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CHROMBIO. 2430

GAS CHROMATOGRAPHIC DETERMINATION OF SERUM BRANCHED-CHAIN α-KETO ACIDS DERIVATIZED BY EXTRACTIVE ALKYLATION

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(First received July 5th, 1984; revised manuscript received October 25th, 1984)

SUMMARY

The procedure presented for gas—liquid chromatographic analysis of α-keto acids is relatively simple, requiring only a few steps for the formation of derivatives suitable for measurement. The recoveries of the branched-chain α-keto acids varied from 92.7% to 106.7%, being sufficiently good especially when smaller amounts of the α-keto acids were added to serum. In addition, the coefficients of variation are satisfactorily small, also for biological samples. The measured values of branched-chain α-keto acids correspond well with those presented earlier by different methods. There exists a slight but insignificant difference between women and men, the values being lower in sera of women for the three branched-chain α-keto acids studied.

INTRODUCTION

Amino acid degradation in man proceeds through deamination yielding the corresponding α-keto acid [1]. Quantitative analysis of these metabolites is of interest from the point of view of metabolic defects as well as of parenteral feeding with nutrients containing amino acids [2]. Also, in the treatment of septic shock, a knowledge of blood levels of α-keto acids is important [3].

There are very few reports available concerning the concentrations of α-keto acids in the blood. The technical difficulties in measuring these compounds are probably the main reasons for this scarcity. α-Keto acid analysis has been performed by paper chromatography of the corresponding 2,4-dinitrophenylhydrazone derivatives [4,5]. Kallio and Linko [6] assayed α-keto acids by gas—liquid chromatography (GLC) of the esterified 2,4-dinitrophenylhydrazone derivatives. In another gas chromatographic procedure [7], derivatization to

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O-methyloxime methyl esters was found useful. Recently, the α-keto acids have been determined as their quinoxalinols by high-performance liquid chromatography [8]. Cree et al. [9] have successfully used silylated quinoxalinol derivatives for GLC.

Extractive alkylation is applicable for various types of organic molecules containing active hydrogen atoms [10]. Methods based on extractive alkylation of the carboxyl group for compounds other than α-keto acids [11,12] have been described earlier. The purpose of the present study has been to develop this technique for the determination of α-keto acids in blood, for clinical use.

MATERIALS AND METHODS

Materials

To determine branched-chain α-keto acids from biological samples, venous blood samples were used. Serum was separated as quickly as possible from cells and stored at 4°C before use unless otherwise stated. For serum values from apparently healthy people, blood samples from twenty women and twenty men were taken after overnight fasting and analysed during the day of sample collection.

α-Ketoisovaleric acid (α-ketovaline) sodium salt (Fluka, Buchs, Switzerland), α-keto-*n*-valeric acid (Sigma, St. Louis, MO, U.S.A.), α-keto-β-methyl-*n*-valeric acid (α-ketoisoleucine) sodium salt (Sigma) and α-ketoisocaproic acid (α-ketoleucine) (Fluka) were used as standards in procedural development tests and in calibration. Tetrabutylammonium hydrogen sulphate (TBA) (E. Merck, Darmstadt, F.R.G.), pentafluorobenzylbromide (PFBBr) (Fluka), dichloromethane (Merck) and petroleum ether 60—80°C (Merck) were used as reagents and solvents.

Sample preparation

The branched-chain α-keto acids were alkylated and extracted from serum samples for gas chromatographic analysis as follows. A 1-ml volume of 0.1 *M* TBA—phosphate buffer (pH 7—8) and 1 ml of 2% PFBBr in methylene chloride were added to 1 ml of serum sample in a stoppered test tube; 10 μl of a solution of internal standard (α-ketovaleric acid, 20 μg/ml) were added to each serum sample. The solution was carefully mixed for 2 h in a mixer and centrifuged for 10 min at 2000 *g*. The aqueous layer was removed and methylene chloride was evaporated in a flow of nitrogen. Then 20—50 μl of petroleum ether were added and the suspension was thoroughly mixed. Direct injection of the methylene chloride layer into the gas chromatograph is not feasible because of serious baseline disturbances caused by high reagent concentrations in this solvent.

Stability of α-keto acids in serum at −20°C was measured by an external standard method using a Shimadzu CR 1A electronic integrator (Tokyo, Japan). By using known concentrations of derivatized α-keto acids in a 2-μl volume as external standards, it was possible to measure the concentrations of α-keto acids weekly from a serum pool stored in separate tubes up to one month.

Instrumentation

A Carlo Erba Fractovap 2350 gas chromatograph (Milan, Italy) fitted with a flame-ionization detector was used in combination with an SE-30 fused-silica capillary column, 24 m × 0.32 mm I.D. (Orion Analytica, Espoo, Finland). Helium was used as carrier gas at a flow-rate of 3 ml/min. Detector flow-rates for hydrogen and air were 30 ml/min and 240 ml/min, respectively. A Grob-type injector was operated in split mode with a split ratio of 1:5. The column oven was temperature programmed from 60°C to 110°C at a rate of 2.5°C/min. The GC electrometer was connected to a Shimadzu CR 1A electronic integrator. Peak identification was performed by gas chromatography—mass spectrometry (GC—MS) using a quadrupole instrument (gas—liquid chromatograph—mass spectrometer 5992A, Hewlett-Packard, Palo Alto, CA, U.S.A.). A representative chromatogram is shown in Fig. 1; the mass spectra of the α-keto acids are displayed in Fig. 2.

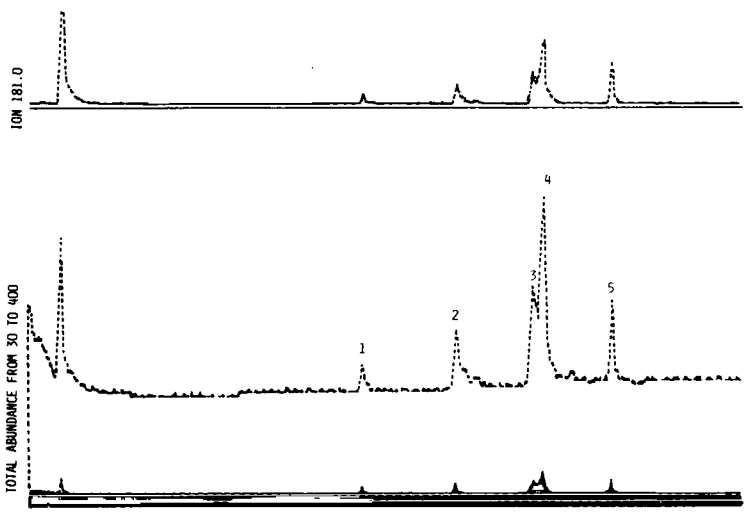

Fig. 1. Chromatogram of α-keto acids obtained using the Hewlett-Packard gas—liquid chromatograph—mass spectrometer with SE-30 silica capillary column, 25 m long. Splitless delay time was 80 sec. The instrument was programmed from 60°C to 110°C at a rate of 2.5°C/min, and operated at 2000 V and 70 eV. Peaks: 1 = α-ketoisovaleric acid, 2 = α-keto-valeric acid (internal standard), 3 = α-keto-β-methylvaleric acid, 4 = α-ketoisocaproic acid, 5 = unidentified acid.

RESULTS AND DISCUSSION

Pentafluorobenzylbromide is one of the best alkylating agents available for extractive alkylation. In the case of the compounds studied here, a complete reaction takes place in about 20—30 min at room temperature, provided that a sufficiently high concentration of PFBBr is present in the reaction mixture [13,14]. In extractive alkylation, the pH value of the aqueous phase should exceed by two units the pK_a values of the compounds to be alkylated [15,16]. The α-keto acids investigated here, with pK_a values ranging from 4.2 to 5.1 will thus easily undergo alkylation of the carboxyl group at the physiological pH

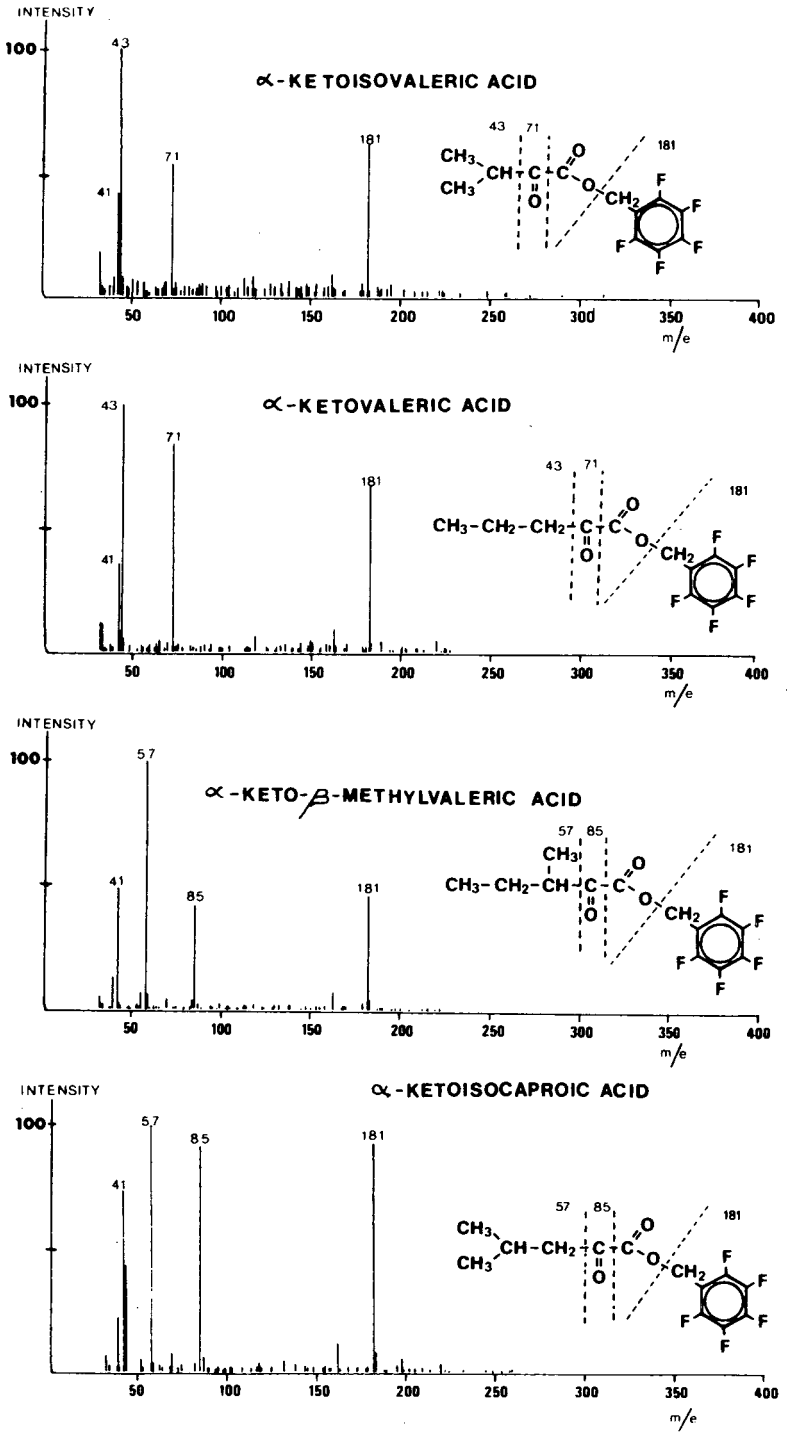

Fig. 2. Mass spectra of α-keto acids. For operating conditions, see Fig. 1.

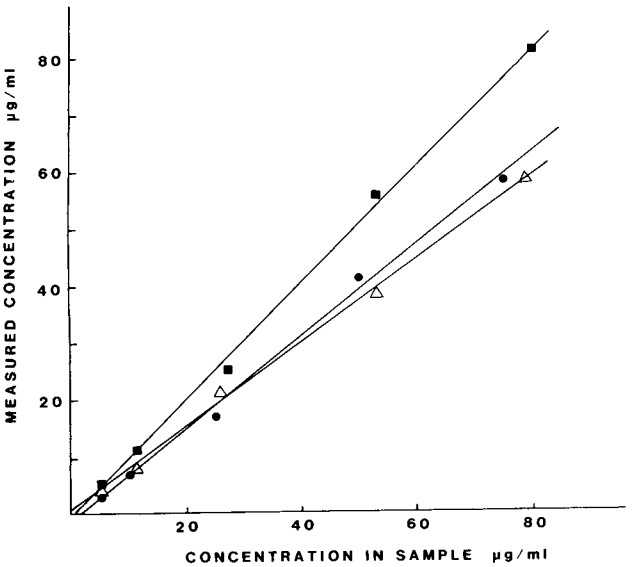

Fig. 3. Linearity curves of α-keto acids: (■) α-ketoisovaleric acid; (●) α-keto-β-methyl-*n*-valeric acid; (△) α-ketoisocaproic acid.

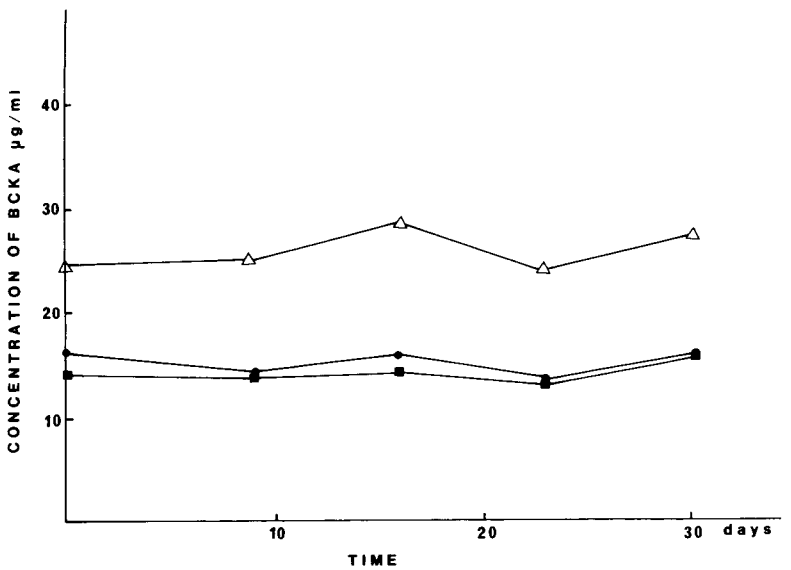

Fig. 4. Stability of branched-chain α-keto acids (BCKA) in serum at −20°C. Results are measured by the external standard method. (■) α-ketoisovaleric acid; (●) α-keto-β-methyl-*n*-valeric acid; (△) α-ketoisocaproic acid.

values of serum samples. The tetrabutylammonium counter-ion was added to the samples in a solution of phosphate buffer (pH 7.5), the pH range 7—8 yielding best values. Linearity of all compounds measured is good up to 80 µg/ml (Fig. 3). Serum samples can be analysed even after one month after derivatization when stored at temperatures of −20°C or below (Fig. 4).

The contribution of sample treatment operations to the variation of the results is reasonably small as determined by ten parallel analyses of a serum sample (Table I). The coefficient of variation is less than 13% for all compounds investigated. Similarly, the instrumental error is quite small, the

TABLE I

MEAN CONCENTRATION, COEFFICIENT OF VARIATION (C.V.) AND VARIATION RANGE OF α-KETO ACIDS OBTAINED BY ANALYSIS OF A SINGLE SERUM SAMPLE

$n = 10$.

	Concentration (μmol/l, mean ± S.D.)	C.V. (%)	Range (μmol/l)
α-Ketoisovaleric acid	13.9 ± 0.9	6.5	12.6—14.8
α-Keto-β-methyl-*n*-valeric acid	25.6 ± 3.1	12.1	20.9—30.2
α-Ketoisocaproic acid	36.9 ± 4.6	12.5	29.2—43.8

TABLE II

MEAN CONCENTRATION, COEFFICIENT OF VARIATION AND VARIATION RANGE OF α-KETO ACIDS OBTAINED BY ANALYSIS OF AN ARTIFICIAL SAMPLE

$n = 10$. Concentrations in artificial sample are: 173.7 μmol/l ketoisovaleric acid, 158.8 μmol/l keto-β-methyl-*n*-valeric acid, 161.4 μmol/l ketoisocaproic acid.

	Concentration (μmol/l, mean ± S.D.)	C.V. (%)	Range (μmol/l)
α-Ketoisovaleric acid	171.1 ± 5.2	3.0%	163.3—181.6
α-Keto-β-methyl-*n*-valeric acid	146.4 ± 3.9	2.7%	139.4—152.6
α-Ketoisocaproic acid	153.9 ± 8.5	5.5%	140.0—163.7

TABLE III

RECOVERY TEST

Serum concentrations of α-keto acids were measured with the external standard method after addition of the corresponding α-keto acids.

Acid	Concentration (μmol/l)			Percentage recovery
	In serum	Added	Found	
α-Ketoisovaleric acid	9	0	9	100
	9	87	96	100
	9	347	330	93
	9	695	678	96
α-Keto-β-methyl-*n*-valeric acid	8	0	8	100
	8	77	85	100
	8	318	302	93
	8	635	604	94
α-Ketoisocaproic acid	31	0	31	100
	31	85	123	106
	31	323	361	102
	31	645	661	98

coefficient of variation being less than 6% for all keto acids, as calculated from ten GC runs of a petroleum ether extract of a serum sample (Table II). The recovery of all compounds studied is quite satisfactory (when a sufficiently long alkylation time is used) (Table III).

The peaks of α-keto-β-methylvaleric and α-ketoisocaproic acids are quite near each other (Fig. 5). When an intelligent integrator is used, reproducible results are obtained with a 24-m capillary column. If a longer column is used, the separation is more complete but the running time is markedly increased.

The results of an investigation of blood levels of α-keto acids in a group of healthy females and males, performed by the method here described, are presented in Table IV. The concentrations of the compounds studied are slightly higher in males. This may be due to higher protein mass found in males. Penttilä [4] found an α-ketoisovaleric acid concentration of 10 ±1 μmol/l (mean ± S.D.) and an α-ketoisocaproic acid + α-keto-β-methylvaleric acid concentration of 49 ± 2 μmol/l, which is in good agreement with our results. In addition, Nissen et al. [8], using high-performance liquid chromatography, reported 28, 18 and 17 μmol/l in venous blood for α-ketoisocaproic, α-keto-β-methylvaleric and α-ketoisovaleric acids, correspondingly.

TABLE IV

NORMAL VALUES OF α-KETO ACIDS IN HUMANS

Acid	Concentration (μmol/l, mean ± S.D.)	
	Female (n = 20)	Male (n = 20)
α-Ketoisovaleric acid	11.3 ± 3.0	13.3 ± 3.3
α-Keto-β-methyl-n-valeric acid	19.0 ± 7.4	23.3 ± 9.5
α-Ketoisocaproic acid	24.5 ± 8.9	37.4 ± 11.5

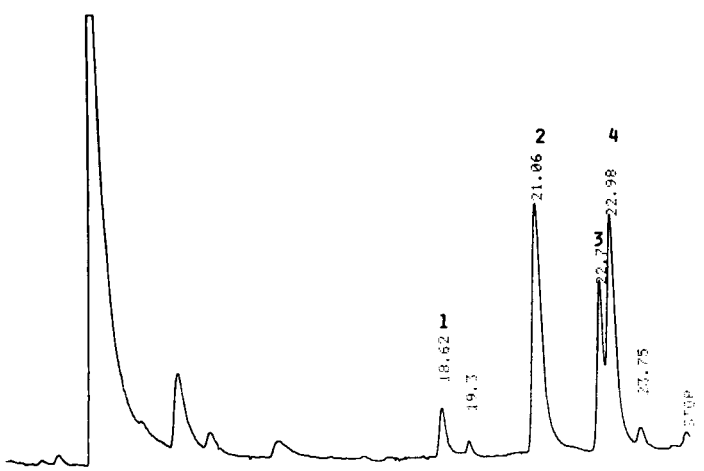

Fig. 5. Chromatogram of α-keto acids. Carlo Erba Fractovap 2350 gas chromatograph with a SE-30 fused-silica capillary column, 24 m long, with flame-ionization detector. Peaks: 1 = α-ketoisovaleric acid, 2 = α-ketovaleric acid (internal standard), 3 = α-keto-β-methylvaleric acid, 4 = α-ketoisocaproic acid.

The present method is very suitable for clinical application. It is easy to handle large numbers of samples, because the extraction and derivatization of the α-keto acids can be performed in the same tube. The combined procedure improves analytical precision by reducing the number of steps associated with sample treatment. It is also interesting to keep in mind that the present method can be used to measure α-keto acids from tissue samples by extractive alkylation to pentafluorobenzyl derivatives for GLC analysis.

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CHROMBIO. 2455

SIMULTANEOUS DETERMINATION OF PROSTANOIDS IN PLASMA BY GAS CHROMATOGRAPHY—NEGATIVE-ION CHEMICAL-IONIZATION MASS SPECTROMETRY

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(First received August 7th, 1984; revised manuscript received October 10th, 1984)

SUMMARY

A method for simultaneous determination of prostaglandin E_2 (PGE_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), and thromboxane B_2 (TxB_2) in plasma was developed. After acidification and addition of ^2H- and ^3H-labelled internal standards, plasma prostanoids were extracted by reversed-phase cartridges and purified by normal-phase high-performance liquid chromatography. The pentafluorobenzyl, methoxime, trimethylsilyl derivatives were formed. Negative-ion chemical-ionization mass spectra with methane as reagent gas show one intense peak at *m/z* (M — pentafluorobenzyl). This ion was used for selective-ion monitoring. Prostanoid plasma concentrations (pg/ml) in five healthy volunteers were: PGE_2 2.0—10.4, $PGF_{2\alpha}$ 2.2—9.8, 6-keto-$PGF_{1\alpha}$ 0.6—1.8, and TxB_2 3.0—45.3. However, there is evidence that the TxB_2 values may frequently be falsely high because of ex vivo production during the sampling procedure.

INTRODUCTION

For the measurement of prostanoids, several methods such as bioassay, radioimmunoassay (RIA), high-performance liquid chromatography (HPLC), gas chromatography and gas chromatography—mass spectrometry (GC—MS) are used. Plasma levels from the low pg/ml to the ng/ml level have been reported [1—10]. Generally, GC—MS data are considered the most reliable [6, 11]. Furthermore, the low values are supported by infusion studies [3, 4]. Published negative-ion chemical-ionization mass spectral (NICI-MS) data are in the low pg/ml range [2, 5].

NICI mass spectra of the trialkylsilyl [trimethylsilyl (TMS) or dimethylpropylsilyl]-pentafluorobenzyl (PFB)-methoxime (MO) prostanoid derivatives contain $(M - PFB)^-$ as the most intense peak [11—13]. The detection limit of

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the TMS-PFB derivative of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is about 200 fg at a signal-to-noise ratio of 5; for the derivatives of the other prostanoids this value is in the range 0.5—1.0 pg. Sensitivity of NICI-MS is about 100-fold higher than electron-impact mass spectrometry (EI-MS).

In this paper we report a sensitive and specific assay suitable to determine $PGF_{2\alpha}$, PGE_2, thromboxane B_2 (TxB_2) and 6-keto-$PGF_{1\alpha}$ simultaneously in a single plasma sample.

EXPERIMENTAL

Apparatus

HPLC. Separation was carried out using a Waters (Eschborn, F.R.G.) HPLC system: a U6K injector, two M6000 pumps, a M660 solvent programmer and a μPorasil HPLC column (10-μm particles, 30 cm × 3.9 mm I.D.).

GC—MS. A modified Finnigan MAT 4021 (Bremen, F.R.G.) quadrupole gas chromatograph—mass spectrometer with a pulsed positive/negative-ion chemical-ionization (PPNICI) module was employed. The gas chromatograph was a Carlo Erba HRGC (Hofheim, F.R.G.) with a J&W on-column injector and a J&W fused-silica capillary column (DB-1, 30 m × 0.259 mm I.D., 0.25 μm film thickness; ICT, Frankfurt, F.R.G.).

Materials

Nanograde solvents were used (chloroform, methanol, and ethyl acetate: Promochem, Wesel, F.R.G.; acetonitrile and water: Baker, Gross-Gerau, F.R.G.; formic acid, p.a., E. Merck, Darmstadt, F.R.G.). Sep-Pak reversed-phase cartridges were obtained from Waters (Gross-Gerau, F.R.G.). O-Methylhydroxyammonium chloride was from Pierce (Günter Karl, Geisenheim-Johannisberg, F.R.G.), N-ethyldiisopropylamine and pentafluorobenzylbromide were from Fluka (Buchs, Switzerland), and bis(trimethylsilyl)trifluoroacetamide (BSTFA) was from Macherey, Nagel & Co. (Düren, F.R.G.). Sephadex LH-20 was supplied by Pharmacia (Uppsala, Sweden), Extrelut by E. Merck.

PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, and TxB_2 and their 3,3′,4,4′-deuterated analogues were kind gifts from Drs. Udo Axen and John Pike (Upjohn, Kalamazoo, MI, U.S.A.). [5,6,8,11,12,14,15(*n*)-^{3}H]PGE_2, [5,6,8,9,11,12,14-15(*n*)-^{3}H]$PGF_{2\alpha}$, and [6-keto-(5,8,9,11,12,14,15(*n*)-^{3}H]$PGF_{1\alpha}$ were obtained from Amersham Buchler (Braunschweig, F.R.G.). [5,6,8,9,11,12,14,15(*n*)-^{3}H]TxB_2 was purchased from New England Nuclear (Dreieich, F.R.G.). Radiochemical purity of ^{3}H standards is about 90% as determined by HPLC.

Sample collection

Venous blood (18 ml) was collected without tourniquet into ice-cold tubes containing 0.5 mg of indomethacin in 2 ml of 3.8% sodium citrate solution and centrifuged immediately at 4°C and 1000 *g*. The plasma volume was determined and the plasma frozen at −80°C. Needles with different inner diameters (1.5 mm and 0.9 mm) were used for venipuncture.

Extraction and purification

Deuterated (1 ng each) and tritiated (25 000 dpm each) PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, and TxB_2 were added to 10 ml of plasma. After acidification with formic acid to pH 3.2, samples were allowed to equilibrate at 0° C for 30 min. Extraction was carried out using a reversed-phase C_{18} Sep-Pak cartridge, which was pre-conditioned with methanol and water. Prostanoids were eluted with ethyl acetate. The solvent was evaporated, the residue dissolved in chloroform and the prostanoids were separated on a normal-phase HPLC column. The initial eluent was chloroform, the final eluent, after 20 min, was 96.8% chloroform, 2.7% methanol, and 0.5% formic acid and the flow-rate 1 ml/min. Fractions of 1 ml were collected. Aliquots of 25 µl were assayed for radioactivity by liquid scintillation spectrometry.

Derivatization

Esterification. The prostanoid-containing fractions were evaporated at room temperature under nitrogen. The PFB derivatives were prepared by dissolving the residue in acetonitrile (50 µl), adding 35% pentafluorobenzyl bromide in acetonitrile (15 µl), and N-ethyldiisopropylamine (15 µl). The mixture was allowed to react at 50° C for 30 min. After evaporation electron-capturing substances were separated by elution through a column (30 mm × 5 mm I.D.) of pre-swollen Sephadex LH-20 with dichloromethane. The solvent was removed as described above.

Methoximation. The fraction containing PGE_2, 6-keto-$PGF_{1\alpha}$, and TxB_2 was dissolved in 100 µl of a O-methylhydroxyammonium chloride in pyridine (20 mg/ml). After reaction for 24 h at ambient temperature the solvent was evaporated. The residue was dissolved in water (0.1 ml) and applied to a short Extrelut column (30 mm × 5 mm I.D.) to remove the derivatization agent. The prostanoid derivatives were eluted with chloroform and the solvent was evaporated.

Silylation. The PFB-MO (PGE_2, 6-keto-$PGF_{1\alpha}$, and TxB_2) and PFB ($PGF_{2\alpha}$) derivatives were silylated with 50 µl of BSTFA at 25° C for 24 h. After evaporation with nitrogen the samples were reconstituted in 10 µl of BSTFA. After 3 h the samples were ready for GC—MS analysis. The derivatives are stable at —20° C for some months. All glassware used in the extraction, purification, and derivatization steps were silanized.

GC—MS conditions

Temperature programme: initial temperature 140° C, increased at 25° C/min to 290° C, then at 7.5° C/min to 320° C, temperature held for 5 min. Carrier gas (helium) pressure: 100 kPa. Sample volume: 2 µl. Temperatures: interface, 300° C; ionizer, 280° C. CI gas (methane) pressure in source: 22 Pa. Electron energy: 70 eV. Emission current: 0.4 mA. Electron multiplier: 1100 V. Conversion dynode: ± 3000 V. Selective-ion monitoring (SIM) at *m/z*: 524.3 (PGE_2), 528.3 ([$^2H_4$]PGE_2), 569.4 ($PGF_{2\alpha}$), 573.4 ([$^2H_4PGF_{2\alpha}$), 614.4 (TxB_2, 6-keto-$PGF_{1\alpha}$), and 618.4 ([$^2H_4$]TxB_2, 6-keto-[$^2H_4$]$PGF_{1\alpha}$).

RESULTS AND DISCUSSION

Our assay was designed to achieve simultaneous quantitation of several prostanoids in a single GC—MS run. This modification renders GC—MS analysis of prostanoids less labour-intensive, thus abolishing its major drawback.

HPLC purification of PGE_2, $PGF_{2\alpha}$, TxB_2 and 6-keto-$PGF_{1\alpha}$ was carried out on a silicic acid column. The chromatogram shows two peaks. The first one belongs to PGE_2, TxB_2 and 6-keto-$PGF_{1\alpha}$, which were not separated [15], the second one to the more polar $PGF_{2\alpha}$ (Fig. 1). It is an advantage of this HPLC system that these three prostanoids co-elute allowing one to save time for derivatization and GC—MS quantitation. Recoveries after the extraction and purification steps are about 45—55%.

After derivatization the prostanoid concentrations in plasma were determined by GC—NICI-MS. Fig. 2 shows the SIM chromatogram of $PGF_{2\alpha}$. The retention time (t_R) is about 11.4 min (200 scans = 1 min). As observed in the other chromatograms the deuterated compound has a somewhat shorter retention time.

PGE_2, TxB_2 and 6-keto-$PGF_{1\alpha}$ were determined in one GC—MS run. The PGF_2 PFB-MO-MTS derivative has the shortest t_R of these prostanoid derivatives. On the GC column the *syn*- and *anti*-oxime isomers were separated (Fig. 3). The concentration of the first eluting isomer (t_R 11.4 min) is about 40% of the second one (t_R 11.8 min). After elution of the PGE_2 derivatives we

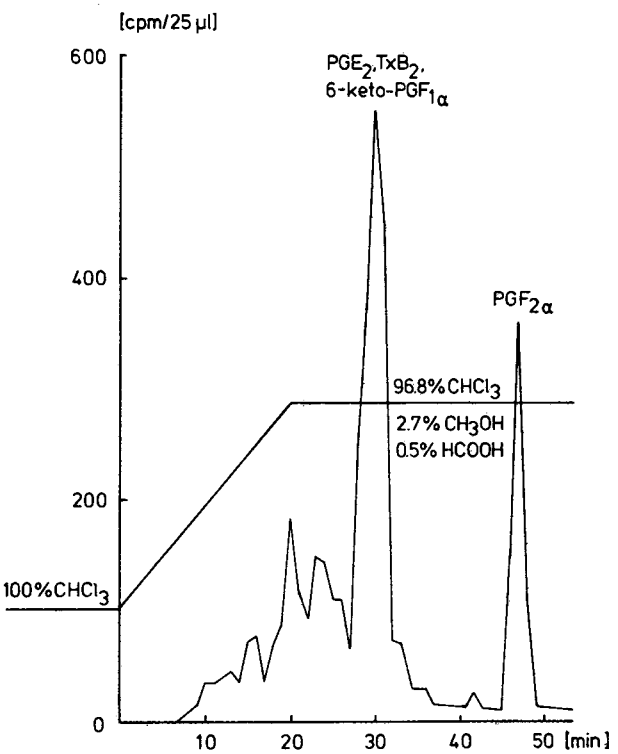

Fig. 1. HPLC radiochromatogram of tritiated PGE_2, TxB_2, 6-keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$.

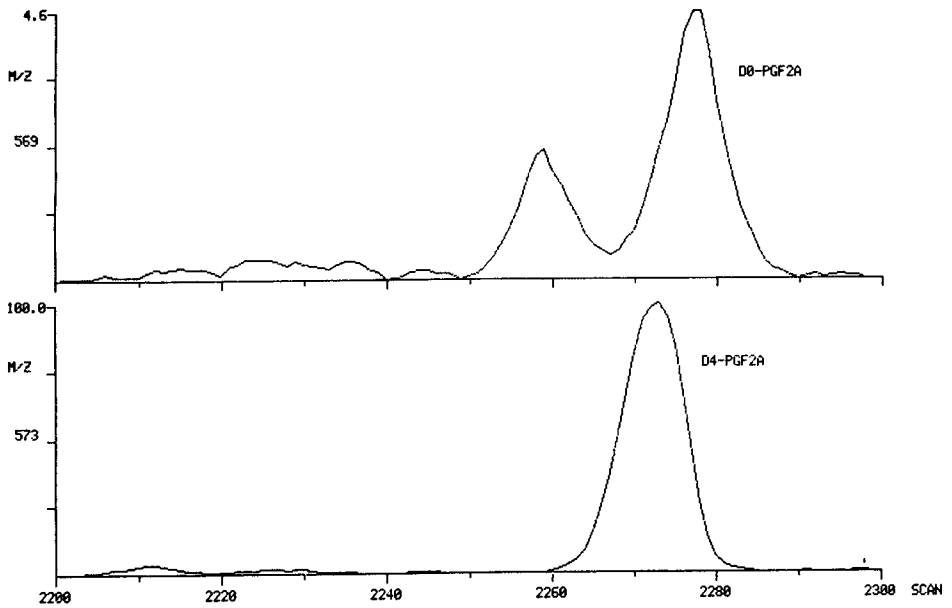

Fig. 2. SIM chromatogram of endogenous $PGF_{2\alpha}$ (m/z 569; relative intensity 4.6%) and deuterated $PGF_{2\alpha}$ (m/z 573; relative intensity 100%). Plasma concentration: 5.6 pg/ml.

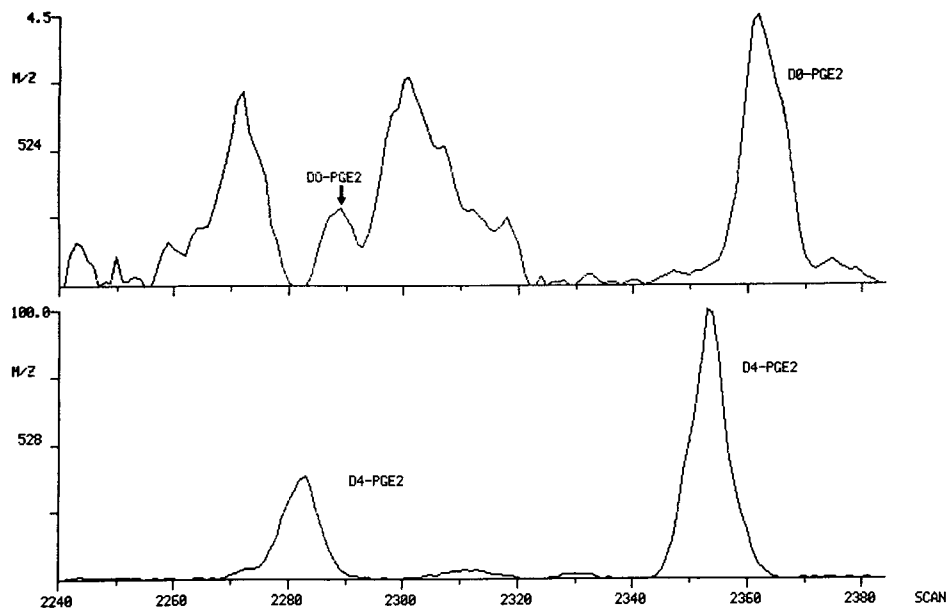

Fig. 3. SIM chromatogram of endogenous PGE_2 (m/z 524; relative intensity 4.5%) and deuterated PGE_2 (m/z 528; relative intensity 100%). Plasma concentration: 4.0 pg/ml.

switched from the SIM descriptor of PGE_2 to that of TxB_2 and 6-keto-$PGF_{1\alpha}$. The PFB-MO-TMS derivatives of these compounds have the same molecular weight. The TxB_2 derivative has a retention time of 12.1 min, the 6-keto-$PGF_{1\alpha}$

derivative elutes after 12.25 min (Fig. 4). The chromatogram shows no separation of the oxime isomers, but the TxB_2 peak is very broad.

For quantitation of endogenous prostanoids standard curves were prepared. They show linearity in the range from 10 pg to 1 ng of prostanoid using 1 ng of the deuterated compound as internal standard. The correlation coefficients (r) are shown in Table I. A blank caused by incomplete deuteration of the internal standards was observed in all investigated prostanoids (Table I). The inter-assay variations (n = 5) were between 8.6% (TxB_2) and 22.8% (6-keto-$PGF_{1\alpha}$) at the low pg/ml range (Table I).

Endogenous plasma concentrations in five healthy male volunteers were determined. The levels were as follows (see also Table II): $PGF_{2\alpha}$ 2.2—9.8 pg/ml, PGE_2 2.0—10.4 pg/ml, 6-keto-$PGF_{1\alpha}$ 0.6—1.8 pg/ml and TxB_2 3.0—45.3 pg/ml. These low values were also obtained in earlier studies using GC—NICI-MS [2, 5] by calculation of plasma levels from prostanoid concen-

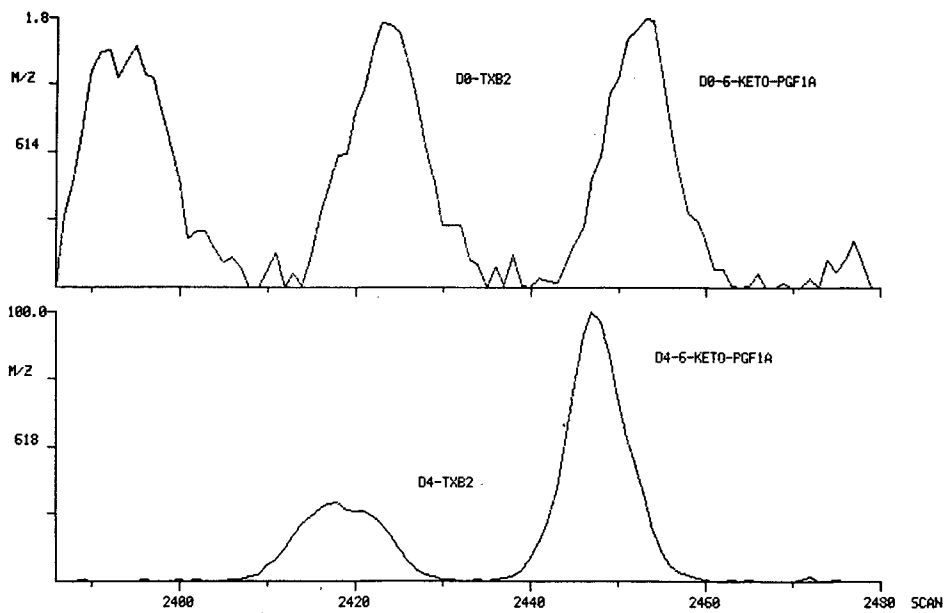

Fig. 4. SIM chromatogram of endogenous TxB_2 and 6-keto-$PGF_{1\alpha}$ (m/z 614; relative intensity 1.8%), and deuterated TxB_2 and 6-keto-$PGF_{1\alpha}$ (m/z 618; relative intensity 100%). Plasma concentrations: TxB_2 3.3 pg/ml, 6-keto-$PGF_{1\alpha}$ 1.0 pg/ml.

TABLE I

CORRELATION COEFFICIENTS (r), BLANKS AND PRECISION OF THE METHOD

Prostanoid	r	Blank (%)	Mean ± S.D. (pg/ml)	R.S.D. (%)
$PGF_{2\alpha}$	0.9998	0.2	4.84 ± 0.80	16.5
PGE_2	0.9999	0.1	2.16 ± 0.37	17.1
TxB_2	0.9990	0.2	5.14 ± 0.44	8.6
6-Keto-$PGF_{1\alpha}$	0.9995	0.5	0.92 ± 0.21	22.8

TABLE II

PROSTANOID CONCENTRATIONS IN PLASMA

Concentrations are expressed in pg/ml.

Volunteer (adult)	Large needle (1.5 mm I.D.)				Small needle (0.9 mm I.D.)			
	PGE_2	$PGF_{2\alpha}$	6-keto-$PGF_{1\alpha}$	TxB_2	PGE_2	$PGF_{2\alpha}$	6-keto-$PGF_{1\alpha}$	TxB_2
1	3.5	2.9	1.2	13.8	2.0	2.9	0.8	32.4
2	2.5	2.2	0.6	9.5	3.5	—	1.0	45.3
3	4.5	2.8	0.8	16.9	2.5	2.6	1.5	24.9
4	10.4	5.7	1.0	3.0	5.8	9.8	1.8	17.2
5					8.7	4.1	1.3	10.0

tration in urine after infusion of precursors [3, 4] and employing RIA after extraction and purification [7]. In contrast, other RIA investigations report very high levels of some hundred pg/ml [10].

For blood collection, needles with different inner diameters (0.9 and 1.5 mm) were used. The concentrations of $PGF_{2\alpha}$, PGE_2 and 6-keto-$PGF_{1\alpha}$ were not significantly affected, whereas TxB_2 levels were unpredictable higher when the smaller needle was used. The concentrations (n = 4) rose from 10.8 ± 6.0 pg/ml to 30.0 ± 12.0 pg/ml. This shows that TxB_2 levels are dependent on sampling conditions. Thrombocytes, endowed with an enormous capacity to synthesize and release TxB_2, are activated during the sampling procedure and increase TxB_2 levels by ex vivo production. The real TxB_2 concentrations in plasma may be much lower than the obtained values. Therefore, plasma TxB_2 levels should be interpreted with great caution unless sampling conditions are clearly described and accounted for.

ACKNOWLEDGEMENTS

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QUANTITATIVE DETERMINATION OF METHYLTESTOSTERONE AND METHYLTESTOSTERONE-d_3 IN SERUM BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A method for the quantitative estimation of methyltestosterone and methyltestosterone-d_3 in biological fluids has been developed using gas chromatography—mass spectrometry—selected-ion monitoring. Methyltestosterone-d_6 was used as an internal standard. Methyltestosterone and methyltestosterone-d_3 in serum were determined based on the peak height ratios of the molecular ions of methyltestosterone, methyltestosterone-d_3 and methyltestosterone-d_6. Sensitivity, specificity, precision, accuracy and reproducibility of the present method were demonstrated to be satisfactory for application to pharmacokinetic and bioavailability studies.

INTRODUCTION

Methyltestosterone (17β-hydroxy-17α-methyl-androst-4-en-3-one) is an orally effective synthetic androgen which has been used in the treatment of eunuchism, eunuchoidism, male impotence and female breast cancer [1—3].

The bioavailability/bioequivalency regulations of the United States Food and Drug Administration (FDA) which became effective on 7 July, 1977, listed 110 drugs and drug dosage forms which were known or suspected of having potential bioavailability/bioequivalency problems [4]. Although methyltestosterone is one of the drugs listed, there appears to be little information on its pharmacokinetic or bioavailability characteristics. Bioavailability and pharmacokinetic studies of methyltestosterone require sensitive, specific and reproducible analytical techniques. Alkalay and co-workers [5, 6] have employed spectrofluorometric determination to assess the bioavailability of methyltestosterone. The method, however, does not provide specificity and sufficient reproducibility.

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The use of gas chromatography—mass spectrometry (GC—MS) and stable-isotope-labelled drugs as diluents has found broad application in pharmacological studies [7—9]. In this technique, stable-isotope-labelled carriers serve as the ideal internal standard to correct for losses of a subtance under study in the initial isolation procedures. Pharmacokinetic and bioavailability studies represent one field in which the sensitivity and specificity of GC—MS offer an advantage. Recently, studies on the relative bioavailability of several different formulations of the same drug have been performed effectively by use of a stable-isotope-labelled variant as the reference with which the test unlabelled formulations are compared [10—12].

We have initiated studies to assess the relative bioavailability of methyltestosterone tablet formulations with coadministration of a stable-isotope-labelled methyltestosterone solution as internal biological standard. The present paper describes an analytical method for the simultaneous quantitative estimation of methyltestosterone and its 17α-methyl-d_3 analogue (methyltestosterone-d_3) in human serum. This method involves GC—MS with selected-ion monitoring (SIM) using methyltestosterone-d_6 as internal standard.

MATERIALS AND METHODS

Chemicals and reagents

17α-Methyl-d_3-testosterone (methyltestosterone-d_3) and 17α-methyl-d_3-testosterone-19,19,19-d_3 (methyltestosterone-d_6) were synthesized in our laboratory as described elsewhere [13]. The isotopic compositions were 99.6 atom% deuterium (d_3, 98.64%; d_2, 1.36%; d_1, 0.00%) for methyltestosterone-d_3 and 99.3 atom% deuterium (d_6, 96.88%; d_5, 2.26%; d_4, 0.86%) for methyltestosterone-d_6. Non-labelled methyltestosterone was purchased from Tokyo Kasei Kogyo, Tokyo, Japan (reagent grade) and was recrystallized from *n*-hexane—ethyl acetate (5:1). All other chemicals and solvents were analytical grade and used without further purification.

Stock solutions

Stock solutions of methyltestosterone (2.494 mg per 250 ml), methyltestosterone-d_3 (2.509 mg per 250 ml) and methyltestosterone-d_6 (2.568 mg per 250 ml) were prepared in ethanol. Storage of these solutions at 4°C did not result in any detectable decomposition for more than six months. All analyses were performed by diluting the stock solutions with ethanol.

Gas chromatography—mass spectrometry—selected-ion monitoring

GC—MS—SIM measurements were made with a Shimadzu LKB-9000B gas chromatograph—mass spectrometer equipped with a Shimadzu high-speed multiple-ion detector—peak matcher 9060S. GC was performed on a glass column (1 m × 3 mm I.D.) packed with 1.5% SE-30 on Chromosorb W (80—100 mesh). The column temperature was 230°C and the temperature of both the injector and separator was 250°C. The temperature of the ion source was 270°C. Helium was used as the carrier gas at a flow-rate of about 25 ml/min. The electron energy was set at 20 eV and the trap current at 60 μA. The multiple-ion detector was focused on the ions at *m/z* 302, 305 and 308.

The recording was made on a Nippon Denshi Kagaku four-pen recorder U-626D5, the chart speed being 10 mm/min.

Sample preparation for GC—MS—SIM

Frozen serum samples were thawed at room temperature. To a PTFE-lined screw-cap culture tube (100 × 16 mm) were added 1.0 ml of serum and 50 ng of methyltestosterone-d_6 dissolved in 20 μl of ethanol. The serum sample was allowed to stand for 30 min at room temperature. After adding 40 μl of 3 *M* sodium hydroxide solution, the serum sample was extracted with *n*-hexane (3 × 3 ml) by vortex-mixing for 10 sec. After centrifugation for 5 min at 1000 *g*, the organic layer was pipetted into a PTFE-lined screw-cap culture tube (100 × 16 mm) and washed with 1 ml of 5% acetic acid by vortex-mixing for 10 sec. After centrifugation for 5 min at 1000 *g*, the organic layer was pipetted into a PTFE-lined screw-cap conical centrifuge tube (100 × 16 mm). The solvent was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 20 μl of ethanol by ultrasonication for 5 min. After centrifugation (1000 *g*, 1 min), a 2—4 μl aliquot of the solution was subjected to GC—MS.

Absolute recovery

To 1.0 ml of human blank serum were added 50 ng of methyltestosterone in 20 μl of ethanol. After the serum sample was allowed to stand for 30 min at room temperature, the same procedures of extraction, washing and evaporation as described under sample preparation for GC—MS—SIM were followed. To the residue thus obtained were added 50 ng of methyltestosterone-d_6 dissolved in 20 μl of ethanol. After ultrasonication and centrifugation, a 2—4 μl aliquot of the solution was subjected to GC—MS. The absolute recovery of methyltestosterone from human serum was estimated by comparing the peak heights of *m/z* 302 and *m/z* 308.

Calibration curves and quantitation

Calibration curves for methyltestosterone or methyltestosterone-d_3 were prepared by adding 0, 1, 2, 5, 20, 50 and 200 ng of methyltestosterone or methyltestosterone-d_3 to 1.0-ml portions of human blank serum. Each sample was prepared in triplicate. The samples were then carried through the entire procedure and the peak height ratios of *m/z* 302 versus *m/z* 308 (d_0/d_6) and *m/z* 305 versus *m/z* 308 (d_3/d_6) were determined in triplicate. The curves were obtained by an unweighted least-squares linear fitting of the peak height ratios versus the mixed molar ratios on each analysis of unknown samples. Serum concentrations were calculated by comparing the peak height ratios obtained from the unknown samples with those obtained from the standard mixtures.

Drug administration

Eight healthy adult male volunteers, ranging in age from 21 to 34 years and in weight from 61.2 to 88.8 kg, participated in the study. The bioavailability tests began at 6.30 a.m. After an overnight fast, they were administered orally either a 10-mg methyltestosterone tablet and a 10-mg methyltestosterone-d_3 solution (100 ml) or a 10-mg methyltestosterone solution (100 ml) and a 10-mg methyltestosterone-d_3 solution (100 ml). The tablet was administered

with 120 ml of water. The solution was followed by a 20-ml water rinse of the container. No food was permitted for 4 h after drug administration. Blood (17 ml) was drawn just before the oral dose and at 0.33, 0.67, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 10 and 12 h after dosing. The samples were allowed to clot and were then centrifuged to separate the serum. The serum samples were stored at −20°C until the time of assay.

On a separate occasion of one week after completion of the first study, the drug administration was repeated with the same subjects receiving the reverse treatment.

RESULTS AND DISCUSSION

The electron-impact mass spectra of methyltestosterone, methyltestosterone-d_3 and methyltestosterone-d_6 demonstrated that the respective relative

TABLE I

PERCENT CONTRIBUTION TO THE ION INTENSITIES OF VARIOUS SPECIES IN CHANNELS MONITORED

Compound	*m/z* 302	*m/z* 305	*m/z* 308
Methyltestosterone	100	0.51	0.29
Methyltestosterone-d_3	0.47	100	0.55
Methyltestosterone-d_6	0.37	0.51	100

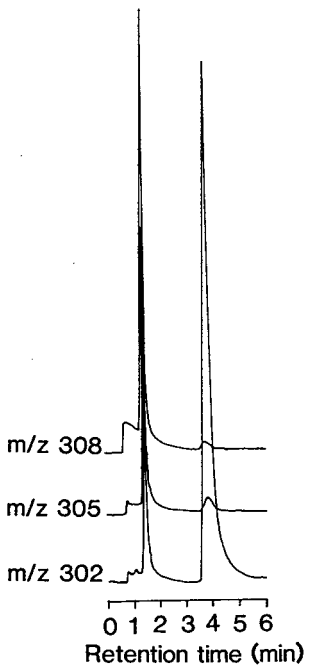

Fig. 1. Selected-ion monitoring of extracts from human serum sample spiked with methyltestosterone, methyltestosterone-d_3 and methyltestosterone-d_6.

abundances of the molecular ions at *m/z* 302, 305 and 308 were prominent. When the molecular ions were monitored, the sensitivity limit of the GC—MS—SIM assay was found to be 50 pg. A signal-to-noise ratio of $\geqslant 2.5$ was used as a criterion for a significant response for the injected methyltestosterone.

Because of the natural abundance of ^{2}H, ^{13}C and ^{18}O, a small peak at *m/z* 305 may appear in the mass spectrum of methyltestosterone. In addition, there is also the possibility that methyltestosterone-d_3 could contribute to the *m/z* 302 and methyltestosterone-d_6 to the *m/z* 302 and/or 305. Precise analysis of the GC—MS—SIM data from pure samples of methyltestosterone, methyltestosterone-d_3 and methyltestosterone-d_6 summarized in Table I indicated that no corrections for overlapping ions among the various isotopic compounds in question were necessary. The labelled compounds possessed sufficiently high isotopic purity and the contributions to the other ions were minor. Since the amount of methyltestosterone-d_6 in the analytical sample was 10- to 100-fold larger than that of methyltestosterone or methyltestosterone-d_3, the contribution of methyltestosterone-d_6 to the *m/z* 302 and 305 might be significant. However, this could be neglected because of the use of a constant amount of methyltestosterone-d_6 throughout all the assay samples.

In the GC—MS—SIM method used in the present study, an appropriate organic extraction solvent had to be chosen to enhance the sensitivity and to avoid the possibility of interferences. The extraction efficiency of methyltestosterone from serum samples was tested with *n*-hexane, diethyl ether, choroform and ethyl acetate. In spite of the high extraction efficiency obtained with diethyl ether, chloroform and ethyl acetate, the presence of numerous interfering peaks limited the use of these solvents. Fig. 1 shows the selected-ion monitoring after processing from a serum sample spiked with methyltestosterone (30 ng), methyltestosterone-d_3 (30 ng) and methyltestosterone-d_6 (50 ng) using *n*-hexane as extraction solvent. The presence of a large peak at 3.5—4.5 min, which corresponded to cholesterol, did not interfere with the analytes. The retention times of the analytes were the same (1.4 min). The total absolute recovery of methyltestosterone extracted from human serum with *n*-

TABLE II

ACCURACY OF SELECTED-ION MONITORING OF METHYLTESTOSTERONE IN SERUM

Added (ng/ml)	Found (ng/ml)					C.V. (%)	Relative error (%)
	Individual values*				Mean ± S.D.		
1.00	0.97	0.96	1.01	0.68	0.91 ± 0.15	16.5	—9.0
2.00	2.10	1.74	1.71	1.98	1.88 ± 0.19	10.0	—6.0
4.99	5.03	5.07	5.42	5.19	5.18 ± 0.18	3.4	3.8
19.95	20.61	19.51	19.79	19.76	19.92 ± 0.48	2.4	—0.2
49.87	50.87	51.60	50.26	49.45	50.55 ± 0.91	1.8	1.4
199.48	203.43	199.19	194.01	200.80	199.34 ± 3.97	2.0	—0.1

*Each individual value represents the mean of triplicate measurements.

TABLE III

ACCURACY OF SELECTED-ION MONITORING OF METHYLTESTOSTERONE-d_3 IN SERUM

Added (ng/ml)	Found (ng/ml)					C.V. (%)	Relative error (%)
	Individual values*				Mean ± S.D.		
1.00	1.10	1.09	0.79	1.07	1.01 ± 0.15	14.7	1.0
2.01	2.08	2.11	2.07	2.07	2.08 ± 0.02	0.9	3.6
5.02	4.83	4.87	4.84	4.89	4.86 ± 0.03	0.6	−3.2
20.07	19.96	20.55	20.53	19.36	20.10 ± 0.56	2.8	0.1
50.18	51.22	51.70	51.66	48.60	50.80 ± 1.48	2.9	1.2
200.72	207.39	203.54	195.43	195.81	200.54 ± 5.90	2.9	−0.1

*Each individual value represents the mean of triplicate measurements.

TABLE IV

DAY-TO-DAY PRECISION

n = 8 for each concentration.

Concentration (ng/ml)	Found (ng/ml)	C.V. (%)	Relative error (%)
Methyltestosterone			
4.99	4.98 ± 0.20	4.0	−0.2
19.95	20.14 ± 0.60	3.0	1.0
49.87	49.75 ± 0.38	0.8	−0.2
Methyltestosterone-d_3			
5.02	4.90 ± 0.09	1.8	−2.4
20.07	20.12 ± 0.33	1.6	0.2
50.18	50.24 ± 0.18	0.4	0.1

hexane was satisfactory, being about 70.4 ± 6.7% (n = 6). The present extraction procedures enabled sensitive quantitative analysis with a detection limit of 500 pg of methyltestosterone or methyltestosterone-d_3 per ml of serum.

Calibration curves were prepared by spiking 1.0 ml of blank human serum with various amounts (1—200 ng) of methyltestosterone or methyltestosterone-d_3 and a constant amount (50 ng) of methyltestosterone-d_6. Each sample was then analysed by monitoring the ions at m/z 302, 305 and 308. When the peak height ratios were plotted against the mixed molar ratios, a good correlation was found between the observed peak height ratios and the mixed molar ratios. Unweighted least-squares regression analysis gave a regression line of y = 1.0129x + 0.0042 (r = 1.0000) for methyltestosterone and y = 1.0317 x — 0.0023 (r = 1.0000) for methyltestosterone-d_3.

The accuracy of measurement was determined for methyltestosterone or methyltestosterone-d_3 added to 1.0-ml aliquots of blank human serum. The serum samples spiked with 1—200 ng of methyltestosterone or methyltestosterone-d_3 were analysed by the present method. The results presented in Tables II and III show that the estimated amounts of methyltestosterone or

methyltestosterone-d_3 were in good agreement with the actual amounts added.

Day-to-day precision of the assay was determined for a period of eight working days by performing triplicate analyses on serum samples containing 5, 20 and 50 ng/ml methyltestosterone or methyltestosterone-d_3. The results listed in Table IV demonstrate the excellent reproducibility.

The present method was applied for the quantitation of serum concentrations of methyltestosterone and methyltestosterone-d_3 after oral administration of a 10 mg + 10 mg mixture of methyltestosterone and methyltestosterone-d_3 solution to a healthy subject. There was no interference from metabolites of methyltestosterone and methyltestosterone-d_3 in the vicinity of the peaks of analytes in the mass fragmentograms. Serum concentrations of methyltestosterone and methyltestosterone-d_3 could be followed up to 8 h; the serum concentration—time curve is shown in Fig. 2. A bioavailability study of methyltestosterone is now in progress and will be described in detail elsewhere.

The present method provided a sensitive, simple and reliable technique for determining the serum levels of methyltestosterone and methyltestosterone-d_3 with good accuracy and precision. The method was confirmed to be applicable for assessing the relative bioavailability of methyltestosterone tablet formulations.

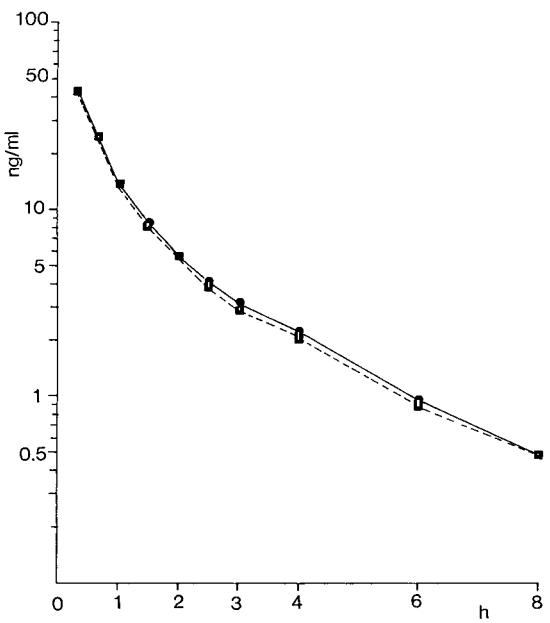

Fig. 2. Serum concentration—time curve for methyltestosterone (•) and methyltestosterone-d_3 (▪) after a single oral dose of a 10 mg + 10 mg mixture of methyltestosterone and methyltestosterone-d_3 solution to a healthy male volunteer.

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SPECIFIC MASS FRAGMENTOGRAPHIC ASSAY FOR 25,26-DIHYDROXYVITAMIN D IN HUMAN PLASMA USING A DEUTERATED INTERNAL STANDARD

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SUMMARY

A specific mass fragmentographic assay for the measurement of 25,26-dihydroxyvitamin D_3 [$25,26(OH)_2D_3$] in human plasma, using a stable isotope labelled internal standard {[$26,27\text{-}^2H_5$]$25,26(OH)_2D_3$}, is described.

Plasma samples (5 ml) were extracted with acetonitrile and applied to a C_{18} Sep-Pak cartridge, from which the vitamin D metabolites were eluted with methanol. The metabolites were then applied to a Sep-Pak SIL cartridge and three fractions were collected. The most polar fraction, containing the polyhydroxylated metabolites, was further purified by high-performance liquid chromatography on Zorbax SIL. The eluent containing $25,26(OH)_2D_3$ was collected, and the 25,26-*n*-butylboronate cyclic ester 3-trimethylsilyl ether derivative was formed. Gas chromatography—mass spectrometry was carried out, monitoring the intensities of the ions at *m/z* 449 and *m/z* 454 (for the internal standard). These ions represent the loss of a methyl group and the 3-silanol group, $(M - 90 - 15)^+$.

The minimum limit of detection of the assay was estimated to be approximately 0.05 μg/l.

0378-4347/85/$03.30

Inter-assay (3.7%) and intra-assay (8.0%) precision was acceptable and added $25,26(OH)_2D_3$, over the concentration range 0.5—1.5 μg/l, was recovered quantitatively. The plasma $25,26(OH)_2D_3$ level was estimated in 26 healthy volunteers and ranged from 0.05 to 1.30 μg/l, with a mean value of 0.54 μg/l.

INTRODUCTION

25,26-Dihydroxyvitamin D_3*, first isolated in 1970 by Suda et al. [1], is one of the polyhydroxylated vitamin D metabolites formed in the kidney [2] and possibly in extra-renal sites [3] by 26-hydroxylation of $25(OH)D_3$. Its precise role is unknown but it is probably a catabolic product formed, like $24,25(OH)_2D_3$, as an alternative to the active calcium homeostatic hormone $1,25(OH)_2D_3$. Although $25,26(OH)_2D_3$ circulates in normal human plasma in low nanomolar concentrations, it has not been widely studied, probably because of the lack of specific methods for assay. It is usually measured by competitive protein binding (CPB) assay using vitamin D binding globulin [4, 5]. This protein is relatively non-specific and therefore extensive purification is necessary prior to assay. A radioimmunoassay using an antiserum for $1,25(OH)_2D_3$ has also been described [6]. Because of the presence of increasing numbers of metabolites of vitamin D which are being described [7], the majority of which involve side-chain modifications, it is becoming more and more difficult to ensure that a sample prepared for assay is free of interfering metabolites. More specific assay techniques for $25,26(OH)_2D$ need to be developed in order to establish whether it possesses any physiological significance. Gas chromatography—mass spectrometry (GC—MS) offers a highly specific physicochemical means of assay and could be used as a definitive reference method against which other assays could be evaluated. This paper is the first report of the use of a method other than protein binding assay or immuno assay for the measurement of $25,26(OH)_2D$ in human plasma. A preliminary report of this procedure has already been published [8].

