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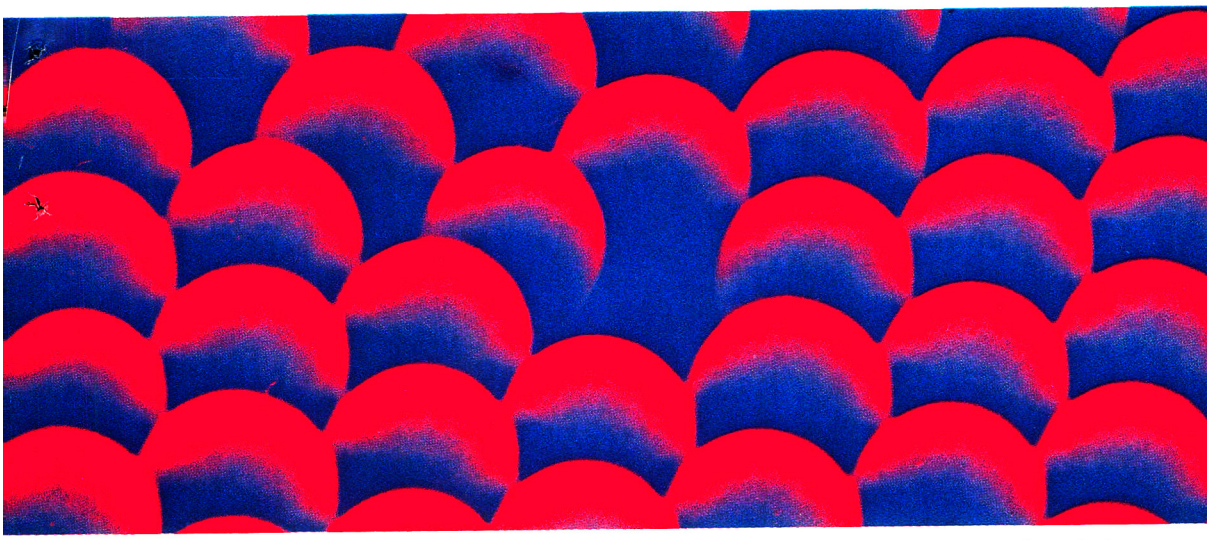

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3-HYDROXYHEXANOIC ACID: AN ABNORMAL METABOLITE IN URINE AND SERUM OF DIABETIC KETOACIDOTIC PATIENTS

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(First received May 22nd, 1984; revised manuscript received August 14th, 1984)

SUMMARY

A new organic acid, 3-hydroxyhexanoic acid, was identified in the urine or serum of five diabetic patients with ketoacidosis. The compound was not detected in the urine and serum of healthy subjects or diabetic patients without ketosis. The compound was also detected in the urine of a non-diabetic ketotic patient with dicarboxylic aciduria, suggesting that the occurrence of the compound is more related to the ketotic state than to "diabetic" ketosis.

INTRODUCTION

Ketoacidosis is a clinical condition caused by the accumulation of increased amounts of ketone bodies, namely 3-hydroxybutyrate, acetoacetate and acetone, in the body fluids. The other acids have been found in large amounts in the urine of ketoacidotic patients. The increased urinary excretion of adipic acid and suberic acid was found in diabetic patients with ketosis [1, 2]. These

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aliphatic dicarboxylic acids are formed through ω-oxidation of free fatty acids followed by β-oxidation. The increased urinary excretion of 3-hydroxyisovaleric acid [3], 3-hydroxyisobutyric acid and 2-methyl-3-hydroxybutyric acid [4] was found in ketoacidosis. The acids are known to be the intermediates of the metabolism of the branched-chain amino acids, leucine, valine and isoleucine, respectively. The increased urinary excretion of these acids is due to the enhanced catabolism of protein in ketoacidosis and to the inhibition of their further metabolic breakdown by the accumulated 3-hydroxybutyric acid and acetoacetic acid [5]. The urinary excretion of 2-hydroxybutyric acid [6, 7] and 2-hydroxyisovaleric acid [8] was increased in patients with lactic acidosis and ketoacidosis. Recently, we found abnormal metabolites, 5-hydroxyhexanoic acid, 3-hydroxyvaleric acid and 2-hydroxy-2-methyllevulinic acid, in the urine of diabetic ketoacidotic patients [9, 10]. In the present study, 3-hydroxyhexanoic acid was demonstrated in the urine and serum of diabetic ketoacidotic patients. To the authors' knowledge this metabolite has never been reported in human physiological fluids to date.

METHOD

Chemicals

3-Hydroxyhexanoic acid was synthesized according to the method of Cornforth et al. [11].

Trimethylsilylating agent, N,O-bis(trimethylsilyl)trifluoroacetamide, was purchased from Pierce (Rockford, IL, U.S.A.). Methoxylamine hydrochloride was obtained from Tokyo Kasei (Tokyo, Japan).

Samples

Urine samples were obtained from five patients with diabetic ketoacidosis, five diabetic patients without ketosis, and five healthy subjects. Serum samples were obtained from five patients with diabetic ketoacidosis, five diabetic patients without ketosis and five healthy subjects.

Case 1 with diabetic ketoacidosis showed hyperglycaemia (398 mg/dl), acidosis (pH 7.26, P_{CO_2} 23 mmHg, P_{O_2} 160 mmHg, base excess −16 mequiv./l under O_2 aspiration), lethargy, glycosuria, ketonuria, and dehydration. Case 2 showed hyperglycaemia (650 mg/dl), acidosis (pH 7.12, P_{CO_2} 43 mmHg, P_{O_2} 181 mmHg, base excess −16 mequiv./l under O_2 aspiration), lethargy, glycosuria, ketonuria, and dehydration. Case 3 showed hyperglycaemia (524 mg/dl), excitation, glycosuria and ketonuria. Gas analysis was not performed before insulin treatment. Case 4 showed hyperglycaemia (556 mg/dl), acidosis (pH 7.21, P_{CO_2} 14 mmHg, P_{O_2} 151 mmHg, base excess −19 mequiv./l under O_2 aspiration), lethargy, glycosuria, ketonuria, and dehydration. Case 5 showed hyperglycaemia (1418 mg/dl), acidosis (pH 7.21, P_{CO_2} 25 mmHg, P_{O_2} 80 mmHg, base excess −16 mequiv./l), coma, glycosuria, ketonuria and dehydration.

Urine was obtained from a 55-year-old, non-diabetic patient with ketosis and normoglycaemia. The patient complained of vomiting, vertigo, paraesthesia, and incontinence.

Sample preparation

Serum was filtered through CF-25 cone membrane filter (Amicon, Lexington, MA, U.S.A.). A 1-ml volume of serum ultrafiltrate or urine was acidified to pH 1 by addition of hydrochloric acid, and saturated with sodium chloride. As an internal standard, 10 μg or 50 μg of *p*-(*n*-amyl)benzoic acid were added to serum ultrafiltrate or urine, respectively. The organic acids were extracted three times with 3 ml of ethyl acetate. The extract was dehydrated over anhydrous sodium sulphate and evaporated with a stream of nitrogen. Methoxylamine hydrochloride (1 mg) in 50 μl of ethyl acetate was added to the extract and allowed to react at 60°C for 30 min. The extract was dried with a nitrogen stream and trimethylsilylated with 20 μl or 40 μl of N,O-bis(trimethylsilyl)trifluoroacetamide for serum samples or for urine samples, respectively. A 3-μl volume of each sample was used for gas chromatography—mass spectrometry (GC—MS).

Gas chromatography—mass spectrometry

A Hewlett-Packard 5710A gas chromatograph combined with a JMS D-300 mass spectrometer (JEOL) was used. The data were processed by a JMA 2000 computer from JEOL. The gas chromatograph was equipped with a 30 m × 0.25 mm I.D. OV-101 open-tubular glass capillary column and a splitless injector. Injection temperature was 250°C. The column temperature was programmed from 70°C to 260°C at 3°C/min.

Electron ionization (EI) mass spectra were recorded at an ionizing energy of 22 eV, an ionization current of 300 μA, and an accelerating voltage of 3 kV.

RESULTS

Fig. 1A shows the gas chromatogram of organic acids in the urine of the diabetic patient with ketoacidosis (case 4). Each component of the gas chromatogram was identified by EI and chemical ionization (CI) mass spectra. The EI mass spectrum of peak 19 is shown in Fig. 2B. The CI mass spectrum showed that the molecular ion of the compound was 276. The ion at *m/z* 261 is due to $[M-CH_3]^+$. The ion at *m/z* 233 is due to $[M-CH_3CH_2CH_2]^+$. The ion at *m/z* 219, $[M-57]^+$, is derived from the loss of CH_3 and CH_2CO, suggesting the structure of 3-hydroxycarboxylic acid [12]. The ion at *m/z* 204 is due to $[M-CH_3CH_2CH_2-CHO]^+$. The intense ion at *m/z* 145 is derived from the loss of CH_2COO-TMS, also suggesting the structure of 3-hydroxycarboxylic acid. Since the TMS (trimethylsilyl) derivative of synthesized 3-hydroxyhexanoic acid and peak 19 in Fig. 1 showed identical retention times on the gas chromatograms (Fig. 1) and identical EI mass spectra (Fig. 2), peak 19 was identified as 3-hydroxyhexanoic acid. 3-Hydroxyhexanoic acid was detected in two urine samples out of five diabetic ketoacidotic patients. The acid was not detected in the urine of five healthy subjects nor in the urine of five diabetic patients without ketosis. In diabetic ketoacidotic patients, the urinary excretion of lactic acid, acetoacetic acid, 2-hydroxybutyric acid, 3-hydroxybutyric acid, 2-hydroxyisovaleric acid, 2-methyl-3-hydroxybutyric acid, 3-hydroxyisovaleric acid, 3-hydroxyvaleric acid, 5-hydroxyhexanoic acid, 2-hydroxy-2-methyllevulinic acid and adipic acid was found to be increased.

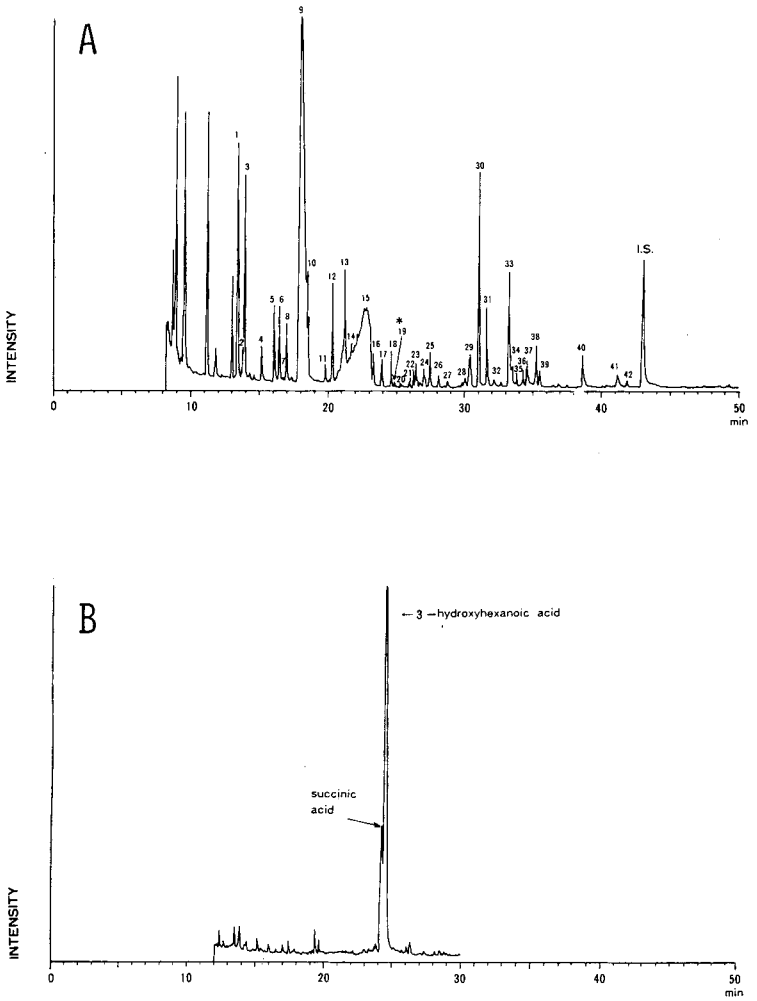

Fig. 1. Gas chromatograms of the methoxime-trimethylsilylated ethyl acetate extract from urine of a diabetic ketoacidotic patient (A) and of trimethylsilylated 3-hydroxyhexanoic acid (B). Peak identification: 1, lactic acid; 2, 2-hydroxyisobutyric acid; 3, glycolic acid; 4, 5, acetoacetic acid; 6, 2-hydroxybutyric acid; 8, 3-hydroxypropionic acid; 9, 3-hydroxybutyric acid; 10, 2-hydroxyisovaleric acid; 11, 2-methyl-3-hydroxybutyric acid; 12, 3-hydroxyisovaleric acid; 13, 2-ethylhydracrylic acid; 14, 3-hydroxyvaleric acid; 15, urea; 16, dimethylmalonic acid; 17, glycerol; 18, succinic acid; 19, 3-hydroxyhexanoic acid; 22, glyceric acid; 23, fumaric acid; 24, 5-hydroxyhexanoic acid; 25, 4-deoxythreonic acid; 28, 3-deoxytetronic acid; 29, 2-hydroxy-2-methyllevulinic acid; 30, 2-deoxytetronic acid; 33, adipic acid; 37, 3-methyladipic acid; 38, 2,3-dideoxypentonic acid; 40, 4-hydroxyphenylacetic acid; 42, isosaccharinolactone.

Fig. 3 shows the gas chromatogram of organic acids in the serum of the diabetic patient with ketoacidosis (case 4). 3-Hydroxyhexanoic acid was also detected in the diabetic ketoacidotic serum. 3-Hydroxyhexanoic acid was detected in four out of five diabetic ketoacidotic patients' sera. The acid was not detected in the sera of five healthy subjects nor in the sera of five diabetic patients with no ketosis. In the diabetic ketoacidotic patients the serum

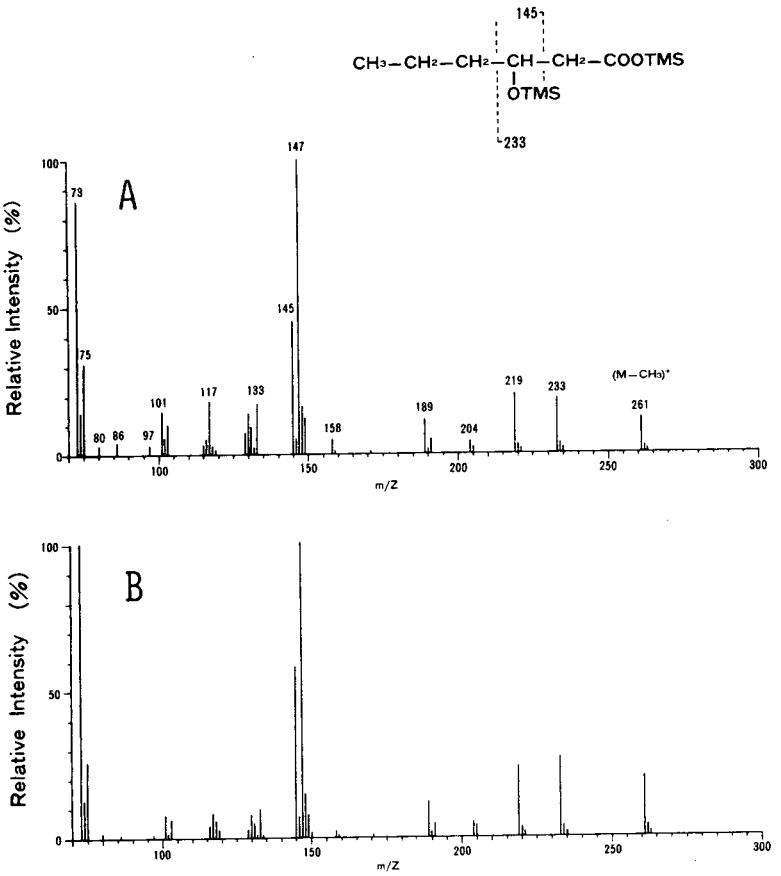

Fig. 2. EI mass spectra of trimethylsilylated 3-hydroxyhexanoic acid (A) and of peak 19 (B) in Fig. 1.

concentrations of lactic acid, 2-hydroxybutyric acid, 3-hydroxybutyric acid, 2-methyl-3-hydroxybutyric acid, 3-hydroxyisovaleric acid, 3-hydroxyvaleric acid, and 2-hydroxy-2-methyllevulinic acid were elevated.

Table I shows the concentrations of 3-hydroxyhexanoic acid in the urine and serum of diabetic ketoacidotic patients. In urine of two diabetic ketoacidotic

TABLE I

CONCENTRATION OF 3-HYDROXYHEXANOIC ACID IN URINE AND SERUM OF DIABETIC KETOACIDOTIC PATIENTS

	Diabetic ketoacidosis					Non-ketotic diabetes (n = 5)	Healthy subjects (n = 5)
	1	2	3	4	5		
Urine (μg/mg creatinine)	0.43	ND*	ND	1.81	ND	ND	ND
Serum (μg/ml)	ND	0.25	1.42	0.95	0.25	ND	ND

*Not detected.

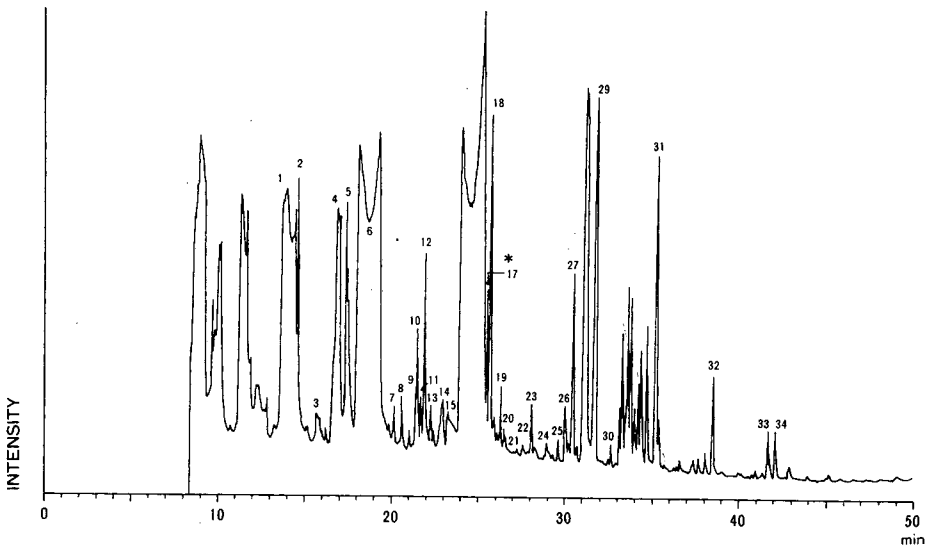

Fig. 3. Gas chromatogram of methoxime-trimethylsilylated organic acids in serum of a diabetic ketoacidotic patient. The peaks were identified as follows. 1, lactic acid; 2, glycolic acid; 4, 2-hydroxybutyric acid; 5, 3-hydroxypropionic acid; 6, 3-hydroxybutyric acid; 7, 2-methyl-3-hydroxybutyric acid; 8, 3-hydroxyisovaleric acid; 9, 2-ethylhydracrylic acid; 11, 3-hydroxyvaleric acid; 14, urea; 17, 3-hydroxyhexanoic acid; 20, glyceric acid; 21, 4-deoxyerythronic acid; 22, 4-deoxythreonic acid; 26, 3-deoxytetronic acid; 27, 2-hydroxy-2-methyllevulinic acid; 28, 2-deoxytetronic acid; 31, 2,3-dideoxypentonic acid; 33, isosaccharinolactone.

patients, 3-hydroxyhexanoic acid was detected at concentrations of 0.43 and 1.81 μg/mg creatinine. In sera of four diabetic patients, 3-hydroxyhexanoic acid was detected at concentrations of 0.25, 1.42, 0.95 and 0.25 μg/ml.

3-Hydroxyhexanoic acid was detected in the urine of a non-diabetic ketotic patient with dicarboxylic aciduria. The compound became undetectable in the patient's urine when ketosis and subjective complaints disappeared.

DISCUSSION

3-Hydroxyhexanoic acid has not been reported to be present in physiological fluids so far. This acid was first detected in the urine and serum of diabetic patients with ketoacidosis. The occurrence of the acid seemed to be correlated to ketosis, since the acid was detected in the urine of a non-diabetic patient with dicarboxylic aciduria and ketosis.

The formation of the compound seems to be closely related to the enhanced β-oxidation of free fatty acids. A possible source of 3-hydroxyhexanoic acid is an intermediate of the enhanced β-oxidation in mitochondria due to the impaired sequence of β-oxidation pathway, which is caused by the relative deficiency of coenzyme A and NAD^+ in mitochondria. Another possible source of the acid is an intermediate of β-oxidation in peroxisome due to the overwhelming supply of free fatty acids for the capacity of β-oxidation in mitochondria, or due to the impaired transport of acyl-coenzyme A into mitochondria, which may be caused by the relative deficiency of carnitine.

Although the occurrence of some higher-molecular-weight 3-hydroxycarboxylic acids (C_8, C_9, C_{10} and C_{12}) in plasma was reported by Pfordt and Spiteller [13], we could not identify these acids except the possible detection of 3-hydroxyoctanoic acid by monitoring its characteristic fragment ions at *m/z* 289 $[M-CH_3]^+$, 247 $[M-CH_3-CH_2CO]^+$, 233 $[M-C_5H_{11}]^+$, and 173 $[M-CH_2COO\text{-}TMS]^+$. A peak suggestive of 3-hydroxyoctanoic acid was detected in four diabetic ketoacidotic sera (cases 2, 3, 4, and 5). It is possible that both acids have a common pathway of formation.

Further study is needed to clarify the metabolic pathway involved in the formation of the compounds and the clinical significance of the metabolites' appearance in body fluids.

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CHROMBIO. 2363

ABSOLUTE CONFIGURATION OF 3-HYDROXYADIPIC ACID IN HUMAN URINE

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SUMMARY

A method involving derivatization and combined gas chromatography—mass spectrometry has been developed to separate the enantiomers of 3-hydroxyadipic acid. By combining this method with asymmetric synthesis of the same acid, it has been shown that 3-hydroxyadipic acid excreted in urine consists of at least 95% of the L-enantiomer. This finding supports the hypothesis that dicarboxylic acids are degraded by ordinary β-oxidation, and indicates that adipic acid may be converted into succinic acid.

INTRODUCTION

The advent of gas chromatography–mass spectrometry (GC–MS) instrumentation has made possible detailed analysis and structure elucidation of urinary organic acids, and this has resulted in the discovery of a variety of "new" metabolic disorders during the last two decades [1]. The urinary excretion of adipic and suberic acid during ketosis has previously been described [2]. Furthermore, increased urinary excretion of the two acids has been observed following the ingestion of longer dicarboxylic [3] and monocarboxylic

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[4, 5] acids. In ketosis, adipic acid and suberic acid may be formed from endogenous long-chain fatty acids, conceivably by tandem ω-oxidation—β-oxidation processes [4, 6].

In addition to adipic and suberic acid, the corresponding 3-hydroxylated acids have been detected in the urine of ketotic patients [7], and of volunteers ingesting dicarboxylic acids [8]. This indicates that 3-hydroxydicarboxylic acids may be intermediates in the degradation of dicarboxylic acids and, furthermore, suggests that the degradation involves an ordinary β-oxidation. If this were the case, the intermediate 3-hydroxylated diacids should be formed in the L-configuration. In order to shed more light on the metabolic degradation of dicarboxylic acids it would therefore be useful to ascertain the configuration of the corresponding 3-hydroxylated metabolites. The present paper describes our successful efforts toward determining the configuration of urinary 3-hydroxyadipic acid (*1*). Essentially, the method employed consists of chiral derivatization and gas chromatographic analysis on an achiral column [9].

EXPERIMENTAL

Chemicals

All of the chemicals used were commercial products of high purity supplied by Fluka (Buchs, Switzerland).

Synthesis of reference compounds

7-Oxabicyclo[4,1,0]hept-3-ene (*2*). A solution of *m*-chloroperbenzoic acid (40 mmol) in chloroform (200 ml) was added dropwise to 1,4-cyclohexadiene (40 mmol) dissolved in chloroform (75 ml). The reaction mixture was stirred at ambient temperature until the starch—iodine test was negative. The mixture was washed (aqueous sodium bicarbonate) and dried (sodium sulphate). The solvent was removed under reduced pressure, and the residue was distilled through a Vigreux column to give 2.42 g (63%) of *2* (lit. [10]: b.p. 72°C at 70 mmHg). The ^{1}H- and ^{13}C-NMR (nuclear magnetic resonance) spectra were in accordance with the literature [10, 11]. IR (infrared) (cm^{-1}): 2990 (s), 2890 (s), 2810 (w), 1480 (w), 1420 (s), 1215 (s), 1010 (m).

3-Cyclohexenol (*3*). A solution of *2* (13.6 mmol) in dry diethyl ether (75 ml) was added to a slurry of lithium aluminium hydride (10 mmol) in dry diethyl ether (50 ml). After stirring at ambient temperature for 5 h, water (5 ml) was added. Water and aluminium compounds were trapped by adding magnesium sulphate. The solid salts were filtered and washed with diethyl ether. The diethyl ether was then removed from the combined filtrates under reduced pressure, and the residue was distilled to give 1.05 g (85%) of *3* (lit. [12]: b.p. 90—94°C at 50 mmHg). IR and ^{1}H-NMR spectra were in accordance with the literature [12]. ^{13}C-NMR ($C^2H_3O^2H$): δ 127.5, 125.2, 67.7, 35.1, 31.9, 25.0. MS [*m/z*, relative abundance (%)]: 98 (2), 97 (2), 83 (7), 80 (100), 79 (24), 70 (8), 69 (10).

3-Cyclohexenol, O-acetyl-D-mandelic ester (*4a*). O-Acetyl-D-mandelic acid was prepared as described in the literature [13]. The corresponding acid chloride was prepared by adding freshly distilled thionyl chloride (100 mmol)

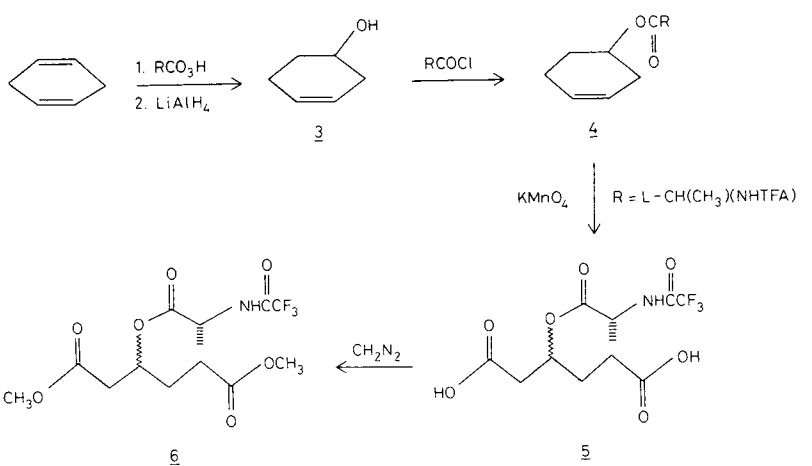
to a solution of O-acetyl-D-mandelic acid (25 mmol) in methylene chloride (10 ml). Unreacted thionyl chloride was evaporated in a stream of dry nitrogen, and the residue was dissolved in methylene chloride (5 ml). 3-Cyclohexenol (10 mmol) was added to this solution, and this mixture was occasionally shaken over a period of four days. When the reaction was complete, water (10 ml) was added. The water phase was made slightly basic by adding aqueous sodium bicarbonate and extracted with diethyl ether (3 × 10 ml). The combined extracts were dried (sodium sulphate) and worked up in the usual way to give a quantitative yield of *4a* which was used for gas—liquid chromatographic (GLC) analysis without further purification.

3-Cyclohexenol, N-trifluoroacetyl-L-phenylalanyl ester (*4b*). N-TFA-L-phenylalanine was made by carefully adding trifluoroacetic anhydride (50 mmol) to L-phenylalanine (10 mmol) which was cooled on an ice-bath. After the amino acid had dissolved, unreacted anhydride was removed by a stream of dry nitrogen. Thionyl chloride (100 mmol) was added, and after 8 h unreacted thionyl chloride was removed as described for compound *4a*. Alcohol *3* was esterified to give *4b* which was worked up as described for ester *4a*.

3-Cyclohexenol, N-TFA-L-prolyl ester (*4c*). N-TFA-L-prolyl chloride was prepared according to literature procedures [14], and the esterification and isolation were performed as described for ester *4a*.

3-Cyclohexenol, N-TFA-L-alanyl ester (*4d*). N-TFA-L-alanyl chloride was prepared according to standard literature procedures [15]. The esterification of *3* and the isolation of *4d* were performed as described for ester *4a*. ^{1}H-NMR (C^2HCl_3): δ 5.6 (2H, m), 4.1 (1H, s), 4.0—3.6 (2H, m), 2.6—1.3 (9H, m). ^{13}C-NMR (C^2HCl_3): δ 171.2, 126.9, 126.8, 123.3, 123.1, 72.1, 72.0, 30.6, 30.5, 27.1, 27.0, 23.0, 22.9, 17.9, 17.8.

Triester 6. This compound was prepared in two steps (Scheme 1). Acid *5* was made by oxidation of *4d* with potassium permanganate according to a literature procedure [16]. Ester *4d* (2.5 mmol) and triethylbenzylammonium chloride were dissolved in benzene (15 ml) and stirred with a solution of potassium permanganate (11 mmol) in water (30 ml) for 2 h. The reaction was quenched by adding sodium bisulphite and hydrazine sulphate, and was



Scheme 1.

worked up in the usual way. The residue (0.72 g, 80%) was essentially pure *5*. ^{1}H-NMR ($C^2H_3O^2H$): δ 5.4 (1H, m), 4.6 (1H, m), 3.6 (1H, m), 2.8—1.6 (6H, m), 1.5 (3H, d). ^{13}C-NMR (C^2HCl_3): δ 175.6, 175.5, 172.6, 171.0, 170.8, 71.6, 49.2, 38.7, 29.7, 29.2, 29.1, 16.8. IR (cm^{-1}): 2970 (s), 2850 (s), 1730 (s), 1700 (s), 1680 (s), 1570 (s).

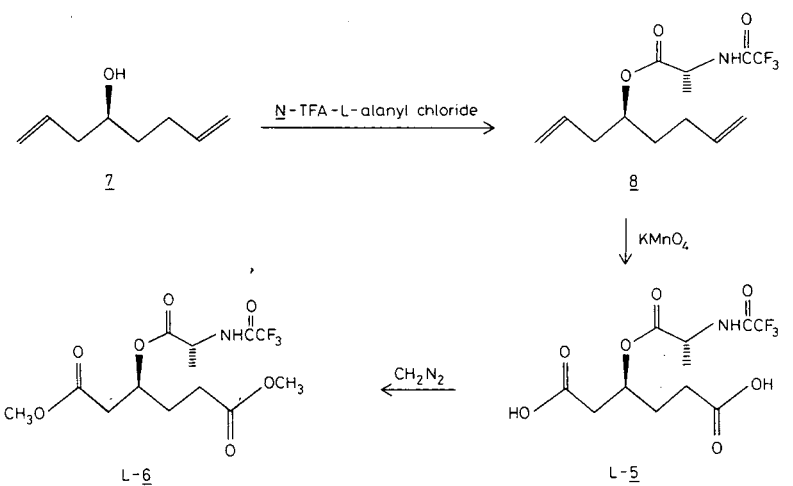
Triester *6* was then obtained in quantitative yield by treating *5* with an ethereal solution of diazomethane. ^{1}H-NMR ($C^2H_3O^2H$): δ 5.2 (1H, m), 4.6 (1H, m), 3.3 (6H, s), 2.8—1.6 (6H, m), 1.5 (3H, d). ^{13}C-NMR ($C^2H_3O^2H$): δ 172.3, 72.4, 39.6, 29.4, 29.1, 29.0, 16.7. IR (cm^{-1}): 3040 (m), 2980 (s), 2880 (m), 1740 (s), 1685 (s), 1550 (s). MS [*m/z*, relative abundance (%)]: 174 (7), 173 (16), 159 (16), 141 (47), 140 (73), 114 (12), 113 (45), 109 (18), 101 (17), 85 (14), 81 (19), 71 (100).

L-1,7-Octadien-4-ol, N-TFA-L-alanyl ester (8). L-1,7-Octadien-4-ol (7) (0.20 g, 1.6 mmol), prepared from L-malic acid as outlined by Bartlett [17], was esterified by N-TFA-L-alanyl chloride (3.25 g, 16 mmol) as described for compound *4d*. The product was obtained in quantitative yield when worked up in the usual way. ^{1}H-NMR ($C^2H_3O^2H$): 5.4 (2H, m), 5.0 (5H, m), 4.5 (1H, m), 2.6–1.1 (9H, m). ^{13}C-NMR ($C^2H_3O^2H$): 172.8, 136.2, 134.0, 118.3, 115.7, 76.1, 39.8, 33.0, 27.4, 16.7. IR (cm^{-1}): 3270 (m), 3030 (m), 2880 (s), 2800 (s), 1715 (s), 1635 (m), 1540 (m), 1345 (m), 1150 (s), 900 (m). MS [*m/z*, relative abundance (%)]: 168 (18), 141 (8), 140 (100), 109 (6), 108 (5), 93 (14), 92 (4).

Triester L-6. Ester *8* (0.100 g, 0.35 mmol) was oxidized as described for compound *4d* to give diacid L-*5* (0.099 g, 88%) which was converted to triester L-*6* in quantitative yield by treatment with diazomethane (Scheme 2). The spectral data of L-*6* were identical to those of the 1:1 diastereoisomeric mixture of *6*, except for the expected intensity differences in the ^{13}C-NMR spectra (see Fig. 2).

Preparation of urine extracts

Urine was collected quantitatively for 12 h from a healthy human given



Scheme 2.

dodecanedioic acid (20 mmol) perorally. An aliquot (100 ml) of the urine was acidified with 1 *M* hydrochloric acid to pH 1 and saturated with sodium chloride. This solution was extracted with ethyl acetate (3 × 100 ml) at 4°C, dried (sodium sulphate), and evaporated under reduced pressure, until a brown viscous oil was obtained. N-TFA-L-alanyl chloride (10 mmol) was added, and this solution was occasionally shaken for three days. Unreacted N-TFA-L-alanyl chloride was hydrolysed by adding water (25 ml) and the solution was extracted with ether (3 × 25 ml), dried (sodium sulphate) and evaporated under reduced pressure. The residue was dissolved in methanol and treated with an ethereal solution of diazomethane prior to GC—MS analysis.

Capillary GLC

A Hewlett-Packard HP-5880A gas chromatograph, equipped with a flame-ionization detector and a fused-silica column (25 m × 0.25 mm I.D.) coated with CP-Sil 19 CB (Chrompack) or OV-101 (Hewlett-Packard) was used for analysis of the reference compounds. Injections were made on a split injector with a split ratio of 1:30. The carrier gas (hydrogen) had a linear gas flow-rate of 45 cm/sec (at room temperature). The injector port and detector temperatures were 230°C and 270°C, respectively, whereas the oven temperature was optimized for each sample.

Gas chromatography—mass spectrometry

The measurements were performed with a VG Analytical MicroMass 7070H double-focusing mass spectrometer equipped with a VG Data System 2050 (PDP 8a computer), a Hewlett-Packard 5710 gas chromatograph, and a digital multiple ion detector unit controlled by the data system. The gas chromatograph was equipped with a fused-silica capillary column (25 m × 0.25 mm I.D.) coated with Carbowax 20M (Hewlett-Packard). Injection port temperature was 250°C, and the helium flow-rate was 2 ml/min. The interface was a direct jet inlet, heated to 250°C. Ionizing and accelerating potentials were 70 eV and 4 kV (standard, otherwise controlled by the multiple ion detector unit), respectively, and the temperature in the ion source was 220°C.

NMR spectroscopy

The ^{1}H-NMR spectra were obtained on a Jeol PMX 60 si spectrometer (60 MHz) at 35°C. The samples were 1—5% by weight in C^2HCl_3 or CCl_4 with tetramethylsilane as internal standard. The ^{13}C-NMR spectra were recorded on a Jeol FX 90 Q instrument (22.50 MHz) at 29°C. The samples, with tetramethylsilane as internal reference, were 5—10% by weight in C^2HCl_3 or $C^2H_3O^2H$ which provided the deuterium signal for the NMR field lock. The spectra were run with a spectral width of 5000 Hz, a pulse width of 5.5 μsec (45°) and a pulse repetition time of 5 sec. The computer operation conditions gave a digital resolution of 0.3 Hz. The C—H decoupling was made by a broad band decoupling pulse at 89.55 MHz.

IR spectroscopy

The infrared spectra were obtained on a Shimadzu IR-420 infrared spectrophotometer either as liquid film or potassium bromide tablet.

RESULTS AND DISCUSSION

In order to be able to determine the configuration of 3-hydroxyadipic acid (*1*) formed by metabolic hydroxylation of adipic acid, it is necessary to develop a method which allows enantiomeric separation of *1* or a derivative of *1*. A common method employed to achieve such separation of racemic alcohols on an analytical scale is to make diastereomeric esters by reacting the alcohols with a chiral acid chloride. Among the best and most convenient reagents for this purpose are N-TFA amino acid chlorides which are readily available and usually give good enantiomeric resolution when the resulting diastereoisomeric mixtures are analysed by GLC [18]. To see if this scheme could be adopted for the analysis of racemic and optically active *1* we first performed exploratory separation experiments with various N-TFA amino acid esters (*4*) of 3-cyclohexenol, an intermediate in the synthesis of racemic *1* (Scheme 1). GLC analysis of the esters on an OV-101 column revealed that the enantiomeric separation varied with the amino acid moiety attached to *3*. Thus, only modest separation was obtained with aromatic esters whereas aliphatic esters showed higher separation ability (Table I). The N-TFA-L-alanyl ester derivative, *4d*, proved to be the best and was chosen for subsequent synthetic transformations.

