A chromatography pump, Model U6K injector and Model 450 variable-wavelength detector. The mobile phase consisted of *n*-butyl chloride—2-propanol—water—diethylamine (92.0:7.9:0.05:0.05). The solvents were mixed and then filtered through a 0.45-μm filter before use. Separations were carried out isocratically in an IBM (Danbury, CT, U.S.A.) 5-μm silica 50 mm × 4.5 mm minicolumn. The mobile phase flow-rate was 1.7 ml/min. The column effluent was monitored at 279 nm for optimal detection of the ring sulfoxides [2]. Chromatograms were recorded using a Hewlett-Packard (Avondale, PA, U.S.A.) 3390A integrator. Instrument operating conditions were maintained for 0.5 h before the first injection was made. Under these conditions, the retention times were 3.7 min for the fast eluting ring sulfoxide, 5.4 min for the slow eluting ring sulfoxide, and 9.8 min for mesoridazine (Fig. 1). Thioridazine and sulforidazine do not interfere with this assay as both compounds elute in the solvent front. Thioridazine disulfoxide, if present, elutes several minutes after mesoridazine.

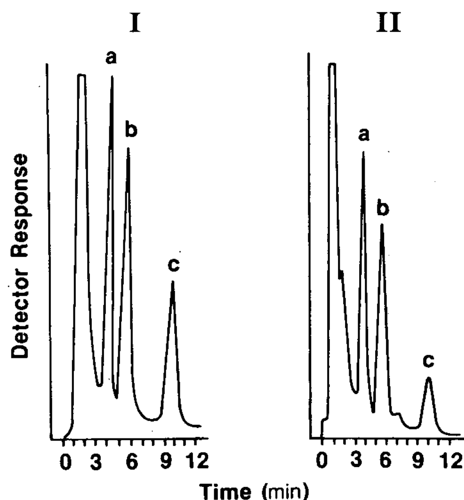

Fig. 1. HPLC chromatograms of (I) a 200 ng/ml serum standard; (II) extracted rat serum 2 h after a 10 mg/kg injection of thioridazine ring sulfoxide. Peaks: a = thioridazine ring sulfoxide fast eluter; b = thioridazine ring sulfoxide slow eluter; and c = internal standard.

Procedure

To 2.0 ml of serum, 3.0 ml of heptane—ethyl acetate (1:1) and 0.40 ml of the internal standard, mesoridazine (3 μg/ml), were added. The mixture was vortexed for 2 min, centrifuged at 1000 *g* for 10 min and the upper (organic) layer aspirated into a new tube. The remaining aqueous phase was reextracted with 3.0 ml of fresh heptane—ethyl acetate. The mixture was again vortexed and centrifuged, and the organic layer drawn off and combined with the organic phase from the first extraction. The combined organic extracts were evaporated to dryness on a steam bath at 40°C under a stream of nitrogen. The residue was dissolved in 0.50 ml of the HPLC mobile phase and 0.10-ml aliquots were injected into the liquid chromatograph.

RESULTS AND DISCUSSION

Standard curves from 5 to 250 ng/ml were constructed from analyses of plasma containing a known concentration of the isomeric ring sulfoxides. The least-squares linear regression equations for the fast eluting thioridazine ring sulfoxide and the slow eluting thioridazine ring sulfoxide were: Y (peak area ratio RSF/internal standard) = $0.022X$ (RSF, ng/ml) + 0.018, (r = 0.991); and Y (peak area ratio RSS/internal standard) = $0.024X$ (RSS, ng/ml) − 0.136, (r = 0.997). The within-run coefficient of variation (C.V.) for RSF (target concentration 250 ng/ml) was 6.31% (n = 9) and that for RSS (target concentration 250 ng/ml) was 4.50% (n = 9). The within-run C.V. for RSF (target concentration 50 ng/ml) was 16.8% (n = 21) and that for RSS (target concentration 50 ng/ml) was 15.0% (n = 21).

The absolute, uncorrected, analytical recovery for both RSF and RSS was calculated by comparison of peak areas obtained from supplemented serum samples with those of non-extracted standards. The mean recoveries over the

concentration range of 10–250 ng/ml of RSF and RSS were 103% (n = 22) and 97% (n = 22), respectively.

The ring sulfoxides are the major metabolites found in the plasma of patients chronically treated with thioridazine. Values range from less than 40 ng/ml to 5 μg/ml of plasma depending on the dose and the duration of thioridazine treatment [5]. Plasma concentrations of the ring sulfoxide have also been correlated with electrocardiographic abnormalities in thioridazine-treated patients [6].

As quantitative separation of the diastereoisomeric ring sulfoxides has only recently been realized [3], this selective and sensitive method is particularly suited for the determination of plasma concentrations of each ring sulfoxide and also for evaluation of the disposition and elimination of the ring sulfoxides in animal models.

The relative retention of the ring sulfoxides and mesoridazine was found to be dependent on the concentration of diethylamine present in the mobile phase. Increasing the concentration of diethylamine only slightly caused a decrease in the retention times of all components. This effect may be due to diethylamine's ability to compete with phenothiazines for acidic binding sites on the silica surface of the column [7, 8].

The HPLC procedure of Wells et al. [3] is also capable of separating the ring sulfoxides. However, their method is cumbersome as it requires post-column oxidation before fluorometric detection. The present method does not require oxidizing reagents, mixing coils, or a fluorometric detector. Instead, non-destructive ultraviolet detection at 279 nm is employed, allowing greater recovery of each isomer for further qualitative determinations, e.g. mass spectroscopy.

The absolute limit of detection using the present method is 5 ng/ml, a value similar to the 2 ng/ml obtainable with fluorometric detection. Our method offers the advantages of sensitive ultraviolet detection of the diastereoisomeric ring sulfoxides and short elution times.

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PUBLICATION SCHEDULE FOR 1985

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

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EVALUATION OF ANALYTICAL METHODS IN BIOLOGICAL SYSTEMS

Part A: Analysis of Biogenic Amines

edited by GLEN B. BAKER *and* RONALD T. COUTTS,
Neurochemical Research Unit, Department of Psychiatry, and Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Canada

TECHNIQUES AND INSTRUMENTATION IN ANALYTICAL CHEMISTRY, 14

This is the first volume of a new multi-volume work entitled "Evaluation of Analytical Methods in Biological Systems" that will evaluate the various analytical techniques and approaches that can be used in a particular bioanalytical field. This volume provides an overview of techniques for the analysis of catecholamines, 5-hydroxytryptamine (serotonin), 'trace amines' and histamine in biological systems.

The authors describe the basic principles underlying the various techniques and discuss their advantages and disadvantages relative to other available methods, thus making this a valuable reference both for those experienced in analysis of biogenic amines who are considering using a new technique, and to those entering the field for the first time.

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