MATERIALS AND METHODS

Materials

25,26-Dihydroxy[26,27-$^2H_5$]vitamin D_3 was synthesised from [26,27-$^2H_6$]cholest-5-ene-3β,25-diol [9] by the route outlined in Fig. 1. Non-labelled standard $25,26(OH)_2D_3$ was also synthesised by the same route using non-deuterated cholest-5-ene-3β,25-diol. Both deuterated and non-deuterated standards were mixtures of the 25*R* and 25*S* isomers. The $25S,26(OH)_2D_3$ was a generous gift from Dr. M. Uskokovic (Hofmann-LaRoche, Nutley, NJ, U.S.A.). Labelled and unlabelled standards were purified by high-performance liquid chro-

*Systematic and trivial names of vitamin D and its metabolites used in this paper are as follows: vitamin D_2 (9,10-seco-ergosta-5,7,10(19),22-tetraen-3β-ol): D_2, vitamin D_3 (9,10-seco-cholesta-5,7,10(19)-trien-3β-ol): D_3, 25-hydroxyvitamin D: 25(OH)D, 24,25-dihydroxyvitamin D: $24,25(OH)_2D$, 25,26-dihydroxyvitamin D: $25,26(OH)_2D$, 1α,25-dihydroxyvitamin D: $1,25(OH)_2D$. The term D is used when there is no need to distinguish between D_2 and D_3.

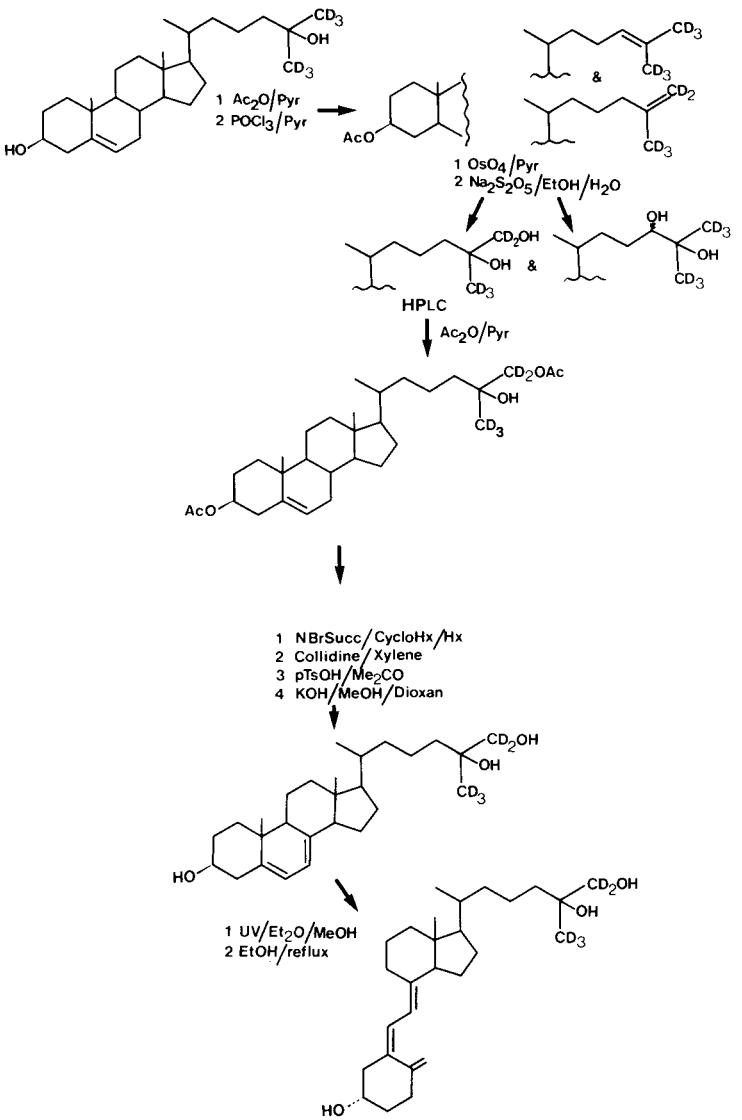

Fig. 1. Outline of the synthesis of $[^2H_5]25,26(OH)_2D_3$ for use as an internal standard. Non-standard abbreviations used are: Ac_2O = acetic anhydride; Pyr = pyridine; EtOH = ethanol; MeOH = methanol; NBrSucc = N-bromosuccinimide; CycloHx = cyclohexane; Hx = hexane; pTsOH = *p*-toluenesulphonic acid, Me_2CO = acetone, UV = ultraviolet light.

matography (HPLC) on receipt and repurified monthly thereafter. Concentrations of 5,7-diene steroids in solution were determined by UV absorbance at 264 nm, assuming a molar extinction coefficient at this wavelength of 18,300 [10]. Extraction solvents (AR grade wherever possible, from BDH , Poole, U.K.) and HPLC solvents (Rathburn Chemicals, Walkerburn, U.K.) were redistilled before use unless otherwise specified. Acetonitrile was shaken with activated charcoal (Sigma London, Poole, U.K.) and filtered immediately before use. Sep-Pak C_{18} and Sep-Pak SIL cartridges were purchased from Waters Assoc. (Northwich,

U.K.) and were used as described previously [11]. *n*-Butyl-, methyl-, and phenylboronic acids and bis(trimethylsilyl)trifluoracetamide (BSTFA) (Pierce and Warriner, Chester, U.K.) were used without further purification.

High-performance liquid chromatography (HPLC) was carried out as described previously [12] using a straight-phase Zorbax SIL column, eluted with isopropanol—methanol—hexane solvent, as described below.

Mass fragmentography was carried out as previously described [13] using a Model 2091 gas chromatograph—mass spectrometer (LKB Instruments, Croydon, U.K.).

All glassware was silanised by soaking overnight in 1% (v/v) dimethyldichlorosilane in toluene and washed with methanol. Blood from apparently healthy volunteer workers was collected into heparinised containers and the plasma separated. Unless analysed immediately, plasma was stored at −20°C under nitrogen.

Sample extraction and purification (Fig. 2)

Approximately 50 ng of [$^2H_5$]25,26(OH)$_2$D$_3$ were added to 5 ml plasma and equilibrated at room temperature for 10 min. An equal amount of freshly charcoal-washed acetonitrile was added and the samples were left to stand for a further 60 min, with occasional vortex-mixing. The precipitate formed was removed by centrifugation (7000 *g*, 15 min) and the supernatant added to 2.5 ml of 0.2 *M* acetate buffer, pH 5.6. This extract was applied to a C_{18} Sep-Pak cartridge which had been pre-washed first with 20 ml methanol and then 10 ml water. After application of the extract, the cartridge was washed with 3 ml methanol—water (60:40, v/v), and the vitamin D metabolites were eluted with 6 ml methanol.

The methanol extract was evaporated to dryness, redissolved in 300 μl isopropanol—hexane (1:99, v/v) and loaded at 4°C onto a Sep-Pak SIL cartridge prewashed with 10 ml methanol and 10 ml isopropanol—hexane (1:99, v/v). The SIL cartridge was washed with 13 ml of the same solvent. Then 10 ml isopropanol—hexane (3:97, v/v) were added to elute 25(OH)D. The polyhydroxylated metabolites, including 25,26(OH)$_2$D$_3$, were eluted with 10 ml isopropanol—hexane (30:70, v/v).

The solvent was evaporated and the residue was redissolved in 100 μl methanol—isopropanol—hexane (3:7:90, v/v/v) and injected onto a Zorbax SIL HPLC column. 24,25(OH)$_2$D$_3$, 25,26(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_3$ were completely separated in this system. The relative retention time of 25,26(OH)$_2$D$_3$ was approximately 11 min. The fractions containing 25,26(OH)$_2$D$_3$ (10—12 min) were collected, evaporated to dryness and either stored in 1 ml ethanol at −20°C or immediately prepared for GC—MS.

Derivatisation and assay

The dried extracts and a series of standards (50 ng deuterated 25,26(OH)$_2$D$_3$ + 0, 3, 5, 8 and 10 ng 25,26(OH)$_2$D$_3$), were converted into *n*-butylboronate (nBBA) cyclic ester 3-trimethylsilyl (TMSi) ether derivatives, by incubating with 30 μl *n*-butyl boronic acid in tetrahydrofuran (1 mg/ml) at room temperature for 30 min. The solvent was removed and the residue taken up in 30 μl BSTFA. After 30 min, 10-μl aliquots were injected onto the GC—MS

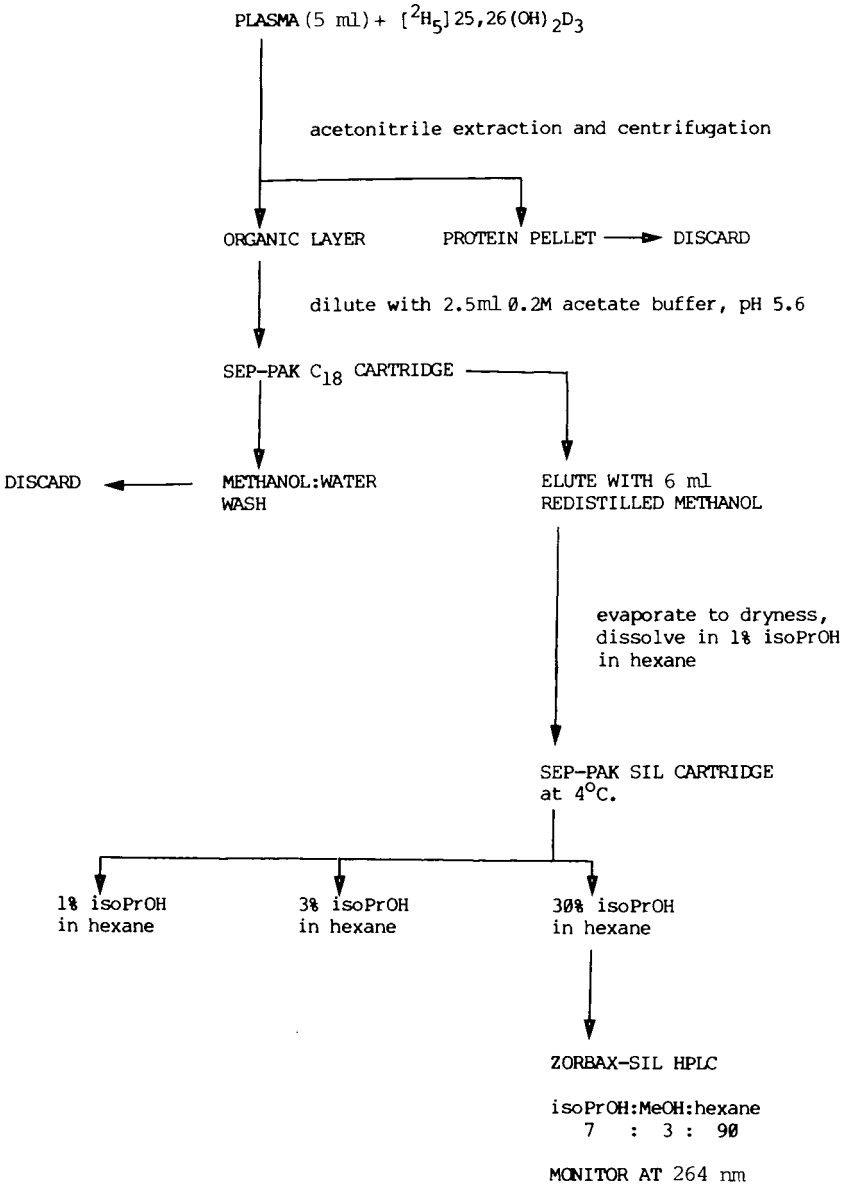

Fig. 2. Flow diagram summarising the extraction and purification of 25,26(OH)$_2$D prior to GC—MS. The HPLC fraction containing 25,26(OH)$_2$D (10—12 min after injection) was collected and analysed by GC—MS as described in the text. IsoPrOH = isopropanol; MeOH = methanol.

system. These derivatives do not appear to be very stable and therefore GC—MS was carried out immediately after formation. The pyro-isomer of the 25,26-*n*-butylboronate cyclic ester 3-trimethylsilyl ether derivative of 25,26(OH)$_2$D$_3$ (25,26(OH)$_2$D$_3$-nBBA-3-TMSi) gave a 20-eV mass spectrum (Fig. 3) with a base peak at *m/z* 449 (*m/z* 454 for the deuterated standard). These ions, $(M - 90 - 15)^+$, are formed from the molecular ion by the loss of a methyl group and the 3-silanol group. The ions at *m/z* 449 and *m/z* 454 were

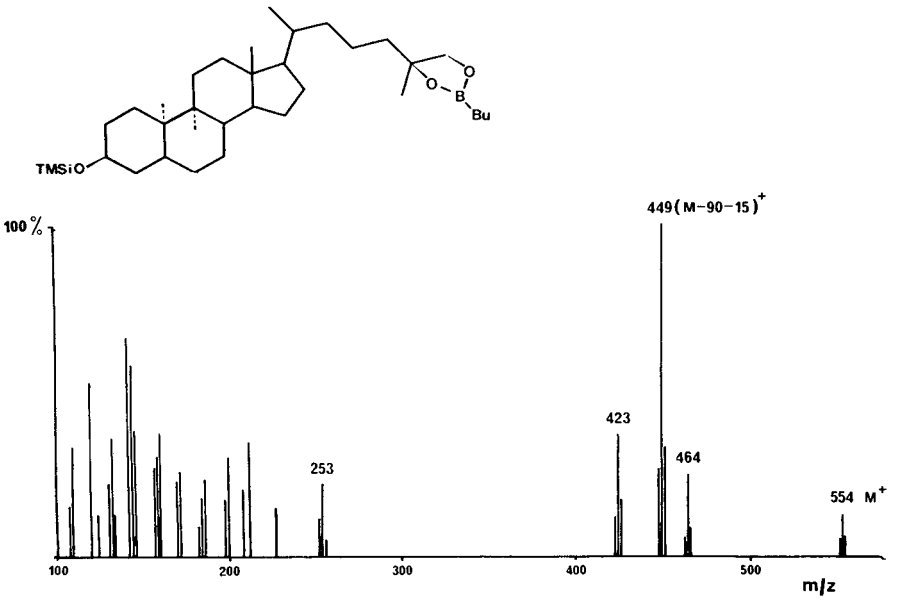

Fig. 3. The 24-eV mass spectrum of the pyro isomer of 25,26(OH)$_2$D$_3$-nBBA-3-TMSi using the LKB 2091 gas chromatograph—mass spectrometer. The derivative was analysed by GC—MS as described in the text and a mass spectrum (*m/z* 100—600) was obtained at the maximum of the emergent peak. Peak heights have been normalised with respect to the base peak (*m/z* 449, 100%). Peaks of less than 5% are not recorded.

monitored and the peak heights of the pyro-isomers of 25,26(OH)$_2$D$_3$ and the internal standard were measured. After correction for channel amplification, the ratio, peak height of ion at *m/z* 449/peak height of ion at *m/z* 454 for the series of standards and the plasma samples was calculated. A standard curve, relating this ratio to the amount of standard 25,26(OH)$_2$D$_3$, was plotted, from which the amount of 25,26(OH)$_2$D$_3$ in each sample could be determined.

Derivatisation

In a previous publication [13] the GC—MS of isotachysterol isomers of some vitamin D metabolites derivatised as trimethylsilyl ethers was described. These derivatives fragment to give a relatively high-intensity molecular ion and preliminary mass fragmentographic studies were carried out on 24,25(OH)$_2$D$_3$. At the time no standard 25,26(OH)$_2$D$_3$ was available and it was suggested that these two metabolites separated in the GC system used. When standard 25,26(OH)$_2$D$_3$ became available, it was discovered that 24,25(OH)$_2$D$_3$ and 25,26(OH)$_2$D$_3$ had identical retention times when applied to the gas chromatograph as tri-trimethylsilyl ethers (tri-TMSi). However, these two metabolites were well separated as *n*-butylboronate cyclic ester 3-trimethylsilyl ethers [14]. The use of these derivatives also increased specificity as cyclical boronates will only form across hydroxyls in close proximity, although not necessarily only on adjacent carbons [15].

Preparation of the trimethylsilyl ether, methylboronate and phenylboronate derivatives of 24,25(OH)$_2$D$_3$ and 25,26(OH)$_2$D$_3$ was also attempted. The

TABLE I

GAS CHROMATOGRAPHIC RETENTION TIMES OF METHYL-, *n*-BUTYL- AND PHENYLBORONATE-3-TMSi DERIVATIVES OF $24,25(OH)_2D_3$ AND $25,26(OH)_2D_3$

Derivative*	Retention time**	
	Pyro isomer	Isopyro isomer
$24,25(OH)_2D_3$		
Methylboronate-3-TMSi	1.6137	2.3357
n-Butylboronate-3-TMSi	2.6250	3.5625
Phenylboronate-3-TMSi	5.9403	8.0450
$25,26(OH)_2D_3$		
Methylboronate-3-TMSi	Not formed	
n-Butylboronate-3-TMSi	3.1797	4.2656
Phenylboronate-3-TMSi	6.6522	8.8551

*The appropriate 24,25- or 25,26-cyclic boronate ester 3-trimethylsilyl ether was prepared as described in the text and analysed by GC using an OV-1 column at 275°C (for methylboronate derivatives) or 300°C.

**Relative to the retention time of pyro-D_2-TMSi (1.7 min at 300°C and 7 min at 275°C).

methylboronate cyclic ester 3-trimethylsilyl ether derivative of $25,26(OH)_2D_3$, however, could not be formed. The mass spectrum obtained from $25,26(OH)_2D_3$ after reaction with methylboronic acid and BSTFA was the same as that obtained from $25,26(OH)_2D_3$ reacted with BSTFA alone, suggesting an inability to form a methylboronate cyclic ester across the hydroxyls on carbons 25 and 26, or subsequent removal by reaction with BSTFA [16]. The phenylboronate derivatives of both metabolites had very long retention times in GC system used (Table I). The mass spectra of methyl- and phenylboronate TMSi derivatives showed similar fragmentations to those shown in Figs. 3 and 4 and no improvement in the intensities of high-mass ions were observed. *n*-Butylboronate-3-TMSi derivatives of $25,26(OH)_2D_3$ were therefore used.

Mass spectrometry

The use of isotachysterol isomers of vitamin D metabolites increases sensitivity due to the formation of a single peak on GC, and, in the case of the tri-TMSi derivatives, by increasing the intensity of the molecular ion, in comparison to that of the pyro isomer [17]. However, using the LKB 2091 gas chromatograph—mass spectrometer, the isotachysterol isomer of $25,26(OH)_2D_3$-nBBA-3-TMSi gave a normalised 24-eV mass spectrum with a base peak at *m/z* 253 and greatly reduced intensities of ions of high mass (Fig. 4). The ion at *m/z* 253 is formed by the loss of the 3-silanol group and the entire C_8 side-chain. This high-intensity ion, however, could not be used for mass fragmentography because the deuterium label on the side-chain was lost during this fragmentation. The pyro isomer was therefore used, monitoring the ion at *m/z* 449 (*m/z* 454 for the internal standard), which gave enhanced sensitivity over that of the molecular ion (*m/z* 632) of $25,26(OH)_2$-isotachysterol$_3$-triTMSi.

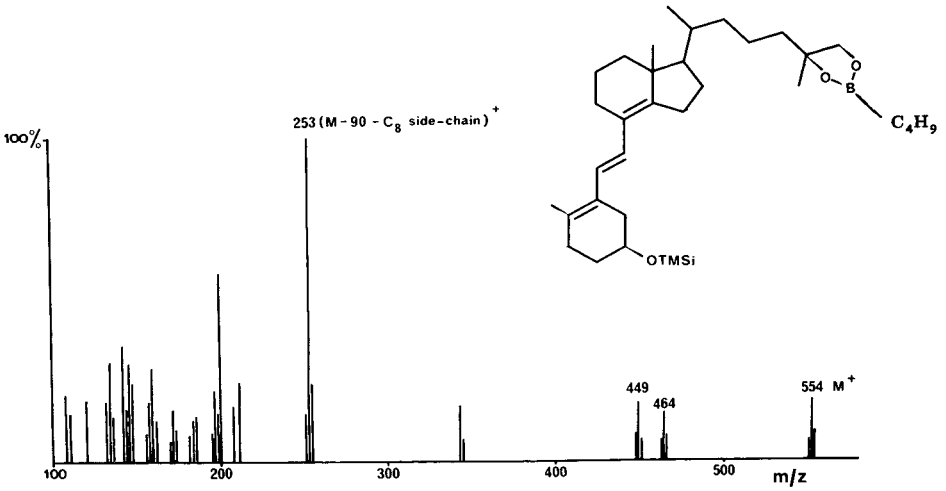

Fig. 4. Normalised mass spectrum of 25,26(OH)$_2$-isotachysterol$_3$-nBBA-3-TMSi obtained at 24 eV using the LKB 2091 gas chromatograph—mass spectrometer. Details are as given in legend to Fig. 3.

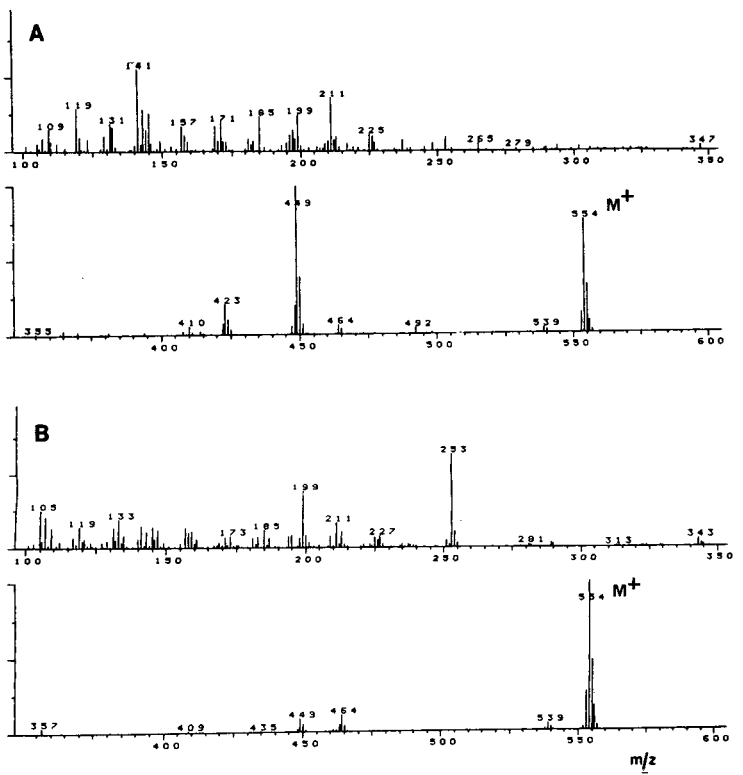

Fig. 5. Normalised mass spectrum of (A) pyro-25,26(OH)$_2$D$_3$-nBBA-3-TMSi and (B) 25,26(OH)$_2$-isotachysterol$_3$-nBBA-3-TMSi obtained at 20 eV using a Nermag R10-10C gas chromatograph—mass spectrometer. Mass spectra were obtained in a similar fashion to those illustrated in Figs. 3 and 4. The peak heights have been normalised with respect to the base peaks (*m/z* 449, 100% for A; *m/z* 554, 100% for B). The fragmentation is qualitatively, but not quantitatively, similar to those obtained in Figs. 3 and 4.

During an evaluation of other mass spectrometers, spectra for $25,26(OH)_2D_3$-nBBA-3-TMSi were obtained on two different quadrupole instruments (Model R10-10C, Nermag SN, Rueil-Malmaison, France and Model 12250, VG Analytical, Manchester, U.K.). Fig. 5 shows the normalised 20-eV mass spectra of pyro and isotachysterol isomers of $25,26(OH)_2D_3$-nBBA-3-TMSi obtained using the Nermag R10-10C. The VG 12250 produced similar mass spectra at 24 eV to those obtained on the LKB 2091 (Figs. 3 and 4), although the pyro isomer showed a molecular ion of greater intensity. At 70 eV, mass spectra produced by the Nermag R10-10C were the same as the 24-eV spectra produced by the LKB 2091 and the VG 12250 (Figs. 3 and 4). On the Nermag R10-10C, the base peak of the 20-eV mass spectrum of the $25,26(OH)_2$-isotachysterol$_3$-nBBA-3-TMSi derivative was the molecular ion m/z 554. It has not yet been possible to assess the relative intensities of the ions produced in the different mass spectrometers. However, it would appear that the Nermag R10-10C offers some advantages since it would allow the use of $25,26(OH)_2$-isotachysterol-nBBA-3-TMSi derivatives, thus avoiding the production of two peaks on the gas chromatograph. Similar differences between the mass spectra obtained on these three instruments were obtained for $24,25(OH)_2D_3$-nBBA-3-TMSi derivatives. Such differences in fragmentation are unexpected and may be due to differences in ion source geometry.

RESULTS

Normal values

The standard curve relating peak height ratio to quantity of $25,26(OH)_2D$ injected was always linear over the range 0—10 ng (e.g. y (peak height ratio) = $0.018x$ (ng of $25,26(OH)_2D$) + 0.0008, correlation coefficient was 1.0017 ($P < 0.001$). Examples of traces obtained from a water blank taken through the procedure, and two plasma samples are illustrated in Fig. 6. Radiolabelled $25,26(OH)_2D$ was not available to us and therefore it was not possible to assess overall recovery with any accuracy. However, rough assessment of the recovery of added deuterated $25,26(OH)_2D_3$ indicated that recoveries were very similar to those previously obtained for $24,25(OH)_2D_3$ [14] at around 60%. The minimum limit of detection of the assay was estimated to be approximately 0.05 μg/l. Plasma concentrations of $25,26(OH)_2D_3$ were measured in fifteen healthy volunteers in the U.K. — a mean value of 0.39 μg/l with a range from 0.05 to 0.79 μg/l was obtained. Samples were also obtained from eleven sun-baked Australians (by courtesy of Professor S. Posen, Miss Dianne Lissner and Mrs Angelike Trube, Royal North Shore Hospital, N.S.W., Australia) and the mean value obtained was 0.76 μg/l with a range from 0.30 to 1.30 μg/l. Estimations of the level of 25(OH)D were also carried out on these plasma samples and linear regression analysis showed a close correlation between the levels of $24,25(OH)_2D_3$, $25(OH)D_3$ and $25,26(OH)_2D_3$ [y ($25,26D_3$) = $0.013x$ ($25D_3$) + 0.137, r = 0.8777; y ($25,26D_3$) = $0.196x$ ($24,25D_3$) + 0.137, r = 0.9234].

Specificity

The extraction procedure prior to derivative formation and GC—MS includes

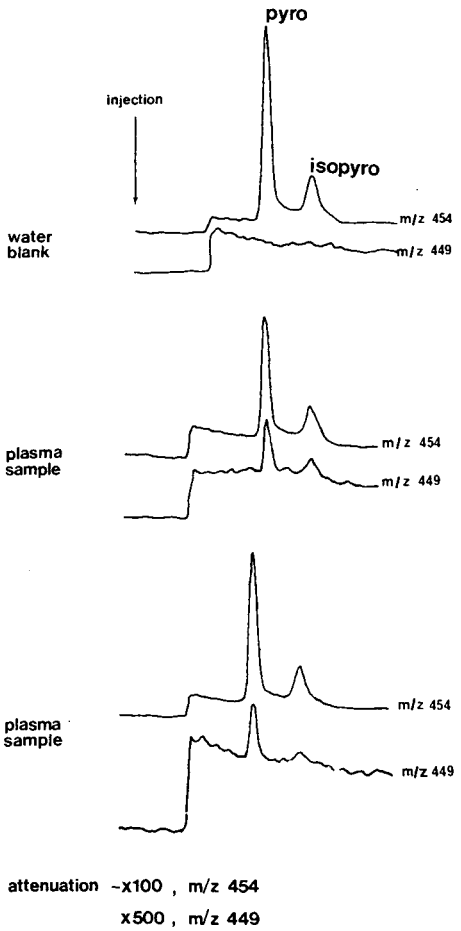

Fig. 6. Representative mass fragmentograms from a water blank and two plasma samples processed as described in the text. No non-deuterated $25,26(OH)_2D_3$-nBBA-3-TMSi was detected in the *m/z* 449 channel of the water blank (upper trace). The plasma samples illustrated had $25,26(OH)_2D_3$ concentrations of 0.81 μg/l (middle trace) and 0.78 μg/l (bottom trace). Amplification settings for each channel are given at the bottom of the Fig. The retention time of the pyro peak was approximately 6 min.

an HPLC step. This is included because this method has been developed as part of a profile procedure in which all the major metabolites of vitamin D can be separated and assayed individually. However, extracts of plasma samples were found to be clean enough after fractionation by Sep-Pak SIL for the $25,26(OH)_2 D_3$ levels to be measured at this stage, together with $24,25(OH)_2D_3$. Some potential contaminants which may interfere in CPB assay also elute from the HPLC in a similar position to $25,26(OH)_2D_3$, but it is unlikely that any would form cyclic boronate esters and would run in the same position on GC and fragment to give similar ions on GC—MS. Absolute specificity cannot however be assured until standards for all the possibly interfering metabolites become available.

An indirect method was used to assess the effect of any $25,26(OH)_2D_2$

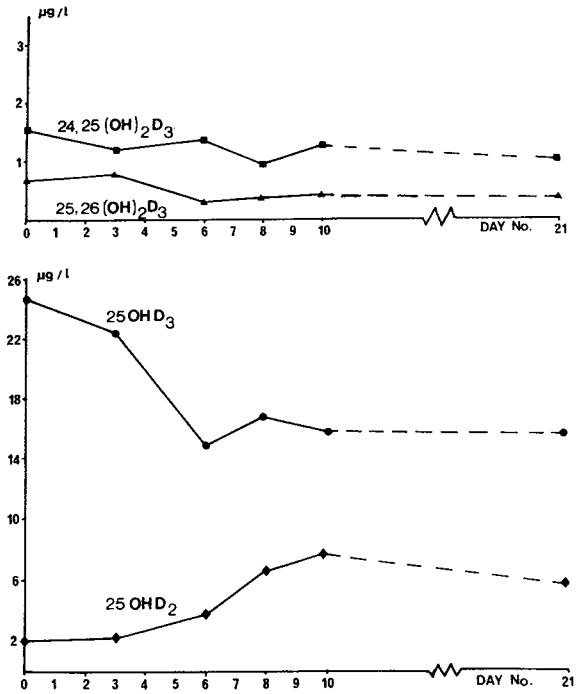

Fig. 7. Plasma concentrations of $25(OH)D_2$, $25(OH)D_3$, $24,25(OH)_2D_3$ and $25,26(OH)_2D_3$ in a normal person taking oral D_2 (day 1 to day 9, 12,000 I.U. per day).

which might be present in the sample, because no standard $25,26(OH)_2D_2$ was available to us. A normal volunteer was dosed with 12,000 I.U. vitamin D_2 per day for nine days, and the varying plasma levels of the vitamin D metabolites were measured during and after this time. The appropriate $(M - 90 - 15)^+$ ion for $25,26(OH)_2D_2$ was monitored, but no peak was detected. Equally, no significant rise in the level of $25,26(OH)_2D_3$ was observed, indicating that if metabolites of D_2 were formed under these conditions, they did not interfere with the assay (Fig. 7).

Reproducibility

Within- and between-batch reproducibility studies were carried out and the results obtained are given in Table II. Plasma samples used in the between-batch study were stored under nitrogen at −20° C and assayed over a period of seven weeks.

TABLE II

REPRODUCIBILITY STUDIES

Two samples were assayed six times. For inter-assay studies the plasma sample was stored at −20° C and analysed over a period of seven weeks.

	Mean value (μg/l)	Standard deviation	Coefficient of variation (%)
Within-batch (intra-assay) precision	0.28	0.01	3.7
Between-batch (inter-assay) precision	0.24	0.02	8.0

TABLE III

RECOVERIES OF STANDARD $25,26(OH)_2D_3$ ADDED TO PLASMA

Values recorded are those from four experiments. Standard $25,26(OH)_2D_3$ was added to 5 ml plasma to obtain the concentrations indicated. The plasma was incubated for 60 min at room temperature and assayed as described in the text.

Concentration (μg/l)		Percentage recovered (mean ± S.D.)
Added	Recovered (mean ± S.D.)	
0.5	0.52 ± 0.04	103.6 ± 7.7
1.0	1.02 ± 0.04	101.8 ± 3.9
1.5	1.55 ± 0.09	103.6 ± 5.6

Recovery experiments

Standard $25,26(OH)_2D_3$ was added to plasma at concentrations of 0.5, 1.0 and 1.5 μg/l and assayed as described above. The recovery of added $25,26(OH)_2D_3$ was calculated and the results are given in Table III.

DISCUSSION

This paper reports the development and evaluation of the first specific assay for $25,26(OH)_2D$ in human plasma which does not utilise relatively non-specific binding proteins or antibodies and does not rely on competitive binding assay. It thus provides the means for the evaluation of the specificity of existing methodology or alternatively offers the opportunity to measure specifically the levels of this metabolite in human plasma under various physiological and pathological conditions. Although the method describes the estimation of $25,26(OH)_2D_3$, it can also be used to measure $25,26(OH)_2D_2$ providing standards are available. Both these compounds can be measured in a single GC run by also monitoring the intensity of the appropriate mass fragment from $25,26(OH)_2D_2$. It has been demonstrated that with a mass spectrometer with sufficient multiple-ion detection channels, it is possible to measure $24,25(OH)_2D$ and $25,26(OH)_2D$ simultaneously immediately after the separation on Sep-Pak SIL without the need for an HPLC step. If a mass spectrometer with many ion monitoring channels is used, it is possible not only to measure the levels of these metabolites in plasma extracts but also to verify the specificity of the peak being measured. If a number of different ions are monitored simultaneously, a pure peak uncontaminated with interfering material will give peak height ratios which are the same as those calculated from the mass spectrum of the pure compound. The more peaks which are monitored and thus the more ratios which agree with those found in the pure compound, the greater the demonstrated specificity. In addition the mass spectrometer can be used in the less sensitive mass chromatography mode and complete mass spectra can be collected at regular intervals throughout the chromatographic run. Peaks with the correct retention time can therefore be checked to ensure that they are homogeneous and that the mass spectrum at the peak corresponds with that of the pure standard. Thus GC—MS is a very

powerful method for the specific measurement of biological compounds and can be used as a definitive method against which other possibly less-specific procedures can be evaluated. A similar method using GC—MS has been described for the measurement of plasma levels of $24,25(OH)_2D$ [14].

It is not clear whether $25,26(OH)_2D$ has any physiological function or whether it represents another catabolic pathway from 25(OH)D. Although $25,26(OH)_2D$ was reported to stimulate the retention of calcium by the intestine [1], it is considerably less effective in this respect than the active calcium homeostatic hormone, $1,25(OH)_2D$. Other physiological roles for $25,26(OH)_2D$ have been suggested [18, 19] but no firm evidence has been provided. The possibility of measuring this metabolite specifically offers the opportunity to establish whether it has any important physiological role or not.

Naturally occurring $25,26(OH)_2D$ has been variously identified as 25*R* [20], 25*S* [21] and as an epimeric mixture of 25*R* and 25*S* isomers [22]. In the GC—MS procedure described here, the deuterated internal standard is a mixture of 25*R* and 25*S* isomers, but this has caused no problems since this isomeric mixture does not separate in any of the chromatographic systems used. The use of the 25*S* isomer or a mixture of 25*S* and 25*R* isomers as standards has given identical results.

The GC—MS results obtained from normal plasma samples are very similar to those reported by Markestad [23] but slightly higher than the values reported using radioimmunoassay [6]. Interestingly, the correlation between the levels of $25(OH)D_3$ and $25,26(OH)_2D_3$ found here is almost exactly the same as that reported by Fraher et al. [6]. Correlation between $24,25(OH)_2D$ and $25,26(OH)_2D$ has also been reported previously [6, 23] but the correlation coefficients are closer to 1.000 in the present study. The minimum detection limit using 5 ml of plasma using radioimmunoassay [6] was 0.04 μg/l which is very similar to that reported here (0.05 μg/l). However, as expected, the precision achieved by GC—MS was substantially better than that of radioimmunoassay. Although absolute specificity has not been demonstrated here, it is clear that GC—MS can provide sensitive and highly specific methods for the assay of $25,26(OH)_2D$ and other metabolites of vitamin D in a variety of body fluids. The use of modern computerised mass spectrometers with improved detection systems, and different methods of ionisation offers the possibility of greatly improved sensitivity without any sacrifice in specificity. Use of high-resolution mass spectrometry can, by reducing the signal-to-noise ratio, also improve sensitivity and a method for the measurement of oestradiol-17β in 2 ml of plasma with a minimum detectable limit of 0.005 μg/l, ten times lower than that described here, has been reported [24].

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ISOLATION AND QUANTITATION OF CARBOHYDRATES IN SHEEP PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The application of ultraviolet detection at 190 nm following chromatography on a Ca^{2+} cation-exchange column with a mobile phase of water enables the low amounts of carbohydrates present in plasma to be quantitated. The separation and quantitation of carbohydrates in maternal and fetal sheep plasma and amniotic fluid are described, as is the application of this method to the determination of specific radioactivities of glucose and fructose in plasma.

INTRODUCTION

The utilisation and metabolic fate of glucose in the fetus and placenta in vivo have been studied by infusion of [2-^{3}H]glucose and [U-^{14}C]glucose via indwelling catheters into the fetus and subsequent collection of samples from both the fetal and maternal circulations for determination of the specific radioactivity of glucose in plasma [1]. As a major metabolic substrate of the gravid uterus, glucose may be metabolised in several ways, including oxidation for energy production or utilisation for biosyntheses via glycolysis, the pentose phosphate pathway and hexose isomerisations. Because the latter pathways are more active in the fetus and/or placenta than in the adult [2], significant incorporation of ^{14}C-radioactivity from glucose into other carbohydrates including fructose may occur [3]. We required a rapid and sensitive method for the determination of the specific radioactivities of glucose and other carbohydrates, particularly fructose and polyols such as dulcitol and sorbitol, in plasma for such studies. A further constraint upon the method was the limited volume of fetal plasma available for analysis.

There are several other approaches to the determination of the specific radio-

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activities of different monosaccharides and their derivatives in plasma, following the administration of radioactive precursors in vivo. Most involve deproteinisation of plasma, removal of charged compounds by ion-exchange chromatography, then separation of the various carbohydrates present. The techniques most commonly used to achieve this separation are paper or thin-layer chromatography [4, 5], electrophoresis [6] or further ion-exchange chromatography of the carbohydrates as borate complexes [7]. In most instances, the carbohydrates must then be located and eluted from the support medium before radioactivity content and the amount present can be quantitated. These techniques are relatively time-consuming and not appropriate to large numbers of samples. In addition, sufficiently sensitive assays for the quantitation of many carbohydrates, particularly polyols, are not available.

In this paper, the successful application of high-performance liquid chromatography (HPLC) to the simultaneous separation and quantitation of carbohydrates in plasma from fetal and maternal sheep for the determination of their specific radioactivities is reported.

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical grade. Carbohydrates, potassium phosphate, glucose oxidase, peroxidase, 2,2′-azino-di(3-ethylbenzthiazoline sulfonic acid), Triton X-100, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] benzene (dimethyl POPOP) and 2,5-diphenyloxazole (PPO) were obtained from Sigma (St. Louis, MO, U.S.A.). Barium hydroxide, zinc sulfate and toluene were obtained from BDH (Victoria, Australia). Millipore-Q reagent-grade water was usd throughout. D-[U-^{14}C] Glucose (10.47 GBq/mmol) and D-[2-^{3}H] glucose (444 GBq/mmol) were obtained from Amersham Australia (Sydney, Australia).

Cation and anion exchangers were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.).

Preparation of samples

Samples of blood and amniotic fluid were obtained from chronically catheterised ewes and their fetuses during late gestation [8]. Following centrifugation at 2500 *g* for 15 min at 4°C, plasma was removed and stored at −20°C until extraction.

Aliquots (0.2—0.5 ml) of plasma or amniotic fluid were deproteinised by the addition of 1.0 ml of 0.16 *M* zinc sulfate. After centrifugation at 2500 *g* for 30 min at 4°C, the supernatant was removed and three aliquots (0.025 ml) reserved for enzymatic assay of glucose.

The remaining supernatant was then deionised by passage through tandem cation (1.0 ml, AG 50W-X8, H^+) and anion (1.0 ml, AG1-X8, CH_3COO^-) exchange columns, followed by two 2.0-ml washes of water. The combined eluent was then washed through C_{18} Sep-Pak (Waters Assoc., Milford, MA, U.S.A.), already equilibrated with water, and freeze-dried. Extracts were reconstituted in water (0.05 ml) for chromatography.

Apparatus

A Waters Model 6000A pump was used for solvent delivery. Injections were made using a Waters U6K universal injector. Eluent was monitored at 190 nm using a Waters Lambda Max Model 480 spectrophotometer and the detector signal plotted and integrated with a Model 730 data module. Eluent fractions were collected using a Gilson Model 202 fraction collector.

Chromatography

Extracts and standards were chromatographed on a Waters Sugar-Pak 1 column (Ca^{2+} cation exchange microparticulate gel) maintained at 65° C by a Waters column temperature controller. The mobile phase of water was filtered through an 0.45-μm HA Millipore filter and degassed under vacuum with sonication for 15 min. A flow-rate of 0.4 ml/min^{-1} was used with a back-pressure of 62 bars. In addition, a guard column containing C_{18} Corasil was placed in-line, preceding the analytical column.

Measurement of radioactivity content in chromatography eluent

Chromatography eluent was collected in 10-sec fractions into scintillation vials for 30 min from the time of injection. Of the scintillation fluid (4 g PPO, 0.1 g dimethyl POPOP, 330 ml Triton X-100 made up to 1 l with toluene) 10 ml were then added to each fraction. For routine determinations, each peak was collected as a single fraction. Samples were stored in the dark for 12 h before measurement of ^{14}C- and ^{3}H-radioactivity in an LKB liquid scintillation spectrometer. Quench curves were constructed using the external standard ratio method and corrections for quenching and cross-over made.

Enzymatic determination of plasma glucose concentration

The concentration of glucose in plasma was determined in triplicate by adding 1.25 ml glucose reagent [0.12 *M* phosphate buffer, pH 7.0; 1.5 U/ml peroxidase, 5.0 U/ml glucose oxidase, 0.92 m*M* 2,2′-azino-di(3-ethylbenz-thiazoline sulfonic acid)] to 0.025 ml deproteinised plasma, water and standards and incubating at 37° C for 60 min. Absorbance at 420 nm was then measured for each sample against water. Intra-assay and inter-assay coefficients of variation were 0.8% and 3%, respectively.

Statistical analysis

Coefficients of variation were calculated as standard deviations expressed as a percentage of mean values.

RESULTS

Preparation of samples

To render samples suitable for HPLC on an ion-modified resin column, plasma was deproteinised, then passed through tandem cation and anion exchangers to remove all charged compounds. Hydrophobic components were then removed by washing the neutral eluent through a C_{18} Sep-Pak before freeze-drying. Recoveries of plasma carbohydrates, based upon that of glucose in the final freeze-dried extract were 94% (S.E.M. 4, $n = 8$).

Separation and quantitation of carbohydrates

Ultraviolet (UV) detection of carbohydrates was carried out at 190 nm since most have an absorption maximum at approximately 188 nm and the slight decrease in absorption by comparison at 190 nm was more than compensated for by a reduction in baseline noise [9]. The offset of the detector at this wavelength typically ranged from 0.36 to 0.51 absorbance units full scale (a.u.f.s.). This was due largely to the temperature-dependent continuous elution of an unknown compound from the column, which limited its use to temperatures below 70° C.

The separation of various mixtures of carbohydrates under the conditions described in Materials and methods is shown in Fig. 1. Their retention times and those of other known neutral plasma components are presented in Table I, together with their response factors in peak area (arbitrary units) per nmol carbohydrate. Hexoses tended to elute before polyols while pentoses eluted with a wide range of retention times.

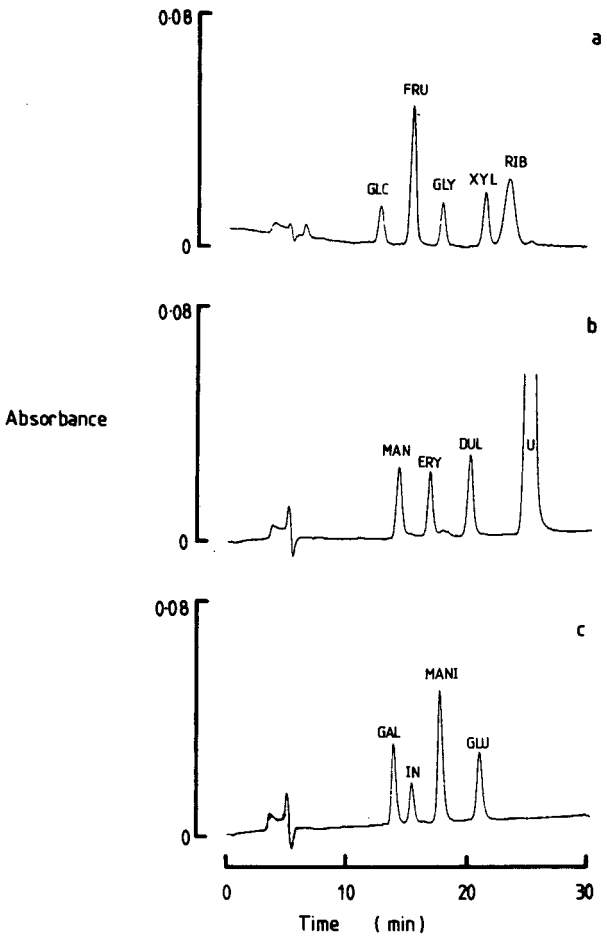

Fig. 1. Chromatographic analysis of carbohydrates. (a) Glucose (GLC), fructose (FRU), glycerol (GLY), xylitol (XYL), ribose (RIB). (b) Mannose (MAN), *i*-erythritol (ERY), dulcitol (DUL), urea (U). (c) Galactose (GAL), inositol (IN), mannitol (MANI), glucitol (GLU). Conditions were as described in Materials and methods.

TABLE I

RETENTION TIMES AND RESPONSE FACTORS OF MONOSACCHARIDES AND THEIR DERIVATIVES

Compound	Retention time (min)	Response factor		Detection limit (nmol)
		Area/nmol	Relative to glucose	
D-Glucose	12.05	20.89	1.0	5.5
L-Sorbose	13.25			
D-Galactose	13.40			3.7
D-Mannose	13.65	41.03	1.96	3.1
D-Fructose	14.70	68.96	3.30	1.6
meso-Inositol	14.90			3.7
i-Erythritol	16.20	23.84	1.14	3.25
Glycerol	17.10	11.28	0.54	
Mannitol	17.25			
Arabitol	17.85			
Dulcitol	19.55	48.07	2.30	1.95
Sorbitol	20.60			2.20
Xylitol	20.70			
d-Ribose	22.90	44.32	2.12	4.75
Urea	23.45	58.62	2.81	1.85

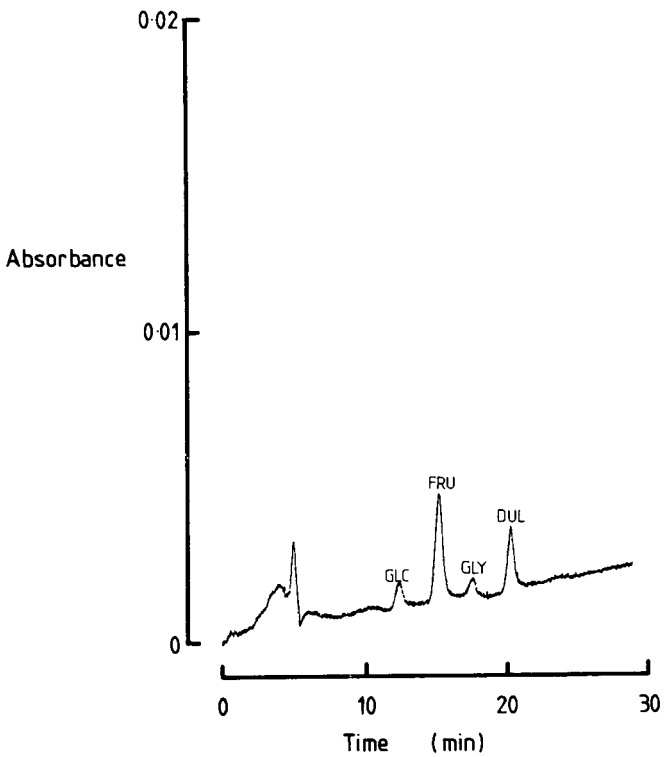

Fig. 2. Chromatographic analysis of 5 nmol each of glucose (GLC), fructose (FRU), glycerol (GLY) and dulcitol (DUL) injected in a 0.015-ml aliquot.

Most of the compounds studied had greater absorbance at 190 nm glucose with the exception of glycerol and *i*-erythritol. This is reflected in their detection limits which were determined as the amount injected in 0.005 ml which gave a peak-height-to-noise ratio of 10 (Table I).

The detection limit of 5.5 nmol for glucose indicates that UV detection under these conditions is at least twenty times more sensitive than detection by refractive index [9]. The chromatogram obtained when 5 nmol of glucose, fructose, glycerol and dulcitol were injected in a 15-μl volume is shown in Fig. 2.

Peak area showed a linear response to concentrations up to approximately 250 nmol for all compounds examined. Coefficients of variation at the 5-nmol level for glucose, fructose and dulcitol were 2.3%, 1.9% and 1.8% respectively.

Determination of specific radioactivities of carbohydrates in sheep plasma

Typical chromatograms obtained when extracts of fetal and maternal plasma were analysed are shown in Figs. 3 and 4. Upon chromatography of extracts of fetal plasma, peaks co-eluting with glucose, mannose, fructose, dulcitol,

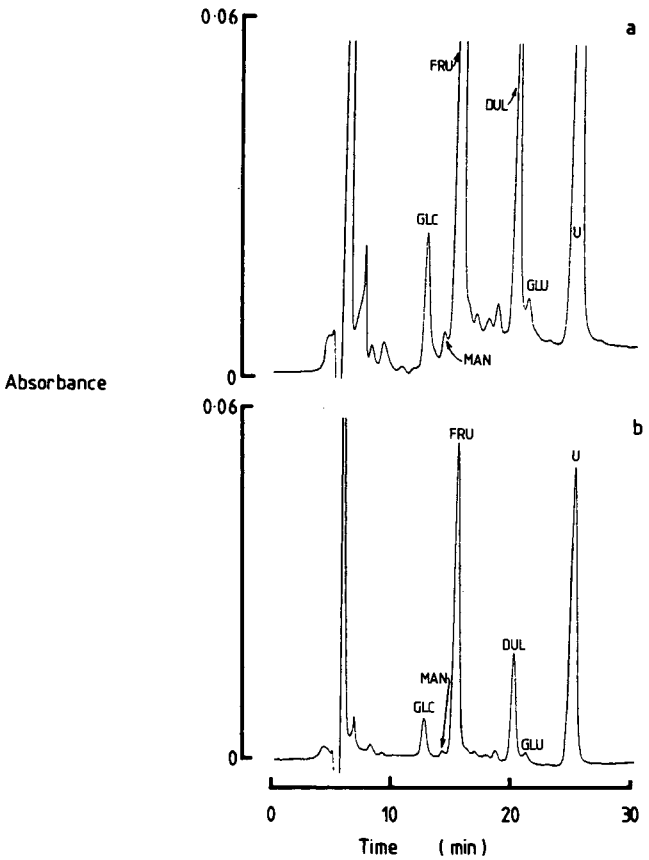

Fig. 3. Chromatographic analysis of fetal plasma: (a) 0.015 ml of fetal plasma extract reconstituted in 0.05 ml of water; (b) 0.005 ml of fetal plasma extract reconstituted in 0.05 ml of water. Conditions as described in Materials and methods. For peak identification, see legend to Fig. 1.

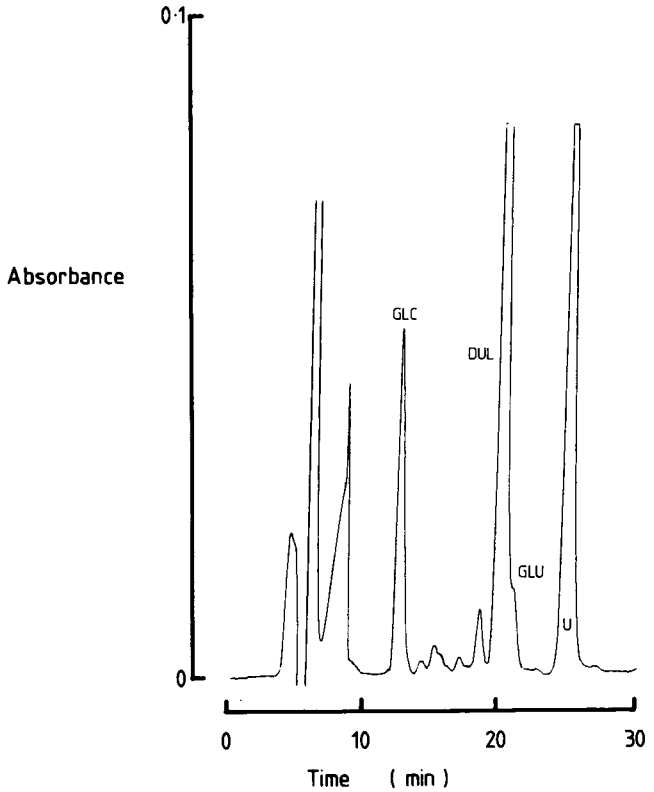

Fig. 4. Chromatographic analysis of maternal plasma (0.015 ml of maternal plasma extract reconstituted in 0.05 ml water). Conditions as described in Materials and methods. For peak identification, see legend to Fig. 1.

sorbitol and urea were observed (Fig. 3). Of these, glucose, dulcitol, sorbitol and urea were separated with baseline resolution. HPLC of maternal plasma extracts revealed a number of peaks, some eluting at the same time as the pure standards, glucose, galactose, mannose, inositol, dulcitol, sorbitol and urea (Fig. 4). All, with the exception of galactose and mannose were usually resolved to the baseline from neighbouring peaks.

The concentration of these compounds in plasma was determined by using glucose as an internal standard whose plasma concentration was measured by enzymatic assay. Where the latter is not required for the purposes of the study, addition of a suitable compound, as an internal standard, to plasma prior to extraction should be a practicable alternative.

The determination of specific radioactivity of glucose in plasma was assessed by adding 0.37 μBq of D-[U-^{14}C]glucose in 0.01 ml to each of ten aliquots of 1.5 ml deproteinised fetal plasma containing 0.60 μmol glucose as determined by enzymatic assay. The specific radioactivity of glucose in plasma was found to be 0.618 μBq/μmol (S.E.M. 0.002, n = 10) compared to the theoretical specific radioactivity of 0.617 μBq/μmol.

This method was then applied to the determination of the specific radioactivity of carbohydrates in fetal and maternal plasma sampled during a

constant infusion of D-[U-^{14}C]glucose and D-[2-^{3}H]glucose (37 μBq/min and 111 μBq/min, respectively) into the tarsal vein of a fetal sheep at 120 days gestation.

The chromatographic profiles of absorbance at 190 nm and of ^{14}C- and ^{3}H-radioactivity of extracted fetal plasma are shown in Fig. 5. In fetal plasma, most ^{3}H-radioactivity co-eluted with glucose while ^{14}C-radioactivity co-eluted with glucose, mannose and fructose. No other significant peaks of radioactivity were observed. The specific activities of glucose and fructose in plasma throughout the infusion, determined in this way, are shown in Fig. 6. The average

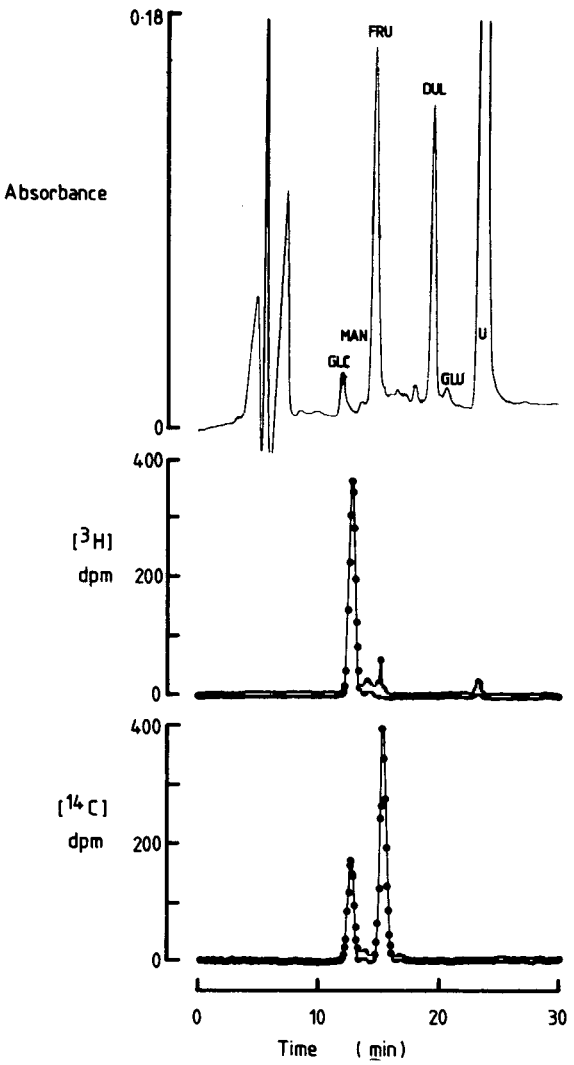

Fig. 5. Chromatographic isolation and quantitation of carbohydrates in plasma obtained from a fetal lamb during the continuous infusion of D-[U-^{14}C]glucose and D-[2-^{3}H]glucose into a chronically catheterised fetal lamb. Fractions (0.1 min) were collected continuously following injection of 0.015 ml of plasma extract reconstituted in 0.05 ml water and ^{14}C and ^{3}H content determined as described in Materials and methods. For peak identification, see legend to Fig. 1.

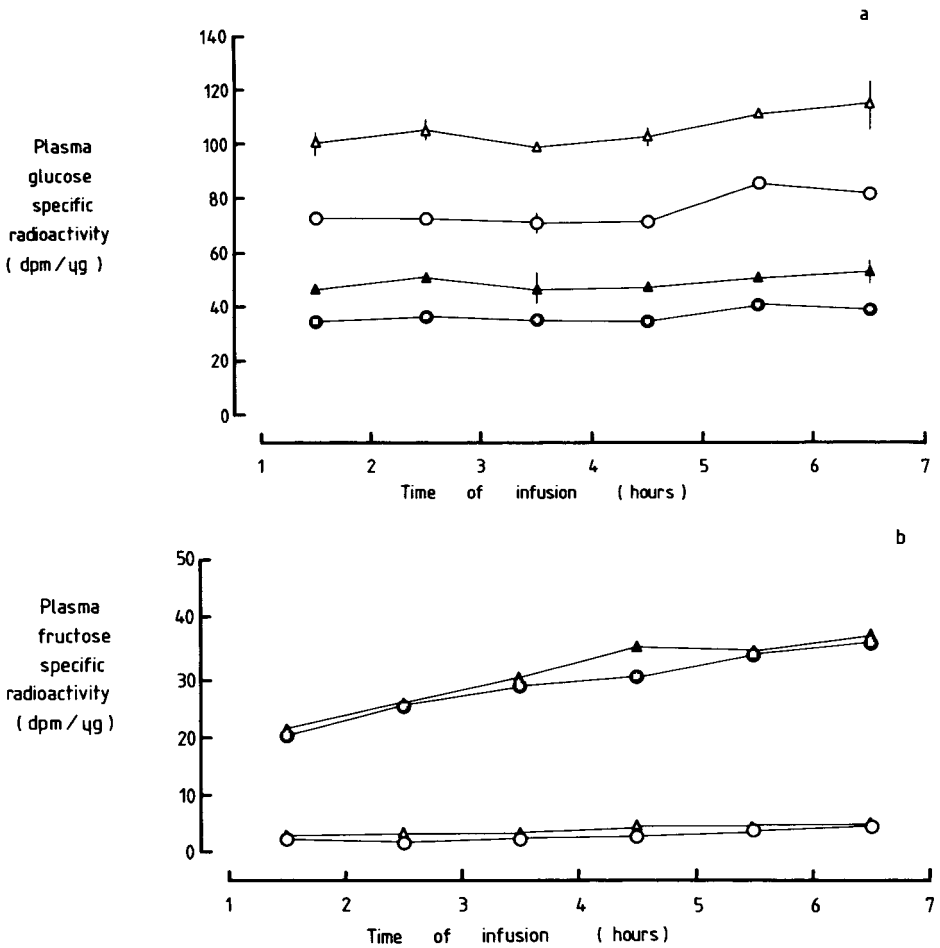

Fig. 6. The specific radioactivities in plasma of (a) glucose and (b) fructose in fetal lamb plasma obtained from the umbilical vein (○) and the femoral artery (△) during a constant intrafetal infusion of D-[U-^{14}C]glucose (●, ▲) and D-[2-^{3}H]glucose (○, △). Each point represents the mean of duplicate determinations with standard deviation shown by bars where sufficiently large.

coefficients of variation for duplicate determinations of specific radioactivity of glucose in fetal plasma were 1.6% for [^{14}C]glucose and 1.7% for [^{3}H]glucose. For duplicate measurements of specific radioactivities of fructose in fetal plasma the average coefficients of variation were 3.11% for [^{14}C]fructose and 4.64% for [^{3}H]fructose.

The chromatographic profiles of absorbance at 190 nm and of ^{14}C-and ^{3}H-radioactivity of extracted maternal plasma are shown in Fig. 7. Both ^{14}C- and ^{3}H-radioactivity co-eluted with glucose only. The specific activities of glucose in maternal plasma glucose determined at intervals throughout the infusion are shown in Fig. 8. The average coefficients of variation for specific radioactivities of [^{14}C]glucose and [^{3}H]glucose in maternal plasma were 1.95% and 2.95%, respectively.

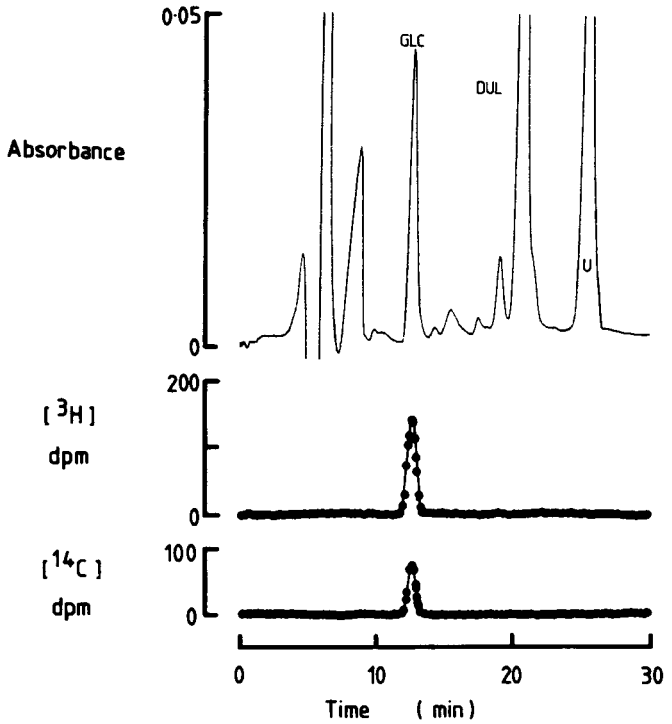

Fig. 7. Chromatographic isolation and quantitation of carbohydrates in plasma of maternal sheep obtained during a continuous infusion of D-[U-^{14}C]glucose and D-[2-^{3}H]glucose into a chronically catheterised fetal lamb. Fractions (0.1 min) were collected continuously following injection of 0.015 ml of plasma extract reconstituted in 0.05 ml of water and ^{14}C and ^{3}H content determined as described in Materials and methods. For peak identification, see legend to Fig. 1.