TABLE I

ENANTIOMERIC SEPARATION OF SOME DERIVATIVES OF 3-CYCLOHEXENOL ON AN OV-101 CAPILLARY COLUMN

Derivative	*R* factor
O-Acetyl-D-mandelic ester	0.6
N-TFA-L-phenylalanyl ester	0.8
N-TFA-L-prolyl ester	1.0
N-TFA-L-alanyl ester	1.2

Permanganate oxidation of *4d* gave a 1:1 diastereoisomeric mixture of the N-TFA-L-alanyl ester *5* of 3-hydroxyadipic acid which was transformed into a diastereoisomeric mixture of the corresponding triester *6* by treatment with diazomethane (Scheme 1). Due to higher polarity compound *6* showed lower enantiomeric separation than ester *4d*; the resolution factor was as low as 0.8, when the GLC analysis was performed on an OV-101 column. However, by increasing the polarity of the column the resolution improved so significantly that baseline separation of the diastereoisomers was achieved (Table II and Fig. 1a).

The diastereoisomeric mixture of *6* can also be analysed by ^{13}C-NMR spectroscopy. The basis for this is that diastereoisomers usually show a pair of signals, one signal for each diastereoisomer, for one or several of the carbon atoms in the molecule. Triester *6* gives rise to three such pairs (Fig. 2a). Two of them, situated at approximately 172 and 176 ppm, are due to the carbon atoms in two of the carboxyl groups and have shift differences of 0.2(2) and 0.1(3) ppm, respectively. The third pair, at approximately 30 ppm, has a shift difference of 0.0(7) ppm and is caused by one of the methylene groups

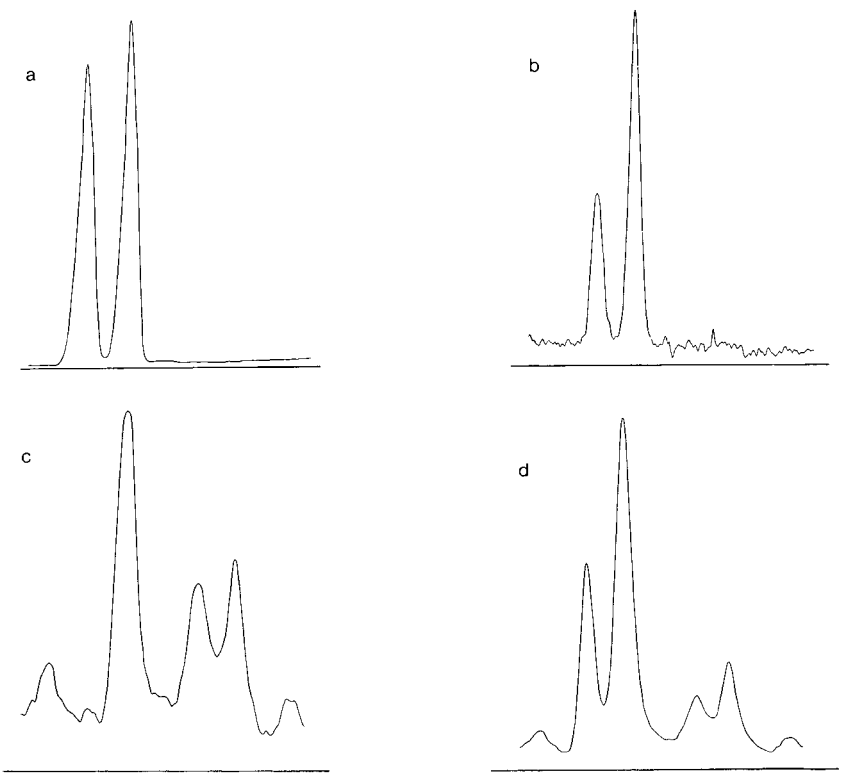


Fig. 1. Multiple ion detection gas chromatograms at m/z = 109 of (a) a 1:1 mixture of D-*6* and L-*6*, (b) a 35:65 mixture of D-*6* and L-*6*, (c) parts of the gas chromatogram of a derivatized ethyl acetate urinary extract, (d) parts of the gas chromatogram of a derivatized ethyl acetate urinary extract to which has been added some of the 1:1 diastereoisomeric mixture of *6*.

TABLE II

ENANTIOMERIC SEPARATION OF 3-HYDROXYADIPIC ACID, N-TFA-L-ALANYL ESTER AS DIMETHYL ESTER ON VARIOUS GLC COLUMNS

Column	*R* factor
OV-101	0.8
CP-Sil 19 CB	1.2
Carbowax 20M	1.5

in the adipic acid moiety. Within each pair the integrals were equal, so there is no significant difference in relaxation times and nuclear Overhauser effects for diastereoisomeric carbons. Thus, two independent methods are available for the analysis of *1*.

To determine the configuration of urinary 3-hydroxyadipic acid it is necessary to synthesize optically active *1* with known configuration. This was done by permanganate oxidation of *8*, prepared from L-1,7-octadien-4-ol (*7*) and N-TFA-L-alanyl chloride (Scheme 2). Optically active *7*, with the L-enantiomer predominating, was synthesized from L-malic acid [17]. The product thus

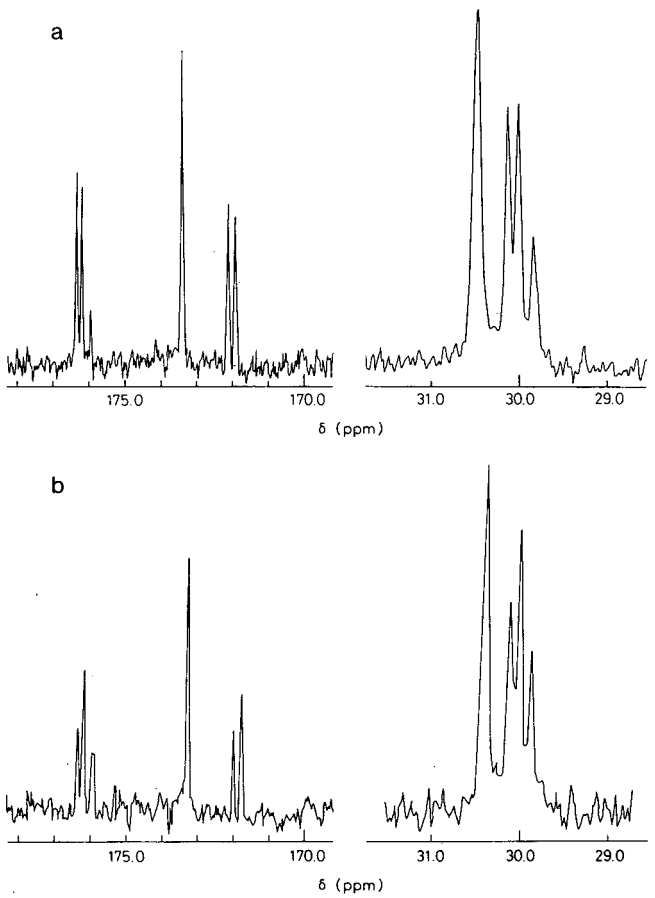


Fig. 2. Parts of the ^{13}C-NMR spectrum of (a) a 1:1 diastereoisomeric mixture of *5*, (b) a 34:66 diastereoisomeric mixture of D-*5* and L-*5*.

obtained, L-*5*, was converted to triester L-*6* in the usual way and analysed by GLC. This analysis revealed that L-*6* was contaminated with 35% of D-*6* and, furthermore, that L-*6* has the longer retention time of the two diastereoisomers under the conditions employed (Fig. 1b). This result was confirmed by a ^{13}C-NMR investigation of the unesterified mixture of *5*; on average the separable peaks due to the two diastereoisomers showed a relative intensity corresponding to a 66:34 mixture of L-*5* and D-*5* (Fig. 2b).

The formation of the D-enantiomer of *5* may have occurred during the preparation of *7* or during its subsequent esterification and oxidation (Scheme 2). In order to clarify this a lanthanide shift experiment was performed on *7*. By addition of a chiral shift reagent, tris[*d*,*d*-dicamphylmethanato]europium-(III), to the alcohol the allylic hydrogen atoms in the dienol moiety of the D-enantiomer of *7* are completely separated from the corresponding hydrogen atoms due to L-*7* [18]. By comparing the integrals of the separated peaks it was established that *7* was a 37:63 mixture of the D- and L-enantiomers, respectively. Thus, racemization took place prior to the esterification and oxidation steps.

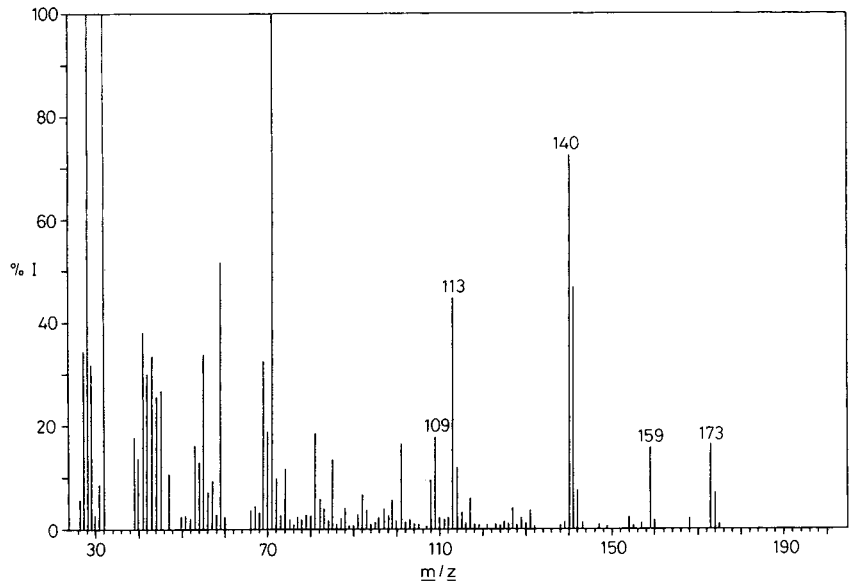


Fig. 3. The electron-impact mass spectrum of a 1:1 diastereoisomeric mixture of *6*.

The mass spectrum of *6* (Fig. 3) showed extensive fragmentation and no molecular ion; furthermore, there was no difference between the mass spectra of the diastereoisomers. The fragment at m/z = 173 is probably due to cleavage of the C—O ester bond in position 3 of the adipic acid moiety. Analogous fragmentation is known from simple *n*-alkyl-N-TFA-L-alanyl esters [19]. The dominant peak at m/z = 140 is characteristic for N-TFA-alanyl esters [20] and is assigned to α-cleavage between the carbonyl group and the α-carbon atom in the alanyl group. Another typical fragmentation process from such esters is α-cleavage on the other side of the carbonyl group, which in this case results in a weak peak with m/z = 168. N-TFA-alanyl esters always have a rather strong peak at m/z = 141, but in the present case this peak may also be due to a loss of methanol from the fragment at m/z = 173. The peak at m/z = 113 originates partly from m/z = 141, by C=O extrusion, and partly from m/z = 173, by elimination of methyl formate (confirmed by metastable daughter-ion scanning from m/z = 173 and 141).

One problem which frequently arises when biological samples are analysed by GC—MS, is that the compound of interest is masked by one or more co-eluting substances. This problem can be avoided by using high-resolution gas chromatography which normally gives well resolved and narrow peaks. Unfortunately, this often generates new problems because the peaks may be so narrow that it is impossible to obtain a full mass spectrum on magnetic scanning instruments. To circumvent these problems magnetic scanning can be replaced by rapid switching of the accelerating and electrostatic analyser voltages between preset values which correspond to characteristic fragments in the mass spectrum of the compound under consideration. This method is extensively used in quantitative measurements (when the number of masses is small), but by increasing the number of m/z values the selectivity can be almost as great as

in the full scanning mode. The main disadvantage with the multiple ion detection method is that the mass spectrum of the compound under consideration must be known, but in the present case this is no obstacle since the necessary reference compounds can be prepared both optically active and as racemates.

The determination of the configuration of metabolic 3-hydroxyadipic acid starts with derivatization of an ethyl acetate extract from a urine sample. Treatment with N-TFA-L-alanyl chloride and diazomethane converts *1* into the same derivatives as the synthetic standards, although some of the acid is lost by lactonization [8]. The sample is now ready for multiple ion detection analysis which was performed on eight of the fragment ions from the mass spectrum of the synthetic standard (Fig. 4). Comparison of the mass selective chromatograms showed that one peak matched all eight ions, the retention time of which was the same as that of the L-enantiomer of the synthetic compound (Fig. 1b and c). This finding was further substantiated by adding some of the 1:1 diastereoisomeric mixture of *6* to the urine sample before injection; this gave an increased amount of the L-enantiomer, together with a new peak with the same retention time as the synthetic D-form (Fig. 1d).

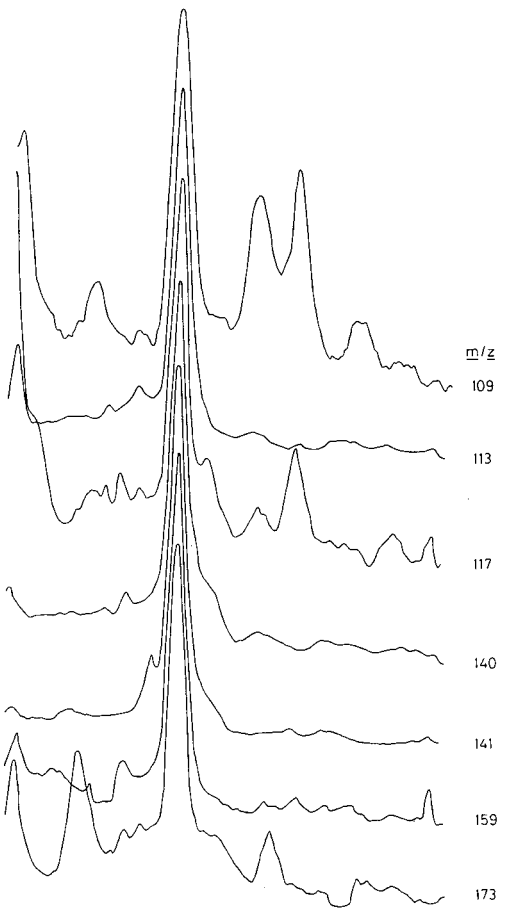


Fig. 4. GC—MS multiple ion detection chromatogram of an ethyl acetate urine extract after derivatization with N-TFA-L-alanyl chloride and diazomethane. The ions selected are the most characteristic ones in the mass spectrum of *6*.

CONCLUDING REMARKS

It has previously been demonstrated that administration of higher (C_8—C_{12}) ^{14}C-labelled dicarboxylic acids to ketotic rats gave a decrease in circulating ketone bodies and an increase in [^{14}C]glucose [21]. Administration of adipic acid gave the same antiketogenic effect, together with a rise in blood glucose and succinic acid [3]. These and other studies have indicated that adipic acid can be further oxidized in the body [22, 23] probably by β-oxidation [8, 24]. Formation of succinic acid, by β-oxidation of adipic acid, may lead to an increased glucose production and be of physiological importance in the regulation of ketosis. Our finding of L-3-hydroxyadipic acid in urine supports the hypothesis that dicarboxylic acids are degraded by ordinary β-oxidation, and indicates that adipic acid may be converted into succinic acid.

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RAPID GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC QUANTITATION OF γ-AMINOBUTYRIC ACID IN BIOLOGICAL SPECIMENS

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SUMMARY

A mass fragmentographic method for γ-aminobutyric acid (GABA) quantitation using the heptafluorobutyryl-cyclohexyl-GABA derivative is described. Both capillary and packed column gas chromatography were used. This procedure employs 2,2[$^2H_2$]GABA as an internal standard and allows the rapid, sensitive, and specific measurement of GABA with a minimum of sample clean-up. Application of the method is demonstrated in mouse embryonic brain, body, and palate and human platelets, plasma, cerebrospinal fluid, and urine.

INTRODUCTION

γ-Aminobutyric acid (GABA) is present in high concentration in the mammalian brain and is believed to be a major inhibitory neurotransmitter [1]. Levels in cerebrospinal fluid (CSF) may reflect altered GABA metabolism in various neurologic and psychiatric diseases [2—6]. High levels are also present in non-neuronal tissue of rat pancreatic islets [7], human insuloma [7], rat ovary [8], and rat Fallopian tube [9], whereas lower GABA concentrations occur in human blood [10] and peripheral tissues [11, 12]. GABA is involved in respiratory regulation [13], cardiovascular control [14], temperature regulation [15], palate morphogenesis [16], and polyamine interrelations during neuronal development [17]. Measurements of GABA in body fluids and tissues will help elucidate its metabolic role and diagnostic value.

Gas chromatography—mass spectrometry—selected ion monitoring

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(GC—MS—SIM) with deuterated GABA as an internal standard provides a sensitive, rapid, and accurate method for GABA quantitation. The heptafluorobutyryl (HFB)-cyclohexyl-GABA derivative described in this paper with its properties of a moderatively high GC retention temperature and abundant high mass ions gives it selected advantages for GABA analysis over derivatives previously described [18—21]. This work reports a rapid method for GABA measurement and its application to a wide variety of biological specimens.

EXPERIMENTAL

Materials

2,2[$^2H_2$]GABA (99%) was purchased from Merck Sharp & Dohme Canada, (Pointe Claire-Dorval, Quebec, Canada); GABA from Sigma (St. Louis, MO, U.S.A.), heptafluorobutyric anhydride from Regis Chemical (Morton Grove, IL, U.S.A.) and hydrogen chloride lecture bottle from Matheson Gas Products (Morrow, GA, U.S.A.). GABA and GABA-d_2 stock solutions of 10 mg/ml of deionized, distilled water were prepared and diluted to 20 ng/ml solutions. A stock solution of 1.5 *M* hydrochloric acid in cyclohexanol was prepared by bubbling hydrogen chloride gas from the lecture bottle into the cyclohexanol [22]. Human CSF specimens were obtained from University and Children's Hospitals (Cincinnati, OH, U.S.A.). Human platelets and plasma were received from the Hoxworth Blood Center (Cincinnati, OH, U.S.A.).

Sample preparation

Palates of day 14.5 embryos of AJ mice were dissected as previously described [16]. Brains and bodies from the same developmental age embryo were also used. Five brains or bodies were homogenized in 1 ml and 50 palate pairs were homogenized in 0.1 ml of deionized, distilled water using a Thomas tissue hand homogenizer (A.H. Thomas, Philadelphia, PA, U.S.A.). Aliquots were taken for protein determination by the method of Lowry et al. [23]. The remaining homogenates were diluted with ethanol to a final concentration of 80%. The internal standard, GABA-d_2 (100 ng), was added to the palate homogenate or aliquots (50—500 μl) of brain or body homogenates. Human plasma (1 ml), platelets (1 ml), and pooled child CSF (1 ml) were lyophilized with 100 ng GABA-d_2 and 0.5 ml of 80% ethanol was added. Samples were centrifuged at 10,000 *g* for 30 min. The supernatant was transferred to glass vials and evaporated to dryness by a stream of nitrogen at 80°C [22]. Other CSF specimens (200 μl) and all urine samples (200 μl), after addition of 100 ng of GABA-d_2, were directly evaporated to dryness at 80°C with nitrogen without lyophilization.

Sample derivatization

Cyclohexyl-GABA was prepared by adding 200 μl of 1.5 *M* hydrochloric acid in cyclohexanol and heating for 15 min at 115°C as previously described [22]. The mixture was evaporated to near dryness at 115°C with a stream of nitrogen and the residue was heated with 100 μl of heptafluorobutyl anhydride in 200 μl of ethyl acetate for 15 min at 80°C. The sample was then evaporated to dryness at 80°C and dissolved in 100 μl of methanol. The HFB-cyclohexyl-

GABA was stable in methanol at room temperature for over three years.

Gas chromatography—mass spectrometry

Initially, GABA quantitation was accomplished using a Finnigan 3200 electron-impact mass spectrometer interfaced to a Finnigan 9500 gas chromatograph. The system was equipped with a Teknivent 29K data system (Teknivent, St. Louis, MO, U.S.A.). A 3% OV-17 column was used for isothermal injections at 185°C [22]. In later experiments quantitation was accomplished using a Hewlett-Packard (HP) 5970A mass selective detector equipped with an HP 5790GC having a 12.5 m × 0.2 mm I.D., cross-linked dimethyl silicone capillary column. The system was interfaced to an HP 2671G printer, an HP 9825B computer and an HP 9134A Winchester disc drive for data storage. After injection of 1—4 μl of sample the column was programmed from 150°C to 275°C at 15°C/min, with GABA eluting at 188°C. Improved sensitivity and specificity were obtained using the capillary column. Quantitation was accomplished by monitoring the ions at *m/z* 282 (GABA) and *m/z* 284 (GABA-d_2) with *m/z* 254, 299 (GABA) and *m/z* 256, 301 (GABA-d_2) as confirming ions. For each day's analysis, levels of GABA were calculated from a standard curve employing at least five points containing 100 ng of GABA-d_2 and increasing amounts of GABA from 10 to 1000 ng.

RESULTS AND DISCUSSION

The mass spectra of derivatized GABA and 2,2[$^2H_2$]GABA are shown in Fig. 1a and b, respectively. The peaks at *m/z* 299 (GABA) and *m/z* 301 (2,2[$^2H_2$]-GABA), representing the $(M—82)^+$ ion, result from the loss of the cyclohexyl group with a hydrogen transfer [22]. A standard curve was prepared by plotting the peak height ratio of *m/z* 282 (GABA)/*m/z* 284 (GABA-d_2) versus increasing amounts of GABA from 10 to 1000 ng. Each sample contained 100 ng of GABA-d_2. A linear regression analysis of the data is described by the equation $y = a + bx$ where the intercept $a = 0.1241$, the slope $b = 0.0098$, and the correlation coefficient $r^2 = 0.9999$. Fig. 2 illustrates the selected ion recordings for GABA quantitation in the mouse embryonic brain, body, and palate obtained on the Finnigan system. HFB-cyclohexyl-GABA gave increased GC retention temperatures compared with other GABA derivatives, such as pentafluoropropionyl-methyl-GABA, HFB-heptafluoro-1-butanyl-GABA [19] and pentafluoropropionyl-hexafluoroisopropanyl-GABA [18]. The increased retention temperature of HFB-cyclohexyl-GABA has the advantage of effecting better separation from other substances which might interfere with the analysis. Pentafluoropropionyl-hexafluoroisopropanyl-GABA was not recommended for analysis of picomole quantities due to variable yields [21]. McCaman et al. [20] prepared the trimethylsilyl derivative of GABA and his procedure, like the work of Faull et al. [19], required a time-consuming ion-exchange column purification step prior to derivatization. Colby and McCaman [21], using the N-dinitrophenyl-ethyl-GABA, with a high GC retention temperature, analyzed GABA without prior ion-exchange column purification although eight extraction steps were employed prior to GC—MS injection.

Improved resolution and sensitivity were obtained using the capillary column.

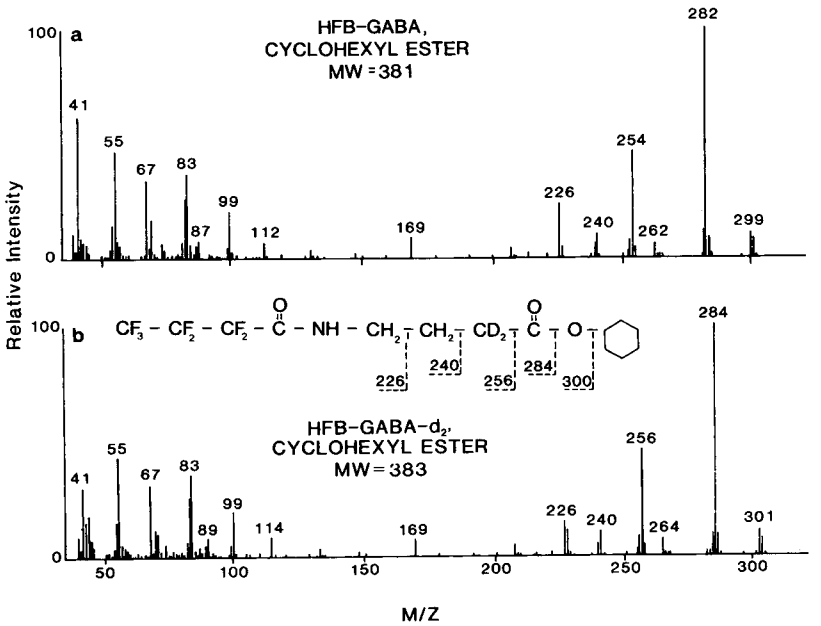

Fig. 1. Electron-impact mass spectra of (a) GABA and (b) GABA-d_2 heptafluorobutyryl-cyclohexyl-GABA derivatives.

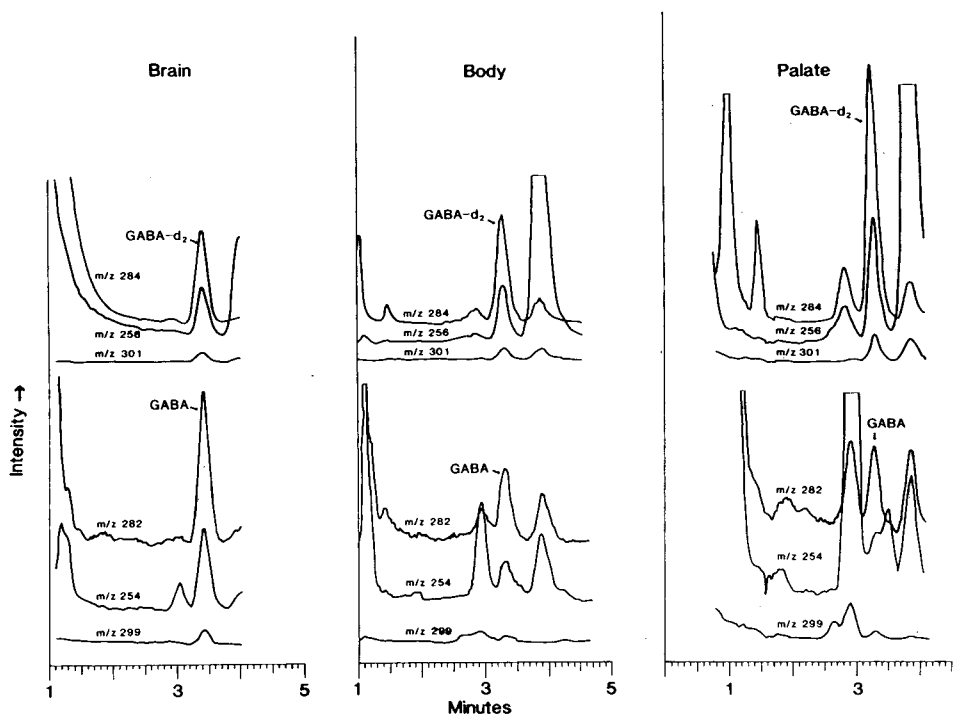

Fig. 2. Selected ion recordings for GABA quantitation in mouse embryonic brain, body, and palate obtained on the Finnigan 3200 GC—MS system.

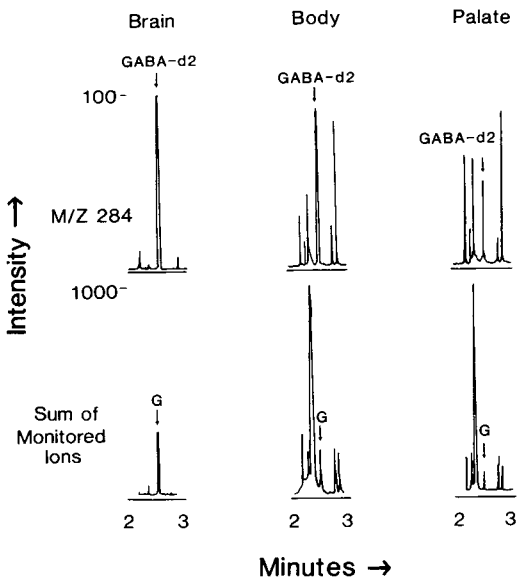

Fig. 3. Selected ion recordings for GABA quantitation in mouse embryo brain, body, and palate obtained on the HP 5970A GC—MS system. The top trace represents the recording for *m/z* 284 (GABA-d_2) and the bottom trace is the sum of the monitored ions. G = GABA (*m/z* 282, 254, 299) and GABA-d_2 (*m/z* 284, 256, 301).

Fig. 3 illustrates the selected ion recordings for GABA analysis of mouse embryonic brain, body, and palate specimens using the HP 5970A GC—MS system. Similar GABA quantitation was accomplished for various human body fluids as shown in Fig. 4. Table I lists the GABA concentration in the various biological specimens. The mean concentration of GABA in mouse embryonic brain of 6.54 is lower than the 14.1 nmol/mg of protein reported for mature mouse brain [24] as might be expected. Concentrations of GABA in mouse embryonic body or palate have not been previously reported. The greater concentration in the body than the palate is not surprising since recent reports have indicated high GABA levels in selected tissues such as pancreatic islets [7], ovary [8], and Fallopian tube [9]. The successful measurement of GABA in the palate illustrates the applicability of the method to the trace levels found in complex biological tissues.

GABA content in blood platelets has not previously been reported to our knowledge; however, GABA-α-oxoglutarate transaminase activity has been demonstrated [25]. GABA uptake in platelets from Down's syndrome patients was shown to be less than control [26]. Ferkany et al. [27] reported GABA concentration in pooled human plasma of 326 ± 28 (n = 7) pmol/ml which is comparable to our value. Stability studies with blood GABA have shown no significant changes in GABA content after 24 h at room temperature [27].

CSF GABA levels shown in Table I are higher than previously reported [4]. CSF GABA exists loosely bound and conjugated in forms such as homocarnosine [28, 29]. The increased GABA content of CSF specimens may reflect the enzymatic hydrolysis of GABA conjugates since CSF GABA increases rapidly at room temperature [30, 31]. More likely, the higher CSF

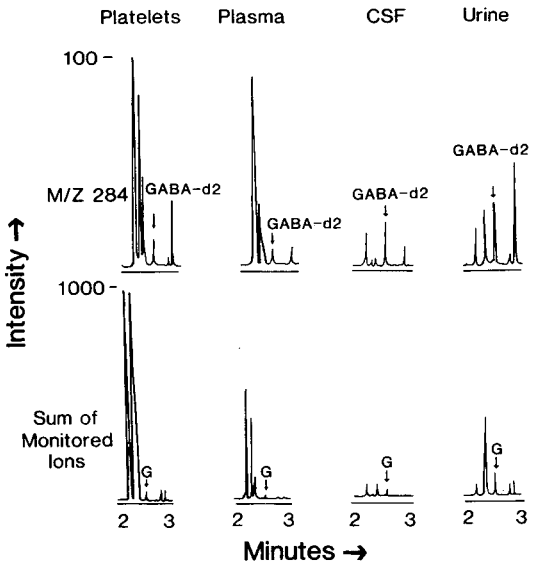

Fig. 4. Selected ion recordings for GABA quantitation in human platelets, plasma, CSF, and urine obtained on the HP 5970A GC—MS system.

TABLE I

LEVELS OF GABA IN VARIOUS BIOLOGICAL SPECIMENS

Specimen	*n*	GABA (nmol ± S.E.)
Mouse embryonic brain (nmol/mg of protein)	14	6.54 ± 0.29
Mouse embryonic body (nmol/mg of protein)	9	0.59 ± 0.02
Mouse embryonic palate (nmol/mg of protein)	9	0.19 ± 0.10
Human platelets (nmol/ml)	2	0.55 ± 0.05
Human plasma (nmol/ml)	4	0.41 ± 0.02
Human CSF adult (nmol/ml)	4	1.08 ± 0.13
Human CSF children pooled (nmol/ml)	1	1.19
Human urine (nmol/mg of creatinine)	2	3.41 ± 0.40

GABA levels shown in Table I result from the derivatization procedure; the acid conditions used for deproteinization liberate loosely bound GABA [28]. Some conjugated GABA may also be hydrolyzed during derivatization. To avoid pitfalls from artifactual increases in free CSF GABA levels, Grove and co-workers [31, 32] and Böhlen et al. [33] suggested that total GABA released, after hydrolysis, representing conjugated plus free, would be a more reliable clinical indicator of brain GABA concentration. In a recent review, Grove et al. [34] concluded that there are no advantages and many disadvantages to measuring free GABA instead of total GABA concentration. The GABA concentration obtained for human urine was 3.41 nmol/mg of creatinine. The clinical significance of GABA excretion is not known.

We have presented a rapid, reliable method for GABA measurement in a wide variety of biological specimens through the GC—MS—SIM analysis of HFB-cyclohexyl-GABA. This methodology provides researchers and clinicians

with the means to study the metabolic role of GABA and to determine the diagnostic importance of GABA in detecting disease states.

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GEL PERMEATION CHROMATOGRAPHY WITH INTERFERENCE REFRACTOMETRY FOR THE RAPID ASSAY OF POLYDISPERSE DEXTRANS IN BIOLOGICAL FLUIDS

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SUMMARY

A semi-automatic system incorporating an ultra-sensitive interference refractometer coupled to a dual-column gel permeation apparatus has been devised for measurement of the molecular size distribution of dextrans in small samples of serum and urine. The system was calibrated with seventeen defined dextran fractions with a range of 1200—250,000 weight average molecular weight ($\bar{M}_w$). Urine samples were prepared for analysis by passage through small ion-exchange columns; serum was pretreated by precipitation with trichloroacetic acid and centrifuged before the ion-exchange treatment. Internal standard (dextran, $2 \cdot 10^6$ $\bar{M}_w$) was added to each sample before pretreatment. Data were obtained in a form suitable for computerised analysis.

INTRODUCTION

The objective of this investigation was to develop a rapid, reliable, semi-automatic method for the separation and estimation of different molecular-weight fractions of neutral dextran in serum and urine. Several groups [1—3] have published methods for the estimation of the molecular size distribution of clinical dextran. The analytical techniques employed by these workers have several disadvantages. The turbidimetric assay of Arturson and Wallenius [1] is too expensive for general use. The conventional gel permeation chromatography employed by other groups [2—4] is slow in operation. Leaching out of dextran-based column packings may contaminate the sample [2]. The colorimetric reactions used for the determination of chromatographed dextran are relatively insensitive, involve handling of large volumes of boiling, concentrated mineral acids and are difficult to automate. A number of the clinical

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studies of membrane permeability have relied on gel permeation chromatography using a heterogeneous calibration mixture of proteins and polysaccharides.

Since the publication of these techniques, mechanically stable column packings have been developed allowing the application of high-speed, high-pressure gel permeation chromatography in aqueous media [5—8]. Estimation of dextran concentration in solutions using interference refractometry has been shown to be highly sensitive, reproducible and to produce results comparable with chemical assays [9]. The combination of these methods was considered likely to offer a significant improvement in speed, sensitivity and cost compared with other existing assays. The continuous measurement of column eluates using a flow-through interference refractometer also allows on-line data collection and reduction using microcomputer techniques. Calibration of gel permeation columns using defined dextran fractions derived from the same organism (*Leuconostoc mesenteroides*) as clinical dextran removes the uncertainty associated with heterogeneous calibration standards.

This communication describes the assembly of the semi-automatic system, its calibration with dextran standards and preparation of the biological samples for analysis using an interference refractometer.

EXPERIMENTAL

Apparatus and materials

Pre-packed columns containing hydrophilic polymer based, semi-rigid gels connected in series were used. TSK columns were used throughout. A pre-column (75 mm × 7.5 mm I.D., GWPH) was followed by two analytical columns (both 600 mm × 7.5 mm I.D., one 5000 PW and one 3000 PW); all columns were purchased from Toyo Soda Manufacturing (Tokyo, Japan). The two analytical columns were water-jacketed and maintained at 33°C. Two in-line 2 μm sieve size filters were incorporated in the stainless-steel lines (1.16 mm O.D. and 0.51 mm I.D.) to protect the columns from contamination; both disposable and re-usable frits were used (Jones Chromatography, Llanbradach, U.K. or Millipore, Harrow, U.K.). The eluent was 0.02% aqueous sodium azide (Fluka, Buchs, Switzerland). To ensure freedom from particulate material and to remove dissolved air it was made up in freshly distilled water and vacuum-filtered with all glass apparatus incorporating disposable filters (Millipore, HAWP 04700). Samples were injected into the system using a sample processor (Model 710B WISP Sample Processor, Waters Assoc., Milford, MA, U.S.A.); samples were prefiltered through 0.45-μm filters (Millex-HV) from Millipore and degassed by sonication for 5 min (Decon Ultrasonics System Model FS100, Hove, U.K.). The volumes of sample injected were programmed in accordance with the anticipated concentration of dextran in the solutions. An interference refractometer equipped with an 8-mm cell using the wavelength of 546 nm and set at × 50 range was used to assay the ratio of the concentrations of dextran in the samples relative to the known concentration in standard solutions (Optilab 902, Tecator AB, Hoganas, Sweden). The detector cell was maintained at 33°C with a water circulator (FH 15 Flow Heater, Grants Instruments Cambridge, Cambridge, U.K.). Two electronic integrators (LDC 308, Laboratory Data

Control, Riviera Beach, FL, U.S.A., or SP 4270, Spectra Physics, San Jose, CA, U.S.A.) were connected to the interference refractometer using 1 V output. Either integrator was set to receive the signal from the interference refractometer and they were programmed to compute the area under the peak and/or in a "slice" of the dextran curve or peak taken every 30 sec. The data from the SP 4270 integrator were transmitted via an RS-232-C interface to an Apple 2+ microcomputer for calculation of results. The analogue signal from the interference refractometer was fed in parallel to an LKB 2210 recorder (Bromma, Sweden) set at 2 mm/min chart speed. The system was pumped at a constant flow-rate (0.81—0.82 ml/min) by means of a Constametric III HPLC unit (Laboratory Data Control). A pulse damper and pressure gauge were connected to the output of the pump by a stainless-steel capillary T-joint; working pressures did not exceed 3.8 MPa (550 p.s.i.). The entire system was started and controlled by the programmable sample processor. Dextran 40 injection BP in 5% dextrose was used as the polydisperse dextran (Lomodex, Fisons Pharmaceutical, Loughborough, U.K.).