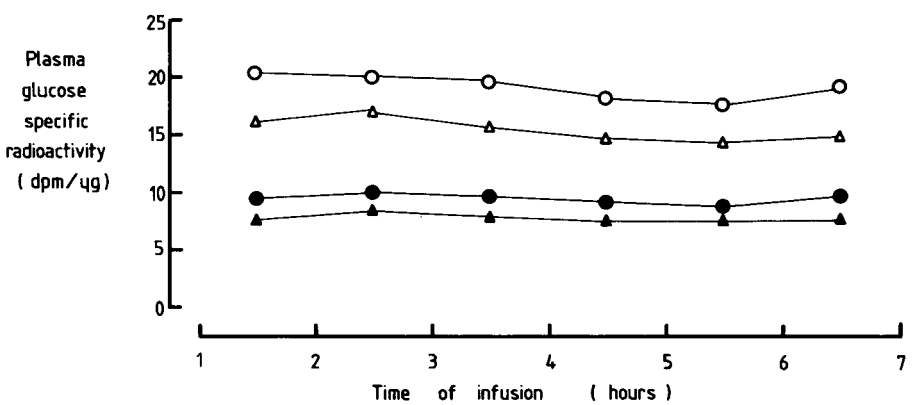

Fig. 8. Specific radioactivities of glucose in plasma of maternal sheep during a constant intrafetal infusion of D-[U-^{14}C]glucose (●, ▲) and D-[2-^{3}H]glucose (○, △). Each point represents the mean of duplicate determinations.

To assess their homogeneity, [^{14}C]glucose, [^{3}H]glucose, [^{14}C]fructose and [^{3}H]fructose peaks were collected and then lyophilised. Following reconstitution in water, an aliquot was chromatographed on an amine—silica column (Waters Assoc.) at 25°C, with a mobile phase of acetonitrile—water (85:15) at a flow-rate of 2 ml/min and detection at 190 nm (a.u.f.s. 0.1). An aliquot of the remainder was counted for ^{14}C- and ^{3}H-radioactivity. Continuous collection of 0.1-ml fractions and measurement of their ^{14}C- and ^{3}H-radioactivity showed recovery of 99% and 102% of ^{14}C- and ^{3}H-radioactivity, respectively, in a peak co-eluting with glucose following rechromatography. Similarly, recovery of ^{14}C-radioactivity in fructose was 99%.

DISCUSSION

Using an HPLC cation-exchange column and a mobile phase of water at a temperature lower than that generally recommended, 65°C, it was possible to quantitate carbohydrates by UV detection and still efficiently separate a wide range of hexoses, pentoses and polyols. In this system, quantitation at 190 nm resulted in detection limits which were lower than previously achieved with UV or refractive index detection [9] and comparable to those reported for post-column derivatisation techniques [10—12].

Other methods of HPLC of carbohydrates largely utilise partition chromatography on silica columns with either chemically or dynamically amine-coated silica stationary phases [13, 14] and detection by refractive index [15]. However, these approaches are in general applicable only to samples with high carbohydrate content due to the insensitivity of this type of detection. In addition, their limited ability to resolve different carbohydrates and polyols requires that relatively few compounds be present in the sample.

Some of these limitations have been overcome by the development of both pre- and post-column derivatisation methods which have in two instances been applied to the analysis of complex biological samples such as human serum [10, 16]. However, these methods are associated with other problems such as variable efficiency of derivatisation and formation of multiple derivatives. In addition, covalent modification of the sample precludes further analysis, in particular the determination of radioactivity content. They also require additional equipment such as pumps, mixing coils and fluorescence detectors as well as the use of highly corrosive reagents.

More recently, heavy-metal cation-exchange resin columns have been used for the separation of carbohydrates in food products and hydrolysates of plant cell walls with refractive index detection [17]. Because such columns use water as a mobile phase, UV detection of carbohydrates at 190 nm appeared feasible. Accordingly, the present method for the extraction of carbohydrates from sheep plasma and their separation and quantitation was developed.

The principal carbohydrates present in the plasma of fetal and maternal sheep were successfully separated and quantitated by this method, using as little as 0.2 ml plasma. In addition, the specific radioactivities of labeled glucose and fructose, present simultaneously in plasma, were accurately determined with good reproducibility.

This technique may also be useful in the analysis of other biological samples

containing a mixture of carbohydrates and polyols. In addition, the measurement of specific radioactivities of various carbohydrates in such samples may be feasible, depending upon their homogeneity following HPLC.

In summary, this technique should be generally applicable where the specific radioactivities of glucose and other carbohydrates and polyols in plasma are to be determined and where measurement of glucose specific radioactivity by conventional methods is made difficult by the presence of other labeled carbohydrates or interfering compounds [3, 18, 19].

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DETERMINATION OF O-PHOSPHOTHREONINE, O-PHOSPHOSERINE, O-PHOSPHOTYROSINE AND PHOSPHATE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic procedure has been developed to measure O-phosphoamino acids (O-phospho-L-serine, O-phospho-L-threonine, O-phospho-L-tyrosine) and phosphate. A strong basic anion-exchange column (Partisil 10 SAX Whatman) enables their separation within 20 min. Detection is made by ultraviolet absorption at 210 nm or measurement of ^{32}P radioactivity. The best separation is obtained with a 0.04 *M* phosphate aqueous mobile phase at pH 4. The performance of the procedure has been tested by analysis of hydrolysed phosphoamino acids and hydrolysed phosphorylated histones; all hydrolysates are purified on Sephadex G-25 before chromatographic analysis.

INTRODUCTION

Protein phosphorylation by protein kinase is a major regulatory mechanism for hormonal regulation of cell function [1]. This phosphorylation is usually studied by measuring the incorporation of [^{32}P]phosphate into amino acids. Among all possible phosphate esters of the natural amino acids, only three, i.e. O-phospho-L-serine (P-Ser), O-phospho-L-threonine (P-Thr) and O-phospho-L-tyrosine (P-Tyr), have reasonable chemical stability over a large pH range [2]. Characterization of phosphoamino acids after protein acid hydrolysis has therefore been restricted to these.

The separation of ^{32}P-labelled amino acids and [^{32}P]phosphate has been studied since at least 1962 [3], and paper [3—7] or thin-layer cellulose plate electrophoresis [8—16] were the most widely used methods. Alternatively, some authors have used high-performance liquid chromatography (HPLC) [17—19] or amino acid analysis [20—22].

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This paper is devoted to the setting up of a rapid analysis involving HPLC on an anion-exchange resin (Partisil 10 SAX Whatman). The performance of the procedure has been tested by measuring phosphate bond hydrolysis rate in P-Thr, P-Ser and P-Tyr and peptidic plus phosphate bond hydrolyses of ^{32}P-phosphorylated histones.

EXPERIMENTAL

Chemical reagents

Potassium dihydrogen phosphate, trichloroacetic acid (TCA), phosphoric acid, hydrochloric acid, diphosphorus pentoxide, barium hydroxide, absolute ethanol, and L-tyrosine (Prolabo, Paris, France) were analytical grade; magnesium chloride, N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES), cyclic AMP, histones from calf thymus type II S, protein kinase, O-phospho-L-serine and O-phospho-DL-threonine were obtained from Sigma (Osi, France); [γ-^{32}P]ATP and [^{32}P]phosphate were obtained from New England Nuclear (U.K.).

Analysis of O-phosphothreonine, O-phosphoserine, O-phosphotyrosine and phosphate by HPLC

The liquid chromatography apparatus consisted of a Model 848 pump module (Dupont, Orsay, France) with a Rheodyne 7125 injection valve, a 100-μl loop (Touzart et Matignon, Vitry sur Seine, France), and an absorbance monitor ISCO Model 1840 (Roucaire, Velizy, France) equipped with an 8-μl flow-cell unit. The chromatographic effluent was collected on an LKB 2212 Redirac fraction collector with a 24-sec delay. The system was operated at room temperature.

Chromatography was performed on a Partisil 10 SAX Whatman column, 25 × 0.4 cm I.D., particle size 10 μm, with a mobile phase of 0.04 *M* potassium phosphate aqueous solution at pH 4. The flow-rate was 1.5 ml/min (80 bars). The O-phosphoamino acids were detected at 210 nm; ^{32}P-radioactive compounds were counted using Aquassure (New England Nuclear) as scintillation fluid in a spectrometric counter SL 4000 (Intertechnique, Montigny Le Brotonneux, France).

Synthesis of O-phosphotyrosine

O-Phosphotyrosine was synthesized as described by Mitchell and Lunan [23]. It was purified by the method of Plimmer [2] by forming a soluble alkaline barium salt, which was separated by filtration from insoluble barium phosphate. O-Phosphotyrosine barium salt was then precipitated by adding an equal volume of absolute ethanol. The isolated solid was homogeneous on cellulose and silica thin-layer chromatography [8, 24]; its UV spectrum showed an absorption maximum at 265 nm as previously described [18, 23].

Synthesis of ^{32}P-phosphorylated histones

Phosphorylated histones were prepared as described [25]. The incubation medium contained: 10 mg/ml histones, 10 m*M* magnesium chloride, 0.05 m*M* cyclic AMP, [γ-^{32}P]ATP (4 μCi/ml), 1.5 mg/ml protein kinase, 50 m*M* HEPES pH 6.8. The reaction was run for 10 min and stopped with TCA (5% final

concentration). After 1 min of centrifugation in a Beckman microfuge, the supernatant was discarded and the precipitated phosphohistones were stored at 4°C.

At 5 min, 40% of the γ-^{32}P of ATP was incorporated into histones and the maximum of the phosphorylation step was reached.

Analysis of unlabelled phosphoamino acids and ^{32}P-phosphorylated histones

Hydrolysis. P-Thr (40 mg) with $^{32}PO_4$ as internal standard (0.25 μCi) was dissolved in 20 ml of 5.6 *M* hydrochloric acid; 2-ml fractions were poured into glass tubes which were sealed under vacuum and heated at 105°C for periods of 0.5, 1, 2, 4, 7, 16 and 24 h. In the same way, P-Ser (40 mg) and P-Tyr (100 μg) together with $^{32}PO_4$ (0.25 μCi) as internal standard were hydrolysed for 0.5, 1, 2, 4, 7 and 16 h. [^{32}P]Phosphohistones (3 mg) were dissolved in 1.5 ml of 5.6 *M* hydrochloric acid; 0.25-ml fractions were hydrolysed for periods of 0.5, 1, 2, 4 and 7 h.

All hydrolysates were dried by evaporation, and the residues dissolved in 150 μl of water and purified on Sephadex G-25 (Pharmacia, Bois d'Arcy, France) before HPLC analysis.

Purification on Sephadex G-25. The hydrolysates were purified on Sephadex G-25 before analysis by HPLC. Sephadex G-25 was equilibrated with 0.04 *M* potassium phosphate aqueous solution (pH 4) and 2 ml of gel were poured into an Econo-Column (Bio-Rad, Touzart et Matignon). The void volume (V_0) of the column was determined using dextran blue. A 100-μl volume of each hydrolysate was deposited on the column; fractions of 180 μl were collected and 45 μl of each were counted. All the fractions containing radioactivity were pooled and lyophilized. The dry residue was dissolved in 200 μl of water.

Chromatography on SAX column (HPLC)

A 50-μl volume of unlabelled phosphoamino acid hydrolysate was injected into the loop to measure the phosphate bond cleavage; 50 μl of hydrolysed [^{32}P]phosphohistones together with 20 μl of non-radioactive O-phosphoamino acids (P-Thr and P-Ser $5 \cdot 10^{-2}$ *M*, and P-Tyr 10^{-4} *M*) as markers were injected into the loop.

Standardization

HPLC assay. The reproducibility and the recovery of the HPLC procedure were tested by making five successive injections of a standard solution consisting of $5 \cdot 10^{-2}$ *M* P-Thr, $5 \cdot 10^{-2}$ *M* P-Ser, 10^{-4} *M* P-Tyr, and [^{32}P]-phosphate (50 000 cpm/ml); 20 μl were injected into the loop at 0.2 a.u.f.s. (absorbance units full scale).

The capacity ratio, k', was defined as $k' = (t - t_0)/t_0$, where t is the retention time of a given compound and t_0 the retention time of the void volume of the column.

RESULTS

HPLC analysis of O-phosphoamino acids and [^{32}P]phosphate

Injection of a mixture of P-Thr, P-Ser, P-Tyr and [^{32}P]phosphate on the

SAX column enabled their clear separation within 20 min using 0.04 *M* potassium phosphate aqueous phase at pH 4 (Fig. 1).

The plot of capacity ratio (k') of O-phosphoamino acids and phosphate as a function of potassium phosphate concentrations at pH 4.5 (Fig. 2) shows a good separation between P-Tyr, P-Ser and P-Thr; phosphate overlapped P-Tyr or P-Ser except at 0.03 *M* and 0.04 *M* phosphate.

The relationship between k' values and the pH of 0.04 *M* potassium phosphate aqueous phase (Fig. 3) indicates that, in the range studied, the best separation is obtained at pH 4 with $k'_{PO_4} > k'_{P\text{-}Tyr} > k'_{P\text{-}Ser} > k'_{P\text{-}Thr}$.

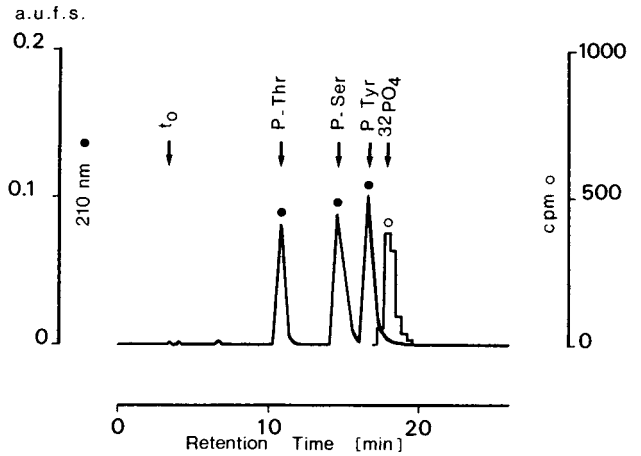

Fig. 1. Chromatogram of standards on Partisil 10 SAX Whatman. Peaks: P-Thr = O-phosphothreonine, 1 μmol; P-Ser = O-phosphoserine, 1 μmol; P-Tyr = O-phosphotyrosine, 2 nmol; $^{32}PO_4$ = phosphate labelled with ^{32}P, 1000 cpm. Injection volume = 20 μl. The mobile phase consisted of 0.04 *M* potassium phosphate aqueous solution, pH 4. Flow-rate = 1.5 ml/min. t_0 = retention time corresponding to the void volume of the column. a.u.f.s. = absorbance unit full scale. cpm = counts per min.

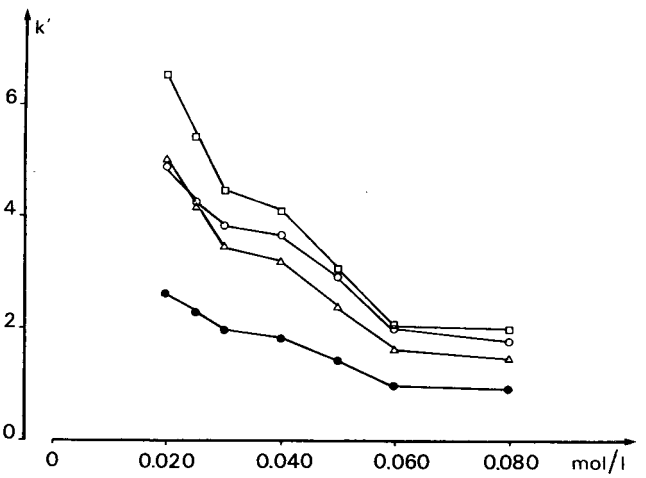

Fig. 2. k' Values of P-Thr, P-Ser, P-Tyr and $^{32}PO_4$ plotted as a function of potassium phosphate concentration at pH 4.5. (□) O-Phosphotyrosine; (○) phosphate labelled with ^{32}P; (△) O-phosphoserine; (●) O-phosphothreonine.

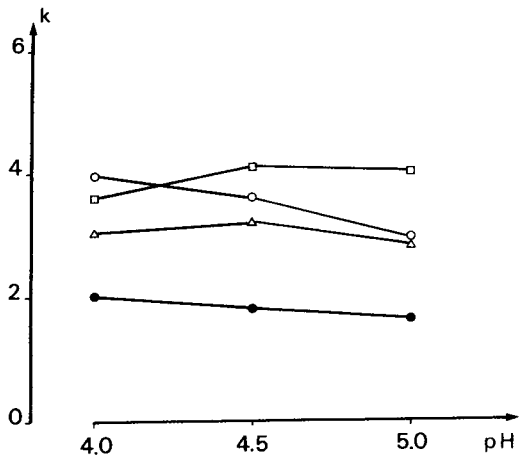

Fig. 3. k' Values of P-Thr, P-Ser, P-Tyr and $^{32}PO_4$ plotted as a function of pH at a phosphate concentration of 0.04 *M*. (□) O-Phosphotyrosine; (○) phosphate labelled with ^{32}P; (△) O-phosphoserine; (●) O-phosphothreonine.

Five repeated injections of the markers (1 μmol P-Thr, 1 μmol P-Ser and 2 nmol P-Tyr) gave relative standard deviations of 1.1%, 1.1% and 1.5%, respectively (n = 5). The recovery of [^{32}P]phosphate was 94% with a relative standard deviation of 2% (n = 5). The limit of detection of [^{32}P]phosphate was 450 cpm per injection.

Analysis of unlabelled phosphoamino acids and [^{32}P]phosphate on Sephadex G25

The recovery of [^{32}P]phosphate from Sephadex G-25 was 94% with a relative standard deviation of 3% (n = 3).

The chromatographic profiles of phosphate and phosphoamino acids overlapped. This was assessed by comparing two HPLC profiles (three experiments); (1) a 5-μl volume of the standard solution was injected into the loop at 0.05 a.u.f.s.; (2) a 100-μl volume of the same standard solution was chromatographed on Sephadex G-25, all radioactive fractions were pooled, adjusted to 1 ml and 50 μl were injected into the HPLC loop at 0.05 a.u.f.s.

The recoveries of phosphoamino acids were: P-Thr 100%, P-Ser 100%, and P-Tyr 97%, with relative standard deviations of 2%, 1.5% and 2%, respectively.

Cleavage of phosphate bonds of P-Thr, P-Ser and P-Tyr

After phosphoamino acid hydrolysis in acid, each sample was purified on a calibrated Sephadex G-25 and analysed by HPLC.

The rates of hydrolysis of P-Thr, P-Ser and P-Tyr are shown in Fig. 4. P-Thr is the most resistant to hydrolysis and P-Tyr the least. After 4 h, 70% of P-Thr, 40% of P-Ser and 20% of P-Tyr remained unhydrolysed.

The recoveries of [^{32}P]phosphate as internal standard were 99 ± 6% after G-25 and 97 ± 7% after HPLC assay (mean ± S.D. of fifteen experiments).

Analysis of hydrolysed phosphorylated histones

The radioactive composition of hydrolysed phosphohistones was studied

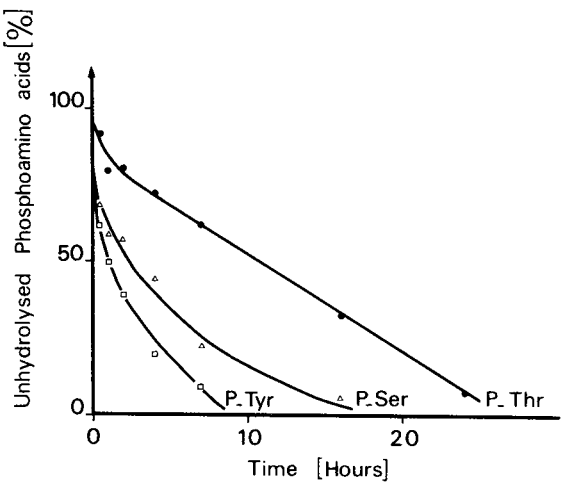

Fig. 4. Measurement of percentage unhydrolysed phosphoamino acid after hydrolysis of O-phosphotyrosine (□), O-phosphoserine (△) and O-phosphothreonine (•) in 5.6 *M* hydrochloric acid at 105° C.

after hydrolysis for 0.5, 1, 2, 4 and 7 h. Each sample was purified on a calibrated Sephadex G-25 prior to its analysis by HPLC. The determinations were made in triplicate.

Analysis on Sephadex G-25. Unhydrolysed phosphohistones eluted at V_0 (Fig. 5a). After 0.5 h of hydrolysis, the broad radioactive peak eluted between V_0 and the PO_4 elution volume (Fig. 5b). After 2 h hydrolysis, the radioactive peak was sharper (Fig. 5c) and eluted as PO_4.

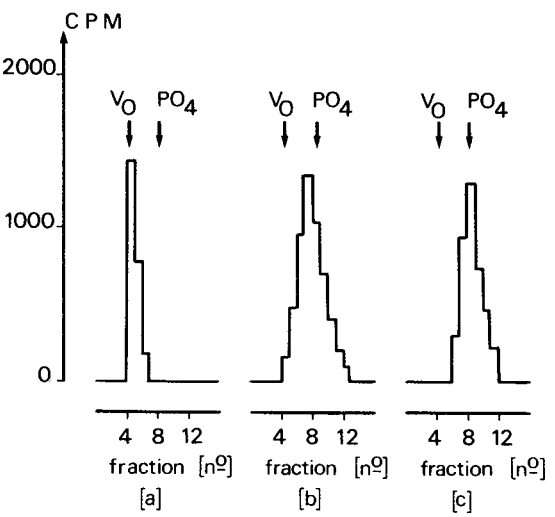

Fig. 5. Chromatographic profiles of Sephadex G-25 (2-ml volume): (a) unhydrolysed [^{32}P]-phosphohistones; (b) [^{32}P]phosphohistone hydrolysis for 0.5 h in 5.6 *M* hydrochloric acid at 105° C; (c) [^{32}P]phosphohistone hydrolysis for 2 h in 5.6 *M* hydrochloric acid at 105° C. V_0 is the void volume of the column. PO_4 indicates the elution volume of [^{32}P]phosphate. Each fraction represents a volume of 180 µl.

These results indicate that unhydrolysed phosphohistones do not contain free [^{32}P]phosphate. At 0.5 h, the elution profile would correspond to an incomplete cleavage of the phosphohistone peptide bonds while these are completely hydrolysed after 2 h hydrolysis.

HPLC analysis. Fig. 6 shows a chromatogram obtained after 1 h of [^{32}P]phosphohistone hydrolysis.

The radioactivity profiles suggest that several products are detected. These can be divided into two groups: (1) two unknown compounds, x and y, with

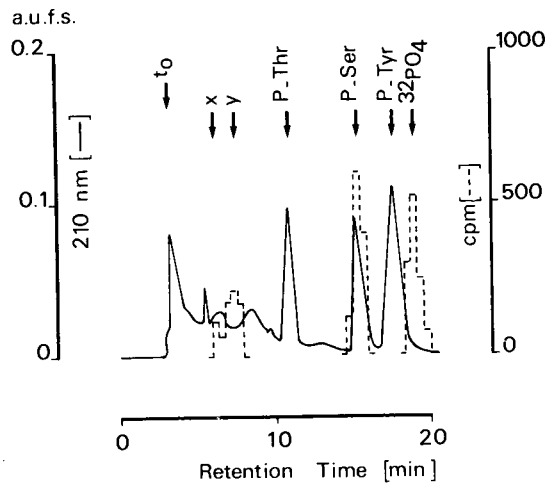

Fig. 6. Chromatogram of hydrolysed [^{32}P]phosphohistones on Partisil 10 SAX Whatman. [^{32}P]Phosphohistones were hydrolysed for 1 h in 5.6 *M* hydrochloric acid at 105°C. The injection volume was 20 μl of the standard solution of unlabelled O-phosphoamino acids and 50 μl of the [^{32}P]phosphohistone hydrolysate (2000 cpm). Peaks: P-Thr = O-phosphothreonine, 1 μmol; P-Ser = O-phosphoserine, 1 μmol; P-Tyr = O-phosphotyrosine, 2 nmol; x and y are undetermined radioactive compounds. The mobile phase consisted of 0.04 *M* potassium phosphate aqueous solution, pH 4. Flow-rate: 1.5 ml/min. t_0 = retention time corresponding to the void volume of the column. a.u.f.s. = absorbance unit full scale. cpm = counts per min.

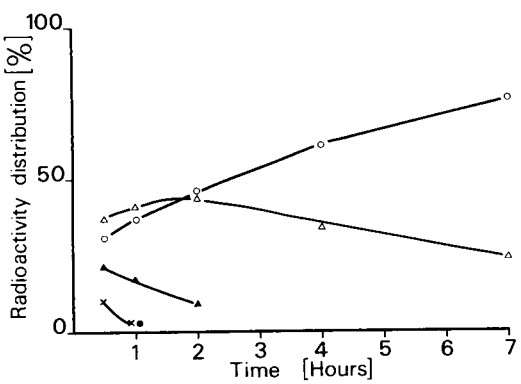

Fig. 7. Radioactivity distribution (%) of hydrolysed [^{32}P]phosphohistones for periods of 0.5, 1, 2, 4, and 7 h in 5.6 *M* hydrochloric acid at 105°C. (○) Phosphate labelled with ^{32}P; (△) O-phosphoserine; (▲) compound y; (×) compound x; (●) O-phosphothreonine.

capacity ratios smaller than those of phosphoamino acids or phosphate (k'_x = 0.95, k'_y = 1.4); (2) three known products, namely P-Thr, P-Ser and PO_4.

The evolution of the HPLC radioactivity profiles as a function of hydrolysis time is shown in Fig. 7; x and y decrease from 0.5 to 1 h and from 0.5 to 2 h, respectively. P-Thr is almost absent while P-Ser increases during the first 2 h and then decreases. PO_4 increases regularly. The recovery of the injected radioactivity is 88 ± 10% (mean ± S.D. of fifteen experiments).

DISCUSSION

The characterization of [^{32}P]phosphoamino acids and phosphate using paper and thin-layer plates has already been described. Thus, Eckhart et al. [8] and Hunter [9] developed methods on thin-layer plates to fractionate phosphoamino acids by electrophoresis in combination with chromatography or by two-dimensional electrophoresis. These methods are indeed adequate but they are time-consuming (some 10 h being required).

Swarup et al. [17] were the first to determine O-phosphoamino acids and phosphate using an anionic resin. However, with this procedure, the time required for complete analysis was 80 min and the separation between P-Ser and P-Tyr was relatively poor (only 2 min apart). In 1983, Swarup et al. [26] reported the advantage of high-voltage electrophoresis (40 min to perform the analysis) over their previous HPLC method.

Alewood et al. [18] described a preparation of O-phosphotyrosine. These authors studied the conversion of tyrosine to phosphotyrosine by HPLC with a C_{18} column, and its optical purity by a modification of the Manning-Moore procedure [27]. However, they did not study the chromatographic profile of other phosphoamino acids.

In 1982, using an anionic resin, Yang et al. [19] were able to reduce the time required for analysis down to 60 min with a good separation. However, other high-voltage electrophoresis techniques appeared [28, 29] which seemed to leave all HPLC procedures aside.

Our previous work on the determination of histamine [30] and 3-methylhistidine [31] in biological samples using a cationic HPLC resin, led us to consider the putative capacities of such techniques when appropriate mobile phases are used.

Our study shows a good resolution between phosphoamino acids. We paid particular attention to the retention time of phosphate because it is always released during phosphoprotein hydrolysis. It was possible to make the phosphate retention time longer than those of O-phosphoamino acids using a 0.04 *M* potassium phosphate aqueous phase at pH 4. The good separation of O-phosphoamino acids and phosphate within 20 min compares favourably with the time reported for high-voltage electrophoresis and other HPLC procedures.

Although P-Thr and P-Ser have a relatively low absorption at 210 nm, unlabelled phosphoamino acids are used as markers because they can be introduced in detectable amounts (1 μmol of each) without affecting the resolving power of the column.

The relative sequence of phosphoamino acid hydrolysis rates is P-Tyr > P-Ser > P-Thr. Nevertheless, in our experiments, the absolute hydrolysis rates of

P-Thr and P-Ser were faster than those described by Bitte and Kabat [4], who reported less than 15% cleavage of the phosphate bond after 2 h heating.

Cooper et al. [28] hydrolysed P-Tyr for 1 h in 1 *M* hydrochloric acid at 100°C or for 5 h in 5 *M* sodium hydroxide at 150°C. We agree that P-Tyr is not very stable in 5.6 *M* hydrochloric acid; it almost disappeared after 7 h of hydrolysis. In fact, Eckhart et al. [8] and Hunter [9] hydrolysed phosphotyrosylproteins and phosphotyrosylpeptides in 5 *M* hydrochloric acid at 110°C for 2 and 1 h, respectively.

After 0.5 or 1 h of phosphohistone hydrolysis, the broad radioactive peak obtained from Sephadex G-25 contained phopshorylated peptides. They were detected by HPLC as the unknown compounds x and y and represented 30% and 20% of the radioactivity distribution, respectively. After 4 or 7 h of hydrolysis, only phosphate and P-Ser appeared. The time course of the appearance of P-Ser from phosphohistone hydrolysis is biphasic. We suggest that it represents the sum of two phenomena: (1) hydrolysis of peptide bonds, which leads to the release of P-Ser, and (2) the cleavage of the P-Ser phosphate bond leading to phosphate release. According to this suggestion, we can use the rate of phosphate bond cleavage of P-Ser (Fig. 4) to calculate the theoretical levels of P-Ser liberated from phosphohistone hydrolysis: after 2 h of hydrolysis, the appearance of 44% of P-Ser would represent 83% of the ^{32}P liberated from the histones as P-Ser; after 7 h of hydrolysis, it would be 96% of the ^{32}P liberated.

Finally, we find that 2 h of phosphohistone hydrolysis corresponded to the best experimental conditions because 83% of the protein bonds were hydrolysed and a high percentage of unhydrolysed P-Ser was seen.

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IDENTIFICATION OF PHENPROCOUMON METABOLITES IN HUMAN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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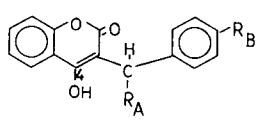
SUMMARY

The oral anticoagulant phenprocoumon is eliminated in urine mainly as the glucuronide conjugate to an extent of 20% of the dose. The urine from patients undergoing phenprocoumon therapy was investigated and the following metabolites were isolated and identified: 7-hydroxyphenprocoumon as the main component, and 4'-hydroxyphenprocoumon and 6-hydroxyphenprocoumon as conjugates. They were characterized by high-performance liquid chromatography and, after methylation, by gas chromatography—mass spectrometry.

The oral anticoagulant phenprocoumon, 4-hydroxy-3-(1-phenylpropyl)-2H-1-benzopyran-2-one (Marcoumar®, Liquamar®), as well as the related drugs warfarin and acenocoumarin (Fig. 1) are clinically indicated in the prophylaxis and treatment of thromboembolic disorders [1—3]. Phenprocoumon plasma levels and urinary excretion data from patients are useful indicators in drug interaction and compliance studies [4—6]; 20% of the phenprocoumon dose



		R_A	R_B
1.	Phenprocoumon	CH_2CH_3	H
2.	Warfarin	CH_2COCH_3	H
3.	Acenocoumarin	CH_2COCH_3	NO_2

Fig. 1. Structures of some 4-hydroxycoumarin anticoagulants.

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is recovered as conjugate in urine [4, 7]. Rats and rat liver microsomes treated with phenprocoumon yield a series of hydroxylated derivatives [8—10] but little is known about the metabolism of the drug in man.

We describe the extraction, purification and identification of phenprocoumon metabolites from human urine, using for characterization high-performance liquid chromatography (HPLC) and gas chromatography—mass spectrometry (GC—MS) after derivatization.

MATERIALS AND METHODS

Apparatus

Liquid chromatography was run with the following system: an automatic injector (WISP 710B), a gradient programmer (M660), two pumps (M6000A), an ultraviolet detector (M440) (all from Waters, Eschborn, F.R.G.) and a fluorimetric detector (Model SFM 23; Kontron, Eching, F.R.G.); the chromatograms were recorded with the printer—plotter R-1B (Shimadzu, Dusseldorf, F.R.G.). For the GC—MS analysis a Model 5986A quadrupole system (Hewlett-Packard, Frankfurt, F.R.G.) was used. Centrifugations were performed at 2000 *g* at room temperature.

Reagents

All test substances were analytical grade; diethyl ether and isooctane were Nanograde quality (Promochem, Wesel, F.R.G.); methyl iodide and 1-methyl-3-nitro-1-nitrosoguanidine (Aldrich, Steinheim, F.R.G.) were practical grade; methanol was spectroscopic grade (E. Merck, Darmstadt, F.R.G.); and deionized water was purified through Norganic cartridges (Millipore, Neu Isenburg, F.R.G.). Phenprocoumon and *p*-chlorophenprocoumon (Fig. 2) were gifts from Hoffmann-La Roche (Grenzach, F.R.G.), and the synthetic monohydroxyphenprocoumons 4′-hydroxy-, 6-hydroxy-, 7-hydroxy- and 8-hydroxy-d_5- (Fig. 2) [11] were gifts from Prof. W. Trager (University of Washington, Seattle, U.S.A.).

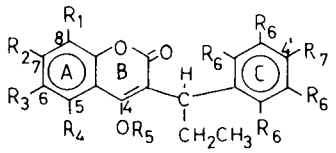
Samples

Urines (24-h) from 35 out-patients undergoing phenprocoumon therapy were collected and frozen at −23°C; samples were thawed and centrifuged before analysis.

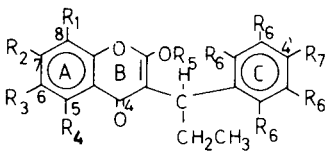
Extraction of metabolites from urine

Extraction a. From each patient a 10-ml sample was hydrolysed as previously described [4], passed through a preconditioned Sep-Pak cartridge (Waters) and washed successively with 5 ml of 1% acetic acid, 5 ml of 40% methanol in 1% acetic acid and 2 ml of methanol. The methanol fraction was evaporated under vacuum and the residue was dissolved in 200 µl of methanol; 20 µl were injected into the HPLC system, or further methylated for GC—MS analysis.

Extraction b. Pooled urine (6.5 l) from three patients was concentrated to 1 l in a vacuum, hydrolysed enzymatically [4], submitted to column chromatography (50 × 1.5 cm; Sep-Pak C_{18} material) and eluted successively with 300-ml portions of 1% aqueous acetic acid solvents with increasing amounts of



		R_1	R_2	R_3	R_4	R_5	R_6	R_7
1. Phenprocoumon		H	H	H	H	H	H	H
4. p-Chloro-	phenprocoumon	H	H	H	H	H	H	Cl
5. 8-Hydroxy-	,,	OH	H	H	H	H	H	H
6. 7-Hydroxy-	,,	H	OH	H	H	H	H	H
7. 6-Hydroxy-	,,	H	H	OH	H	H	H	H
8. 4'-Hydroxy-	,,	H	H	H	H	H	H	OH
9. 8-Hydroxy-d_5-	,,	OH	H	H	H	H	D	D
10. 4-Methyl-	,,	H	H	H	H	Me	H	H
11. 4-Methyl-7-methoxy-	,,	H	OMe	H	H	Me	H	H
12. 4-Methyl-6-methoxy-	,,	H	H	OMe	H	Me	H	H
13. 4-Methyl-4'-methoxy-	,,	H	H	H	H	Me	H	OMe



		R_1	R_2	R_3	R_4	R_5	R_6	R_7
14. 2-Methyl-	phenprocoumon	H	H	H	H	Me	H	H
15. 2-Methyl-7-methoxy-	,,	H	OMe	H	H	Me	H	H
16. 2-Methyl-6-methoxy-	,,	H	H	OMe	H	Me	H	H
17. 2-Methyl-4'-methoxy-	,,	H	H	H	H	Me	H	OMe

Fig. 2. Structures of phenprocoumon metabolites and derivatives. d_5 = pentadeutero (formula 9).

methanol (20, 40, 60, 80 and 100%). The 60% and 80% methanol fractions were evaporated, dissolved in methanol and centrifuged. The 60% methanol extract was used for the micropreparative isolation of 4'-hydroxyphenprocoumon (Fig. 2). 6-Hydroxyphenprocoumon, 7-hydroxyphenprocoumon and phenprocoumon (Fig. 2) were isolated after injecting the 80% methanol extract several times (5 × 20 µl) into the HPLC system, peak collection and solvent evaporation.

Methylation

Synthetic reference compounds (Fig. 2; formulae 1, 4, 6, 7, 8, 9), urine extracts (extraction a or b), or fractions isolated by HPLC, were dissolved in methanol, dried under nitrogen in conical vials, dissolved in dry acetone (100 µl) and alkylated with methyl iodide (50 µl) in the presence of potassium carbonate (15 mg) and potassium iodide (2 mg) at 37° C overnight after capping with PTFE-lined discs. The solvent was evaporated, the residue was dissolved in

100 μl of water and 100 μl of 3 *M* hydrochloric acid, and extracted twice with 200 μl of chloroform. The chloroform extracts were evaporated; the residue was dissolved in isooctane (10—50 μl) and injected into the GC—MS system. Diazomethane solutions obtained as described by Fales et al. [12] were also used for the methylations.

Chromatographic conditions

High-performance liquid chromatography. Linear gradient elution (44—100% solvent B in 50 min; flow-rate 2.0 ml/min) was used; solvent B was methanol and solvent A was a mixture of methanol—0.5% acetic acid (neutralized to pH 4.8 with ammonia) (100:900). Each run lasted 20 min (15 min analysis time and 5 min equilibration delay at initial conditions) and was performed at room temperature. A reversed-phase C_{18} column was employed for the separations (LiChrosorb RP-18 with 10-μm irregular particles; 250 × 4 mm I.D.; Hibar RT 250-4; Merck); a guard column (30 × 4 mm) was packed with Bondapak C_{18}-Corasil (35—50 μm spherical particles; Waters). After a series of analyses the column was washed with water and methanol. Peaks were detected fluorimetrically (excitation and emission wavelengths 310 and 390 nm, respectively; sensitivity setting low, 100%).

Gas chromatography—mass spectrometry. A gas chromatograph 5840A provided with a 1835C capillary inlet system, splitless injector injector liner and a quartz capillary column (OV-1; 12.5 m × 0.2 mm) (all from Hewlett-Packard) were used for the separations. After the sample injection (1 μl), the oven temperature was maintained for 1 min at 80°C followed by a temperature gradient (30°C/min) to 250°C (injector temperature 250°C). The gas chromatograph was coupled to the mass spectrometer with an open split interface at 250°C. Purified helium was used as carrier gas, at a column pressure of 1.5 bars. The mass spectrometer was operated under electron-impact (EI) conditions both in the scanning mode for the mass spectra recording of the GC peaks and in the selected-ion monitoring (SIM) mode the specified ions. Typical parameters used for the EI settings were source temperature 200°C, emission 300 μA, electron energy 70 eV, multiplier 1400 V.

Thin-layer chromatography (TLC). HPTLC silica gel 60 F_{254} plates (E. Merck) were used with solvents 1 (dichloroethane—acetic acid, 96:4) and 2 (*tert.*-butanol—benzene—aqueous ammonia—water, 45:20:9:3) [11]. The spots were visualized with UV light at 254 nm.

RESULTS AND DISCUSSION

The following parameters were determined for the patients, who received a weekly dose of 15.5 ± 6.3 mg of phenprocoumon: plasma phenprocoumon concentration, 2.04 ± 0.68 mg/l; phenprocoumon urinary excretion (as conjugate), 0.44 ± 0.22 mg per 24 h; thrombotest (9.6 ± 4.4%) [7]. The characterization of 7-hydroxyphenprocoumon as the main metabolite and of 4'-hydroxyphenprocoumon and 6-hydroxyphenprocoumon found in patients' urine after enzymatic hydrolysis was accomplished mainly by HPLC and GC—MS.

High-performance liquid chromatography

Fig. 3a shows the separation of a mixture of synthetic substances (4′-OH-, 6-OH-, 8-OH- and 7-OH-phenprocoumon, phenprocoumon and *p*-chloro-phenprocoumon) using radient elution HPLC and specific detection by fluorimetry; Fig. 3b shows the separation of an extract from blank urine spiked with 4′-OH-, 6-OH- and 7-OH-phenprocoumon and phenprocoumon. The fluorescence responses from the different metabolites at the same concentrations in relation to phenprocoumon were 0.40, 1.48 and 0.31, respectively. Fig. 3c shows the chromatogram from a patient's urine after hydrolysis and work-up as described under *Extraction a*, in which 4′-OH-, 6-OH- and 7-OH-phen-procoumon and phenprocoumon could be detected. Each of the 35 patients' urines was analysed in the same way with similar results. HPLC peaks from the urine extracts were qualitatively identified by their retention times compared to those of authentic substances, as well as by fractional collection of the peaks, methylation of each fraction as described above and analysis by GC—MS in the SIM mode as described below.

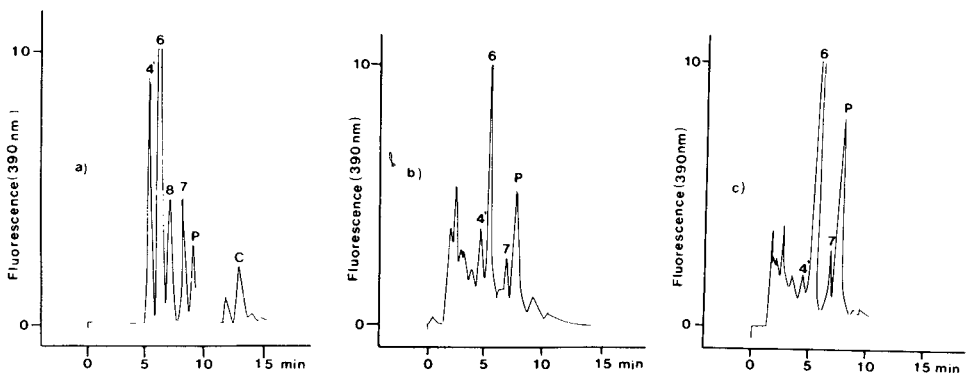


Fig. 3. HPLC profiles of phenprocoumon and metabolites. (a) Mixture of pure compounds: 4′ = 4′-hydroxyphenprocoumon (retention time, t_R = 4.24 min, capacity factor, k' = 2.85), 6 = 6-hydroxyphenprocoumon (t_R = 5.07, k' = 3.61), 8 = 8-hydroxy-d_5-phenprocoumon (t_R = 6.05, k' = 4.50), 7 = 7-hydroxyphenprocoumon (t_R = 6.65, k' = 5.05), P = phen-procoumon (t_R = 7.45, k' = 5.77), C = *p*-chloro-phenprocoumon (t_R = 11.39, k' = 9.33). (b) Extract from blank urine spiked with 1 μg/ml each of 4′-hydroxy-, 6-hydroxy- and 7-hydroxyphenprocoumon and phenprocoumon. (c) Extract from patient's urine.

Although the analytical recoveries of phenprocoumon and metabolites in spiked urine using extraction a were high (90—95%) and reproducible (± 5%) and the fluorimetric detection was more specific than the ultraviolet previously used [4], accurate quantitative results could not be obtained with this method for patients' urine due to interfering endogenous compounds and/or other drugs.

HPLC was also used for the micropreparative isolation of phenprocoumon and metabolites (extraction b) for further characterization by mass spectrometry (see below).

Gas chromatography—mass spectrometry

Prior to the analysis of phenprocoumon and the hydroxylated metabolites

by GC—MS it was necessary to derivatize them by methylation; mixtures of the 4-O-methyl isomers (Fig. 2, formulae 10—13) with small amounts of the 2-O-methyl isomers (Fig. 2, formulae 14—17) were produced (due to the tautomerism in the 4-hydroxycoumarin series) [13, 14] and could be separated with capillary GC columns. Methyl iodide alkylation [15] instead of the earlier diazomethane treatment [12] was used preferentially as higher yields of methylated products and less 2-O-methyl isomers were produced. The individual isomers could be characterized after methylating each synthetic substance (Fig. 2, formulae 1, 6—9), submitting the reaction mixtures to HPLC and identifying each isolated peak by UV spectroscopy [13, 14] and GC—MS. It was found that the 4-O-methyl isomers have longer GC retention times than the corresponding 2-O-methyl isomers.

By processing a large amount of patients' urine using extraction b, purified samples were obtained which contained substances identified later by GC—MS as phenprocoumon, 7-OH-, 6-OH- and 4'-OH-phenprocoumon.

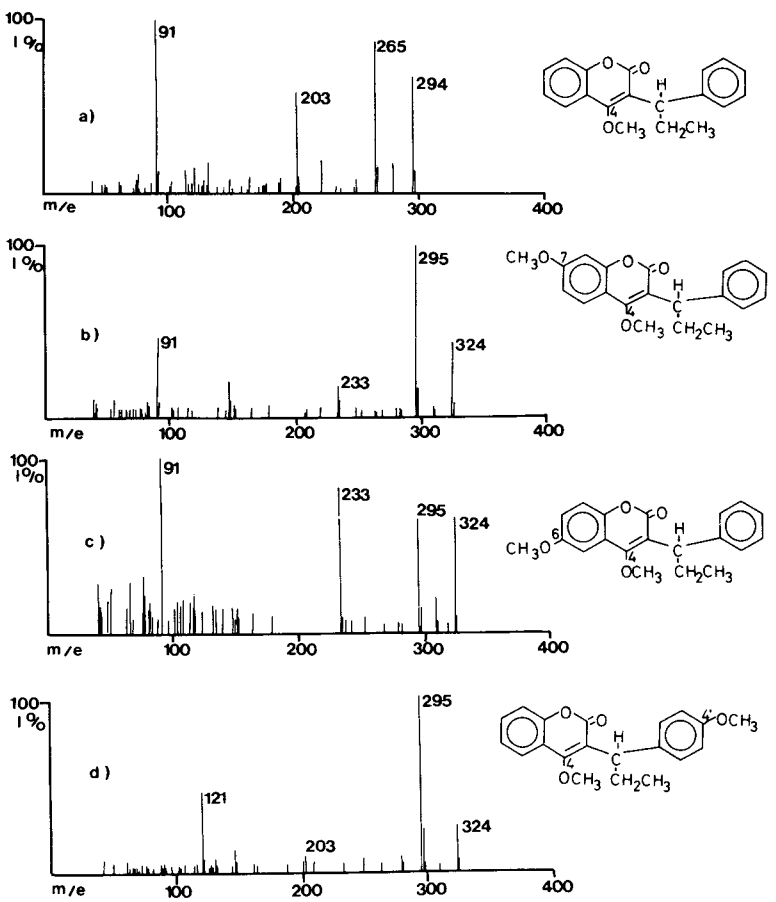


Fig. 4. Mass spectra of phenprocoumon and metabolites isolated from patient's urine (see under *Extraction b*) after methylation and GC—MS. (a) 4-O-methylphenprocoumon, (b) 4,7-dimethyl-7-hydroxyphenprocoumon, (c) 4,6-dimethyl-6-hydroxyphenprocoumon, (d) 4,4'-dimethyl-4'-hydroxyphenprocoumon.

Characteristic mass spectra were obtained for the 4-O-methylated phenprocoumon (Fig. 4a) and the metabolites (Fig. 4b—d). The molecular ion of methylated phenprocoumon (M^+, *m/e* 294) was displaced to *m/e* 324 for the methylated metabolites, indicating an additional oxygen in the latter. Fig. 4a—c shows an intense ion at *m/e* 91 characteristic for the benzylic side-chain, while Fig. 4d shows an *m/e* 121 peak indicating a methoxylated side-chain. This was corroborated by the appearance of the ion $m^+ - 91$ for spectra a—c and $M^+ - 121$ for spectrum d (*m/e* 203, 233, 233 and 203, respectively). Moreover, all spectra show a characteristic and intense $M^+ - 29$ peak (*m/e* 265 for phenprocoumon and 295 for the metabolites) from the loss of an ethyl group from the phenylpropyl side-chain, which was useful for GC—MS in the SIM mode. The retention times in GC—MS as well as their mass spectra (Fig. 4) were identical with those of synthetic phenprocoumon, and 7-OH-, 6-OH- and 4′-OH-phenprocoumon methylated by the same procedure. A detailed account of the mass spectrometric fragmentation of phenprocoumon and derivatives will be published elsewhere [16]. The structures of the ions could be corroborated by the use of several substituted and deuterated compounds.

The main metabolite, 7-OH-phenprocoumon, as well as phenprocoumon, were also identified by the measurement of ultraviolet [11] and fluorescence spectra in acid and alkaline solution which are characteristic for the different isomers, and by TLC in several solvents, which were identical with those of authentic material [11].

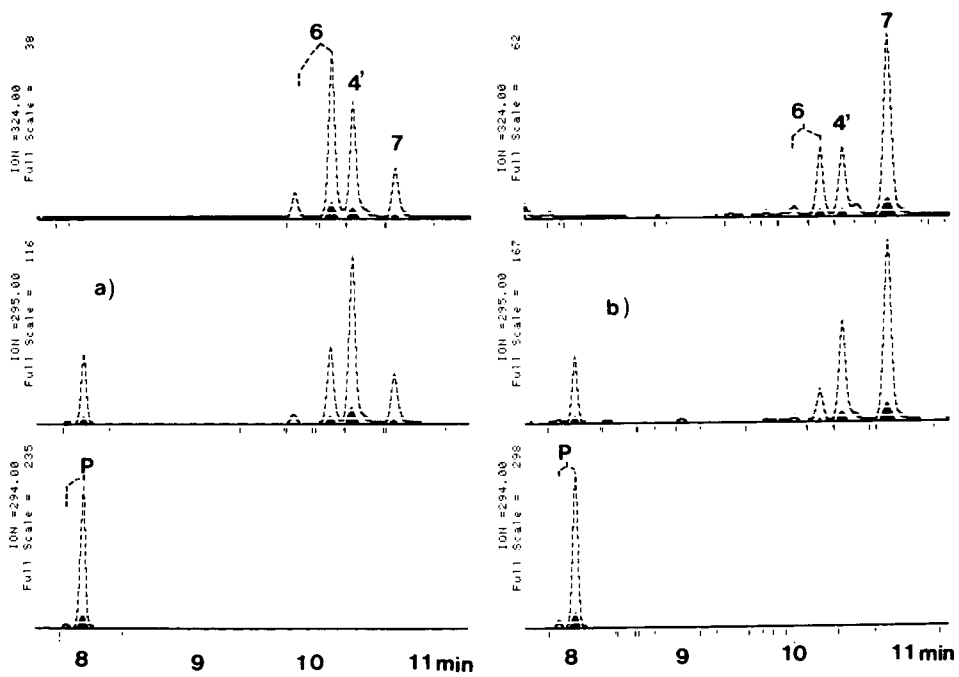


Fig. 5. GC—MS analysis in the SIM mode of phenprocoumon and metabolites after methylation. (a) Urine extract spiked with 1 μg/ml each of phenprocoumon (P), 6-hydroxyphenprocoumon (6), 4′-hydroxyphenprocoumon (4′) and 7-hydroxyphenprocoumon (7). (b) Extract from patient's urine.

The same urine extracts from 35 patients previously analysed by HPLC were methylated and subjected to GC—MS analysis in the SIM mode. Fig. 5a shows a GC—MS chromatogram from a mixture of synthetic 6-OH-, 4′-OH- and 7-OH-phenprocoumon and phenprocoumon after methylation. The molecular ions at *m/e* 294 and 324 for the methylated phenprocoumon and metabolites, as well as the *m/e* 295 ($M^+ - 29$) peak, characteristic of the methylated metabolites, were chosen for ion monitoring. Fig. 5b shows the chromatogram from a patient's urine extract (the same shown in Fig. 3c) after methylation; the methylated 6-OH-, 4′-OH- and 7-OH-phenprocoumon and phenprocoumon were identified by their retention times and intensity ratios for the specified ions. All 35 samples showed the same qualitative pattern. 8-OH-phenprocoumon (whose 4-O-methyl derivative has a shorter GC retention time than the other metabolites) could not be detected. Detection limits were in the order of 10 ng/ml.

An evaluation of the relative amounts of metabolites was obtained by adding *p*-chlorophenprocoumon as internal standard to the extracts, methylating and analysing the products by GC—MS in the SIM mode. The *m/e* 299 trace ($M^+ - 29$ for *p*-chlorophenprocoumon) was used for the normalization and the *m/e* 295 for monitoring the metabolites. This semi-quantitative method showed for the ratios metabolites/phenprocoumon median values [17] of 0.9, 0.2 and 0.1 (Table I) for the 7-OH, 4′-OH and 6-OH compounds, respectively, which has also been observed independently in volunteers [18]. Considerable interindividual variability was seen for the patients, and median means were calculated to take into account the extreme values in the range [17] (Table I).

Analysis of urine before and after enzymatic hydrolysis showed that only trace amounts of unconjugated substances were eliminated. No methoxylated derivatives from phenprocoumon, 6-OH-, 4′-OH- or 7-OH-phenprocoumon could be detected as direct metabolites in patients' urine by HPLC or GC—MS.

TABLE I

RATIO OF URINARY EXCRETED METABOLITES TO PHENPROCOUMON

Metabolite	Median ± S.E.M.	Range
7-Hydroxyphenprocoumon	0.92 ± 0.06	0.20—1.70
4′-Hydroxyphenprocoumon	0.23 ± 0.03	0.02—1.90
6-Hydroxyphenprocoumon	0.10 ± 0.03	0.02—2.00

The elimination of phenprocoumon, metabolites and warfarin

In vivo metabolic studies with rats using radioactive phenprocoumon have been published previously by Haddock et al. [8], who showed that the drug and hydroxylated metabolites were excreted in urine and faeces (20% and 59%, respectively, from the total radioactivity after twelve days). The elimination pattern was found to be 6-OH-phenprocoumon > 4′-OH-phenprocoumon > phenprocoumon > 7-OH-phenprocoumon > 8-OH-phenprocoumon. Phenprocoumon is metabolized in the rat by liver microsomes and this drug has been used, as well as warfarin, as a probe to categorize the effect of inducing agents on microsomal hydroxylases [8—10].

Earlier studies on the excretion of phenprocoumon in man [4, 7] have shown that only a fraction of the applied dose (20%) is eliminated in urine as conjugate; as we have shown here, 7-OH-, 6-OH- and 4′-OH-phenprocoumon are produced and also eliminated as conjugates. The total amount of phenprocoumon plus metabolites excreted in urine accounts for somewhat more than the half of the ingested drug; or unknown metabolites are produced which could not be detected, or a considerable amount is excreted in the faeces as has been previously described for rats [18]. Biliary excretion of phenprocoumon and metabolites has been reported for rats and is presumed in humans as studied in the interaction with cholestyramine [19]. Phenprocoumon and the metabolites are excreted in urine as conjugates, and enzymatic hydrolysis prior to extraction and analysis has to be done to measure the total amount eliminated.

Warfarin and metabolites have been characterized previously in human urine (warfarin, 7-hydroxy-warfarin, 6-hydroxy-warfarin, two diastereoisomeric warfarin alcohols and benzylic hydroxywarfarin) [20—24] and plasma (warfarin, warfarin alcohols, 7-hydroxywarfarin and 6-hydroxywarfarin [23, 25, 26]. The acetonyl side-chain (instead of ethyl in phenprocoumon) is the main cause for the different physicochemical properties of these compounds [27—29], which is also reflected in differences in the pharmacokinetics and metabolism of both compounds.

Many drugs interact with warfarin, phenprocoumon and other coumarin anticoagulants [30—32]. However, drug interactions with warfarin or phenprocoumon are sometimes quite different, as in the case of cimetidine and sulfinpyrazone [5, 6, 33—36] and are of clinical relevance.

Further work is being undertaken to develop a specific and sensitive assay for the quantitation of phenprocoumon metabolites for further clinicopharmacological investigations.

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GAS CHROMATOGRAPHIC ANALYSIS OF TRIETHYLENETHIOPHOSPHORAMIDE AND TRIETHYLENEPHOSPHORAMIDE IN BIOLOGICAL SPECIMENS

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SUMMARY

Comprehensive pharmacokinetic studies could realise a greater potential for the anti-tumour agent triethylenethiophosphoramide (ThioTEPA), and these would be aided by the development of a selective and sensitive assay. After extraction of ThioTEPA and its metabolite, triethylenephosphoramide (TEPA), from plasma using Sep-Pak C_{18} cartridges, the compounds were separated by capillary chromatography, detected using a nitrogen detector and quantified by reference to an internal standard, hexaethylphosphoramide. The limits of sensitivity were 1—5 ng/ml. Analytical recoveries were 74 and 95%, for TEPA and ThioTEPA, respectively, in the therapeutic range. At similar concentrations, extents of protein binding, determined by ultrafiltration, were not significant. Preliminary investigations of the elimination of ThioTEPA show that drug loss occurs more quickly in mice than in humans and in both species the metabolite is extensively recycled.

INTRODUCTION

The cytotoxic drug triethylenethiophosphoramide (ThioTEPA, Fig. 1), is being used currently in a controlled randomised trial to examine the contri-

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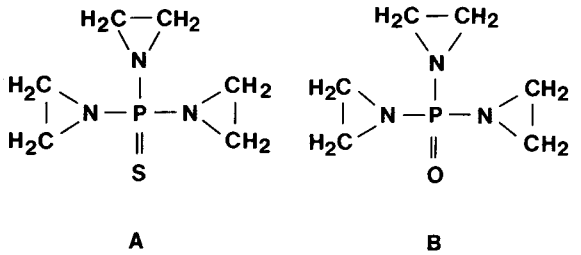

A B

Fig. 1. Molecular structures of (A) ThioTEPA and (B) its metabolite, TEPA.

bution of nandrolone decanoate (Deca-durabolin, Organon) to chemotherapy regimes for ovarian and breast cancer. Studies, both clinical [1] and with animal models [2], indicated that anabolic steroids may stimulate regeneration of peripheral leukocytes. A generalised anabolic effect, however, may not account completely for the increased therapeutic index [1]. It is possible that the synergism of the combination could be explained in part by a pharmacokinetic interaction. For purposes of this investigation a sensitive assay for ThioTEPA and its metabolite, triethylenephosphoramide (TEPA, Fig. 1), also a potent antitumour agent, was required.

Previous studies of the elimination of ThioTEPA were performed using the fluorescent properties of the drug and metabolite [3] or after administration of the isotopically labelled compounds [4—7]. Laborious solvent extractions or chromatographic separations, respectively, were necessary to achieve specificity. Pharmaceutical preparations of ThioTEPA have been analysed using infrared [8] and proton magnetic resonance [9] spectrometry, but these methods are unsuitable for routine determinations of drug levels in plasma. The vapour pressures of TEPA and ThioTEPA, an important consideration in their use as chemosterilants, were determined by gas chromatography (GC) with a flame ionization detector [10].

Retention indices of ThioTEPA have been obtained using various GC conditions and nitrogen detection [11]. Recently, the stability of ThioTEPA in urine and buffer solutions was investigated by solvent extraction of the compound and degradation products before analysis utilizing GC with nitrogen detection [12]. Quantitative determination of TEPA and ThioTEPA in body fluids using GC has not been evaluated previously.

We investigated the application of reversed-phase extraction to sample preparation prior to analysis using capillary chromatography and nitrogen detection. The primary aim was to achieve adequate sensitivity to enable pharmacokinetic characterisation of the drug. Initial application of the method to studies in patients and mice of the elimination of TEPA and ThioTEPA, and their protein binding in vitro is described.

EXPERIMENTAL

Materials

Spectroscopic grade ethanol (BDH, Poole, U.K.) and triple-distilled water were used. Other reagents were of analytical grade. Buffer solutions were phosphate-buffered saline (PBS) which contained $1.34 \cdot 10^{-1}$ *M* NaCl, 5.36 ·

10^{-3} *M* KCl, $1.46 \cdot 10^{-2}$ *M* NaH_2PO_4, $8.16 \cdot 10^{-2}$ *M* Na_2HPO_4, $9.01 \cdot 10^{-4}$ *M* $CaCl_2$ and $1.04 \cdot 10^{-3}$ *M* $MgCl_2$, or 0.5 *M* sodium cacodylate—HCl, pH 7.4.

ThioTEPA was a gift from Lederle Labs. (Gosport, U.K.). TEPA was synthesized by a method similar to that given for the radiolabelled substance by Craig and Jackson [13]; the synthetic compound produced satisfactory analytical and proton magnetic resonance data, and was chromatographically homogeneous. Triethylenemelamine was obtained from Lederle Labs., hexaethylphosphoramide was purchased from Fluorochem (Glossop, U.K.), hexamethylphosphoramide from BDH and tris-(N,N-tetramethylene)-phosphoric acid triamide from Fluka (Buchs, Switzerland); these compounds were examined as possible internal standards for the GC assay.

Human serum albumin (HSA), containing less than 0.005% fatty acid, and human α-, β- and γ-globulins were purchased from Sigma (St. Louis, MO, U.S.A.). Time-expired blood was obtained from the Haematology Department, Bradford Royal Infirmary. Blood was centrifuged at 2000 *g* for 15 min and the separated plasma was stored at −20° C.

Ultrafiltration

TEPA and ThioTEPA (500 mg) were added to PBS (5.0 ml) alone or containing HSA (58.0 mg/ml), α-globulins (15.8 mg/ml), β-globulins (9.3 mg/ml) or γ-globulins (9.6 mg/ml). The mixtures were incubated for 2 h at 37° C and aliquots (1.1 ml) were retained for extraction and GC analysis of total drug or metabolite. The remaining solutions were ultrafiltered at 37° C using a Multimicro concentrator (Amicon, MA, U.S.A.) and Amicon UM10 Diaflo membranes (25 mm diameter). A flow-rate of 0.8 ml/h was maintained using mass flow control of a nitrogen supply. As the initial ultrafiltrate is diluted by buffer solution which remains within the void volume of the membrane after washing, the first portion (500 μl) was discarded and an aliquot (1.1 ml) was then taken for extraction and GC analysis.

Extraction of drug and metabolite

Sep-Pak cartridges containing particles of C_{18}-coated silica (Waters Assoc., Northwich, U.K.) were activated by passing through ethanol (5 ml) and then distilled water (5 ml) under pressure using a plastic syringe. Biological specimens or control samples of plasma (1 ml) were mixed with cacodylate buffer or TEPA and ThioTEPA in cacodylate buffer (100 μl). Hexaethylphosphoramide (50 or 500 ng) in cacodylate buffer or PBS (50 μl) was added to plasma specimens, protein solutions, ultrafiltrates or control samples, which were applied to Sep-Pak cartridges at a flow-rate less than 2 ml/min. The cartridges were washed with distilled water (5 ml) and the solutions remaining were evacuated. Ethanol (2 ml) was passed through the cartridges and the effluents were collected in tapered plastic tubes. Volumes of solvent were reduced to approximately 100 μl by evaporation under a stream of nitrogen with the tubes immersed in a water bath at 55° C. Samples (0.8 μl) were taken up into a gas-tight syringe containing ethanol (0.2 μl) and injected into the chromatograph.