Calibration of the system

Direct calibration of the system was achieved by the use of a series of seventeen dextran fractions (Dr. R. Gibbs, Fisons Pharmaceutical, Crewe, Cheshire, U.K., and Dr. K. Granath, Pharmacia Chemicals, Uppsala, Sweden). Each fraction was prepared by weighing the dry powder and made up as a 10% (w/v) stock solution in 0.02% sodium azide; all stock solutions were held at 8°C. Working concentrations of 0.1% were prepared just prior to running on the columns. Each calibration fraction was subjected to analysis at least nine times, and at least three different volumes of sample were injected: 25, 50 and 75 μl. The calibration standards were loaded on to the columns in amounts ranging from 0.01 to 0.07 mg. The weight average molecular weight ($\overline{M}_w$) ranged from 1200 to 250,000; these values had been determined by low-angle laser beam analysis (Fisons). Using the data of Granath and Kvist [2] (Table I) Stokes radii were calculated for those dextran fractions having $\overline{M}_w$ values within the published range. A dextran polymer with an $\overline{M}_w$ in excess of 2,000,000 was used to determine the void volume (V_0), while glucose (the monomer of which dextran is a polymer) was used to define the total separation volume (V_t) of the series of columns incorporated in the system.

TABLE I

LITERATURE DATA USED TO CALCULATE STOKES RADII OF THE DEXTRAN CALIBRATION FRACTIONS [2]

$\overline{M}_w$	Stokes radius (nm)	$\overline{M}_w$	Stokes radius (nm)
10,000	2.33	58,000	5.30
13,200	2.65	76,000	6.05
19,300	3.18	96,000	6.75
27,800	3.77	130,000	7.80
36,000	4.25	147,000	8.20
48,300	4.85		

For each defined fraction the elution volume at which 50% of the material had emerged was designated the 50% volume (V_{50}). Using these values a distribution coefficient (K_{av}) was determined after the concept developed by Laurent and Killander [10].

$$K_{av} = \frac{V_{50} - V_0}{V_t - V_0}$$

The values obtained when the sized dextran fractions were run separately were used to calibrate the dual-column system; these calculations can also be made on the basis of retention time.

Preparation of serum samples

Serum samples were stored at −70°C. The effective removal of serum components which could contaminate the analytical columns or invalidate the assay of the content of dextran in the sample posed considerable difficulties. Precipitation with zinc sulphate failed to clear the serum of residual components as evidenced by the uninterpretable chromatograms. It was, however, found that serum could be effectively prepared for analysis by first precipitating it with 10% trichloroacetic acid (TCA). A solution of a large dextran fraction ($>10^6$ $\overline{M}_w$) as for V_0 was used as an internal standard to allow correction for dilution or losses during sample preparation and as a check on chromatographic conditions. Samples were treated as follows: 0.25 ml distilled water (or an aliquot of dextran infusate), 0.25 ml internal standard, 0.50 ml serum, and 3.50 ml of 10% TCA were well mixed after each addition and finally homogenised with a Vortex mixer; after standing for 10 min the suspension was centrifuged (840 *g*) for 5 min and the supernatant was decanted. Recovery volume was 4.0 ± 0.1 ml.

As the dual-column packing could be damaged by excessive acid, serum samples had to be neutralised prior to being run on the analytical system. Removal of TCA by extraction with diethyl ether was found to be unsatisfactory. A suitable means of neutralising the samples was found by filtering them through separate anion and cation resins. Analytical-grade anion-exchange resin, AG 2×8 (Cl^-) and cation-exchange resin AG 50W-X2 (H^+) both 100—200 mesh obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.) were used. The resins were pretreated with 1 mol/l sodium hydroxide (anion) or 1 mol/l hydrochloric acid (cation); 2.0 ml of resin was then placed in a polypropylene column with a total volume of 11 ml (Econo-Column, Bio-Rad Labs.); resins were washed with freshly prepared boiled, glass-distilled water, and washing was continued until the eluent from the column was neutral when tested with Universalindikator pH 1—14 (E. Merck, Darmstadt, F.R.G.). Serum supernatant was poured on to the anion column and passed directly to the cation column from which it emerged at a neutral pH.

Preparation of urine samples

Urine samples were held at 8°C. Removal of urine components which interfered with the detection of the dextran was undertaken. Precipitation with zinc sulphate completely failed to yield a solution capable of being analysed by means of interference refractometery. Methanol precipitation, with or without

addition of sodium chloride to the urine, was also ineffective. Treatment using the same type of ion-exchange columns as for serum was found to yield satisfactory results. The urine test samples were prepared as follows: 0.25 ml internal standard and 4.25 ml urine.

The urine preparation was passed through the cation column before entering the anion column. All samples were then filtered through Millex filters, sonicated and loaded into the autosampler which was already programmed for analysis.

RESULTS

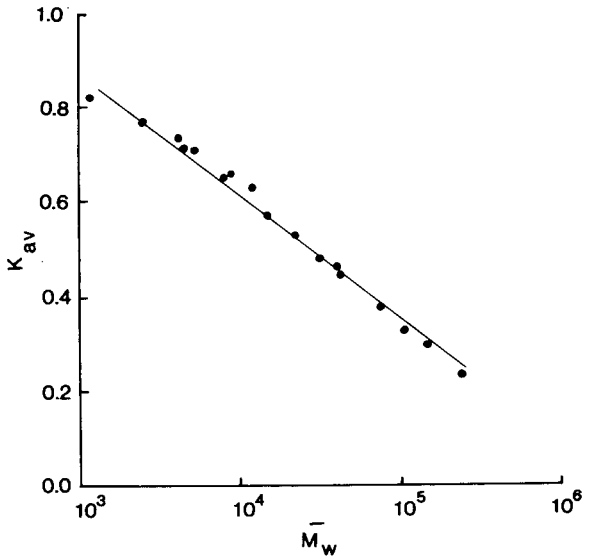
The elution patterns of the sized fractions of dextran used to calibrate the dual columns were Gaussian. Table II illustrates the V_{50} values found for the elution of the standards and the K_{av} values calculated therefrom. A plot of K_{av} versus log $\overline{M}_w$ is linear (Fig. 1), whereas the relationship between K_{av} and Stokes radius is log—log (Fig. 2). The Stokes radius of $\overline{M}_w$ of unknown sizes of dextran can be estimated using the appropriate plot.

Fig. 3 shows the elution curves obtained with serum extracts containing internal standard compared with samples containing clinical dextran demonstrating the removal of interfering substances in the region of interest. The elimination of the interfering materials in the dextran portion of the elution pattern obtained from urine is illustrated in Fig. 4. Co-precipitation of dextran with serum proteins might be expected to be dependent on the molecular size. Fig. 5 illustrates that the recovery of dextran with Stokes radii between 3 and 6 nm agrees well with expected values, although smaller and

TABLE II

CALCULATED STOKES RADII, ELUTION VOLUMES AND DISTRIBUTION COEFFICIENTS FOR THE DEFINED DEXTRAN FRACTIONS USED FOR CALIBRATION OF THE TSK (5000PW + 3000PW) DUAL-COLUMN SYSTEM

$\overline{M}_w$	Stokes radii (nm)	V_{50} ($x \pm$ 1S.D.) (ml)	K_{av}
1170	—	35.1 ± 0.3	0.83
2500	—	33.9 ± 0.4	0.77
4100	—	33.3 ± 0.4	0.71
4500	—	32.7 ± 0.4	0.71
5250	—	32.6 ± 0.3	0.71
7900	—	31.5 ± 0.3	0.65
8825	—	31.6 ± 0.2	0.66
11,500	2.48	30.9 ± 0.2	0.62
14,700	2.80	29.9 ± 0.3	0.57
21,975	3.41	29.0 ± 0.3	0.53
31,900	4.02	28.0 ± 0.3	0.48
39,200	4.40	27.4 ± 0.2	0.45
42,150	4.50	27.4 ± 0.2	0.45
73,625	5.72	25.9 ± 0.2	0.37
104,450	6.80	24.9 ± 0.2	0.32
144,960	8.10	24.3 ± 0.2	0.29
239,825	—	23.2 ± 0.2	0.24



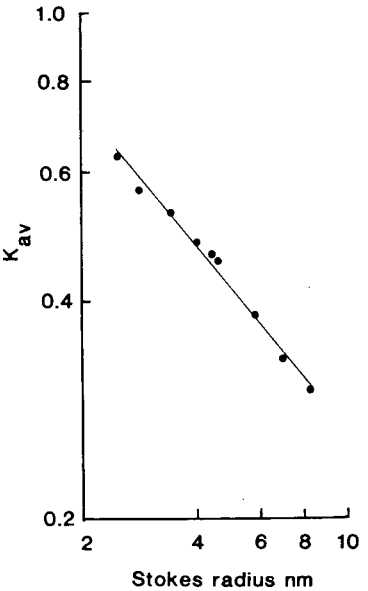


Fig. 1. Relationship between K_{av} and $\bar{M}_w$ for defined dextran fractions using the dual-column TSK (5000PW + 3000PW) system.

Fig. 2. Plot of K_{av} versus Stokes radius on log scale for defined dextran fractions using the dual-column TSK (5000PW + 3000PW) system.

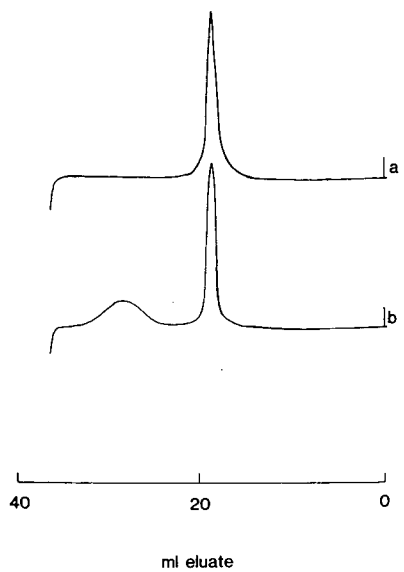
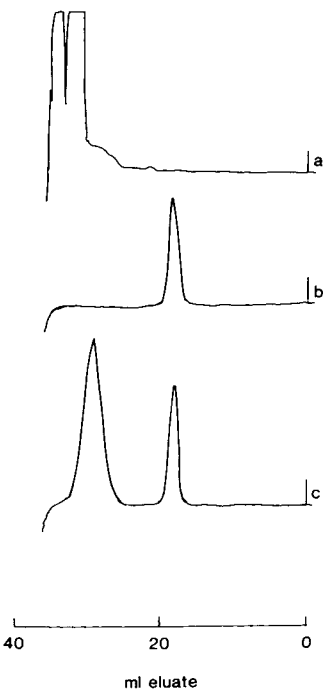


Fig. 3. Elution curves of (a) an extract of serum with internal standard but no clinical dextran; and (b) an extract of serum from a subject recently infused with clinical dextran.

larger fractions may be slightly underestimated. There is no evidence for a molecular size related variation in apparent recovery despite the use of a very-high-molecular-weight internal standard. Losses during sample preparation may be readily corrected through the incorporation of the internal standard.



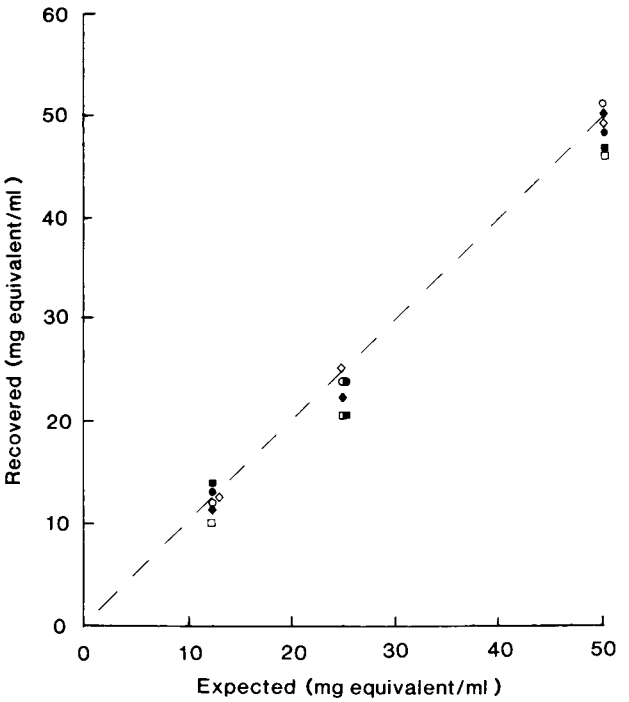


Fig. 4. Elution curves of (a) an ineffectively treated urine; (b) fully prepared urine with internal standard but containing no clinical dextran; and (c) fully prepared urine from a subject recently infused with clinical dextran.

Fig. 5. Comparison of expected and observed recoveries of known amounts of clinical dextran added to serum. The recovery for fractions of the following Stokes radii are shown: 7.2 (□), 5.7 (○), 4.6 (◇), 4.4 (●), 3.0 (◆) and 2.4 (■) nm.

Internal standard recoveries from serum ranged from 57% to 84% (mean 66%), while from urine the range was 57—67% (mean 63%). The variation in retention time of the internal standard from sample to sample was ± 0.2%.

The limit of detection of the method was determined by chromatographing a series of dilutions of dextran under standard assay conditions. The standard deviation (S.D.) of the detector response over the range of interest was calculated and a signal greater than 2 S.D. above baseline was considered significant. Under these conditions the smallest amount of clinical dextran measurable was 6 μgequiv., over the range of Stokes radii between 2.4 and 7.2 nm. This corresponds to 0.08 mgequiv./ml.

DISCUSSION

Among the intended applications of the new method was the investigation of renal glomerular function in infants and small children. This focused attention on the development of methods applicable to very small sample sizes, yet maximising the precision of the data obtained.

The measurement of the dextran components by the interference refractometer offers a substantial improvement in terms of sensitivity, labour-intensive operations, cost and time. The analysis of a single sample requires 70 min, as

compared with many hours using previously described techniques.

The volume of serum used in these studies was 0.5 ml, but as only 0.075 ml of the final preparation was loaded on to the analytical system, this sample size could be substantially reduced. Urine volumes were also amenable to reduction as only 0.050 ml of the neutralised, filtered specimens were used to charge the columns for analysis. In addition, the sensitivity of the interference refractometer could be increased 25-fold. The range of Stokes radii that can be detected is dependent upon the molecular size distribution of the polydisperse dextran preparation used. In these experiments less than 5% of the material has a Stokes radius below 2.4 nm or above 7.2 nm. Thus, the molecular size distribution of polydisperse clinical dextran infusates in the above range are readily measured using minimal sample volumes making the semi-automatic system the method of choice when studying infants or a paediatric population.

An additional advantage of the semi-automatic system described in this communication derives from the possibility of changing the exclusion limits through selection of different grades of pre-packed columns with a concomitant change of marker molecule.

Incorporation of an electronic integrator provides a continuous print-out of data which can be analysed by hand or by means of a specially written computer programme.

The development of the methodology to assay clinical dextrans in biological fluids by means of an interference refractometer depends upon an efficient sample preparation scheme. The interfering substances in both serum and urine were entirely removed using the method described above.

Limited clinical studies with healthy volunteers have yielded excellent results and will be more completely described in the clinical literature to follow.

ACKNOWLEDGEMENTS

The authors express their gratitude for the helpful and constructive discussions and assistance of Drs. Raymond Baker and Cynthia Lyne. The interest shown and data given by Dr. Ralph Gibbs of Fisons Pharmaceutical and Dr. Kirsti Granath of Pharmacia are gratefully acknowledged. The financial support of the Special Trustees of Guy's Hospital and Friends of Guy's is appreciated. Special thanks are due to Carol Switft for preparation of this manuscript.

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ANALYSIS OF D-PENICILLAMINE BY GAS CHROMATOGRAPHY UTILIZING NITROGEN—PHOSPHORUS DETECTION

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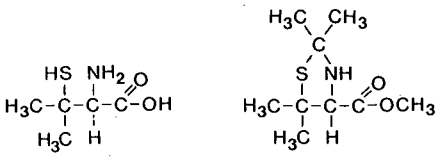
SUMMARY

A method is presented for the analysis of the "orphan" drug D-penicillamine (D-Pa), which is used for the treatment of the inherited rare copper metabolism dysfunction known as Wilson's disease, by assaying a derivative of the compound by gas chromatography employing a rubidium sensitized nitrogen—phosphorus detector. Analytical procedures are described for the analyses of residues of D-Pa · HCl salt in animal feed and for the analyses of the salt or free base from aqueous solutions by utilizing a single-step double derivatization with diazomethane—acetone. Stability data for D-Pa · HCl in animal feed and for the free base in water are presented. An ancillary fluorescence derivatization procedure for the analysis of D-Pa in water is also reported.

INTRODUCTION

Wilson's disease is a rare inherited metabolic disorder resulting in the accumulation of excess copper, first in the liver, then in the cornea, brain and kidneys and finally overflowing into the urine. The untreated course of the disease is cirrhosis of the liver, destruction of motor coordination, eclipsing of the intellect, renal failure and eventual death of the patient.

The idea that the disease could be arrested by the removal of copper from patients suffering from Wilson's disease was credited to Cumings [1] of London, who suggested in 1948 that the chelating agent 2,3-dimercapto-propanol (BAL) developed to detoxify trivalent arsenic be used for the elimination of copper as the chelated copper complex. However, the BAL treatments were not successful due to most patients developing tachyphylaxis while each successive painful intramuscular injection of BAL exhibited diminishing efficacious results. In 1956, Walshe [1] introduced the oral administration of penicillamine (Fig. 1, I), as a copper-chelating agent for the treatment of



I

II

Fig. 1. Structures of D-penicillamine (I) and of a diazomethane—acetone derivative (II).

Wilson's disease and obtained only limited success due to its toxicity. In 1957, the toxicity observed for penicillamine was attributed to impurities. Further testing with purified D(−)-penicillamine (D-Pa) proved it to be a remarkably effective therapeutic agent for the elimination of copper. In addition to the disease being arrested, the patients were eventually rendered essentially symptom-free with real prospects of leading an otherwise normal life when they maintained daily dosages of D-Pa [1]. Unfortunately, a small percentage of Wilson's disease patients developed an intolerance to D-Pa [2] and the drug therapy had to be withdrawn (a life-threatening situation).

Studies have been proposed to be conducted at the National Center for Toxicological Research (NCTR) for the evaluation of the toxicology of D-Pa. Prerequisite for these toxicological studies was development of analytical methodology for chemical characterization, dose certifications and stability evaluations of the drug in the dosage forms. A search of the chemical literature for analytical techniques for characterization and analysis of D-Pa yielded a few limited analytical procedures which were inadequate for our proposed toxicological studies [3—5]. We, therefore, developed new analytical methodology for the analysis of D-Pa which incorporated a single-step double derivatization with detection by gas chromatography (GC) utilizing a rubidium sensitized nitrogen—phosphorus detector.

This paper, therefore, describes new analytical procedures for the analysis of D-Pa and residues of D-Pa · HCl in animal feed at 1.6% and 0.016% by weight. Stability data for D-Pa in water, D-Pa · HCl in animal feed, and an ancillary fluorescence derivatization procedure for the analysis of D-Pa in water are also included. Structures of D-Pa and its diazomethane—acetone derivative are shown in Fig. 1.

EXPERIMENTAL

Reagents

D-Pa was obtained from Merck Sharp & Dohme (Rahway, NJ, U.S.A.). The Diazald™ and carbitol [2-(2-ethoxyethoxy)ethanol] were from Aldrich (Milwaukee, WI, U.S.A.) and the diethyl ether from Fisher Scientific (Pittsburgh, PA, U.S.A.). The N,N-diethylaniline was from Chem Service (West Chester, PA, U.S.A.), the Fluram™ from Roche (Nutley, NJ, U.S.A.), and the animal feed Type 5010M from Ralston Purina Company (St. Louis, MO, U.S.A.). The acetone and hexane were grades suitable for GC. All other chemicals were CP grade.

Preparation of D-Pa · HCl from D-Pa

Approx. 30 g of D-Pa were added to 200 ml of methanol in a 500-ml round bottom flask. The bulk of the material was insoluble. Anhydrous hydrochloric acid was bubbled through a sintered glass sparge into the methanol with frequent swirling of the solvent to facilitate mixing. The D-Pa base was converted rapidly to the soluble D-Pa · HCl resulting in a clear solution. The excess hydrochloric acid and most of the methanol solvent were removed by rotary evaporation at room temperature by using water pump vacuum. The flask was chilled in a refrigerator (ca. 5°C) overnight and the resulting crystals were collected by decanting the excess solvent. The flask was placed back on the rotary evaporator to remove additional methanol from the crystalline matrix. This material was then placed in a small flat-bottomed dish and enclosed in a vacuum oven at 55°C at a pressure of 250 mmHg for 1 h. The resulting white crystalline cake of D-Pa · HCl was ground using a mortar and pestle and placed back in the vacuum oven at room temperature for an additional two days. The D-Pa · HCl salt (approx. 30 g) was sealed in an amber bottle and stored in a dessicator for subsequent use.

Preparation of diazomethane

Diazomethane was produced in a diazomethane generator consisting of a round glass tube, 12 cm × 2 cm I.D. fitted with a 19/22 Wheaton two-arm adapter with one arm extending within approx. 2 cm of the bottom of the tube and its upper end capped or connected to a nitrogen purge source. The other arm was joined by a 2-cm PTFE tube to a Pasteur pipette. All joints of the apparatus were constructed of clear glass fittings as diazomethane has been reported to occasionally explode when distilled in ground-glass apparatus [6]. For safety precautions diazomethane was generated in an efficient fume hood behind a shield.

To the generator 2 ml of 60% potassium hydroxide, 1 ml of carbitol and 1 ml of diethyl ether were added. Approx. 1 g of Diazald was added and the apparatus was quickly capped. Sufficient diazomethane was generated to methylate several samples. Additional diazomethane was expelled from the apparatus using a gentle nitrogen purge.

Purity and structural characterization

Purity and structual characterization of D-Pa and the D-Pa · HCl were assessed by the following analytical techniques: polarimetry, high-resolution gas chromatography with flame-ionization detection (GC—FID), high-resolution gas chromatography with mass spectroscopy (GC—MS) and nuclear magnetic resonance (NMR) spectrometry.

A Rudolph and Son (Caldwell, NJ, U.S.A.) Model 80 polarimeter was used to obtain the specific rotation $[\alpha]_D^{25}$ of a 5% solution of D-Pa (free base) in 1 *M* sodium hydroxide. A Finnigan (San Jose, CA, U.S.A.) Model 4023 mass spectrometer equipped with a Finnigan Model 9610 gas chromatograph and a J & W (Rancho Cordova, CA, U.S.A.) 30 m × 0.25 mm DB-1 bonded fused-silica column (0.25 μm film thickness) was used to obtain electron impact (EI) and ammonia chemical ionization (CI) spectra of D-Pa derivatized with diazomethane—acetone. The D-Pa free base in 1 ml of water was diluted with 9 ml of

acetone containing 0.1 ml of concentrated hydrochloric acid and a 1-ml aliquot was derivatized with diazomethane, extracted into hexane (9.4 ml) and reserved for subsequent injection of an aliquot into the GC—MS system. Purity analysis of the free base was performed by 500-MHz ^1H NMR in ^2H_2O on a Bruker (Fallanden, Switzerland) WM-500 NMR spectrometer.

Purity of the D-Pa · HCl salt was assessed by diazomethane—acetone derivatization followed by high-resolution GC—FID analysis and by ^{13}C NMR analysis of the salt itself. A Tracor (Austin, TX, U.S.A.) Model 560 gas chromatograph equipped with a flame-ionization detector, a J & W on-column injector and a J & W 30 m × 0.25 mm DB-1 capillary column was used to determine the purity of the D-Pa · HCl salt. A 1-μl aliquot of 1 mg/ml concentration of D-Pa · HCl derivatized with diazomethane—acetone was injected directly on column for analysis. The column was held at 60°C for 1 min and then temperature-programmed at 20°C/min to 220°C and maintained there for 10 min. The helium carrier flow-rate was 1.8 ml/min and the resulting retention time (t_R) for the derivative of D-Pa · HCl was 6.37 min. The purity of the D-Pa · HCl salt was also determined in ^2H_2O from the 67.9 MHz ^{13}C NMR spectrum obtained on a Bruker WH270 NMR spectrometer.

Derivatization of D-Pa · HCl in animal feed and D-Pa base in water

Animal feed. Triplicate samples of 1.6, 0.16 and 0.016% by weight of D-Pa · HCl in animal feed were prepared by adding 160, 16 and 1.6 mg of the salt, respectively to 10 g of animal feed contained in 250-ml screw topped Erlenmeyer flasks. The flasks were sealed with PTFE-lined screw caps, shaken to facilitate mixing and placed in the dark for two days. Acetone (100 ml) was added to each flask and the flasks were then shaken on an Eberbach (Ann Arbor, MI, U.S.A.) mechanical shaker for 1 h at 200 excursions per min. Aliquots (1 ml) of the 1.6% samples were diluted to 10 ml with acetone and 1-ml portions of these dilutions were transferred to 12-ml PTFE-lined screw cap tubes. Aliquots of 1 ml of the 0.16% samples were directly transferred to 12-ml tubes. Aliquots of 10 ml of the acetone extract of the 0.016% samples were rotary evaporated at room temperature in a 50-ml round-bottom flask and quantitatively transferred to 12-ml tubes by using 4 × 0.25-ml washes. These latter samples were evaporated to dryness using a stream of nitrogen and reconstituted in exactly 1-ml vols. of acetone. A 1-ml aliquot of a standard of 160 μg/ml of D-Pa · HCl in acetone was used for quantitation purposes. All 1-ml samples and the standard were derivatized by bubbling diazomethane into the samples which immediately turned the solvent yellow. The samples were capped and allowed to stand for 5 min, whereupon 1 ml of water was added to each to stop the derivatization. The addition of 1 ml of water caused the expulsion of the gaseous diazomethane with considerable effervescence. Hexane (9.4 ml) was added, the tubes were capped, shaken for 2 min by hand and allowed to stand approx. 2 min to allow the immiscible layers to separate. The upper layer now consisted of 10.0 ml in volume due to the partitioning of the acetone between the hexane and water layers. The hexane, upper layer, containing the diazomethane—acetone derivative of D-Pa · HCl was then reserved for analysis by GC. For GC analyses utilizing a nitrogen—phosphorus detector, an appropriate amount of N,N-diethylaniline was added in this hexane layer as an internal injection standard.

Water. The derivatization of D-Pa base in water was accomplished in a similar manner with minor modifications. A 1-ml aliquot of a 1 mg/ml water solution of the D-Pa free base was diluted with 9 ml of acetone containing 0.1 ml of concentrated hydrochloric acid thus converting the base to the hydrochloride salt. A 1-ml aliquot was then derivatized as described above and reserved for GC analysis. Quantitation was by comparison to a freshly prepared D-Pa free base standard prepared in water and derivatized as described above.

Gas chromatography

A Tracor Model 560 gas chromatograph was equipped with a nitrogen—phosphorus detector and a 90 cm × 2 mm I.D. glass column containing 5% SP2100 (Supelco, Bellefonte, PA, U.S.A.) on Supelcoport 100—120 mesh. The injection port, column oven and nitrogen—phosphorus detector temperatures were 140°C, 110°C and 280°C, respectively. The helium carrier gas flow-rate was 25 ml/min and the hydrogen and air flow-rates to the nitrogen—phosphorus detector were 2.0 and 110 ml/min, respectively. Samples of diazomethane—acetone derivatized D-Pa · HCl extracts from animal feed or derivatized D-Pa free base from water were injected in 2 μl of hexane. The t_R of the derivative was 2.0 min. Samples of diazomethane—acetone derivatized D-Pa · HCl extracts from animal feed were also analyzed by GC using flame-photometric detection (GC—FPD) on a Perkin-Elmer (Norwalk, CT, U.S.A.) Model 3920B GC equipped with a linearizer for the sulfur mode. The 180 cm × 2 mm I.D. glass column contained 10% SP 2100 on Supelcoport 100—120 mesh. The injection port, column and detector temperatures were 180°C, 160°C and 200°C, respectively. The helium carrier flow-rate was 25 ml/min and the hydrogen and air flow-rates for the flame-photometric detector were 70 and 100 ml/min, respectively. All injections were in 2 μl of hexane. The t_R of the derivative was also 2.0 min.

Stability experiments

Tests were performed to determine the stability of the test chemical at the highest and lowest proposed dose levels under simulated animal test conditions (i.e., opened container, in light) and under storage conditions (i.e., closed container, in dark). In animal feed, D-Pa · HCl was blended at levels of 0.016% and 1.6% by weight by the addition of 160 mg of the salt to 1 kg of feed and 16 g to 984 g of feed, respectively. These were blended for 30 min in a Model LV Twin-Shell Lab Blender (Patterson-Kelly, East Stroudsburg, PA, U.S.A.) operated at 20 rpm with the intensifier bar turned off. Each dosage level was then divided in half. One portion of each was then placed in an open container under incandescent lighting and the other portion stored in a sealed container in the dark. Extraction and analyses of D-Pa · HCl in animal feed were determined as previously described with the exception that the 0.016% D-Pa · HCl in animal feed samples were not concentrated ten-fold by rotary evaporation prior to the diazomethane—acetone derivatization of 1 ml of the extract.

The stability of D-Pa in distilled deionized water was determined for 100, 10 and 1 mg/ml concentrations, respectively, at ambient temperature under laboratory lighting conditions. The D-Pa solutions were sampled at 6, 24 and

48 h after preparation and analyzed as previously described. Samples were quantitated by comparison to a freshly prepared standard of D-Pa in water.

Preparation of fluorescent derivative of D-Pa in water

Triplicate 1-ml samples of 100, 50, 20, 10, 5, 2 and 1 μg/ml of D-Pa in water were placed in 8-ml screw-top tubes containing 1 ml of 0.2 *M* borate buffer, pH 9. Acetonitrile (3 ml) containing 150 μg/ml Fluram was added, the tube capped, and immediately vortexed before proceeding to the next tube. After allowing the samples to stand for 20 min, their fluorescence was read on an Aminco-Bowman (Silver Spring, MD, U.S.A.) spectrofluorometer. The excitation wavelength (λ_{ex}) was 350 nm and the emission wavelength (λ_{em}) was 490 nm. The Aminco-Bowman spectrofluorometer was calibrated to yield a response of 50% at 10× when using a quinine sulfate standard of 0.3 μg/ml in 0.05 *M* sulfuric acid with λ_{ex} = 350 nm and λ_{em} = 450 nm.

RESULTS AND DISCUSSION

Upon receipt of the D-Pa (base, MW 149) from Merck Sharp & Dohme, chemical characterization and purity of the material were assessed. The D-Pa was found to have a specific rotation $[\alpha]_D^{25}$ of −54° which compared well with the Merck Index listed value of −55°. When reacted with ninhydrin or phosphotungstic acid, D-Pa also produced the characteristic blue color described under the identification section for D-Pa in The United States Pharmacopeia Twentieth Revision [7]. As previously described, the number of analytical techniques reported in the chemical literature for the analysis of D-Pa were extremely limited, were non-specific for the chemical or did not meet our specific needs. We, therefore, developed a simple, rapid analytical procedure applicable to the analysis of D-Pa in water or D-Pa · HCl in animal feed or water. D-Pa is not amenable to GC analysis per se, but can be analyzed by GC after methylation with diazomethane in acetone under acidic conditions. The derivative was chromatographed on a fused-silica capillary column and the EI and ammonia CI spectra subsequently obtained on a Finnigan mass spectrometer. EI data (Fig. 2) indicated the molecular ion of the derivative to be at *m/z* 203 and CI data indicated a corresponding M+1 ion at *m/z* 204. Mass spectral data were consistent with the interpretation that D-Pa not only reacted with the diazomethane but also reacted with the solvent acetone. Proton NMR spectral data of this derivative indicated that acetone caused ring closure to a five-membered ring (thiazolidine); structure shown in Fig. 1. Proton NMR data of the original D-Pa free base in ^2H_2O indicated a purity of 99%. The purity of the D-Pa · HCl by high-resolution GC—FID and ^{13}C NMR analyses also indicated a purity in excess of 99%. The purities of the D-Pa and D-Pa · HCl were judged sufficient for the proposed toxicology studies.

The highest and lowest proposed levels for penicillamine as the hydrochloride salt in animal feed were 1.6% and 0.016% by weight, respectively. In Fig. 3, GC chromatograms of the D-Pa · HCl extracted from animal feed and derivatized with diazomethane—acetone are shown for the 0.016% level. Triplicate assays at the two levels by both FPD and nitrogen—phosphorus

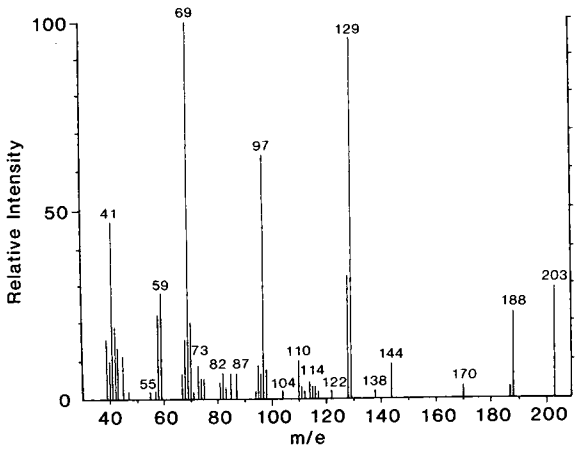


Fig. 2. Mass spectrum (EI) of the diazomethane—acetone derivative of D-penicillamine.

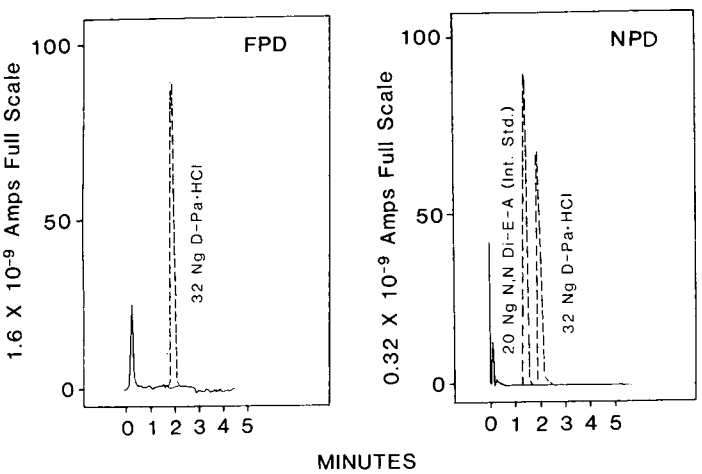


Fig. 3. Chromatograms of D-Pa • HCl as the diazomethane—acetone derivative by GC with flame-photometric detection (FPD) and with nitrogen—phosphorus detection (NPD); (—) responses from derivatized control animal feed samples, (- - -) response from 0.016% by weight of D-Pa • HCl in animal feed, N,N-diethylaniline (N,N-Di-E-A) included as an internal injection standard for nitrogen—phosphorus detector as indicated.

detection gave similar results and showed no interfering peaks. The flame-photometric detector output signal was electronically linearized because the sulfur mode is a non-linear square root function while the nitrogen—phosphorus detector output signal is inherently linear. Also, the flame-photometric detector baseline was found to be considerably noisier than the baseline of the nitrogen—phosphorus detector as shown in Fig. 3. Therefore, analysis by GC with nitrogen—phosphorus detection was the procedure of choice.

Recovery experiments were also performed in which (1) D-Pa free base and (2) D-Pa · HCl in methanol were added to animal feed. However, these experiments were unsuccessful in recovering the original material. The D-Pa free base was found to be essentially insoluble in the extraction solvent (acetone) as opposed to the salt which is very soluble indicating that the base

does not significantly react with acetone. Recovery of D-Pa · HCl in methanol spiked into animal feed was also unsuccessful (ca. 0% recovery). This was probably due to the intimate contact between the D-Pa · HCl and the animal feed. The D-Pa · HCl salt as well as the D-Pa free base are not chemically inert. In fact, they have several reactive functional groups within the molecule that could react with animal feed components. Therefore, by blending the D-Pa · HCl dry with the animal feed, intimate contact with the feed was reduced resulting in much improved recoveries of 75% and 95% from the 0.016% and 1.6% by weight of the D-Pa · HCl in the animal feed, respectively.

The proposed levels for penicillamine as the base in water were 100, 10 and 1 mg/ml to be administered by gavage. Analysis of D-Pa in water was similar to that previously described for analysis of D-Pa · HCl in feed with minor modifications. An aliquot of the D-Pa in water was diluted with acetone—hydrochloric acid before derivatization with diazomethane. The acetone serves to dilute the water therefore allowing the diazomethane to dissolve in solution and the D-Pa free base was converted to the salt form by the hydrochloric acid thereby allowing the diazomethane—acetone derivatization to proceed smoothly. GC data of diazomethane—acetone derivatized D-Pa from water, however, did show a minor secondary peak that eluted after the main peak and was completely separated from it. Ammonia CI data of this peak indicated a M+1 ion of *m/z* 218 as opposed to the main peak at *m/z* 204. Since this is a difference of 14 MU it is proposed that the diazomethane reacted to add a second CH_2 group into the molecule. In addition, the diazomethane—acetone derivatization reaction was found to be linear for various quantities (0.05—10

TABLE I

STABILITY OF D-Pa · HCl IN ANIMAL FEED SPIKED AT TWO LEVELS

Sampling interval		D-Pa · HCl recovered* (%)	
		Spiked at 1.6% by weight	Spiked at 0.016% by weight
*Short-term study***			
Days:	0	94.9 ± 1.1	74.6 ± 0.4
	1	97.9 ± 2.2	70.0 ± 1.2
	4	96.5 ± 1.2	62.2 ± 8.3
	8	93.3 ± 1.2	48.3 ± 1.1
	14	90.1 ± 0.1	N.D.***
Long-term study §			
Weeks:	0	94.9 ± 1.1	74.6 ± 0.4
	1	94.5 ± 3.0	49.6 ± 1.8
	2	91.1 ± 2.5	N.D.
	4	89.1 ± 2.6	N.D.
	8	84.6 ± 1.6	N.D.