Gas chromatography

GC was performed using a PU4500 capillary chromatograph equipped with

a Grob-type injection system and nitrogen detector (Pye-Unicam, Cambridge, U.K.). Components were separated using a quartz column (25 m × 0.25 mm I.D.), wall-coated (0.2 μm) with SE-30 (Pye-Unicam). Thermal conditions were: injector temperature, 270°C; detector temperature, 300°C; temperature of the column oven was programmed from 180°C to 220°C at 6°C/min. Flow-rates of gases were: flame gases, (hydrogen), 30 ml/min and (air), 300 ml/min; make-up gas (nitrogen), 30 ml/min; carrier gas (hydrogen), 2 ml/min. The split ratio was 5:1.

Patient and animal protocols

Three patients for whom ThioTEPA was part of their medical treatment at the Bradford Royal Infirmary and the Yorkshire Clinic (Bingley, U.K.) participated with informed consent. Each was given the drug (30 mg) as an intramuscular (i.m.) injection. Patients R.A. and L.R. received 150 and 200 mg nandrolone decanoate, two and seven days before ThioTEPA dosage and ampicillin (1 g daily) and dexamethazone (10 mg daily), respectively, were administered concomitantly. Blood specimens (3 ml) were taken by venipuncture into heparinized tubes just before and at 1, 3, 5, 7, 9 and 24 h after dosage.

The experimental animals used were female NMRI mice from our inbred colony. ThioTEPA (10 or 20 mg/kg) was given by intraperitoneal (i.p.) injection. Mice were bled in triplicate by cardiac puncture and the specimens were pooled at hourly intervals during periods up to 12 h after drug administration.

Plasma was separated and stored at −20°C.

Pharmacokinetic analysis

The half-life of elimination ($t_{½}$) was determined graphically as the time taken for drug concentration in plasma to decline by 50%. The area under the plasma concentration versus time curve (AUC) was calculated using the trapezoid rule [14].

RESULTS AND DISCUSSION

Analytical considerations

Choice of internal standard. Hexaethylphosphoramide was chosen from the compounds, structurally related to TEPA and ThioTEPA, which were examined as possible internal standards, since it was separated completely from other components in the assay yet eluted within a reasonable time for analysis. Retention times of TEPA and ThioTEPA, relative to hexaethylphosphoramide, were 0.68 and 0.76, respectively. In addition, ThioTEPA had a similar response relative to hexaethylphosphoramide (0.85), but that of TEPA (0.51) was considerably less. Hexamethylphosphoramide fulfilled some of the criteria of a suitable internal standard for TEPA assay, having a similar response and proximate retention time (0.62 and 0.53, relative to hexaethylphosphoramide, respectively), but was rejected on the basis of high volatility [10].

Elution characteristics. The chromatogram shown in Fig. 2A is typical of the fast and efficient separation of components obtained. Retention times of TEPA, ThioTEPA and hexaethylphosphoramide were 3.1, 3.45 and 4.6 min,

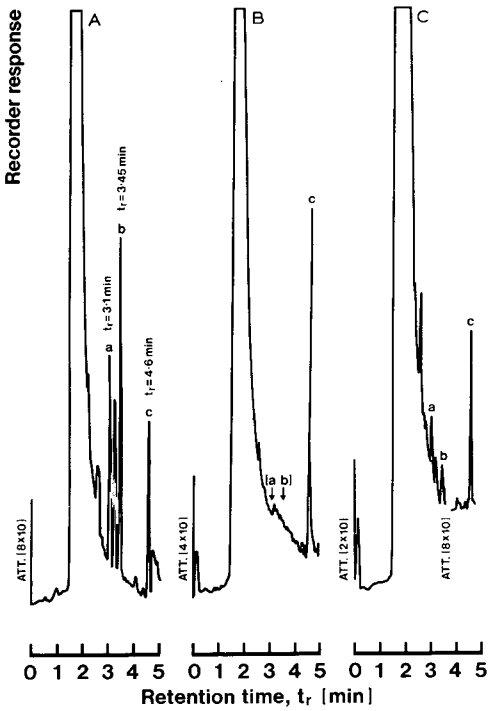

Fig. 2. Gas chromatograms of extracts of plasma (1 ml) containing 50 ng hexaethylphosphoramide: A, control plasma with 100 ng TEPA and ThioTEPA added; B, patient plasma before drug dosage; C, patient plasma, 24 h after i.m. administration of 30 mg ThioTEPA, with concentrations of TEPA (5.3 ng/ml) and ThioTEPA (2.5 ng/ml). Peaks: a = TEPA; b = ThioTEPA; c = hexaethylphosphoramide (internal standard).

respectively. An endogenous component of control human plasma had a retention time intermediate between that of TEPA and ThioTEPA. This extra peak was large in chromatograms of extracts of some specimens of the time-expired plasma used in control experiments (for example, Fig. 2A). The interfering substance, however, was not present in significant quantity in any sample of freshly prepared patient plasma (for example, Fig. 2B) and so did not affect the accuracy of measurement of low levels of drug and metabolite.

Interferences. Nandrolone, the major product in serum after extravascular administration of the decanoate ester, and methotrexate, which is also included often in combination regimes with ThioTEPA, are extracted with greater than 78% efficiency using the Sep-Pak procedure. The cytotoxic drug, its primary metabolite, 7-hydroxymethotrexate, and steroid compounds, however, do not interfere in the GC assay of ThioTEPA, as they are not sufficiently volatile.

Recovery and reproducibility. The mean recovery of hexaethylphosphoramide from plasma using the Sep-Pak procedure and hexamethylphosphoramide as external standard, was 96.4 ± 4.9% (S.E.) at a concentration of 500 ng/ml. Analytical recoveries of TEPA and ThioTEPA and their precision using hexaethylphosphoramide as internal standard are shown in Table I. Recoveries of ThioTEPA were acceptable but those of TEPA were significantly lower, which may reflect in part the more polar nature of the metabolite [3]. The

TABLE I

RECOVERIES OF TEPA AND ThioTEPA FROM PLASMA AND THEIR REPRODUCIBILITIES AFTER SEP-PAK C_{18} EXTRACTION AND GC DETERMINATION USING HEXAETHYLPHOSPHORAMIDE AS INTERNAL STANDARD

Concentration of TEPA or ThioTEPA (ng/ml)	*n*	Recovery (%) (mean ± S.E.)	
		TEPA	ThioTEPA
50.0	12	73.6 ± 5.6*	95.2 ± 7.5*
50.0	12	74.2 ± 6.5**	93.6 ± 7.8**
500.0	6	75.2 ± 5.7*	97.5 ± 5.5*
500.0	6	74.1 ± 7.0**	96.9 ± 8.0**

*Intra-assay statistical variations.
**Inter-assay statistical variations.

greater polarity of TEPA might result in larger binding to active sites on the silica particles of the Sep-Pak material or the metabolite might not partition as strongly to the hydrophobic moiety of the reversed-phase packing, and so may be partly eluted during the aqueous wash. It would be expected, however, that the greater volatility of TEPA than of ThioTEPA [10] would account mainly for the losses of TEPA, which must occur during the concentration stage of sample preparation. Evaporation to dryness of ethanolic solutions of TEPA and ThioTEPA (1 μg/ml), which were then reconstituted in ethanol (100 μl) containing internal standard, resulted in losses of 52.5 ± 4.2% (S.E.) and 34.9 ± 3.9% (S.E.), respectively. According to the method of Dijkhuis [15], isoamylacetate (2.5%, v/v) was added to ethanolic solutions to inhibit evaporation when a volume of 50—100 μl remained. There was no significant increase in recovery of the metabolite or drug and so the ester was omitted and evaporation was performed routinely under supervision until extracts were almost dry.

Linearity. The effect of concentration of TEPA and ThioTEPA on peak height ratio was linear over the ranges, 0—0.2 and 0—1.0 μg/ml, utilizing 50 and 500 ng hexaethylphosphoramide, respectively. Calibration plots, obtained by least squares linear regression analysis of replicate determinations ($n = 6$), were described by: $y = 10.21x - 0.007$ ($r = 0.9999$) for TEPA and $y = 22.38x - 0.03$ ($r = 0.9999$) for ThioTEPA in the lower range of concentration; $y = 1.20x - 0.005$ ($r = 0.9999$) for TEPA and $y = 2.37x - 0.01$ ($r = 0.9999$) for ThioTEPA in the higher range of concentration. Standard deviations in the slopes of the plots were 0.50, 0.88, 0.04 and 0.08, respectively. The total range of the calibration was adequate for estimation of drug and metabolite levels in patient plasma during 24 h after treatment. Because of the greater amounts of the substances in mice, and the lesser volumes of mouse blood obtainable, plasma (100 μl) was diluted to 1 ml with control mouse plasma and estimations were performed over the same range of concentration as for patient samples.

Sensitivity. The lower limit for accurate detection of TEPA and ThioTEPA was 1—5 ng/ml, corresponding to peak height ratios of twice those observed for analyses of control specimens (for example, Fig. 2B). Fig. 2C shows the GC analysis of patient plasma, taken 24 h after ThioTEPA administration, in which concentrations of drug and metabolite are close to the limit of assay sensitivity.

Pharmacokinetic investigations

Humans. Preliminary investigations were performed in three patients after i.m. administration of 30 mg ThioTEPA and these results are shown in Fig. 3. The excretion kinetics of ThioTEPA were apparently first-order in only one patient and the plasma half-life is given in Table II. The elimination kinetics of the metabolite were nonlinear in all cases. Increases in levels of TEPA occurred after its initial peak concentrations were observed and were coincident with the reductions in the rate of ThioTEPA elimination.

Patients who receive nandrolone decanoate are treated using slightly different schedules of combination therapy. In order to assess any possible influence of the steroid on drug or metabolite clearance in a larger study, it will be necessary to determine the plasma levels of nandrolone that exist simultaneously with the measured TEPA and ThioTEPA concentrations. It is intended to implement a radioimmunoassay for this purpose.

Mice. Pharmacokinetic profiles of TEPA and ThioTEPA after administration of higher doses of the drug than were used clinically are shown in Fig. 4. Intraperitoneal administration of 20 mg/kg ThioTEPA resulted in the unusual pattern of metabolite clearance observed in the patient study, but this feature was not so marked at the lower dosage of 10 mg/kg. Significant indications of a dose dependence of ThioTEPA elimination were that on doubling the dose, the half-life did not remain constant but increased two-fold and there was a disproportionate increase in AUC value (Table II).

When administered concomitantly, nandrolone decanoate was shown to

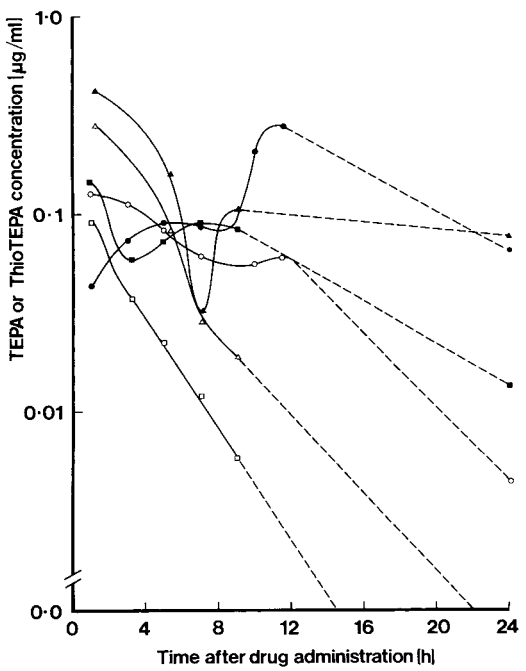

Fig. 3. Plasma levels of drug (open symbols) and metabolite (closed symbols) in patient E.C. (circles), patient R.A. (squares), and patient L.R. (triangles) after i.m. administration of 30 mg ThioTEPA.

TABLE II

ELIMINATION PARAMETERS OF TEPA AND ThioTEPA IN PATIENTS AND MICE

	Dose (mg/kg)	$t_{1/2}$ (min)	AUC* (μg h/ml)		
			TEPA	ThioTEPA	TEPA/ThioTEPA
Patients					
R.A.	0.50	123.0	0.75	0.30	2.54
E.C.	0.32	ND**	0.77	0.76	1.02
L.R.	0.59	ND**	1.76	1.07	1.65
Mice					
	10.0	15.2	25.8***	5.1***	5.06
	20.0	29.1	41.8***	14.7***	2.83
	20.0	28.0	ND§	12.8***	ND§

*Calculated to 9 h after drug dosage.
**Not determined as pharmacokinetics nonlinear.
***Values obtained by interpolating the plasma concentration versus time curve between 0 and 1 h to maxima at 16.0 and 3.3 min for TEPA and ThioTEPA, respectively, as shown in pilot studies of i.p. drug administration in mice.
§Not determined as data inadequate.

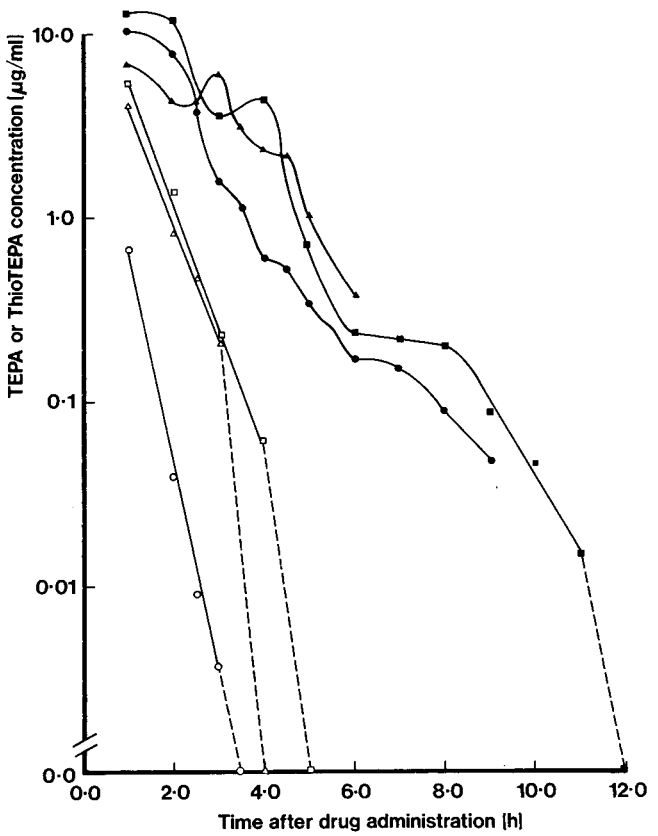

Fig. 4. Plasma levels of drug (open symbols) and metabolite (closed symbols) in three experiments in mice after ThioTEPA administration (i.p.) of 10 mg/kg (circles) and 20 mg/kg (squares and triangles).

significantly decrease the clearance of TEPA but not of ThioTEPA and the reduction was greater in female than in male mice. The detailed results are in preparation for publication.

Species variation. Half-lives of elimination were smaller in mice (Table II) such that drug levels in plasma were undetectable 5 h after dosage. Also, the larger ratios of areas under the drug and metabolite profiles in mice than in patients (Table II) reflect an increased rate of elimination of ThioTEPA rather than decreased clearance of TEPA. In comparison with other species, the mouse is remarkable in its ability to metabolise ThioTEPA completely to inorganic phosphate [7].

Mechanism of elimination. From a comparison of the rate of disappearance of TEPA and ThioTEPA in both patients (Fig. 3) and in mice (Fig. 4), it is apparent that the elimination of TEPA is much slower than that of ThioTEPA. The slower decay of TEPA levels has been observed previously in dogs after an intravenous injection of 3 mg/kg ThioTEPA [3]. As a general rule, lipid solubility is the limiting factor in urinary excretion of drugs [16]. ThioTEPA is more lipid soluble than TEPA, which is extremely soluble in water and would be expected to be excreted more quickly than the parent drug. The fluctuations in the plasma profiles of TEPA particularly in mice (Fig. 4) give some indication of an explanation for reduced metabolite clearance. The declining parts of the curves show irregularly spaced peaks due to loss from and reabsorption into the systemic circulation. The recycling of TEPA may explain the

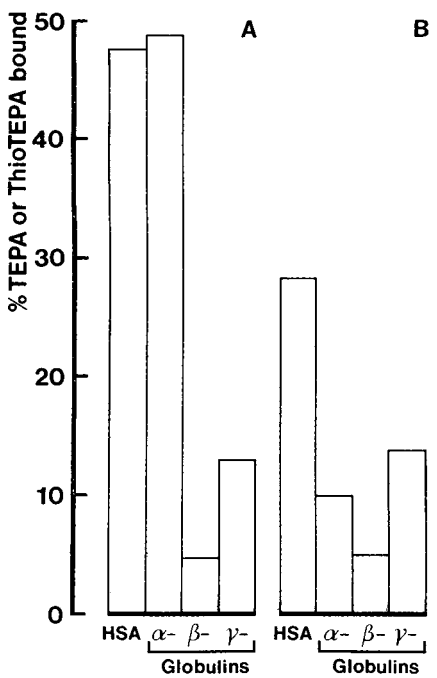

Fig. 5. Extents of binding of A, TEPA and B, ThioTEPA (100 ng/ml) to HSA (58.0 mg/ml), α-globulins (15.8 mg/ml), β-globulins (9.3 mg/ml) and γ-globulins (9.6 mg/ml), corrected for per cent rejection by the membrane.

delayed pharmacological effect of ThioTEPA, which may be manifest in marked myelosuppression up to 30 days after 5—7 day courses [17].

Protein binding. In control experiments in PBS, mean recoveries were 100.5 and 99.2% and intra-assay variations (S.E.) were 6.9 and 2.9% (n = 8) for TEPA and ThioTEPA (100 ng/ml), respectively, after incubation but before ultrafiltration: after ultrafiltration, recoveries were 92.7 and 90.9% and intra-assay variations were 7.7 and 3.9%. These results indicate that TEPA and ThioTEPA solutions at 37°C and pH 7.4 are stable over 2 h and therefore should not degrade to any significant degree during the period of ultrafiltration. In addition, the extents of rejection by the membrane of the drug (8.3%) and metabolite (7.8%) were similar.

The degree of binding of therapeutic concentrations of TEPA and ThioTEPA to human plasma components under physiological conditions are shown in Fig. 5. TEPA was bound to HSA to a greater extent (48%) than ThioTEPA (29%). ThioTEPA did not bind greatly to globulin fractions but the proportion of TEPA bound to α-globulins was significant (49%) by comparison with the degree of binding to β- and γ-globulins (< 15%). Electrophoretic investigations using human plasma in vitro have shown the complete association of ^{14}C-labelled ThioTEPA with protein [4]. Also, greater than 90% of the radioactivity in patient plasma after dosage with ^{32}P-labelled TEPA could be precipitated with 80% (v/v) aqueous acetone [18]. The compounds, therefore, may be associated with protein fractions in addition to those studied here, such as lipoproteins. Contrary to the present findings, Maxwell [19] reported that ThioTEPA was bound extensively and solely to γ-globulins both in vitro and in vivo. Further in vitro studies are required, initially, to clarify the role of the different protein fractions particularly in TEPA binding, before in vivo investigations to ascertain if it has any pharmacological significance.

ACKNOWLEDGEMENT

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PLASMA DETERMINATION OF 3-METHYLCLONAZEPAM BY CAPILLARY GAS CHROMATOGRAPHY

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SUMMARY

A capillary gas—liquid chromatographic method for the determination of 3-methylclonazepam in plasma was developed. This method involved a single extraction by butyl acetate followed by analysis of the organic extract on a CP-Sil 5 glass capillary column with detection by electron capture. The detection limit was about 0.1 ng/ml, and the inter- and intra-assay precision did not exceed 8% for the concentration range 0.1—6.0 ng/ml. Specificity towards some of the possible metabolites in human plasma was demonstrated. This method was used for the measurement of the pharmacokinetic parameters of 3-methylclonazepam in healthy volunteers after a single intravenous administration of 1 mg, and oral administrations of 1 and 4 mg.

INTRODUCTION

3-Methylclonazepam (Ro 11-3128, compound I) (Fig. 1) is a new benzodiazepine which exhibits very interesting anxiolytic [1] and antiparasitic properties [2]. The anxiolytic effect is reached with low doses of 1 or 2 mg per os. Therefore, the pharmacokinetic characterization of such a compound requires very sensitive and reproducible methods for its quantification in plasma or urine samples. Due to the electronegative character of some of the substituent groups of I, only two techniques were considered suitable for this purpose: gas—liquid chromatography (GLC) with electron-capture detection (ECD), and GLC coupled with mass spectrometry (GC—MS) operating in the negative chemical ionization mode [3—6]. The GC—MS procedures are, undoubtedly, the most specific and sensitive, but routine determinations could not be performed with these methods. Thus, a rapid and highly sensitive GLC procedure had to be developed, adapted to the analysis of large number of samples. This assay needed to be sensitive enough to measure precisely the

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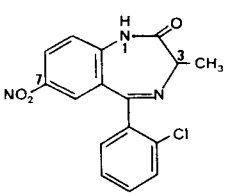
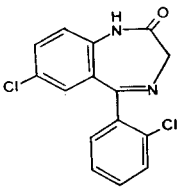


compound I



compound II

Fig. 1. Chemical structure of 3-methylclonazepam (I) and its internal standard (II).

small concentrations of unchanged I in plasma after oral administration of 1 mg of this compound.

MATERIALS AND METHOD

Materials

Capillary columns. Capillary wall-coated superior-capacity open tubular (WSCOT) glass columns (25 m × 0.5 mm I.D.), with an apolar polydimethylsiloxane stationary phase (CP-Sil 5), supplied by Chrompack (Orsay, France), were used for the determination of I. These columns were primed each day with an injection of 1 μl of a methanolic solution of cholesterol (1%) and slow temperature programming (2°C/min) from 280°C to 300°C.

Chromatographic analysis. GLC analysis was performed on a GLC—ECD system from Girdel (Suresnes, France), equipped with a solid injection system (moving glass needle injector). The carrier gas was helium N55 at a head column pressure of 0.75 bar; the auxiliary gas was argon—methane (95:5) at a flow-rate of 10 ml/min. The temperature settings for injection port and detector were 300°C. Respective retention times for compound I and its

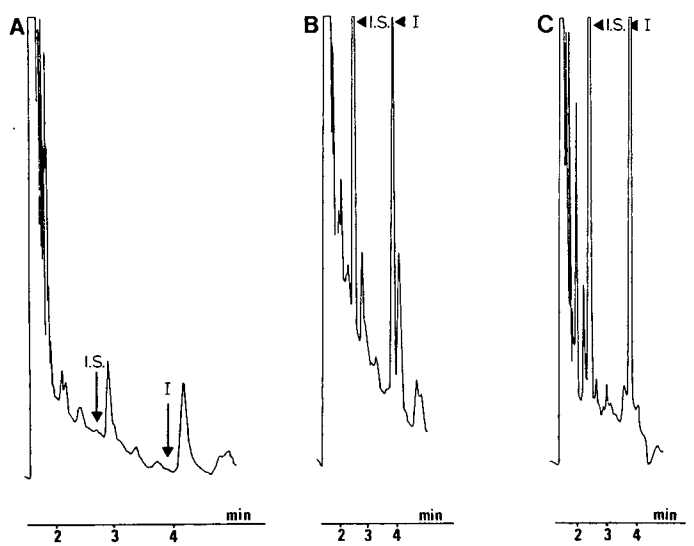


Fig. 2. Chromatograms obtained after extraction of (A) control plasma, (B) control plasma spiked with 2.5 ng/ml I, and (C) subject's plasma 0.5 h (6.7 ng/ml I) following a 1-mg intravenous bolus injection (I.S. 1.38 ng).

internal standard at an oven temperature of 280°C were 3.9 and 2.6 min (Fig. 2).

Standard solutions. The standard solutions of compound I and its internal standard, a desalkylated benzodiazepine (compound II) (Fig. 1), were prepared by carefully weighing about 10 mg into a 10-ml volumetric flask, and dissolving in methanol. Dilutions were also prepared in methanol.

Reagent. Only butyl acetate, Purex brand, supplied from S.D.S. (Peypin, France), was used for the extraction of I from plasma.

Method

Extraction procedure. An adequate volume of a methanolic solution of the internal standard was concentrated to a volume of about 20—25 μl in tapered 10-ml tubes under a gentle stream of pure nitrogen. Then 0.5 ml of plasma to be analysed was added, and extracted for 3 min with 0.3 ml of butyl acetate; the tubes were gently shaken on a vortex system to avoid serious emulsion formation. After centrifugation for 15 min at 4500 *g*, the organic phase was transferred to a 1.0-ml minivial (Pierce, Rockford, IL, U.S.A.), and then concentrated to a volume of 20—100 μl, depending on the range of expected concentrations, prior to GLC analysis.

Quantification of unknown samples. Data relating to plasma concentrations of I were obtained from least-square linear regression curves, established daily from four or five calibration points. Peak height ratios were computed by means of a SP 4270 system (Spectra Physics, Orsay, France). Quality control samples were also analysed together with the unknowns to confirm the assay accuracy.

RESULTS AND DISCUSSION

Reproducibility

The intra-day reproducibility of the method was evaluated over a concentration range of 0.6 to about 6 ng of I per 0.5 ml of plasma. The data presented in Table I indicate that the precision (given by the relative standard deviation) and the accuracy (defined by the difference between found and expected concentrations) were acceptable over this concentration range.

TABLE I

INTRA-ASSAY REPRODUCIBILITY AND ACCURACY

I · HCl concentration (ng per 0.5 ml of plasma)		*n*	Relative standard deviation* (%)	Difference between found and added concentration (%)
Added	Found			
0.63	0.62	7	8.0	−1.6
1.25	1.23	7	3.3	−1.6
3.12	3.15	7	3.2	+1.0
4.68	4.63	9	3.0	−1.1
6.25	6.14	5	2.1	−1.8

*95% Confidence.

TABLE II

INTER-ASSAY REPRODUCIBILITY AND ACCURACY

I • HCl concentration (ng per 0.5 ml of plasma)		*n*	Relative standard deviation* (%)	Difference between found and added concentration (%)
Added	Found			
0.61	0.52	13	7.4	−14.7
1.10	1.08	11	10.4	−1.8
2.49	2.35	25	3.8	−5.6
2.80	2.62	16	3.8	−6.4

*95% Confidence.

The inter-assay reproducibility was estimated from quality control samples analysed during routine determinations of the unknowns (about 10% of the analysed samples were quality controls). The results of these determinations, reported in Table II, were acceptable, except the inter-assay accuracy at 1.2 ng/ml which showed a "significant" loss of about 15%.

Limit of detection

The limit of detection, defined by a signal-to-noise ratio of 4—5, was about 0.1 ng of compound I per ml of plasma (Fig. 3), corresponding to an absolute amount of 4—5 pg per injection. The intra-assay reproducibility near this detection limit was better than 5% (Table III).

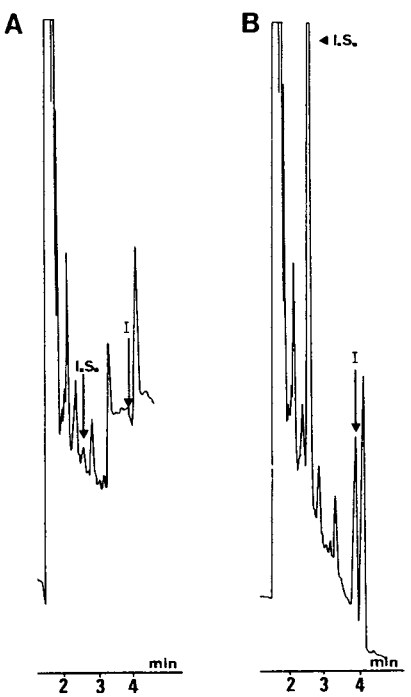


Fig. 3. Chromatograms illustrating the detection limit of I. (A) Control plasma; (B) control plasma spiked with 0.125 ng/ml I • HCl (I.S. 0.55 ng).

TABLE III

INTRA-ASSAY REPRODUCIBILITY NEAR THE DETECTION LIMIT

I · HCl plasma concentration (ng/ml)	*n*	Relative standard deviation* (%)
0.13	5	4.8
0.25	7	3.3

*95% Confidence.

Linearity

The linearity of the method was checked for concentrations of I in the range of 0.3 to about 9.4 ng per 0.5 ml of plasma. Some linearity test results are presented in Table IV. The difference, at each concentration, between the actual amounts used and the amounts found by the linear regression did not exceed 5—6%; the correlation coefficients were in the range 0.9986—0.9995, and the intercepts of the calibration curves did not differ significantly from zero.

TABLE IV

LINEARITY OF PLASMA DETERMINATION OF 3-METHYLCLONAZEPAM

I · HCl concentrations added (ng per 0.5 ml of plasma)	Equation of the non-weighted linear regression curve	Correlation coefficient	Found/added concentration ratio (mean value)
0.312, 0.625, 1.250, 1.875	$0.4368x - 0.012$	0.9986	0.994
0.625, 1.56, 3.12, 6.25	$0.3382x - 0.003$	0.9993	0.980
3.12, 6.25, 9.36	$0.1867x - 0.009$	0.9995	0.986

Recovery

The extraction of compound I from 0.5 or 1.0 ml plasma using 0.3 ml of butyl acetate was nearly quantitative up to the maximum investigated plasma concentration of about 10 ng per 0.5 ml. But, due to the formation of an emulsion during plasma extraction, it was not possible to obtain more than

TABLE V

EXTRACTION RECOVERY OF 3-METHYLCLONAZEPAM

I · HCl concentration (ng per 0.5 ml of plasma)	*n*	Extraction recovery (%)	Relative standard deviation* (%)
11.3	6	56.8	9.3
5.65	6	64.0	8.2
2.82	6	54.7	4.5
1.41	6	60.0	6.4

*95% Confidence.

0.2 ml of the organic phase. Under these conditions, the recovery of I from plasma was about 60—70%, independent of the concentration or of the plasma volume (Table V).

Specificity

Possible metabolites in man [1] would be the 7-amino derivative (Ro 12-5520, III), the 3-hydroxymethylclonazepam (Ro 11-5564, IV) and the 7-amino-3-hydroxymethylclonazepam (Ro 12-8063, V). In relation to underivatized III, the two peaks (t_R = 2.2 and 3.75 min) observed before extraction by butyl acetate were identified by GC—MS as non-chlorinated compounds; therefore, this amino metabolite could not be chromatographed in the unchanged form. Moreover, no interference from the unchanged hydroxylated compounds, IV and V, was seen under the analytical conditions described.

Stability in human plasma

Compound I was added to blank plasma at different concentrations (0.6, 2.4 and 5.6 ng per 0.5 ml plasma), and stored at different temperatures for different periods of time (one day at 20° C, three months at —20° C). Then, a set of six to seven freshly prepared control samples was analysed together with the same number of stored samples of the same concentration. The results are presented in Table VI. For plasma concentrations above 2 ng per 0.5 ml, no significant difference was detectable between the control plasmas and the plasmas stored in glass tubes. But the plasma samples at concentrations below 1 ng per 0.5 ml, stored for either three months at —20° C in glass tubes, or one day at 20° C in glass or in polypropylene tubes, showed that a "significant" decrease occurred during the storage period. That did not seem to be the case for storage during three months at —20° C in polypropylene tubes, even for very low plasma levels.

TABLE VI

STABILITY OF 3-METHYLCLONAZEPAM IN HUMAN PLASMA

Sample	Storage tube	Experimental I · HCl concentration* (ng per 0.5 ml of plasma)	*n*	Difference from control (%)	Confidence limits** (%)
1. Freshly spiked		2.42 ± 0.07 (2.54)***	6		
Stored one day at 20° C	Glass	2.33 ± 0.10 (2.54)	6	—3.9	—6.7 to —1.0
2. Freshly spiked		0.61 ± 0.04 (0.61)	7		
Stored one day at 20° C	Glass	0.51 ± 0.04 (0.61)	7	—15.8	—21.1 to —10.1
3. Freshly spiked		0.55 ± 0.03 (0.56)	7		
Stored one day at 20° C	Polypropylene	0.48 ± 0.06 (0.56)	6	—14.0	—22.7 to —4.4
4. Freshly spiked		5.59 ± 0.18 (5.50)	7		
Stored three months at —20° C	Glass	5.36 ± 0.12 (5.50)	6	—4.0	—0.7 to —7.2
5. Freshly spiked		0.63 ± 0.06 (0.55)	6		
Stored three months at —20° C	Glass	0.48 ± 0.04 (0.55)	5	—24.4	—31.6 to —16.4
6. Freshly spiked		0.55 ± 0.02 (0.55)	7		
Stored three months at —20° C	Polypropylene	0.59 ± 0.05 (0.55)	6	+7.4	+0.5 to +14.7

*95% Confidence.
**90% Confidence.
***Theoretical value.

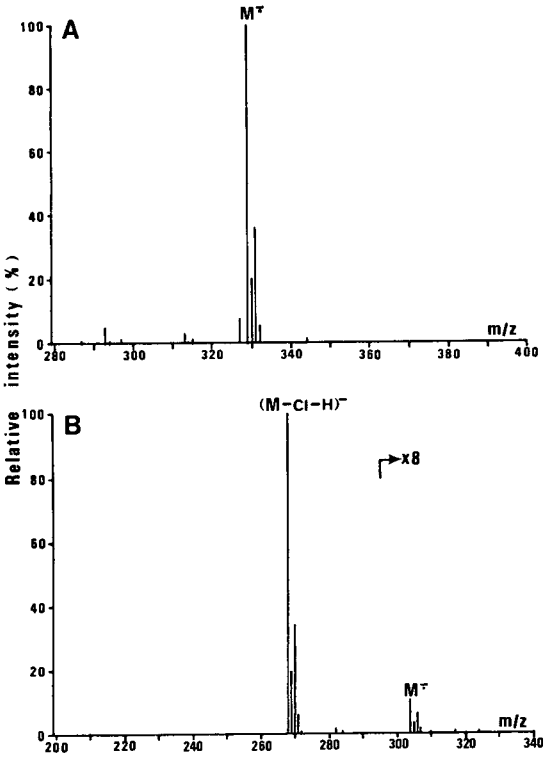


Fig. 4. Mass spectra of compound I (A) and compound II (B).

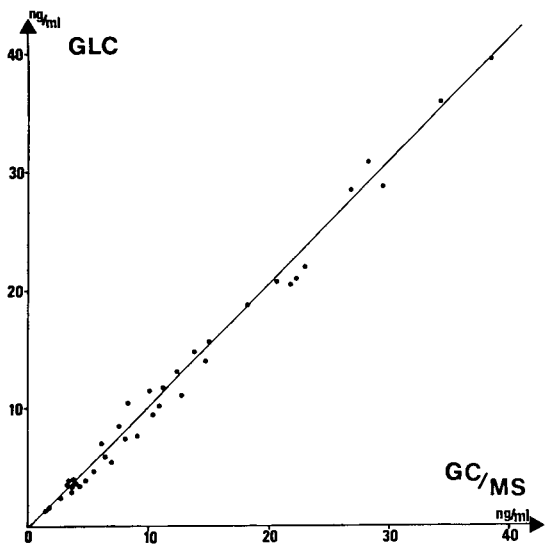


Fig. 5. Correlation between GLC and GC—MS for determination of I in plasma.

Gas chromatographic—mass spectrometric analysis

An assessment of the assay quality was realized by GLC coupled with mass spectrometry (GC—MS). The experiments were performed on a Hewlett-Packard apparatus (HP 5985 connected to a data system HP 1000) operating in the negative chemical ionization (NCI) mode. GLC analysis were carried out using a CP-Sil 5 glass (WSCOT) capillary column (8 m × 0.5 mm I.D.)

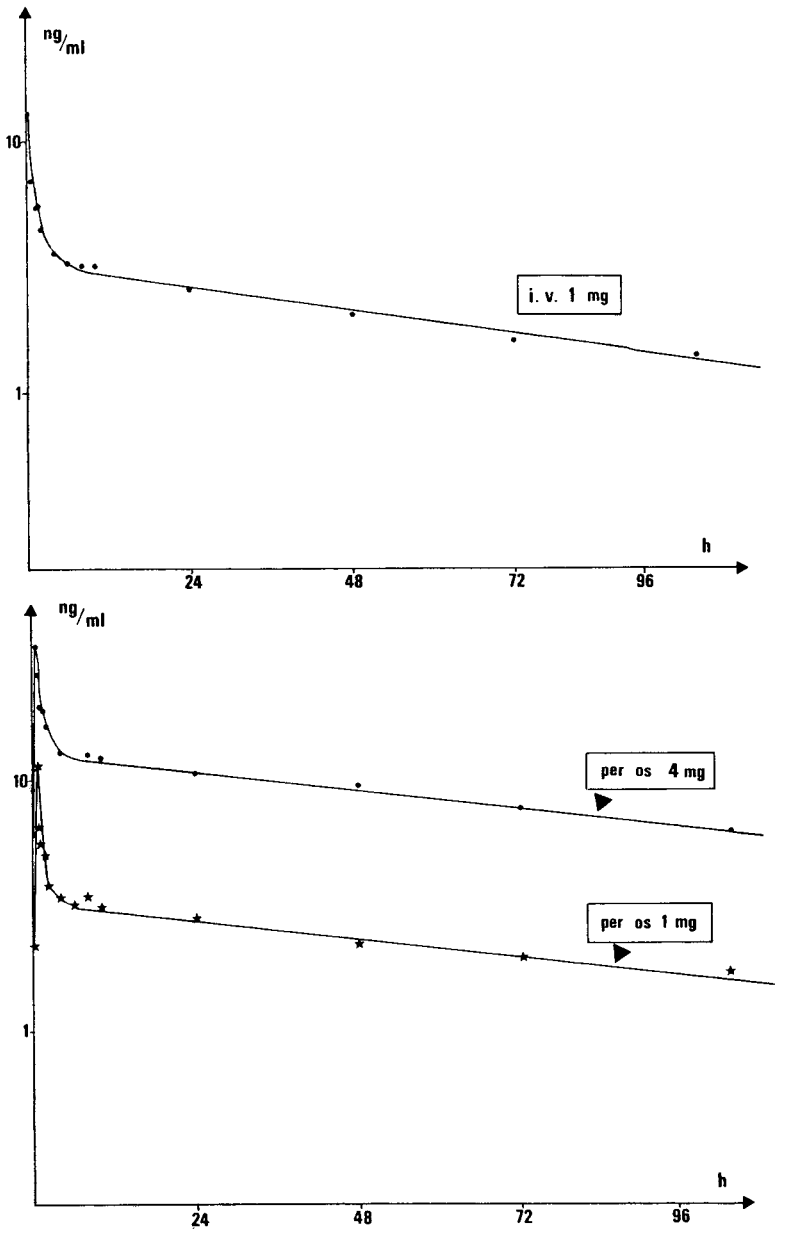


Fig. 6. Concentration—time course of I after intravenous (i.v.) administration of 1 mg and oral administration of 1 and 4 mg of this compound to a healthy volunteer.

programmed from 240°C to 280°C in steps of 15°C/min. The solid injector, ion source and GC—MS interface (transfer platinum line) temperatures were 280°C, 150°C and 285°C, respectively. Helium and methane were used as carrier gas (head column pressure of 0.8 bar) and reagent gas (ion source pressure of 1 Torr), respectively. The mass spectrometer was operated with an emission current of 300 μA and an electron energy of 230 eV.

Under these conditions, NCI mass spectra of compound I and its internal standard, presented in Fig. 4, were characterized principally by the molecular ions M^- for compound I and $(M - Cl - H)^-$ for compound II. These ions, the most intense, were used for plasma measurements by mass fragmentography in selective ion monitoring mode.

Plasma extracts of the same subject were simultaneously analysed by GLC—ECD and GC—MS: correlations between these two techniques, for about 40 plasma concentrations ranging from 1.5 to 40 ng per ml of plasma, are reported in Fig. 5 (regression curve $y = 1.029x - 0.050$, $r = 0.9929$). The mean value of the differences between each GLC and GC—MS result was 9.0 ± 1.8% (95% confidence). This validates the GLC—ECD methodology previously described.

APPLICATION

The described method was used for the analysis of plasma from healthy volunteers who had received compound I doses of 1 mg intravenously (i.v.) and of 1 and 4 mg per os in a cross-over design. Fig. 6 shows a typical plasma concentration—time course after each of these different administrations in a representative subject. Extensive treatment of these experimental data will be the subject of a further publication.

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DETERMINATION OF THE ANTIBIOTIC FLUDALANINE IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING A PACKED-BED, POST-COLUMN REACTOR WITH *o*-PHTHALALDEHYDE AND 2-MERCAPTOETHANOL

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SUMMARY

Fludalanine is a novel anti-bacterial agent active against gram-negative and gram-positive bacteria. A high-performance liquid chromatographic assay has been developed using ion-pair chromatography to resolve fludalanine and the internal standard 3,3-difluoro-D-alanine from plasma and urine background. The mobile phase contains sodium dodecyl sulfonate and methanol in a phosphate buffer. Fludalanine is derivatized post-column with *o*-phthalaldehyde via a packed-bed chemical reactor. The adduct is detected fluorometrically. The plasma and urine assays are sensitive to 0.25 and 0.5 μg/ml, respectively.

INTRODUCTION

Fludalanine, 2-deutero-3-fluoro-D-alanine (I), is a broad spectrum antibiotic [1]. The antibacterial action results from a sequential blockade of the biosynthesis and incorporation of D-alanine into the bacterial cell wall [2]. The metabolism of fludalanine in animals (Fig. 1) involves its initial oxidation to 3-fluoropyruvate (II); this metabolite is then reduced to 3-fluoro-L-lactate (III) [3]. In addition, inorganic fluoride is an end metabolite [4].

Fludalanine is administered with pentizidone, D-4-(4-oxo-2-pentene-2-amino)-3-isoxazolidinone (IV), a prodrug of cycloserine. The combination potentiates the individual antimicrobial activity of fludalanine and of cycloserine. The combination also eliminates the self-reversal phenomenon of fludalanine [1, 4].

A chemical assay for the quantification of fludalanine in plasma and urine has not been available. Generally, the detection of an amino acid by high-performance liquid chromatography (HPLC) involves either pre- or post-

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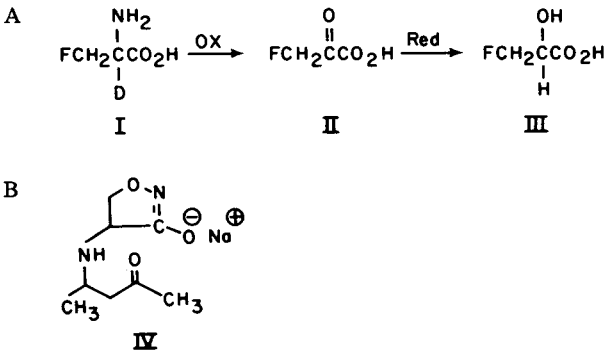

Fig. 1. (A) Metabolism of fludalanine in animals. (B) Structural formula of pentizidone (IV).

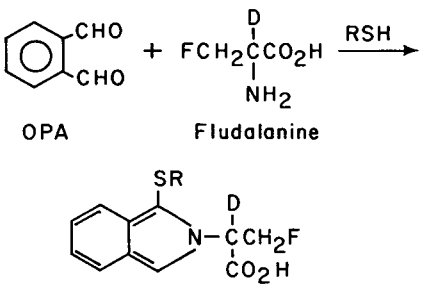

Fig. 2. *o*-Phthalaldehyde reaction with fludalanine and a mercaptan.

column derivatization [5, 6]. An HPLC assay is described for fludalanine that involves post-column derivatization* with *o*-phthalaldehyde (OPA) and 2-mercaptoethanol (Fig. 2). Optimal conditions for this post-column, packed-bed chemical reactor are described.

EXPERIMENTAL

Reagents

Sodium dodecyl sulfonate and OPA were obtained from Regis. Fludalanine, 3,3-difluoro-D-alanine (internal standard), cycloserine and sodium pantizidone hemihydrate were synthesized at Merck Sharp & Dohme Research Labs. [1, 7].

Chromatography

The liquid chromatographic system consists of one M6000A solvent delivery

*Attempts at pre-column derivatization of fludalanine in deproteinated plasma with fluorescamine, ninhydrin or *o*-phthalaldehyde (OPA) were not successful. It appeared that the derivatives were not resolved from biological background on reversed phase. Of the derivatizing reagents tested, OPA appeared to be the most reactive, yielding the greatest fluorescent response. Attempts were then made with different mercaptans and OPA (pre-column) to vary the S-alkyl group of the isoindole adduct of fludalanine in order to resolve it from background (Fig. 2). Selectivity, however, was not achieved and many of the mercaptans commercially available were not suitable (e.g. insolubility). Therefore, a method was developed involving the initial chromatography of fludalanine followed by post-column derivatization.

system for the mobile phase, a Model 710B autosampler (WISP), a Model 720 system controller and an M730 Data Module, all from Waters Assoc. The OPA reagent is delivered by a Perkin-Elmer Series 10 liquid chromatograph. The effluents from the analytical column and from the reagent pump mix in a low internal volume tee (1/16 in.) from Scientific Science and exit into a packed-bed reactor. The reactor consists of a stainless-steel column (25 cm × 4.6 mm I.D.) packed with 40-μm glass beads from Whatman. It is heated at 40°C (Temperature Control Unit, Model III, Elilex Labs.). The effluent from the reactor is monitored by a Perkin-Elmer 650-10S fluorescent detector (excitation wavelength 340 nm, emission 455 nm).

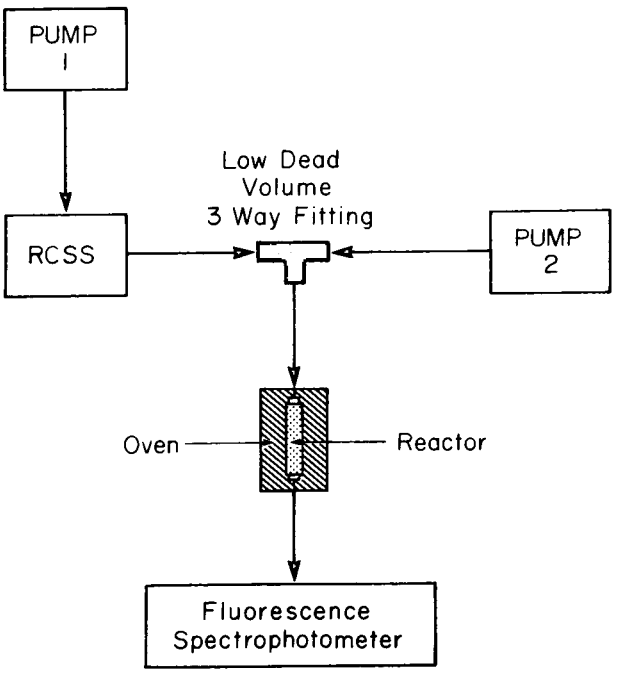

Fig. 3. HPLC set-up with a post-column reactor.

A diagram of the post-column system is shown in Fig. 3. The reagent is pumped through the high-sensitivity damper at a flow-rate of 1 ml/min to the tee. The mobile phase flow-rate is 2 ml/min through a Radial Compression Separation System (RCSS): an RCM-100 containing a Guard-Pak C_{18} and a Radial-Pak C_{18} cartridge (10 cm × 8 mm I.D., 5 μm particle size) from Waters. Radial-Pak C_{18} is LiChrosorb C_{18} (10 μm particle size), irregular packing.

The mobile phase contains 50 mg of sodium dodecyl sulfonate, 100 ml of methanol and 2 ml of 85% orthophosphoric acid. The solution is diluted to 1000 ml with water and titrated to pH 2.5 with 1.0 *M* potassium hydroxide. The solution is then filtered (0.45-μm Nylon-66 membrane filters).

The derivatizing reagent is a mix containing OPA and 2-mercaptoethanol in a borate buffer: 1 g of OPA and 0.5 ml of 2-mercaptoethanol are dissolved in 10 ml of 100% ethanol. A borate buffer is prepared with 3.0 g boric acid, 160 ml of 1 *M* potassium hydroxide diluted with water to 1 l. It is filtered (0.45-μm

Nylon-66 membrane filters). The buffer and the alcoholic solution are mixed and kept under a helium atmosphere in an amber reservoir.

The analytical column is conditioned sequentially with methanol (200—300 ml), methanol—water (1:9) and the mobile phase. The column is equilibrated when the retention time of fludalanine has stabilized (12—24 h).

Sample clean-up

Plasma. A 1-ml aliquot is mixed with 50 μl of difluoroalanine solution (0.90 mg/ml). The aliquot is transferred to a Centriflo ultrafilter (CF50A from Amicon) and centrifuged for about 15 min at 657 *g* (Dynac II centrifuge). The filtrate is analyzed.

Urine. A 0.3-ml urine aliquot is mixed with 25 μl of difluoroalanine solution (10 mg/ml). The aliquot is mixed with 2 ml of acetonitrile and loaded onto a dry silica Sep-Pak (Waters Assoc.). The mini-column is washed with 3 ml of acetonitrile, and fludalanine is eluted with 1.0 ml of water. Any silica in the eluate is centrifuged to the bottom of the receiving tube (about 10 min, 657 *g*) before analysis.

Standard calibration curves

Stock solutions of fludalanine and internal standard are prepared in water (kept cold during storage). Standards are mixed by diluting aliquots from an aqueous fludalanine stock solution (2 mg/ml) to appropriate working concentrations with water. Of each working stock solution 100 μl are mixed with 1.0 ml of plasma to achieve concentrations of 0.25, 0.5, 1.0, 2.5, 5.0, 10 and 20 μg/ml; for urine, 30 μl of each working stock solution are mixed with 300 μl of urine for concentrations of 0.5, 1.0, 2.5, 5.0, 10, 25, 50, 100 and 200 μg/ml.

RESULTS AND DISCUSSION

Post-column reaction

The post-column HPLC system for fludalanine detection (Fig. 3) involves mixing the eluate from the analytical column and the OPA reagent in a tee and then passing the mixture through a packed-bed column reactor to form a fluorescent isoindole (Fig. 2). The bed reactor consists of an empty Whatman column (25 cm × 4.6 mm I.D.) packed-dry with 40-μm glass beads. The residence time for fludalanine in the reactor is 0.5 min. The post-column reaction with fludalanine at room temperature appears to be incomplete. This was observed by stopping the flow of the reagent and the mobile phase during the elution of derivatized fludalanine from the reactor into a Schoeffel fluorometer (Fig. 4) and following the detector's response with time. The fluorometric response slowly increased from the level observed when the flow was stopped (fludalanine and OPA continued to react in the cell of the fluorometer) to a new level in 20.8 min. From this level, the response dropped only 5% over 60 min, revealing a stable fluorescent derivative.

Conditions of the post-column reactor were studied in order to optimize the reaction yield and peak resolution of fludalanine: comparisons were made of the packed-bed reactor's length, inner diameter, bead characteristic and temperature (Table I). Basically, peak width at one-half peak height remained

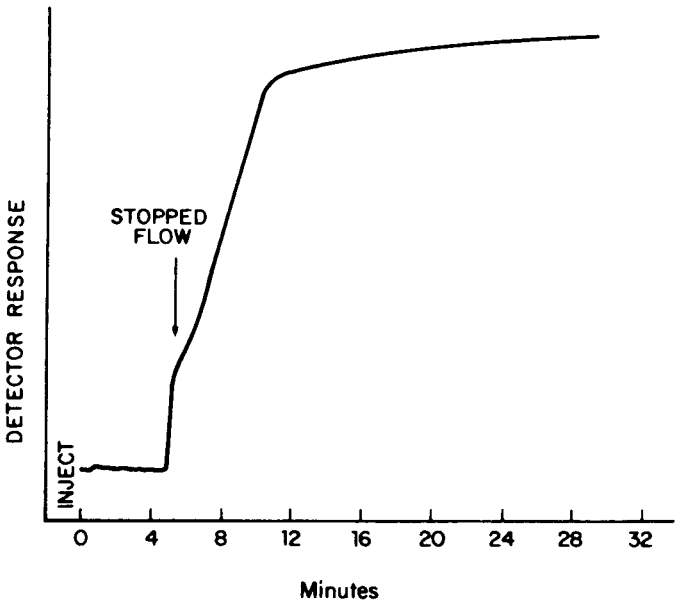

Fig. 4. A profile of the OPA-SH—fludalanine reaction with time.

TABLE I

CHARACTERISTICS OF PACKED-BED CHEMICAL REACTORS AT ROOM TEMPERATURE

Reactor	Length (cm)	I.D. (mm)	Residence time (min)	Peak height (cm)	Peak width (cm)	Peak area*
Glass beads						
1	25.0	4.6	0.50	9.2	0.6 (0.6864595)**	1.00
2	50.0	4.6	1.0	13.2	0.7 (8130531)	1.79
3	25.0	2.0	0.20	5.05	0.6 (6703337)	0.55
*Silanized glass beads****						
4	25.0	4.6	0.53	8.5	0.6 (6611166)	0.89

*The peak areas were normalized using the glass-bead reactor No. 1.
**Integrated area from a Waters data module divided by peak height in cm.
***The column was packed dry with the glass beads. Surfasil (Pierce) was forced through the column; it remained in the column at room temperature for ca. 1 h and was subsequently removed from the column with dichloromethane and ethanol. The above was repeated. Finally, the column was cleaned with water.

constant for all variations of the column make-up, except when the column was doubled in length. Also the detector response due to the post-column fludalanine reaction seems to be proportional to the residence time of the reaction in the column. A plot of the response against residence time for each of the different glass-bead columns is linear, $y = 0.243 + 1.55x$ ($r^2 = 0.9998$).

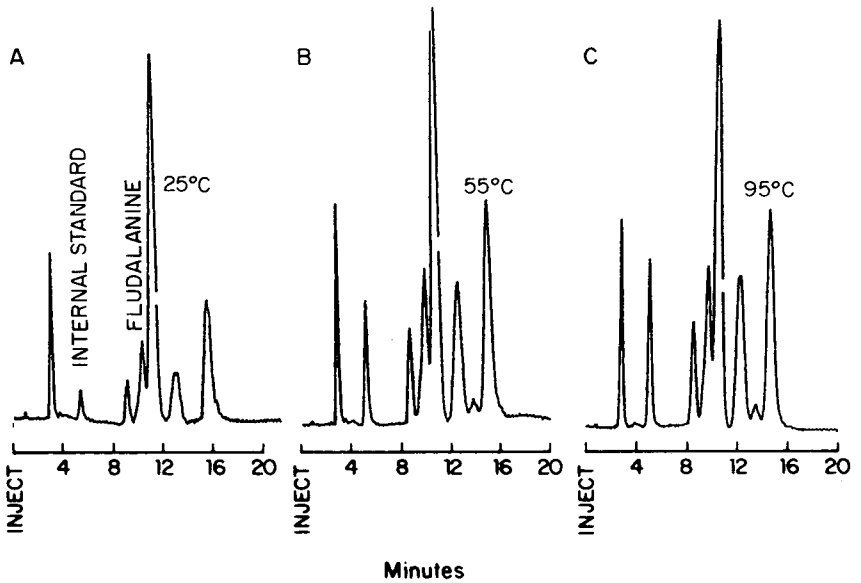

Fig. 5. Chromatograms of a plasma sample containing fludalanine (25 μg/ml) and internal standard. (A) The sample was passed through the packed-bed reactor at 25°C; (B) the same sample passed through the reactor at 55°C; (C) the same sample as the above at 95°C.

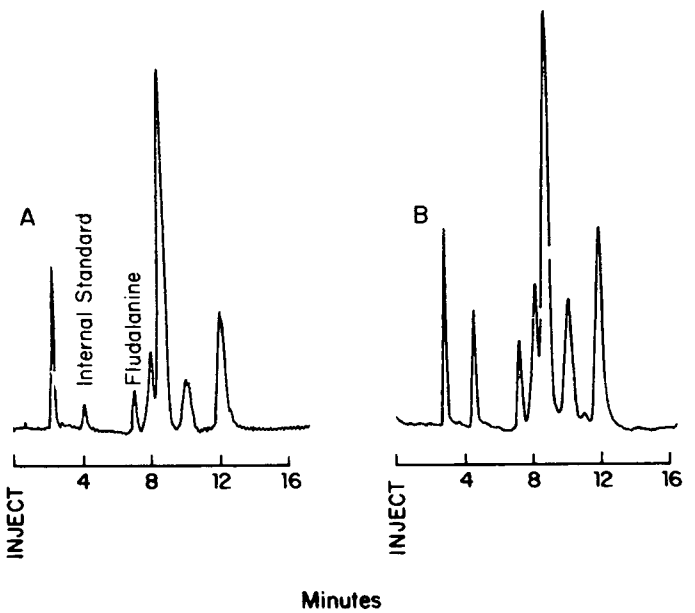

Fig. 6. Chromatograms of a plasma sample containing fludalanine (25 μg/ml and internal standard. (A) The sample was passed through a packed-bed reactor of 25 cm × 4.6 mm I.D.; (B) the same sample passed through a reactor of 50 cm × 4.6 mm I.D.

However, the response from the silanized glass-bead column compared to the regular glass-bead column was lower and gave an unexpectedly longer residence time. Raising the temperature of the reactor from 25°C to 95°C gave a

logarithmic plot of response versus temperature. At 95°C, the peak height was increased by 2.3 times. Peak width remained constant. Chromatograms of plasma standards in Figs. 5 and 6 support the above observations. Raising the temperature of the reactor from ambient improved the peak response of fludalanine but not its resolution from plasma background. The peak band width at one-half height remained the same. Doubling the length of the bed reactor improves the detector's response for fludalanine and internal standard but not resolution.

TABLE II

CHARACTERISTICS OF A PACKED-BED REACTOR VERSUS A TUBULAR COIL

Mobile phase and reagent flow-rates are 2 ml/min and 1 ml/min, respectively. Chart-speed 2 cm/min.

Reactor	Residence time (min)	Peak height (cm)	Peak width at ½ peak height (cm)	Peak area*
PTFE coil**	0.53	7.8	0.8 (8846373)***	1.09
Bed reactor§	0.50	9.2	0.6 (6864959)	1.00

*Peak areas are normalized. The areas are integrated by a Waters data module.
**Tubular column, 510 mm × 0.5 mm I.D., 62 turns with a diameter of 2.54 cm.
***Integrated area from a Waters data module divided by peak height in cm.
§Packed column, 250 mm × 4.6 mm I.D.

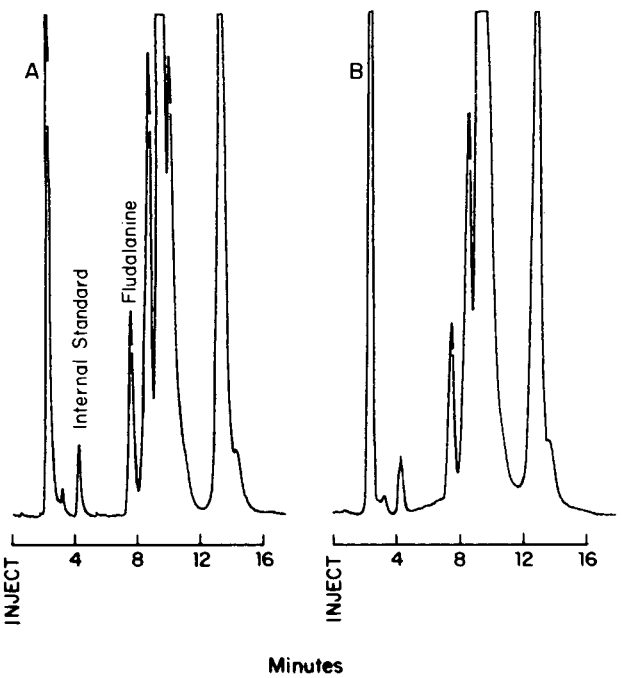

Fig. 7. Chromatograms of a plasma sample containing fludalanine (5.5 μg/ml) and internal standard. (A) The sample was passed through a packed-bed reactor (25 cm × 4.6 mm I.D.); (B) the same sample passed through a PTFE coil (510 cm × 0.5 mm I.D.).

A comparison was made of the packed-bed reactor with a PTFE, tubular coil (510 mm × 0.5 mm I.D., residence time of 0.5 min) with respect to reaction yield and peak broadening of fludalanine. Peak widths measured at one-half peak height (in cm) revealed broader peaks with the coil reactor (Table II and Fig. 7). The detector response of the coil was comparable to the packed-bed reactor. Precision of fludalanine at 2 μg/ml using the bead reactor was 6.3% (n = 5); using the coil, 6.9% (n = 5). Recently, Kratos introduced a reactor unit consisting of epoxy-supported PTFE tubing with a unique configuration (encased in a metal cylinder). The Kratos' unit (1.5 ml) produced sharper peaks but lower reaction yield when compared to the packed-bed reactor (Table III).

TABLE III

KRATOS' TUBULAR REACTOR VERSUS A PACKED-BED

Reactor	Peak height (cm)	Band width (cm)	Area*
Tubular**	11.7	0.30	$3.05 \cdot 10^8$
Packed-bed column	11.2	0.35	$4.21 \cdot 10^8$

*Generated from a Waters Data Module.
**Dead volume of 1.5 ml.

Chromatography

An ion-pairing agent in the mobile phase is needed to increase the retention time of fludalanine significantly beyond the void volume using a radially compressed, reversed-phase column*. The ion-pairing agent dodecyl sulfonate in methanol and water provides a chromatographic window for fludalanine and its internal standard difluoroalanine, clearly separated from the plasma or urine background (Fig. 8 and 9). Shorter-chain-length alkylsulfonates do not give the requisite separation.

The assay does not give a detector response for the co-administered prodrug of cycloserine, pentizidone. Cycloserine and the degradation products of cycloserine and fludalanine do not interfere. Plausable by-products of fludalanine via loss of deuterium fluoride are D-serine and pyruvic acid [8]. Fludalanine elutes before serine and most other amino acids (e.g. alanine, glycine, aspartic acid, etc.) Cycloserine and the dimer of cycloserine, which is a by-product [9], elute much later.

Assay parameters

The plasma assay for fludalanine is linear from 0.25 to 20 μg/ml with a least-squares analysis of $r^2 = 0.9998$, $y = 0.0213 + 0.0213x$. The urine assay is linear from 0.5 to 200 μg/ml with $r^2 = 0.9997$, $y = 0.0099 + 0.0138x$.

Intra-day reproducibility over the above plasma range has a mean value of 6.14 ± 2.0%; for urine standards, the mean is 5.18 ± 2.25% (Table IV). Inter-

*Stainless-steel columns of comparable or of better end-capped packing (e.g. Altex Ultrasphere-IP ODS) did not provide sufficient resolution for fludalanine from biological background.

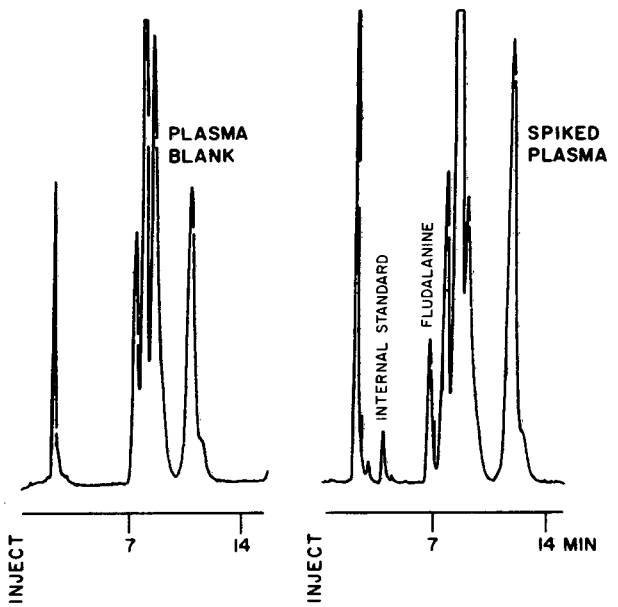

Fig. 8. Chromatograms of a plasma blank sample and of a plasma sample containing fludalanine (5.5 μg/ml) and internal standard.