*Mean ± standard error from triplicate assays.
**Open container, incandescent lighting, and ambient temperature.
***N.D. = not determined.
§Sealed container, light-tight cabinet, and ambient temperature.

TABLE II

STABILITY OF D-Pa IN WATER SPIKED AT THREE LEVELS

Sampling interval (h)	D-Pa recovered* (%)		
	Spiked at 100 mg/ml	Spiked at 10 mg/ml	Spiked at 1 mg/ml
6	100	102	101
24	100	97.7	94.0
48	102	96.4	83.5
pH	5.2	5.1	4.5

*Sealed container, incandescent lighting, and ambient temperature.

mg) of D-Pa · HCl even in the presence of 1 g equivalent of feed extract. A similar linear response was also found for the diazomethane—acetone derivatization of the D-Pa free base from water.

Tables I and II indicate the stability of D-Pa · HCl in animal feed and the stability of D-Pa free base in water. The D-Pa · HCl concentrations in animal feed were found to decrease with time as would be expected for the chemically active D-Pa · HCl as it reacts with the animal feed components. The data also indicate that this decrease was independent of whether the samples were in the light in an open container or in a closed container in the dark. A decrease in the concentration of D-Pa free base in water with respect to time was also noted. Therefore, both dosage forms must be prepared and used immediately to provide assurance that the requisite dosages are administered to the test animals.

Fig. 4 represents data from an ancillary procedure for the spectrofluorometric analysis of D-Pa from water after derivatization with Fluram

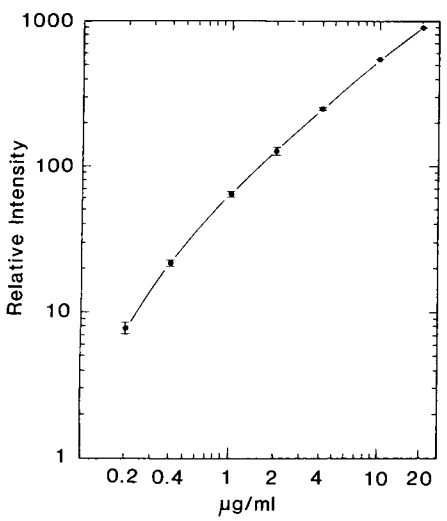


Fig. 4. Fluorescence response for the derivatization of D-penicillamine in water using Fluram; triplicate samples.

(a non-fluorescent chemical that hydrolyzes in aqueous media to non-fluorescent products) [8]. Initially, a high fluorescence reading resulted which subsided to an almost constant response. Therefore, for accurate quantitation, timing of the fluorescence readings was a critical factor.

In summary, this paper describes a new, rapid GC procedure for the analysis of D-Pa · HCl in animal feed and for the analysis of D-Pa · HCl or D-Pa free base in water.

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CHROMBIO. 2349

GAS CHROMATOGRAPHIC DETERMINATION OF MEFLOQUINE IN HUMAN AND DOG PLASMA USING ELECTRON-CAPTURE DETECTION

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SUMMARY

A sensitive and selective gas—liquid chromatographic method for the determination of plasma levels of mefloquine in human and dog plasma is described. The drug and internal standard were extracted from plasma at pH 9.0 into isopropyl acetate. After evaporation of the solvent, the residue was taken up in toluene and derivatised with heptafluorobutyrylimidazole. The derivative was quantified by gas—liquid chromatography on a 3% GC GE-SE30 column with electron-capture detection. The limit of detection for mefloquine in plasma was 10 ng/ml. The mean overall recovery from plasma was 102.7 ± 3.3%. The method was shown to be specific for mefloquine without any interference from endogenous compounds in plasma or from the drugs pyrimethamine and sulfadoxine (compounds often administered in combination with mefloquine).

The assay described was successfully applied to the determination of plasma levels of mefloquine in man and dog following oral and intravenous administration, respectively.

INTRODUCTION

Mefloquine, racemic *erythro*-α-(2-piperidyl)-2,8-bis(trifluoromethyl)-4-quinolinemethanol (Fig. 1), is an antimalarial drug used for the treatment and prevention of chloroquine-resistant *Falciparum malaria*. Several methods have

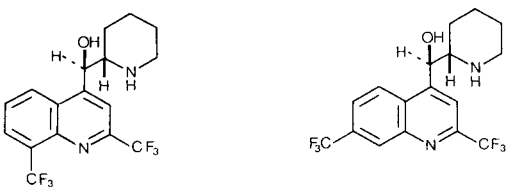

Fig. 1. Chemical structures of mefloquine and internal standard.

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been proposed for the determination of mefloquine in blood, plasma and urine [1—5]. All these methods lack sufficient sensitivity and/or suitability for routine use.

In this paper we describe a chromatographic method for the quantitation of mefloquine in plasma, without interference from either sulfadoxine or pyrimethamine. The method involves solvent extraction, derivatisation with heptafluorobutyrylimidazole and electron-capture detection.

EXPERIMENTAL

Reagents and solvents

Mefloquine and the internal standard (I.S.) [racemic erythro-α-(2-piperidyl)-2,7-bis(trifluoromethyl)-4-quinolinemethanol] were provided by Hoffmann-La Roche (Basle, Switzerland). Glycine (AnalaR), sodium chloride (GPR), sodium hydroxide (CVS), toluene (AnalaR) and ammonia solution (35% AnalaR) were purchased from BDH (Poole, U.K.). Isopropyl acetate (Puriss p.a.) was obtained from Fluka (Buchs, Switzerland). Heptafluorobutyrylimidazole (HFBI) was purchased from Pierce (Chester, U.K.) and methanol (HPLC grade) was obtained from Fisons Scientific (Leicester, U.K.). Aqueous solutions were prepared with nanopure water (deionised water obtained by reverse osmosis, Barnstead System).

Standards

Mefloquine hydrochloride (2.631 mg; equivalent to 2.4 mg mefloquine) was dissolved in 100 ml methanol to yield the stock solution (24 μg/ml).

A series of standard solutions containing 18, 12, 9, 6, 3, 1.5, 0.75, 0.375, 0.187 and 0.094 μg/ml mefloquine was prepared by serial dilution of the stock solution with methanol. Plasma standards were freshly prepared each day by spiking blank plasma with working standards at a ratio of 1:10 to yield mefloquine concentrations ranging from 9.4 to 2400 ng/ml in plasma. A stock solution of internal standard was prepared by dissolving 0.5 mg in 50 ml methanol. This solution was further diluted to give concentrations of 1 and 2 μg/ml.

All standard solutions were stored at 4°C for up to two months. Drug-free plasma was obtained from healthy volunteers, kept at 4°C and used within one week. The buffer solution was prepared by dissolving glycine (7.5 g) and sodium chloride (5.85 g) in nanopure water (1 l) and adjusting to pH 9.0 with sodium hydroxide (1 *M*).

Instrumentation

A Pye-Unicam Series 104 gas chromatograph equipped with ^{63}Ni electron-capture detector in pulse mode (period of 150 μsec) was used. The column (1.5 m × 4 mm I.D., glass) was packed with 3% GC GE-SE-30 on Gas-Chrom Q (100—120 mesh) and conditioned overnight at 280°C with nitrogen at a flow-rate of 30 ml/min.

The gas chromatographic conditions used in this assay were as follows: carrier gas: nitrogen at a flow-rate of 40 ml/min; column temperature: 180°C for 9 min, raised to 280°C for 7 min; detector temperature: 350°C.

Extraction procedure

Plasma (0.25, 0.50 or 1.0 ml depending on the expected concentration of mefloquine) spiked with mefloquine standard (25, 50 or 100 μl, respectively) and internal standard (50 μl, 1 or 2 μg/ml) was mixed with 1 ml buffer solution in a glass-stoppered tube. After the addition of isopropyl acetate (8 ml), the drugs were extracted by rotating gently for 20 min on a mechanical rotator, followed by centrifugation for 10 min at 700 *g* at 5°C. The organic phase was transferred into a glass culture tube which was placed in a water bath at 40°C and the solvent was evaporated under a gentle stream of oxygen-free nitrogen. The residue was transferred quantitatively into a small narrow-bore culture tube of 5 ml capacity using an aliquot of 200 μl and followed by a second aliquot of 100 μl isopropyl acetate. The tube was placed in a water bath at 40°C and the solvent was evaporated under nitrogen.

Samples were extracted exactly as described above for standards except in place of the mefloquine standards an equivalent amount of methanol was added.

Derivatisation procedure

Toluene (100 μl) and HFBI (30 μl) were added to the dried residue and the tube was capped immediately, vortexed and left in a water bath at 37°C for 30 min. The excess of derivatising reagent was removed by addition of water (1 ml) and vortexing for 30 sec followed by addition of toluene (100 μl) and 2% ammonia solution (1 ml). This was further vortexed for another 30 sec. The tube was capped and centrifuged for 3 min at 1000 *g*. The toluene layer was transferred into another tube and 0.5—1 μl was injected into the gas chromatograph.

Calibration and calculation

Evaluation of the assay was carried out using five-point calibration standards in the concentration range 37.5—600 ng/ml mefloquine in plasma. The calibration curves were obtained by linear regression of the peak height ratios of mefloquine/I.S. versus concentrations of mefloquine.

Because of the wide concentration range found for mefloquine in plasma samples, different calibration curves were set up, each consisting of at least four points and the amount of internal standard was accordingly adjusted. These calibration curves were then used to interpolate the concentrations of mefloquine in plasma from the measured peak height ratio of mefloquine/I.S.

RESULTS AND DISUCSSION

Stability of acyl derivatives

It is known that acyl derivatives should be stored in the reaction medium until they are required for chromatography [6—8]. Derivatives of mefloquine and internal standard exhibit satisfactory stability in the presence of water and this allowed the use of aqueous washes to remove excess derivatising reagent. However, one technical point should be observed after removing the excess of derivatising reagent. The derivatives of internal standard and mefloquine should be analysed within 8 h after removal of the excess reagent if kept at room

temperature. However, if for any reason the excess of reagent is removed but samples cannot be analysed, addition of 10 μl HFBI will prolong the storage time. Excess of derivatising reagent should again be removed before analysis.

Selectivity

The emergence of plasmodial resistance to many currently applied antimalarials is becoming a severe problem in many parts of the world, especially to chloroquine, one of the most frequently used antimalarials. A similar resistance phenomenon may be anticipated for mefloquine when given alone, but to a lesser degree when combined with other drugs [9, 10]. The use of the triple combination of mefloquine (MQ), pyrimethamine (PY) and sulfadoxine (SX) results in a marked delay of resistance development and because lower individual doses may be given, is better tolerated with fewer side-effects.

To determine whether or not pyrimethamine and sulfadoxine interfere with the determination of mefloquine the following study was carried out. Drug-free plasma samples were spiked with each of the following: (1) PY (500 ng/ml); (2) SX (500 ng/ml); (3) PY plus I.S. (200 ng/ml); (4) SX plus I.S.; and (5) PY plus SX plus I.S. plus MQ (150 ng/ml). These samples, including a drug-free sample, were taken through the entire extraction and derivatisation procedure and injected into the gas chromatograph. Under the described procedural conditions no interference with mefloquine or internal standard was observed from either pyrimethamine or sulfadoxine. Many endogenous compounds present in plasma elute later than mefloquine and the internal standard and therefore a rise in temperature was necessary to elute all these compounds before the next injection was made.

Limit of detection

Under procedural conditions the limit of detection using a 1-ml plasma sample and 0.8-μl injection was 10 ng/ml, where the signal-to-noise ratio was greater than 3:1. A calibration curve consisting of 9.38, 18.75, 37.5 and 75 ng/ml mefloquine in plasma demonstrated a correlation coefficient of 0.990 and the regression equation was $y = 0.0107x - 0.0209$.

Recovery

The overall recovery was calculated in two different ways (Table I). First by comparing the peak heights of a series of mefloquine-spiked samples after its extraction from plasma and derivatisation, with the peak heights of a series of unextracted, derivatised reference standards. Secondly, by comparing the slope (determined by linear regression analysis) of a processed (extracted, derivatised) standard curve to that of the reference (derivatised) standards. Using these methods in the concentration range 37.5—600 ng/ml the mean overall recoveries were 102.7% and 103.1%, respectively.

Precision and accuracy

The data presented in Table II show the precision and accuracy of this assay. Intra-assay precision was determined at five concentrations in quadruplicate analysis, at levels of 37.5, 75, 150, 300 and 600 ng/ml mefloquine in plasma. Inter-assay precision was determined singly at the same five concentrations in

TABLE I

RECOVERY

Method 1

Concentration (ng/ml)	Mefloquine peak height (cm) (mean of duplicates)		Recovery (%)
	Set A: derivatised authentic standards	Set B: standards extracted from plasma and derivatised	
Blank	N.D.*	N.D.	—
37.5	1.25	1.25	100.0
75	2.75	2.90	105.4
150	6.45	6.40	99.2
300	12.70	13.55	106.7
600	24.40	25.00	102.4

Mean overall recovery ± S.D. = 102.7 ± 3.3%

Method 2

Regression line for Set A: $y = 0.0411x - 0.0428$, $r = 0.999$

Regression line for Set B: $y = 0.0424x - 0.0271$, $r = 0.999$

$$\text{Overall recovery} = \frac{0.0424}{0.0411} \times 100 = 103.1\%$$

Mean recovery determined by Methods 1 and 2 = 102.9%.

*N.D. = Not detectable.

TABLE II

PRECISION AND REPRODUCIBILITY

Concentration added (ng/ml)	Mean (n = 4) concentration found ± S.D. (ng/ml)	C.V. (%)	Difference between added and found concentration (%)
Intra-assay precision (repeatability)*			
37.5	41.6 ± 0.0	0.0	10.9
75	76.8 ± 5.0	6.5	2.4
150	139.4 ± 6.0	4.3	−7.1
300	292.7 ± 10.1	3.4	−2.4
600	607.3 ± 22.7	3.7	1.2
	Mean C.V. =	3.6%	
Inter-assay precision (reproduciblity)			
37.5	39.4 ± 4.4	11.3	5.1
75	72.6 ± 3.2	4.5	−3.2
150	136.7 ± 8.0	5.9	−8.9
300	303.6 ± 18.1	6.0	1.2
600	600.5 ± 8.8	1.5	0.1
	Mean C.V. =	5.8%	

*Linear regression line for intra-assay precision: $y = 0.0028x - 0.0174$ and correlation coefficient (r) = 0.999.

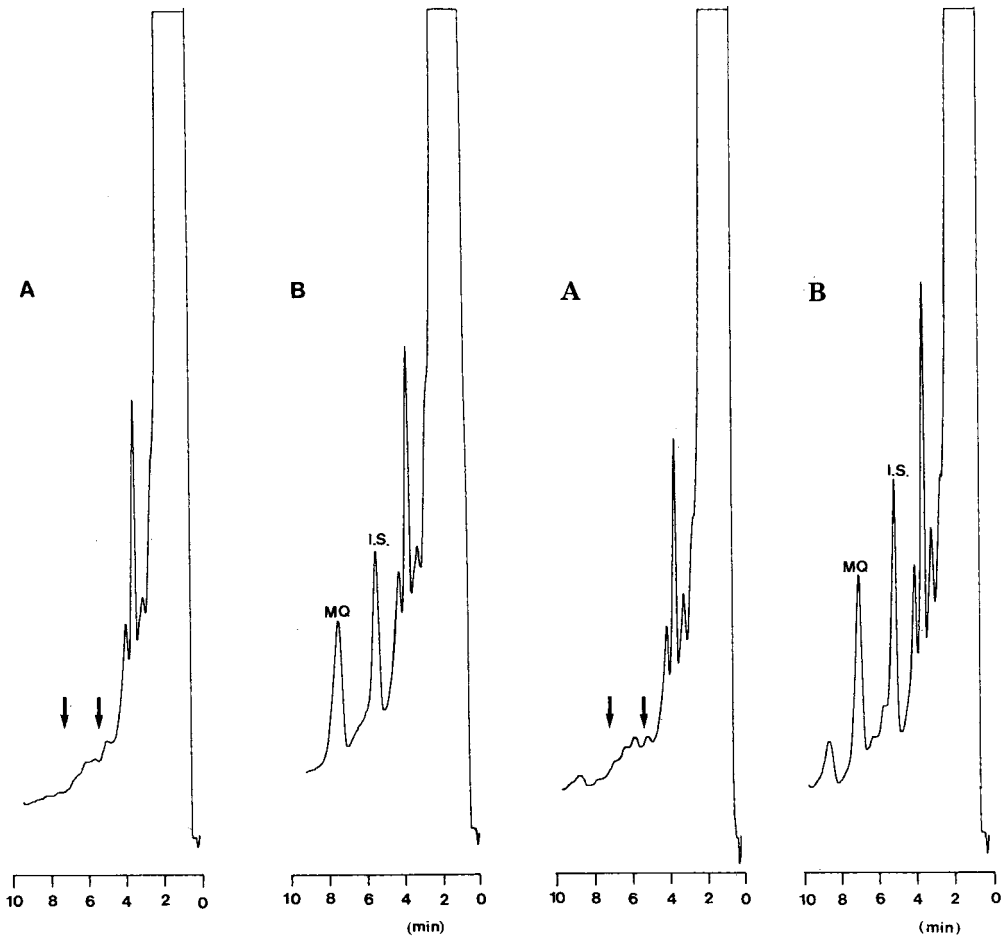

Fig. 2. Chromatograms of a human drug-free plasma (A) and a human drug-free plasma spiked with internal standard and 75 ng/ml mefloquine (B). Arrows indicate the retention times of internal standard (I.S.) and mefloquine (MQ).

Fig. 3. Chromatograms of a dog drug-free plasma (A); and a dog drug-free plasma spiked with internal standard and 75 ng/ml mefloquine (B). Arrows indicate the retention times of internal standard (I.S.) and mefloquine (MQ).

four replicate runs. The precision of the method (mean coefficient of variation, C.V.) was 3.6% and 5.8% for intra- and inter-assay, respectively. The accuracy (mean percentage differences between added and measured amounts) for the values of recovered standards, when calculated as unknowns against the linear regression line were between 0.1—10.9% over the concentration range investigated.

Separation

Figs. 2, 3 and 4 show typical chromatograms of mefloquine as determined in extracts of human and dog plasma, respectively. The mean retention times of internal standard and mefloquine were 5.2 and 7.3 min, respectively.

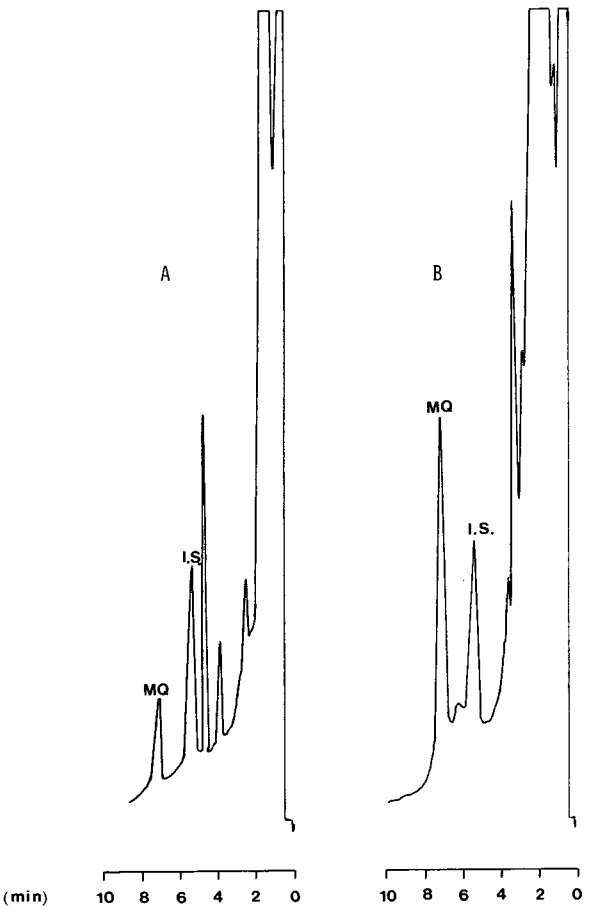

Fig. 4. Chromatograms of a human plasma sample (A) 21 days after receiving a single oral dose of a tablet containing MQ (750 mg), SX (1500 mg) and PY (75 mg); and a dog plasma sample (B) 3 days after receiving a single intravenous dose of MQ (100 mg).

Linearity

A measure of linearity is given under intra-assay precision, Table II. The correlation coefficient (r) was 0.999 and the intercept did not differ significantly from zero.

Application of the method to biological samples

The described method has been successfully applied to plasma samples from humans and dogs dosed with mefloquine. Table III presents the plasma concentrations of mefloquine in human and dog plasma, respectively. Determinations were carried out singly, while standard curves were evaluated in duplicate.

TABLE III

PLASMA CONCENTRATIONS OF MEFLOQUINE IN HUMAN AND DOG SAMPLES

The human subject received a single oral dose of a tablet containing mefloquine (750 mg), sulfadoxine (1500 mg) and pyrimethamine (75 mg). The dog received a single intravenous dose of mefloquine (100 mg).

Human plasma		Dog plasma	
Time after dose	Concentration (ng/ml)	Time after dose	Concentration (ng/ml)
2 h	620	10 min	2900
4 h	560	20 min	1720
6 h	860	40 min	1915
8 h	910	1 h	2155
10 h	570	2 h	1820
Day 1	560	3 h	1810
2	500	4 h	2260
4	470	6 h	1600
7	365	8 h	1625
14	350	12 h	1575
21	175	25 h	675
28	160	Day 2	405
35	105	3	265
42	120	4	140
49	55	6	70
56	40	7	50
63	20	8	35

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CHROMBIO. 2339

REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF TRICYCLIC NUCLEOSIDE AND TRICYCLIC NUCLEOSIDE 5'-PHOSPHATE IN BIOLOGICAL SPECIMENS

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SUMMARY

An isocratic, sensitive, high-performance liquid chromatographic assay was developed for the determination of the tricyclic nucleoside 1,4,5,6,8-pentaazaacenaphthylene-3-amino-1,5-dihydro-5-methyl-1-β-D-ribofuranosyl 5'-monophosphate (TCN-P; NSC 280594) and its dephosphorylated metabolite TCN (NSC 154020). Separation was obtained using a C_{18} Sep-Pak precolumn, a reversed-phase column, and a mobile phase of phosphate buffer—methanol (87.5:12.5, v/v) containing 0.0025 *M* tetrabutylammonium hydroxide. The absorbance of both TCN and TCN-P was monitored at 280 nm with a sensitivity limit of 10 ng/ml. The recovery was 54 ± 6% and 51 ± 8% (mean ± S.D.) from plasma for TCN and TCN-P, respectively. Rapid enzymatic dephosphorylation of TCN-P in plasma and tissue samples was prevented by adding a large excess of adenosine 5'-monophosphate. The assay was used to determine plasma and tissue concentrations of TCN-P and TCN after administration of TCN-P to cancer patients in a Phase I clinical study.

INTRODUCTION

The tricyclic nucleoside, 1,4,5,6,8-pentaazaacenaphthylene-3-amino-1,5-dihydro-5-methyl-1-β-D-ribofuranosyl (TCN; NSC 154020) is a new anticancer agent in the class of nucleosides active against implanted murine tumors and L1210 leukemia [1]. Tricyclic nucleoside 5'-phosphate (TCN-P; NSC 280594) is the more water-soluble phosphate ester of TCN (Fig. 1). Both agents inhibit DNA synthesis and are toxic to cells in the S phase of the growth cycle. TCN-P is enzymatically dephosphorylated by ecto-5-nucleotidase, actively transported into cells, and then rephosphorylated by adenosine kinase to the cytotoxic form [2—4]. TCN-P is currently being investigated in five major cancer centers

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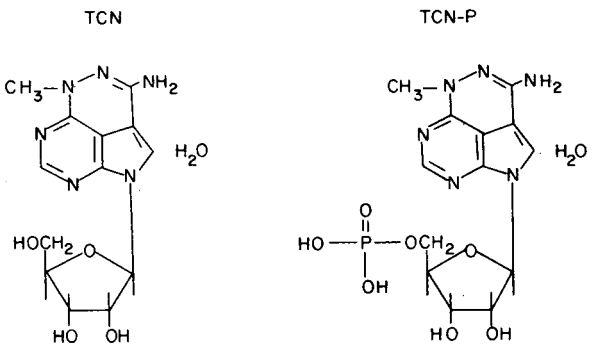

Fig. 1. Clinical names and structures of 1,4,5,6,8-pentaazaacenaphthylene-3-amino-1,5-dihydro-5-methyl-1-β-D-ribofuranosyl (TCN, left) and its 5′-monophosphate (TCN-P, right).

in the U.S.A. as part of Phase I clinical studies. A method was needed to determine both TCN-P and its metabolite TCN in biological fluids and tissues at concentrations below 100 ng/ml.

The method described herein uses a concentration step to attain the required sensitivity, a reagent to retard dephosphorylation of TCN-P, and an ion-pair reagent to prolong retention times and allow the use of an isocratic system. Previously published methods did not address the problem of dephosphorylation in a biological matrix, and were not sufficiently sensitive for a pharmacokinetic study [5, 6].

MATERIALS AND METHODS

Reagents

TCN and TCN-P were obtained from Investigational Drug Branch Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, U.S.A.; Lot No. 3, Lot No. MS-01-188). Adenosine 5′-monophosphate (AMP) was supplied by Sigma (St. Louis, MO, U.S.A.), HPLC-grade methanol, acetonitrile and water by J.T. Baker (Phillipsburg, NJ, U.S.A.), reagents for sodium phosphate buffer by Fisher Scientific (Fairlawn, NJ, U.S.A.) and tetrabutylammonium hydroxide (TBA) (1 *M* in methanol) by Eastman-Kodak (Rochester, NY, U.S.A.).

Chromatographic equipment

The chromatographic system consisted of a Waters Model 6000A solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) at a flow-rate of 1 ml/min, a Rheodyne injector (Rheodyne, Berkeley, CA, U.S.A.) or a Waters Model U6K injector with a 100-μl loop, a precolumn, a Waters μBondapak C_{18} column (300 × 4.6 mm I.D.), a Waters Model 440 detector set at 280 nm, and a Linear strip chart recorder (Linear Instruments, Irvine, CA, U.S.A.). Precolumns were either silica or C_{18} Sep-Pak cartridges obtained from Waters Assoc. The evaporator was an N-EVAP Model 112 obtained from Organization Assoc. (South Berlin, MA, U.S.A.).

Solutions

TCN and TCN-P stock solutions containing 50,000 ng/ml were prepared

every second day in deionized water and stored at 2°C. Stock solutions were serially diluted into the working range of 10—10,000 ng/ml. These solutions were injected directly onto the column. AMP was dissolved in water to a concentration of 10 mg/ml and stored at 2°C; its retention time of 6.3 min did not interfere with drug detection.

Plasma standards contained 2 ml blank plasma (Harper Hospital Blood Bank, Detroit, MI, U.S.A.), 0.2 ml TCN or TCN-P aqueous standards over a concentration range of 10—1000 ng/ml, and 0.2 ml AMP solution. This mixture was vortexed for 30 sec. Standards of each drug were used to establish a calibration curve for quantification of concentrations from patient specimens.

Mobile phase

To 1 l of 0.01 *M* phosphate buffer, pH 7.0, were added 2.5 ml of 1.0 *M* TBA. The pH of the solution was adjusted to 7.0 with phosphoric acid and poured through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.). To 875 ml of this solution were added 125 ml methanol for the final mobile phase. Prior to use, the mobile phase was degassed by stirring under vacuum for 15 sec.

Extraction procedure

Because TCN-P was unstable in blood plasma, specimens were chilled in ice immediately after collection, centrifuged at 2800 rpm for 5 min (Beckman Instruments, Palo Alto, CA, U.S.A.; Model TJ5 centrifuge), AMP was added, and samples were stored at −18°C until analysis. Plasma (2 ml) containing 0.2 ml AMP solution was applied to a conditioned C_{18} Sep-Pak column, washed with 5 ml of 0.0025 *M* TBA—0.005 *M* phosphate buffer adjusted to pH 7.0 with phosphoric acid and eluted with 3 ml methanol. The methanol was evaporated in a water bath at 40°C under a stream of nitrogen, and the residue was redissolved in 100 μl water.

A 50-μl aliquot was injected onto the column.

Patients received 70 or 96 mg TCN-P per m^2 as a 5-min intravenous infusion. Blood samples were collected via an intravenous catheter and transferred to heparinized test tubes before therapy and at selected times thereafter.

From one patient who died 61 days after a single dose of 70 mg TCN-P per m^2, pleural fluid was obtained by pleuracentesis twenty days after dosage, and tissue specimens were obtained at autopsy.

Three male Sprague—Dawley rats were obtained from Charles River (Wilmington, MA, U.S.A.). All animals were anesthetized with 50 mg pentobarbital per kg body weight, given intraperitoneally. Tissue and plasma samples from one animal served as blanks. Two rats were administered 50 mg TCN-P per kg by rapid injection into a femoral vein and the animals were killed 15 min later by cervical dislocation. Selected tissue specimens were removed, mechanically disintegrated (Polytron, Brinkman Instruments, Westbury, NY, U.S.A.), an aliquot was taken, AMP added, centrifuged, and immediately extracted and analyzed such as plasma samples.

Calculations

The concentration of TCN-P or its metabolite TCN in plasma or tissue was calculated by dividing the peak height by the slope of the appropriate plasma

standard curve. The slope of each standard curve was determined as the mean ± S.D. of response factors (peak height divided by concentration) calculated for each standard concentration. Extraction efficiency (recovery) was determined by comparing peak heights of extracted plasma standards to peak heights of directly injected aqueous standards of corresponding concentrations.

RESULTS

Stability of aqueous standards

At 2°C TCN-P standards in aqueous solutions were stable for up to three days, but long-term studies revealed a complete decomposition after six months; TCN aqueous standards were stable over a four-week period at 2°C, and afterwards additional peaks were detected at 4 min retention time. TCN-P aqueous standards (10 μg/ml) were unaltered by heat (60°C) over 4 h. Long-term (three months) ultracold storage (—60°C) led to degradation. Light-protected samples were stable for one week at room temperature and for two months when stored at 2°C. We routinely prepared fresh aqueous standards every two days, and these were stored at 2°C.

Stability of plasma standards

In heparinized blood bank plasma TCN-P (1.0 μg/ml) was rapidly dephosphorylated with a half-life of 40 min (Table I). The disappearance of TCN-P was correlated in time to the appearance of TCN, and by 3 h the dephosphorylation reaction was nearly complete. When a 1000-fold excess of AMP was added to the plasma (Table I), the TCN-P concentration did not decrease during the 3-h study, and no TCN could be detected. When this experiment was repeated using fresh plasma from a volunteer, essentially the same results were obtained.

Chromatograms

Sample chromatograms of aqueous standards (A), control blood bank plasma

TABLE I

PERCENTAGE OF ADDED 1.0 μg TCN-P PER ml PLASMA AND APPEARANCE OF TCN AFTER INCUBATION AT ROOM TEMPERATURE

Time (min)	Without added AMP		With added AMP	
	TCN-P	TCN	TCN-P	TCN
0	100*	0	100	0
15	67.0**	14.9***	100.0	0
75	17.5	63.2	105.5	0
135	5.6	95.4	107.1	0
185	2.0	82.8	86.6	0

*Theoretical.
**Spiked plasma samples were prepared at time zero and extracted at designated times.
***TCN concentrations were converted to TCN-P equivalents which are shown here as a percentage of zero-time TCN-P.

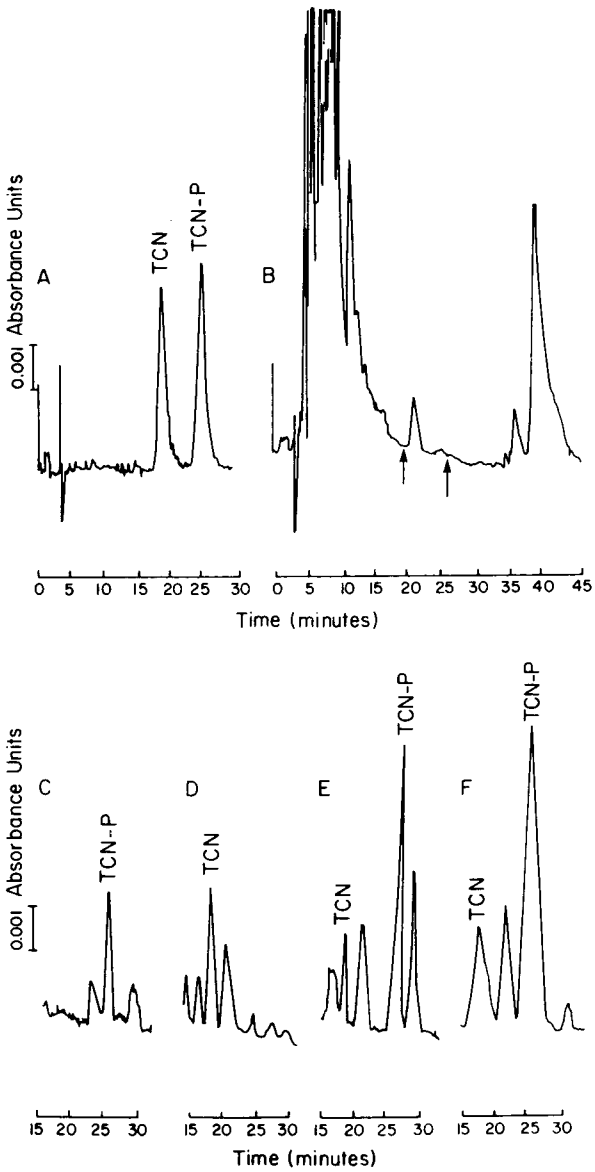

Fig. 2. Chromatograms obtained from (A) aqueous standards prepared with 10 μg/ml each; (B) control blank plasma; (C) control plasma containing 1.0 μg/ml TCN-P and 1.0 mg/ml AMP, processed 1 h after preparation; (D) patient's plasma 21 days, (E) pleural effusion fluid 20 days, and (F) liver metastases at autopsy 61 days after treatment with a single dose of 70 mg TCN-P per m^2 body surface area. The retention times for TCN and TCN-P were 18.6 and 25.5 min, respectively.

(B), spiked blood bank plasma (C) and patient plasma (D and E) are shown in Fig. 2. No interfering peaks were seen in control plasma, patient plasma obtained before administration of TCN-P, or in any of the blank tissue samples obtained from rats. The retention times for TCN-P and TCN were 25.5 and 18.6 min, respectively.

TABLE II

LINEARITY AND PRECISION OF THE HPLC ASSAY OF TCN-P ADDED TO WATER OR PLASMA

TCN-P aqueous standard (ng/ml)		Coefficient of variation (%)	TCN-P plasma standard (ng/ml)		Coefficient of variation (%)	Recovery[§] (%)
Theoretical	Found*		Theoretical**	Found***		
100	115.3	15.3	10	11.4	14.0	50
300	288.2	3.9	30	28.4	5.3	50
500	576.4	15.3	50	42.6	14.8	38
1000	1008.6	0.9	100	108.0	8.0	54
3000	2536.0	15.5	300	289.8	3.4	58
5000	4149.9	17.0	500	505.7	1.1	62
10,000	10,489.9	4.9	1000	1000.0	0.0	48
Mean ± S.D.		10.4 ± 6.84			6.7 ± 5.9	51 ± 8

*The slope calculated was 0.0347 ± 0.0046 mm/(ng/ml) (mean ± S.D.).
**Theoretical concentrations were based on the amount of TCN-P in water added to 2 ml plasma. AMP (1 mg/ml) was added to each milliliter of plasma to prevent dephosphorylation.
***The concentration found was calculated from the calibration curve for each of two plasma samples per concentration. The slope calculated was 0.352 ± 0.032 mm/(ng/ml) (mean ± S.D.).
[§]The percentage recovery was calculated from peak heights of plasma standards containing AMP divided by peak heights of standard solutions in water containing the corresponding concentrations.

TABLE III

LINEARITY AND PRECISION OF THE HPLC ASSAY OF TCN ADDED TO WATER OR PLASMA

TCN aqueous standard (ng/ml)		Coefficient of variation (%)	TCN plasma standard (ng/ml)		Coefficient of variation (%)	Recovery[§] (%)
Theoretical	Found*		Theoretical**	Found***		
300	321.3	7.1	50	50.0	0.0	45
1000	1164.7	16.5	100	123.1	23.1	55
5000	4819.3	3.6	500	446.2	10.8	58
10,000	8032.1	19.7	1000	876.9	12.3	57
Mean ± S.D.		11.7 ± 7.6			11.6 ± 8.2	54 ± 7

*The slope calculated was 0.0249 ± 0.0039 mm/(ng/ml) (mean ± S.D.).
**Theoretical concentrations were based on the amount of TCN in water added to plasma.
***The concentration found was calculated from the calibration curve for each of two plasma samples per concentration. The slope calculated was 0.260 ± 0.042 mm/(ng/ml) (mean ± S.D.).
[§]The percentage recovery was calculated from peak heights of plasma standards divided by peak heights of standard solutions in water containing the corresponding concentrations.

Standard curves

Representative standard curves for TCN-P and TCN are shown in Tables II and III, respectively. For TCN-P the slopes of the aqueous (0.0347) and plasma (0.352) standard curves were essentially parallel, and the ten-fold difference in slope was due to the twenty-fold concentration step and a 51% recovery. The linearity of these curves was assessed by linear regression analysis of concentration versus peak height; for TCN-P the correlation coefficients were 0.9991 for aqueous standards and 0.9998 for plasma standards.

For TCN the slopes of aqueous and plasma standard curves were 0.0249 and 0.260, respectively, and the corresponding correlation coefficients were 0.9949 and 0.9974. The mean recovery from extraction was 54%.

Table IV describes the tissue distribution of both TCN and TCN-P studied in rats. The assay allowed detection of TCN in brain tissue and high concentrations of both drugs in the pancreatic gland.