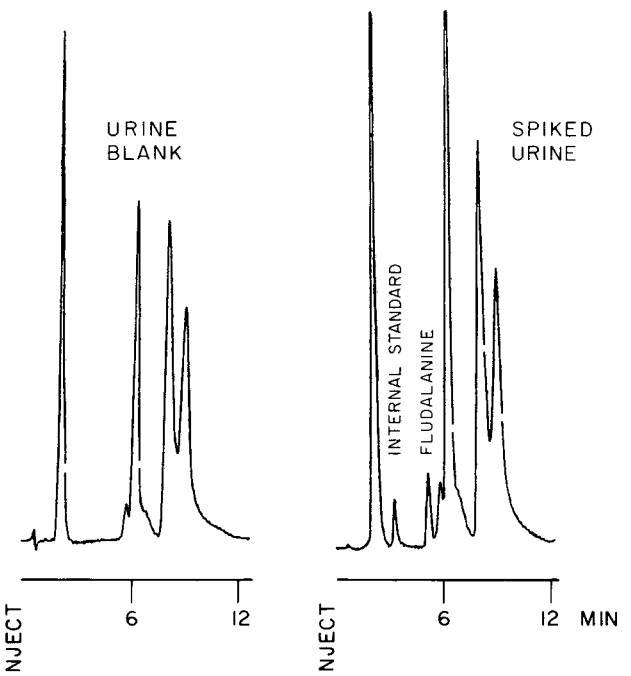

Fig. 9. Chromatograms of a urine blank and of a urine sample containing fludalanine (10.0 μg/ml) and internal standard.

TABLE IV

COEFFICIENTS OF VARIATION OF REPLICATE ANALYSES (n = 6) OF FLUDALANINE

Standard (μg/ml)	C.V. (%)
Plasma	
0.25	6.8
0.50	3.3
1.0	9.3
2.5	4.5
5.0	5.7
10	7.7
20	5.7
Urine	
0.50	7.4
1.0	7.1
2.5	5.6
5.0	5.3
10	4.4
25	1.9
50	2.0
100	4.6
200	8.3

day reproducibility for the plasma assay is 8.7% at 12.5 μg/ml; for the urine it is 8.3% at 100 μg/ml. Recovery of fludalanine from plasma by ultrafiltration prior to HPLC is 48.9 ± 3.0%; recovery from urine via adsorption chromatography prior to HPLC is 66.5 ± 4.3%. The values are based on peak heights of treated biological standards compared to aqueous standards.

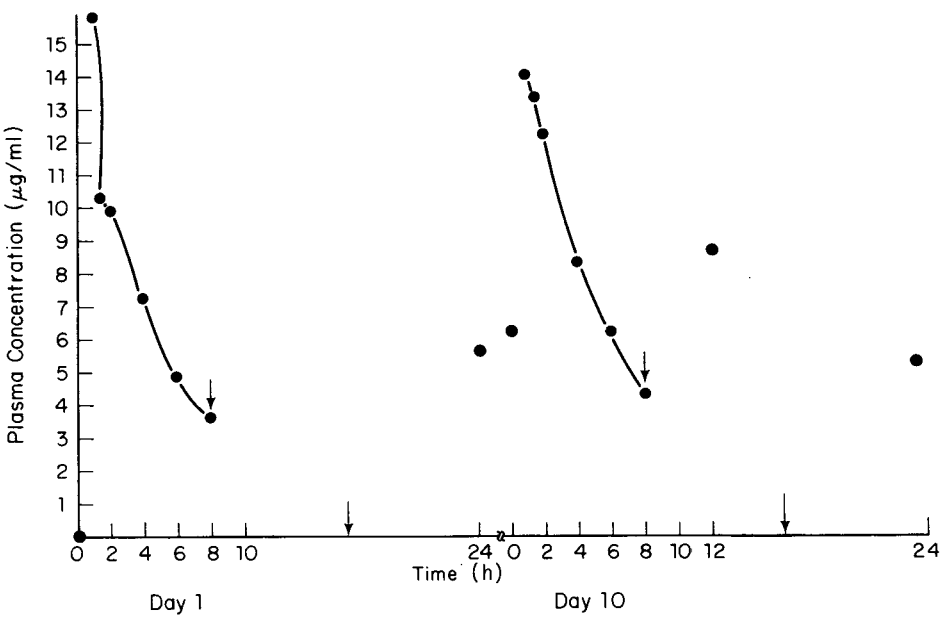

Fig. 10. Human plasma levels of fludalanine plotted against time from volunteers receiving 500 mg fludalanine orally every 8 h for ten days.

Application

The assay methodology for fludalanine has been applied to samples from a multiple-dose study. Male volunteers were orally administered 500 mg of fludalanine three times a day for ten days. Blood samples were collected on day 1 and on day 10 up to 24 h. Plasma concentrations for one volunteer ranged from just under 16.0 μg/ml at 1 h post dose to 3.6 μg/ml at 8 h on day 1 (Fig. 10). The apparent half-life was about 3.4 h.

The urine concentrations from the same volunteer during the first 8 h of collection (time intervals: −1−0, 0−1, 1−2, 2−4, 4−6, 6−8) ranged from 34.1 to 173.0 μg/ml. Dose recovery was 34.1%. During the remaining treatment period (time intervals: days 2−9 with 0−24 h collections; day 10 with −1−0, 0−1, 1−2, 2−4,, etc. collections) concentrations ranged from 13.2 to 375 μg/ml. Dose recovery at steady state was 53.1%.

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CHROMBIO. 2432

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF DIMETHYLXANTHINE METABOLITES OF CAFFEINE IN HUMAN PLASMA

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SUMMARY

A normal-phase high-performance liquid chromatographic assay of caffeine and its metabolites, theophylline, theobromine and paraxanthine, in human plasma is described. The two internal standards ethyltheophylline and 1,3,7-trimethyluric acid are used simultaneously and cover the range of different polarities from caffeine to the three dimethylxanthines. Plasma (0.5 ml) in the presence of ammonium sulphate is extracted with chloroform—isopropanol (1:1, v/v). The extract is chromatographed with a LiChrosorb Si 60 5-μm column and a mobile phase of dichloromethane containing 2.5% of a formate buffer in methanol. Calibration is performed with six different calibration mixtures which take into account the large plasma concentration differences between caffeine and its metabolites in man. The method is suitable for the simultaneous determination of caffeine and its dimethylxanthine metabolites in plasma of healthy and diseased persons.

INTRODUCTION

Caffeine (1,3,7-trimethylxanthine), an exogenous substance extensively consumed in beverages and foodstuffs, undergoes practically complete absorption [1], is metabolized almost exclusively in the liver [2] and single doses (equivalent to two to three cups of coffee) may be considered innocuous. Its pharmacokinetics in healthy man are now well elucidated [1,3]. The recent surge of interest in caffeine is based on its potential therapeutic and diagnostic application. Thus, caffeine has been shown to be effective in the treatment of apnoea in the newborn [4,5]; further, its bronchodilating activity has been confirmed in adult asthmatics [6]. On the other hand, caffeine has been investigated as a probe to assess liver function [7,8].

Since caffeine is metabolized to the pharmacologically active dimethyl-

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xanthines (DMX) theobromine (3,7-DMX), theophylline (1,3-DMX) and paraxanthine (1,7-DMX), a more comprehensive knowledge of its effects in man requires the simultaneous measurement of these main metabolites. Therefore, a high-performance liquid chromatographic (HPLC) method was developed for the determination of caffeine and dimethylxanthines in human plasma. Because of the difference of polarity between caffeine and dimethylxanthines, the method described in this paper uses two internal standards with different polarities.

MATERIALS AND METHODS

Apparatus

Analysis was performed with a high-performance liquid chromatograph (Waters Model M6000A) equipped with an automatic sample injection system (Waters Model 710B WISP), a variable-wavelength absorbance detector (Kratos Spectro-Flow 773) and a recorder—integrator (Hewlett-Packard 3390 A). A Gerhardt shaking apparatus (Model LS 20), a Hettich Mikro Rapid centrifuge and a Sorvall RC-5 refrigerated centrifuge with HS-4 swinging bucket were used.

Reagents

All reagents were of analytical grade. Dichloromethane (HPLC grade), methanol (HPLC grade) and 1,3,7-trimethyluric acid were purchased from Fluka (Buchs, Switzerland). Hibar® RT 250-4 columns filled with LiChrosorb Si 60, 5 μm particle size, were obtained from E. Merck (Darmstadt, F.R.G.). 7-Ethyltheophylline and [3-methyl-^{14}C]caffeine were a kind gift from Dr. M.J. Arnaud (Nestlé Research Department, La-Tour-de-Peilz, Switzerland).

Mobile phase

The mobile phase of Midha et al. [9] and Sved and Wilson [10] was used. Ammonium formate (0.2 g) and 15 μl of formic acid were added to 100 ml of methanol; 25 ml of this solution were mixed with 975 ml of dichloromethane. The composition of the mobile phase formate buffer—dichloromethane varied slightly according to the age of the HPLC column between 35:965 (new column) and 18:982 (old column). The mobile phase was degassed with a gentle stream of helium during analysis.

Chromatographic conditions

The flow-rate decreased with the age of the column (from 2 to 1.2 ml/min). The back-pressure was approximately 7 MPa. UV detection was performed at 280 nm (0.06 a.u.f.s.). The injected volume varied between 25 and 50 μl and was kept constant throughout one series of samples. Peak heights were measured.

Extraction procedure

Ammonium sulphate (0.2 g) was weighed into a screw-capped glass centrifuge tube (20 ml) with a PTFE liner. Plasma (0.5 ml) and 0.10 ml of an aqueous solution of the internal standards containing 5 nmol of 7-ethyltheo-

phylline and 5 nmol of 1,3,7-trimethyluric acid were added, followed by 10 ml of a mixture of chloroform—isopropanol (1:1, v/v). The mixture was shaken for 30 min and thereafter centrifuged at 0°C and 3000 *g* (4000 rpm) for 10 min. The organic layer was decanted into a glass centrifuge tube and evaporated to dryness under a stream of nitrogen at 70°C. The residue was dissolved in 0.5 ml of mobile phase, shaken for 30 sec, transferred to a conical plastic tube (1-ml eppendorf) and centrifuged at 4°C (5 min, 11 000 *g*) in order to remove solid particles which could damage the HPLC system. The supernatant was transferred to a glass tube of the autosampler, and an aliquot was injected.

The recovery of caffeine was assessed by extraction of 0.5 ml of bovine plasma spiked with 150 000 dpm of [3-methyl-^{14}C] caffeine (in 50 μl of water) and 100 μl of calibration mixture 3 (Table I). The radioactivity in the sample was determined before and after the extraction procedure. The chloroform—isopropanol mixture in the counter vials was evaporated with a stream of nitrogen before counting in order to avoid quenching. Scintillation cocktail (10 ml) (Lumagel®, Lumac, The Netherlands) was added. A Packard Tri-Carb® 2660 liquid scintillation system (Packard Instrument International, Zurich, Switzerland) with the external channels ratio was used.

Calibration

Different stock solutions were prepared by dissolving each methylxanthine in 100 ml of water, as indicated in Table I. Aliquots of these stock solutions were mixed together (upper figures in Table I) yielding six calibration mixtures with four methylxanthines and two internal standards. For the calibration procedures either xanthine-free human or bovine plasma was used, which yielded no difference in the slopes or intercepts of the resulting calibration curves. Portions of 0.5 ml of this plasma were spiked with 0.1 ml of the calibration mixtures and analysed as described above. The ratio peak height of caffeine to peak height of 7-ethyltheophylline (internal standard 1) was plotted against the amount of caffeine in the spiked plasma samples (lower figures in Table I). Accordingly, the ratio of peak height of dimethylxanthine to peak height of 1,3,7-trimethyluric acid (internal standard 2) was plotted against the amount of dimethylxanthine. Peak height ratios were measured in the patient samples, and concentrations of methylxanthines were calculated from the calibration curves by linear regression.

RESULTS AND DISCUSSION

Fig. 1 shows a blank chromatogram and a chromatogram of methylxanthine standards from a plasma sample which was spiked with a calibration mixture and extracted. Baseline separation was achieved within 12 min. 7-Ethyltheophylline (internal standard 1) and caffeine elute after each other, which indicates similar polarities. The most polar xanthine in the sample is 1,3,7-trimethyluric acid (internal standard 2) which elutes after the three dimethylxanthines. The plasma used for calibration curves was free of interferences at the retention time of the methylxanthines (Fig. 1a). Retention times remained stable within one day. Calibration was performed every day. The calibration curves plotting peak height ratios were linear within the range given in Table I,

TABLE I

PREPARATION OF CALIBRATION MIXTURES

Compound	Molecular weight	Stock solution (mg per 100 ml)	Volume of stock solution in calibrator mixture (ml) Amount of methylxanthine per 100 µl of calibration mixture (nmol)						Internal standards for patient samples
			Calibrator 1	Calibrator 2	Calibrator 3	Calibrator 4	Calibrator 5	Calibrator 6	
Caffeine	194.2	77.6	0.625	1.25	2.5	5.0	10.0	20.0	
			2.5	5.0	10.0	20.0	40.0	80.0	
Theobromine	180.2	18.0	0.25	0.5	2.5	5.0	10.0	20.0	
			0.25	0.5	2.5	5.0	10.0	20.0	
Theophylline hydrate	198.2	19.8	0.25	0.5	2.5	5.0	10.0	20.0	
			0.25	0.5	2.5	5.0	10.0	20.0	
Paraxanthine	180.2	36.0	0.5	1.25	2.5	5.0	10.0	20.0	
			1.0	2.5	5.0	10.0	20.0	40.0	
7-Ethyltheophylline (internal standard 1)	208.2	10.4	10.0	10.0	10.0	10.0	10.0	10.0	10.0
			5.0	5.0	5.0	5.0	5.0	5.0	5.0
1,3,7-Trimethyluric acid (internal standard 2)	210.2	10.5	10.0	10.0	10.0	10.0	10.0	10.0	10.0
			5.0	5.0	5.0	5.0	5.0	5.0	5.0
Water (ml)			78.38	76.5	70.0	60.0	40.0	—	80.0
Total volume (ml)			100	100	100	100	100	100	100

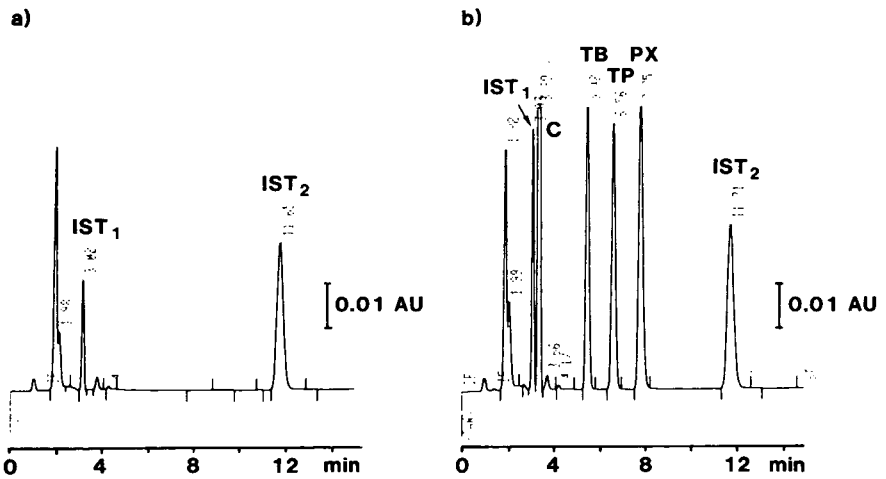

Fig. 1. (a) Chromatogram of a blank bovine plasma sample spiked with 7-ethyltheophylline (IST_1) and 1,3,7-trimethyluric acid (IST_2). (b) Chromatogram of calibration mixture 4 extracted from 0.5 ml of plasma spiked with 7-ethyltheophylline (IST_1), caffeine (C), theobromine (TB), theophylline (TP), paraxanthine (PX) and 1,3,7-trimethyluric acid (IST_2).

with linear correlation coefficients of >0.997. Inter-day reproducibility of the assay (Table II) measured with plasma of a healthy volunteer after coffee intake was below 4% coefficient of variation for caffeine, and below 8% for the metabolites, with the exception of theophylline. The higher coefficient of variation of the theophylline measurements presumably reflects the low theophylline concentration in this plasma sample.

There is a considerable difference of polarity between the trimethylxanthine caffeine and the dimethylxanthines theobromine, theophylline and paraxanthine. Therefore, the less polar internal standard ethyltheophylline was used for analysis of caffeine. Mean recovery of caffeine after the extraction procedure was 85% (n = 5), as determined with ^{14}C-labelled caffeine.

The more polar internal standard 1,3,7-trimethyluric acid was employed for analysis of all three dimethylxanthines (Fig. 2). This compound is a well known degradation product of caffeine, but no measurable concentration in human plasma has been reported, so far. Only less than 1% of an administered dose of caffeine can be found as 1,3,7-trimethyluric acid in urine [3,11]. In our laboratory no measurable peak could be detected in human samples (newborns, patients with liver disease, controls) at the retention time of this internal standard; therefore interference with the endogenous compound can be excluded.

Large variations in methylxanthine levels in humans had to be taken into account when the assay was developed. In liver disease the impaired metabolism of methylxanthines is reflected in increased plasma levels. Table III depicts the fasting plasma levels in ten patients with liver cirrhosis and ten healthy controls. Not only caffeine, but also its main metabolites may accumulate up to 40-fold compared to mean fasting concentrations in normal man. Total methylxanthines were increased on the average five-fold in the cirrhotic group. Representative chromatograms of each group are shown in Fig. 2.

TABLE II

REPRODUCIBILITY OF METHYLXANTHINE DETERMINATION

Compound	Number of determinations	Concentration* in plasma (μmol/l)	Coefficient of variation (%)
Intra-day			
Caffeine	15	27.3 ± 0.6	2.3
Theobromine	15	7.9 ± 0.3	4.3
Theophylline	15	1.4 ± 0.1	7.1
Paraxanthine	15	6.5 ± 0.2	3.5
Inter-day			
Caffeine	11	31.2 ± 1.1	3.7
Theobromine	11	3.7 ± 0.3	7.7
Theophylline	11	2.5 ± 0.3	11.2
Paraxanthine	11	14.0 ± 1.0	7.1

*Mean ± standard deviation. Different plasma samples of two healthy volunteers after coffee consumption.

TABLE III

FASTING PLASMA METHYLXANTHINE LEVELS IN PATIENTS WITH LIVER DISEASE AND CONTROLS

Values are expressed as μmol/l (mean ± S.D.), range in parentheses.

	Caffeine	Theobromine	Theophylline	Paraxanthine	Total methylxanthines
Cirrhosis (n = 10)	26.0 ± 23.1 (9.6—80.9)	13.7 ± 12.0 (2.0—38.2)	2.0 ± 1.6 (0.7—6.1)	9.1 ± 9.1 (0—30.6)	50.8 ± 24.4 (15.1—93.6)
Controls (n = 10)	2.0 ± 1.3 (0.1—4.1)	3.1 ± 2.0 (1.0—8.1)	0.9 ± 0.5 (0.2—1.8)	2.2 ± 1.8 (0—5.9)	9.3 ± 2.1 (2.1—14.4)

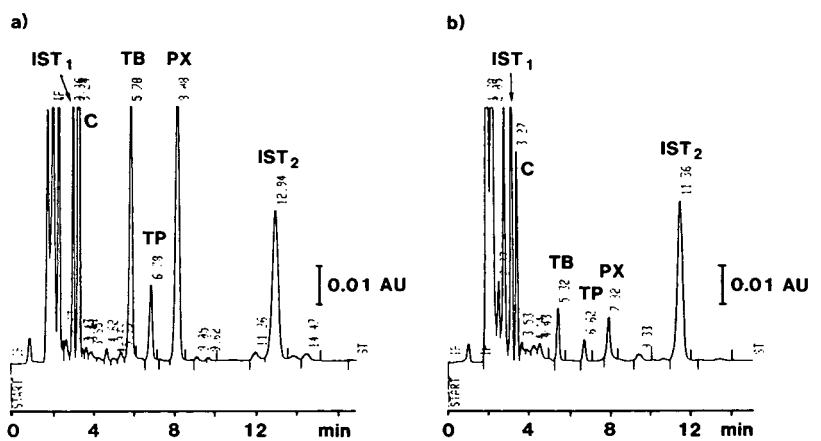

Fig. 2. Chromatogram of methylxanthines extracted from 0.5 ml of plasma of a patient with liver cirrhosis (a) and a healthy control (b). Peak symbols are the same as in Fig. 1.

These chromatograms demonstrate also the marked difference in concentration, especially between caffeine and theophylline.

Several HPLC methods for the analysis of methylxanthines in plasma have been published, most of them using reversed-phase HPLC columns [12—15]. However, baseline separation of paraxanthine and theophylline in the reversed-phase mode is difficult. Muir et al. [16] used an ion-pairing reversed-phase HPLC assay which allowed the separation of these two dimethylxanthines. The normal-phase solvent system presented here has the major advantage that the dimethylxanthines are well separated from the front peaks and also from each other, therefore allowing determination of caffeine and its main metabolites in one single isocratic run. This HPLC method facilitates the assessment of the metabolic pattern of methylxanthines and its clinical application in various fields, such as pulmonary diseases (treatment of asthma [6]), psychiatry (anxiety disorders [17]), paediatrics (treatment of apnoea in newborn [4,5]) and hepatology. Caffeine and dimethylxanthine metabolism in patients with different forms of liver disease is now under investigation using this HPLC method.

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CHROMBIO. 2441

ANALYSIS OF N-BENZOYL-L-TYROSYL-*p*-AMINOBENZOIC ACID (BENTIROMIDE) METABOLITES IN URINE BY ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method is described for the analysis of bentiromide metabolites in urine. The procedure involves no more than direct injection of the diluted urine sample, obviating the need for an extraction step or an internal standard. A μBondapak C_{18} column is used with a mobile phase of 0.01 *M* tetrabutylammonium chloride (pH 7.4)—methanol (9:1). A flow-rate of 1.4 ml/min, detection at 254 nm and column temperature of 40°C are employed. These conditions were achieved by investigating the effects of mobile phase pH, and concentrations and types of organic modifiers, buffers and ion-pairing agents on the resolution of the metabolites. The analysis time is 18 min per sample and the coefficient of variation on replicate assays is less than 10% for most concentrations studied. Analytical recoveries were between 95 and 100% throughout the appropriate concentration ranges and no interferences were obtained with the exception of *p*-acetamidobenzoyl glucuronide which could be eliminated by treatment of the samples with β-glucuronidase. Concentration profiles of the metabolites were studied in normal subjects, and the method was found to be potentially useful for clinical situations in which the existing bentiromide test leads to ambiguous results because of small bowel and hepatic dysfunctions.

INTRODUCTION

Urine analyses of *p*-aminobenzoic acid (PABA) derived from N-benzoyl-L-

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tyrosyl-*p*-aminobenzoic acid (bentiromide, Fig. 1) have been proposed as a screening test for human pancreatic function [1—3]. The cleavage of PABA from bentiromide takes place through the action of the pancreatic endopeptidase (E.C. 3.4.21.1, chymotrypsin) in the small bowel following an oral dose of the drug. The PABA marker is then absorbed from the small bowel, metabolized by the liver and excreted in the urine. The urinary concentrations of PABA and its metabolites are therefore reflective of chymotrypsin activity. Exocrine pancreatic insufficiency is indicated by low recoveries of these substances.

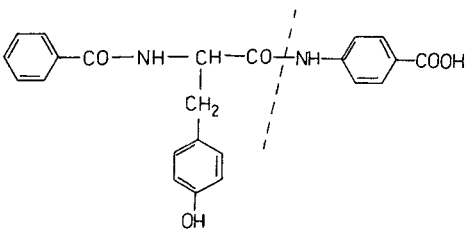

Fig. 1. Structure of bentiromide showing the point of cleavage by chymotrypsin (- - - - -).

p-aminobenzoic acid (PABA) p-aminohippuric acid (PAHA)

p-acetamidobenzoic acid (PAABA) p-acetamidohippuric acid (PAAHA)

Fig. 2. Structures of *p*-aminobenzoic acid and metabolites.

Quantification of total urinary PABA may be accomplished by hydrolysis of PABA metabolites to the parent compound followed by treatment with chromogenic reagents and spectrophotometric measurement [4, 5]. More recently a liquid chromatography method has been described for the determination of PABA using electrochemical detection [6]. Abnormalities in small bowel and hepatic function however, may give rise to ambiguous results in vivo [7], since current methods detect only total PABA and it is not possible to differentiate low recovery caused by pancreatic insufficiency from that caused by small bowel or liver diseases. If individual PABA metabolites are quantified, concentration patterns or fingerprints may be established that

would indicate the source of low PABA recovery and thus differentiate false positives from true pancreatic dysfunction.

p-Acetamidobenzoic acid (PAABA), *p*-aminohippuric acid (PAHA) and *p*-acetamidohippuric acid (PAAHA) (Fig. 2) have been identified as PABA metabolites in animals [8, 9] and are likely candidates as human metabolites. We have developed an ion-pair high-performance liquid chromatographic (HPLC) method for urinary analysis of these metabolites as well as the parent compound and applied it to a study of metabolite concentration profiles in normal subjects.

EXPERIMENTAL

Chemicals and reagents

PABA, PAABA and PAHA were obtained from Sigma, PAAHA was synthesized by the reduction of acetic anhydride by PAHA as described by Vogel [10]. The tetrabutylammonium (TBA) chloride was 95% pure from Fisher Scientific as were the tetramethylammonium chloride, tetraethylammonium chloride and hexadecyltrimethylammonium bromide. HPLC-grade organic modifiers (methanol and acetonitrile) were also supplied by Fisher. All other chemicals (KH_2PO_4, phosphoric acid and sodium hydroxide) were analytical grade from various sources. The water was distilled and deionized by a Watts Model M (Lawrence, MA, U.S.A.) water purifier.

Human studies

Bentiromide (500 mg) (Chymex, Adria Labs. Columbus OH, U.S.A.) was administered in 250 ml of water to five healthy adult volunteers (four males, one female, ages 21—31 years) who were fasted overnight. Prior to dosing, the subjects were instructed to empty their bladders and consume 500 ml of water. An additional 250-ml volume of water was given at 2 h and 4 h after administration of the drug and the subjects fasted throughout the test. Urine samples were collected at 30 min and 1, 2, 3, 4, 5 and 6 h after receiving the dose. Each sample was mixed by inversion, the volume was measured, and aliquots stored at 4°C for no more than three days prior to analysis.

Sample preparation

Individual urine samples were centrifuged and then diluted with deionized water so that the metabolite concentrations were within the range of the standard curve. The extent of dilution was based on the results of preliminary experiments. Samples smaller than 500 ml were diluted 100-fold whereas volumes voided which were between 50 and 100 ml, 100 and 150 ml and above 150 ml were diluted 50-, 20- and 10-fold respectively. The diluted samples were injected without further preparation.

Chromatography

A Waters Model M-6000A liquid chromatograph (Milford, MA, U.S.A.) equipped with a Rheodyne 7105 loop injector and fixed wavelength detector (254 nm) was used for the analyses. Chromatography was performed on a Waters μBondapak (30 × 0.4 cm, 10 μm) column. An Omniscribe Model 3532

strip chart recorder (Houston Instruments, Austin, TX, U.S.A.) was used. The column temperature (40°C) was controlled with an Alltech water jacket (Deerfield, IL, U.S.A.) connected to a Temptrol 153 (Precision Scientific, Chicago, IL, U.S.A.) circulating-water bath. The ultraviolet spectra of PABA and its metabolites were obtained using a Beckman Model 35 spectrophotometer (Beckman Instruments, Irvine, CA, U.S.A.). The chromatographic conditions used for analysis of bentiromide metabolites in urine are summarized in Table I.

TABLE I

CHROMATOGRAPHIC CONDITIONS

Parameters	Conditions
Column	μBondapak C_{18}
Mobile phase	Methanol—0.01 *M* TBA (10:90)
Wavelength	254 nm
Flow-rate	1.4 ml/min
Temperature	40°C
Chromatography time	18 min
a.u.f.s.	0.01
Sample volume	20 μl

RESULTS AND DISCUSSION

Separation

Organic modifier. The influence of methanol and acetonitrile concentration on the retention of the compounds of interest by the column was studied. The aqueous component of the mobile phase was buffered at pH 4 with a 0.01 *M* phosphate buffer and no pairing ions were added at this stage. In all cases, decreasing the concentration of organic modifiers increased the retention of the solutes by the column (Figs. 3 and 4). However, acetonitrile had a more selective effect on retention than had methanol. For example, lowering the acetonitrile concentration from 15 to 5% increased the capacity ratios (k') of PAABA and PAAHA from 1.0—2.4 and 0.4—1.3, respectively. The corresponding increase in the k' values of PABA and PAHA were only 1.0—1.3 and 0.4—0.6 respectively. Consequently, whereas 5% acetonitrile provided satisfactory overall retention characteristics, the resolution of PABA and PAAHA was poor (Fig. 3). In comparison, methanol demonstrated a less selective effect on the k' values of PAABA and PAAHA which increased from 2.6—4.1 and 0.9—2.3, respectively, when the methanol concentration was decreased from 20 to 5% (Fig. 4). The corresponding increases in the k' values of PABA and PAHA were 1.0—1.4 for PABA and 0.4—0.5 for PAHA. With concentrations of either organic modifier below 5%, the solutes eluted too slowly from the column to be analytically useful.

The results indicate that methanol is a stronger solvent, having less effect on analyte retention, than acetonitrile for PAABA and PAAHA but not for their non-acetylated counterparts. A possible explanation for this is that the amides (PAABA and PAAHA) are better proton acceptors than the amines

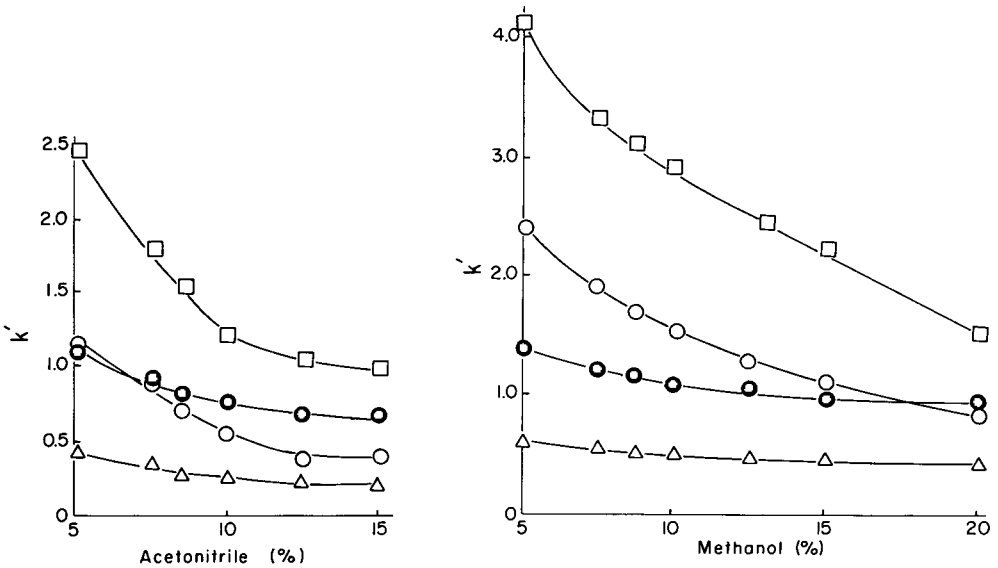

Fig. 3. The effect of mobile phase acetonitrile concentration on capacity factors for *p*-aminobenzoic acid and metabolites. The chromatographic conditions were the same as those listed in Table I except the flow-rate was 1.2 ml/min and the column temperature was 25° C. • = PABA; △ = PAHA; ○ = PAAHA; □ = PAABA.

Fig. 4. The effect of mobile phase methanol concentration on capacity factors for *p*-aminobenzoic acid and metabolites. The symbols and conditions are the same as for Fig. 3.

(PABA and PAHA) [11] and could be expected to interact more strongly with a proton donating solvent. Methanol can serve as both a proton donor and acceptor [12] making it a stronger solvent than acetonitrile for hydrogen-bonding interactions [13].

With a mobile phase containing 10% methanol the resolution [14] of all of the peaks of interest was greater than 1.00. Although a mobile phase containing 10% methanol in 0.01 *M* phosphate buffer (pH 4.0) adequately resolved the peaks of interest, interference from endogenous components of urine was observed. Resolution was optimized by investigating the effect of pH and the addition of cationic ion-pairing agents to the mobile phase.

pH Optimization. The dependence of retention volume on pH was studied with a mobile phase containing 10% methanol. The pH of the aqueous component of the mobile phase (0.01 *M* KH_2PO_4) was adjusted by addition of phosphoric acid or sodium hydroxide. Fig. 5 shows that the retention of all the compounds decreased with increasing pH above 3.5 because of deprotonation of the carboxylic groups of the solutes [15]. Changes in elution order with changing pH may be attributed to differences in the pK_a values of the carboxyl groups. For example, the acidic pK_a values for PABA and PAHA are 4.9 and 3.6, respectively [16]. No literature values for the pK_a values of PAABA and PAAHA are available.

Good separation of the metabolites was achieved at pH 4.0. However, under these conditions components of human urine persisted, indicating that an alternative strategy was necessary, to selectively enhance the retention of the

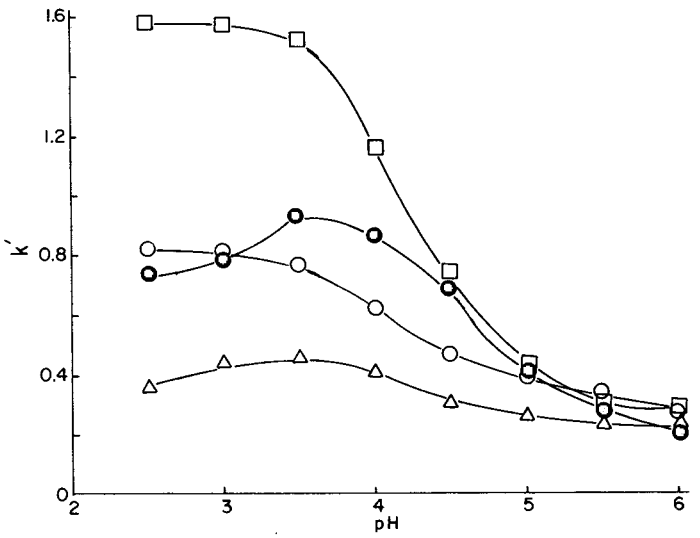

Fig. 5. The effect of pH on capacity factors for *p*-aminobenzoic acid and metabolites. Conditions and symbols as in Fig. 3 except the mobile phase contained 10% methanol and 0.01 *M* KH_2PO_4.

compounds of interest without affecting the endogenous components of urine.

Ion-pair chromatography. The four solutes of interest possess ionizable carboxylate groups suggesting that their retention may be selectively enhanced by the addition of an oppositely charged, cationic, pairing-ion to the mobile phase [17]. The retention of all the solutes was found to increase in a sigmoidal manner [17] with increasing concentration of TBA chloride (0.001—0.01 *M*) in a mobile phase consisting of methanol—0.01 *M* phosphate buffer

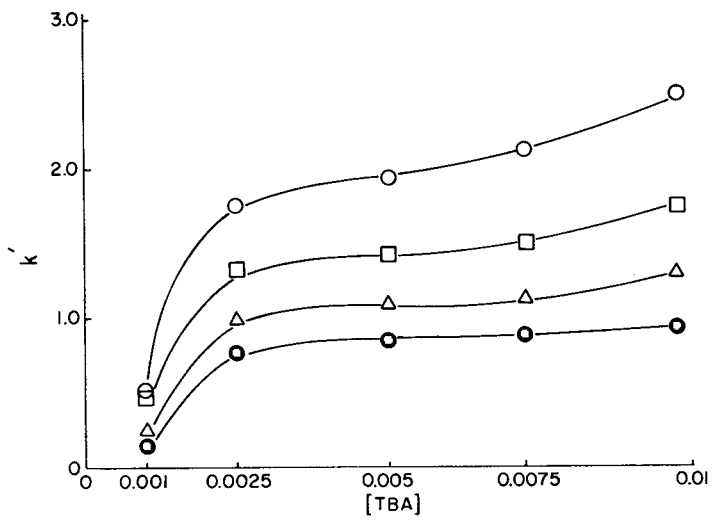

Fig. 6. The effect of tetrabutylammonium chloride concentration on the capacity factors of *p*-aminobenzoic acid and metabolites. The symbols and conditions are the same as those for Fig. 5 and the pH of the mobile phase was adjusted to 6.0.

(pH 6.0) (10:90) (Fig. 6). No changes in elution order were observed with changes in the TBA concentrations. This is consistent with previous observations [17] that selectivity is independent of pairing-ion concentration, provided that the solutes are fully ionized. The influence of ion-pairing on the retention of the solutes was investigated further by studying the effect of pairing-ion hydrophobicity (carbon number) at a constant concentration (0.01 *M*) in a mobile phase consisting of methanol—0.01 *M* phosphate buffer (pH 6.0) (10:90) (Fig. 7). Three symmetrical cationic pairing ions (TMA, TEA, TBA) of general formula $(C_nH_{2n+1})_4N^+$ ranging in total carbon number from 4 to 16 (n = 1—4) were studied and compared with a long chained cationic surfactant, hexadecyltrimethylammonium bromide (HTAB). In the case of the symmetrical quaternary ammonium ions there was a linear relationship between the logarithm of the capacity ratio of the solutes and the total number of carbon atoms in the pairing ions. In contrast with the effect of changing pairing-ion concentration (Fig. 6), which produced no changes in selectivity, the selectivity of the system and hence, the elution order of the solutes was markedly influenced by the hydrophobicity of the pairing ion. The two hippuric acid analogues (PAHA, PAAHA) exhibited a stronger dependence on pairing-ion size than their benzoic acid counterparts (Fig. 7). This effect may be attributed to the greater distances between the ionized carboxylate groups and the phenyl ring in PAHA and PAAHA than in PABA and PAABA. The influence of steric effects on the interaction between the solutes and the pairing ions is further demonstrated by the effect of HTAB on the retention of the solutes which is greater than would have been predicted by the relationships between log k' the number of carbons in the symmetrical pairing ions (TMA, TEA and TBA).

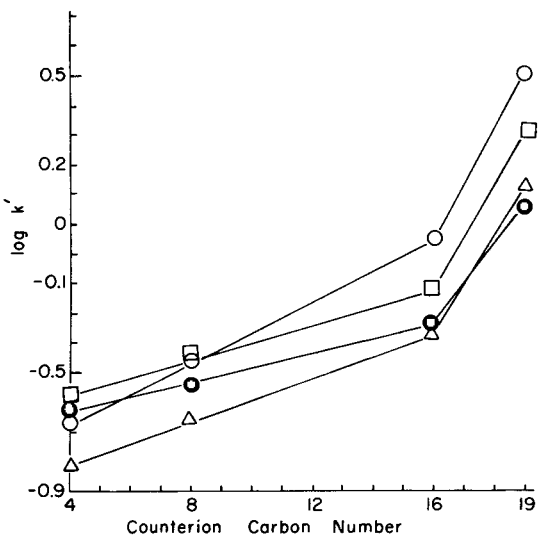

Fig. 7. Counter-ion size effect on capacity factors for *p*-aminobenzoic acid and metabolites. Tetramethylammonium (TMA) chloride = 4 carbons; tetraethylammonium (TEA) chloride = 8 carbons; tetrabutylammonium chloride = 16 carbons and hexadecyltrimethylammonium bromide = 19 carbons. The symbols and conditions are the same as for Fig. 6.

In the presence of TBA, retention of the solutes was much less influenced by changing the pH of the mobile phase (pH 4—5.8) (Fig. 8). Over the pH range studied, the degrees of ionization of the solutes could be expected to change with decreasing pH of the mobile phase and a change in retention mechanism would take place. At higher pH values the solutes would be retained as a result of interaction with the ion-pairing agent added to the mobile phase (0.01 *M* TBA), and at lower pH values the solutes would be retained by the column as neutral or positively charged species. Fig. 8 indicates that for three of the solutes (PABA, PAHA, PAAHA) the two contributions to retention appear to be about equal. In contrast the neutral form of PAABA is retained to a greater extent than its corresponding ion-pair with TBA.

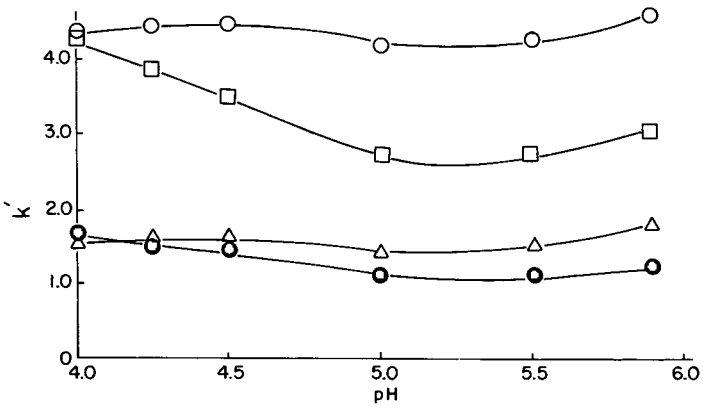

Fig. 8. The effect of pH on capacity factors for *p*-aminobenzoic acid and metabolites with 0.01 *M* tetrabutyl ammonium chloride added to the mobile phase. Other conditions as in Fig. 6; symbols as in Fig. 3.

The addition of 0.01 *M* TBA to the mobile phase at pH values above 5.8 had little if any effect on the elution of the components of urine which previously interfered with the compounds of interest. The optimum conditions for the separation and determination of bentiromide metabolites in urine are summarized in Table I. The optimization of the chromatographic conditions was performed with a phosphate buffer in the mobile phase. It was found that this component could be eliminated from the mobile phase without any detrimental effect on the separation and peak shapes (Fig. 9). Under these conditions the pH of the aqueous component of the mobile phase (0.01 *M* TBA) was found to be 7.4.

The studies described above were performed at ambient temperature (25°C) with a flow-rate of 1.2 ml/min. A reduction in the overall analysis time and a slight improvement in peak shape was obtained by operating at a flow-rate of 1.4 ml/min and 40°C. Further increases in flow-rate (up to 2 ml/min) and temperature (up to 70°C) resulted in incomplete resolution of the compounds of interest.

Linearity. There was a linear relationship between peak height and concentration of the solutes in aqueous solutions over the following ranges: PABA (0.13—1.0 mg/l), PAHA (0.18—1.42 mg/l), PAABA (1.63—13.04 mg/l) and PAAHA (3.23—25.86 mg/l). The mean slopes of the graphs and corresponding

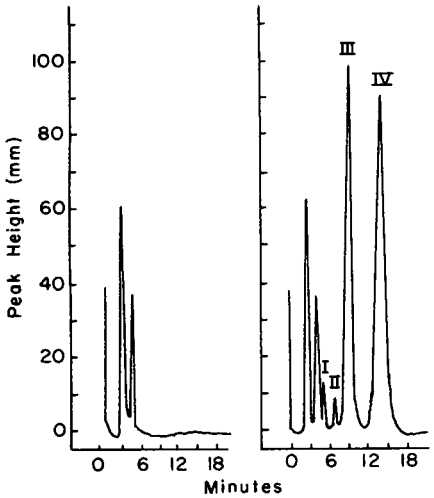

Fig. 9. Typical chromatograms under the conditions in Table I, of a pooled blank urine (left) and a spiked urine containing 0.5 mg/l PABA (I), 0.71 mg/l PAHA (II), 6.52 mg/l PAABA (III), and 12.93 mg/l PAAHA (IV).

standard deviations of the mean of six measurements were 31.7 ± 0.44 for PABA; 17.1 ± 0.20 for PAHA; 17.7 ± 0.19 for PAABA and 8.5 ± 0.06 for PAAHA. The limits of detection, taken as the peak height corresponding to twice the baseline noise, were found to be 0.06 mg/l for PABA, 0.12 mg/l for PAHA, 0.11 mg/l for PAABA and 0.24 mg/l for PAAHA.

Recovery. Various amounts of each compound were added to pooled blank urine collected from fasting subjects under test conditions. The recoveries were determined by replicate analyses (n = 6) at each concentration. The mean slopes of the peak height versus concentration curve for spiked urine were compared with those curves obtained by direct injection of identical aqueous standards. The results are summarized in Table II.

TABLE II

RECOVERY STUDIES (n = 6)

Compound	Concentration range (mg/l)	Mean slope	Standard error	Recovery (%)
PABA	0.13— 1.0	30.0	0.23	95
PAHA	0.18— 1.42	16.7	0.38	98
PAABA	1.63—13.04	17.7	0.13	100
PAAHA	3.23—25.86	8.4	0.03	100

Precision. The between-day precision was assessed by analysing samples containing four different concentrations of each solute on five consecutive days. The coefficient of variation (C.V.) for each compound was less than 10% for all urine concentrations tested (Table III) except for very low concentrations of PABA and PAHA. The within-day variation in peak height was less than 5% (C.V.) at the lowest and highest standard concentrations for all compounds tested (n = 5).

TABLE III

BETWEEN-DAY PRECISION (n = 6)

Compound	Mean concentration (mg/l)	C.V. (%)
PABA	0.98	3.0
	0.48	9.2
	0.25	9.2
	0.13	16.7
PAHA	1.40	3.6
	0.67	2.3
	0.32	5.4
	0.18	14.0
PAABA	12.4	6.5
	6.5	3.8
	3.3	3.2
	1.6	8.3
PAAHA	26.0	2.9
	12.8	1.4
	6.5	1.4
	3.3	5.3

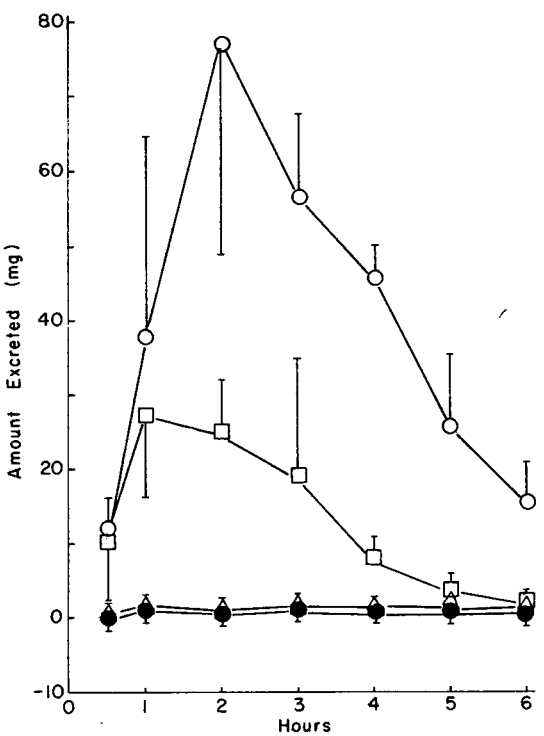

Fig. 10. Urinary excretion versus time curves for p-aminobenzoic acid and metabolites (n = 5). See Fig. 3 for symbols and Table I for conditions.

Selectivity. Good selectivity of the method under test conditions was indicated by the fact that no interfering peaks were observed in pooled blank urine (Fig. 9). The y intercept of curves constructed from spiked urine was 0.039 ± 0.13 for PABA, −0.66 ± 0.32 for PAHA, 1.78 ± 0.98 for PAAHA and −0.059 ± 0.50 for PAAHA (n = 6 in each case) also indicating no significant interferences. Blank urine from non-fasting subjects who were also receiving medication, however, did demonstrate several interfering peaks which suggests that test conditions must be rigorously controlled for good selectivity.

Patient samples

Bentiromide (500 mg) was administered to five normal volunteers and the amount of each metabolite excreted in urine versus time is presented in Fig. 10. While the concentration profiles of PAABA and PAHA are too low to be useful in the normal subjects tested, it is possible that a study of hepatic or small bowel diseased patients may produce higher levels. The profiles of PAAHA and PAABA demonstrate concentration maxima at 3 and 2 h, respectively and potential differentiation of liver and small bowel versus pancreatic disease in a single sample would probably be best achieved over a collection interval of 0—3 h, since any metabolic lag time could best be detected prior to the concentration maximum, and levels would be high enough for easy quantitation. Also, a peak which interfered with PAABA was observed in samples collected after 4 h. This was confirmed as PAABA glucuronide by treatment of the sample with β-glucuronidase which eliminated the interfering peak and caused an increase in PAABA peak height.

CONCLUSIONS

An accurate and reproducible method is described for the measurement of PABA and its metabolites in human urine from patients undergoing the bentiromide test for pancreatic function. This method was applied to a study in normal subjects to determine the appropriate sampling interval for metabolite differentiation. This work will permit clinical studies involving small bowel, hepatic and pancreas diseased patients to determine whether differential diagnosis can be made on the basis of metabolite concentration patterns. The large standard error found in normal subjects reflects individual variation. The methodology described may also be applicable to pharmacokinetic studies designed to reveal a better understanding of the mechanism of PABA metabolism.

ACKNOWLEDGEMENTS

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DETERMINATION OF METHYL PARAOXON IN DOG PLASMA BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method for the determination of methyl paraoxon in plasma has been developed. Disodium EDTA and aluminon are used to inhibit hydrolysis of methyl paraoxon in plasma. Methyl paraoxon and the internal standard fenitrooxon are extracted from plasma into methylene chloride. Chromatography is performed on a reversed-phase C_{18} column, connected with a fixed-wavelength ultraviolet detector at 280 nm; the compounds are eluted in about 5 min with tetrahydrofuran—acetonitrile—0.01 *M* sodium phosphate buffer, pH 7.4 (12:25:63, v/v/v). Concentrations down to 5 ng/ml methyl paraoxon in plasma can be determined with good precision and accuracy. The method was applied to plasma samples from dogs after intravenous administration.

INTRODUCTION

Methyl paraoxon [O,O-dimethyl (*p*-nitrophenyl) phosphate] is the activated oxygen analogue of methyl parathion, an organophosphate insecticide which exerts its acute toxicity by inhibition of acetylcholinesterase [1]. The main metabolite of methyl paraoxon is *p*-nitrophenol. The structures of the compounds are given in Fig. 1. Knowledge of the plasma concentration of organophosphates could be helpful in the management of intoxicated patients.

Analysis of methyl paraoxon by electron-capture gas chromatography (GC)

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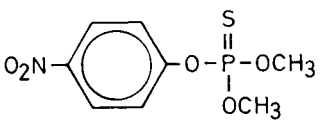
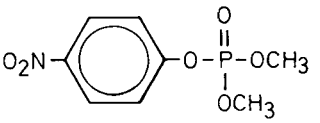
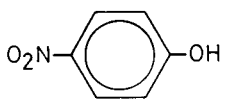


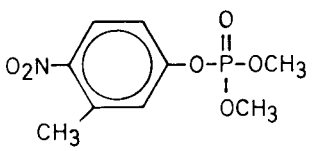
Methyl parathion



Methyl paraoxon



p-Nitrophenol



Fenitrooxon

Fig. 1. Structure of methyl parathion, methyl paraoxon, *p*-nitrophenol and fenitrooxon.

[2—4], alkali flame-ionization GC [5] and GC with combination of both detection systems [6] has been described. As methyl paraoxon has a very high UV absorbance, high-performance liquid chromatography (HPLC) with UV detection can be used. An HPLC method, using a column packed with gels and UV monitoring at 260 nm, for the separation of solutions containing methyl parathion and fenitrothion metabolites has been reported [7]. This procedure, however, was not applied to biological samples.

The present paper describes a selective HPLC method for the determination of methyl paraoxon in dog plasma with a simple and rapid extraction procedure, using fenitrooxon [O,O-dimethyl (3-methyl-4-nitrophenyl) phosphate] as the internal standard (Fig. 1). As methyl paraoxon is not stable in plasma, an adequate inhibitor of its hydrolysis was sought. The method has been used to determine plasma concentrations in the dog after intravenous administration of methyl paraoxon.

EXPERIMENTAL

Reagents

Methyl paraoxon and fenitrooxon were supplied by Ehrenstorfer (Augsburg, F.R.G.) and were purified using preparative column chromatography. Aluminon (aurin tricarboxylic acid ammonium salt) was obtained from Aldrich-Europe (Beerse, Belgium). Disodium EDTA, sodium dihydrogen phosphate, disodium hydrogen phosphate and methylene chloride were supplied by E. Merck (Darmstadt, F.R.G.) and were of analytical grade. Spectrophotometric grade acetonitrile and tetrahydrofuran were used (Carlo Erba, Milan, Italy).

Standard solutions

Stock solutions, corresponding to 1 μg/μl in methanol, were prepared for methyl paraoxon and fenitrooxon. Standard solutions were obtained by diluting the stock solutions to concentrations of 1 or 10 ng/μl in methanol.

Sample preparation

Inhibition. As methyl paraoxon and fenitrooxon are not stable in plasma and

blood, inhibitors mentioned in the literature [8], disodium EDTA (10^{-2} *M*) and a mixture of disodium EDTA (10^{-2} *M*) and aluminon (10^{-3} *M*), were tested. The influence of these inhibitors and of temperature on the stability of the product and the internal standard was evaluated by incubation for 1 h at 0°C, 25°C and 37°C with or without inhibition. Samples were taken at regular time intervals.

Extraction. A 1-ml volume of inhibited plasma (unknown samples, product-free samples or samples containing known amounts of the product) is transferred to a glass-stoppered 5-ml silanized glass tube spiked with either a high (750 ng) or a low (75 ng) amount of the internal standard, dissolved in 75 µl of methanol, depending on the expected concentration range of methyl paraoxon. The sample is extracted with 3 ml of methylene chloride by shaking for 5 min at 50 Hz. After centrifugation at 3000 *g* and 4°C for 10 min, the organic phase is transferred to a 5-ml silanized glass conical tube and evaporated to dryness at room temperature under nitrogen. The residue is stored at −20°C until chromatography.

Chromatography

The chromatography is performed on a microprocessor-controlled Spectra Physics SP-8000 high-performance liquid chromatograph with a fixed-wavelength UV detector operating at 280 nm (Spectra Physics SP-8210), an automatic injector, and a 10-µl sample loop. Separations are achieved using a reversed-phase C_{18} column (250 × 4.6 mm I.D.) packed with 5 µm particle size Spherisorb 5 ODS (Chrompack, Merksem, Belgium) and thermostatted at 40°C. Just prior to chromatography the various extraction residues are redissolved in 30 µl of acetonitrile by vigorous vortexing, and 10 µl are injected onto the HPLC column. With a degassed mixture of tetrahydrofuran—acetonitrile—0.01 *M* sodium phosphate buffer, pH 7.4 (12:25:63, v/v/v), the samples are eluted within 5 min, at a constant flow-rate of 1.5 ml/min (170—210 bars). The use of buffer is necessary to elute *p*-nitrophenol, the main metabolite of methyl paraoxon, with the solvent front. Chromatograms are plotted on a Hewlett-Packard 3390A reporting integrator.

Calibration

For the lower range, inhibited blank plasma samples (1 ml) were spiked with increasing amounts (5—75 ng) of methyl paraoxon and with 75 ng of the internal standard, using standard solutions of 1 ng/µl in methanol. The higher range was calibrated with samples of inhibited blank plasma (1 ml) spiked with increasing amounts (50—1200 ng) of methyl paraoxon and with 750 ng of the internal standard, using standard solutions of 10 ng/µl in methanol. These calibration samples were then taken through the extraction procedure and chromatography described above.

Sample concentrations are calculated by determining peak height ratios of the product, related to the internal standard, and comparing these ratios with the standard curves obtained after analysis of calibration samples.

Absolute recovery

Absolute recovery of methyl paraoxon from plasma is determined by adding

the internal standard after extraction, and comparing peak height ratios with the peak height ratios of a calibration curve. This calibration curve is obtained by injecting acetonitrile solutions containing a fixed amount of the internal standard and varying amounts of methyl paraoxon. Absolute recovery of fenitrooxon from plasma is determined using methyl paraoxon as the internal standard.

RESULTS AND DISCUSSION

Inhibition of hydrolysis, absolute recovery, precision and accuracy

The in vitro stability of methyl paraoxon in dog blood and plasma was studied at several temperatures. An example of the hydrolysis of methyl paraoxon in dog plasma at 0° C, 25° C and 37° C is shown in Fig. 2. Each point represents the result of a sample worked up in duplicate. At 37° C, the half-life of degradation is about 10 min in dog blood, about 5 min in dog plasma; at 25° C it is about 20 and 10 min for blood and plasma, respectively. Even at 0° C a considerable loss of methyl paraoxon is observed, with a half-life of 90 and 60 min for blood and plasma, respectively. Therefore, for accurate determination of methyl paraoxon in dog plasma the hydrolysis of the product has to be inhibited. Very few data on this problem are found in the literature. Most authors quantitate *p*-nitrophenol, the main metabolite of methyl paraoxon, or apply their method to solutions. Erdös and Boggs [8], however, tried to inhibit the splitting of another organophosphate, paraoxon [O,O-diethyl (*p*-nitrophenyl) phosphate], in human plasma. Disodium EDTA alone was not effective, but degradation was inhibited adequately by adding 10^{-2} *M* disodium EDTA and 10^{-3} *M* aluminon. In our study the same mixture was used to inhibit the degradation of methyl paraoxon and fenitrooxon. After 1 h, 80% of

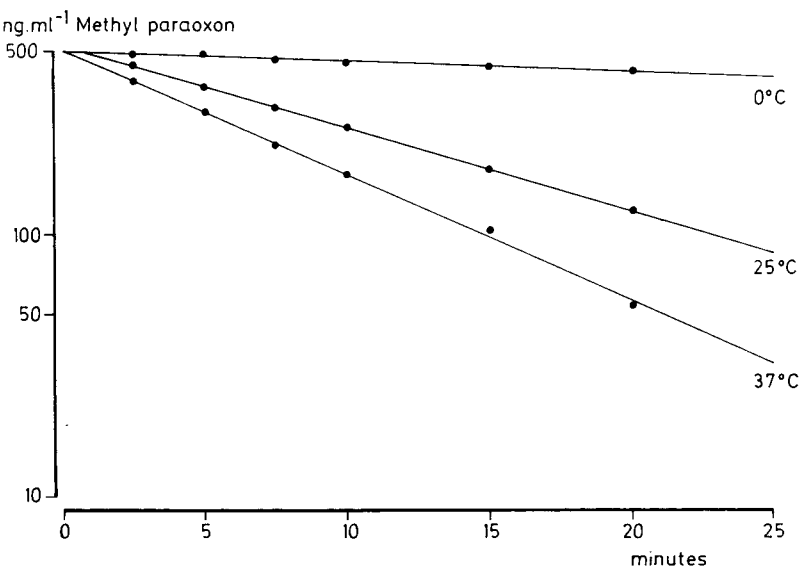


Fig. 2. Hydrolysis of methyl paraoxon in dog plasma at 0° C, 25° C and 37° C as a function of time.

TABLE I

ABSOLUTE RECOVERY OF METHYL PARAOXON AND FENITROOXON FROM PLASMA

$n = 6$.

Amount added* (ng)	Mean recovery (%)	C.V. (%)
Methyl paraoxon		
10	80.4	7.6
30	81.3	4.9
50	85.4	11.9
150	89.4	6.4
400	87.4	6.4
Fenitrooxon		
75	88.7	4.1
500	90.5	5.3

*Quantity added to 1 ml of dog plasma.

TABLE II

WITHIN-RUN ACCURACY AND PRECISION OF THE METHYL PARAOXON ASSAY

Methyl paraoxon* (ng)	n	Relative error (%)	C.V. (%)
15	12	+0.7	5.9
50	12	−4.0	2.7
100	11	+4.4	2.2
400	11	−1.9	3.4

*Quantity added to 1 ml of dog plasma.

methyl paraoxon is recovered at 37°C from blood and plasma, 90% at 25°C. At 0°C no hydrolysis is observed over 1 h. Similar observations were made for fenitrooxon.

The extraction with methylene chloride gives good recoveries (80—91%) of methyl paraoxon and fenitrooxon (Table I).

Plasma standard curves are linear for the concentrations tested, ranging from 5 to 1200 ng/ml plasma. The average slope for twenty calibration curves assayed over three months is 0.00243 ± 0.00029 (mean ± S.D.), with an average intercept of 0.00495 ± 0.01724 and an average correlation coefficient of 0.9993 ± 0.0014. The accuracy and precision of the procedure was ascertained by adding different amounts of both compounds to blank inhibited plasma and analysing a number of samples with the method described. The results are summarized in Table II.

The lowest acceptable concentration is about 5 ng/ml for a plasma sample of 1 ml, as can be seen in Fig. 3, which shows two chromatograms of extracted plasma (1 ml): (A) blank inhibited dog plasma and (B) blank inhibited dog plasma spiked with 5 ng of methyl paraoxon and 75 ng of the internal standard.

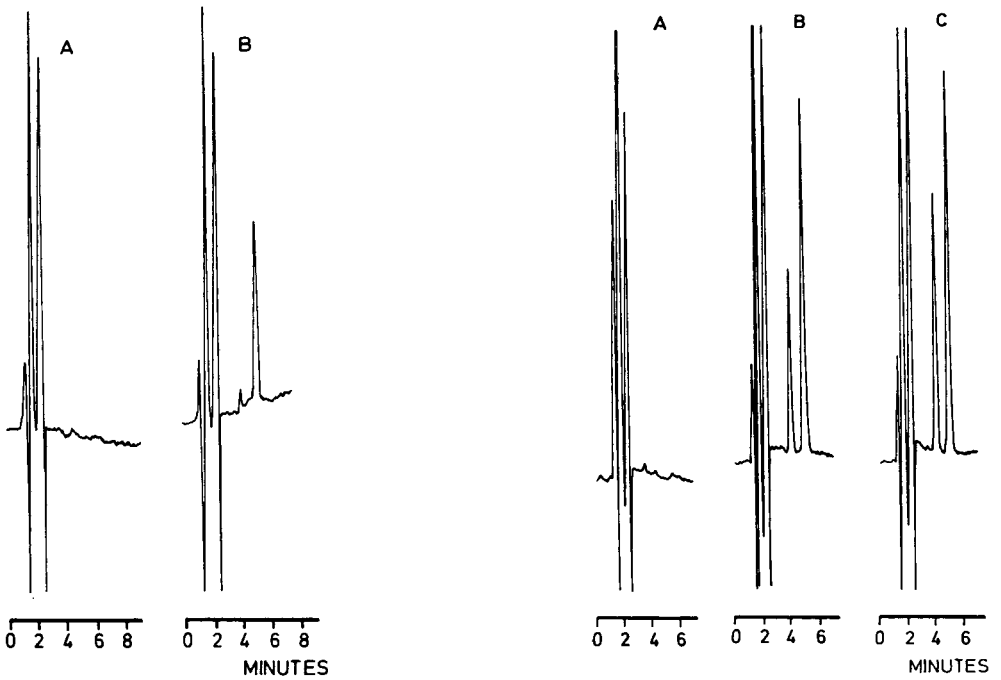


Fig. 3. Chromatograms of extracted plasma: (A) blank inhibited dog plasma; (B) blank inhibited dog plasma spiked with 5 ng of methyl paraoxon (t_R = 3.95 min) and 75 ng of the internal standard, fenitrooxon (t_R = 4.90 min).

Fig. 4. Chromatograms of extracted plasma: (A) blank inhibited dog plasma; (B) blank inhibited dog plasma spiked with 200 ng of methyl paraoxon (t_R = 3.95 min) and 750 ng of the internal standard, fenitrooxon (t_R = 4.90 min); (C) inhibited plasma sample, obtained from a dog 30 min after the injection of 2.5 mg/kg methyl paraoxon; this sample was spiked with 750 ng of the internal standard, fenitrooxon. Note that the sensitivity in this figure is four times lower than in Fig. 3.

Selectivity

As can be seen in Fig. 4A, which shows a representative chromatogram of an extract of blank inhibited dog plasma, there are no interfering peaks. Fig. 4B shows a chromatogram of an extract of blank inhibited dog plasma spiked with 200 ng of methyl paraoxon and 750 ng of the internal standard. The two products are well separated, within a short analysis time. Fig. 4C represents a chromatogram of an extract of inhibited dog plasma, 30 min after the injection of 2.5 mg/kg methyl paraoxon, spiked with the internal standard. There is no interference of hydrolysis products of methyl paraoxon. If a mobile phase containing water instead of buffer pH 7.4 is used, *p*-nitrophenol, the main metabolite of methyl paraoxon, interferes in the HPLC procedure; so the use of buffer is necessary to elute *p*-nitrophenol with the solvent front.

Preliminary toxicokinetic investigations

The method described has been used to measure plasma levels of methyl paraoxon in dogs. Atropine (1 mg/kg) was given intramuscularly 30 min before

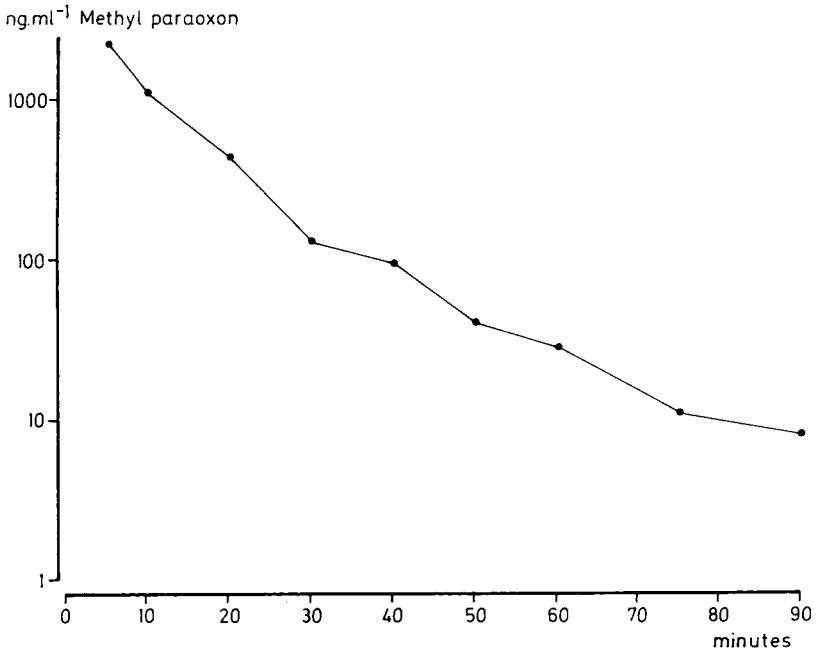


Fig. 5. Semilogarithmic plot of methyl paraoxon plasma concentrations versus time after intravenous injection of 2.5 mg/kg of the product.

intravenous injection of methyl paraoxon at a dose of 2.5 mg/kg. The plasma concentration—time curve in a dog is depicted in Fig. 5. The concentration of methyl paraoxon can be determined accurately, since dog blood samples can be collected with a syringe and added to tubes containing disodium EDTA and aluminon placed in an ice bath, without loss of methyl paraoxon.

The proposed method allows toxicokinetic studies of methyl paraoxon, since inhibition of the hydrolysis, extraction from plasma, sensitivity, analytical recovery and selectivity are satisfactory.

ACKNOWLEDGEMENTS

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Note

Assay of 3-methoxy-4-hydroxyphenylglycol in human plasma using high-performance liquid chromatography with amperometric detection

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The measurement in plasma of 3-methoxy-4-hydroxyphenylglycol (MHPG), a major metabolite of norepinephrine, may provide a useful tool for assessing central and peripheral noradrenergic activity in humans. Since the half-life of the metabolite is 45—75 min, its plasma levels reflect the average norepinephrine metabolism over an interval of several hours, providing a more integrated measure of presynaptic neuron activity than does the parent amine [1]. MHPG levels in plasma have thus been examined in various disorders in which the noradrenergic system appears to be involved, such as anxiety, hyperactivity and some neuropsychiatric diseases [2—4].

The only method with a sufficient degree of sensitivity and specificity employed for determination of MHPG in plasma has been gas chromatography—mass spectrometry (GC—MS) [5—7]. Unfortunately, not many clinical and research laboratories have the financial and technical means required for this technique.

Recently, reversed-phase high-performance liquid chromatography (HPLC) coupled with electrochemical detection (ED) gave good results when applied to urinary MHPG measurement [8—11]; in plasma the determination of MHPG is more difficult because of the low levels of the metabolite in this fluid.

In this note we describe a method for the determination of unconjugated MHPG in human plasma based on protein precipitation, solvent extractions and isocratic HPLC with ED. High precision was achieved using 3-hydroxy-4-methoxyphenylglycol (iso-MHPG) as internal standard.

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EXPERIMENTAL

Apparatus and liquid chromatographic conditions

A Knauer HPLC pump, Model 64 (Knauer, Berlin, F.R.G.) and a Model 7125 injector from Rheodyne (Cotati, CA, U.S.A.) were used. The electrochemical detector from Bioanalytical Systems (West Lafayette, IN, U.S.A.) consisted of an LC 4B controller and a TL-3 cell packed with CP-0 carbon paste; the electrode potential was set at +0.75 V versus an Ag/AgCl reference electrode. The chromatographic column was a prepacked Ultrasphere ODS column (250 × 4.6 mm, 5 μm particle size) with a self-packed ODS precolumn (Altex, Berkeley, CA, U.S.A.).

The mobile phase was a mixture of 0.09 mol/l sodium acetate with 0.009 mol/l citric acid buffered to pH 5.0 and containing 10% methanol, degassed under vacuum by filtration through a Millipore 0.2-μm membrane and delivered at a flow-rate of 1.0 ml/min.

Chemicals and reagents

All reagents used were of the highest purity (A.C.S. certified grade). MHPG hemipiperazine salt was obtained from Sigma (St. Louis, MO, U.S.A.); iso-MHPG was from Paesel (Frankfurt, F.R.G.).

Solutions of standards were prepared in distilled deionized water and kept frozen until use.

Procedure

Plasma samples (2 ml), obtained from heparinized blood, were transferred into polycarbonate tubes each containing 100 μl of iso-MHPG in water (0.2 ng/μl). After brief mixing and incubation on ice (10 min), 200 μl of cold 4 mol/l perchloric acid were added to each tube. The samples were vigorously mixed and centrifuged at 30 000 *g* for 10 min at 4°C. The supernatants were removed and adjusted to pH 6.5 with cold 5 mol/l potassium hydroxide in 0.5 mol/l phosphate buffer and then, after saturation with sodium chloride, extracted twice with 3 ml of ethyl acetate for 1 min in a vortex mixer. In order to facilitate the separation between the phases, the test tubes were placed in glycol antifreeze solution at −25°C for 3 min and the separated ethyl acetate pools were re-extracted twice with 1 ml of 0.1 mol/l phosphate buffer, pH 5.3. Each aqueous phase was washed with 4 ml of diethyl ether, its pH adjusted to 9.0 with 5 mol/l sodium hydroxide and then extracted twice with 2 ml of ethyl acetate. The ethyl acetate extracts were evaporated to dryness under reduced pressure using a rotavapor; each residue was reconstituted with 250 μl of mobile phase and 100 μl were injected into the HPLC column.

Quantitative analysis

Quantitative analysis was performed using the internal standard addition method. A plasma pool was made from ten different subjects and increasing amounts of pure MHPG (2, 4, 8, 16 ng) were added to each 2 ml sample of the pool; these, after addition of internal standard (100 μl of a 0.2 ng/μl iso-MHPG solution), were subjected to the whole procedure.

After chromatography, ratios of responses of MHPG to those of iso-MHPG

were calculated for each sample and were plotted versus the amount of metabolite added. Linear regression analysis was performed to determine the best linear graph. The equation for the standard curve was $y = 0.146x + 0.492$ ($r = 0.999$). The concentrations of MHPG in the unknown samples were calculated according to the equation

$$\text{Concentration of MHPG (ng/ml)} = \frac{\text{MHPG peak height}}{\text{iso-MHPG peak height}} \times \frac{1}{\text{slope of standard curve}}$$

RESULTS AND DISCUSSION

Despite the high resolving power of HPLC and the selectivity of the electrochemical detector, the determination of MHPG in plasma requires at least partial purification before chromatographic analysis.

Recently, Scheinin et al. [12] adopted a preliminary extraction of the compound in ethyl acetate. However, this step alone was not sufficient to obtain a good separation of both MHPG and EHPG (3-ethoxy-4-hydroxyphenylglycol, internal standard) from interfering substances, unless rigorously optimized chromatographic conditions (mainly pH and temperature) were adopted. Injections of methanol at the end of the chromatographic analysis were also necessary to reduce the analysis time.

In the present procedure we introduced a purification step based on re-

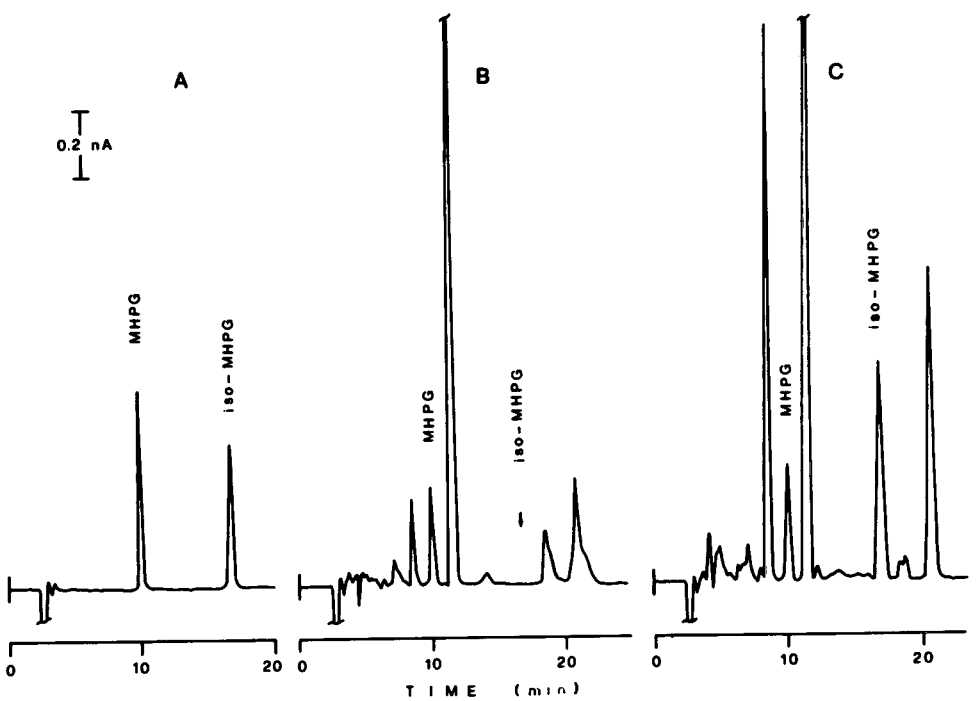

Fig. 1. Typical chromatograms of human plasma from healthy subjects: (A) MHPG and iso-MHPG standards; (B) pooled plasma sample; (C) plasma sample (3.66 ± 0.055 ng/ml, mean ± S.D. of five observations) with iso-MHPG added. For chromatographic conditions, see text.

extraction of the metabolite with phosphate buffer and subsequent washing with diethyl ether to eliminate any trace of ethyl acetate from the aqueous phase. Of considerable importance also is the final extraction into ethyl acetate at pH 9.0. The result was that, despite a lower recovery of MHPG, we obtained chromatograms free of interfering peaks and with a straight baseline, with the possibility of using higher sensitivity levels.

The usefulness of iso-MHPG as internal standard for the determination of MHPG and its possible presence in man, had been previously discussed [10]. Even if in some chromatograms a very small peak was detected with a retention time corresponding to that of iso-MHPG, nevertheless the height of this peak was negligible compared with the height of the internal standard (always less than 2%), so that correction was unnecessary.

Although an increase in the oxidation potential above 0.75 V results in an increase in the electrochemical response of compounds, this potential was adopted in our procedure because it allows a highly sensitive analysis of MHPG and iso-MHPG without loss of selectivity.

Typical chromatograms of a mixture of MHPG and iso-MHPG and endogenous free MHPG in plasma without and with added iso-MHPG are shown in Fig. 1.

The absence of late peaks in this assay allows injection of samples every 25 min with isocratic elution. The chromatographic peak was identified as MHPG since the retention time and voltage curve were similar to those of authentic MHPG.

The recovery of MHPG added to plasma was 35.1 ± 1.7% (mean ± S.D. of five observations). The sensitivity of the assay is suitable for clinical studies, with a detection limit of 0.18 ng/ml, based on a signal-to-noise ratio of 2. The precision of the assay was evaluated by analysing samples of a plasma pool. The intra-assay coefficient of variation was 4.1% while the inter-assay coefficient of variation was 5.6% (n = 10).

The concentrations of free MHPG in plasma samples of fifteen apparently healthy subjects (volunteers or laboratory personnel, aged between 20 and 65 years) are reported in Table I: these results agree well with recently published data obtained by GC—MS and by HPLC—ED [5, 6, 12].

TABLE I

FREE MHPG CONCENTRATIONS IN PLASMA OF HEALTHY SUBJECTS

Values are given in ng/ml.

n	Males	n	Females
1	3.66	9	3.53
2	2.66	10	3.55
3	3.67	11	3.83
4	2.83	12	3.70
5	4.14	13	3.85
6	4.71	14	2.51
7	3.11	15	3.01
8	4.08		
Mean	3.60		3.42

In conclusion, this method provides a sensitive and precise means for routine determination of the metabolite 3-methoxy-4-hydroxyphenylglycol in laboratories where GC—MS is not available.

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Note

Simultaneous assay of 3,4-dihydroxyphenylethylene glycol and norepinephrine in human plasma by high-performance liquid chromatography with electrochemical detection

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The ratio of norepinephrine (NE) to its deaminated metabolite free 3,4-dihydroxyphenylethylene glycol (DHPG) in plasma has recently been suggested to be of use in diagnosis of pheochromocytoma [1, 2]. However, only a few assays have been described which can simultaneously measure NE and DHPG [1—4]. These have either used radioenzymatic techniques [1, 2] or high-performance liquid chromatography (HPLC) with electrochemical detection (ED) [3, 4]. HPLC—ED assays have advantages over radioenzymatic techniques in that they are simpler and cheaper to perform. However, only one previously described HPLC—ED method [3] has measured DHPG and NE simultaneously in human plasma, and this method did not allow ideal resolution of NE from the solvent front. We have previously described a simultaneous method [4] for DHPG and catecholamines in rat brain and now report modifications to the technique that allow the simultaneous assay of DHPG and NE in human plasma.

EXPERIMENTAL

The chromatographic system consisted of a Pye-Unicam LC-XPS pump, a precolumn (40 × 1 mm) packed with LiChroprep (Merck), and a 25 cm × 4.0 mm I.D. reversed-phase column. The column was packed with Spherisorb 5-μm silica (Phase Separations). The mobile phase was 70 m*M* NaH_2PO_4 (pH 3.0) (Fisons) with 1.85 m*M* octanesulphonic acid (Fisons) and 13.4 m*M* EDTA (BDH, AnalaR). The flow-rate was 1 ml/min and the column effluent was passed through a TL3 cell (Bioanalytical Systems) with a glassy carbon

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electrode where the catechols were detected using an LC4 amperometric detector (Bioanalytical Systems). The applied potential was set at +0.66 V relative to the Ag/AgCl reference electrode. The sensitivity of the detector was set at 1.0 nA/V, and the background current was 1.2 nA.

Alpha-methyl norepinephrine (αMNE), (—)-NE bitartarte and DHPG used as standards in the assay were purchased from Sigma (St. Louis, MO, U.S.A.).

Fresh human blood was collected from normal volunteers after 5 min resting supine. Rabbit blood was obtained by ear artery cannulation. Blood samples were collected into lithium heparin tubes containing 1 mg sodium metabisulphite and centrifuged immediately at 1000 *g* for 5 min at 4°C to separate plasma. Plasma (2.5 ml) was placed in 10-ml conical polycarbonate tubes, then 4 ng of αMNE (internal standard) was added along with 500 μl of 1.5 *M* Tris (pH 8.6) to adjust the pH of the mixture to 8.6. Acid-washed alumina (50 mg) was added and the sample mixed gently for 30 min. The alumina was allowed to settle and the plasma aspirated. The alumina was then washed twice with 10 ml of twice distilled water. Catechols were eluted by vigorously mixing the alumina with 250 μl of 0.2 *M* perchloric acid. A 200-μl aliquot of the eluate was injected into the liquid chromatograph.

RESULTS AND DISCUSSION

The sensitivity of the system (twice background) was 30 pg for DHPG, NE and αMNE. While this sensitivity was adequate for the routine measurement of DHPG and NE, it was inadequate to reliably detect epinephrine. The retention times were 4.9 min for DHPG, 11.8 min for NE, 19.6 min for αMNE and 23.6 min for epinephrine. Dopamine had a retention time of 57.0 min, but was not detected in plasma samples assayed. The usual run time was, therefore, 30 min per sample. The recoveries of 4 ng of each of the catechols from alumina were DHPG, 54.1 ± 2.1%; NE, 59.4 ± 3.1%; and αMNE 65.1 ± 2.5% (mean ± S.D., $n = 8$). The recovery of DHPG was significantly lower ($p < 0.001$, Student's *t*-test) than that of the internal standard, as has previously been reported [3]. However, over the concentration range usually assayed (0.5—5 ng) the recovery of DHPG correlated linearly with that of αMNE ($r = 0.9772$, $p < 0.001$, $n = 11$). The within-day coefficients of variation were 4.1% for DHPG and 3.4% for NE ($n = 8$) while the between-day coefficients of variation were 8.9% for DHPG and 4.6% for NE ($n = 6$).

A typical chromatogram from human plasma is presented in Fig. 1. Human plasma DHPG and NE levels were 5.16 ± 0.98 n*M* and 1.38 ± 0.35 n*M*, respectively (mean ± S.D., $n = 6$). These results are similar to those reported using previous techniques [1—3]. The identity of the DHPG peak was further validated by demonstrating that it was greatly reduced by pretreating rabbits with the monoamine oxidase inhibitor, pargyline, 100 mg/kg 5 h prior to blood sampling (Fig. 1).

The system described differs from the previously described method for brain tissue [4] in the column used, the lower concentration of ion-pairing reagent and the absence of methanol in the mobile phase. These changes were necessary to resolve DHPG from the solvent front and NE from uric acid. The internal standard (αMNE) was used in preference to dihydroxybenzylamine [3, 4]

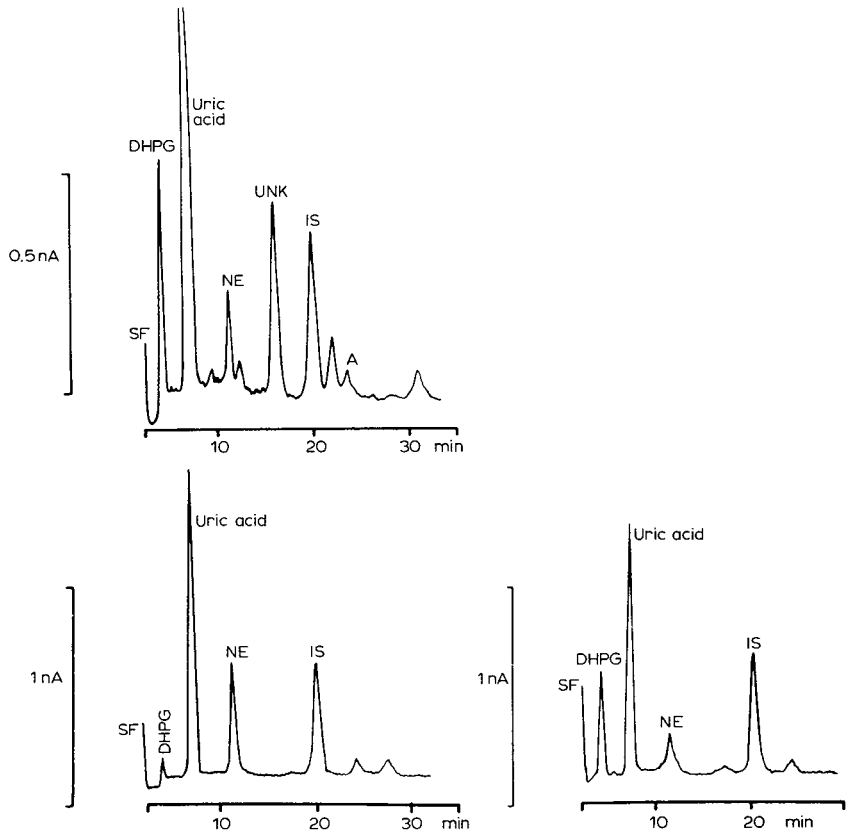

Fig. 1. Top: typical chromatogram of human plasma. Bottom, left: chromatogram of rabbit plasma 5 h after treatment with the monoamine oxidase inhibitor pargyline (100 mg/kg intraperitoneally) showing a marked reduction in the DHPG peak and associated increase in the NE peak compared to a chromatogram of normal rabbit plasma (bottom, right). Peaks: IS = internal standard (αMNE); UNK = unidentified peak; SF = solvent front; A = epinephrine.

because in the present system dihydroxybenzylamine co-chromatographed with epinephrine. This method allows the simple, simultaneous determination of NE and its major metabolite DHPG in human plasma by HPLC—ED. The technique will be useful for studies of sympathetic activity in man and possibly for the diagnosis of pheochromocytoma [1, 2].

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CHROMBIO. 2437

Note

Clinical analysis of steroids

XXXII*. New method for the determination of hapten number of the antigen prepared for the radioimmunoassay of steroidal sulphates using ion chromatography

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Measurement of steroidal conjugates in biological fluids has conventionally been done by prior hydrolysis or solvolysis and the subsequent analysis of deconjugated steroids. Recently, the conjugated steroids have been determined without prior hydrolysis, directly by gas chromatography—mass spectrometry, high-performance liquid chromatography or radioimmunoassay.

Because of its simplicity, convenience and ability to deal with many samples, radioimmunoassay is widely used for the measurement of steroidal sulphates. The first stage in establishing a radioimmunoassay of the sulphates is the preparation of antigen by coupling the sulphates with appropriate carrier protein, usually bovine serum albumin (BSA).

Since the antigens prepared should contain a proper number of haptens in order to become immunogenic, it is necessary to determine the hapten number (hapten/protein molar ratio of the antigen) prior to the immunization of animals. Hapten numbers are usually calculated by measuring the spectrometric absorption due to the chromophore originally present in the steroidal skeleton [1]. This procedure is not sufficient in the case of sulphates, because it is not clear whether the hapten is coupled with BSA without cleavage of the ethereal sulphate group during the reaction. In the radioimmunoassay of steroidal sulphates, therefore, it is necessary to determine the sulphate group in the antigen molecule. This paper describes the procedure for the determination of

*For part XXXI, see K. Watanabe and I. Yoshizawa, J. Chromatogr., 337 (1985) 114.

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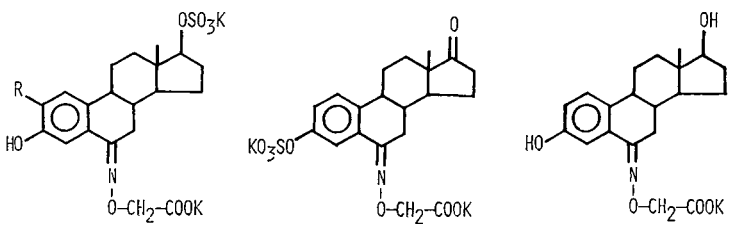
sulphate group in the antigen prepared for radioimmunoassay of steroidal sulphates. The principle of this new method is based on the ion chromatographic measurement of SO_4^{2-} liberated by the hydrolysis of prepared antigens.

MATERIALS AND METHODS

Materials

Potassium 6-carboxymethoxyimino-3-hydroxyoestra-1,3,5(10)-trien-17β-yl sulphate (I) [2], potassium 6-carboxymethoxyimino-2,3-dihydroxyoestra-1,3,5(10)-trien-17β-yl sulphate (II) [3], potassium 6-carboxymethoxyimino-2-methoxy-3-hydroxyoestra-1,3,5(10)-trien-17β-yl sulphate (III) [4], potassium 6-carboxymethoxyimino-17-oxooestra-1,3,5(10)-trien-3-yl sulphate (IV) [4] and 6-carboxymethoxyiminooestra-1,3,5(10)-trien-3,17β-diol (V) [5] were prepared in this laboratory according to the known methods as described previously (see Fig. 1 for formulae). BSA (fraction V) was obtained from Armour Pharmaceutical Co. (Kankanee, IL, U.S.A.). Isobutyl chloroformate and tri-*n*-butylamine were obtained from Tokyo Seikagaku Kogyo (Tokyo, Japan). Visking seamless cellulose tubes (Union Carbide, IL, U.S.A.) were used after refluxing in 1% sodium carbonate for 30 min followed by washing with distilled SO_4^{2-}-free water. Other reagents used were of analytical grade.



I: R = H
II: R = OH
III: R = OCH_3

IV

V

Fig. 1. Chemical formulae of I—V.

Instruments

The ion chromatograph equipped with an electric conductivity detector was a Type 2000i (Dionex, Midland, MI, U.S.A.) with a stainless-steel column (5 cm × 4.6 mm, I.D.) packed with TSK Gel IC-620 SA (Toyo Soda, Tokyo, Japan). The column was maintained at 30°C. A solution (pH 8.5) containing 1.3 m*M* sodium borate and 1.3 m*M* gluconic acid was used as the mobile phase at a flow-rate of 1.2 ml/min and a pressure of 8 kg/cm^2. Ultraviolet (UV) absorption spectra were recorded on a Model 200-20 spectrometer (Hitachi, Tokyo, Japan).

Preparation of antigens

Hapten compounds (I—V) were coupled with BSA by the mixed anhydride method under the usual conditions [1]. For hapten I, three kinds of antigen (antigen-I_1, antigen-I_2 and antigen-I_3) were prepared in the following manner. Isobutylchloroformate (3.0 mg for antigen-I_1, 6.0 mg for antigen-I_2 and 9.0 mg

for antigen-I_3) was added to a solution (2.0, 4.0 and 6.0 ml) containing hapten (8.0, 15.0 and 25.0 mg) and tri-*n*-butylamine (5.0, 10.0 and 15.0 mg) in a mixture of dioxane and dimethylformamide (1:2). After the mixtures were stirred under cooling for 1 h, BSA (80 mg) dissolved in 60% aqueous dioxane (6.0 ml) was added to each mixture. The whole was stirred under cooling for 5 h followed by additional stirring at room temperature for 6 h. The solution was kept neutral by occasional addition of a few drops of 1 *M* potassium hydroxide. The resulting solution was dialysed against SO_4^{2-}-free water at 4°C for 72 h. The steroid—protein conjugates were obtained by lyophilization as white powder; 77 mg, 83 mg and 87 mg for antigen-I_1, antigen-I_2 and antigen-I_3, respectively.

Antigens of other haptens (II—V) were prepared in the same way as described in the preparation of antigen-I_2 except for the neutralization by 1 *M* potassium hydroxide for hapten II, where 0.05 *M* potassium carbonate was used. Lyophilized antigens of II—V were obtained as white powder in the amount of 67—88 mg. The antigens obtained from haptens II, III, IV and V, are referred to hereafter as antigen-II, antigen-III, antigen-IV and antigen-V, respectively.

Determination of hapten number

The steroid/protein molar ratio of the antigens prepared was determined spectrometrically in methanol at a wavelength 270 nm using the previous method [1].

Determination of SO_4^{2-}

An exact amount (ca. 3.0 mg) of the hapten—BSA conjugates was dissolved in 2 *M* hydrochloric acid (3.0 ml), and the solution was refluxed for 2 h. After being cooled to room temperature, the solution was neutralized by addition of 2 *M* potassium hydroxide, followed by addition of a known amount of disodium hydrogen phosphate as an internal standard. The whole was diluted with SO_4^{2-}-free water to 10 ml, and centrifuged at 1500 *g* for 10 min to remove the precipitate, followed by extraction with ethyl acetate (3 × 2 ml). The aqueous layer was centrifuged again at 1500 *g*, and injected into the chromatograph with a microlitre syringe. Ten experiments were carried out on each antigen.

Standard curve

To obtain a standard curve, standard solutions of 1.0, 2.0, 3.0, 5.0, 10.0, 20.0, 30.0 and 50.0 ppm of SO_4^{2-} (sodium sulphate) containing 10 ppm of HPO_4^{2-} in each were prepared. Each mixture was injected into the chromatograph. The peak areas were determined by the half-band width method.

RESULTS AND DISCUSSION

In order to establish radioimmunoassay methods for oestradiol 17-sulphate, 2-hydroxyoestradiol 17-sulphate and 2-methoxyoestradiol 17-sulphate, the antigens of these sulphates were prepared by coupling with BSA by the mixed anhydride method. By difference of the hapten contents in the antigen, three

kinds of antigens for hapten I were prepared: antigen-I_1, antigen-I_2 and antigen-I_3. Similarly, antigen-II and antigen-III were prepared by coupling haptens II and III, respectively, with BSA.

The hapten numbers in these antigens were easily obtained from the absorbance at 270 nm due to the benzophenone oxime chromophore of the steroidal skeleton (UV method). However, it is uncertain whether these antigens contain theoretical numbers of ethereal sulphate groups because of the possibility of partial solvolysis of sulphate during the reaction. In order to confirm this, we developed a method to determine the sulphate group present in the antigen molecule. For this purpose, the antigens were hydrolysed to give the SO_4^{2-}, which was determined by ion chromatography.

Initially, the separation of anions was investigated by using several kinds of solvents as mobile phase. When a solution (pH 8.5) of 1.3 m*M* sodium borate and 1.3 m*M* gluconic acid was used, the anions concerned were separated satisfactorily as shown in Fig. 2.

Secondly, the quantification of SO_4^{2-} was investigated under the above conditions. The calibration curve was constructed by plotting the peak area of SO_4^{2-} to that of the internal standard against the amount of the former, and a satisfactory linearity was observed in the range of 1—50 ppm of SO_4^{2-}.

Application of the present method to oestradiol 17-sulphate—BSA conjugate was then carried out. The steroid numbers of antigen-I_1, antigen-I_2 and antigen-I_3 were determined spectrometrically and compared with the SO_4^{2-} values of each antigen. Oestradiol—BSA conjugate (antigen-V) was used as blank. The results obtained for these antigens are shown in Table I, from which it can be seen that, at any content of hapten residue in the antigens, there is no substantial difference between the UV method and the ion chromatography procedure. This means that no cleavage of the sulphate group at C-17 of the

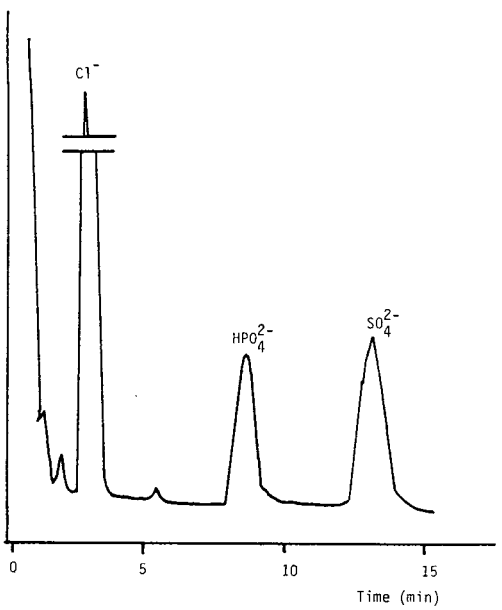


Fig. 2. Ion chromatogram showing the separation of the anions Cl^-, HPO_4^{2-} and SO_4^{2-}.

TABLE I

COMPARISON OF HAPTEN NUMBERS DETERMINED BY THE UV METHOD AND AMOUNTS OF SULPHATE ION (SO_4^{2-}) DETERMINED BY ION CHROMATOGRAPHY FOR THREE KINDS OF OESTRADIOL 17-SULPHATE—BSA CONJUGATES

Antigen	Hapten number	Molar ratio of SO_4^{2-} to BSA		Coefficient of variation (%)
		Mean	S.D. (n = 10)	
Antigen-I_1	10.7	11.3	0.23	2.04
Antigen-I_2	18.9	19.8	0.33	1.67
Antigen-I_3	30.2	31.4	0.42	1.34
Antigen-V	22.6	0.21	0.005	2.38

TABLE II

COMPARISON OF HAPTEN NUMBERS AND AMOUNTS OF SULPHATE ION (SO_4^{2-}) FOR VARIOUS KINDS OF ANTIGENS

Antigen	Hapten number	Molar ratio of SO_4^{2-} to BSA		Coefficient of variation (%)
		Mean	S.D. (n = 10)	
Antigen-I_2	18.9	19.8	0.33	1.67
Antigen-II	20.2	21.4	0.34	1.59
Antigen-III	19.4	19.8	0.29	1.46
Antigen-IV	22.6	7.8	0.43	5.51
Antigen-V	22.6	0.21	0.005	2.38

hapten residue occurred during the coupling reaction. Although a trace amount of SO_4^{2-} was detected in the case of antigen-V, this may be due to contamination during the course of experiments. The reproducibility is considered to be satisfactory as shown in Table I, where the C.V. values are between 1.3% and 2.0% except for antigen-V.

Similar experiments were carried out on four kinds of antigens. The results are summarized in Table II, and imply that the ethereal sulphates of the alcoholic hydroxyl group are not hydrolysed in the conditions of the reaction; thus the SO_4^{2-} values agree with the steroid numbers obtained by the UV method. In contrast, oestrone sulphate—BSA conjugate (antigen-IV) showed a big difference between these two values. Because the blank value is negligible, the result shows that a majority of the phenolic sulphate is solvolysed in the course of coupling with BSA. The mixed anhydride method is thus not suitable for preparing antigens of phenolic sulphate. This has been already demonstrated by Nambara et al. [6], who succeeded in obtaining oestrone sulphate—BSA conjugate by the activated ester method instead of the mixed anhydride method.

The detection limit of SO_4^{2-} by the present method using electric conductivity detector was 0.4 ppm (signal-to-noise ratio = 4.0 at full scale). Provided that an antigen contains twenty sulphate groups in the molecule, the amount of antigen required for the hydrolysis is calculated theoretically as about 0.2 mg (on the basis of a molecular weight of BSA of 66 210 [7]). Thus, the sensitivity of the present method is considered to be satisfactory.

In order to develop a radioimmunoassay for sulphoconjugated steroids, measurement of the sulphate group of the antigen is necessary. Many investigators have used antigens for immunization of animals without prior confirmation of the complete retention of the sulphate group, and only checked the molar amount of steroid nucleus by the UV method. The antibodies they obtained were, fortunately, specific for the steroidal sulphates, but they were not specific for the deconjugated haptens. This means simply that the sulphate group of the hapten residue was not hydrolysed inadvertently.

In planning a radioimmunoassay for endogenous steroidal sulphates, the present method may be applicable to confirmation of the characteristics of the antigen because of its simplicity, reproducibility and sensitivity. This procedure may also be useful in the radioimmunoassay of sulphoconjugated foreign compounds.

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Note

Simple purification procedure of rat α-fetoprotein by a combination of Cibacron Blue gel affinity chromatography and anion-exchange high-performance liquid chromatography

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α-Fetoprotein (AFP), a glycoprotein produced by the yolk sac, liver and gastrointestinal tract, is the principle plasma protein of the foetus [1, 2]. It is found only in very low concentration in normal adult serum [3]. Elevated levels of this protein in adult serum can be detected during pregnancy and in certain diseases, particularly liver cell carcinoma and germ cell tumours [4—7].

Previously, we have used a combination of Cibacron Blue gel affinity chromatography and anion-exchange high-performance liquid chromatography (HPLC) for the isolation of bovine AFP from foetal calf serum [8]. In the present paper, we report the adaptation of this methodology for the purification of AFP from rat amniotic fluid and rat foetal homogenate.

MATERIALS AND METHODS

Materials

Wistar rats of 15—19 days' gestational age were obtained from Charles River (St. Constant, Canada). Rat serum albumin (RSA) and rat gamma-globulin

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(RGG) were purchased from Sigma (St. Louis, MO, U.S.A.). Rat AFP (RAFP) and anti-RAFP and anti-RSA antibodies were gifts from Dr. H.F. Deutsch (University of Wisconsin, Madison, WI, U.S.A.). Cibacron Blue—Sepharose was obtained from Pharmacia (Montreal, Canada). All other reagents used were of analytical or reagent grade and purchased from local suppliers.

Instrumentation

HPLC was performed on a Pharmacia fast protein liquid chromatography (FPLC) system equipped with two pumps and capable of generating a gradient or step gradient elution profile. Sample injections were carried out using a V-7 valve and chromatograms were recorded by monitoring the absorbance at 280 nm using a single-path UV-1 monitor fitted with a 10-mm path cell. The absorbance unit full scale (a.u.f.s.) was set between 0.05 and 2.0 units as appropriate. Fractions were collected with a FRAC-100 fraction collector. The FPLC system was operated at room temperature.

Chromatography

The Cibacron Blue—Sepharose gel was washed first with 6 *M* urea and then with 6.5 m*M* 1,3-bis[tris(hydroxymethyl)methylamino]propane (Bis-Tris propane) buffer, pH 7.0. The washed gel was packed into a Pharmacia HR10/10 column (100 × 10 mm I.D.) and chromatographed using the FPLC system. A sample was injected, the column eluted with 6.5 m*M* Bis-Tris propane buffer, pH 7.0, and the unretained fraction collected. The fraction retained by the blue gel was then eluted using the same buffer containing 1.4 *M* sodium chloride. The column was regenerated with 6 *M* urea and equilibrated with the starting buffer before the next run.

The unretained protein fraction from above was used directly for HPLC on a Pharmacia Mono-Q SI anion-exchange column (50 × 5 mm I.D., 10 μm particle size). Buffer A was 6.5 m*M* Bis-Tris propane, pH 7.0. Buffer B was buffer A containing 0.35 *M* sodium chloride. A preprogrammed linear gradient was used for the chromatography and the appropriate peak fractions were collected, dialyzed against distilled water, and lyophilized.

Rat foetal material

Rat amniotic fluid was obtained from the amniotic sacs of rats. Each pregnant rat was anesthetized with diethyl ether and the uterus opened with a clean pair of scissors. The intact amniotic sac was punctured and the fluid collected. Approximately 4 ml of undiluted amniotic fluid was obtained from each rat. Contaminating blood cells were removed by centrifugation. The amniotic fluid was dialyzed against distilled water and lyophilized. Weighed samples were dissolved in 6.5 m*M* Bis-Tris propane buffer, pH 7.0, and used for Cibacron Blue gel chromatography.

Rat foetal homogenate was prepared from the foetuses by homogenizing them in 1 volume of 6.5 m*M* Bis-Tris propane buffer, pH 7.0. After centrifugation at 12,000 *g* for 10 min and Millipore filtration (0.22 μm), the supernatant was used for Cibacron Blue gel chromatography.

Protein assay

Total protein concentrations were determined by the Bio-Rad protein assay

method [9] using bovine serum albumin as a standard, or by UV absorption at 280 nm. RSA and RAFP concentrations were assayed by the radial immunodiffusion method of Mancini et al. [10]. The purity of the protein was determined by disc polyacrylamide gel electrophoresis (PAGE) [11]. Bands in the gels were quantified by scanning with a DCD-16 digital densitometer (Gelman, Ann Arbor, MI, U.S.A.).

RESULTS

We initially attempted to establish an HPLC condition for the separation of RAFP from RSA, the latter protein being an anticipated major contaminant of RAFP which has similar physicochemical properties. However, no satisfactory resolution of the two proteins could be achieved; by employing the optimal HPLC conditions described under Materials and methods, we could only partially resolve RAFP and RSA. It was therefore evident that before subjecting rat foetal material to HPLC separation, a pre-purification step to remove the RSA was necessary.

Fig. 1 shows the elution profile of rat amniotic fluid on a Cibacron Blue—Sepharose column. This affinity column was found to be effective in removing RSA from the injected sample. The unretained fraction I showed a solvent peak (a) and a major protein peak (b). Analyses of peak (b) by disc PAGE and radial immunodiffusion plate revealed that it contained RAFP

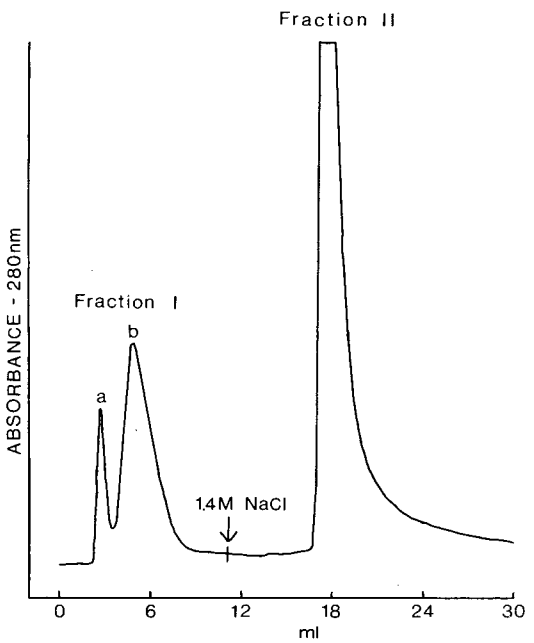

Fig. 1. Chromatography of rat amniotic fluid (10 mg) on an HR 10/10 Cibacron Blue-Sepharose column using 6.5 m*M* Bis-Tris propane buffer, pH 7.0, as the eluent (flow-rate, 0.2 ml/min, a.u.f.s., 2.0). Fraction I is the unretained fraction and fraction II is the retained fraction eluted with 1.4 *M* sodium chloride. Peaks: a = solvent; b = RAFP and other proteins.

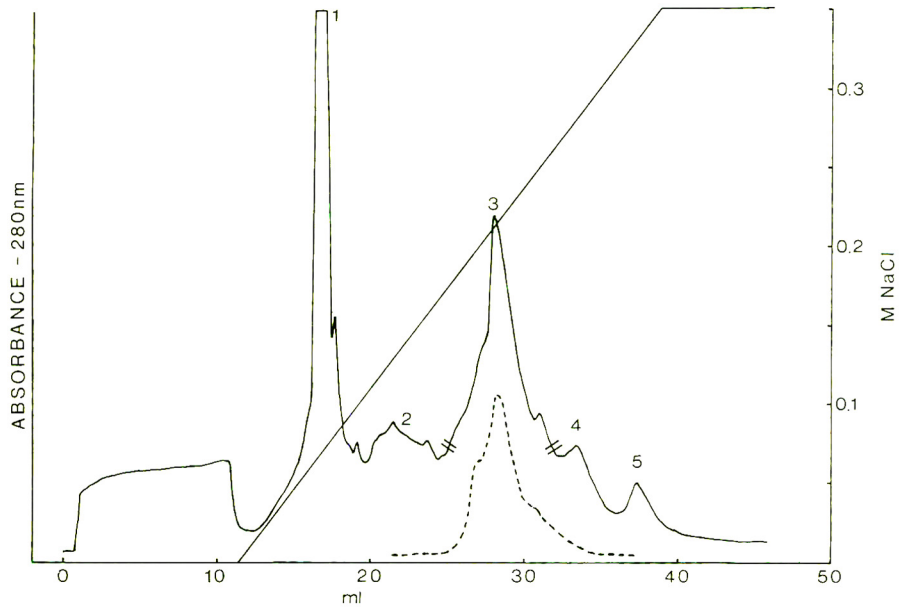

Fig. 2. HPLC separation of fraction I (Fig. 1) on a pre-packed HR 5/5 Mono-Q SI column. Buffer A: 6.5 m*M* Bis-Tris propane, pH 7.0; buffer B: buffer A containing 0.35 *M* sodium chloride. Flow-rate, 1 ml/min; a.u.f.s., 0.5. The RAFP fractions collected are indicated by hash marks in the elution profile. - - -, Elution profile of a standard solution of RAFP.

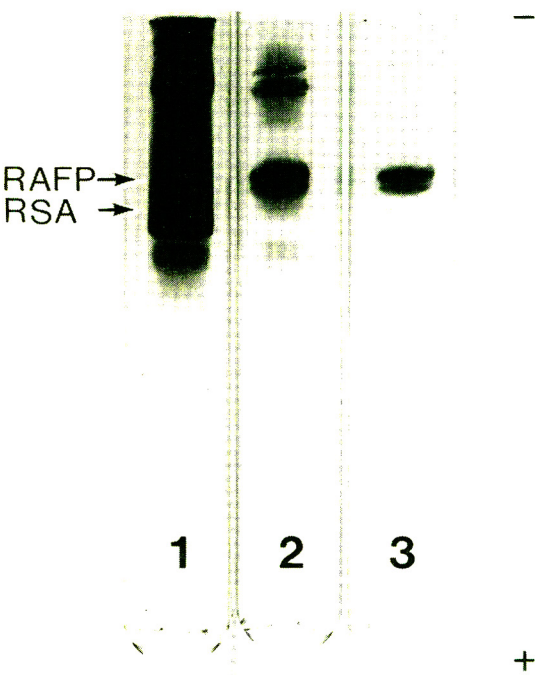

Fig. 3. Disc PAGE of (1) rat amniotic fluid; (2) rat amniotic fluid after Cibacron Blue—Sepharose chromatography (fraction I, Fig. 1); and (3) rat amniotic fluid after Cibacron Blue—Sepharose chromatography, anion-exchange HPLC and repeat Cibacron Blue—Sepharose chromatography. The arrows indicate the relative positions of standard RAFP and RSA.

(and other proteins) but no detectable amount of RSA. The retained fraction II (eluted with 1.4 *M* sodium chloride), on the other hand, contained RSA almost exclusively.

The RSA-depleted fraction (I) of rat amniotic fluid was next subjected to anion-exchange HPLC. A total of five peaks was resolved on the chromatogram (Fig. 2). Peak 1 was identified as RGG by comparison with a standard sample. RAFP was eluted as peak 3 at a sodium chloride concentration of between 0.17—0.25 *M*. The identities of the other minor peaks were not established.

The purity of the RAFP thus isolated was analyzed by disc PAGE, the results of which are shown in Fig. 3, with those of neat rat amniotic fluid and RSA-depleted fraction I also shown for purposes of comparison. The purified RAFP was quantified to have a purity of > 95% with no detectable amount of RSA. It shows the familiar pattern of fast and slow bands [12]. Its extinction coefficient ($E^{1\%}_{1\,cm}$, 280 nm) was 4.1, similar to that reported for RAFP isolated by an immunoabsorbent method ($E^{1\%}_{1\,cm}$, 278 nm = 4.27) [12]. The maximum loading capacity of rat amniotic fluid through this two-step chromatographic procedure was 20 mg, giving a yield of 3 mg (50% overall yield) of purified RAFP per injection.

The methodology was also tested for the purification of RAFP from rat foetal homogenate. Fig. 4A depicts the chromatogram of the Cibacron

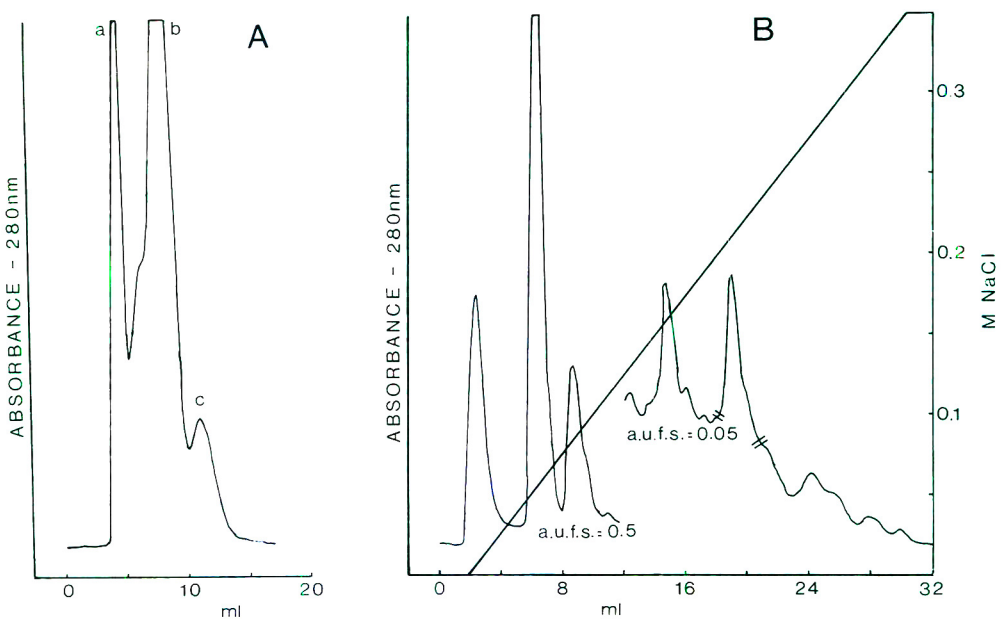

Fig. 4. (A) Unretained fraction of rat foetal homogenate (125 µl) chromatographed on an HR 10/10 Cibacron Blue—Sepharose column using 6.5 m*M* Bis-Tris propane, pH 7.0, as the eluent. Flow-rate, 0.2 ml/min; a.u.f.s., 0.5. Peaks: a = solvent; b = RAFP and other proteins; c = predominantly haemoglobin. (B) HPLC separation of peak b (Fig. 4A) on a pre-packed HR 5/5 Mono-Q SI column. Buffer A: 6.5 m*M* Bis Tris propane, pH 7.0; buffer B: buffer A containing 0.35 *M* sodium chloride. Flow-rate, 1 ml/min. The RAFP fractions collected are indicated by the hash marks in the elution profile.

Blue—Sepharose unretained fraction of rat foetal homogenate while Fig. 4B shows its chromatographic profile after anion-exchange HPLC. Because of the greater complexity of the protein constituents in rat foetal homogenate, the RAFP thus isolated was found to have a purity of only 80—90%. The purity, however, could be increased by re-chromatography on the anion-exchange column.

DISCUSSION

The binding properties of AFP have been extensively studied, but there exist in the literature discrepant reports of these binding functions, involving, for example, the number of types of oestrogen-binding sites and the oestrogen-binding specificity of human AFP [13]. A possible explanation for this confusion may lie in the different methods of AFP purification used, which may select sub-populations of AFP or cause modification due to harsh conditions. Thus it is reasonable to suggest that for studying the binding properties of AFP, a simple and mild method capable of purifying the protein in its most native form is highly desirable.

Rat amniotic fluid has been found to be a good source of RAFP, as RAFP constitutes about 30—40% of the total protein in this fluid [14]. RAFP has been purified from rat amniotic fluid by isoelectric focusing [14] and preparative PAGE [15], but these procedures are tedious and often give low recoveries of the product. In this respect, purification by a direct immunoadsorbent affinity chromatography [16, 17] and an indirect immunoadsorbent precipitation [18] method is fast and efficient and offers high recovery of RAFP. However, immunochemical methods are initially costly and time consuming, as they require the production of specific antibodies; they also have the inherent difficulties associated with immunoadsorbent chemistry, such as the low pH used in the recovery of ligand from the immunoadsorbent. In contrast with these methods of purification, the procedure described here is simple, mild, and efficient; using it, one can obtain milligram quantities of purified RAFP in less than 1 h.

RAFP and RSA are difficult to separate by physicochemical means because of their similarities in physical and chemical properties. Attempts to develop an HPLC condition for the complete resolution of these proteins proved unsuccessful. The Cibacron Blue affinity column, on the other hand, was found to be efficient in separating these proteins since RSA, but not RAFP, was retained by the column. These observations have previously been reported by other workers [19, 20].

In the present purification procedure, the efficiency of the Cibacron Blue gel column is critical if one is to obtain completely albumin-free RAFP. We have occasionally observed albumin contamination of the sample which we attribute to the deterioration of the affinity column after prolonged use. This contamination, however, is usually small ($< 2\%$) and can be completely removed by re-chromatography of the sample on the Cibacron Blue gel column.

A major difficulty in the purification of AFP lies in obtaining large quantities of the protein. Of the existing methods reported for the isolation of RAFP, the immunoabsorbent technique is probably the only one that can

readily give milligram quantities of the product. By employing the presently described methodology, one can obtain milligram quantities of purified RAFP through repeated injections. Furthermore, the present method has the potential to be automated for the production of RAFP by running the two chromatographic steps in series; we are now working toward this end.

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Note

Rapid large-scale isolation of biologically active molecules using reversed-phase "flash" chromatography: initial purification of endogenous Na^+,K^+-ATPase inhibitors from human urine

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An increased activity or concentration of an endogenous Na^+,K^+-ATPase inhibitor or inhibitors has been reported to be associated with hypertension in humans [1—3] and animals [4]. The analytical approaches to the separation of these Na^+,K^+-ATPase-active substances has varied. The reported methods include chromatography on Sephadex G-25 [5, 7], ion-exchange chromatography [8] and combinations of ion-exchange and reversed-phase chromatography [9—11]. While these methods have been utilized on an analytical scale, they have not been successfully adapted to the preparative scale needed for isolation and identification of these substances.

We have recently reported a reversed-phase high-performance liquid chromatographic (HPLC) method for the isolation on an analytical scale of endogenous Na^+,K^+-ATPase inhibitors from human plasma and urine [12]. This method utilizes an octadecyl support and an acetonitrile—water gradient. We have now adapted this method for preparative work using "flash" chromatography [13], which is a low-pressure-driven hybrid of conventional gravity column chromatography and high-pressure preparative systems. The chromatography was carried out using a 450-ml heavy walled glass column packed with

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40-μm octadecylsupport, a stepwise acetonitrile—water gradient and low nitrogen pressure. In this manner we have been able to process 1—2 l of unconcentrated human urine in a single run with a significant initial purification of the desired inhibitors.

EXPERIMENTAL

Apparatus

The chromatography was carried out using a glass column (52.7 cm × 33 mm I.D.) fitted with a pressure-regulating flow controller and a 500-ml solvent reservoir; the column was filled with 100 g of 40-μm octadecyl reversed-phase column packing (J.T. Baker, Deventer, The Netherlands). The chromatographic fractions were collected using an LKB 2211 Superrac (LKB, Bromma, Sweden) and freeze-dried in a Lyovac GT2 (Leybold-Heraeus, France). Sodium, potassium and calcium ion concentrations in the collected fractions were measured by flame ionization using an Eppendorf Gerätebau (Netheler and Hinz, Hamburg, F.R.G.).

Materials

HPLC-grade water, methanol, acetonitrile and trifluoroacetic acid were purchased from J.T. Baker.

Biological samples

Blood samples and total 24-h urines were collected from essential hypertensive patients and normotensive subjects. The essential hypertensive patients came from the Hypertension Unit of Necker Hospital and had been off medication for at least one week. The normotensive subjects were also free of any form of medication for at least a week.

The blood samples were tested for their capacity to inhibit both dog kidney Na^+,K^+-ATPase activity [14, 15] and ouabain binding to human erythrocytes [2, 14]. On the basis of these tests each subject was placed in one of three groups: inhibiting hypertensive (I), inhibiting normotensive (II) and non-inhibiting (III). The urine samples were pooled by group and chromatographed.

Chromatography

The mobile phases were prepared using HPLC-grade solvents and contained 0.1% trifluoroacetic acid.

Before each chromatographic run, the column was washed with 500 ml of methanol followed by 500 ml of water. A pooled urine sample (1—2 l containing 0.1%, v/v trifluoroacetic acid) was placed on the column and chromatographed using a step-gradient of: 0, 15, 25, 30, 35, 50 and 100% acetonitrile in water. A flow-rate of 15 ml/min was maintained using 2—3 bars nitrogen pressure; 30-ml fractions were collected.

Bioassays

The HPLC fractions were freeze-dried and the residues reconstituted to 1/30 of the initial collected volume with 10^{-3} *M* acetic acid. The HPLC residues were then assayed for their capacity to inhibit Na^+,K^+-ATPase activity

following previously published procedures [8, 12] and for their phosphate [16] and sodium, potassium and calcium ion content. Inhibiting fractions were also tested for cross-reactivity with antidigoxin antibodies following the method described by Smith et al. [17].

RESULTS AND DISCUSSION

The chromatograms resulting from the flash chromatography of inhibiting urine from hypertensive patients (I), inhibiting urine from normotensive subjects (II) and non-inhibiting urine (III) are presented in Fig. 1. Each sample contained a large number of fractions which inhibited Na^+,K^+-ATPase activity. The sodium, potassium, calcium and phosphate ion concentrations of the inhibiting fractions were determined, and significant concentrations were found in fractions 1—20. Since these ions are known to affect Na^+,K^+-ATPase activity [18, 19] the fractions were discarded and are not included in the chromatograms.

The remaining inhibitory fractions were assessed for their capacity to cross-react with antidigoxin antibodies. Cross-reactivity with antidigoxin antibodies coupled with the capacity to inhibit Na^+,K^+-ATPase activity has been used as an indicator of the presence of endogenous Na^+,K^+-ATPase inhibitors [9, 12, 20, 21]. In this manner, the fractions eluted with 15% acetonitrile in the mobile phase (urines I, II and III) and the fractions eluted with 30—35% acetonitrile in the mobile phase (urines I and II) were found to contain in the desired activities. Thus, there appears to be a significant difference between inhibiting and non-inhibiting urine based on the presence or absence of activity in the fractions eluted with 30—35% acetonitrile in the mobile phase.

These results are consistent with our previous data [12] comparing the concentration of endogenous Na^+,K^+-ATPase inhibitors in inhibiting and non-inhibiting urines. The samples were analysed on an analytical scale using a reversed-phase octadecyl column and an acetonitrile—water gradient. The resulting chromatograms contained two peaks which inhibited Na^+,K^+-ATPase activity and cross-reacted with antidigoxin antibodies. The peaks eluted at acetonitrile concentrations of 18% and 28%.

The inhibitory capacity of the first peak (18% acetonitrile) was the same for both the inhibiting and non-inhibiting urine. However, the inhibitory activity of the second peak (28% acetonitrile) was significantly greater in the inhibiting urines. These results suggest, as do the results from the preparative-scale flash chromatography, that the second peak contains a substance which is specific for essential hypertension.

The endogenous Na^+, K^+-ATPase inhibitors are present in minute concentrations in the plasma and urine. An initial accumulation of the crude material is necessary before further purification and eventual identification is possible. Flash chromatography using a reversed-phase octadecyl support and an acetonitrile step gradient appears to be a viable and reproducible first step for the rapid, large-scale initial purification of these inhibitors. It is now routinely used in our laboratory.

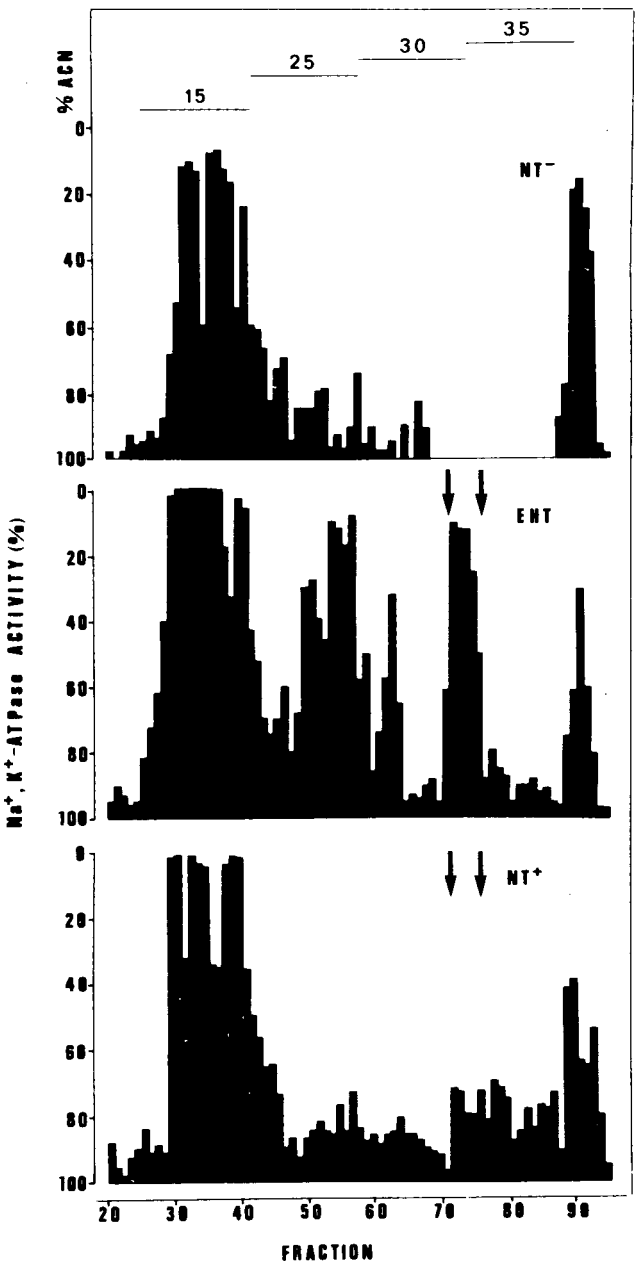

Fig. 1. Na^+,K^+-ATPase inhibitory activity of fractions from the flash chromatography of pooled urines from essential hypertensive patients (I, EHT), normotensive subjects with inhibiting plasma (II, NT^+) and normotensive subjects without inhibiting plasma (III, NT^-). Particular activity found in urine of EHT patients and NT^+ subjects lies inside arrows. These are profiles of repeated chromatographic runs (n = 12). ACN = Acetonitrile.

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Note

Estimation of plasma hydroxychloroquine by high-performance liquid chromatography with ultraviolet detection

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The use of antimalarial aminoquinoline drugs is well established in treatment of rheumatoid arthritis. Toxicity with prolonged use is often associated with binding to melanin-rich tissue, e.g. the retinal epithelium [1—3]. However, the relationship between blood levels and likely therapeutic efficacy or toxicity is not resolved. Laaksonen et al. [4] provided evidence of such a relationship for hydroxychloroquine (HCQ) and chloroquine (CQ), suggesting a maximum safe serum concentration of HCQ of around 1.5 μmol/l. Wollheim et al. [5], however, failed to observe any relationship between plasma CQ levels and therapeutic response.