TABLE IV

TCN AND TCN-P CONCENTRATIONS IN VARIOUS BODY FLUIDS AND TISSUES OF TWO RATS 15 min AFTER RECEIVING 50 mg TCN-P PER kg INTRAVENOUSLY AS A PUSH INJECTION

Material	Concentrations (μg/ml of μg/g)	
	TCN	TCN-P
Red blood cells	5.6	12.9
Blood plasma*	2.6, 6.1	6.6, 6.0
Brain	4.4	n.d.**
Pancreas	43.2	118.5
Spleen	9.6	n.d.
Kidneys	36.9	n.d.
Small intestine	8.7	31.7
Large intestine	17.3	36.0
Carcass	2.1	24.4

*Separate determinations for specimens from two animals.
**n.d. = concentrations not detected.

DISCUSSION

A variety of mobile phases revealed short retention times below 20 min and were insufficient to separate the drugs from interferences. However, when 0.0025 *M* tetrabutylammonium hydroxide was added to the aqueous buffer—methanol mobile phase, satisfactory sensitivity and resolution were achieved. A previously described extraction procedure employed 20% trichloroacetic acid (TCA) to precipitate proteins [5]. Repeated trials in this laboratory, however, revealed dephosphorylation and degradation of TCN-P immediately after TCA was added. Polar solvents, e.g. methanol, ethyl acetate or diethyl ether, allowed recovery of only 10% of added TCN-P from plasma. A silica Sep-Pak column conditioned with acetonitrile—water (90:10, v/v) and eluted with water revealed a drug recovery of only 18%. The most reproducible, sensitive results were obtained using an ion-pair reagent and C_{18} Sep-Pak precolumn.

The method described here was not subject to interferences from a number of concomitantly administered drugs such as digitalis or penicillin. In a further application of this assay, we have measured concentrations of TCN-P as small as 100 ng/ml incubated in human tumor stem cell assays [7, 8]. The method described herein is practical for use in any laboratory with HPLC capability for clinical plasma and tissue drug level measurement, as well as preclinical animal and in vitro studies.

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CHROMBIO. 2362

SIMULTANEOUS DETERMINATION OF CYTOSINE ARABINOSIDE, ITS NUCLEOTIDES AND METABOLITES BY ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Currently available high-performance liquid chromatographic assays for cytosine arabinoside (ara-C) and its metabolites suffer from two major shortcomings: inability to resolve both ara-C and its nucleotides in a single chromatographic step and/or inadequate sensitivity to allow quantitation of intracellular cytosine arabinofuranoside-5′-triphosphate (ara-CTP) without the use of radiolabelled drug. In this paper, we describe a new ion-pairing high-performance liquid chromatographic assay for ara-C in biological samples that can separate ara-C from its nucleotides, metabolites, and naturally occurring ribonucleotides in a single chromatographic step with a lower limit of quantitation of 5 pmol for ara-C and 10 pmol for ara-CTP. Examples of the utility of this assay are shown in studies of intracellular pharmacokinetics of ara-C in cultured human breast cancer cells and in analysis of plasma nucleoside levels in patients receiving high-dose thymidine chemotherapy. We conclude that this assay provides a rapid and versatile system that can be applied to the study of both cellular and plasma nucleoside pharmacokinetics.

INTRODUCTION

Cytosine arabinoside (1-β-D-arabinofuranosyl cytosine, ara-C) is a drug of established value in the treatment of acute non-lymphocytic leukemia [1]. Its active metabolite, cytosine 1-β-D-arabinofuranoside-5′-triphosphate (ara-CTP), inhibits DNA synthesis both by inhibition of DNA polymerase and by incorporation into the DNA molecule [2—4]. Key elements in the cellular sensitivity to ara-C are the extent of ara-CTP formation, the activity of the

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deaminase pathways that inactivate ara-C and the duration of drug exposure [5].

Studies of the biochemical and clinical pharmacology of ara-C have generally focused on two major areas; intracellular ara-CTP formation and the plasma pharmacokinetics of ara-C and its major metabolite, uracil arabinoside (ara-U). The development of specific high-performance liquid chromatographic (HPLC) assays for ara-C and ara-CTP has resulted in a rapid expansion of our knowledge of the pharmacology of this drug. Yet, currently available HPLC assays suffer from two major shortcomings: inability to resolve both ara-C and its nucleotides in a single chromatographic step and/or inadequate sensitivity to allow quantitation of intracellular ara-CTP without the use of radiolabelled drug. Other necessary attributes of a useful HPLC assay for ara-C are a capacity to separate ara-C and its nucleotides from naturally occurring pyrimidines as well as a capacity to separate and quantitate the uracil arabinoside metabolites of ara-C.

The most commonly used HPLC assays for ara-CTP employ anion-exchange columns and are able to detect unlabelled drug with a sensitivity as low as 25 pmol [6—11]. These assays, however, fail to accurately resolve free bases and nucleosides. Separation of nucleosides from nucleotides can be accomplished using a reversed-phase HPLC system to resolve nucleosides and an anion-exchange system to separate nucleotides but this procedure is cumbersome and time-consuming. Simultaneous separation of ara-C nucleosides and nucleotides has been reported using a C_{18} amine column but baseline separation between all peaks was not achieved and the sensitivity of the assay was not reported [12]. Recently, Crowther et al. [13] demonstrated that the use of tetrabutylammonium phosphate as an ion-pairing agent allows rapid and complete resolution of naturally occurring nucleosides and nucleotides. Using this ion-pairing agent, we have developed a new HPLC assay for ara-C that allows resolution of parent drug, its nucleotide derivatives and its metabolites in a single chromatographic step. This assay is applicable to the study of both intracellular and plasma ara-C pharmacokinetics.

MATERIALS AND METHODS

Chemicals

Cytosine 1-β-D-arabinofuranoside (ara-C), cytosine 1-β-D-arabinofuranoside 5′-monophosphate (ara-CMP), cytosine 1-β-D-arabinofuranoside 5′-triphosphate (ara-CTP), uracil-1-β-D-arabinofuranoside (ara-U), cytidine 5′-monophosphate (CMP), cytidine 5′-diphosphate (CDP), cytidine 5′-triphosphate (CTP), uridine 5′-monophosphate (UMP), uridine 5′-diphosphate (UDP), and uridine 5′-triphosphate (UTP) were purchased from Sigma (St. Louis, MO, U.S.A.). Tetrabutylammonium phosphate (TBAP) (Kodak Chemical), potassium phosphate monobasic and HPLC-grade acetonitrile were purchased from Fisher Scientific (St. Louis, MO, U.S.A.) and [5-^{3}H]cytosine 1-β-D-arabinofuranoside ([^{3}H]ara-C), specific activity 11.2 Ci/mmol, was purchased from Amersham (Arlington Heights, IL, U.S.A.). All other chemicals used were of analytical grade.

Cell culture supplies

Earles minimum essential medium without L-glutamine (MEM), L-glutamine, non-essential amino acids, penicillin and streptomycin and heat-inactivated fetal bovine serum were purchased from KC Biological (Lenexa, KS, U.S.A.). Sterile pipets and 75-cm^2 tissue culture flasks were purchased from Fisher Scientific.

Instrumentation and HPLC columns

A Waters Assoc. HPLC system (Milford, MA, U.S.A.) consisting of two Model M6000A pumps, a U6K injector, and a Model 440 absorbance detector with 280-nm filter is used in this assay. The mobile phase is prepared by a Model 660 solvent programmer mixing the effluents of these two pumps. Mobile phase A consists of 100 m*M* potassium dihydrogen phosphate with 5 m*M* TBAP, pH 4.5 and mobile phase B is 100% acetonitrile. Authentic standards in a volume of 25 μl or cell or plasma extracts are injected on to a Radial-Pak 5-μm C_{18} cartridge in an RCM 100 radial compression module connected to a Z module radial compression system also containing a 5-μm C_{18} cartridge. Elution is carried out at room temperature at a flow-rate of 2 ml/min using curve No. 8 on the Model 660 solvent programmer. The gradient is run from initial conditions of 0% pump B to 10% pump B for 30 min following which elution is continued with 10% acetonitrile for an additional 14 min. A 15-min re-equilibration period using initial conditions is necessary before injection of the next sample.

Ultraviolet absorbance is monitored at 280 nm and standard chromatograms are recorded on a Houston Instruments Omniscribe recorder. Fractions of cell extracts prepared after incubation with [^{3}H]ara-C are collected every 0.2 min for the first 14 min of the assay and then every 0.5 min for the next 30 min for determination of radioactive peaks. Radioactivity is determined using a Beckman LS-6800 scintillation counter.

Propagation of cells in culture

MCF-7 human breast cancer cells are grown in MEM supplemented with 10% fetal bovine serum, L-glutamine at 584 μg/ml, penicillin at 124 μg/ml and streptomycin at 270 μg/ml under 5% carbon dioxide at 37°C. The human derivation, hormonal responsiveness and growth characteristics of the cells have been described [14].

Preparation of cell extracts

Following in vitro incubation with the desired concentration of ara-C, half the drug-containing medium is removed from the flask and set aside and the cells are scraped off the flask surface with a rubber policeman into the remaining medium. The cell suspension is centrifuged at 400 *g* for 2 min at 4°C, the supernatant is removed and the cell pellet is immediately resuspended in 500 μl of the drug-containing medium set aside earlier. This concentrated cell suspension is then layered over a silicone oil—mineral oil (84:16) interface in a 1.5-ml polypropylene microfuge tube and immediately centrifuged at 500 *g* for 1 min in a table-top centrifuge. The cells, but not the medium, travel to the tip of the tube which contains 500 μl of 1 *M* perchloric acid. The tubes are then quickly frozen and cut at the oil—perchlorate interface. The perchlorate

extract is transferred to another tube and neutralized with 8 *M* potassium hydroxide following which the potassium perchlorate is removed by centrifugation and the supernatant is stored at −80°C until injection into the HPLC system.

Preparation of plasma samples for analysis

Plasma nucleoside levels are determined by addition of 1 ml of 1 *M* perchloric acid to 1 ml plasma, removal of the precipitate by centrifugation and injection of the supernatant on a Sep-Pak C_{18} cartridge previously prepared by injection of 10 ml acetonitrile followed by 10 ml of 1 m*M* perchloric acid. The cartridge is then washed with 10 ml of water and the nucleosides are eluted with 4 ml of acetonitrile, evaporated to dryness under nitrogen and resuspended in the HPLC mobile phase A prior to injection.

RESULTS

HPLC analysis

The separation of ara-C, its nucleotides and metabolites from naturally occurring ribonucleotides is shown in Fig. 1. Following injection of a standard mixture of authentic nucleosides and nucleotides, baseline separation is

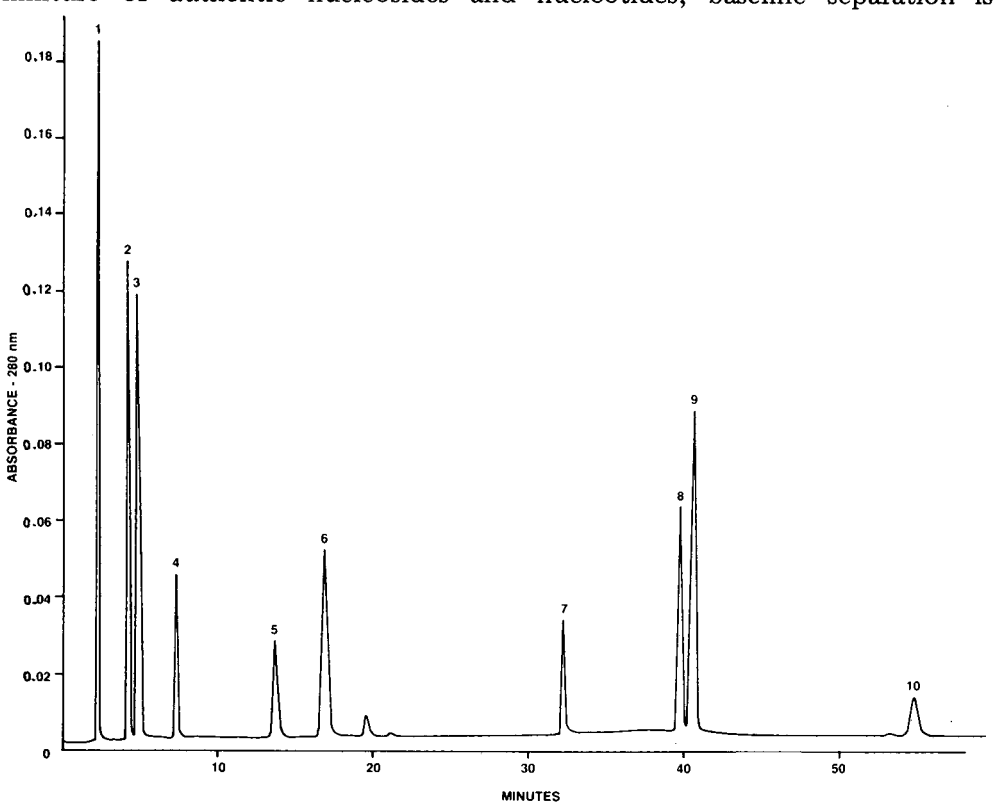

Fig. 1. HPLC separation of a standard mixture of cytosine arabinoside and its metabolites from naturally occurring ribonucleotides. Peaks: 1 = ara-C; 2 = CMP; 3 = ara-CMP; 4 = ara-U; 5 = UMP; 6 = CDP; 7 = UDP; 8 = CTP; 9 = ara-CTP; 10 = UTP.

achieved for all compounds and R factors for all peaks exceed 0.90. Variation in retention time for all peaks is less than 10%. Ara-C is well separated from both its nucleotides and its deamination products and quantitation can be achieved to levels as low as 10 pmol for ara-CTP and 5 pmol for ara-C.

Intracellular ara-C pharmacokinetics in vitro

This assay method can easily be applied to the study of intracellular ara-C pharmacokinetics in vitro. MCF-7 human breast cancer cells were incubated with 1 μM [^{3}H]ara-C and harvested at various times as described above. The time course of ara-C uptake and metabolism in these cells is shown in Fig. 2. Total intracellular drug increases linearly during a 60-min incubation with [^{3}H]ara-C. This increase is due entirely to the formation of phosphorylated derivatives of ara-C, i.e., intracellular levels of ara-C itself remain constant during the period of drug exposure. Ara-CTP is the first nucleotide derivative to accumulate in the cell. As ara-CTP levels increase and eventually plateau, ara-CMP and ara-CDP begin to accumulate suggesting that phosphorylation to the triphosphate proceeds very rapidly initially then subsequently slows allowing accumulation of the mono- and diphosphate derivatives. Neither ara-U nor its phosphorylated derivatives were detected intracellularly during this period of drug exposure.

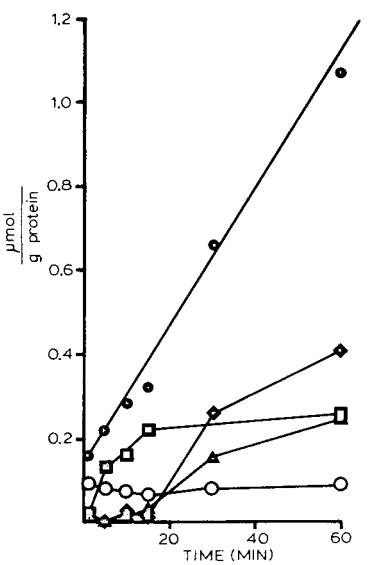

Fig. 2. Time course of [^{3}H]ara-C uptake and metabolism in MCF-7 cells. MCF-7 human breast cancer cells were incubated with 1 μM [^{3}H]ara-C and harvested at various times for HPLC analysis of cellular contents. (●) Total drug; (○) ara-C; (◆) ara-CMP; (▲) ara-CDP; (■) ara-CTP.

Fig. 3 depicts the dose—response relationship for accumulation of ara-C and its metabolites in human breast cancer cells during a 15-min incubation with ara-C. Total intracellular drug increases rapidly with increasing extracellular drug concentration and this is due, again, largely to the formation of ara-C nucleotides, particularly ara-CTP. As the extracellular drug concentration is

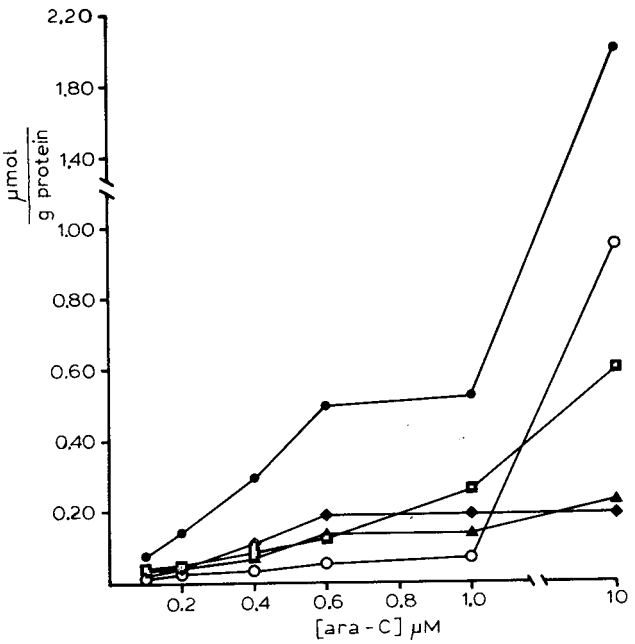

Fig. 3. Uptake and metabolism of [^{3}H]ara-C in MCF-7 cells. MCF-7 human breast cancer cells were incubated with varying concentrations of [^{3}H]ara-C for 15 min then harvested for HPLC analysis of cellular contents. (•) Total drug; (○) ara-C; (♦) ara-CMP; (▲) ara-CDP; (▪) ara-CTP.

increased from 1 to 10 μM however, further increases in intracellular drug are due primarily to a marked (twelve-fold) increase in intracellular ara-C, while ara-CMP and ara-CDP levels remain relatively constant and ara-CTP levels increase by only two-fold.

Analysis of plasma nucleosides

This assay can also be easily modified to allow rapid analysis of plasma nucleoside concentrations. As an example of its utility in this area, we have used the assay to measure plasma thymidine levels in patients receiving high doses of this nucleoside as part of an investigational treatment protocol. Plasma extracts are injected on to a 5-μm C_{18} cartridge in an RCM 100 radial compression system. Initial buffer conditions (buffer A) are 100 mM potassium phosphate with 5 mM TBAP and elution is done isocratically for 4 min. A step gradient is then used to immediately bring final buffer conditions to buffer A + 12% acetonitrile and elution is carried out for an additional 11 min. Total assay time is 15 min at a flow-rate of 2 ml/min. Fig. 4 depicts a representative chromatogram of plasma obtained from a patient receiving high-dose thymidine. Deoxyinosine was added during the extraction procedure to serve as an internal standard. A chromatogram of a pretreatment plasma sample is shown for comparison and demonstrates a small peak which co-chromatographs with authentic thymine. No absorbing materials are detected at 6 min or 8 min, the retention times of authentic deoxyinosine and thymidine, respectively. To determine plasma thymidine concentrations, a standard curve is constructed by

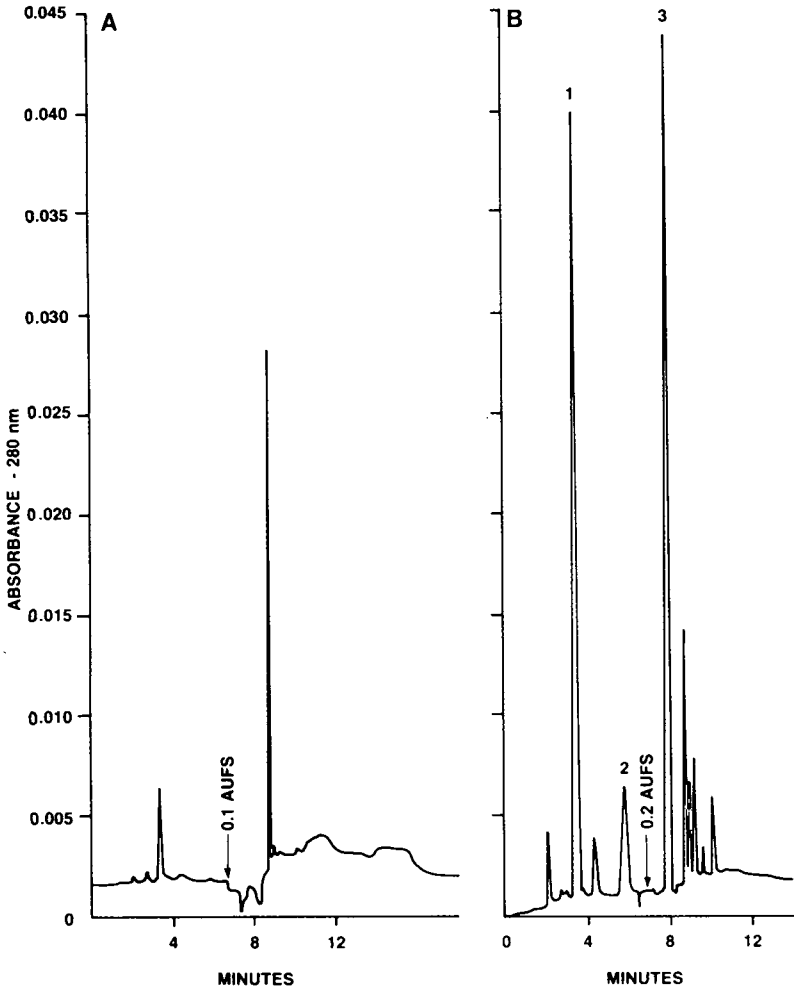

Fig. 4. HPLC analysis of plasma thymidine concentrations. (A) Patient plasma prior to treatment and (B) patient plasma during continuous infusion of thymidine at 24 g/m^2/day. Peaks: 1 = thymine; 2 = deoxyinosine (internal standard); 3 = thymidine.

plotting the ratio of peak heights for thymidine and deoxyinosine versus known thymidine concentrations. Coefficients of variation for these ratios are 7.4% and 10.2% at thymidine concentrations of 25 μM and 250 μM, respectively. A similar standard curve is constructed for determination of plasma thymine levels. In the example shown in Fig. 4, the patient was receiving 24 g/m^2 thymidine as a continuous intravenous infusion. Steady-state plasma thymidine levels were 160 ± 10 μM and steady-state thymine levels were 406 ± 147 μM. In another patient receiving 40 g/m^2 thymidine, steady-state plasma thymidine levels rose to 381 ± 85 μM. Plasma thymidine concentrations as low as $5 \cdot 10^{-8}$ M can be detected using this assay. The isocratic portion of this assay can also be used to determine plasma ara-C levels with a lower limit of quantitation of 5 pmol injected. The retention time for ara-C in this system is 2 min.

DISCUSSION

The development of specific and sensitive HPLC assays for many antineoplastic drugs has significantly enhanced our understanding of the biochemical and clinical pharmacology of these agents. We have previously applied the concept of ion-pairing in the development of an HPLC assay for methotrexate and its polyglutamate derivatives [15] and, in the present study, we describe a new ion-pairing HPLC assay for nucleosides and nucleotides which is particularly valuable for study of the biochemical and clinical pharmacology of cytosine arabinoside. Unlike the commonly used HPLC assays for ara-C, this ion-pairing assay provides a method for separation of ara-C, its nucleotides and deamination products in a single chromatographic step. This capability provides a unique opportunity for the study of the intracellular pharmacokinetics of ara-C in vitro since ara-C and its mono-, di- and triphosphates can all be determined reliably with a single-extraction procedure and HPLC run thereby eliminating the variability inherent in using separate HPLC assays to quantitate these materials as is commonly done.

The sensitivity of this assay (5 pmol for ara-C and 10 pmol for ara-CTP) compares favorably to that reported for other HPLC assays used to quantitate intracellular ara-CTP without the use of radiolabelled drug. Application of this technique thus offers the potential to accurately determine cellular ara-CTP levels in patients receiving ara-C therapy and to correlate these levels with other important parameters such as plasma ara-C pharmacokinetics and clinical response to treatment.

As an example of the utility of the assay in the study of cellular ara-C pharmacokinetics, human breast cancer cells were incubated in vitro with [^{3}H]ara-C and the time course and dose response of ara-C uptake and metabolism were studied. As shown in Figs. 2 and 3, use of this HPLC assay allows simultaneous analysis of the intracellular distribution of ara-C, ara-CMP, ara-CDP and ara-CTP over time and following exposure to varying extracellular ara-C concentrations. Of particular interest is the finding that ara-CTP is the first nucleotide derivative to accumulate in the cell with ara-CMP and ara-CDP appearing only as ara-CTP levels plateau suggesting that phosphorylation to the triphosphate proceeds rapidly initially but slows as ara-CTP accumulates in the cell then resulting in the accumulation of the mono- and diphosphate derivatives of ara-C. Fig. 3 depicts the dose-dependent nature of ara-C nucleotide formation but reveals that, at high extracellular ara-C concentrations, increases in total intracellular drug are due primarily to accumulation of ara-C rather than to increases in ara-CTP formation. Information such as this might be useful in assessing the potential clinical utility of high dose ara-C therapy in the treatment of solid tumors.

This ion-pair HPLC assay can also easily be modified for rapid determination of plasma nucleoside levels. As shown in Fig. 4, we have successfully applied this assay to measurement of plasma thymidine and thymine concentrations in patients receiving high-dose thymidine as part of an experimental treatment protocol. The assay can be used with equal facility for determination of plasma ara-C and ara-U levels and thus provides a rapid and versatile system which can be applied to the study of both cellular and plasma nucleoside pharmacokinetics.

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CHROMBIO. 2348

ION-PAIRED HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF MITOXANTRONE IN PHYSIOLOGICAL FLUIDS

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SUMMARY

Mitoxantrone is one of the newer anthracenedione derivatives which has already been studied in phase I and II trials, where it has shown significant antitumour activity against a variety of human tumours. To determine the prolonged terminal half-life of mitoxantrone, we developed a sensitive high-performance liquid chromatographic method, providing a detection limit of 1 ng/ml of extracted serum. This system uses a C_{18} reversed-phase column. The mobile phase consists of a mixture of acetonitrile (30%, v/v) and an ammonium formate buffer (70%, v/v) with a pH of 2.7. Hexane sulphonic acid is added as an ion-pair former. Detection at a wavelength of 658 nm provides a highly selective system, showing no interfering peaks. Ametantrone, another anthracenedione derivative, is used as an internal standard. The extraction procedure for serum also uses hexane sulphonic acid in an ion-paired system. Because of the highly selective detection wavelength, urine samples can be injected without a sample clean-up procedure.

This very sensitive method, combined with high selectivity and a fast and inexpensive serum clean-up procedure, has allowed us to document the prolonged terminal plasma half-life of mitoxantrone (levels of 2—5 ng/ml of plasma can still be detected six days after an

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intravenous infusion of 15 mg/m² over 30 min). In urine an as yet unidentified metabolite was discovered.

INTRODUCTION

Mitoxantrone (Fig. 1), 1,4-dihydroxy-5,8-bis{[2-[(2-hydroxyethyl)amino]-ethyl]amino}-9,10-anthracenedione dihydrochloride, or DHAD (NSC-301739), is an anthracenedione derivative which has shown significant antitumour activity in animal models [1—4]. It has also been studied in phase I and II studies and proved to have equal activity in breast cancer to that of doxorubicin, but with lower cardiotoxicity [5—11]. Previous reports dealing with the determination of mitoxantrone in plasma have two major disadvantages: either they have poor sensitivity [12—15] or else they lack an internal standard [16].

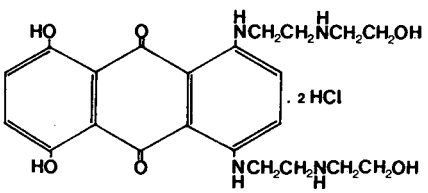


Fig. 1. Structural formula of mitoxantrone.

For those reasons and in order to evaluate the prolonged terminal half-life of this drug we tried to develop a new high-performance liquid chromatographic (HPLC) method with a sensitivity of at least 1 ng/ml of serum and using an internal standard.

MATERIALS AND METHODS

Materials

Mitoxantrone was kindly provided by American Cyanamid (Louvain-la-Neuve, Belgium) and ametantrone by the Drug Synthesis and Chemistry Branch of the National Cancer Institute, Bethesda, MD, U.S.A. (Dr. J.P. Davignon).

All glasswork was siliconized using Glass-treat (5% solution in hexane) from Chrompack (Middelburg, The Netherlands). Formic acid, 25% ammonia and sodium tetraborate · $10H_2O$ were purchased from E. Merck (Amsterdam, The Netherlands) and were analytical grade. Hexane sulphonic acid was obtained from Kodak (Oostdijk, The Netherlands), and *l*-ascorbic acid from Brocacef B.A. Holland (Maarssen, The Netherlands); both were analytical grade. Acetonitrile and dichloromethane were purchased from Pharmachemie (Haarlem, The Netherlands) and were HPLC grade.

Human plasma containing citrate—phosphate—dextrose as anticoagulant was kindly provided by the National Blood Bank of The Netherlands (Amsterdam, The Netherlands).

All aqueous solutions were filtered through a 0.22-μm filter (Sartorius SM

11107) and the organic solutions through a 0.22-μm filter (Sartorius SM 11607).

Sample clean-up procedure

Blood samples were collected at time 0, 15, 30, 40, 45, 60, 90 and 120 min after the beginning of the infusion, and afterwards at 4, 8, 12, 24, 36, 48 h up to six days after time 0.

After centrifugation at 1500 *g*, and separation, the plasma was immediately frozen and stored at −20° C.

Plasma samples were warmed up quickly just prior to extraction and mixed on a vortex for 10 sec. One millilitre of a solution containing hexane sulphonic acid (0.01 mg/ml), ascorbic acid (0.5 mg/ml) and ametantrone (1.2 mg/ml) as internal standard was added to 1 ml of plasma. After vortexing for 30 sec, 1 ml of a 0.1 *M* borax buffer (pH 9.5) was added and again vortexed for 30 sec. Extraction was performed with 5 ml of dichloromethane; after centrifugation at 1500 *g*, and separation, the organic layer was dried at 40° C under nitrogen. The dry residue was kept at −20° C until HPLC analysis was performed.

Urine was prepared as follows: 1 ml of frozen urine was brought to room temperature, mixed with 1 ml of an aqueous solution containing the internal standard and 100 μl were directly injected.

HPLC analysis

Plasma or urine was assayed for mitoxantrone by reversed-phase HPLC using an ion-pair system.

The HPLC system employed a Spectra Physics chromatography pump Model 740B or a Waters Assoc. solvent delivery system Model 6000A, and a Waters Assoc. injector Model U6K.

Separation of mitoxantrone, possible metabolites and the internal standard was carried out using a 30 × 0.39 cm μBondapak C_{18} column (Waters Assoc.) with a particle size of 10 μm. A guard column (7 × 0.21 cm) packed with the same material was used to protect the chromatographic column. The packing was changed every two weeks or when necessary. The mobile phase consisted of a mixture of acetonitrile (30%) and 0.16 *M* ammonium formate buffer (70%) pH 2.7.

Hexane sulphonic acid was added in a concentration of 0.025 *M*. The flow-rate was 1.6 ml/min.

Detection at 658 nm was performed either on a Pye Unicam LC-UV type variable-wavelength spectrophotometer or a Waters Assoc. fixed-wavelength detector Model 441. The peaks were recorded on a Hewlett-Packard integrator recorder Model 3380 A.

Quantitation, recovery and precision

Quantitation was done by the internal standard method, using the peak heights as well as the integrated areas under the peak.

Plasma standard curves were established for mitoxantrone in aqueous solution, ametantrone in aqueous solution, mitoxantrone extracted from aqueous solution and from plasma, in absolute values and after plotting against the internal standard level.

Recovery was calculated by comparing the measured levels of spiked serum to those of standard aqueous solutions.

Five samples, spiked with 100, 250, 500 or 1000 ng of mitoxantrone per ml of serum, were assayed.

Three additional samples with concentrations of 5, 10 or 20 ng of mitoxantrone per ml of serum were used to calculate the recovery data in the range close to the detection limit.

Precision and variation were determined by assaying five to ten samples of spiked serum.

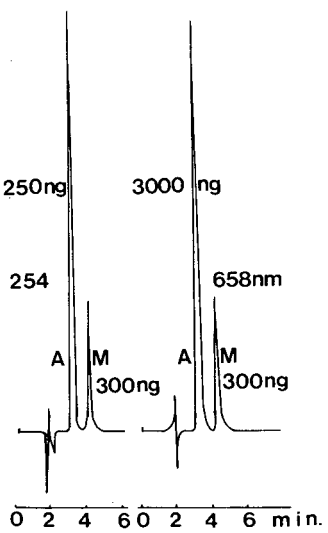
All experiments were done in triplicate and repeated on different occasions.

RESULTS

Fig. 2 shows the chromatogram of an aqueous solution containing 250 ng of ametantrone and 300 ng of mitoxantrone, when determined at 254 nm; or 3000 ng of ametantrone and 300 ng of mitoxantrone when assayed at 658 nm. Here there is a ten-fold decrease in peak height for ametantrone when the wavelength is changed from 254 to 658 nm.

A representative chromatogram at 0.005 a.u.f.s. of both drugs extracted from plasma is shown in Fig. 3. This sample of serum contained 250 ng of ametantrone and 5 ng of mitoxantrone per ml. This demonstrates that a sensitivity of 1 ng/ml of extracted serum can easily be reached, knowing that only half of the reconstituted extract is injected.

A blank serum showed no peaks at the position of the internal standard or mitoxantrone. The coefficient of variation, estimated on five samples, varied



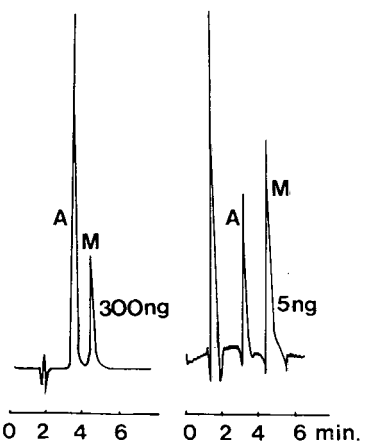


Fig. 2. Chromatograms of mitoxantrone (300 ng) and ametantrone (250 ng) in aqueous solution at 254 nm, and of mitoxantrone (300 ng) and ametantrone (3000 ng) in aqueous solution at 658 nm. Peaks: A = ametantrone; M = mitoxantrone.

Fig. 3. Chromatograms of mitoxantrone and ametantrone at 658 nm, after extraction from serum; serum was spiked with 300 ng or 5 ng of mitoxantrone. Peaks: A = ametantrone; M = mitoxantrone.

TABLE I

COEFFICIENT OF VARIATION FOR EXTRACTED PLASMA SAMPLES

Concentration (ng/ml)	*n*	C.V. (%)
5	10	11.9
10	10	14.6
20	10	8.5
200	5	2.7
400	5	2.3
1000	5	2.7
2000	5	2.24

from 2.24% for a concentration of 2000 ng of mitoxantrone per ml of extracted serum to 23% for 10 ng mitoxantrone per ml. When five additional samples were assayed for the low concentrations (5, 10 and 20 ng) the respective coefficients of variation were 11.9%, 14.6% and 8.5% (Table I).

The recovery of the sample clean-up was 62 ± 2% at concentrations of 100, 250, 500 or 1000 ng/ml, and 58 ± 7.6% at concentrations of 5, 10 or 20 ng/ml.

Fig. 4 shows the result of the determination of a urine sample. Note the absence of other peaks, except for the one before the internal standard which could represent a still unidentified metabolite. This chromatogram can only be measured at 658 nm; at 254 nm there are a lot of disturbing peaks.

Fig. 5 shows the various standard curves. All correlation coefficients were above 0.9995. Note the difference in slope between curve 4 (absolute values of mitoxantrone after extraction from plasma) and curve 5 (internal standard — corrected values); this is due to the recovery of the sample clean-up of 62%.

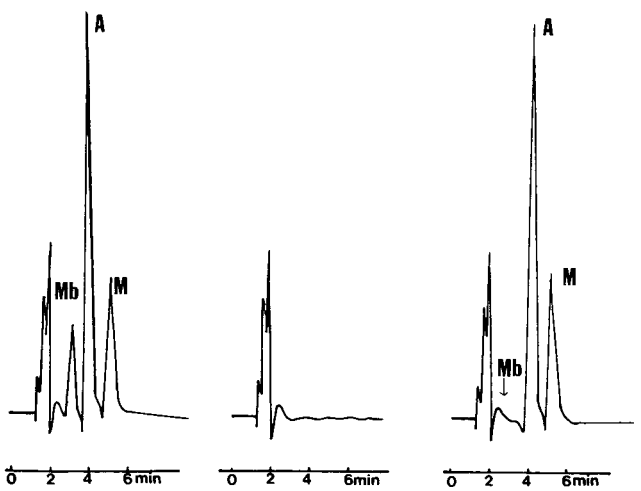


Fig. 4. Chromatograms of urine samples of a patient treated with mitoxantrone (15 mg/m² intravenous infusion over 30 min). The left chromatogram is of a sample of urine containing a metabolite of mitoxantrone (Mb), ametantrone (A) as internal standard and unchanged mitoxantrone (M). The centre chromatogram is representative of blank urine, and the right is of spiked urine containing ametantrone and mitoxantrone.

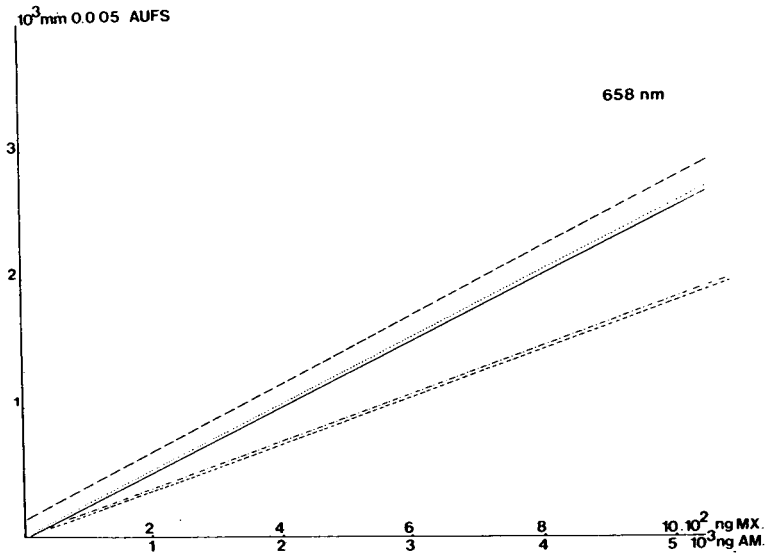


Fig. 5. Standard curves of mitoxantrone (MX) in aqueous solution (——), ametantrone (AM) in aqueous solution (- - - -), mitoxantrone extracted from aqueous solution (. . . . .) in absolute values and after plotting against the internal standard, mitoxantrone extracted from plasma in absolute values (— · — · — · —) and after plotting against the internal standard (— — — —).

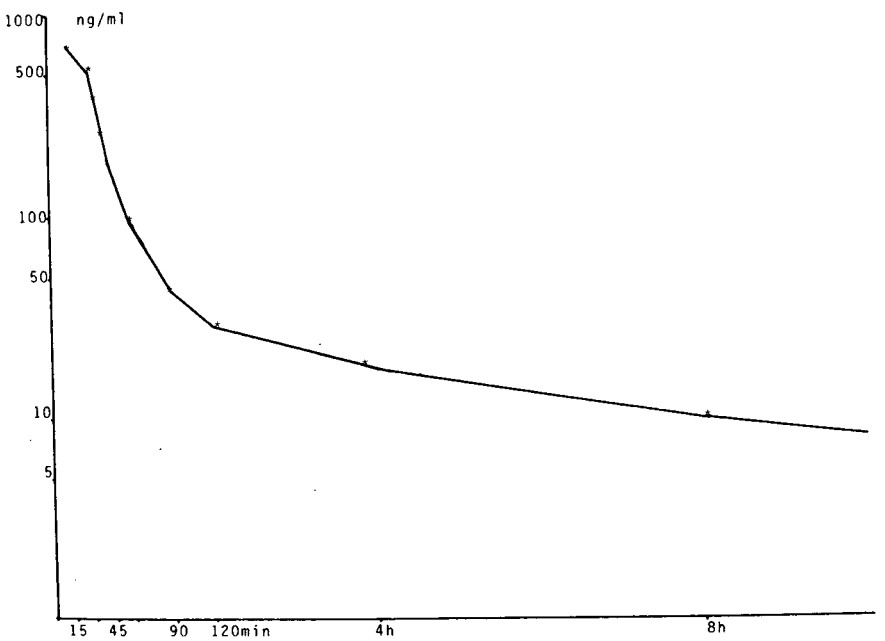


Fig. 6. Representative plasma elimination curve from twenty patients (mean values), treated with 15 mg/m^2 mitoxantrone, intravenously, over 30 min. Levels of 3—5 ng/ml plasma are still found 48 h to six days after the infusion.

After plotting against the internal standard, the slope of the curve is the same as that representing mitoxantrone in aqueous solution. Fig. 6 represents a typical plasma elimination curve, which shows the mean values of twenty patients treated with an infusion of 15 mg/m^2 mitoxantrone over 30 min. Mitoxantrone concentrations of 2—5 ng/ml of serum can still be detected after six days (not shown in curve).

DISCUSSION

We used ametantrone, another antracenedione derivative, as the internal standard. This method leads to more accuracy than the use of cresyl violet [14], because ametantrone is structurally related to mitoxantrone and the total elution time is less than 5 min. Ostroy and Gams [14] used cresyl violet as internal standard but the elution time is more than 50 min after mitoxantrone. Hulhoven and Desager [15] also used ametantrone as internal standard but their sensitivity level was not below 15 ng/ml. We could determine 1 ng of mitoxantrone per ml of plasma, even without using available electronic methods to magnify the recorded peaks.

On the other hand, Peng et al. [16] reached the same sensitivity level of 1 ng/ml but they used the external standard method which adds considerably to the cost [16]. Their system is based on the same constituents for the mobile phase: acetonitrile and an ammonium buffer. But when using an ammonium formate buffer with a pH of 2.7 instead of an ammonium acetate buffer with a pH of 4.0 we had better results. Reduction of pH from 4.0 to 2.7 gave better peaks, but adding hexane sulphonic acid to the mixture led to a spectacular increase in peak sharpness, and an increase in recovery of 15—20%.

We chose the wavelength of 658 nm to determine mitoxantrone for two major reasons. First, it is a very specific wavelength since commonly taken drugs do not absorb at 658 nm. Secondly, we could use a fixed-wavelength detector since there is a mercury-lamp filter available for the 658 nm wavelength. Other absorption peaks of the mitoxantrone spectrum are 254, 546 and 611 nm. The two cited wavelengths of 546 and 611 nm are somewhat less sensitive and a variable-wavelength detector is necessary to reach them.

The sensitivity at 254 nm and 658 nm is almost equal for mitoxantrone but differs by a factor of ten for ametantrone which has better absorption peaks at 254, 589 and 618 nm. Ascorbic acid was used by Peng et al. [16] to protect against degradation of mitoxantrone in solutions. We had an increase of about 10% in recovery when adding ascorbic acid to the sample before the clean-up procedure. On the other hand, adding it to the fresh sample, before freezing, made no difference, compared to samples directly frozen at —20° C. The whole sample clean-up procedure takes about 30 min.

There were no interfering peaks in the urine chromatogram, so no clean-up was used. The peak preceding the ametantrone peak (Fig. 4) can only be seen in urine from patients treated with mitoxantrone; it was absent in urine spiked with the internal standard or mitoxantrone and in blank urine. For these reasons we think it is a metabolite which we have not yet been able to identify. It must be a substance structurally related to mitoxantrone and ametantrone, since it also absorbs at 658 nm and it is localized near the peaks of these two anthracenedione derivatives.

Possible reasons why other authors until now could not visualize such a metabolite are the lack of a very sensitive HPLC method to separate the substances and possible loss or alteration during sample clean-up procedures.

The cumulative urinary excretion of unchanged mitoxantrone was about 6—12% after 48 h. The possible metabolite was seen only during the first 24 h. Along with the remaining plasma levels this suggests that mitoxantrone is probably stored in a third space from where it is afterwards slowly released. Some data suggest that it could be stored partly in red blood cells [17].

Assay of samples of haemolysed blood showed peak levels six to eight times higher than non-haemolysed samples (unpublished data).

In conclusion, we have developed an HPLC method and sample clean-up procedure based on the property of ion-pair formation by hexane sulphonic acid. It has allowed us to document the prolonged terminal half-life of mitoxantrone in plasma and we could also demonstrate a possible metabolite in urine. The sample clean-up method is inexpensive, fast and reliable. Another advantage is that the same technique can be used to determine the pharmacokinetics of ametantrone, using mitoxantrone as internal standard.

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CHROMBIO. 2351

DETERMINATION OF SERUM METHOTREXATE AND 7-HYDROXYMETHOTREXATE CONCENTRATIONS

METHOD EVALUATION SHOWING ADVANTAGES OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The use of large doses of methotrexate (MTX), > 3 g/m^2, for the treatment of some malignant disorders requires careful monitoring of serum concentrations. A simple and sensitive method for the separation of MTX and 7-hydroxymethotrexate (7-OH-MTX) by reversed-phase high-performance liquid chromatography (HPLC) is described. The method involves deproteinizing the serum sample on a Sep-Pak C_{18} cartridge, followed by separation on a C_{18} column and detection at 313 nm. The extraction efficiency of free MTX from serum is 70% and the maximum sensitivity is $2.2 \cdot 10^{-8}$ *M*. A high degree of correlation was obtained between the HPLC method of serum MTX determination and an enzyme multiplied immunoassay technique. The HPLC method separates MTX from its analogues, or drugs which may be administered concomitantly with MTX. Concentrations of MTX and 7-OH-MTX achieved over a 24-h period during high-dose therapy, (500—1000 mg/m^2), and over 48 h for very-high-dose methotrexate therapy (8—12 g/m^2) are described. A significant observation is the presence of 7-OH-MTX in sera of patients 6 h after commencement of infusion.

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This method was also utilized for monitoring cerebrospinal fluid MTX concentrations.

INTRODUCTION

Methotrexate (MTX), amethopterin, 4-amino-N^{10}-methylpteroylglutamic acid, is a folate antagonist which acts by inhibiting intracellular dihydrofolate reductase. The consequence of this inhibition is the depletion of intracellular stores of reduced folates required for the 'de novo' synthesis of purines and pyrimidines, and for the metabolism of amino acids.

In combination with other antineoplastic agents, MTX is frequently used for the treatment of acute lymphoblastic leukemia, lymphomas, osteogenic sarcoma, and other forms of cancer. When using high doses of MTX, delayed excretion may lead to toxicity and therefore careful monitoring of serum MTX is important for optimal patient management [1]. In addition, the measurement of its less soluble major metabolite, 7-hydroxymethotrexate (7-OH-MTX) may be important, as it has been implicated in the development of renal toxicity [2].

Several methods have been reported for measuring concentrations of MTX in plasma and serum. These include enzyme inhibition [3, 4], protein binding [5], radioimmunoassay (RIA) [6, 7], fluorescence [8, 9], and high-performance liquid chromatography (HPLC) [10—13]. Apart from the method of detection by HPLC, none of these techniques employ separation steps capable of resolving or quantitating 7-OH-MTX. In the present paper, we report a relatively simple and sensitive method of detecting MTX and 7-OH-MTX by HPLC in sera and cerebrospinal fluid (CSF) of children receiving high-dose and very-high-dose MTX therapy.

MATERIALS AND METHODS

Chemicals

The chemicals utilized in these studies were obtained from the following sources: MTX and folinic acid in clinical use from Lederle Labs. (Pearl River, NY, U.S.A.); 7-OH-MTX and N-{4-[[(2,4-diamino-6-quinazolinyl)methylamino]benzoyl]}aspartic acid were generous gifts from Dr. David Johns and Dr. Harry B. Wood, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD, U.S.A.); folic acid, prednisone and prednisolone from Protea (Sydney, Australia); 4-aminoacetophenone from E. Merck (Darmstadt, F.R.G.); trimethoprim from Wellcome Australia (Sydney, Australia); daunorubicin (cerubidin) from May and Baker (Footscray, Australia); purified MTX, cytosine arabinoside and dexamethasone from Sigma (St. Louis, MO, U.S.A.); *p*-aminobenzoic acid from Ajax (Sydney, Australia); HPLC-grade methanol and acetonitrile from Waters Assoc. (Milford, MA, U.S.A.); EMIT methotrexate assay kits from Syva (Palo Alto, CA, U.S.A.). All other chemicals and reagents were of analytical grade.

Chromatographic equipment

HPLC was performed using a Model ALCGPC 204 liquid chromatograph equipped with a U6K injector, a Model 6000A pump and a Model 440

absorbance detector fitted with a 313-nm filter. Chromatography was performed on a Radial-Pak C_{18} column (particle size 10 μm, 10 cm × 5 mm I.D.) fitted into a RCM-100 radial compression separation system. The above pieces of equipment were obtained from Waters Assoc.

Solvent system

The solvent system for column equilibration and drug elution consisted of 0.15 *M* ammonium phosphate buffer (pH 4.85) containing 11% (v/v) acetonitrile. Column elution was performed at room temperature using a flow-rate of 1 ml/min and a pressure of 3500 kPa.

Serum collection

Blood samples were obtained from patients undergoing MTX therapy as a part of the current treatment protocol being utilized in the Section of Paediatric Haematology and Oncology (Prince of Wales Children's Hospital, Sydney, Australia). High-dose methotrexate (HD-MTX), in a dosage of 500—1000 mg/m^2 (one-third of the dose administered in the first 0.5—1 h and the remainder over the next 23 h), was administered intravenously to children with acute lymphoblastic leukemia or lymphoma. Very-high-dose methotrexate (VHD-MTX), in a dosage of 8—12 g/m^2 was administered as an infusion over 4—6 h to children with osteogenic sarcoma. The blood samples were allowed to clot and the serum obtained by centrifugation at 2500 *g* for 15 min.

Sample preparation

Deproteinization of serum samples was performed by passage through a Sep-Pak cartridge (Waters Assoc.,) which is a miniature C_{18} column. This column was previously activated by washing with 10 ml of methanol followed by 10 ml of water. Serum (1 ml) was loaded on to the column and the eluent discarded. Subsequently, the column was washed with 5 ml of water to remove serum proteins and serum contaminants. The free MTX which remained bound to the column was then eluted with 3 ml of methanol. The methanol fraction was dried at 45°C under a jet of nitrogen, and then dissolved in 100 μl of methanol—water (20:80, v/v). This procedure concentrates the MTX present in 1 ml of serum by ten-fold. A 20-μl aliquot of this sample was injected into the chromatograph, and MTX concentrations were determined by measurement of peak heights on HPLC tracings. A standard curve was prepared daily, ranging from $0.11 \cdot 10^{-6}$ *M* to $11 \cdot 10^{-6}$ *M* MTX dissolved in normal drug-free serum. If concentrations in patient sera exceeded the range of the standard curve, they were diluted appropriately. Due to the commercial scarcity of pure 7-OH-MTX, multiple injections of 20 nmol of the pure compound was utilized as a reference standard.

Enzyme multiplied immunoassay technique (EMIT)

Serum MTX concentrations were measured using EMIT [14]. In this procedure, antibodies to MTX, glucose-6-phosphate and the co-enzyme, nicotinamide adenine dinucleotide (NAD), are pipetted into tubes containing sera (50 μl) obtained from patients undergoing either HD-, or VHD-MTX

therapy. Under these conditions, the MTX present in the sera would bind to the added MTX antibodies. In the second step of the assay, the enzyme glucose-6-phosphate dehydrogenase (G6PDH), coupled to MTX, is added to the tubes. The G6PDH—MTX complex binds to any free binding sites still available on the MTX antibodies and thereby becomes inactive in its ability to oxidize glucose-6-phosphate to 6-phosphoglucono-δ-lactone. Therefore a measure of the G6PDH reaction would be in direct proportion to the amount of MTX present in the serum.

$$\text{glucose-6-phosphate} \xrightleftharpoons[\text{NAD}\quad\text{NADH} + \text{H}^+]{\text{G6PDH}} \text{6-phosphoglucono-}\delta\text{-lactone}$$

The G6PDH reaction is determined in a spectrophotometer by measuring the conversion of NAD to NADH, at a wavelength of 340 nm, and at a temperature of 30°C. This method is used to measure MTX concentrations between $3 \cdot 10^{-7}$ and $3 \cdot 10^{-3}$ *M*. Interference from serum G6PDH activity is avoided by use of the co-enzyme NAD, which is active only with the bacterial enzyme (*Leuconostoc mesenteroides*) employed in the assay.

RESULTS

Extraction efficiency of free MTX from serum using the HPLC system

Table I shows that between 20% and 28% of the total MTX was washed off the Sep-Pak during the water wash. Since the binding of added MTX to serum proteins was not measured, we cannot state whether the loss of MTX in the water wash was due to the free MTX, or to protein-bound MTX. Theoretically, the protein-bound drug would not bind to the column and hence would be washed away. Between 67% and 72% of the total MTX was eluted with methanol. The remaining 4—10% was lost, either during serum elution or during the solubilisation procedure. The overall extraction efficiency of free MTX from serum was approx. 70%.

TABLE I

EXTRACTION OF FREE MTX FROM SERUM

Appropriate amounts of MTX (220, 22 or 2.2 nmol) were added to 1 ml of serum and the free MTX was separated and quatitated as described in Materials and methods. The results are expressed as a mean of three experiments.

Amount of MTX (nmol)	220	22	2.2
Amount lost in water wash	62.3	4.3	0.44
Amount eluted with methanol	148.1	16.0	1.54
Miscellaneous loss	9.6	1.7	0.22

Separation of MTX from 7-OH-MTX and other compounds

Fig. 1 shows chromatograms obtained from normal drug-free serum (a); serum spiked with MTX (b); serum spiked with both MTX and 7-OH-MTX (c); serum from a patient 24 h after HD-MTX treatment (d). The present procedure is capable of separating MTX from other contaminants in serum. The retention

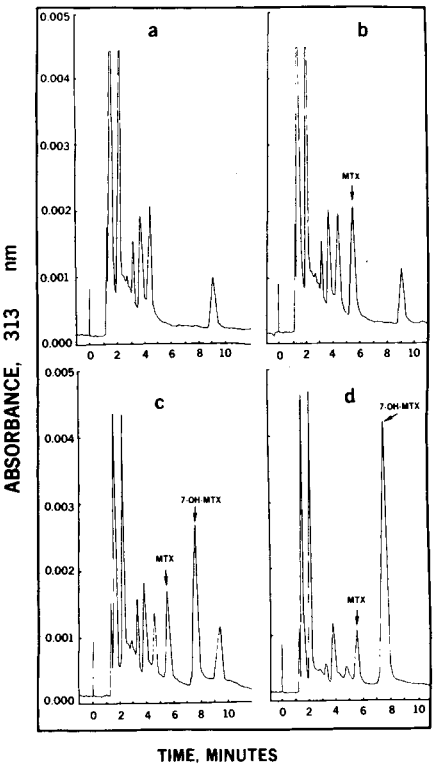

Fig. 1. Representative HPLC chromatograms of normal drug-free serum (a); serum spiked with MTX (b); serum spiked with both MTX and 7-OH-MTX (c); serum from a patient 24 h after HD-MTX treatment (d). Chromatography was performed on a Radial-Pak C_{18} column, as described in Materials and methods.

TABLE II

RETENTION TIMES OF VARIOUS DRUGS AND MTX ANALOGUES

Compound	Retention time (min)
MTX	5.4
7-OH-MTX	7.4
Folic acid	6.6
Folinic acid	2.1
N-{4-[[(2,4-Diamino-6-quinazolinyl)methylamino]-benzoyl]}aspartic acid	4.2
p-Aminoacetophenone	11.8
p-Aminobenzoic acid	3.7
Trimethoprim	16.0
Vincristine	13.6
Daunorubicin	NE*
Cystosine arabinoside	13.2
Dexamethasone	ND**
Prednisone	ND
Prednisolone	ND

*NE = Not eluted 30 min after injection.
**ND = Not detectable at 313 nm.

time of MTX (5.4 min) is sufficiently different from 7-OH-MTX (7.4 min) to be able to accurately quantitate each compound present in serum.

The limit of sensitivity was found to be 4.4 pmol of MTX injected onto the column in a total volume of 20 μl. As the MTX present in 1 ml of serum is concentrated ten-fold, the present method is capable of measuring concentrations of MTX down to $2.2 \cdot 10^{-8}$ *M*. The within-assay coefficient of variation (C.V.) for MTX assay was 1.69%, aand 3.69% for 7-OH-MTX ($n = 6$; 20 nmol). The day-to-day C.V. for MTX was 6.44%, and 7.34% for 7-OH-MTX ($n = 6$; 20 nmol). The C.V. value due to the usage of different Sep-Pak cartridges was investigated. A known concentration of MTX (20 nmol) was injected into a

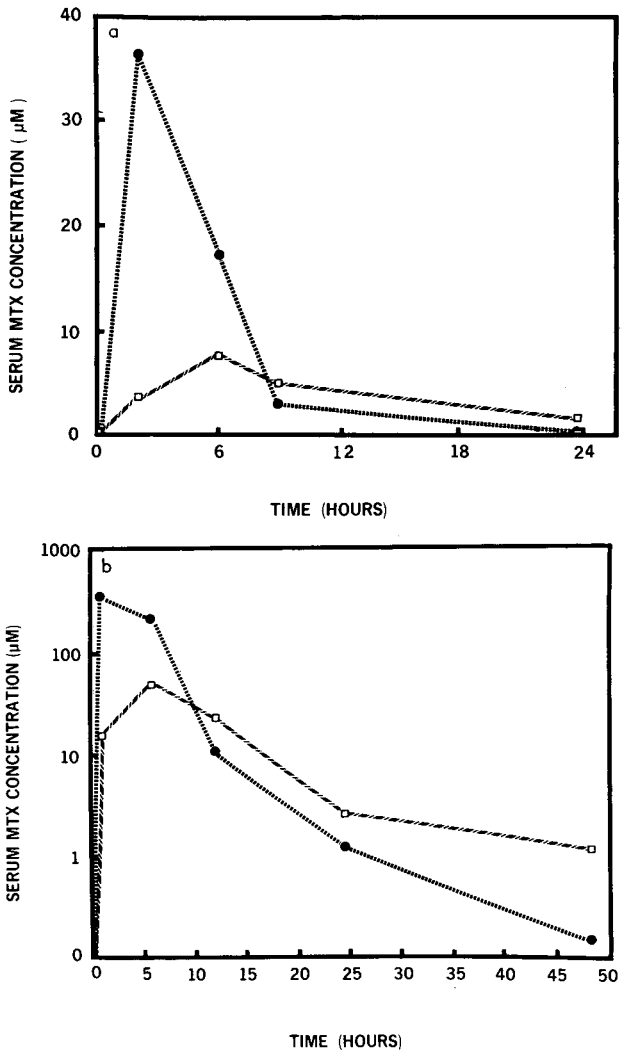

Fig. 2. MTX and 7-OH-MTX concentrations in sera of a patient at selected time intervals following infusion with (a) HD-MTX, 500—1000 mg/m^2; or (b) VHD-MTX, 8—12 g/m^2. The MTX (•) and the 7-OH-MTX (▫) concentrations were determined using HPLC as described in Materials and methods.

series of Sep-Pak cartridges and the concentration of eluted drug monitored. The C.V. was found to be 3% (n = 6). This was confirmed by using the radioactive labelled MTX and monitoring the eluted counts.

Table II lists several drugs which may be administered concomitantly with MTX, or compounds with structural similarities to MTX. The steroids do not absorb at 313 nm. Each of the other drugs listed has a distinct retention time to clearly separate it from MTX or 7-OH-MTX.

Concentrations of MTX and 7-OH-MTX in patients

The serum MTX concentration in our patients (Fig. 2 and Table III) were similar to those in other published results [4, 10] and will be the subject of a separate paper [15]. Depending on the actual dosage and body surface area, the peak concentrations achieved in the high-dose regimens ranged from $5 \cdot 10^{-6}$ to $40 \cdot 10^{-6}$ M, and in the very-high-dose regimens these concentrations ranged from $200 \cdot 10^{-6}$ to $600 \cdot 10^{-6}$ M.

TABLE III

SERUM CONCENTRATIONS OF MTX AND 7-OH-MTX IN PATIENTS

MTX and 7-OH-MTX concentrations were measured in the sera of patients at selected time intervals following infusion with HD-MTX, or VHD-MTX.

Patient	Dose*	Time (h)	MTX concentration (μM)	7-OH-MTX concentration (μM)
1	HD	3	8.7	ND**
		6	8.7	ND
		11	6.2	ND
		24	1.1	ND
2	HD	3	37.0	3.5
		6	14.0	7.3
		9	3.2	4.6
		24	0.18	1.0
3	VHD	6	434.0	92.0
		12	16.0	21.0
		24	1.1	2.5
		48	0.19	0.6
4	VHD	6	230.0	106.0
		12	18.0	40.0
		24	1.1	6.5
		48	0.23	2.5
5	VHD	1	550.0	22.0
		6	323.0	70.0
		12	14.0	32.0
		24	1.7	3.7
		48	0.21	1.5

*HD = high-dose treatment, 500—1000 mg/m^2; VHD = Very-high-dose treatment, 8—12 g/m^2.

**ND = not determined.

The administered MTX is metabolised to 7-OH-MTX in vivo, with peak concentrations appearing 6 h after commencement of infusion (Fig. 2 and Table III). The peak concentrations of 7-OH-MTX observed in patients receiving VHD-MTX therapy is high (approx. $70 \cdot 10^{-6}$ *M*). This is a direct consequence of the massive doses of MTX utilized. The effect of such concentrations of 7-OH-MTX on the entry of MTX into the cell, and its effect on the cell remain to be clarified. However, the present experiments do indicate that the excretion rate of 7-OH-MTX is slower than MTX (Fig. 2). This observation would be particularly important with regard to the known low solubility of the drug [2].

The CSF concentrations of MTX 3 h after commencement of high-dose infusion are in the order of 10^{-6} to 10^{-7} *M*. In exceptional cases CSF concentrations of $3 \cdot 10^{-6}$ to $4 \cdot 10^{-6}$ *M* have been found.

Comparison between HPLC and EMIT for determination of MTX concentrations in serum

Serum MTX concentrations were determined concurrently using EMIT and HPLC. Linear regression analysis of the data in Fig. 3 shows a very high degree of correlation ($r = 0.97$, $p < 0.001$) between the two procedures. The gradient of the graph being close to unity ($m = 0.99$) suggests that the two methods are furnishing similar values for the MTX concentrations.

The cross-reactivity of 7-OH-MTX binding site on the MTX antibody was investigated using EMIT. A known concentration of 7-OH-MTX ($2.2 \cdot 10^{-6}$ *M*) was incubated with the MTX antibody. The MTX concentration determined

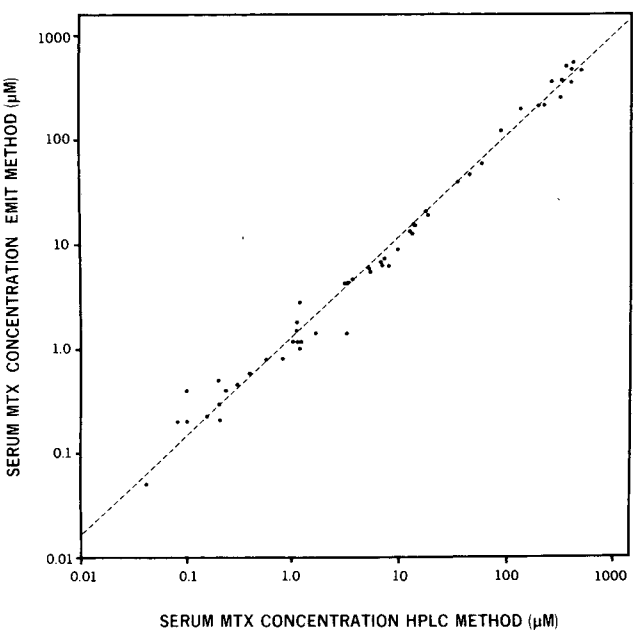

Fig. 3. Comparison between HPLC and EMIT for determination of MTX concentrations in serum. MTX concentrations were determined by HPLC and EMIT in sera obtained from patients at selected time intervals following either HD-, or VHD-MTX infusion ($r = 0.97$, $p < 0.001$).

was $0.08 \cdot 10^{-6}$ *M*, i.e. a cross-reactivity of 4%. This shows that 7-OH-MTX present in sera does not bind to the MTX antibody, and therefore does not influence the levels of MTX determined in the sera by EMIT.

DISCUSSION

Measurement of MTX concentrations in serum by methods such as enzyme inhibition [3, 4], protein binding [5], RIA [6, 7] and fluorescence [8, 9] are useful for clinical investigations, but are of limited value for metabolic studies. Use of HPLC allows for the measurement of metabolites such as 7-OH-MTX [10—13]. In patients receiving HD-MTX therapy, 7-OH-MTX is present in significant quantities. However, the biochemical and clinical importance of this compound still needs to be clarified.

With HPLC, serum samples have to be deproteinized prior to injection onto a column. When methods such as organic extraction [10], ammonium sulphate precipitation [13], and acid precipitation [11, 12] are used, deproteinization can be laborious and time-consuming. The utilization of Sep-Pak C_{18} cartridges for deproteinization allows for the removal of serum proteins and other polar metabolites in a single step. The MTX and 7-OH-MTX which are retained on the column can be concentrated from 2—5 ml of serum, allowing low levels of the drug and its metabolites to be measured.

In our method, the overall efficiency of free MTX extraction from serum is 70% (Table I). This compares favourably with other reported values which range from 46% to 82% [10, 11, 13]. The limit of sensitivity is $2.2 \cdot 10^{-8}$ *M*. This is similar to the value of $1 \cdot 10^{-8}$ to $2 \cdot 10^{-8}$ *M* reported by Canfell and Sadée [10], and is ten-fold better than other reports [11, 13]. The increased sensitivity can be attributed to the ten-fold concentrating step achieved by using Sep-Pak, the use of acetonitrile rather than methanol in the elution buffer, and the increased absorbance of MTX at 313 nm in comparison to 254 nm (the absorbance maximum of methotrexate is 305 nm).

We have measured MTX concentrations in serum using RIA [16], EMIT and HPLC, using both the radial compression module (RCM) and stainless-steel columns. They all furnish similar results and each method has particular advantages and disadvantages. The EMIT and RIA methods appear to be suited to clinical laboratories dealing with large numbers of samples. The HPLC method appears to be better suited to research laboratories. We found the RCM module particularly attractive as compared with stainless-steel columns; the cartridges are cheaper, easier to attach, and easier to store without any apparent loss in efficiency. A particular disadvantage of the RIA and EMIT methods is that the kits are quite expensive and have expiry dates for radioisotopes, enzymes and antibodies. This can result in significant waste when only small numbers of samples are being processed.

We believe HPLC provides a method for measuring MTX and its metabolites, which is technically straightforward, economical, but with a high degree of specificity and sensitivity. In addition the present method saves time, requiring less than 15 min to process each sample.

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Note

High-performance liquid chromatographic analysis of bile acids in hamster bile

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During the course of studies on biliary lithiasis in the golden hamster, we analysed the pattern of conjugated bile acids present in the bile of these animals. The golden hamster has been used as an experimental model in this type of study [1, 2] and the pattern of conjugated bile acids found in the hamster was compared with that of human bile [3, 4]. Although the analysis of the bile acid composition of bile has been achieved with several analytical techniques, high-performance liquid chromatography (HPLC) is clearly the method of choice [5—13], especially since fluorometric detection methods have been introduced [14—18]. When gas chromatography was used for the analysis of the bile acid composition of hamsters the glyco and tauro derivatives could not be identified or determined, and the results obtained always refer to cholic, deoxycholic, chenodeoxycholic, ursodeoxycholic and lithocholic acids.

In the present report we describe the separation of ten conjugated bile acids found in hamster bile which are identical to those found in human bile.

MATERIALS AND METHODS

Reagents

Methanol and acetonitrile were HPLC grade (E. Merck, Darmstadt, F.R.G.). Glass-bidistilled water was used in all the experiments. All other reagents used were HPLC grade (Fisher Scientific, New Jersey, U.S.A.). Solvents were filtered through a 0.45-μm Millipore membrane and degassed.

The sodium salts of taurocholic acid (TCA), taurochenodeoxycholic acid

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(TCDCA), taurodeoxycholic acid (TDCA), taurolithocholic acid (TLCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA) and glycolithocholic acid (GLCA) were obtained from Sigma (St. Louis, MO, U.S.A.). The sodium salts of tauroursodeoxycholic acid (TUDCA) and glycoursodeoxycholic acid (GUDCA) were a gift from Tokyo Tanabe (Tokyo, Japan). Dexamethasone (Sigma) was used as internal standard. Sep-Pak C_{18} cartridges (Waters Assoc., Milford, MA, U.S.A.) were employed to purify the bile samples.

Animals

Golden hamsters (Centro Panamericano de Zoonosis, Buenos Aires, Argentina) were used. Six females and six males, each weighing 90 ± 10 g were kept in individual cages with water and standard roden Chow (Purina Labina, Buenos Aires) during 40 days. They were weighed twice a week. The room temperature was 24°C and the lights were turned off from 8 p.m. to 8 a.m. Hamsters were fasted for 16 h before being killed. They were in good health at the time of death.

Equipment

A liquid chromatograph Varian Model 5020 (Palo Alto, CA, U.S.A.) equipped with a spectrophotometric detector (Vari-chrom VUV 10 Varian), a recorder (Model 9176, Varian), and a data processor (CDS-111 L Chromatography Data System, Varian) were used. A Micropack MCH-5 column (300 mm × 4 mm I.D., particle size 5 μm) was employed.

Operating conditions

The mobile phase consisted of two solvents: solvent A was 0.3% ammonium dihydrogen phosphate, pH 7.5, and solvent B was acetonitrile. Gradient elution profile: solvent B increased from 28% to 35% during the first 19 min and then to 40% during the next 11 min. Flow-rate: 0.8 ml/min. Temperature: 32°C. Injection volume: 10 μl. Detection was performed at 210 nm and 0.05 a.u.f.s.

Sample preparation

The animals were killed by a blow on the head between 9 a.m. and 11 a.m., and the gallbladders were removed.

A normal saline solution (50 μl) was injected into each gallbladder and 130—230 μl of bile were aspirated. The bile samples were kept at —20°C and processed as soon as possible. A 100-μl volume of bile was diluted with 2 ml of 0.5 *M* phosphate buffer (pH 7.0) and this solution was passed through a Sep-Pak C_{18} cartridge (2 drops per sec) previously washed with 2 ml of methanol and 5 ml of water. The cartridges were then washed with 8 ml of water, 2 ml of 1.5% ethanol and finally with 4 ml of methanol which eluted the bile acids. The methanolic solution was evaporated to dryness in vacuo at 40°C. The residue was dissolved in 1 ml of methanol with 100 μg of internal standard (dexamethasone) and filtered through a 0.2-μm Sartorius membrane filter before being injected into the chromatograph.

RESULTS

Quantitative analysis of bile acids in hamster bile

A reference methanolic solution of ten standard conjugated bile acids containing 140—200 μg/ml of each one and 100 μg/ml of internal standard were chromatographed according to the operating conditions (Fig. 1). The

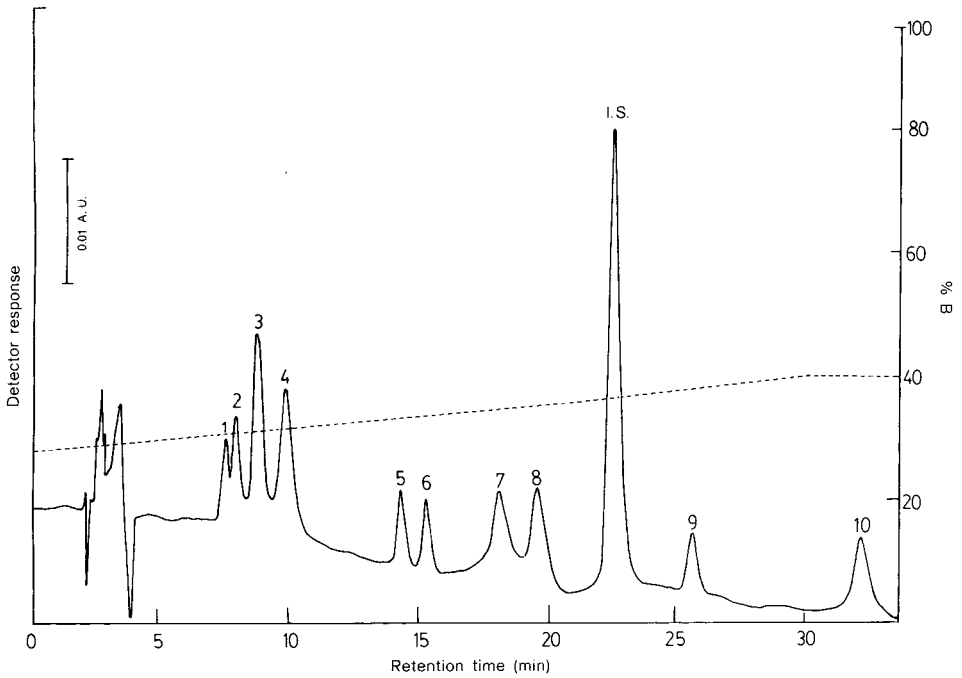

Fig. 1. Chromatogram of a reference solution of conjugated bile acids. Mobile phase: solvent A = 0.3% ammonium dihydrogen phosphate (pH 7.5), solvent B = acetonitrile. Peaks: 1 = TUDCA; 2 = TCA; 3 = GUDCA; 4 = GCA; 5 = TCDCA; 6 = TDCA; 7 = GCDCA; 8 = GDCA; 9 = TLCA; 10 = GLCA; I.S. = dexamethasone.

TABLE I

RELATIVE RETENTION TIMES OF CONJUGATED BILE ACIDS REFERRED TO TAURODEOXYCHOLIC ACID

Bile acid	Relative retention time
Tauroursodeoxycholic acid	0.49
Taurocholic acid	0.52
Glycoursodeoxycholic acid	0.57
Glycocholic acid	0.64
Taurochenodeoxycholic acid	0.94
Taurodeoxycholic acid	1.00
Glycochenodeoxycholic acid	1.19
Glycodeoxycholic acid	1.29
Taurolithocholic acid	1.68
Glycolithocholic acid	2.10
Dexamethasone (internal standard)	1.48

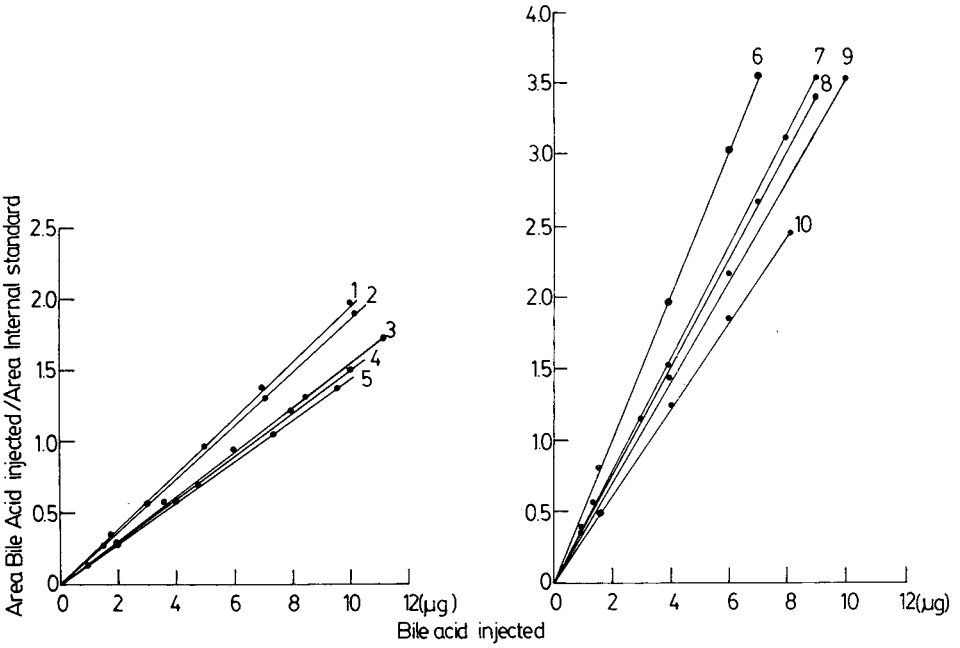

Fig. 2. Calibration curves for conjugated bile acids with dexamethasone as internal standard. Correlation coefficients of linear regression ranged between 0.996 and 0.999. 1, TUDCA; 2, TDCA; 3, TLCA; 4, TCA; 5, TCDCA; 6, GUDCA; 7, GLCA; 8, GCA; 9, GCDCA; 10, GDCA.