Many of these early studies relied on relatively non-specific, poorly standardised fluorimetric methods which would not distinguish parent drug from oxidation products or any other component fluorescing at the chosen wavelength. More recently more selective chromatographic methods have been developed to determine CQ and its de-ethylated metabolites in plasma [6—8].

The present paper reports a selective reversed-phase high-performance liquid chromatographic (HPLC) method which requires a single solvent extraction in sample preparation, and can detect the parent drug (HCQ) in plasma at least fourteen days after a single oral 400-mg dose. The results of five such single-dose studies illustrate the application of the method in rheumatoid patients.

MATERIALS AND METHODS

Reagents

All reagents were of analytical grade and aqueous solutions prepared in glass-

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distilled water. Aqueous solutions of HCQ sulphate (Sterling Pharmaceuticals, Australia) and CQ diphosphate (20 μmol/l, Sigma) were stored at 4°C and prepared fresh monthly. The extracting solvent, chloroform (Mallinckrodt, Australia), was used without further distillation. A solution of sodium tetraborate (2.28 g) was prepared in 100 ml of 1 mol/l sodium hydroxide.

Standards

Stock solutions of HCQ (6 and 60 μmol base per l) were used to prepare calibration standards. Known amounts were added to plasma, previously shown to contain no compounds which interfered with the chromatography, to give a concentration range of 0.03—15 μmol/l. The internal standard, CQ, was employed to allow for inter-sample variability in recovery through the extraction procedure.

Chromatography

The analysis was performed using a Spectra-Physics (SP8000B) high-performance liquid chromatograph coupled with an SP8400 UV—VIS detector set at 340 nm (range of 0.002—0.08 a.u.f.s.). The separation was performed at 40°C on a 10-μm 30 cm × 3.9 mm μBondapak™ Phenyl column (Waters Assoc., Australia, Part No. 27198). The mobile phase was acetonitrile (Burdick & Jackson Labs., HPLC grade)—0.001 *M* disodium orthophosphate buffer (pH = 3.5) (9:1). These solutions were filtered under vacuum through a 0.45-μm filter (Millipore) before use and degassed continuously with helium. The flow-rate was 2 ml/min and injection volume was 100 μl.

Metabolite analysis

The oxidiation metabolites, N-desethyl chloroquine, N,N-didesethyl chloroquine and chloroquine-diol (Winthrop Labs.) at concentrations of 0.03, 0.06 and 0.13 μmol/l, respectively, were extracted using the present method and subjected to the same chromatographic separation to assess potential interference and quantitation of these compounds.

Sample preparation

Aliquots (2 ml) of heparinised plasma were added to glass extraction tubes. The internal standard (100 μl) and sodium hydroxide—borate mix (250 μl) were added and briefly vortexed before adding 5 ml of chloroform. The tubes were then capped and shaken horizontallly for 10 min at 100 oscillations per min. The two phases were separated by centrifugation at 1000 *g* for 10 min. The upper aqueous layer was aspirated to waste.

In order to avoid traces of aqueous contamination, the chloroform phase was tipped firstly into one glass tube (75 × 12.5 mm) and then to a second similar tube. This latter fraction was evaporated to dryness under a gentle stream of nitrogen in a heating block at 45°C. As soon as the chloroform had evaporated, each tube was removed from the heating block and reconstituted in either 125 μl or 250 μl of the same mobile phase used for the chromatographic separation.

The performance of the above technique was assessed by assaying in a single assay run five replicates of plasma samples spiked with HCQ to give final concentrations 0.03, 1.5 and 15 μmol/l. The variability between assay runs was

assessed by monitoring the standard curve stability in five successive assays. The recoveries of HCQ and CQ were estimated by comparing the peak heights obtained in six extracted samples (1.5 and 1.0 μmol/l, respectively), with the peak heights of six replicate injections of unextracted aqueous solutions of HCQ and CQ. These extracts were subsequently reinjected after sitting at room temperature (20°C) for 24 h to assess potential sample deterioration over the time course of a long assay run.

Patient studies

Venous blood samples (10 ml) were drawn into lithium heparin (125 I.U.) tubes at 0, 0.5, 1.0, 1.5, 3, 5, 9, 12 h and 1, 2, 3 and 4 days following a single oral 400-mg dose of Plaquenil™ from five patients with rheumatoid arthritis and who were both clinically assessed to be candidates for HCQ therapy and had not previously received HCQ therapy. These blood samples were centrifuged for 15 min (1000 *g*) and the plasma fraction was stored at −20°C until assayed. These patients were hospitalised for the first 24 h to facilitate sample collection. Subsequent samples were drawn in an outpatient clinic. Patients participating in the study were invited in advance to complete an informed consent form before being admitted to the study.

RESULTS

Fig. 1 shows sample chromatograms obtained using the method described above. Fig. 1a shows the result of injecting an aqueous mixture of HCQ and CQ (peaks 1 and 2, respectively) which had not been subjected to the extraction procedure described. Fig. 1b, shows the result of an extracted plasma sample spiked with HCQ (0.15 μmol/l) and CQ (1.0 μmol/l). Fig. 1c and d show extracts of patient plasma samples (spiked with the internal standard, CQ) before and 1.5 h after an oral 400-mg dose of Plaquenil, respectively. Fig. 1e, shows a mixture of the oxidation products N,N-didesethylchloroquine (peak 5), chloroquine-diol (peak 4), N-desethylchloroquine (peak 3), HCQ (peak 1), and CQ (peak 2).

Using the ratio of the height of peaks (HCQ:CQ) obtained from spiked plasma standards, a calibration curve was constructed in each assay run and showed a linear relationship between the peak height ratios and HCQ concentration over the range 0.03—15 μmol/l (r^2 = 0.998, slope = 2.137). The detection limit of the method was 0.01 μmol/l.

The performance and reproducibility of the method, assessed by comparing five replicate estimations obtained using three concentrations within a single assay and five concentrations between assay runs are presented in Table I. This shows a within-run coefficient of variation ranging from 9.1% (at 0.03 μmol/l) to 6.7% (at 15 μmol/l). The corresponding between-run values were 7.4% and 6.6%, respectively. The mean recoveries of HCQ (1.5 μmol/l) and CQ (1.0 μmol/l) through the extraction procedure were 91.5 ± 3.9% (n = 6) and 84.3 ± 4.4% (n = 6), respectively. Samples were found to be stable at room temperature in mobile phase for at least 24 h, as no deterioration or alteration in chromatography could be detected when samples were injected a second time on the day following the initial assay.

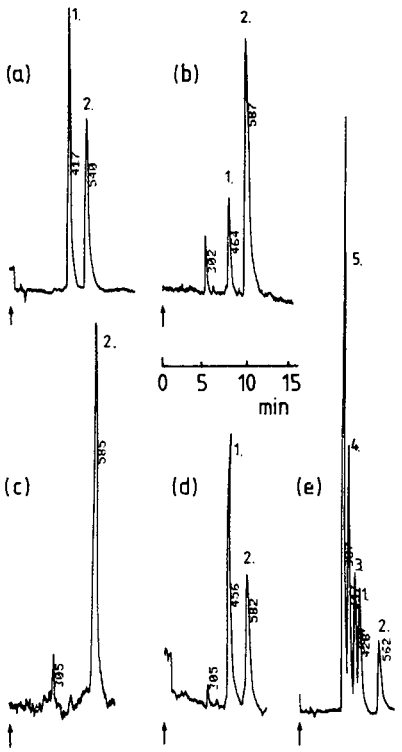

Fig. 1. Sample chromatographic traces obtained using the method described. (a) An unextracted aqueous mixture of HCQ and CQ; (b) a plasma extract spiked with 0.15 μmol/l HCQ and the internal standard, CQ (1.0 μmol/l); (c) a pre-HCQ dose plasma sample blank spiked with internal standard; (d) a patient plasma sample 1.5 h after a 400-mg dose of HCQ (Plaquenil); (e) a mixture of HCQ, CQ, N,N-didesethylchloroquine, chloroquine-diol and N-desethylchloroquine. Peaks: 1 = HCQ; 2 = CQ; 3 = N-desethylchloroquine; 4 = chloroquine-diol; and 5 = N,N-didesethylchloroquine.

TABLE I

PRECISION OF THE METHOD (n = 5)

HCQ concentration (μmol/l)	Coefficient of variation (%)	
	Between-assay	Within-assay
0.03	7.4	9.1
0.3	7.2	—
1.5	5.1	5.5
7.5	7.5	—
15	6.6	6.7

Patient studies

The results of the single oral Plaquenil (400-mg) dose studies (Fig. 2) illustrate the application of the method to patient samples. Fig. 2 shows means and standard errors of plasma HCQ levels (in nmol/l) estimated using the above assay technique in five rheumatoid patients. It can be seen that mean time to

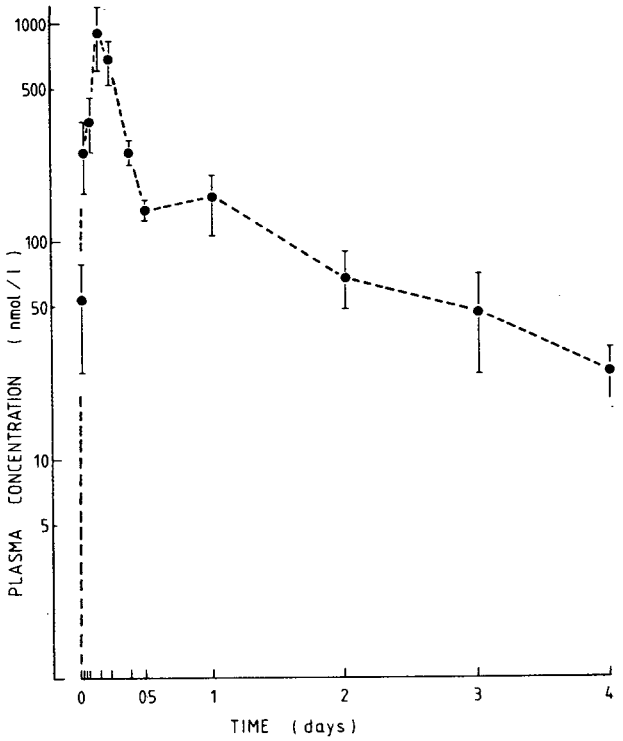

Fig. 2. Shows the estimated mean (± S.E.M.) plasma HCQ concentrations (nmol/l) up to four days following single oral 400-mg doses of Plaquenil in five rheumatoid patients, not previously medicated with HCQ, using the assay methodology described.

peak was between 3.0 and 5.0 h after the dose. The terminal phase is indicative of at least two compartments over the time course indicated. Three of the five patients had further samples drawn at seven and fourteen days following the dose. Plasma levels of HCQ could still be detected in each of these patients at fourteen days and in each case the level was less than or equal to the lowest standard (30 nmol/l) and approached the limit of detection of the present method (10 nmol/l).

DISCUSSION

The HPLC method presented allows a convenient and sensitive estimation of HCQ levels in heparinised plasma samples. The recovery and reproducibility of the extraction procedure are acceptable. No interfering compounds have yet been found either in samples assayed so far from patients receiving concominant therapy (including naproxen, aspirin, indomethacin, methyl-clothiazide, cimetidine and diclofenac) or as demonstrated with the three oxidation products presented.

Most previous methods for measuring HCQ have relied on non-specific fluorimetric methods and so could be criticised for the potential interference from metabolites and/or other fluorescing compounds. In the present HPLC method, fluorescence detection was avoided as it was found that these com-

pounds only fluoresce significantly at a pH greater than 8, which is inconsistent with HPLC column packing stability. An alternative approach could be to invoke a post-column pH adjustment to facilitate fluorescence. However, this approach obviously requires the dedication of a second pumping system which may not only be unavailable or inconvenient in many laboratories, but also introduces peak broadening and hence potential loss of sensitivity.

The single-dose studies in five patients presented suggests at least two compartments in the terminal phase, one with a half-life in excess of one day. As seen in the standard error bars in Fig. 2, the inter-patient variability in this latter compartment appears to be considerably greater than that of the first compartment. This could suggest that patients with the slower terminal half-life may achieve higher trough plasma levels at steady-state and, speculatively, be more likely to experience toxicity. These hypotheses will be tested in the next phase of the study where these and further patients, having been studied for two weeks following a single oral dose, progress to chronic HCQ therapy. Steady-state trough levels will be estimated for each patient to test whether these correlate with a slower terminal half-life in the single-dose study and the possible presentation of side-effects, especially retinal toxicity.

Further, it is hoped that the method, possibly in a modified form, may be employed to measure tissue levels of HCQ (e.g. leucocytes) as these may have a more direct relationship with disease suppression or the expression of toxicity than do plasma levels.

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CHROMBIO. 2442

Note

High-performance liquid chromatographic method for quantitative determination of Yutac®, a new antiarrhythmic agent, in dog plasma

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(First received July 27th, 1984; revised manuscript received September 17th, 1984)

Among the wide variety of chemical compounds used in the therapy of cardial arrhythmia, bispidine derivatives [1—3] belong to the first group of the classification of Vaughan Williams [4]. As a bispidine derivative, 3-methyl-7-ethyl-9-α-ol-(4-chlorobenzoyloxy)-3,7-diazabicyclo (3.3.1)-nonane monohydrochloride (I) (Fig. 1) acts mainly via membrane stabilization, but possesses calcium-antagonistic activity as well [5]. The compound is soluble in methanol and water, with a molecular weight of 322.8. The pK_a values of the functional N atoms are 1.67 and 12.3, respectively. The maximum UV absorbance occurs at 243 nm and does not show any pH dependence.

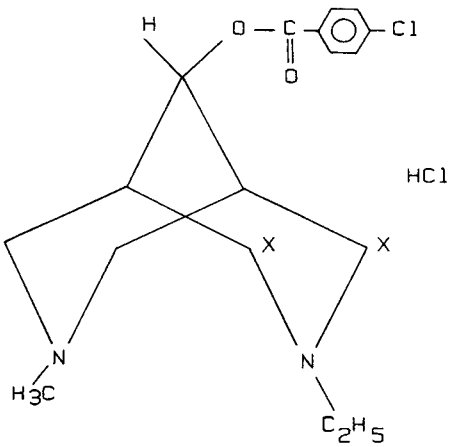

Fig. 1. Structural form of I. The position of the ^{14}C label is marked by X.

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In the present paper a method for the extraction of I from plasma and a high-performance liquid chromatographic (HPLC) procedure suitable for pharmacokinetic studies in dogs are described.

EXPERIMENTAL

Chemicals

I and internal standard [II, 3,7-dimethyl-9-(2′-naphthoyloxy)-3,7-diazabicyclo (3.3.1)-nonane dimethane sulphonate] were produced by the Chemical Works of Gedeon Richter (Budapest, Hungary). ^{14}C-Labelled I (specific radioactivity: 334.42 MBq/mmol) was prepared by the Central Research Institute for Chemistry, Hungarian Academy of Sciences (Budapest, Hungary). Methanol, acetonitrile, diethyl ether and Extrelut were purchased from E. Merck (Darmstadt, F.R.G.). All other chemicals used were the products of Reanal (Budapest, Hungary) and were of analytical purity.

Sample treatment and preparation of calibration curves

To 0.8 ml of dog plasma, an aqueous solution of 50 ng of II (internal standard) and 0—500 ng of ^{14}C-labelled I were added. The pH of plasma samples was adjusted to 9 using 20 μl of conc. NH_4OH. The samples were applied to the top of the chromatographic columns (15 × 5 mm) filled with 500 mg of Extrelut, allowed to soak for 5 min and eluted with 7 ml of diethyl ether. The recovery of I was checked by liquid scintillation counting. Diethyl ether was evaporated under a stream of nitrogen; the dry residue was dissolved in 0.1 ml of methanol by shaking and 30-μl aliquots were injected onto the analytical column.

Radioactivity of samples was counted in a Packard Tri-Carb 2660 liquid scintillation spectrometer. The ^{14}C radioactivity in 100-μl plasma aliquots was measured in Insta-Gel® (Packard, Downers Grove, IL, U.S.A.) and that of the diethyl ether samples (100-μl aliquots) in a toluene-based liquid scintillation solution (5 g of 2,5-diphenyloxazole, 0.1 g of dimethyl 1,4-bis(5-phenyloxazolyl-2)benzene, 1000 ml of toluene).

Chromatographic conditions

HPLC assay was performed on a Hewlett-Packard 1081 B chromatograph equipped with a variable-wavelength Cecil 2112 UV detector (Cecil Instruments, Cambridge, U.K.) and a 3380 S integrator. A Chromsil Si 60 prepacked column (25 × 4.6 mm I.D.), 10 μm particle size (Labor-MIM, Budapest, Hungary), was used for the separation of the compounds. The flow-rate was 1.3 ml/min at a pressure of 0.7 kPa; the temperature was 30°C. The absorbance of the effluent was monitored at 243 nm.

In order to study the influence of pH and water content of eluent on column efficiency, the pH of eluents, consisting of methanol—acetonitrile—0.01 *M* sodium perchlorate (100:100:2) were changed from pH 3.7 to pH 2.7 (pH 3.7, 3.4, 3.0, 2.7) using perchloric acid, and water content of the eluent was changed from 0.1% to 2.0% (0.1, 0.5, 1.0, 2.0%) of the total volume.

In vivo experiments

Male and female Beagle dogs were used as experimental animals. I in physiological saline was administered intravenously to dogs into the cephalic vein or orally in capsules at a dose of 2 mg/kg of body weight in randomized "cross-over". Blood samples were taken from the cephalic vein at times indicated in Fig. 6 and collected into heparinized test-tubes. To the plasma obtained from blood after centrifugation at 1500 *g* for 10 min, 50 ng of II in 1 μl of water were added and the samples were processed as above.

RESULTS AND DISCUSSION

Fig. 2 shows the values of capacity and selectivity factors for I and internal standard at different pH values. At pH 3.7 the retention time was too long (k' = 7.0) and the width of the peaks was fairly large. The theoretical number of plates was also very low (N = 1550). By increasing the acid concentration of the eluent, the capacity factor decreased (k' = 2.0) and the theoretical number of plates increased (N = 2700). Alteration of the pH did not essentially change the values of the selectivity factors. Decreasing the pH resulted in an increase of N, but it was smaller than required.

The influence of water on k' and N is shown in Figs. 3 and 4. The k' value for both derivatives increased significantly with increasing water content in

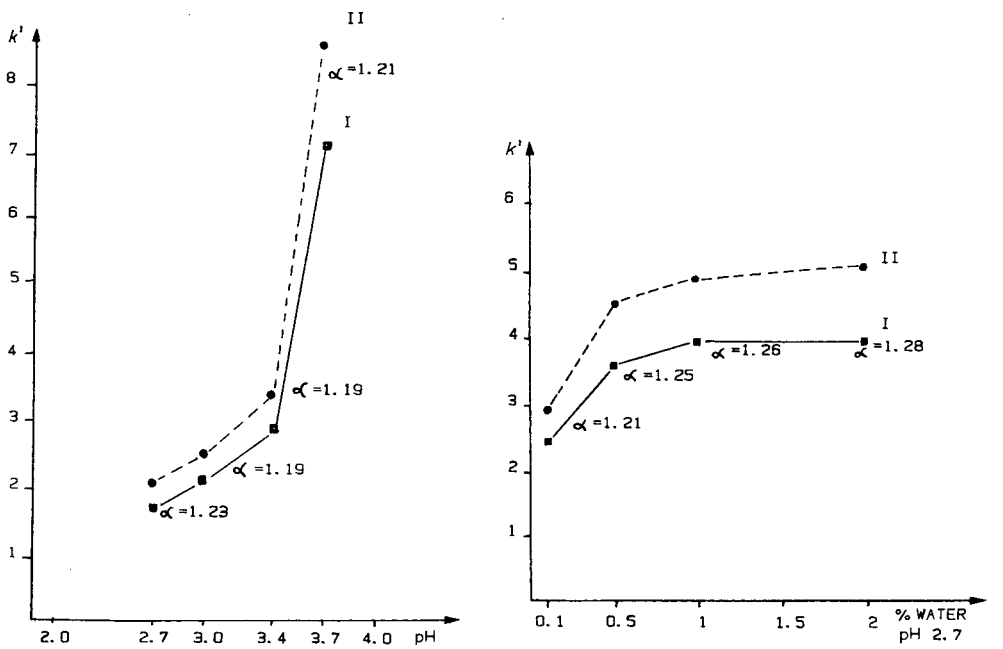

Fig. 2. Effect of pH on values of capacity (k') and selectivity (α) factors of I and II. Mobile phase: acetonitrile—methanol—0.01 *M* sodium perchlorate pH 2.7—3.7 (100:100:2).

Fig. 3. Effect of water on values of capacity (k') and selectivity (α) factors of I and II. Mobile phase: acetonitrile—methanol—0.01 *M* sodium perchlorate (100:100:0.2) in water (0.1—2%) at pH 2.7.

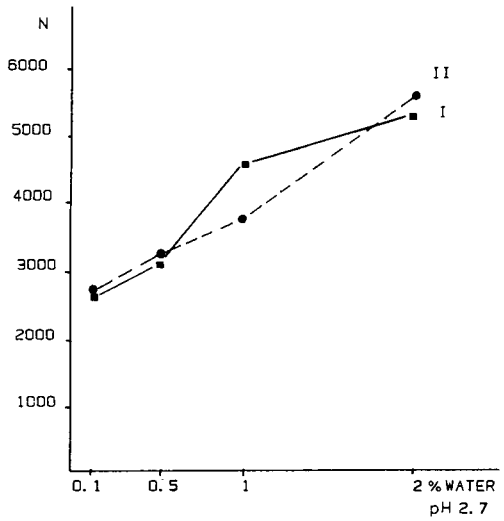

Fig. 4. Effect of amount of water (as a moderator) on theoretical number of plates (N) of the column.

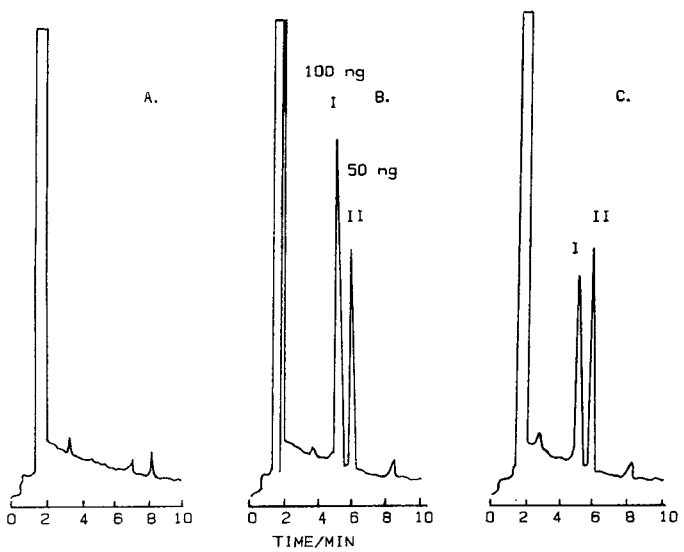

Fig. 5. Chromatograms of dog plasma extracts: (A) blank plasma extract; (B) plasma spiked with 100 ng of I and 50 ng of II; (C) plasma extract after intravenous administration of I at a dose of 2 mg/kg. Separation of two bispidine derivatives on a Chromsil Si 60 (10 μm particles size) column. Eluent: methanol—acetonitrile—0.01 M sodium perchlorate/perchloric acid (100:100:2) at pH 2.7.

the range of 0.1—2% of water (Fig. 3). The theoretical number of plates also increased (Fig. 4). The best separation was obtained when the eluent consisted of 100 ml of methanol, 100 ml of acetonitrile and 2 ml of 0.01 M sodium perchlorate/perchloric acid (pH 2.7).

The calibration curves were linear in the concentration range of interest (15—500 ng/ml). The linear regression curve for the data used for calibration

could be described by the equation $y = 0.006x + 0.018$ ($r = 0.994$) where y is the ratio of peak heights of I to II and x is the concentration of I.

The day-to-day coefficient of variation of the slope of the calibration curves was 3.6% ($n = 6$). Coefficients of variation for identical samples were 11.2% at 25 ng/ml, 7.6% at 100 ng/ml, 6.3% at 200 ng/ml ($n = 5$ at each concentration). The detection limit was found to be 15 ng of I per ml of plasma. Recovery of I from plasma was checked using radiolabelled drug and was found to be 73.1 ± 3.08% (S.D.) ($n = 6$).

Fig. 5 shows chromatograms of dog plasma extracts. Under the HPLC conditions used no interference by endogenous compounds has been observed.

The pharmacokinetic curve in Fig. 6 illustrates the successful application of the method. The results presented demonstrate that the method is appropriate for routine analysis and pharmacokinetic studies.

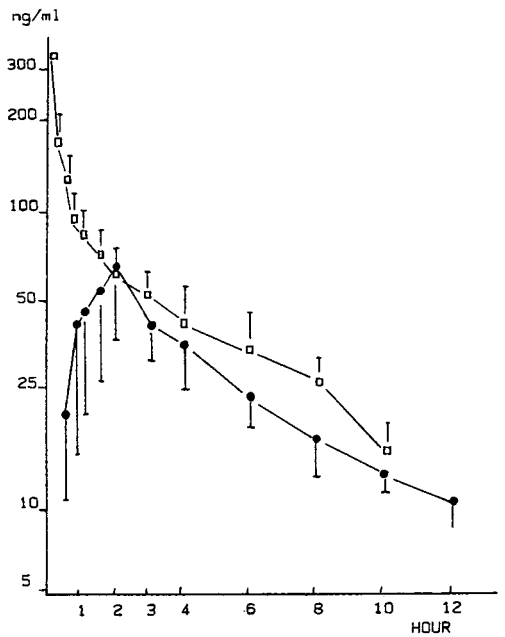

Fig. 6. Pharmacokinetic curve of I after intravenous (□) and oral (•) administration of the drug at a dose of 2 mg/kg of body weight.

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Thanks are due to Miss Zsuzsa Bárkányi for her skilful technical assistance.

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CHROMBIO. 2443

Note

Determination of naftidrofuryl in human plasma by high-performance liquid chromatography with fluorescence detection

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(First received August 3rd, 1984; revised manuscript received September 19th, 1984)

Naftidrofuryl (nafronyl) is a vasodilator used for the treatment of cerebral and peripheral vascular disorders [1—3]. Plasma concentrations of this drug have been determined both by fluorimetry [4, 5] and by high-performance liquid chromatography (HPLC) [6]. By their nature, fluorimetric assays tend to be less specific than those involving chromatography; they are also more likely to be affected by interference from metabolites and other substances.

The HPLC method described in this paper has been modified from that reported previously [6]. In the previous procedure [6], the internal standard may be subject to metabolite interference. Accordingly, the conditions for chromatography have been modified and fluorescence detection has been used in preference to ultraviolet absorption detection, in order to improve the specificity of the assay procedure whilst maintaining its sensitivity.

This present method differs in several respects from the HPLC method recently employed by Garrett [7] to investigate the pharmacokinetics of naftidrofuryl in the dog. Although both methods require fluorescence detection, they differ in the wavelengths selected, columns used and the internal standards utilised; furthermore, sub-sampling the plasma into ammonia solution (with the subsequent extraction procedure described in this method) was found to prevent the decomposition of naftidrofuryl in the plasma of those species in which this compound has a shorter half-life due to plasma esterase activity. The method here described has been successfully applied to the analysis of naftidrofuryl in both human and animal plasma.

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EXPERIMENTAL

Materials

Acetonitrile was HPLC grade (Fisons, Loughborough, U.K.). All other reagents were of analytical grade and all inorganic reagents were prepared in freshly glass-distilled water.

Standard solutions of naftidrofuryl [3-(1-naphthyl)-2-tetrahydrofurfuryl-propionic acid 2-(diethylamino)ethyl ester as the oxalate salt, Fig. 1], and the internal standard [3-(1-naphthyl)-2-tetrahydrofurfurylpropionic acid 2-(dimethylamino)ethyl ester, also as the oxalate salt, Fig. 1], were prepared in acetonitrile and stored in the dark at 4°C throughout the study. Analytical standards of these compounds were supplied by Lipha (Lyon, France).

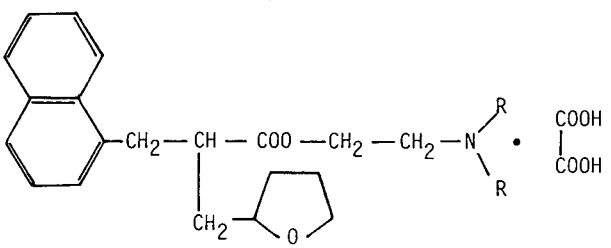

Fig. 1. Structure of naftidrofuryl (R = C_2H_5) and internal standard (R = CH_3) as their oxalate salts.

Extraction procedure

Plasma samples (1 ml), taken into balanced oxalate (12 mg ammonium oxalate and 8 mg potassium oxalate) tubes, were transferred into screw-capped centrifuge tubes (10 ml capacity) containing ammonia solution (10%, 1 ml), and spiked with internal standard (500 ng in 50 μl acetonitrile). Where plasma concentrations of naftidrofuryl exceeded the calibration range, aliquots (0.5 ml) of plasma were made up to volume (1 ml) with control plasma and analysed in the normal manner. The samples were extracted into freshly redistilled diethyl ether (5 ml) by rotary mixing for 5 min, then centrifuged for 5 min. The organic layers were removed into conical tubes and evaporated to dryness at room temperature, under a stream of nitrogen. Residues were reconstituted in mobile phase (60 μl), transferred to autosampling vials and aliquots (20 μl) were chromatographed.

Calibration procedure

Aliquots of blank human plasma (1 ml), taken into balanced oxalate tubes, were transferred into tubes containing ammonia solution (10%, 1 ml) and spiked with naftidrofuryl at concentrations equivalent to 20, 200, 400, 600 and 800 ng free base per ml and with internal standard at a fixed concentration of 500 ng/ml. Samples were then submitted to the extraction procedure described previously.

Instrumentation

The liquid chromatograph consisted of an M6000A pump (Waters Assoc., Northwich, U.K.) coupled to an LS-3 fluorescence detector (Perkin-Elmer,

Beaconsfield, U.K.) operated at an excitation wavelength of 286 nm and an emission wavelength of 335 nm. Injection was via an automatic injector (Waters' Intelligent Sample Processor, WISP 710A). Chromatograms were recorded using an HP 3380A computing integrator (Hewlett-Packard, Slough, U.K.). Peak heights and peak height ratio measurements were made manually since this gave greater precision of measurement than the computing facility of the integrator.

Chromatography

The column used for the analysis of the plasma samples was constructed of stainless steel (12.5 cm × 0.5 cm I.D.) pre-packed with Spherisorb ODS (mean particle diameter 5 μm, Hichrom, Reading, U.K.). A pre-column (5 cm × 0.2 cm I.D.) constructed of stainless steel and dry-packed with pellicular Co:Pell® ODS (particle diameter 25—37 μm, Whatman, Maidstone, U.K.) was installed in front of the analytical column to protect it from contamination.

Chromatography was performed in the reversed-phase mode, using a mobile phase consisting of acetonitrile (70%, v/v) in sodium dihydrogen orthophosphate buffer (0.2%, w/v, containing 3 ml/l of 1 *M* orthophosphoric acid). The mobile phase was passed through the column at a flow-rate of 3 ml/min.

Fig. 2 illustrates the separation of the internal standard and naftidrofuryl, the retention times of which were 6.5 and 8.6 min, respectively. Retention times were sensitive to both the pH and phosphate concentration in the mobile phase which were, therefore, carefully controlled.

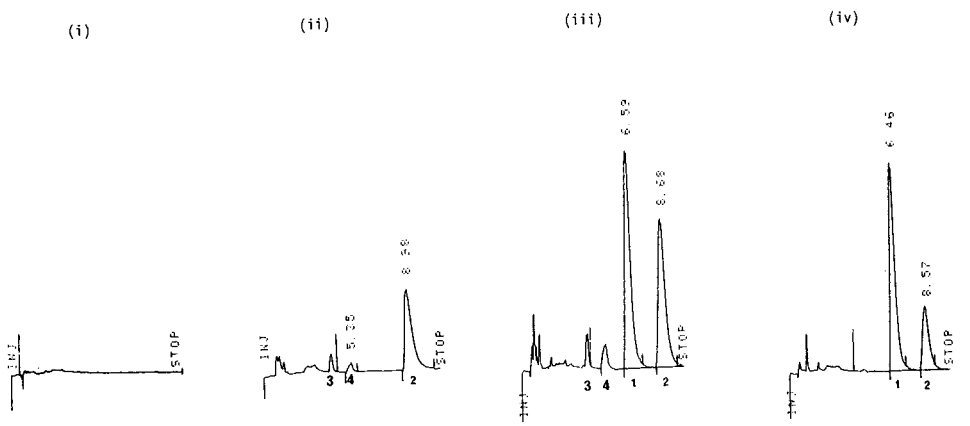

Fig. 2. Chromatograms of (i) pre-dose (control) plasma; (ii) post-dose plasma (no internal standard added); (iii) post-dose plasma plus internal standard; (iv) spiked calibration standard, 200 ng/ml naftidrofuryl plus internal standard. Conditions: column, Spherisorb ODS (12.5 cm × 0.5 cm I.D.); mobile phase, acetonitrile (70%, v/v) in aqueous sodium dihydrogen orthophosphate (0.2%, w/v, containing 3 ml/l of 1 *M* orthophosphoric acid); flow-rate 3 ml/min; fluorescence excitation 286 nm, emission 335 nm.

Studies in human subjects

The method of analysis was applied to plasma samples obtained during a study of naftidrofuryl administered as 300 mg of the oxalate salt (Praxilene®, Lipha) to five human subjects. The conditions of the study were similar to those described previously [8].

RESULTS AND DISCUSSION

Precision and accuracy

Extraction and measurement at each of three concentrations in plasma over the calibration range, was repeated on five separate occasions. The precision of measurement of the assay, as indicated by the coefficient of variation of peak height ratio measurements of drug to internal standard, was ± 10% at 20 ng/ml, ± 1% at 300 ng/ml and ± 1% at 500 ng/ml.

Daily calibration lines were constructed from duplicate measurements at each of six concentrations over the calibration range 0 to 800 ng/ml. Plots of peak height ratio against concentration were linear ($y = a + bx$), where y is the peak height ratio and x is the concentration of naftidrofuryl free base. The mean standard error of the fitted lines, an index of the accuracy of the measurement, was ± 28 ng/ml.

Recovery

The recovery of internal standard (500 ng/ml) from plasma (1 ml) was determined by comparison of peak height ratio measurements of internal standard to naftidrofuryl, of standards taken through the extraction procedure, to those injected into the chromatograph without extraction. The mean recovery of internal standard was 92 ± 3% S.D. ($n = 11$). The mean recovery of naftidrofuryl from plasma was determined by comparison of peak height ratios of extracted standards corrected for 100% recovery of internal standard, with those of non-extracted standards; the mean recovery was 88 ± 2% S.D. (Table I).

TABLE I

RECOVERY OF NAFTIDROFURYL ADDED TO PLASMA OVER THE CONCENTRATION RANGE 100—500 ng/ml

Concentration of naftidrofuryl (free base) (ng/ml)	Mean peak height ratios of non-extracted standards	Mean peak height ratios of standards extracted from plasma	Mean relative recovery (%)	Mean overall recovery of naftidrofuryl*
100	0.17	0.16	94	86
200	0.33	0.32	97	89
300	0.49	0.46	94	86
400	0.64	0.61	95	87
500	0.79	0.78	99	91

*Corrected for 100% recovery of the internal standard.

Specificity and limits of detection

No peaks with retention times similar to those of naftidrofuryl or internal standard were present in extracts of pre-dose (control) plasma. No peak with the same retention time as the internal standard was present in extracts of post-dose plasma analysed without the addition of internal standard (Fig. 2), indicating the absence of metabolite interference with the internal standard used.

The limit of detection of naftidrofuryl free base was taken as the lowest point on the calibration line (20 ng/ml). This was the minimum concentration detected by the assay based on the analysis of 1 ml of plasma using 500 ng/ml internal standard and maintaining all peaks on scale. Where a narrower calibration range was desired, the method was successfully applied to achieve a lower limit of detection of naftidrofuryl of 4 ng/ml.

Concentrations of naftidrofuryl in human plasma

The mean concentrations of naftidrofuryl in the plasma of five human subjects after single oral doses of 300 mg of naftidrofuryl oxalate, analysed by the HPLC method described are presented in Table II. Plasma concentrations declined monoexponentially with a half-life of 2.0 ± 0.4 h S.D., similar to that reported previously [6].

TABLE II

MEAN CONCENTRATIONS (± S.D.) OF NAFTIDROFURYL FREE BASE (ng/ml) IN THE PLASMA OF FIVE HUMAN SUBJECTS AFTER ADMINISTRATION OF SINGLE ORAL DOSES OF 300 mg OF NAFTIDROFURYL OXALATE

Time (h)	Concentration (ng/ml, ± S.D.)
Pre-dose	<20
0.25	156 ± 54
0.5	548 ± 227
0.75	600 ± 293
1	559 ± 115
1.5	446 ± 98
2	313 ± 89
3	162 ± 48
4	104 ± 31
5	63 ± 19
6	49 ± 12
8	<20

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We are grateful to Lipha, Lyon, France, for financial support.

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CHROMBIO. 2451

Note

Analytical method for the quantification of famotidine, an H_2-receptor blocker, in plasma and urine

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(First received August 2nd, 1984; revised manuscript received October 25th, 1984)

The new H_2-receptor blocker famotidine (Fig. 1) is a potent inhibitor of gastric acid secretion in man [1,2]. In anticipation of the analysis of biological samples from clinical studies, a selective and sensitive analytical method was developed for the quantification of famotidine both in plasma and urine samples. A straightforward high-recovery sample preparation procedure was effected by using silica-gel cartridges to adsorb the hydrophilic drug from the biological matrices. The described sample preparation scheme permitted the quantification of famotidine in plasma (1 ml) by UV detection (λ_{max} = 267 nm, $\epsilon = 1.58 \cdot 10^4$) alone. Resolution of famotidine from endogenous interferences was accomplished on an RP-8 column with a 0.019 *M* phosphoric acid—acetonitrile (90:10) mobile phase.

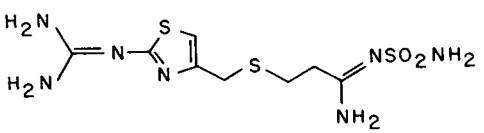

Fig. 1. Chemical structure of famotidine.

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EXPERIMENTAL

Materials

All extractions were performed on a Baker-10 extraction system using Bond-Elut 2.8-ml silica columns (Analytichem International, No. 601303). The water was of Milli-Q quality (18 M Ω cm resistivity), cartridge sequence: Super-C, Ion-Ex, Ion-Ex, Organex-Q. Glacial acetic acid and phosphoric acid (HPLC grade) were purchased from Fisher. Methanol, acetonitrile, and N,N-dimethylformamide (DMF), all HPLC grade, were obained from Burdick & Jackson Labs. Frozen heparinized control plasma was purchased from Sera-Tec Biologicals (New Brunswick, NJ, U.S.A.). Control human urine consisted of pooled urine collections from Merck laboratory personnel and placebo treatment groups from clinical studies. A famotidine analytical standard was supplied by the Chemical Data Section of Merck Sharp & Dohme (Rahway, NJ, U.S.A.). The prepared samples were evaporated in a Organomation analytical evaporator.

Glassware

All glassware was washed in a laboratory dishwasher with detergent at pH 12, rinsed with distilled water and dried at 60° C.

Instrumentation and accessories

The high-performance liquid chromatographic (HPLC) instrumentation consisted of Waters Assoc. 703 HPLC system equipped with a 730 data module, a 720 system controller, a WISP 710B automatic injector (limited-volume inserts), a Model 441 UV detector (254 nm and 0.01 a.u.f.s. for urine) or a Model 480 UV detector (267 nm and 0.002 a.u.f.s. for plasma). The analytical column was an Altex RP-8, particle size 5 μm (25 cm × 4.6 mm). The pre-column was purchased from Brownlee Labs: RP-8, 10 μm, 4 cm Spherisorb.

Mobile phase preparation

The analyses were performed at room temperature (22—25° C). The mobile phase consisted of a mixture of 0.019 *M* phosphoric acid—acetonitrile (90:10, v/v). The mobile phase components were filtered through a 0.45-μm Nylon 66 membrane separately. After sparging the mobile phase with helium or argon for 5 min, the mobile phase containing bottle was covered. Long-term sparging resulted in evaporation of acetonitrile from the mobile phase.

Stability in plasma

Famotidine was stable in plasma for at least five weeks at −15° C. When stored at −15° C for a period of one year, the amount of famotidine remaining in the sample was approximately 80% of the original concentration.

Stability in urine

Drug was unstable in urine at room temperature (22—25° C); there was a 10—15% loss of drug when stored at room temperature for a period of 4 h. Famotidine was stable in urine for at least five weeks at −15° C and for at

least two days at 5°C. The amount of famotidine remaining in the sample after storage at −15°C for one year was between 84% and 94%.

Famotidine was stable in methanol for at least one week at 5°C. All stock standards were prepared weekly and all working standards were prepared daily from the appropriate stock standard.

Preparation of urine sample

Frozen (−15°C) specimens were thawed to ⩽ 5°C, shaken and sampled. To a 13-ml centrifuge tube were added 1.0 ml of urine and 0.1 ml of methanol and the sample was mixed by vortex. To a 2.8-ml silica cartridge, attached to a Baker-10 extraction system under vacuum, were added 1.0 ml methanol, to activate the cartridge, 1.0 ml water, 0.5 ml of the urine—methanol mixture prepared above, followed by 3.0 ml water. The vacuum was relieved and 2-ml collection tubes were positioned under the cartridges. The system was evacuated and 2.0 ml DMF—water (50:50) mixture were added to elute famotidine. The eluate was placed in WISP limited-volume insert and 150 μl were injected in the HPLC system. The pre-column was changed every 100—125 injections.

Preparation of plasma sample

The frozen plasma samples were thawed by placing them at approximately 5°C overnight. The next day the specimens were mixed by vortex and centrifuged. To a 2.8-ml silica cartridge, attached to a Baker-10 extraction system under vacuum, were added 1.0 ml methanol, to activate the cartridge, 1.0 ml water, 1.0 ml plasma, and 5.0 ml water. The vacuum was relieved and 2-ml collection tubes were positioned under the cartridges. The system was evacuated and 2.0 ml of acetonitrile were added to elute famotidine. The eluate was evaporated to dryness under a stream of dry nitrogen at 40°C. The residue was reconstituted in 0.2 ml of 0.017 *M* glacial acetic acid. The acid was transferred to a WISP limited-volume insert and 150 μl were injected in the HPLC system.

Quantification

A standard curve was prepared and assayed daily with the unknown samples. The standards were prepared by mixing 1.0 ml of control fluid with a famotidine standard prepared in methanol. At the start of the clinical study, known concentrations of famotidine in biological fluid were prepared and frozen at −15°C. With each days' analyses, these quality control standards were assayed with the unknown samples. Unknown sample concentrations were calculated from an unweighted (weight, $W = 1$) linear regression analysis of the standard curve data (urine = peak area; plasma = peak height).

Selectivity

Famotidine, itself, was well resolved from endogenous interferences, as shown in Figs. 2 and 3. However, the metabolism of famotidine results in the formation of small amounts of an S-oxide metabolite. The famitodine S-oxide was not retained on the analytical column described in this method. The metabolite can be retained and adequately resolved from chromatographic

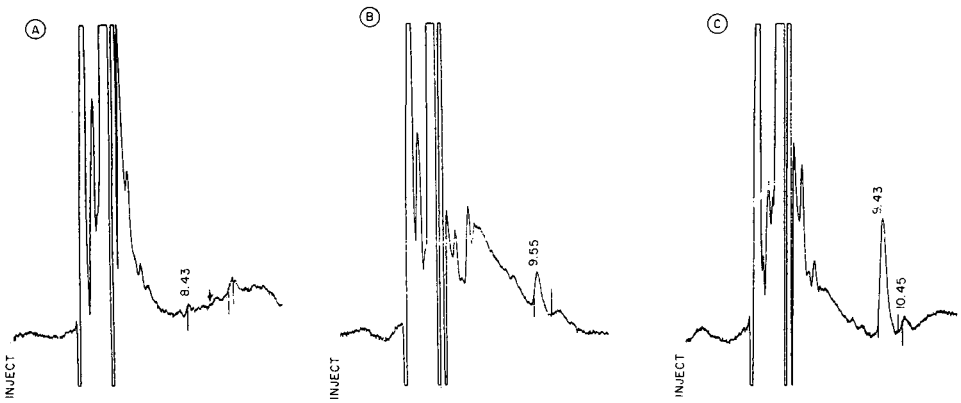

Fig. 2. Chromatograms of famotidine in plasma. (A) Plasma blank; (B) 5 ng/ml standard of famotidine in plasma; (C) normal subject dosed with 10 mg famotidine, 7 h post-dose, 13.6 ng/ml.

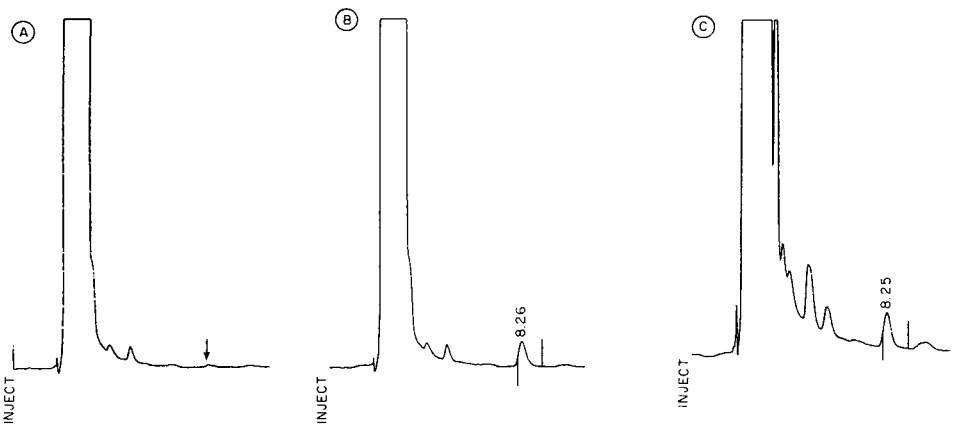

Fig. 3. Chromatograms of famotidine in urine. (A) Urine blank; (B) 500 ng/ml standard of famotidine in urine; (C) normal subject dosed with 20 mg famotidine, 12—24 h collection post-dose, 0.68 μg/ml.

interferences on a silica column. Unfortunately, metabolite concentrations in human urine were too low for reliable quantification.

RESULTS AND DISCUSSION

Anticipating peak plasma famotidine levels in the 5—10 ng/ml range for a 5-mg dose of the drug, initial detection schemes were centered around pre- and post-column derivatization of famotidine with fluorescence or UV tags. No acceptable derivatization method was developed for either the sulfonamide or the guinidine moieties [3—6]. Oxidation of famotidine on either glassy or porous carbon also was unsuitable for routine analysis.

Apparently, famotidine was so highly associated with plasma proteins that any attempt to clean-up plasma by protein precipitation resulted in low drug recovery.

TABLE I

SOLUBILITY OF FAMOTIDINE AT 20°C

Solvent	Solubility (%, w/v)
N,N-Dimethylformamide	80
Glacial acetic acid	50
Methanol	0.3
Water	0.1
Ethanol (100%)	<0.01
Ethyl acetate	<0.01
Chloroform	<0.01

TABLE II

INTRA- AND INTER-DAY VARIABILITY OF FAMOTIDINE

Sample	Intra-day		Inter-day*		
	Mean**	C.V. (%)	Mean***	*n*	C.V. (%)
Urine § *(μg/ml)*					
0.5	0.5	4.8			
1.0	1.0	1.6	0.9 (1.0)	31 (24)	6.0 (12.3)
2.0	2.0	5.9			
5.0	5.0	2.1			
10.0	10.0	4.1	9.0	32	3.8
15.0	15.1	1.0			
20.0	19.8	4.1	17.7 (19.8)	32 (22)	4.0 (4.7)
30.0	29.9	4.7			
Plasma (ng/ml)					
5.0	5.2	8.9			
10.0	9.8	5.7	10.8 (10.4)	9 (38)	9.0 (9.1)
20.0	19.8	5.9			
30.0	29.3	7.2			
40.0	40.0	7.6	40.6 (39.3)	9 (38)	5.3 (6.0)
50.0	51.0	6.7			

*Values determined from an additional study, new quality control preparations, are given between parentheses.
**Mean calculated concentration, $n = 6$.
***Quality control standards.
§ Nominal concentration.

The solubility of famotidine in many organic solvents is low (see Table I) and extraction of the drug ($pK_a = 6.7$) from plasma (pH 4, 6 or 8) with ethyl acetate gave highly variable recoveries (20—60%). In addition, these extracts, when evaporated and reconstituted with mobile phase, contained many endogenous interferences which could not be resolved from famotidine using a variety of HPLC columns. Liquid—solid extraction of drug from plasma or urine on different bonded phase cartridges (Baker) was less than adequate, yielding either low recovery or background interferences. On the other hand, silica cartridges were adequate, since famotidine, containing polar substituents

like other H_2-receptor blockers, adsorbs well to silica [7, 8]. Endogenous chromatographic peaks in the eluate from the silica cartridges were resolved from famotidine using a C_8 analytical column. The use of two different supports, silica and C_8, for the method, in toto, likely contributes to the very clean backgrounds. The mean recoveries of famotidine over the range of the standard curve were 90.3 ± 1.0% and 90.2 ± 3.7% for plasma and urine, respectively. The recovery was based upon the peak height (area) of a direct injection of the corresponding standard.

The limit of reliable quantification was 5.0 ng/ml for plasma and 500 ng/ml for urine. Drug was stable in the injection solvent for 24 h at 20—22°C. Analytical method accuracy and precision data are presented in Table II. The calibration curves were linear within the concentration range of interest (urine 0.5—30.0 μg/ml; plasma 5.0—50.0 ng/ml). The mean regression line (fifteen standard curves) for urine had a correlation coefficient of 0.99992 and for plasma (seven standard curves) 0.99839.

This method affords a simple, sensitive and direct method for the determination of famotidine levels in clinical study specimens.

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Note

Determination of levobunolol and dihydrolevobunolol in blood and urine by high-performance liquid chromatography using fluorescence detection

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Levobunolol, 5-[*tert.*-butylamino)-2'-hydroxypropoxy]-3,4-dihydro-1(2*H*)-naphthalenone hydrochloride (LB), and its pharmacologically active metabolite dihydrolevobunolol, 5-[(*tert.*-butylamino)-2'-hydroxypropoxy]-1,2,3,4-tetrahydro-1-naphthol (DHLB), are effective β-adrenoreceptor antagonists [1, 2] (Fig. 1). The biotransformation of LB has been investigated in several animal species as well as in humans [2—6].

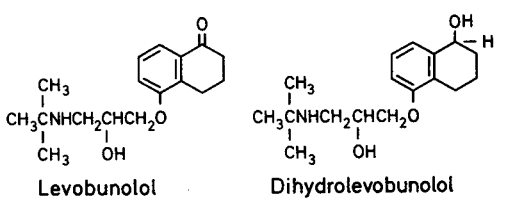

Fig. 1. Structures of levobunolol and its active metabolite dihydrolevobunolol.

Because of its high potency, LB may be administered in very low oral doses (1—8 mg per day) [1]. Therefore, for pharmacokinetic studies a sensitive and specific assay method was required for the determination of the active compounds in biological material at the lower nanogram level.

Several methods have been described for the determination of β-adrenoceptor-blocking agents. These methods comprise either the use of gas—liquid chromatography after appropriate derivatization [7, 8], gas—liquid chromatography combined with mass spectrometry [9], or high-performance liquid chromatography (HPLC) with spectrofluorometric or UV-absorption detection [10].

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Initial studies indicated insufficient sensitivity when analysing for LB or DHLB by means of HPLC using UV detection. The lower detection limit for LB at 255 nm was 100 ng/ml of plasma or urine, for DHLB only 1000 ng/ml.

DHLB exhibits a strong intrinsic fluorescence, thus permitting its sensitive detection. Although LB does not show native fluorescence, it can be detected after chemical reduction of its acetophenone-like structure, resulting in the formation of DHLB.

This paper describes an HPLC method for the determination of the sum of LB and DHLB as well as of DHLB in blood and urine at high sensitivity, using fluorometric detection. The applicability to pharmacokinetic studies is demonstrated by typical results.

EXPERIMENTAL

Standards and reagents

Levobunolol · HCl and dihydrolevobunolol · HCl were obtained from the Chemical Department, Gödecke Research Institute, Freiburg, F.R.G.; metoprolol tartrate (internal standard) was commercially purchased (Beloc®, Astra, F.R.G.). Solvents and chemicals were of analytical grade and used without further purification, except in the case of methanol, where "Uvasol"-grade (E. Merck, Darmstadt, F.R.G.) was used, and benzene which was distilled prior to use. Aqueous solutions were prepared with "Millipore" water (Millipore, Neu Isenburg, F.R.G.).

Chromatographic system

A Model 2/2 liquid chromatograph (Perkin-Elmer, Überlingen, F.R.G.) fitted with a 7105 injection valve (Rheodyne, Cotati, CA, U.S.A.) was used with a column packed with μBondapak C_{18} (250 mm × 4.6 mm I.D., 10 μm) (Waters, Königstein, F.R.G.). The mobile phase consisted of methanol—water (48:52) containing 0.4% phosphoric acid and 0.2% heptane sulphonic acid sodium salt at a flow-rate of 2 ml/min. Elution peaks were detected by means of a 650-10 LC fluorimeter (Perkin-Elmer, Überlingen, F.R.G.) set at an excitation wavelength of 225 nm and emission at 295 nm. The slit-width in both cases was 10 nm.

Retention times and peak areas were determined using an Autolab System I computing integrator (Spectra Physics, Darmstadt, F.R.G.).

Sample preparation

Blood samples were collected in pre-heparinized syringes and added directly to Pyrex tubes containing sufficient acetonitrile to obtain a 20% acetonitrile concentration in blood (5:1). The closed tubes were immediately shaken vigorously for 10 sec. The tubes were frozen at −20° C in an upright position.

Determination of DHLB in blood

A 1-ml blood sample containing 20% of acetonitrile was placed in a 10-ml conical glass tube; 20 ng of metoprolol (10 μl of an aqueous solution) were added as an internal standard. After dilution with 1 ml of water the pH was

adjusted to 9.8—10.2 by adding seven to ten drops of 0.1 *M* sodium hydroxide. The mixture was extracted twice with 2 ml of benzene for 30 min and 20 min, respectively, by means of an automatic shaking machine.

After each extraction step, the phases were separated by centrifugation. The combined benzene phases were evaporated to dryness in a tapered flask on a rotary evaporator at 30°C. The residue was dissolved in about 50 μl of the mobile phase; 10—50 μl were injected onto the HPLC column.

Determination of the sum of LB and DHLB (total amount) in blood

A 1-ml sample of blood containing 20% of acetonitrile was fortified with the internal standard metoprolol and cleaned up when analysing for DHLB. The benzene phase was evaporated in a 10-ml conical tube on a rotary evaporator to dryness, redissolved in 200 μl of methanol and approximately 5 mg of sodium borohydride were added. The closed tube was left at room temperature for 30 min. After reduction, the sample was diluted with 1 ml of water, about 0.3 g of sodium chloride was added and the mixture was extracted for 20 min with 3 ml of benzene. After centrifuging, the benzene layer was transferred into a 5-ml tapered flask and evaporated. The residue was analysed for DHLB as described above. LB levels were calculated by the difference of the total content (LB + DHLB) and the measured amount of DHLB.

Determination of LB and DHLB in urine

To 0.02—1 ml of urine (volume depending on the expected concentration, to ensure detector linearity) were added 200 ng of metoprolol followed by 1 ml of 0.2 *M* sodium borate buffer pH 10.2 (Sörensen) and, if necessary, the pH was adjusted to 9.8—10.2 using 0.1 *M* sodium hydroxide. After the addition of approximately 0.5 g of sodium chloride, the mixture was extracted for 30 min with 4 ml of benzene and analysed accordingly for DHLB, or LB and DHLB after reduction, as described for the analysis of blood samples.

Quantitation

Standard curves were prepared by adding LB and DHLB (2—80 ng/ml) to drug-free samples of blood (containing 20% of acetonitrile) or urine, and processing these standards according to the assay procedure.

The precision of this method was validated by replicate analysis (n = 5) of spiked blood samples (Table I).

RESULTS AND DISCUSSION

Metoprolol proved to be well suited as an internal standard, having similar extraction properties and chromatographic and fluorescence characteristics to DHLB. In addition, when reducing LB to DHLB no chemical changes occurred.

The elution times of DHLB and metoprolol under the conditions described above were approximately 3.8 min and 4.6 min, respectively. No interfering peaks were visible in blank human blood samples at the retention times of DHLB and metoprolol, respectively. Representative chromatograms are shown in Fig. 2.

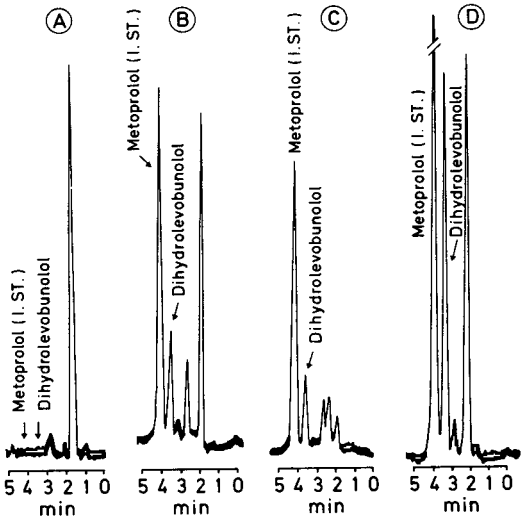

Fig. 2. Chromatograms of DHLB in blood samples. (A) Control human blood sample; (B) human blood sample spiked with 10 ng of LB and 20 ng of internal standard, metoprolol, after subsequent reduction to DHLB; (C) human blood sample analysed directly for DHLB 6 h after oral administration of 12 mg of LB · HCl to a human volunteer (DHLB concentration was found to be 7 ng/ml); (D) dog blood sample analysed for its total content (LB + DHLB) derived 3 h after oral administration of 10 mg/kg LB · HCl to a dog (concentration was found to be 106 ng/ml) (50 ng of internal standard used).

The quantification of LB was achieved by two separate steps: first, DHLB was determined directly by native fluorescence (*A*); secondly, the sum of LB and DHLB was determined following the reduction of LB to DHLB with sodium borohydride in methanol (*B*). LB levels were obtained by the difference between *B* and *A*.

TABLE I

MEANS, STANDARD DEVIATIONS AND RELATIVE STANDARD DEVIATIONS OF PEAK HEIGHT RATIOS OF DIHYDROLEVOBUNOLOL AND LEVOBUNOLOL (AFTER SUBSEQUENT REDUCTION) TO METOPROLOL IN HUMAN BLOOD

Concentration (ng/ml)	Mean peak height ratio	S.D.	C.V. (%)
Dihydrolevobunolol (n = 5)			
2	0.069	0.002	2.9
5	0.163	0.011	6.75
10	0.303	0.002	0.66
20	0.590	0.010	1.70
80	2.264	0.04	1.77
Levobunolol (n = 5)			
2	0.091	0.005	5.50
5	0.196	0.012	6.12
10	0.366	0.018	4.91
20	0.718	0.008	1.11
80	2.48	0.060	2.42

The lower limit of detection was approximately 0.5—1 ng/ml in blood or urine. The recoveries for LB, DHLB as well as metoprolol from blood and urine were about 90%. The reduction of LB to DHLB was quantitative.

Calibration curves for DHLB and LB showed a linear correlation between peak area ratios and the concentrations described (r = 0.999), the intercepts being practically zero.

The coefficient of variation obtained from replicate analysis (n = 5) ranged from 1% to 7% (Table I).

Earlier studies disclosed that LB present in blood is susceptible to enzymatic reduction resulting in the formation of DHLB [11], even in vitro. To avoid falsification of the in vivo concentration of DHLB or LB in blood, it was important to stop the enzymatic conversion of LB to DHLB immediately after withdrawal of blood samples. This could be achieved by adding 20% of acetonitrile to the blood samples instantly after withdrawal, thus ensuring the separate determination of total levobunolol and dihydrolevobunolol. The addition of acetonitrile to blood does not impair the assay method. Because of the risk of enzymatic reduction taking place in blood, e.g. during centrifugation, we refrained from using plasma samples.

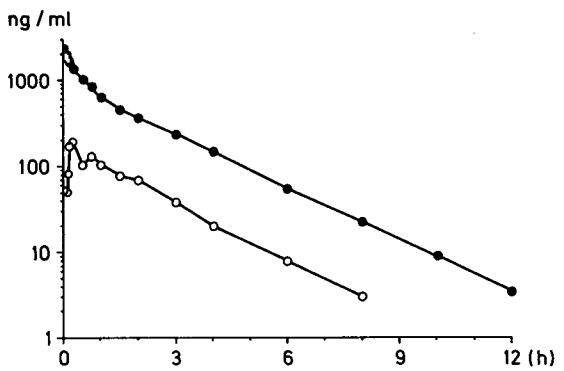

Fig. 3. Blood levels of total levobunolol (LB + DHLB) (•) and dihydrolevobunolol (DHLB) (○) following an intravenous injection of 5 mg/kg levobunolol · HCl to a beagle dog.

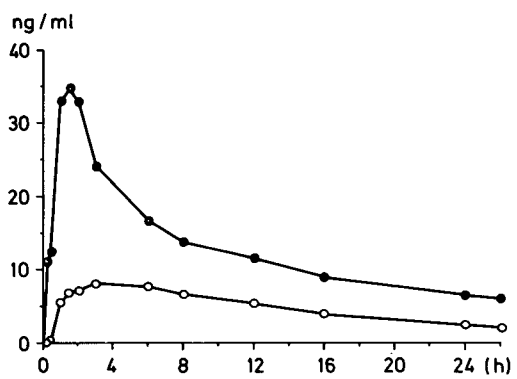

Fig. 4. Blood levels of total levobunolol (LB + DHLB) (•) and dihydrolevobunolol (○) following an oral dose of 12 mg of levobunolol to a human volunteer.

The enzymatic conversion of LB in blood mentioned above was confirmed by the following in vitro studies: 10 min after the addition of 100 ng/ml LB · HCl to freshly withdrawn human blood samples, 6—7% of the compound was converted to DHLB. After an incubation time of 90 min, the amount of DHLB had increased to 20—25% and a steady state was reached. However, in blood samples spiked with DHLB no reverse reaction to form LB was observed under the above conditions. In experiments for testing the stability of LB and DHLB, blood samples containing 20% of acetonitrile spiked separately with both substances (50 ng/ml) were analysed 24 h after remaining at room temperature, as well as after having been stored for a two-month period at —20°C. No changes in the concentrations were seen.

The applicability of the method is demonstrated by the blood level profiles obtained following an intravenous dose of 5 mg/kg LB · HCl to a dog (Fig. 3) and after oral administration of 12 mg to a human volunteer (Fig. 4).