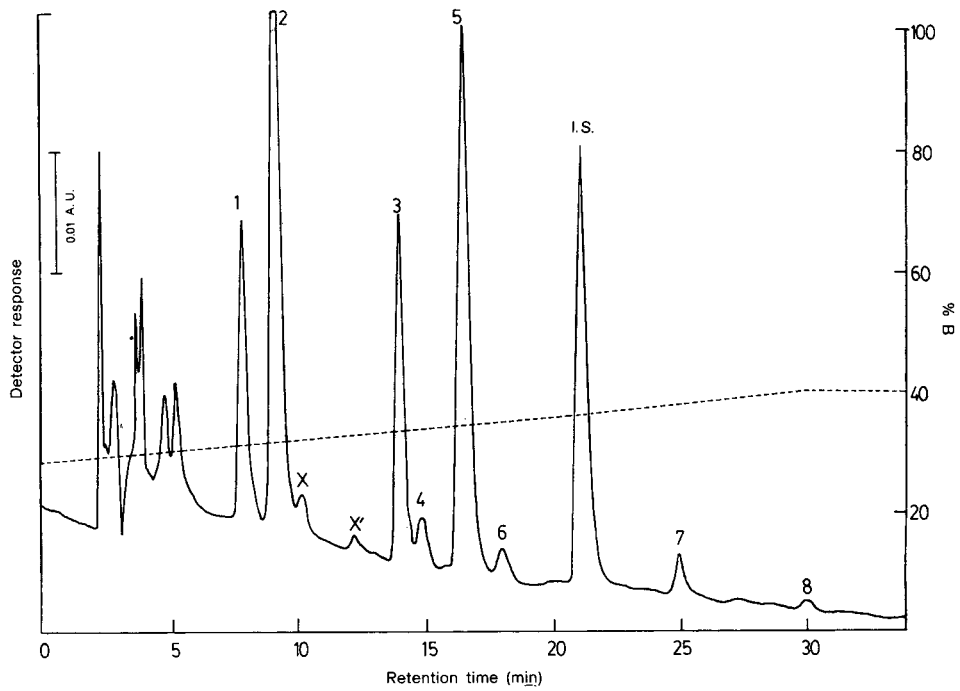

Fig. 3. Bile acid composition of hamster bile. Mobile phase: solvent A = 0.3% ammonium dihydrogen phosphate (pH 7.5), solvent B = acetonitrile. Peaks: 1 = TCA; 2 = GCA; 3 = TCDCA; 4 = TDCA; 5 = GCDCA; 6 = GDCA; 7 = TLCA; 8 = GLCA; X, X' = unknown; I.S. = dexamethasone.

TABLE II

BILE ACID COMPOSITION IN BILE OF GOLDEN HAMSTERS

Sample	Sex*	Percentage of total bile acids										TBA (mg/ml)
		TCA	GCA	TCDCA	GCDCA	TDCA	GDCA	TLCA	GLCA	TCBA	GCBA	
1	M	13.4	19.9	21.2	36.6	6.2	2.7	trace	—	40.8	59.2	9.7
2	M	28.9	16.1	24.2	22.9	5.2	0.6	1.3	0.8	59.6	40.4	21.1
3	M	18.7	22.9	25.5	26.2	3.2	1.0	2.5	trace	49.9	50.1	28.5
4	M	15.5	23.2	26.1	28.0	4.2	3.0	trace	—	45.8	54.2	27.0
5	M	10.3	10.5	35.7	36.0	2.6	2.1	trace	2.8	48.6	51.4	9.4
6	M	20.5	20.1	26.8	19.7	9.5	2.4	0.4	0.6	57.2	42.8	24.6
7	F	4.2	28.8	22.4	44.6	trace	trace	—	—	26.6	73.4	12.0
8	F	14.4	18.2	32.0	28.1	4.4	0.4	2.2	0.3	53.0	47.0	27.6
9	F	5.2	20.3	31.3	42.5	trace	0.7	trace	—	36.5	63.5	17.5
10	F	8.3	29.2	21.1	32.3	5.8	3.3	trace	—	35.2	64.8	14.4
11	F	6.3	28.7	33.7	30.4	trace	0.9	trace	—	40.0	60.0	11.8
12	F	20.3	16.8	41.6	19.9	trace	trace	1.4	trace	63.3	36.7	17.4

*M = male; F = female.

TABLE III

COMPARISON OF ANALYTICAL RESULTS FOR BILE ACIDS IN HAMSTER BILE OBTAINED BY HPLC WITH SEP-PAK C_{18} CARTRIDGES AND WITH ETHANOL IN THE CLEAN-UP OF THE SAMPLE

Sample		Concentration (mg/ml)							
		TCA	GCA	TCDCA	GCDCA	TDCA	GDCA	TLCA	GLCA
Bile A	Sep-Pak C_{18}	3.98	5.01	8.82	7.74	1.20	0.12	0.61	0.090
	Ethanol	4.12	5.25	8.87	7.92	1.24	0.14	0.60	0.088
Bile B	Sep-Pak C_{18}	1.20	4.20	3.04	4.64	0.85	0.47	Trace	—
	Ethanol	1.05	4.06	2.95	4.55	0.98	0.41	Trace	—

TABLE IV

REPRODUCIBILITY OF DETERMINATION OF BILE ACIDS IN HAMSTER BILE BY HPLC

$n = 5$.

Bile acid	Mean ± S.D. (mg/ml)
TCA	3.25 ± 0.11
GCA	4.63 ± 0.10
TCDCA	5.12 ± 0.09
GCDCA	6.01 ± 0.09
TDCA	1.12 ± 0.07
GDCA	0.63 ± 0.05
TLCA	1.05 ± 0.06

relative retention times are shown in Table I. A linear calibration response to each bile acid in the range of 0.3—10 μg is shown in Fig. 2. The detection limits found were in the range of 75 ng for GUDCA to 200 ng for GDCA and 250 ng for TUDCA to 350 ng for TCDCA. Twelve hamster bile samples were chromatographed according to the procedure previously described (Fig. 3). The results are summarized in Table II. Similar HPLC bile acid patterns were obtained when the bile samples were deproteinized with ethanol, centrifuged at 1000 *g* for 5 min, washed and dried in vacuo at 40°C. There was a good correlation between the results obtained with these samples and the samples analysed

directly as described above (Table III). In order to examine the recovery of bile acids known quantities of standard bile salts were added to a hamster bile sample, before the purification step with Sep-Pak C_{18} cartridge. The recovery values were 90% for GCA, 92% for GCDCA and 99% for TCA. The reproducibility of the analysis was tested on a hamster bile pool for $n = 5$ with standard deviations of 1.5—7.9% (Table IV).

DISCUSSION

Reversed-phase HPLC performed on individual samples of hamster bile allowed the separation of glyco and tauro conjugates of bile acids in 30 min using dexamethasone as internal standard. This separation could not be achieved with an isocratic system [13], but with the gradient system reported in this paper good reproducibility and recovery values were obtained. The use of the Sep-Pak C_{18} cartridge allowed direct clean-up of the sample without previous deproteinization. The reported HPLC separations of conjugated bile acids required longer elution times [8, 10—12], more elaboration [7, 9, 12, 19, 20], or had less sensitivity [5—7]. More elaborate techniques require a more complicated sample purification which increases analysis time and costs [14—18]. Our method is well adapted to the small amount of bile present in the hamster.

Loss of resolution of the column was observed with time of use, but the column could be regenerated by washing first with methanol—water (70:30), then with tetrahydrofuran, chloroform, tetrahydrofuran and finally with methanol—water (70:30).

The composition of hamster bile showed appreciable variations among the samples examined. The conjugation ratio with glycine and taurine showed great variability (Table II). TUDCA was not detected in the bile samples and GUDCA was identified in two samples in trace amounts. In hamster bile a major proportion of GCDCA and TCDCA was observed compared to GCA and TCA. TDCA and GDCA were present in low proportions. These results are similar to those reported in human bile by other authors [12, 19, 20] using HPLC.

Two unknowns were present in most of the chromatograms (Fig. 3). These peaks could be related to bile acid conjugated forms reported by Bergman et al. [21]. Work is in progress to identify them.

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Note

Quantification of branched-chain α-keto acids as quinoxalinols: importance of excluding oxygen during derivatization

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The quantitative analysis of branched-chain α-keto acids (BCKA), the first intermediates in the oxidation of the essential amino acids L-leucine, L-isoleucine and L-valine in mammals, is of considerable widespread interest [1—17]. Several methods have been developed for this using gas chromatography (GC) [1—8], high-performance liquid chromatography (HPLC) [9—12], or enzymatic determination [13]. The last is rapid and sensitive but does not distinguish between α-keto-β-methylvalerate, α-ketoisocaproate and α-ketoisovalerate. In our experience the HPLC method of Hayashi and co-workers [9, 10] with fluorescence detection is highly sensitive and has a single derivatization procedure yielding stable quinoxalinols. Quinoxalinol formation is also frequently used with GC methods of BCKA analysis prior to a secondary derivatization with bis(trimethylsilyl)acetamide (BSTFA) to more volatile products [1, 2, 4, 6—8].

Over a period of several weeks we obtained standard curves using procedures described by Hayashi et al. [9] but observed variations in the ratio of the peak areas for each BCKA to that for the internal standard, α-ketooctanoic acid. The presence of oxygen during derivatization was found to have a considerable effect and this report demonstrates that exclusion of oxygen improves the reliability of the derivatization procedure and gives a significant increase in the sensitivity of the method for α-ketoisovaleric acid.

EXPERIMENTAL

Preparation of quinoxalinols

Sodium salts of D,L-α-keto-β-methylvaleric (KMV), α-ketoisocaproic (KIC) and α-ketoisovaleric (KIV) acids and the free α-ketooctanoic acid (KOA)

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(Sigma, London, U.K.) were dissolved together in water each at a concentration of 50 nmol ml^{-1} and frozen at −20°C. Solutions of *o*-phenylenediamine were prepared daily by dissolving 40 mg of the dihydrochloride (Sigma) and 100 μl of mercaptoethanol in 20 ml of 2.0 *M* hydrochloric acid. Nitrogen (oxygen-free) and oxygen (containing 5% carbon dioxide) were from BOC. The α-keto-acid solution (0.5 ml) and *o*-phenylenediamine solution (2.0 ml) were mixed in 160 × 16 mm screw-capped Sovirel tubes and heated at 80°C for 2 h. The tubes were cooled with a large volume of water at approx. 15°C. Saturated sodium sulphate solution (4 ml) and ethyl acetate (5 ml) were added and the quinoxalinols extracted into the upper, organic phase by shaking for 5 min. Almost all the upper phase was pipetted onto anhydrous sodium sulphate (approx. 100 mg) for drying at 1—4°C overnight before being evaporated to dryness. For chromatography, the residue was dissolved in dimethylformamide (40 μl) and water (100 μl) and 20—40 μl aliquots were injected onto the column.

The BCKA were analysed after derivatization under five different oxidation conditions: experiment A: mixture under oxygen and without mercapto-ethanol; experiment B: mixture under oxygen with mercaptoethanol; experiment C: mixture under air with mercaptoethanol (i.e. as in ref. 9); experiment D: mixture under nitrogen with mercaptoethanol; and experiment E: mixture under nitrogen with mercaptoethanol and 1—2 mg of sodium dithionite added 3—5 sec before the end of gassing with nitrogen. The dithionite produced some turbidity but this did not interfere with the chromatography.

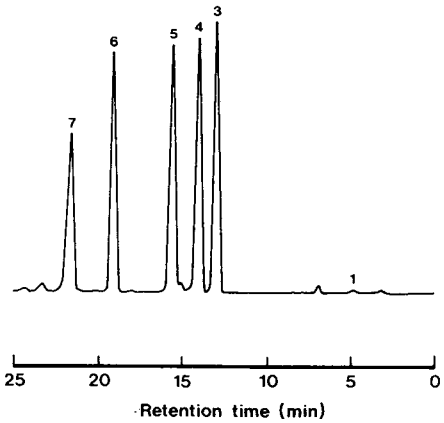
Chromatography

The apparatus comprised a Model 3B pump, an LC-100 oven at 50°C, a Rheodyne 7125 valve injector (100-μl loop) and a Model 3000 fluorescence spectrophotometer fitted with a 16-μl flow cell (all Perkin-Elmer). Excitation and emission wavelengths were set at 322 and 391 nm, respectively. An on-line degasser (Erma Optical Works, Model 3310) was used to reduce possible quenching of fluorescence by oxygen dissolved in the mobile phase. The column (250 mm × 4.6 mm I.D.) was stainless steel packed with 5-μm LiChrosorb RP-8 bonded silica (HPLC Technology). A silica pre-column was used to enrich the mobile phase with silica.

The mobile phase system of Hayashi et al. [9] was used with omission of the ion-pairing reagent and consisted of acetonitrile—water (4:1, solution A) and acetonitrile—water—0.1 *M* sodium dihydrogen phosphate—sodium hydroxide buffer, pH 7.0 (1:12:7, solution B). A linear gradient of 30—80% A was run over 30 min, at a flow-rate of 1.5 ml min^{-1}.

RESULTS AND DISCUSSION

A typical separation of BCKA quinoxalinols, prepared in the absence of oxygen (experiment E) is shown in Fig. 1. This contrasts with Fig. 2 which shows their separation when prepared under oxygen (experiment A) and also shows an almost complete loss of the KIV peak and a considerable increase in the size of the KIC peak. The reason for the increase is not known although the formation of a co-eluting species when oxygen is not excluded, seems possible.



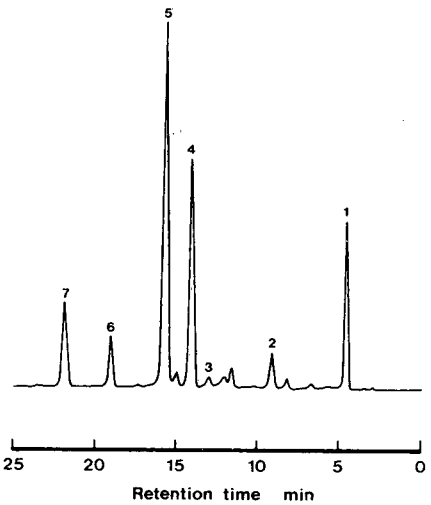


Fig. 1. HPLC chromatogram of the α-keto acids derivatized in the absence of oxygen (experiment E). Peaks: 3 = KIV; 4 = KMV; 5 = KIC; 7 = KOA; 1 and 6 are unidentified.

Fig. 2. HPLC chromatogram of the α-keto acids derivatized in the presence of oxygen (experiment A). Peaks: 3 = KIV; 4 = KMV; 5 = KIC; 7 = KOA; 1, 2 and 6 are unidentified.

The presence of oxygen produced little change in the peak area of the KMV derivative but a reduction in the peak area for the derivative of the internal standard, KOA. Peaks labelled 1 and 2 appeared only when oxygen was present during derivatization. The nature of these components was not investigated but the appearance of peak 1 was a sensitive indication of the presence of oxygen during derivatization of the standard mixture of BCKA (this indication does not neccessarily apply to some biological samples).

The effects of decreasing the extent of the oxidizing conditions on the peak area ratios are shown in Fig. 3. All three BCKA derivatives had similar peak area ratios when prepared under nitrogen and in the presence of mercaptoethanol and sodium dithionite. With increasing oxygenation of the reaction mixture the peak area ratios showed increasing divergence. Variations between peak area ratios from sample to sample were generally smallest in the absence of oxygen. Some of the variability was probably due to the inclusion of varying amounts of oxygen in the different tubes in each experiment since the volume of the Sovirel tubes used for derivatization differed from one tube to another, even though tubes measuring 160 × 16 mm were always used. Gassing with nitrogen alone was not always sufficient to remove all the oxygen as judged by the appearance of a small peak at position 1 in some instances, and divergence of the peak area ratios from closely similar values. A separate experiment had been performed similar to experiment D but with gassing with nitrogen for a prolonged time (10 min, approximately twenty times longer). This served to remove all effective oxygen as indicated by only very small peaks at position 1 on the chromatograms of four replicates. In view of the volatility of the α-keto acids at room temperature under the acidic conditions present during the gassing stage, prolonged gassing would not seem advisable.

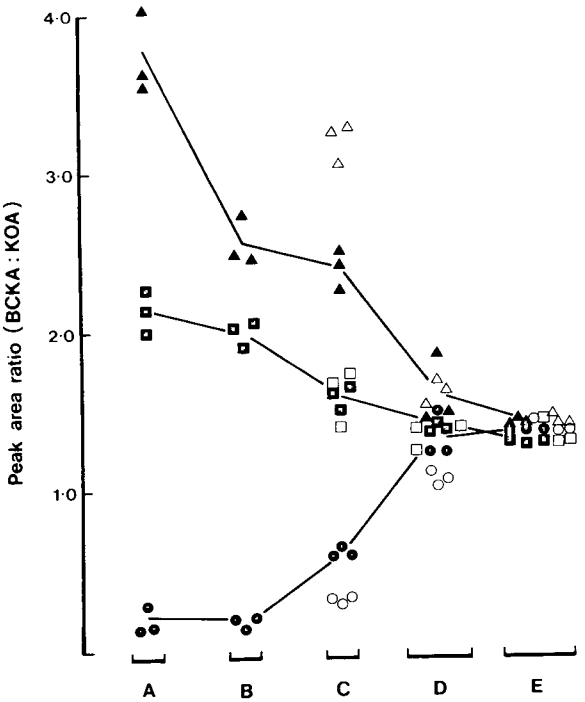


Fig. 3. Effects of progressively decreasing the extent of the oxidizing condition during derivatization on the peak area ratios for BCKA:KOA. Results are for experiments A (most oxidizing) to E (least oxidizing) (see Experimental). Symbols represent: (△, ▲) KIC; (□, ■) KMV; (○, ●) KIV. Closed symbols are for experiments and replicates for which derivatives were prepared simultanenously and open symbols represent experiments conducted on separate days but replicates derivatized simultaneously in each experiment. The lines join points which are the mean values of the data represented by the closed symbols.

Browning of the reaction mixture occurred when oxygen was present during derivatization. A colourless solution did not, however, indicate adequate exclusion of oxygen, since some colourless solutions of derivatives (mostly from experiment D and some from experiment C) showed sizeable peaks at position 1 on the chromatograms, divergence of the peak area ratios from similar values and diminution of the peak area for the KIV derivative, all indicating some effective oxygen had been present. We have no evidence of a mechanism by which oxygen interferes in the derivatization and oxidation of precursors or products would be presumptive.

It is interesting to determine whether the presence of oxygen has interfered with the analysis of BCKA in other published work. We have no indication from the literature of attempts to exclude oxygen from the derivatization mixture although it is possible that mercaptoethanol has been used [9, 10] to reduce auto-oxidation of the *o*-phenylenediamine [18]. The stoppered tube as described by Hayashi et al. [9] for derivatization contains a large air space above the derivatization mixture suggesting oxidation was possible. Some indication of the effect the contained oxygen might have had can be gained by an examination of the pattern of the relative peak area ratios. The peak area

ratio for the KMV derivative is least affected by oxygen during derivatization; if the peak area ratio for this keto-acid is then taken as unity, the relative peak area ratios for the KIC, KMV and KIV derivatives (calculated from ref. 10) are found to be approx. 2.5, 1.0, 0.75, respectively. These values correspond to 1.5, 1.0 and 0.4, respectively in the present study for samples derivatized under air. Rather similar relative peak area ratios are obtained using GC methods when *o*-phenylenediamine is used as the primary derivatizing reagent: from the data of Woolf et al. [8] calculations show relative values of 1.3, 1.0 and 0.8 and from that of Cree et al. [2] relative values of 1.2, 1.0 and 0.7. As KIC and KMV are isomeric, similar responses would be expected, particularly in GC methods where a flame-ionization detector is used. Variations in the relative peak area ratios between different laboratories will reflect differences in instrumentation as well as the extent of interference by oxygen during derivatization. However, the similarity in the pattern of relative peak area ratios suggests that interference by oxygen during the derivatization step may be a common but previously unrecognized problem.

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Note

Separation of cadaverine from putrescine, histamine and polyamines in rat kidney by phosphocellulose chromatography

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The aliphatic diamine cadaverine (1,5-diaminopentane) is a chemical analogue of putrescine (1,4-diaminobutane) and, like putrescine, the biosynthesis of cadaverine appears to be maximal during periods of rapid tissue proliferation [1].

For several years, numerous analytical methods have been devised for the separation and determination of the biogenic di- and polyamines [2]. Among these, high-performance liquid chromatography (HPLC) coupled with fluorometric detection presents strong sensitivity and good specificity as an analytical method for di- and polyamines [3, 4]. However, the superb resolution of HPLC must be offset, in part, by the inherent disadvantages including expensive equipment and limited loading capacity.

This paper describes a rapid and simple method of separating cadaverine from putrescine and other polyamines using phosphocellulose column chromatography. The applicability of the method is demonstrated on the kidney of a castrated rat treated with an anabolic steroid.

MATERIALS AND METHODS

Chemical reagents

Phosphocellulose was obtained from Brown (Berlin, NH, U.S.A.) (Lot No. M3G8190, capacity 1.04 mequiv./g). Fluorescamine [4-phenylspirofluran-2(3H),1′-phthalan-3,3′-dione, Lot No. 23F-0249] was obtained from Sigma (St. Louis, MO, U.S.A.). Durabolin (nandrolone phenpropionate, Lot No. 130755) was from Organon Teknika (Oss, The Netherlands). *o*-Phthalaldehyde

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(OPA, Lot No. M2M1045) was from Nakarai (Kyoto, Japan). All other reagents used were reagent grade and obtained from Wako (Tokyo, Japan).

Animal experiment and sample preparation

Male Wistar rats, weighing about 200 g and obtained from Shizuoka Lab-Animal Center (Shizuoka, Japan) were used. Castration was performed one week before the actual experiment. Durabolin, an anabolic steroid, was suspended in olive oil and injected subcutaneously 5 mg per 100 g daily for three days as reported previously [5]. Controls were given vehicle only. After the last injection, the experimental animals were sacrificed at different intervals and their kidneys were removed immediately and frozen on dry-ice. After homogenization in 5 vols. of ice-cold 0.4 *M* perchloric acid, the kidneys were centrifuged at 1600 *g* for 5 min at 4° C and the supernatants were neutralized to pH 5—6 with ice-cold potassium hydroxide. After neutralization the sample was centrifuged again and the supernatant was used for further analysis.

Column chromatography on phosphocellulose

The sample (0.5 ml of the above final supernatant) was applied to a phosphocellulose column (0.6 X 3.5 cm) equilibrated with phosphate buffer pH 6.2. First the column was washed stepwise with 1 ml of 0.01 *M* phosphate buffer (pH 6.2), 5 ml of 0.05 *M* phosphate buffer (pH 6.2) and 12 ml of 0.02 *M* borate buffer (pH 8). The diamines and polyamines were then eluted also in a stepwise manner: 6 ml of 0.1 *M* borate buffer (pH 8) were needed to elute histamine, 5 ml of 0.1 *M* borate buffer, pH 8, containing 0.015 *M* sodium chloride were needed for cadaverine, 9 ml of 0.1 *M* borate buffer, pH 8, containing 0.1 *M* sodium chloride for putrescamine, 3 ml of 0.2 *M* borate buffer, pH 8, containing 0.1 *M* sodium chloride for a contaminating compound (unknown), 9 ml of 0.2 *M* borate buffer, pH 8, containing 0.3 *M* sodium chloride for spermidine, and 9 ml of 0.2 *M* borate buffer, pH 8, containing 0.6 *M* sodium chloride for spermine. The mobile phase temperature was always maintained above 20° C. The content of putrescine, cadaverine, spermidine and spermine in the respective fractions was determined fluorometrically with fluorescamine using Endo's method [6]; the histamine fraction was quantitated using the method of Shore et al. [7]. All fluorescence measurements were made with a Shimadzu spectrofluorophotometer RF-500 (Shimadzu, Kyoto, Japan).

Recovery

To estimate the recoveries of biogenic amines, the tissue homogenate was divided into two fractions. Known amounts of the respective amines were added to the first fraction while the other fraction was used as control. The recoveries were determined by subtracting the values obtained for control. The recovery experiment was performed with a healthy kidney with quite satisfactory results as shown in Table I.

High-performance liquid chromatography

For the purpose of ascertaining the results, diamines and polyamines in the same samples were determined using an HPLC system. An aliquot of the final

TABLE I

PRECISION OF RECOVERY OF BIOGENIC AMINES USING THE EXTERNAL STANDARD METHOD

Amine standards were added to kidney homogenates prior to preparation of sample and separation.

Biogenic amine	Amount added (nmol)	Recovery (%) (Mean ± S.D., $n = 6$)
Histamine	1	90.6 ± 6.99
Cadaverine	10	92.8 ± 3.72
Putrescine	10	91.9 ± 7.25
Spermidine	50	91.7 ± 8.50
Spermine	50	92.0 ± 4.04

supernatant (0.5 ml) was applied to a phosphocellulose column (0.6 × 3.5 cm) equilibrated with pH 6.2 phosphate buffer. The column was washed in the same stepwise manner as described above. Then histamine was eluted by 6 ml of 0.1 *M* borate buffer (pH 8) and other amines were eluted with 12 ml of 0.3 *M* borate buffer (pH 9). The histamine fraction was reacted with OPA according to the method of Shore et al. [7] and was injected into the HPLC system. The fraction containing the other amines was reduced to 1 ml and reacted with Dns chloride [8]. The Dns amines were extracted with benzene and evaporated to dryness. The residue was dissolved in acetonitrile and injected into the HPLC system. A Shimadzu LC-3A liquid chromatograph equipped with a spectrofluorometer system (Shimadzu RF-530) was used for this purpose. The column was μBondapak C_{18} (Waters Assoc., Milford, MA, U.S.A.) and the mobile phase acetonitrile—water (70:30, v/v). The spectrofluorometer was set at 358/446 nm for histamine estimation and at 360/510 nm for the estimation of other amines.

RESULTS AND DISCUSSION

Fig. 1 shows the elution patterns of authentic amines. Several techniques have been described for the separation of cadaverine, putrescine and histamine [2]. In most cases, however, expensive equipment such as an HPLC system or amino acid analyser have been adopted. Low-pressure liquid chromatography uses simpler equipment and is cheaper to use than the previous systems; unfortunately it is not possible to separate cadaverine from putrescine in this way [6, 9, 10]. In the present study, cadaverine was separated clearly from other diamines, histamine and putrescine, using the phosphocellulose method. Our application of phosphocellulose to the analysis of cadaverine and other amines is based on the earlier work of others [6, 9, 10]. The resolution of cadaverine, putrescine and histamine was optimized by altering the ionic strength of the eluting buffers. For the fluorometric estimation, histamine was treated with OPA while other amines were treated with fluorescamine. The fluorescamine reaction is more sensitive, simple and gives a more stable

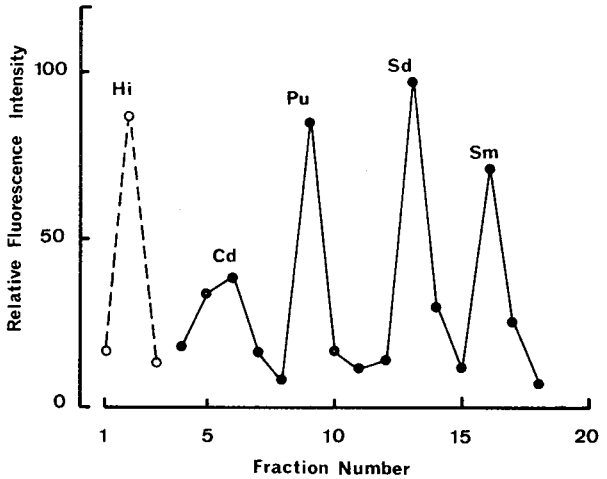

Fig. 1. Elution patterns of authentic diamines and polyamines. A standard solution (1 ml) containing histamine (Hi, 1 nmol), cadaverine (Cd, 10 nmol), putrescine (Pu, 10 nmol), spermidine (Sd, 50 nmol) and spermine (Sm, 50 nmol) was applied to the phosphocellulose column. Elution and estimation were carried out as described under Materials and methods; 3 ml of each fraction (except histamine and cadaverine fractions) were used for estimation. For histamine 2 ml were collected, for cadaverine 1 ml, and each made up to 3 ml with buffer and estimated. Control fluorescence intensities in the OPA and fluorescamine reactions were 50 for 0.5 nmol of histamine and 50 for 5 nmol of putrescine.

product than other methods [6]. Unknown amines, however, eluted near the region where histamine appears. Since OPA is known to react with histamine selectively and sensitively [7], this means of derivatization was used to avoid contamination of histamine with unknown amines. These considerations about the most suitable derivatives hold, however, for the stepwise elution during the phosphocellulose method. With HPLC, Dns derivatives of all polyamines (except histamine) are preferred [2].

Table II shows a comparison between the concentrations of diamines obtained by the phosphocellulose method and the HPLC method. The agreement between the two methods was acceptable with the sample set checked.

TABLE II

COMPARISON BETWEEN DIAMINE CONCENTRATIONS IN CASTRATED RAT KIDNEY UNDER THE INFLUENCE OF ANABOLIC STEROID AS DETERMINED BY THE PHOSPHOCELLULOSE AND HPLC METHODS

Animals received anabolic steroid subcutaneously (5 mg per 100 g, every day) for three days; 24 h after the last injection, they were sacrificed. Concentrations are expressed as nmol/g wet weight of tissue.

Biogenic diamine	Phosphocellulose	HPLC
Histamine	1.60 ± 0.15	1.68 ± 0.28
Cadaverine	8.74 ± 2.71	7.68 ± 1.60
Putrescine	54.62 ± 10.24	59.30 ± 22.14
Spermidine	655.6 ± 43.95	643.5 ± 33.99

The retention times with the phosphocellulose method were about 120 min, while with the HPLC method they were about 45 min; however, more than 30 samples could be separated at the same time by the phosphocellulose method using several columns side by side. Thus, the phosphocellulose method described here represents a very rapid and inexpensive procedure for separating the diamines and polyamines in biological samples.

Fig. 2 shows a typical chromatographic pattern of endogenous diamines and polyamines in the kidney of a castrated rat treated with an anabolic steroid, and indicates the presence of cadaverine in this sample. Henningsson et al. [5] reported that an anabolic steroid induced cadaverine synthesis in castrated

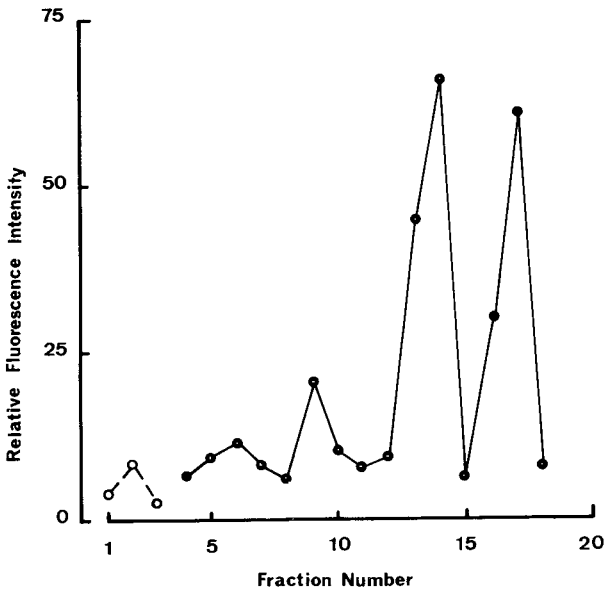

Fig. 2. Separation of endogenous diamines and polyamines in castrated rat kidney under the influence of anabolic steroid for three days. The separation and estimation procedures were described in Materials and methods.

TABLE III

RELATIONSHIP BETWEEN THE BIOGENIC AMINE CONCENTRATIONS IN CASTRATED RAT KIDNEY AND TIME AFTER THE LAST STEROID INJECTION

Concentrations are expressed as nmol/g wet weight of tissue. Each value is the mean ± S.D. of six or eight samples.

Time (h)	Histamine	Cadaverine	Putrescine	Spermidine	Spermine
0	1.06 ± 0.33	3.32 ± 1.16	41.7 ± 10.82	485.4 ± 48.17	681.9 ± 74.63
2	1.14 ± 0.20	5.71 ± 1.02*	47.8 ± 4.02	481.3 ± 41.12	719.4 ± 38.52
4	1.20 ± 0.11	5.46 ± 4.17	53.9 ± 4.71	509.3 ± 27.76	766.3 ± 17.98*
6	1.61 ± 0.35*	9.62 ± 4.36*	64.7 ± 6.82*	553.8 ± 28.60*	761.7 ± 19.56*
12	1.10 ± 0.16	7.84 ± 5.23	57.6 ± 5.00*	512.8 ± 20.78	696.6 ± 28.65

*Statistically significant difference between the mean of 0 h and experimental values, $p < 0.05$.

TABLE IV

CONCENTRATIONS OF DIAMINES AND POLYAMINES IN RAT KIDNEY AND DOSE OF ANABOLIC STEROID

Animals received anabolic steroid or vehicle every day for three days; 24 h after the last injection, they were sacrificed. Each value is the mean ± S.D. of six or seven rats. Concentrations are expressed as nmol/g wet weight of tissue.

Drug dose (mg per 100 g)	Histamine	Cadaverine	Putrescine	Spermidine	Spermine
Normal rats					
0	1.32 ± 0.15	2.57 ± 2.82	48.99 ± 4.15	617.8 ± 27.12	777.5 ± 56.61
2.5	1.12 ± 0.31	3.69 ± 3.70	41.85 ± 13.18	592.6 ± 19.36**	705.0 ± 45.47**
5	1.20 ± 0.29	5.93 ± 3.92	49.67 ± 6.97	614.8 ± 26.78	713.7 ± 57.56**
10	1.02 ± 0.11*	6.78 ± 5.42	47.41 ± 5.18	599.2 ± 27.09	701.8 ± 58.51**
Castrated rats					
0	1.30 ± 0.26	1.49 ± 1.86	43.99 ± 3.44	581.3 ± 43.44	729.6 ± 60.36
2.5	1.16 ± 0.29	6.12 ± 3.38*	48.11 ± 5.47	559.7 ± 56.84	756.7 ± 44.01
5	1.16 ± 0.15*	8.74 ± 2.71*	54.62 ± 10.24*	655.6 ± 43.95*	768.0 ± 122.22
10	0.94 ± 0.15*	8.94 ± 1.81*	49.27 ± 6.11**	617.9 ± 35.02	766.6 ± 55.41

*$p < 0.05$, ** $p < 0.1$: statistically significant difference between the means of control and steroid-treated rats.

mouse kidney. The same phenomenon was observed in rat kidney. Table III shows the relationship between the content of renal polyamines and diamines and their time dependency after steroid injection. Six hours after the last injection, all the amines reached their maximum and decreased from then on. Cadaverine increased markedly. Table IV shows the relationship between the content of renal amines and the drug dose. The renal diamines and polyamines increased dose-dependently, and reached their maximum when 5 mg per 100 g were injected.

It is well known that anabolic steroids induce the rapid growth of kidneys of castrated animals. Salzman and Stepita-Klauco [1] suggested that the synthesis of cadaverine appears to be maximal during periods of rapid tissue proliferation and the major physiological role of cadaverine could be an endogenous modulator of polyamine metabolism. If this speculation is true, cadaverine must play an important role in mammalian cell proliferation. The present method appears to be a good technique for studying the role of diamines and polyamines in cell physiology.

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CHROMBIO. 2355

Note

Development of a system to simplify identification of peptides and their catabolites in a high-performance liquid chromatographic effluent

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(First received February 1st, 1984; revised manuscript received August 22nd, 1984)

Increasing usage of peptides as drugs has focused attention on the in vivo fate of these labile compounds. Both endo- and exopeptidases operate on peptides to produce numerous catabolites. Frequently, the spectroscopic properties of these catabolites are similar to each other as well as to the parent peptide, making identification after high-performance liquid chromatographic (HPLC) separation difficult. If, in the course of analyzing a series of samples, the retention time of a component changes, identification of this compound may be speculative.

In response to these problems, a new configuration of equipment and software has been developed. Samples containing radioactive peptide and resulting catabolites are co-chromatographed with a mixture of unlabeled standards. The HPLC effluent is passed sequentially through a spectrophotometer and a radioactive flow monitor. Data are gathered by each instrument and transferred to a microcomputer, where elution profiles are constructed and superimposed. Superimposition of the radioactivity and absorbance data facilitate identification of catabolites in a complex mixture.

MATERIALS AND METHODS

Equipment

Components of the HPLC system are: high-performance liquid chromato-

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graph (Model 110A, Beckman Instruments, Palo Alto, CA, U.S.A.), spectrophotometer (Model 8450A, Hewlett-Packard, Palo Alto, CA, U.S.A.) [1], radioactive flow monitor (TRACE 7140, United Technologies, Chicago, IL, U.S.A.), micro-computer (Model 85, Hewlett-Packard), plotter (Model 7225B, Hewlett-Packard).

System design

The system is arranged as shown in Fig. 1. Effluent from the HPLC column passes through the spectrophotometer and radioactive flow monitor sequentially. This arrangement, rather than the reverse, was chosen to minimize band spreading caused by the larger volume of the flow cell in the radioactivity monitor (160 μl versus 8 μl). Two serial interfaces connect the spectrophotometer and flow monitor to the computer.