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Note

High-performance liquid chromatographic procedure for the determination of a new positive inotropic agent, 3,4-dihydro-6-[4-(3,4-(dimethoxybenzoyl)-1-piperazinyl]-2(1*H*)-quinolinone, in human plasma and urine

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3,4-Dihydro-6-[4-(3,4-(dimethoxybenzoyl)-1-piperazinyl]-2(1*H*)-quinolinone (I; OPC-8212) has been reported to have a positive inotropic action [1—3]. Compound I preferentially increases myocardial contactile force without affecting heart rate [2]. Heart failure has been treated with digitalis and catecholamines, but digitalis elicits a variety of responses from different individuals, has a very narrow therapeutic margin and involves the risk of occasional fatal arrhythmia [4—6], and catecholamines are available only in intravenous dosage forms [7, 8]. Compound I does not present these problems and so was selected for further evaluation [2]. It was found to elicit pharmacological activities different from those of other compounds of similar clinical application. It is also devoid of an arrhythmia effect and is considered to be a potential selective, positive inotropic agent for oral use [9, 10]. We have already reported the pharmacokinetics of I in several animals [11]. Therefore, to study the pharmacokinetics in humans, an attempt was made to develop a method to quantitate I in human plasma and urine that would be simple, suitable for routine analysis and highly sensitive.

In this paper, we describe a simple method for the determination of I in human plasma and urine using high-performance liquid chromatography (HPLC). The results of the HPLC assay for plasma and urine concentrations of I after a single oral dose to healthy male subjects are also described.

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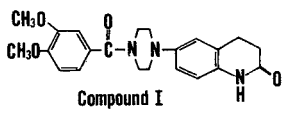
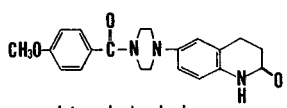
MATERIALS AND METHODS

Drug and chemicals

Compound I (Fig. 1) and the internal standard, 3,4-dihydro-6-[4-(4-methoxybenzoyl)-1-piperazinyl]-2(1*H*)-quinolinone (Fig. 1), were supplied by the Laboratories of Medicinal Chemistry, Tokushima Research Institute, Otsuka Pharmaceutical (Tokushima, Japan). A standard solution of I was prepared in a hydrochloric acid—methanol solution (1 mg/ml) and was diluted with methanol as required before use. The internal standard solution was prepared in a methanol solution. Acetonitrile, chloroform, methanol, acetic acid, sodium hydroxide and potassium nitrate were of analytical-reagent grade and were purchased from Wako (Tokyo, Japan). Human control plasma was supplied by the Japan Red-Cross Blood Supply Center, Tokushima Branch (Tokushima, Japan).



Compound I



Internal standard

Fig. 1. Chemical structure of I and internal standard.

Chromatography

The HPLC separations were carried out using a Waters Assoc. ALP/GCP 204 compact routine system (Model 6000A pump, Model 440 absorbance detector equipped with 280-nm filter kit, WISP 710B autosampler; Waters Assoc., Milford, MA, U.S.A.). The peak height ratio was calculated by a Shimadzu Chromatopac C-R1B integrator system (Shimadzu Seisakusho, Kyoto, Japan). A μBondapak C_{18} reversed-phase column (30 cm × 3.9 mm I.D., particle size 10 μm, Waters Assoc.) was used with acetonitrile—0.01 *M* potassium nitrate—acetic acid (30:70:1) as the mobile phase at a flow-rate of 1.0 ml/min.

Sample preparation

The internal standard (400 ng for plasma, 2 μg for urine) was added to 1 ml of human plasma or 0.5 ml of human urine (in the case of high concentrations, plasma or urine was diluted with control plasma or urine, respectively). The contents were stirred on a vortex mixer followed by the addition of 1 ml of 1 *M* sodium hydroxide and 5 ml of chloroform and shaken mechanically for 10 min. The mixture was centrifuged for 10 min at 1700 *g* and 4 ml of the chloroform layer were evaporated to dryness under a stream of air. The residue was redissolved in methanol (100 μl), and an aliquot (20 μl) was injected into the HPLC system and analysed.

Preparation of the calibration curve

The diluted standard I solution was added to a portion of plasma or urine.

Four replicate plasma samples were prepared in the concentration ranges of 20–1000 ng/ml for plasma and 0.2–5.0 μg/ml for urine, and extracted using the extraction procedures described above. After HPLC analysis the peak height ratio of I to the internal standard calculated from the chromatograms was plotted on the ordinate, and unchanged I concentrations were plotted on the abscissa. The calibration curve was constructed by a least-squares regression method from the peak height ratio versus the corresponding unchanged concentration of I in the test samples.

RESULTS AND DISCUSSION

Chromatograms obtained by the above procedures using plasma or urine samples with and without I and the internal standard are given in Figs. 2 and 3. No significant interference was observed in the regions for I and the internal standard on the chromatogram. Good chromatographic separation was obtained with distinct retention times for I and the internal standard of 8.5 and 12.0 min, respectively. The time required for analysis of a single sample was 14 min. The linearity, reproducibility and precision of the calibration curves are given in Tables I and II. Calibration curves were linear in concentrations of 20–1000 ng/ml of plasma and 0.2–5 μg/ml of urine, and passed through the origin. At a plasma concentration of 100 ng/ml the peak height ratio was 0.37

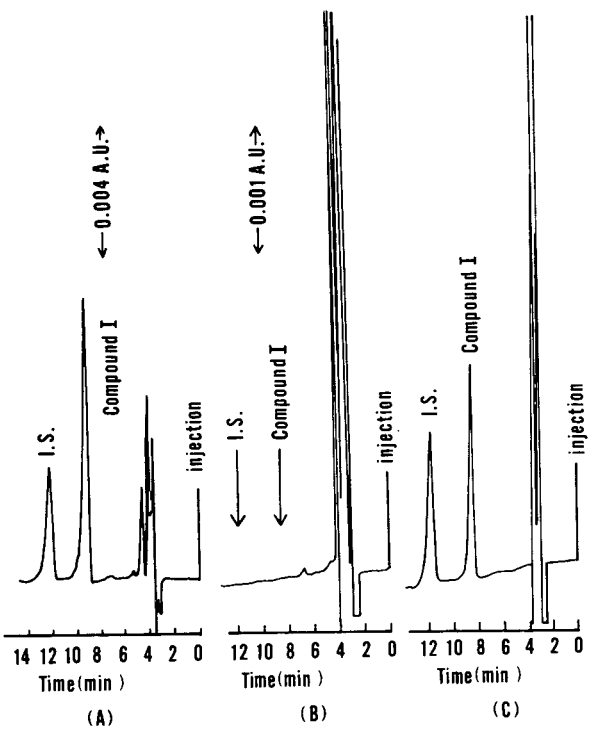


Fig. 2. (A) Representative chromatogram of human plasma spiked with internal standard (400 ng/ml) after the administration of the drug. (B) Chromatogram of blank human plasma. (C) Chromatogram of I (20 ng) and internal standard (20 ng).

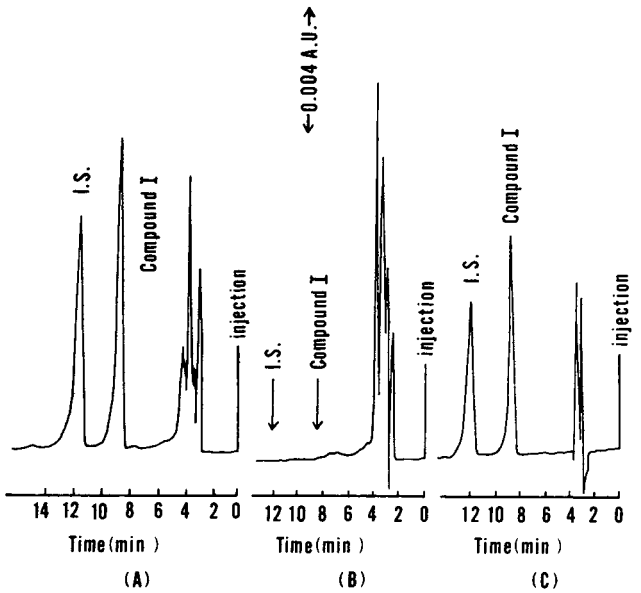


Fig. 3. (A) Representative chromatogram of human urine spiked with internal standard (2 μg/ml) after the administration of the drug. (B) Chromatogram of blank human plasma. (C) Chromatogram of I (100 ng) and internal standard (100 ng).

± 0.02 with a coefficient of variation (C.V.) of 4.1%. At other concentrations the C.V. was smaller than 4.1% indicating little deviation of the peak height ratio. The equation for the resulting line was $y = 271.9945x + 1.8947$ with a correlation coefficient of 0.9995. At a urine concentration of 1.0 μg/ml the peak height ratio was 0.37 ± 0.01 with a coefficient of variation of 1.3%. At other concentrations the C.V. was smaller than 1.3% indicating little deviation from the peak height ratio. The equation for the resulting line was $y = 2.5988x + 0.0278$ with a correlation coefficient of 0.9999. The plasma and urine concentrations of I calculated from the calibration curve were comparable to the corresponding amount of the drug added to the plasma, being 97—118% and

TABLE I

LINEARITY AND PRECISION OF HPLC ASSAY OF I IN HUMAN PLASMA WITH THE INTERNAL STANDARD METHOD

A 1-ml volume of plasma was used.

Concentration of the drug added to plasma (ng/ml)	Peak height ratio (mean ± S.D.)*	C.V. (%)	Recalculated concentration (ng/ml)	Percentage of theoretical concentration
20	0.08 ± 0.00	0.0	23.7	118
50	0.19 ± 0.01	2.7	52.8	106
100	0.37 ± 0.02	4.1	101.7	102
500	1.77 ± 0.05	2.9	483.9	97
1000	3.70 ± 0.05	1.4	1007.5	101

*Results from four replicate samples were used.

TABLE II

LINEARITY AND PRECISION OF HPLC ASSAY OF I IN HUMAN URINE WITH THE INTERNAL STANDARD METHOD

A 0.5-ml volume of urine was used.

Concentration of the drug added to urine (μg/ml)	Peak height ratio (mean ± S.D.)*	C.V. (%)	Recalculated concentration (μg/ml)	Percentage of theoretical concentration
0.2	0.07 ± 0.00	0.0	0.21	105
1.0	0.37 ± 0.01	1.3	0.99	99
2.0	0.76 ± 0.01	0.7	1.99	100
5.0	1.92 ± 0.02	1.0	5.00	100

*Results from four replicate samples were used.

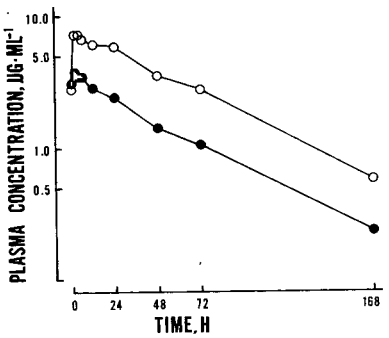


Fig. 4. Plasma concentration of I after oral administration of I to human male subjects. •, Single oral dose of 60 mg per body; peak level 3.77 μg/ml; half-life 44.50 h; AUC 228.93 μg h ml^{-1}. ○, Single oral dose of 120 mg per body; peak level 7.38 μg/ml; half-life 49.28 h; AUC 553.36 μg h ml^{-1}.

99—105% of amount of the drug added to the plasma and urine, respectively. The plasma and urine concentrations of I were determined as described above.

Fig. 4 shows the time course of the plasma concentration of I after single oral doses of 60 and 120 mg per body in healthy male subjects. The plasma levels 4 h after oral doses of 60 and 120 mg per body reached peak levels of 3.77 and 7.38 μg/ml and then declined with apparent biological half-lives of 44.50 and 49.28 h, respectively. The areas under the curve (AUC) until ∞ h calculated by the trapezoidal rule were 228.93 and 553.36 μg h ml^{-1}, respectively. It was recognized that the plasma concentration and urinary excretion were closely related. The detection limit (< 20 ng/ml in plasma, < 0.2 μg/ml in urine) of this procedure was considered sufficient to determine plasma and urine concentrations of I, since the dose of 120 mg per body used in the present study was smaller than the predicted clinical dose which yields plasma and urine concentrations within the range of the calibration curve constructed here. For higher concentrations of I in plasma, a smaller sample than that used in this study is required. In view of these results, it was concluded that this HPLC method is a simple, highly sensitive, reproducible procedure for the determination of plasma and urine concentrations of I, and therefore a valuable

tool in the investigation of the clinical pharmacokinetics and bioavailability of this compound.

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Note

High-performance liquid chromatographic procedure for the determination of a new antithrombotic and vasodilating agent, cilostazol, in human plasma

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(First received August 8th, 1984; revised manuscript received October 5th, 1984)

Cilostazol (OPC-13013), 6-[4-(1-cyclohexyl-1*H*-tetrazol-5-yl)butoxy]-3,4-dihydro-2(1*H*)-quinolinone, is a newly synthesized compound by Nishi et al. [1] and a potential antithrombotic and vasodilating drug. Its effectiveness and mechanism of action have been described previously [2—5]. It has been reported that cilostazol has both antithrombotic and cerebral vasodilating effects [2], and one of the mechanisms is the selective inhibition of platelet cyclic AMP phosphodiesterase [3—5]. Consequently, it was suggested that cilostazol has clinical potential in the management of post-stroke syndrome and the prevention of recurrent stroke, and cilostazol is currently being evaluated in clinical studies. We were interested in the determination of plasma cilostazol levels in man for the routine therapeutic monitoring of this compound, which is valuable for the elucidation of its therapeutic effect and mechanism of action. Therefore, the establishment of a simple assay technique for routine analysis was studied.

In this paper, we describe a simple, highly sensitive and selective method for the determination of plasma cilostazol levels in man using reversed-phase high-performance liquid chromatography (HPLC). This paper also describes how plasma cilostazol levels after a single oral administration of 100 mg to healthy male volunteers were determined by this method.

MATERIALS AND METHODS

Chemicals

Cilostazol (Fig. 1A) and the internal standard (OPC-13012, Fig. 1B) were

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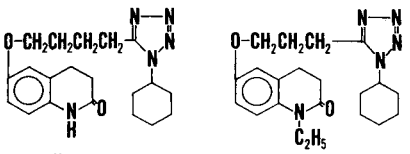

Fig. 1. Chemical structure of cilostazol (A) and internal standard (B).

supplied by the Laboratories of Medicinal Chemistry, Otsuka Pharmaceutical (Tokushima, Japan). All another reagents and solvents were of analytical grade (Wako, Tokyo, Japan).

Chromatography

The HPLC system consisted of a Waters Assoc. ALC/GPC 204 compact system (Model 6000A pump, Model 440 UV detector equipped with a 254-nm filter kit), Waters 710B autosample processor (Waters Japan, Tokyo, Japan) and Shimadzu Chromatopack C-R1B (Shimadzu, Kyoto, Japan). A μBondapak C_{18} reversed-phase column (30 cm × 3.9 mm I.D., particle size 10 μm, Waters) was used with the mixture of acetonitrile—water (42:58, v/v) as the mobile phase at a flow-rate of 1.7 ml/min.

Sample preparation

The internal standard (600 ng per 10 μl of methanol) and acetonitrile (4.0 ml) were added to human plasma (1.0 ml). The contents were stirred on a vortex mixer and centrifuged at 1700 *g* for 10 min. The supernatant was transferred to another centrifuge tube, and the acetonitrile was evaporated under a stream of air. To the residue, 0.2 *M* sodium hydroxide (1.0 ml) and chloroform (5.0 ml) were added and the mixture was shaken and centrifuged at 1700 *g* for 10 min. The chloroform layer was transferred to another centrifuge tube, and the chloroform evaporated to dryness under a stream of air. To the residue, 0.2 *M* sodium hydroxide (1.0 ml) and diethyl ether (5.0 ml) were added. The mixture was again shaken and centrifuged, and the diethyl ether layer was transferred to another centrifuge tube and evaporated to dryness. The residue was redissolved in methanol (100 μl), and an aliquot (40 μl) of the methanol solution was injected into the HPLC system. The calibration curve was constructed at cilostazol levels of 25—2000 ng/ml of plasma.

Healthy male volunteers received a single oral dose of 100 mg of cilostazol. Blood samples were collected at scheduled intervals and centrifuged at 1700 *g* for 10 min to obtain plasma samples.

RESULTS AND DISCUSSION

Typical chromatograms of the extracts from plasma containing cilostazol (250 ng/ml), blank plasma and sample plasma after a single oral dose of 100 mg of cilostazol obtained in the above procedure are shown in Fig. 2. No significant interference was observed in the regions for cilostazol and the internal standard on the chromatogram. The retention time was 7.5 min for cilostazol and 12.0 min for the internal standard.

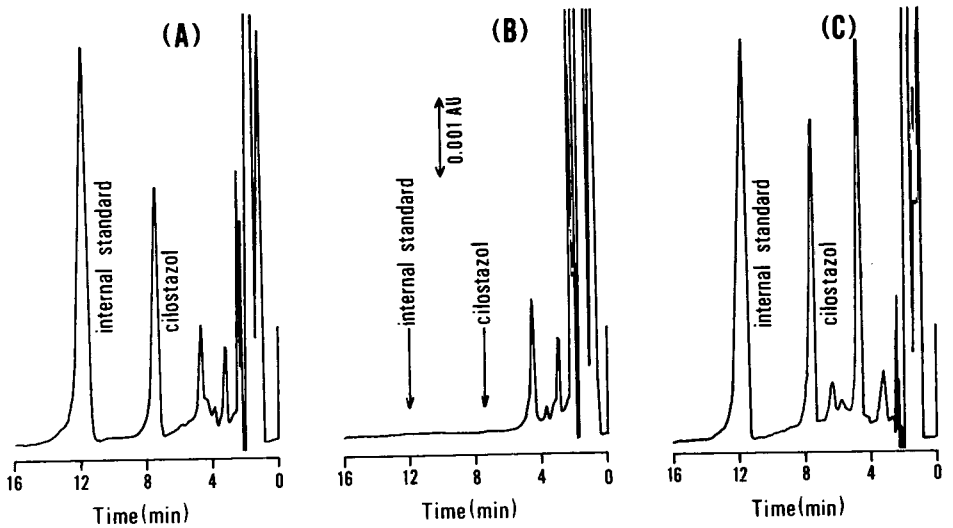

Fig. 2. (A) Chromatogram of human plasma containing cilostazol (250 ng/ml) and internal standard (600 ng/ml). (B) Chromatogram of blank human plasma. (C) Chromatogram of sample human plasma after a single oral dose of 100 mg of cilostazol.

The linearity of the calibration curve constructed for the determination of cilostazol at levels of 25—2000 ng/ml is shown in Table I. The precision of the HPLC assay was determined by calculating a mean peak height ratio ± standard deviation (S.D.) for each of the five standards. The peak height ratio was 0.059 ± 0.002 with a coefficient of variation (C.V.) of 3.6% at 25 ng/ml, and correspondingly 0.122 ± 0.006 and 4.6% at 50 ng/ml. At levels higher than 50 ng/ml the C.V. values were smaller than 1.6%, which were very much smaller than at 25 and 50 ng/ml, showing very little deviation of the peak height ratio. The equation for the resulting line was $y = 0.00259x - 0.01371$ with a correlation coefficient of 0.999. Plasma cilostazol levels calculated from the calibration curve were comparable to corresponding amounts of cilostazol

TABLE I

LINEARITY AND PRECISION OF HPLC PROCEDURE FOR CILOSTAZOL IN HUMAN PLASMA BY INTERNAL STANDARD METHOD

Concentration of cilostazol added to plasma (ng/ml)	Peak height ratio (mean ± S.D.)*	C.V. (%)	Recalculated concentration (ng/ml)	Percentage of theory	Recovery (%)
25	0.059 ± 0.002	3.6	27.9	112	69
50	0.122 ± 0.006	4.6	52.3	105	74
100	0.246 ± 0.004	1.6	100.2	100	79
250	0.628 ± 0.005	0.8	247.2	99	77
500	1.280 ± 0.018	1.4	498.6	100	77
1000	2.571 ± 0.022	0.9	996.4	100	73
2000	5.180 ± 0.039	0.8	2002.4	100	70

*Results from five replicate samples were used.

added to the plasma. They agreed well, being 99—112% of the amounts added to the plasma.

The recovery of cilostazol in this assay procedure was determined by comparing peak heights of the compound from processed samples to heights of other prepared reference solutions. Recoveries were greater than 69% at any level.

This assay system was applied to the study of the pharmacokinetics of cilostazol in man. A plasma cilostazol level versus time curve after a single dose of 100 mg orally is shown in Fig. 3. Cilostazol was rapidly absorbed, and reached peak plasma levels of 764 ng/ml at 3 h. The plasma elimination half-life was 2.2 h (α-phase) and 18.0 h (β-phase). The quantifiable limit (25 ng/ml) of this procedure was considered sufficient to determine plasma cilostazol levels, since the dose of 100 mg used in this study will be equal or lower than intended daily clinical doses of cilostazol [6].

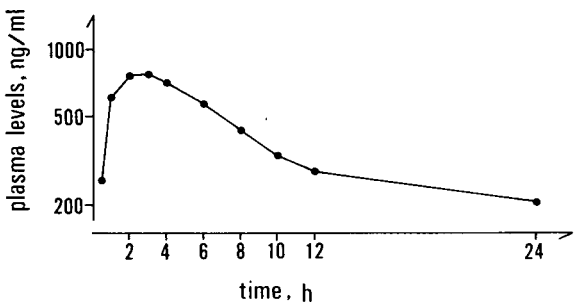

Fig. 3. Plasma cilostazol levels after a single oral dose of cilostazol (100 mg per body) in healthy male volunteers (n = 12).

In view of these results, it was concluded that HPLC is a simple, sensitive and reproducible procedure for the determination of plasma cilostazol levels and, therefore, a suitable and valuable tool in the investigation of the clinical pharmacokinetics and bioavailability of cilostazol.

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CHROMBIO. 2439

Letter to the Editor

Rapid extraction method for ethosuximide and other antiepileptics in serum for determination by high-performance liquid chromatography

Sir,

Our recently published high-performance liquid chromatographic method [1] for the simultaneous determination of eleven antiepileptic compounds showed a rather high day-to-day coefficient of variation (C.V.) of 9.8% for ethosuximide, the other substances having a mean C.V. of 5.4%. The high C.V. for ethosuximide can be partly attributed to the high water-solubility of this drug, which reduces the extraction into an organic solvent. Equal extraction conditions were guaranteed by shaking the standards and tests simultaneously [1]. The low melting point of ethosuximide (64—65°C) and possibly low vapour pressure also contribute to the high C.V., although the evaporation was done at 37°C by means of a direct air stream [1]. The extraction method described has a more pronounced salting out effect than the earlier method [1] and the evaporation step is omitted. The method described by Kabra et al. [2], where the extraction step is also left out, was not selected because we believe that the present extraction results in a cleaner and a more concentrated injection solution.

Our method is as follows: 400 μl of serum, 400 μl of saturated sodium dihydrogen phosphate in water (ca. 850 g of $NaH_2PO_4 \cdot H_2O$ dissolved in 1 l of water, pH 3.4) and 600 μl of acetonitrile containing the internal standard (45 mg of hexobarbital in 1 l of acetonitrile) are placed in a 100 × 15 mm glass tube. The tubes are vortex-mixed for 15 sec and centrifuged for 10 min at 3000 *g*. Due to the high salt content, an acetonitrile extract separates from the water phase and 300 μl of supernatant can be pipetted away from the protein pellet; 8 μl of this extract are injected into a Hewlett-Packard 1084 B high-performance liquid chromatograph. The liquid chromatographic settings are the same as published [1], except for the wavelength which is 204 nm.

A chromatographic separation of a drug standard which was added to drug-free serum and extracted as a patient serum is shown in Fig. 1. Fig. 2 shows a chromatogram of the Seronorm Pharmaca AED control serum (toxic level, diluted 1:1 with water; Nygaard, Norway), which was used for the within-day precision of the described method (Table I). The day-to-day C.V. was 5.6% (425.1 ± 25.3 μmol/l, n = 27) and was determined by analysing pooled serum

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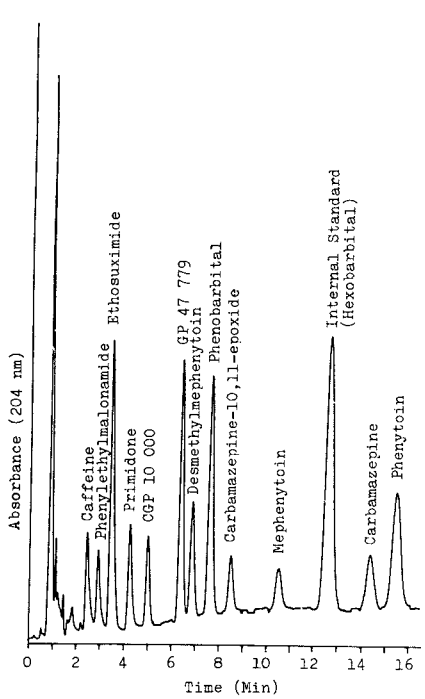

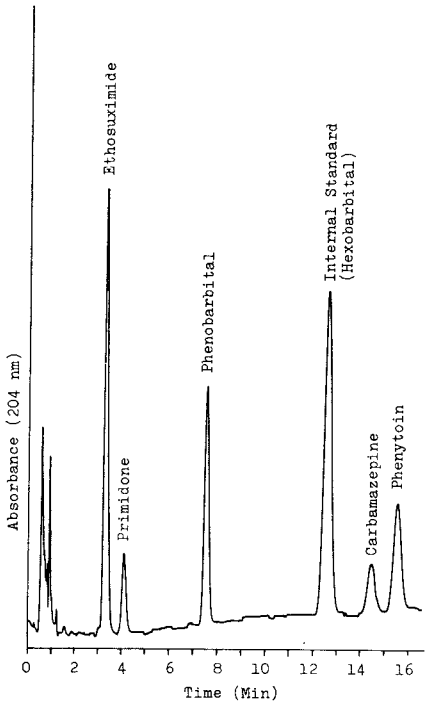

Fig. 1. Chromatographic separation of the drug standard [1] which was added to drug-free serum and extracted as a patient serum. Column: LiChrosorb RP-8, 10 μm, 25 × 0.4 cm. Gradient elution with 17—23% acetonitrile in water for 6 min, remaining isocratic for the rest of the run. Flow-rate: 2.3 ml/min. Temperature: 35°C. Wavelength: 204 nm.

Fig. 2. Chromatographic separation of Seronorm Pharmaca AED control serum. The concentrations are those listed in Table I. Conditions are the same as in Fig. 1.

TABLE I

WITHIN-DAY PRECISION OF THE METHOD

n = 32 in every case.

Drug	Concentration (mean ± S.D.) (μmol/l)	C.V. (%)
Carbamazepine	30.5 ± 0.9	2.9
Ethosuximide	442.3 ± 11.0	2.5
Primidone	30.1 ± 2.5	8.3
Phenobarbital	89.5 ± 1.3	1.5
Phenytoin	45.6 ± 1.6	3.5

containing only ethosuximide. The determination of primidone is not recommended with these liquid chromatographic settings, because in some cases an unidentified peak with the same retention time as primidone can appear with drug-free serum.

Swiss Epilepsy Center,
Zürich (Switzerland)

N. WAD

1 N. Wad, J. Chromatogr., 305 (1984) 127—133.
2 P.M. Kabra, D.M. McDonald and L.J. Marton, J. Anal. Toxicol., 2 (1978) 127—133.

(First received July 27th, 1984; revised manuscript received September 28th, 1984)

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CHROMBIO. 2433

Book Review

Clinical liquid chromatography, Vol. I, Analysis of exogenous compounds, and *Vol. II, Analysis of endogenous compounds*, edited by P.M. Kabra and L.J. Marton, CRC Press, Boca Raton, FL, 1984, XIII + 212 pp. (Vol. I), XIII + 226 pp. (Vol. II), prices: Vol. I: US$ 63.00 (U.S.A.), US$ 72.00 (other countries), Vol. II: US$ 72.00 (U.S.A.), US$ 83.00 (other countries), ISBN: Vol. I: 0-8493-6637-2, Vol. II: 0-8493-6638-0.

After the three years that have passed since Kabra and Marton's "Liquid Chromatography in Clinical Analysis" was published [for a review see J. Chromatogr., 231 (1982) 222], there has now appeared a book on similar topics, but different in its approach. Whereas the earlier book gave an overview of some topics of the area of exogenous and endogenous compounds, these new volumes give detailed procedures used for the analysis of selected compounds presented in such a way that they can be immediately applied in a clinical chemistry laboratory. Most of the individual chapters are devoted to a single compound rather than the analysis of a number of compounds. In those instances where there are several equal alternatives, the procedures suitable for the analysis of the same compound are presented in parallel chapters (e.g., the analysis of theophylline, quinidine, homovanillic acid and cortisol).

It is admirable that the Editors have succeeded in unifying the structure of individual chapters (Introduction, Principle, Materials and Methods — Equipment, Reagents, Standards, Procedure, Calculation, Results — Optimization, Linearity, Recovery, Reproducibility, Accuracy, Sensitivity, Interferences and Comments). Each chapter is illustrated by practical examples of chromatograms. Unfortunately, there is much less uniformity in the bibliography part: some authors quote only a single paper (mainly their own) that constitutes the basis of the method (Kabra, Schmidt, etc.), whereas others present much more complete bibliographies (Seiler). In general, the literature cited covers the period up to the end of 1982, with a few papers from 1983.

Although in comparison with the 1981 book there are many new compounds or groups of compounds, the reader is still likely to miss some categories such as sugars, lipids and the determination of enzymic activities. This comment refers mainly to the second volume.

It can be concluded that these two volumes will distinctly enrich the range of books available in the area of clinical applications of high-performance liquid chromatography, and clinical chemists will appreciate the fact that the methods are described in sufficient detail to help them to solve their particular problems and help them to introduce reliable procedures into clinical practice. In this respect both the Editors and the authors can be congratulated on the result.

Prague (Czechoslovakia) KAREL MACEK

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CHROMBIO. 2448

Book Review

Methods of protein analysis, edited by I. Kerese, Ellis Horwood Chichester, Halsted (Wiley), New York, 1984, 371 pp., price £ 39.50, ISBN 0-85312-176-1 (Ellis Horwood), 0-470-27497-2 (Halsted Wiley).

This book, dedicated to the memory of the pioneer in protein chemistry research, Prof. W. Grassmann, attempts to present an overview of current methods in protein analysis. This, due to the vastness and diversity of the field, is a difficult thing to do. The authors and the editor have limited the coverage somewhat by emphasizing the separation procedures, which represent a crucial step in any analysis of proteinaceous materials. Thus, readers would miss some aspects of protein analysis they might find useful in their own research or clinical work. Thus, for instance, very little attention, if any at all, is paid to sequencing methods, determination of amino acid derivatives or methods of specific protein cleavage. Since in the Introduction it is stated that "this book is intended to serve a practical purpose", the reviewer is tempted to ask why methods exploiting the determination of marker amino acids are hardly touched on in spite of being of extreme practical value. Another aspect that I particularly dislike about this book is the compilation of methods without a critical evaluation. Thus, for example the newcomer to the field will not learn of the difficulties currently encountered in reversed-phase chromatography of proteins; he is unlikely to realize that isotachophoresis is at present far from being a widely accepted method for protein or peptide analysis; the *o*-phthaldehyde method of amino acid analysis is given less than a paragraph though it is at the moment the most progressive procedure. While there is a tiny chapter (p. 117) devoted to the interpretation of one-dimensional electrophoreograms, the reader will not find any information about the problems encountered in evaluating two-dimensional protein patterns. The most sensitive and advanced method of staining proteins separated in gels is the silver stain procedure; but this, according to the present volume, does not exist. Since there are several pages about the Kjeldahl method of nitrogen determination, one would expect at least a mention about automation of this method as this is the area where interest is now focused. Again, nothing in this respect.

When opening this book I expected some sort of a return of books like that of Bailey's Methods in Protein Chemistry (Elsevier, Amsterdam, 1962); the result was, however, a disappointment.

In conclusion, the book summarizes classical methods of protein analysis, a topic elaborated in many books already. The last part, devoted to the description of actual procedures, may be useful and indeed may serve as a laboratory guide. However, in general, for an expert there is little in-depth discussion; for a newcomer a more critical approach would be useful.

Prague (Czechoslovakia) Z. DEYL

Related articles published in *Journal of Chromatography*, Vols. 319—320

Volume 319

Volume 320

Author Index

Subject Index

Erratum

J. Chromatogr., 337 (1985) 301—309
Page 303, 16th line from the bottom, "1 mg/l" should read "1 mg/ml".

Vol. 338, No. 2

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

MEETINGS

DEVELOPMENTS IN ANALYTICAL METHODS IN THE PHARMACEUTICAL AND BIOMEDICAL SCIENCES, CAMERINO, ITALY, JUNE 17–20, 1985

An International Conference of Developments in Analytical Methods in the Pharmaceutical and Biomedical Sciences will be organized by the Italian Group for Mass Spectrometry in Biochemistry and Medicine and by the Department of Chemical Sciences of the University of Camerino, Italy. The Conference will be held on June 17–20, 1985 in the Lecture Rooms of the Department of Chemical Sciences of the University of Camerino.

The conference will illustrate and discuss all aspects of NMR, mass spectrometry, chromatography and chromatography–mass spectrometry and their areas of application, including biochemistry, pharmaceutical chemistry, medicine, toxicology, drug research, nutrition science and food safety, forensic science, clinical chemistry, and pollution. The conference will consist of lectures by prominent speakers, contributed papers, and discussions. Facilities will be available for participants to display posters. A book and instrument exhibition on NMR, mass spectrometry and chromatography will also take place.

The registration fee is US$ 220 (for Italian participants Lit. 350.000 + IVA). The registration fee for accompanying persons is US$ 120 (for Italian participants Lit. 180.000 + IVA). Payment can be made by cheque or by banker's draft payable to the Italian Group for Mass Spectrometry in Biochemistry and Medicine and crossed "Not Negotiable".

For further information contact: Professor Ippolito Antonini, Department of Chemical Sciences, University of Camerino, Via S. Agostino 1, 62032 Camerino (MC), Italy; Tel. (0737) 3243/3018, Telex 560024.

6th INTERNATIONAL BIOANALYTICAL FORUM, GUILDFORD, U.K., SEPTEMBER 10–13, 1985

The 6th International Bioanalytical Forum (bioactive analytes including antipsychotics and peptides) will be held at the University of Surrey, Guildford, during September 10–13, 1985. There will be the usual emphasis on analyzing biological samples for drugs and their metabolites, e.g. prostaglandins, G–I hormones and (on 13 September, allowing one-day attendance) CNS-active drugs. 'State-of-the-art' topics on 11 and 12 September include advances in capillary GC and in HPLC including MS and other detection modes, photodiode array approaches, chiral separations, and sample processing.

For information contact: Dr. E. Reid, Guildford Academic Associates, 72 The Chase, Guildford, Survey GU2 5UL, U.K.; Tel. 0483-65324.

CALENDAR OF FORTHCOMING EVENTS

Apr. 29–May 1, 1985 Brussels, Belgium	**XXXIIIrd Colloquium – Protides of the Biological Fluids** Contact: Colloquium – Protides of the Biological Fluids, Institute for Medical Biology, Alsembergsesteenweg 196, B-1180 Brussels, Belgium. Tel.: 32-2-348-05 11. Telex: CDHBRU 26501.
May 13–15, 1985 Virginia Beach, VA, U.S.A.	**Infant Formula Conference** Contact: Dr. James Tanner, Food and Drug Administration, HFF-266, 200 C Street, S.W., Washington, DC 20204, U.S.A. Tel.: (202) 472-5384.
May 14–16, 1985 Riva del Garda, Italy	**6th International Symposium on Capillary Chromatography** Contact: Dr. P. Sandra, Laboratory of Organic Chemistry, University of Ghent, Krijgslaan 281 (S.4), B-9000 Ghent, Belgium. (Further details published in Vol. 298, No. 1.)
May 27–31, 1985 Urbino, Italy	**The A.J.P. Martin Honorary Symposium** Contact: Dr. F. Bruner, University of Urbino, Urbino, Italy. (Further details published in Vol. 315.)
June 3–6, 1985 Oslo, Norway	**21st International Symposium on Advances in Chromatography** Contact: Prof. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. Tel.: (713) 749-2623. Telex: 76278. (Further details published in Vol. 310, No. 1.)
June 11–14, 1985 Budapest, Hungary	**Budapest Chromatography Symposium – the 5th Annual American Eastern European Symposium on Liquid Chromatography** Contact: Dr. Huba Kalász, Department of Pharmacology, Semmelweis University of Medicine, P.O. Box 370, H-1445 Budapest, Hungary. (Further details published in Vol. 315.)
June 17–20, 1985 Camerino, Italy	**Developments in Analytical Methods in the Pharmaceutical and Biomedical Sciences** Contact: Prof. Ippolito Antonini, Dept. of Chemical Sciences, University of Camerino, Via S. Agostino 1, 62032 Camerino (MC), Italy. Tel.: (0737) 3243/3018. Telex: 560024.
June 17–21, 1985 Amsterdam, The Netherlands	**Amsterdam Summerschool on HPLC** Contact: The Municipal Congress Bureau, Oudezijds Achterburgwal 199, 1012 DK Amsterdam, The Netherlands. Tel.: (020) 552.3458 or (020) 552.3459. (Further details published in Vol. 321, No. 1.)
July 1–5, 1985 Edinburgh, U.K.	**HPLC '85. 9th International Symposium on Column Liquid Chromatography** Contact: 9th ISCLC Secretariat, CEP Consultants Ltd., 26 Albany Street, Edinburgh EH1 3QH, Scotland, U.K. Tel.: (031) 557 248. (Further details published in Vol. 310, No. 1.)
July 17–19, 1985 Oxford, U.K.	**Joint Meeting of the British Electrophoresis Society and the Techniques Group of the Biochemical Society** Contact: Dr. M.J. Dunn, Muscle Research Unit, Royal Postgraduate Medical School, DuCane Road, London W12 0HS, U.K. Tel.: 01-743-2030 ext. 338.

Aug. 11–16, 1985 Espoo, Finland	**XIVth International Conference on Medical and Biological Engineering and VIIth International Conference on Medical Physics** Contact: Hannu Seitsone, Secretary General, P.O. Box 105, 00251 Helsinki, Finland. Tel.: 358-0-4713070.
Sept. 1–6, 1985 Prague, Czechoslovakia	**6th International Symposium on Bioaffinity Chromatography and Related Techniques** Contact: Dr. J. Turková, Institute of Organic Chemistry and Biochemistry ČSAV, Flemingovo No. 2, CS-166 10, Prague 6, Czechoslovakia. Tel.: (422) 324541, int. 080. (Futher details published in Vol. 308.)
Sept. 2–6, 1985 Siófok, Hungary	**International Conference on High-Performance Chromatographic and Electrophoretic Techniques in Biochemistry** Contact: Hungarian Biochemical Society, MTESZ, Biochemical Separation Conference, P.O. Box 240, H-1368 Budapest, Hungary. Telex: 225369 mtesz h (Biochem. Soc.).
Sept. 5–8, 1985 Birmingham, U.K.	**Flow Analysis III – An International Conference on Flow Analysis** Contact: Flow Analysis III, Dr. A.M.G. Macdonald, Department of Chemistry, The University, P.O. Box 363, Birmingham B15 2TT, U.K. (Further details published in Vol. 288, No. 2.)
Sept. 8–13, 1985 Chicago, IL, U.S.A.	**190th National Meeting of the American Chemical Society** Contact: Meetings Department, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.
Sept. 9–13, 1985 Manchester, U.K.	**30th International Congress of Pure and Applied Chemistry** Contact: The Royal Society of Chemistry, Burlington House, London W1V 0BN, U.K.
Sept. 10–13, 1985 Guildford, U.K.	**6th International Bioanalytical Forum** Contact: Dr. E. Reid, Guildford Academic Associates, 72 The Chase, Guildford, Surrey GU2 5UL, U.K. Tel.: 0483-65324.
Sept. 22–Oct. 1, 1985 Rome, Italy	**FEBS Course: Advanced Electrophoretic Techniques** Contact: Dr. G. Minozzi, Lab. Biologia Cellulare CNR, Via Romagnosi 18A, Rome 00196, Italy. Tel.: (06)3611407.
Sept. 25–27, 1985 Rostock, G.D.R.	**9th Symposium on Biomedical Applications of Chromatography** Contact: Dr. Joachim Wagner, Medical Clinic of the K. Marx University, Johannisallee 32, DDR-7010 Leipzig, G.D.R. (Further details published in Vol. 337, No. 1.)
Oct. 3–4, 1985 Pont à Mousson, France	**Two-Dimentional Electrophoresis Satellite Symposium to the 6th International Colloquium on Prospective Biology** Contact: Prof. Marie-Madeleine Galteau, Centre du Médicament, 30, rue Lionnois, 54000 Nancy, France. Tel.: (8) 332.29.23, Telex: CMP 961640 F.
Oct. 24–25, 1985 Freiburg, F.R.G.	**2nd Symposium on Handling of Environmental and Biological Samples in Chromatography** Contact: Workshop Office IAEAC, M. Frei-Hausler, Postfach 46, CH-4123 Allschwil 2, Switzerland. (Further details published in Vol. 337, No. 1.)
Nov. 11–16, 1985 Yalta, U.S.S.R.	**5th Danube Symposium on Chromatography** Contact: Dr. L.N. Kolomiets, The Scientific Council of Chromatography, Academy of Sciences of the U.S.S.R., Institute of Physical Chemistry, Lenin-Prospect 31, Moscow 117312, U.S.S.R. (Further details published in Vol. 281.)

May 18–23, 1986 San Francisco, CA, U.S.A.	**New Frontiers in HPLC. 10th International Symposium on Column Liquid Chromatography** Contact: Ms. Shirley Schlessinger, 400 E. Randolph Drive, Chicago, IL 60601, U.S.A.
Sept. 21–26, 1986 Paris, France	**16th International Symposium on Chromatography** Contact: G.A.M.S., 88, Boulevard Malesherbes, F-75008 Paris, France. Tel.: (1) 563 93 04. (Further details published in Vol. 310, No. 1.)

NEW BOOKS

Affinity chromatography and biological recognition, edited by I.M. Chaiken, M. Wilchek and I. Parikh, Academic Press, London, 1983, 544 pp., price US$ 43.50, ISBN 0-12-166580-1.

Two-dimensional gel electrophoresis of proteins – Methods and applications, edited by J.E. Celis and R. Bravo, Academic Press, London, 1984, 504 pp., price US$ 69.50, ISBN 0-12-164720-X.

Cost/benefit and predictive value of radioimmunoassay (*Symposia of the Giovanni Lorenzini Foundation,* Vol. 18), edited by A. Albertini, R.P. Ekins and R.S. Galen, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, XIII + 270 pp., price Dfl. 167.00, US$ 61.75, ISBN 0-444-80618-0.

Monoclonal antibodies and new trends in immunoassays, edited by Ch.A. Bizollon, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, X + 310 pp., price Dfl. 160.00, US$ 59.25, ISBN 0-444-80619-9.

Pharmaceutical analysis – Modern methods, Part B (*Drugs and the Pharmaceutical Sciences Series,* Vol. 11), edited by J.W. Munson, Marcel Dekker, New York, Basel, 1984, XII + 496 pp., price US$ 79.75 (U.S.A. and Canada), US$ 95.50, SFr 222.00 (rest of world), ISBN 0-8247-7251-2.

Microspheres and drug therapy. Pharmaceutical, immunological and medical aspects, edited by S.S. Davis, L. Illum, J.G. McVie and E. Tomlinson, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, XVIII + 448 pp., price Dfl. 285.00, US$ 109.50, ISBN 0-444-80577-X.

The pharmacology of inflammation (*Handbook of Inflammation,* Vol. 5), edited by I.L. Bonta, M.A. Bray and M.J. Parnham, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, *ca.* 400 pp., price Dfl. 240.00, US$ 92.25, ISBN 0-444-90312-7.

Pharmacological and chemical synonyms, compiled by E.E.J. Marler, Elsevier, Amsterdam, Oxford, New York, Tokyo, **8th revised edition,** 1984, *ca.* 550 pp., price Dfl. 245.00, US$ 94.25 ISBN 0-444-90359-3.

The diabetes annual, edited by K.G.M.M. Alberti and L.P. Krall, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, *ca.* 450 pp., price Dfl. 190.00, US$ 73.00, ISBN 0-444-90343-7.

Fluoropyrimidines in cancer therapy (*International Congress Series,* No. 647), edited by K. Kimura, S. Fujii, M. Ogawa, G.P. Bodey and P. Alberto, Excerpta Medica, Amsterdam, New York, 1984, 588 pp., price Dfl. 295.00, US$ 113.50, ISBN 0-444-80612-1.

Wilm's tumor: clinical and biological manifestations, edited by C. Pochedly and E.S. Baum, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, *ca.* 515 pp., price Dfl. 250.00, US$ 65.00, ISBN 0-444-00857-8.

Liver and lipid metabolism (*International Congress Series,* No. 632), edited by S. Calandra, N. Carulli and G. Salvioli, Excerpta Medica, Amsterdam, New York, 1984, 228 pp., price Dfl. 160.00, US$ 61.50, ISBN 0-444-80608-3.

Ferritins and isoferritins as biochemical markers (*Symposia of the Giovanni Lorenzini Foundation,* Vol. 19), edited by A. Albertini, P. Arosio, E. Chiancone and J. Drysdale, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, X + 208 pp., price Dfl. 109.00, US$ 40.50, ISBN 0-444-80607-5.

Progress in pesticide biochemistry and toxicology, Vol. 4, edited by D.H. Hutson and T.R. Roberts, Wiley, Chichester, New York, 1985, X + 368 pp., price £ 42.00, ISBN 0-471-90460-0.

New horizons in microbiology, edited by A. Sanna and G. Morace, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, X + 340 pp., price Dfl. 178.00, US$ 68.50, ISBN 0-444-80623-7.

Vol. 338, No. 2

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

MEETINGS

DEVELOPMENTS IN ANALYTICAL METHODS IN THE PHARMACEUTICAL AND BIOMEDICAL SCIENCES, CAMERINO, ITALY, JUNE 17–20, 1985

An International Conference of Developments in Analytical Methods in the Pharmaceutical and Biomedical Sciences will be organized by the Italian Group for Mass Spectrometry in Biochemistry and Medicine and by the Department of Chemical Sciences of the University of Camerino, Italy. The Conference will be held on June 17–20, 1985 in the Lecture Rooms of the Department of Chemical Sciences of the University of Camerino.

The conference will illustrate and discuss all aspects of NMR, mass spectrometry, chromatography and chromatography–mass spectrometry and their areas of application, including biochemistry, pharmaceutical chemistry, medicine, toxicology, drug research, nutrition science and food safety, forensic science, clinical chemistry, and pollution. The conference will consist of lectures by prominent speakers, contributed papers, and discussions. Facilities will be available for participants to display posters. A book and instrument exhibition on NMR, mass spectrometry and chromatography will also take place.

The registration fee is US$ 220 (for Italian participants Lit. 350.000 + IVA). The registration fee for accompanying persons is US$ 120 (for Italian participants Lit. 180.000 + IVA). Payment can be made by cheque or by banker's draft payable to the Italian Group for Mass Spectrometry in Biochemistry and Medicine and crossed "Not Negotiable".

For further information contact: Professor Ippolito Antonini, Department of Chemical Sciences, University of Camerino, Via S. Agostino 1, 62032 Camerino (MC), Italy; Tel. (0737) 3243/3018, Telex 560024.

6th INTERNATIONAL BIOANALYTICAL FORUM, GUILDFORD, U.K., SEPTEMBER 10–13, 1985

The 6th International Bioanalytical Forum (bioactive analytes including antipsychotics and peptides) will be held at the University of Surrey, Guildford, during September 10–13, 1985. There will be the usual emphasis on analyzing biological samples for drugs and their metabolites, e.g. prostaglandins, G–I hormones and (on 13 September, allowing one-day attendance) CNS-active drugs. 'State-of-the-art' topics on 11 and 12 September include advances in capillary GC and in HPLC including MS and other detection modes, photodiode array approaches, chiral separations, and sample processing.

For information contact: Dr. E. Reid, Guildford Academic Associates, 72 The Chase, Guildford, Survey GU2 5UL, U.K.; Tel. 0483-65324.

CALENDAR OF FORTHCOMING EVENTS

Apr. 29–May 1, 1985 Brussels, Belgium	**XXXIIIrd Colloquium – Protides of the Biological Fluids** Contact: Colloquium – Protides of the Biological Fluids, Institute for Medical Biology, Alsembergsesteenweg 196, B-1180 Brussels, Belgium. Tel.: 32-2-348-05 11. Telex: CDHBRU 26501.
May 13–15, 1985 Virginia Beach, VA, U.S.A.	**Infant Formula Conference** Contact: Dr. James Tanner, Food and Drug Administration, HFF-266, 200 C Street, S.W., Washington, DC 20204, U.S.A. Tel.: (202) 472-5384.
May 14–16, 1985 Riva del Garda, Italy	**6th International Symposium on Capillary Chromatography** Contact: Dr. P. Sandra, Laboratory of Organic Chemistry, University of Ghent, Krijgslaan 281 (S.4), B-9000 Ghent, Belgium. (Further details published in Vol. 298, No. 1.)
May 27–31, 1985 Urbino, Italy	**The A.J.P. Martin Honorary Symposium** Contact: Dr. F. Bruner, University of Urbino, Urbino, Italy. (Further details published in Vol. 315.)
June 3–6, 1985 Oslo, Norway	**21st International Symposium on Advances in Chromatography** Contact: Prof. A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004, U.S.A. Tel.: (713) 749-2623. Telex: 76278. (Further details published in Vol. 310, No. 1.)
June 11–14, 1985 Budapest, Hungary	**Budapest Chromatography Symposium – the 5th Annual American Eastern European Symposium on Liquid Chromatography** Contact: Dr. Huba Kalász, Department of Pharmacology, Semmelweis University of Medicine, P.O. Box 370, H-1445 Budapest, Hungary. (Further details published in Vol. 315.)
June 17–20, 1985 Camerino, Italy	**Developments in Analytical Methods in the Pharmaceutical and Biomedical Sciences** Contact: Prof. Ippolito Antonini, Dept. of Chemical Sciences, University of Camerino, Via S. Agostino 1, 62032 Camerino (MC), Italy. Tel.: (0737) 3243/3018. Telex: 560024.
June 17–21, 1985 Amsterdam, The Netherlands	**Amsterdam Summerschool on HPLC** Contact: The Municipal Congress Bureau, Oudezijds Achterburgwal 199, 1012 DK Amsterdam, The Netherlands. Tel.: (020) 552.3458 or (020) 552.3459. (Further details published in Vol. 321, No. 1.)
July 1–5, 1985 Edinburgh, U.K.	**HPLC '85. 9th International Symposium on Column Liquid Chromatography** Contact: 9th ISCLC Secretariat, CEP Consultants Ltd., 26 Albany Street, Edinburgh EH1 3QH, Scotland, U.K. Tel.: (031) 557 248. (Further details published in Vol. 310, No. 1.)
July 17–19, 1985 Oxford, U.K.	**Joint Meeting of the British Electrophoresis Society and the Techniques Group of the Biochemical Society** Contact: Dr. M.J. Dunn, Muscle Research Unit, Royal Postgraduate Medical School, DuCane Road, London W12 0HS, U.K. Tel.: 01-743-2030 ext. 338.

Aug. 11–16, 1985 Espoo, Finland	**XIVth International Conference on Medical and Biological Engineering and VIIth International Conference on Medical Physics** Contact: Hannu Seitsone, Secretary General, P.O. Box 105, 00251 Helsinki, Finland. Tel.: 358-0-4713070.
Sept. 1–6, 1985 Prague, Czechoslovakia	**6th International Symposium on Bioaffinity Chromatography and Related Techniques** Contact: Dr. J. Turková, Institute of Organic Chemistry and Biochemistry ČSAV, Flemingovo No. 2, CS-166 10, Prague 6, Czechoslovakia. Tel.: (422) 324541, int. 080. (Futher details published in Vol. 308.)
Sept. 2–6, 1985 Siófok, Hungary	**International Conference on High-Performance Chromatographic and Electrophoretic Techniques in Biochemistry** Contact: Hungarian Biochemical Society, MTESZ, Biochemical Separation Conference, P.O. Box 240, H-1368 Budapest, Hungary. Telex: 225369 mtesz h (Biochem. Soc.).
Sept. 5–8, 1985 Birmingham, U.K.	**Flow Analysis III – An International Conference on Flow Analysis** Contact: Flow Analysis III, Dr. A.M.G. Macdonald, Department of Chemistry, The University, P.O. Box 363, Birmingham B15 2TT, U.K. (Further details published in Vol. 288, No. 2.)
Sept. 8–13, 1985 Chicago, IL, U.S.A.	**190th National Meeting of the American Chemical Society** Contact: Meetings Department, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.
Sept. 9–13, 1985 Manchester, U.K.	**30th International Congress of Pure and Applied Chemistry** Contact: The Royal Society of Chemistry, Burlington House, London W1V 0BN, U.K.
Sept. 10–13, 1985 Guildford, U.K.	**6th International Bioanalytical Forum** Contact: Dr. E. Reid, Guildford Academic Associates, 72 The Chase, Guildford, Surrey GU2 5UL, U.K. Tel.: 0483-65324.
Sept. 22–Oct. 1, 1985 Rome, Italy	**FEBS Course: Advanced Electrophoretic Techniques** Contact: Dr. G. Minozzi, Lab. Biologia Cellulare CNR, Via Romagnosi 18A, Rome 00196, Italy. Tel.: (06)3611407.
Sept. 25–27, 1985 Rostock, G.D.R.	**9th Symposium on Biomedical Applications of Chromatography** Contact: Dr. Joachim Wagner, Medical Clinic of the K. Marx University, Johannisallee 32, DDR-7010 Leipzig, G.D.R. (Further details published in Vol. 337, No. 1.)
Oct. 3–4, 1985 Pont à Mousson, France	**Two-Dimentional Electrophoresis Satellite Symposium to the 6th International Colloquium on Prospective Biology** Contact: Prof. Marie-Madeleine Galteau, Centre du Médicament, 30, rue Lionnois, 54000 Nancy, France. Tel.: (8) 332.29.23, Telex: CMP 961640 F.
Oct. 24–25, 1985 Freiburg, F.R.G.	**2nd Symposium on Handling of Environmental and Biological Samples in Chromatography** Contact: Workshop Office IAEAC, M. Frei-Hausler, Postfach 46, CH-4123 Allschwil 2, Switzerland. (Further details published in Vol. 337, No. 1.)
Nov. 11–16, 1985 Yalta, U.S.S.R.	**5th Danube Symposium on Chromatography** Contact: Dr. L.N. Kolomiets, The Scientific Council of Chromatography, Academy of Sciences of the U.S.S.R., Institute of Physical Chemistry, Lenin-Prospect 31, Moscow 117312, U.S.S.R. (Further details published in Vol. 281.)

May 18–23, 1986 San Francisco, CA, U.S.A.	**New Frontiers in HPLC. 10th International Symposium on Column Liquid Chromatography** Contact: Ms. Shirley Schlessinger, 400 E. Randolph Drive, Chicago, IL 60601, U.S.A.
Sept. 21–26, 1986 Paris, France	**16th International Symposium on Chromatography** Contact: G.A.M.S., 88, Boulevard Malesherbes, F-75008 Paris, France. Tel.: (1) 563 93 04. (Further details published in Vol. 310, No. 1.)

NEW BOOKS

Affinity chromatography and biological recognition, edited by I.M. Chaiken, M. Wilchek and I. Parikh, Academic Press, London, 1983, 544 pp., price US$ 43.50, ISBN 0-12-166580-1.

Two-dimensional gel electrophoresis of proteins – Methods and applications, edited by J.E. Celis and R. Bravo, Academic Press, London, 1984, 504 pp., price US$ 69.50, ISBN 0-12-164720-X.

Cost/benefit and predictive value of radioimmunoassay (*Symposia of the Giovanni Lorenzini Foundation,* Vol. 18), edited by A. Albertini, R.P. Ekins and R.S. Galen, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, XIII + 270 pp., price Dfl. 167.00, US$ 61.75, ISBN 0-444-80618-0.

Monoclonal antibodies and new trends in immunoassays, edited by Ch.A. Bizollon, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, X + 310 pp., price Dfl. 160.00, US$ 59.25, ISBN 0-444-80619-9.

Pharmaceutical analysis – Modern methods, Part B (*Drugs and the Pharmaceutical Sciences Series,* Vol. 11), edited by J.W. Munson, Marcel Dekker, New York, Basel, 1984, XII + 496 pp., price US$ 79.75 (U.S.A. and Canada), US$ 95.50, SFr 222.00 (rest of world), ISBN 0-8247-7251-2.

Microspheres and drug therapy. Pharmaceutical, immunological and medical aspects, edited by S.S. Davis, L. Illum, J.G. McVie and E. Tomlinson, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, XVIII + 448 pp., price Dfl. 285.00, US$ 109.50, ISBN 0-444-80577-X.

The pharmacology of inflammation (*Handbook of Inflammation,* Vol. 5), edited by I.L. Bonta, M.A. Bray and M.J. Parnham, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, *ca.* 400 pp., price Dfl. 240.00, US$ 92.25, ISBN 0-444-90312-7.

Pharmacological and chemical synonyms, compiled by E.E.J. Marler, Elsevier, Amsterdam, Oxford, New York, Tokyo, **8th revised edition,** 1984, *ca.* 550 pp., price Dfl. 245.00, US$ 94.25 ISBN 0-444-90359-3.

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Wilm's tumor: clinical and biological manifestations, edited by C. Pochedly and E.S. Baum, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, *ca.* 515 pp., price Dfl. 250.00, US$ 65.00, ISBN 0-444-00857-8.

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Progress in pesticide biochemistry and toxicology, Vol. 4, edited by D.H. Hutson and T.R. Roberts, Wiley, Chichester, New York, 1985, X + 368 pp., price £ 42.00, ISBN 0-471-90460-0.

New horizons in microbiology, edited by A. Sanna and G. Morace, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, X + 340 pp., price Dfl. 178.00, US$ 68.50, ISBN 0-444-80623-7.

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Microprocessor Programming and Applications for Scientists and Engineers

by **R.R. SMARDZEWSKI,** *Surface Chemistry Branch, Naval Research Laboratory, Washington, DC, USA*

This is a much-needed, clear and straightforward explanation of the fundamentals of microprocessing including machine-language programming and its research applications. Most of the literature on the general subject of microprocessors has been written with the electrical engineer or computer hobbyist in mind. This book, however, has been written by a research chemist for those scientists, engineers and hobbyists outside the electrical engineering community, who seek a basic understanding of microprocessors and their applications in laboratory environments.

First principles and elementary concepts are illustrated throughout the text by a series of 35 programmed instructional experiments. The particular microprocessor chosen for examination is the popular 6502 which is currently employed in a variety of microcomputing systems (Apple, Acorn, BBC, Commodore, Rockwell, and others). Actual programming examples contained in the text demonstrate the basic principles and ideas of 6502 machine-language programming and applications in research/laboratory situations.

The book is a must for the modern scientist and engineer working in physical chemistry, physics, analytical chemistry, applied engineering, design, CAD-CAM, process control, and bioengineering. As *no prior background in computer science or programming* is assumed, it will serve as an ideal elementary text for the beginner. At the same time, the advanced machine-language programmer will find it an invaluable reference handbook.

CONTENTS: **Chapter 1. Computer Organization.** Bits, Bytes and Nibbles. Instructions. Locations in Memory. Locations of I/O Devices. Data. Programming Languages. AIM 65 Computer. **2. Number Systems/Code Conversions.** Number Systems: Decimal Numbers. Binary Numbers. Octal and Hexadecimal Numbers. Fractional Numbers. BCD Numbers. Binary-to-Decimal. Decimal-to-Binary. Binary Addition. Binary Subtraction. Signed Binary Numbers. Binary Multiplication. Binary Division. Register Shifts. Code Conversions: Lookup Tables. Number Base Conversion. Hardware Devices. **3. Logic Gates.** Inverter (NOT) Gate. AND Gate. OR Gate. NAND Gate. NOR Gate. XOR Gate. Diode AND Function. Diode OR Function. Transistor Inverter. Equivalent Gates. Inverted Inputs, Outputs. Summary Table. Flip-Flops. Decoders. Multiplexers. Open-Collector Logic. Tri-State Logic. MOS and CMOS Devices. **4. The 6502 MPU.** Architecture. Execution. Mnemonics. Registers. Paging. Instructions and Addressing Modes. **5. The 6522 VIA.** Input/Output. Timing. Shifting. Function Control. Interrupt. Control. **6. Monitor Routines. 7. Data Acquisition.** Sensors. Signal Conditioners. Digital-> Analog, Analog-> Digital Conversions. Hardware A/D Converters. Other A/D Conversion Schemes. Beyond 8-bits. Sample-and-Hold Circuits. **8. Control.** Solid State Relays. Stepper Motors. Programmable-Gain Amplifiers. Thyristors (SCR's and Triacs). Power MOSFET's. **9. Data Communication Interfaces.** Centronics Parallel Interface. RS-232C Serial Interface. IEEE-488 Parallel Interface. Backplane Busses. **10. Program Development.** Assemblers. The FORTH Language. Structured Programming. Flowcharts. Development Systems. **Selected References. Appendix A. Reference Information.** R650X, R651X Microprocessors. R6522. Versatile Interface Adapter. AIM 65 Microcomputer. **Appendix. B. 6502 Instructions.** Descriptions. Addressing Modes. Internal Registers. Mnemonics/Op-Codes. Execution Times. **Index.**

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MONTH	N 1984	D 1984	J 1985	F	M	A	M	
Journal of Chromatography	312 314	315 316 317	318/1 318/2 319/1	319/2 319/3 320/1	320/2 321/1 321/2 322/1	322/2 322/3 323/1 323/2		The publication schedule for further issues will be published later
Chromatographic Reviews		313						
Bibliography Section				335/1		335/2		
Biomedical Applications		336/1 336/2	337/1	337/2 338/1	338/2	339/1	339/2 340	

INFORMATION FOR AUTHORS

(Detailed *Instructions to Authors* were published in Vol. 295, No. 2, pp. 555-558. A free reprint can be obtained by application to the publisher.)

Types of Contributions. The following types of papers are published in the *Journal of Chromatography* and the section on *Biomedical Applications:* Regular research papers (Full-length papers), Short communications and Notes. Short communications are preliminary announcements of important new developments and will, whenever possible, be published with maximum speed. Notes are usually descriptions of short investigations and reflect the same quality of research as Full-length papers, but should preferably not exceed four printed pages. For review articles, see page 2 of cover under Submission of Papers.

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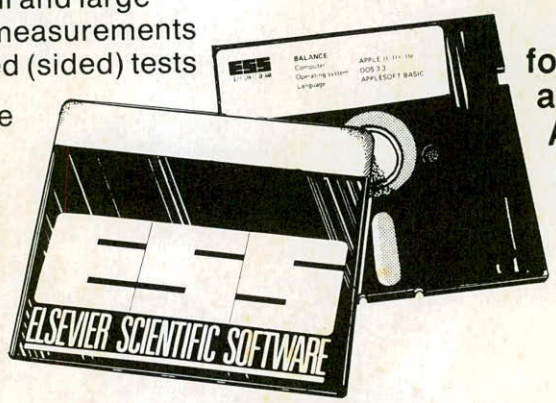

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