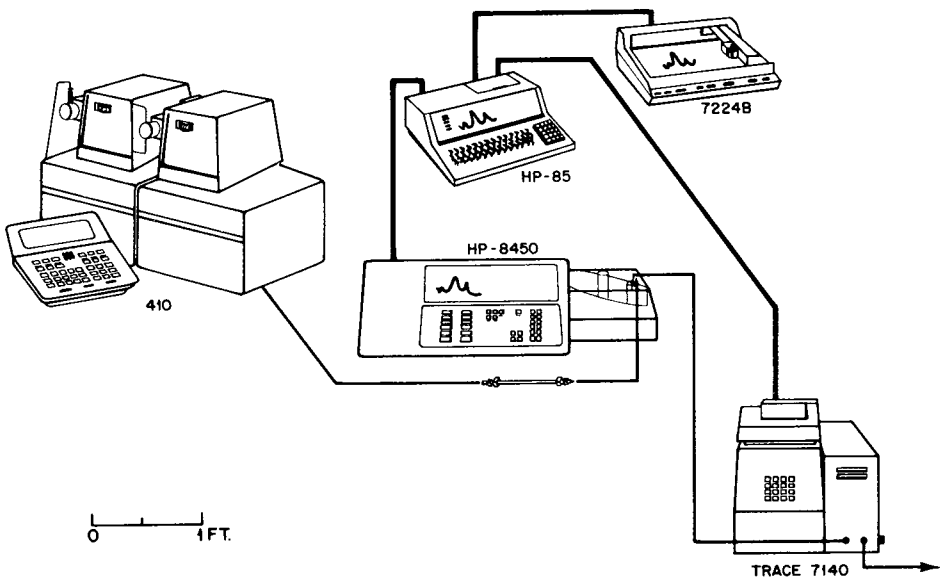

Fig. 1. Arrangement of the HPLC system which measures both absorbance (200—800 nm) and radioactivity of a column effluent and constructs superimposed elution profiles.

Programs

A series of menu-driven, linked programs converts the radioactivity and ultraviolet (UV) absorbance data into comparable elution profiles. Programs are MASTER, DATAQ, and GRAPH*. MASTER establishes the desired parameters, DATAQ controls the actual run, and GRAPH plots acquired data. The programs are written in HP BASIC and occupy 16 kilobytes of memory.

MASTER is an interactive program which prompts the operator to choose data collection start and finish times, frequency of radioactivity and absorbance readings, type of radioactivity to be monitored, spectral range, reference wavelengths, and number of wavelengths monitored. These variables

*Either HP-85 tapes containing the programs or program listings are available from the authors.

are printed and may be stored for subsequent runs involving the same parameters. MASTER provides the user with detailed operational instructions and checks all entered parameters for compatability.

DATAQ obtains the parameters designated in MASTER and allows for repeated background checks on both the radioactive flow monitor and the spectrophotometer. While running, DATAQ plots either absorbance or radioactivity versus time on the cathode-ray tube and stores both data types. Upon completion of data acquisition, DATAQ insures that all apparatus are turned off.

GRAPH gathers the stored data collected by DATAQ, displays data parameters available for plotting, and allows for alteration of plotting parameters such as run time and maximum ordinate. The finished plot includes radioactivity data, absorbance up to five wavelengths, and the eluent gradient. GRAPH accommodates a variable dead volume for determination of the effluent gradient and radioactivity/absorbance lag-times.

RESULTS

An HPLC system, in which both the absorbance and radioactivity (^{3}H, ^{14}C, etc.) of a column effluent are measured, the elution profiles constructed, superimposed and plotted, has been developed to simplify identification of labeled catabolites by co-chromatography with unlabeled standards. The program, MASTER, is written in BASIC and initiates interfacing between a computer, spectrophotometer, and radioactivity flow monitor. MASTER establishes and stores run parameters and then chains the program DATAQ

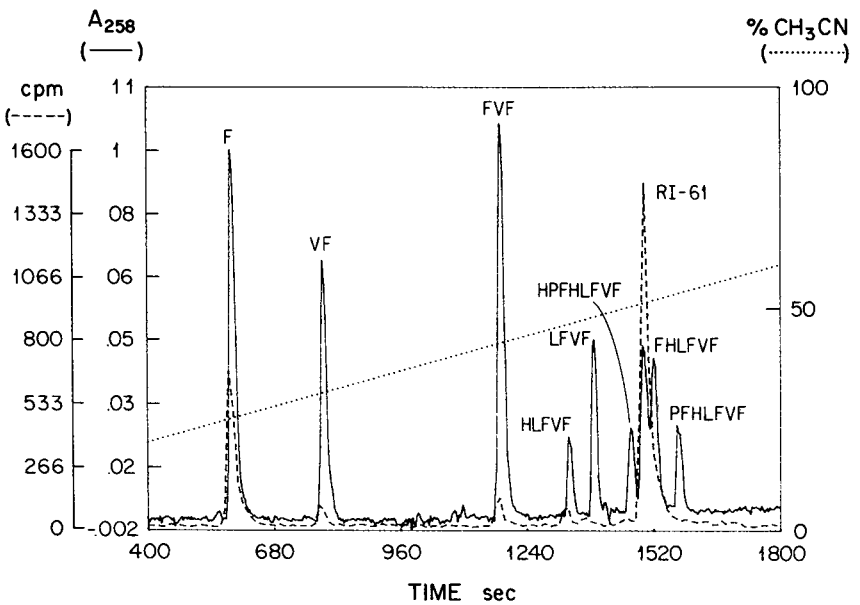

Fig. 2. Co-chromatography of partly catabolized RI-61 (Pro-His-Pro-Phe-His-Leu-Phe-Val-[^{3}H]Phe, 30 Ci/mol) with unlabeled catabolites that could arise from cleavage of the peptide chain.

to acquire data. DATAQ reads the desired values, checks for baseline consistency on the spectrophotometer and radioactivity flow monitor, gathers and stores the specified data, and plots either radioactivity or absorbance on the computer's cathode-ray tube during chromatography. The program GRAPH interfaces the computer and plotter to superimpose and plot the data along with the eluent gradient of the HPLC effluent.

An example of HPLC in which the UV absorbance (258 nm) of unlabeled peptides is used to identify radioactive catabolites is plotted in Fig. 2. The labeled peptide renin inhibitor RI-61 (Pro-His-Pro-Phe-His-Leu-Phe-Val-[^{3}H]Phe) [2] was exposed to the mucosal side of rabbit jejunum for 15 min [3]. Unlabeled RI-61 and the eight possible labeled catabolites which can arise by cleavage of the peptide chain have been co-chromatographed with the labeled inhibitor (100 Ci/mol). As can be seen, inactivation of the renin inhibitor occurs largely by removal of the C-terminal phenylalanyl residue, although small quantities of other cleavage products are also observed. Detailed catabolic patterns for the renin inhibitor in the presence of various inhibitors have been determined and will be reported elsewhere [4].

With minor changes in the interfacing portions, these programs can be adapted to function in any system able to communicate in BASIC.

ACKNOWLEDGEMENT

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CHROMBIO. 2340

Note

Clinical analysis of steroids

XXXI*. Assay of oestradiol 17-sulphate 4-hydroxylase activity by high-performance liquid chromatography with electrochemical detection

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Since the discovery of 2-methoxyoestrone [1, 2], the main metabolism of oestrogens has long been recognized as the introduction of an oxygen function at the C-2 position to produce 2-hydroxyoestrogens. Later, a similar biological hydroxylation was found to occur also at the C-4 position, by which another type of catechol, 4-hydroxyoestrogens, are formed [3]. These catechol oestrogens are now being widely investigated in numerous laboratories, because they are not just inactive metabolites but act physiologically as neurotransmitters [4—6].

Previously, we demonstrated the sulphate-specific, and sex-dependent 2-hydroxylation of oestradiol 17-sulphate (E-17-S) by rat liver microsomes with an NADPH-generating system [7]. Namely, liver microsomes from male rats converted E-17-S to 2-hydroxyoestradiol 17-sulphate (2-OH-E-17-S) as the sole product. In contrast, no such regulating effect was observed in the microsomes from female rats, and multiple kinds of hydroxylated products including a 2-hydroxylated metabolite were formed. Recently, this catechol formation from E-17-S was found to be not so specific because another type of catechol, 4-hydroxyoestradiol 17-sulphate (4-OH-E-17-S), was produced, although in extremely small amounts [8].

In a recent paper of this series, we reported an assay method for E-17-S 2-hydroxylase employing high-performance liquid chromatography (HPLC) [9]. Application of this HPLC method to induction studies has revealed that the 2-hydroxylation of E-17-S by rat liver microsomes is induced by

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cytochrome P-450 inducer [10]. Whether such an induction could occur also for the 4-hydroxylation is an interesting problem to be investigated. For this purpose, it is necessary to establish an assay method for E-17-S 4-hydroxylase.

The present paper deals with an assay method for E-17-S 4-hydroxylase activity by HPLC with electrochemical detection (ED).

EXPERIMENTAL

Materials

E-17-S [11], 2-OH-E-17-S [12], 4-OH-E-17-S [8], and 2-methoxyoestradiol 17-sulphate [13], were prepared in this laboratory according to the known methods. Glucose 6-phosphate (G-6-P), NADP, and G-6-P dehydrogenase were obtained from Oriental Yeast (Osaka, Japan). Sep-Pak C_{18} cartridges and column guards were obtained from Waters Assoc. (Milford, MA, U.S.A.) and Millipore (Bedford, MA, U.S.A.), respectively. All other reagents and solvents used were purchased commercially. Methanol and water used for elution of steroid conjugates through cartridges were bubbled with nitrogen gas to remove any dissolved oxygen, followed by addition of ascorbic acid (5 mg in each 100 ml).

High-performance liquid chromatography

HPLC was carried out using a Model 803 chromatograph equipped with an EC-8 electrochemical detector (Toyo Soda, Tokyo, Japan) at 0.8 V versus the Ag/AgCl reference electrode. A stainless-steel column (25 cm × 4.6 mm I.D.) packed with TSK-Gel ODS-120 A (5 μm) (Toyo Soda) was used and maintained at 40° C in a circulating water bath. A mixture of 0.5% ammonium dihydrogen phosphate (pH 3.0) and methanol (60:40, v/v) was used as mobile phase. The flow-rate of the mobile phase was 1.0 ml/min and the column pressure 130 kg/cm^2.

Animals

Wistar rats, ranging in age from 58 ± 7 days after birth for male (230—280 g) to 62 ± 7 days after birth for female (190—220 g) were used. All the animals were starved for 18 h prior to decapitation.

Preparation of microsomes

Rat liver microsomes were prepared by the method described previously [7, 8, 10]. Microsomal protein was determined by the method of Lowry et al. [14] using bovine serum albumin as reference standard.

Assay procedure

The standard incubation was carried out using the following conditions. Ice-cold reaction vessels contained microsomal protein (0.5 ml), an NADPH-generating system (NADP, 0.5 m*M*; G-6-P, 5 m*M*; magnesium chloride, 5 m*M*; G-6-P dehydrogenase, 0.6 U/ml; potassium chloride, 90 m*M*; EDTA, 0.1 m*M*; and E-17-S, 0—400 μ*M*). The mixture was diluted with Tris—HCl buffer (pH 7.4, 50 m*M*) to 3.0 ml as a final volume and was incubated for 0—60 min at 37° C under aerobic conditions. The reaction was terminated by heating the

incubation vessels in boiling water for 1 min, followed by addition of ascorbic acid (5 mg) as antioxidant and a known amount of 2-methoxyoestradiol 17-sulphate as internal standard (1.0—10 μg), and finally was diluted with 10 ml of water. For the control experiment, the incubation was performed using boiled microsomes (100° C for 1 min) using the same procedure as described above.

The incubation mixtures were centrifuged at 1500 *g* for 20 min, and the precipitates were suspended in water and again centrifuged. The combined supernatants were passed through Sep-Pak C_{18} cartridges. After washing with 2.0 ml of water, the conjugated fractions were obtained by elution with methanol (4.0 ml). The eluates were passed through column guards and the methanolic filtrates were evaporated under a nitrogen stream at 40° C to give the residues, which were subjected to HPLC as methanolic solutions. The enzyme activity of 2-hydroxylase was determined by a slight modification of the method described previously [9].

RESULTS AND DISCUSSION

The methanolic eluates of the incubation mixture passed through Sep-Pak C_{18} cartridges were subjected to HPLC, the results of which are compared with the chromatogram of authentic specimens in Fig. 1. Three peaks (1, 2 and 3) in both sexes are coincident with those of the authentic conjugates, E-17-S, 2-OH-E-17-S and 4-OH-E-17-S, respectively. A group of peaks (4) composed of three kinds of peaks appeared when female rats were used, and they were identified as E-17-S metabolites hydroxylated at C-6β, C-7β and C-16α positions [7].

As described previously [7, 8], these hydroxylations were found to proceed without removal of the conjugated group at C-17 of the substrate used. Since control experiments using boiled microsomes showed no product formation, these hydroxylations should occur enzymatically.

Because 2-OH-E-17-S was obtained in a fairly large amount and could be measured by ultraviolet detection as well as the more sensitive ED, the assay method for E-17-S 2-hydroxylase was established without difficulty [9]. The amount of 4-OH-E-17-S formed from E-17-S, on the other hand, was extremely small. The minimum amount of 4-OH-E-17-S produced in a series of kinetic studies was about 5 ng per incubation sample, and one-tenth of these samples was injected for HPLC. The amount of the catechol injected is, thus, nearly equal to the detection limit of 4-OH-E-17-S by ED (0.2 ng, signal-to-noise ratio = 3.5 at 1 nA full scale) (Fig. 1d). Therefore, ED is absolutely necessary for performing enzymatic studies on E-17-S 4-hydroxylase.

Since peak 3 was confirmed to be composed solely of 4-OH-E-17-S [8], development of a method for quantification of this catechol by determining the peak height became possible. The calibration curve for 4-OH-E-17-S was constructed by plotting the peak height of the material to that of the internal standard against the amount of the former, and a satisfactory linearity was observed in the range of 1—100 ng of the catechol.

In order to confirm the validity of the present method for the determination of 4-OH-E-17-S, a recovery test was undertaken using authentic sample. A known amount of the conjugate was added to the medium, and the conjugate

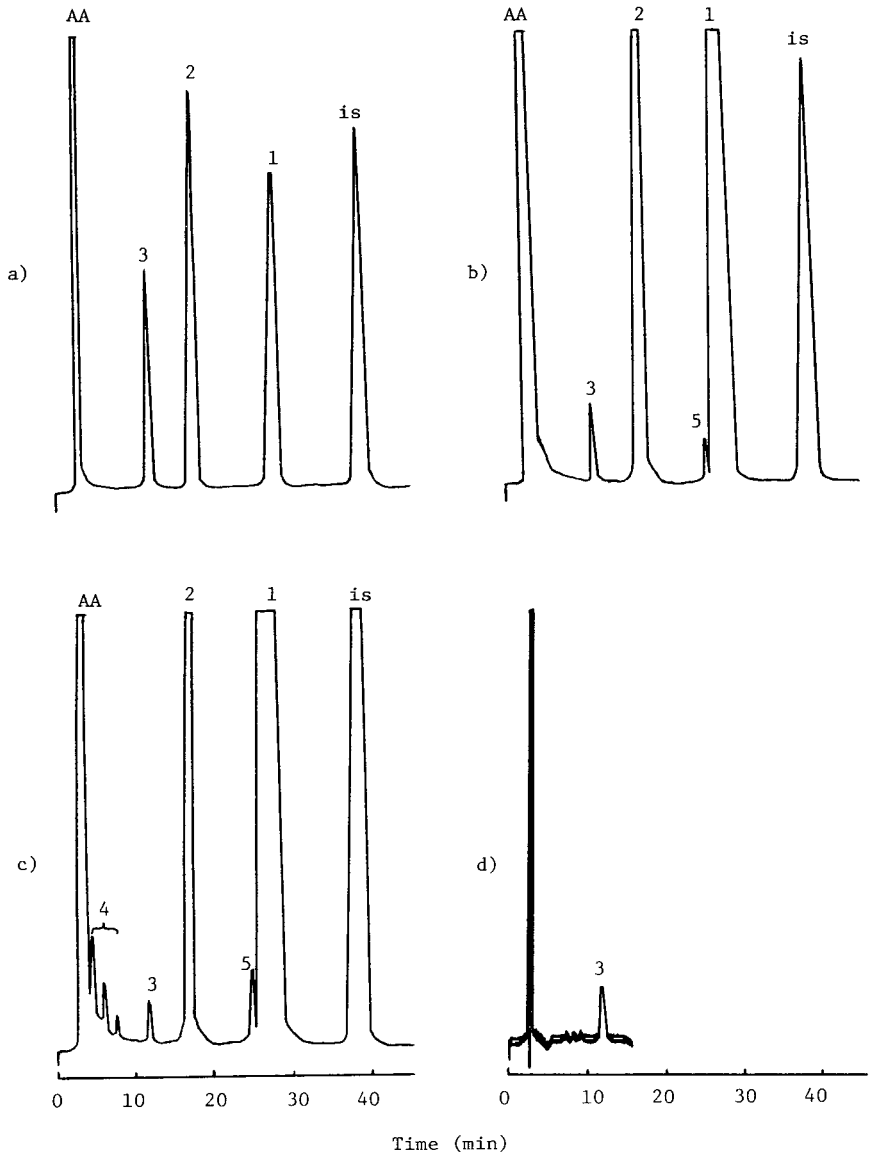

Fig. 1. High-performance liquid chromatograms of authentic conjugates (a), incubation products of oestradiol 17-sulphate of liver microsomes from male (b) and female (c) rats, and detection limit of 4-hydroxyoestradiol 17-sulphate (d) using electrochemical detection. Peaks: 1, oestradiol 17-sulphate; 2, 2-hydroxyoestradiol 17-sulphate; 3, 4-hydroxyoestradiol 17-sulphate; 4, group composed of metabolites of oestradiol 17-sulphate hydroxylated at 6β, 7β and 16α; 5, peak derived from microsomes; is, 2-methoxyoestradiol 17-sulphate (internal standard); AA, ascorbic acid.

recovered through the whole clean-up procedure was determined. The results (Table I) indicate that 4-OH-E-17-S is recovered to a satisfactory extent.

Some kinetic parameters of E-17-S 4-hydroxylase were measured by the present method. Fig. 2 shows the influence of the incubation time (a), enzyme concentration (b) and hydrogen ion concentration (c) on the production of

TABLE I

RECOVERIES OF 4-HYDROXYOESTRADIOL 17-SULPHATE FROM THE INCUBATION MEDIUM AND AFTER THE WHOLE CLEAN-UP PROCEDURE

Steroid was dissolved in the incubation medium (3.0 ml) and the mixture was immediately heated for 1 min in boiling water, followed by the same treatment as described in the text. Each value is expressed as the mean % ± S.D. (n = 6).

Amount added (μg)	Recovery (%)
10	99.1 ± 3.2
5	98.0 ± 2.4
2	97.7 ± 2.0
0.2	100.1 ± 2.9

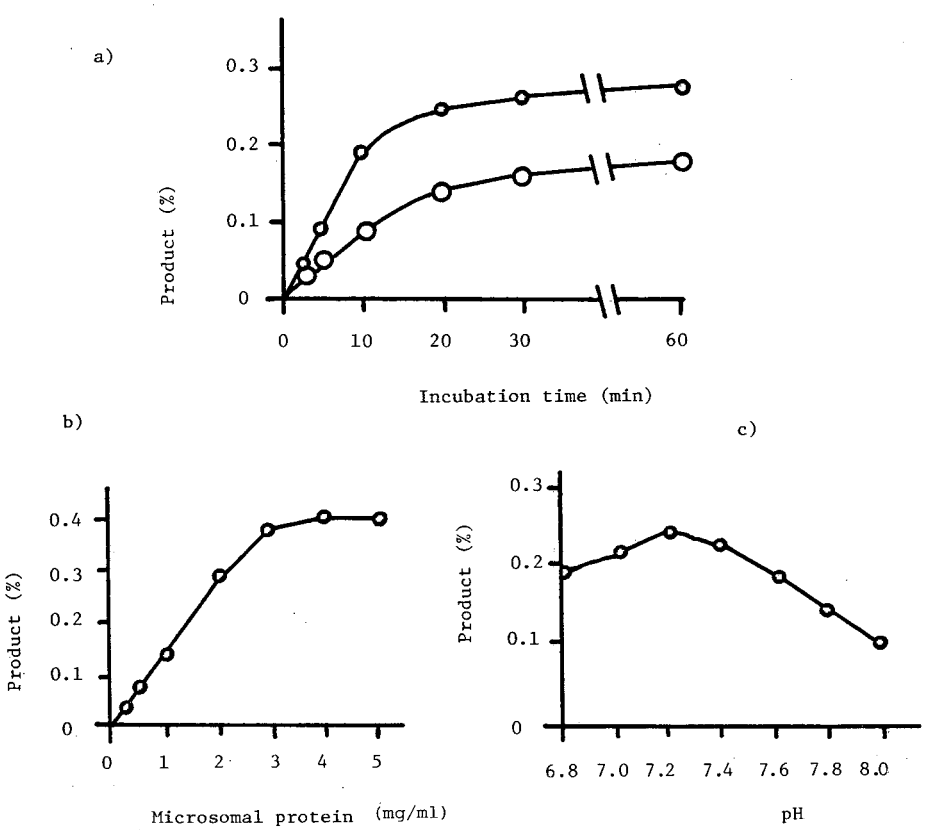

Fig. 2. Effect of incubation time (a), microsomal protein concentration (b) and hydrogen ion concentration (c) on the 4-hydroxylation of oestradiol 17-sulphate by male rat liver microsomes. Oestradiol 17-sulphate (200 μM) was incubated with an NADPH-generating system at 37°C at the following conditions: (a) pH 7.4 using protein concentration of 2.0 mg/ml (•) and 1.0 mg/ml (○); (b) pH 7.4 for 30 min; (c) for 30 min using a protein concentration of 2.0 mg/ml. Each point represents the mean value of six experiments.

4-OH-E-17-S by male rat liver microsomes. The enzyme activity was linear up to 10 min incubation and up to 2.0 mg/ml protein. The effect of pH on the product formation was studied, and the optimum pH was shown to be about 7.2. Similar results were obtained when female rats were used.

The enzyme kinetics of E-17-S 4-hydroxylase from the microsomes of male rat liver follow classical Michaelis—Menten kinetics producing a Lineweaver—Burk plot (Fig. 3). Analogous results were obtained from the experiments with female rat liver microsomes.

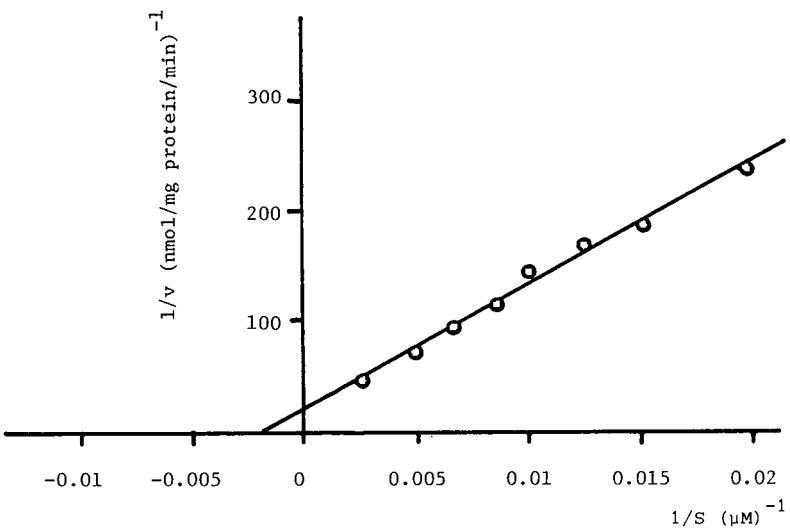

Fig. 3. Lineweaver—Burk plot for oestradiol 17-sulphate 4-hydroxylase of male rat liver microsomes.

TABLE II

COMPARISON OF APPARENT K_m (μM) AND V_{max} (nmol/mg PROTEIN/min) VALUES FOR OESTRADIOL 17-SULPHATE 2- AND 4-HYDROXYLASES IN RAT LIVER MICROSOMES

Sex	4-Hydroxylase		2-Hydroxylase	
	K_m	V_{max}	K_m	V_{max}
Male	540	0.047	185	1.35
Female	570	0.050	200	0.41

The E-17-S 4-hydroxylase activity was measured from a series of six separately prepared samples using the same rat liver microsomes. Table II shows a comparison of the apparent K_m and V_{max} values for E-17-S 2- and 4-hydroxylases in rat liver microsomes from either sex under these conditions. From these results, there seems to be some difference between the two enzymes, especially in their K_m and V_{max} values. A sex difference between the two enzymes is observed in the case of 2-hydroxylation. Such a sex difference of 2-hydroxylation was also observed in the metabolism of free oestradiol [15].

The K_m and V_{max} values of 2-hydroxylase in Table II are different from

those reported in the previous paper [9], the reported values being 85.5 μM and 0.64 nmol per mg protein per min in the male rats, and those in female rats were 117 and 0.19, respectively. The discrepancy between the previous and the present results may be attributable to the age of the animals studied. This is estimated by the recent report by Theron et al. [16] who showed that the enzyme kinetics of oestradiol 2-hydroxylase undergo a dramatic change during the development of the animals. Since we have used animals of almost the same age in the present experiments, in contrast to the previous report [9], the results in Table II on 2-hydroxylase should be more reliable. Further investigations of the influence of age on the 2- and 4-hydroxylation of E-17-S are needed.

Utilization of the present method in biological and endocrinological studies of the C-17 conjugated oestradiol will be the subject of a future communication.

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Note

Identification of 1,3-diphenyl-2-aminopropane metabolites by gas chromatography, gas chromatography with Fourier transform infrared spectroscopy and gas chromatography—mass spectrometry

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1,3-Diphenyl-2-aminopropane (DAP), which was synthesized for use as a central nervous stimulant [1], has been used as a model compound for the study of the liver monooxygenase system [2].

In the present study, the metabolites of DAP were isolated from bile and urine, and after enzymatic hydrolysis purified by column chromatography and thin-layer chromatography (TLC). The prepurified sample was analysed by gas chromatography (GC), gas chromatography with Fourier transform infrared spectroscopy (GC—FTIR) and gas chromatography—mass spectrometry (GC—MS). The following metabolites have been identified by comparison with authentic samples: 1-(4′-hydroxyphenyl)-3-phenyl-2-aminopropane, 1,3-diphenylpropane-2-on-oxime, 1,3-diphenylpropane-2-on, 1,3-diphenyl-2-hydroxypropane.

EXPERIMENTAL

Samples of bile (0—5 h) and urine (0—24 h) were hydrolysed enzymatically with β-glucuronidase—arylsulphatase (isolated from *Helix pomatia*, Calbiochem) [3] in an acetate buffer (0.1 *M*, pH 4.5). The metabolites were then prepurified on Amberlite XAD-2 resin, using methanol for the final elution [4]. The resin was purified before use. One volume of resin was stirred for 1 h with each portion of the following washing solutions: twice with 5 vols. of methanol—concentrated hydrochloric acid (100:1), twice with 5 vols. of

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methanol—water—acetone (45:45:10), and finally three times with 10 vols. of water (distilled twice from glass, or HPLC quality) [5]. The purified resin was stored under methanol.

The methanolic eluates of the samples were divided into two parts, and evaporated to dryness in a nitrogen stream. The first part of the sample was dissolved in 200 μl of ethyl acetate, and chromatographed on a short column. The column, constructed in the laboratory from PTFE, was 5 × 0.5 cm and packed with Merck LiChroprep Si 60 (25—40 μm). The column was first eluted with 10 ml of ethyl acetate to eliminate impurities, followed by 5 ml of ethanol which eluted the metabolites. The samples obtained were analysed by on-line GC—FTIR [6, 7] after derivatization. For this purpose flash methylation with Methelute (Pierce) was used [8].

The second part of the divided sample was chromatographed on home-made TLC plates (Kieselgel HF_{254}, Merck 20 × 20 cm, 0.25 mm thickness). The metabolites were separated from polar impurities using benzene—ethanol (5:1) as solvent. Separation from apolar compounds was achieved after elution of the spots and rechromatography in benzene—diethyl ether (1:1) on another plate. Spots were detected by ultraviolet light. The spots were eluted from the plates and the solutions analysed by capillary GC. The metabolites were identified by GC—MS.

Apparatus

For GC—FTIR a Varian 3700 GC coupled with a Nicolet 7199 FT-IR was used [9—11]. The column was of silanized glass, 2 m × 2 mm, packed with 3% SE-30 on Chromosorb W AW DMCS (100—120 mesh). The carrier gas was nitrogen at a flow-rate of 20 ml/min. Temperatures were: injector 300°C, transfer line 305°C, light pipe 305°C; temperature programme was 210—290°C (8°C/min).

A Perkin-Elmer F22 was used for capillary GC with flame-ionization or nitrogen—phosphorus detection. The column, 25 m × 0.25 mm, was of Pyrex glass coated with SE-30. Carrier gas was nitrogen at a flow-rate of 3 ml/min. Injections were made in the splitless mode (split closed 30 sec) [12]. Temperatures were: injector 240°C, detector 240°C; temperature programme was 1 min at 70°C, then 70—140°C (30°C/min), 140—205°C (4°C/min), 16 min at 205°C.

For GC—MS a Pye-104 gas chromatograph was coupled with an MM-12 F 1A (V.G. Micromass) type single-focusing mass spectrometer. The column, 10 m × 0.25 mm, was of Pyrex glass coated with OV-101. Carrier gas was helium at 2 ml/min. Injections were splitless. Temperatures were: injection 205°C, ionization chamber 200°C; temperature programme was 5 min at 95°C, then 95—145°C (25°C/min), 145—200°C (5°C/min), 20 min at 200°C. The mass spectra of the samples were recorded using electron-impact ionization of 70 eV and 3 kV accelerating voltage.

RESULTS

GC and TLC data suggested that the major metabolic pathway produced an aromatic hydroxylated metabolite. We could not determine the exact position

of the hydroxyl group in the aromatic ring by GC—MS. In order to determine the exact position of the hydroxyl group we used on-line GC—FTIR; however, the sensitivity of the GC—MS method was better by two orders of magnitude than GC—FTIR in our hands. The GC—FTIR results were obtained as Chemigrams™. The Chemigram is produced by the Nicolet instrument on-line, in real time, by monitoring five preselected wavelength regions (windows) and these are recorded both on the screen and by the plotter [9, 10]. Five windows were selected corresponding to the expected bands of functional groups (e.g. 850—750 ν window corresponds to *para*-substitution), and the infrared absorbances were monitored in these windows (Fig. 1). The complete infrared spectra of the main metabolite and 1-(4′-hydroxyphenyl)-3-phenyl-2-aminopropane standard were identical.

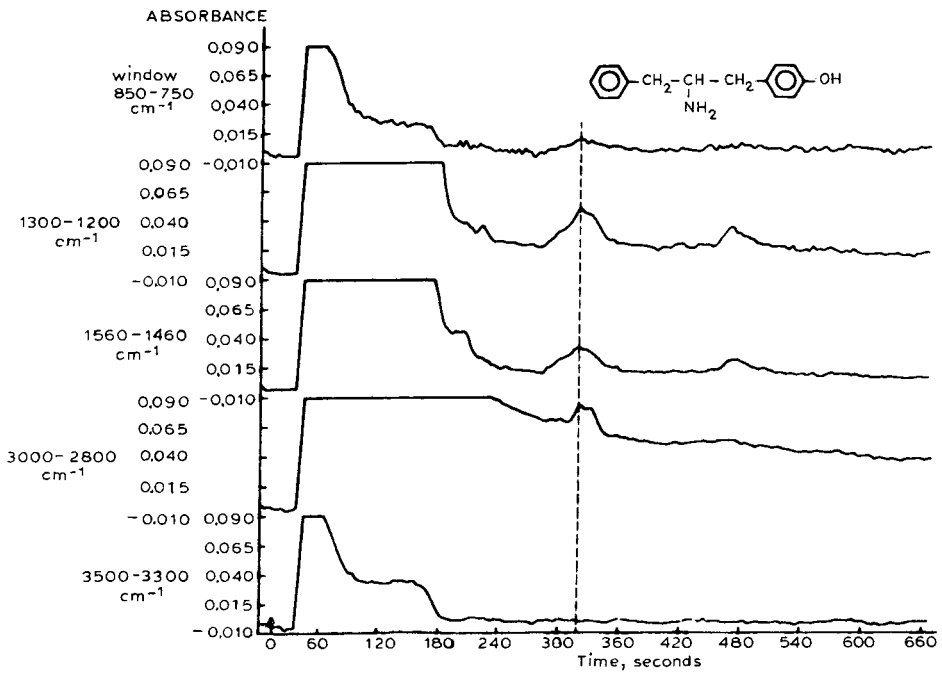

Fig. 1. Chemigram of DAP main metabolite (t_R = 310 sec) 1-(4′-hydroxyphenyl)-3-phenyl-2-aminopropane. Windows correspond to the expected functional groups. For parameters see text.

Flash methylation was chosen as the derivatization method for several reasons. In our laboratory flash methylation is routinely used. Preliminary experiments were made with trifluoroacetyl derivatives, but experiments were not promising in case of the biological samples, of which we had only limited quantitaties. We favoured flash methylation against trifluoroacetylation to avoid evaporation of a further solvent, which further contaminated the samples. The samples were already contaminated with the residues of several solvents and chemicals used during preceding operations. Methylation was not complete under our experimental conditions. The amino group was only partially derivatized, therefore double peaks were found. This did not influence

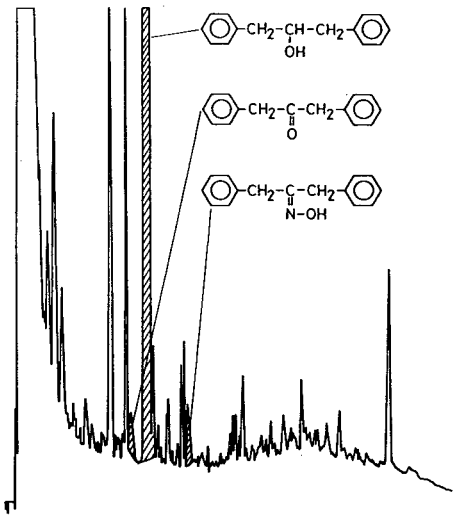

Fig. 2. One of the typical glass capillary GC analyses (flame-ionization detection) for minor metabolites in a urine extract. For parameters see text.

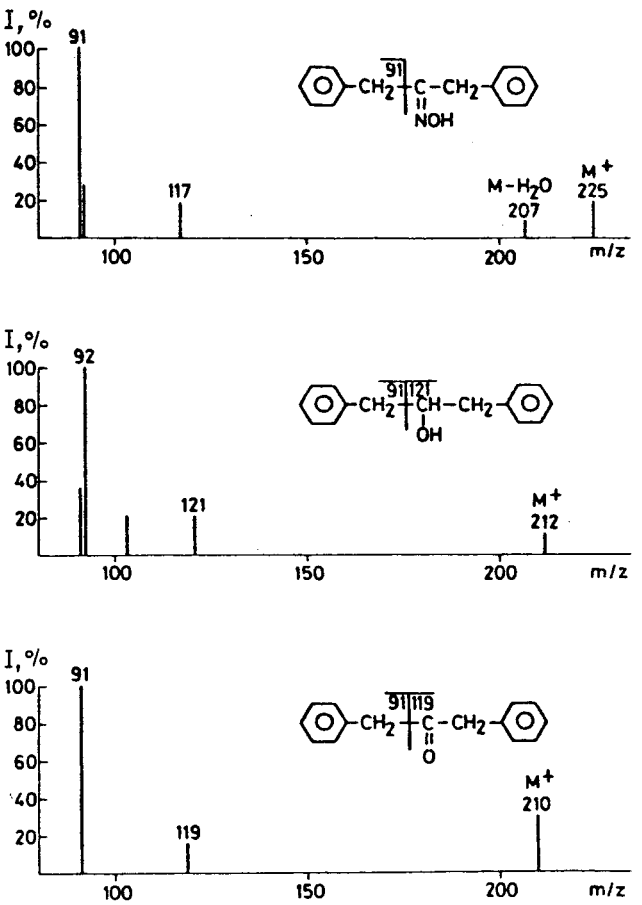

Fig. 3. Mass spectra of the minor metabolites. For parameters see text.

the identification of 1-(4′-hydroxyphenyl)-3-phenyl-2-aminopropane. The presence of another hydroxylated compound was shown by GC—MS, but its amount was insufficient for identification by GC—FTIR. According to TLC R_F values and GC retention times, one of the trace materials is probably 1-(2′-hydroxyphenyl)-3-phenyl-2-aminopropane by comparison with standard compound.

Some other minor metabolites eluted from TLC spots were analysed by glass capillary GC (Fig. 2) and GC—MS. The identification was based on comparison with synthetic standards. We found the following compounds: 1,3-diphenylpropane-2-on-oxime, 1,3-diphenylpropane-2-on, 1,3-diphenyl-2-hydroxypropane. The mass spectra of these compounds are shown in Fig. 3.

ACKNOWLEDGEMENTS

We would like to thank Mr. J. Szammer for synthesizing the reference compounds, and Mr. G. Alexander for preparing the capillary columns.

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CHROMBIO. 2342

Note

Simple determination of trichloroacetic acid in urine using head space gas chromatography: a suitable method for monitoring exposure to trichloroethylene

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(First received June 26th, 1984; revised manuscript received August 9th, 1984)

Trichloroethylene is a relatively frequent industrial toxic substance. It is a well known fact [1] that 60—75% of trichloroethylene absorbed by the exposed organism is mainly metabolized to trichloroacetic acid (TCA), and to trichloroethanol as well. Perchloroethylene is also metabolized to TCA to a limited extent [2].

TCA has a longer half-time of excretion (100 h) compared with trichloroethanol (12 h) [3]. Hence the concentration of TCA in the urine characterizes a week's exposure, while that of trichloroethanol characterizes the exposure of the previous working shift [4]. In evaluating the permanent but mostly variable exposure to trichloroethylene, the determination of TCA in the urine is more significant if compared with that of trichloroethanol. Moreover, during exposure to perchloroethylene, trichloroethanol is not present in the urine at all.

Hence we paid particular attention to developing a method suitable for the determination of TCA in the urine in our current screening of workers' exposure to trichloroethylene or perchloroethylene. In the literature can be found many methods. Photometric determination based on Fujiwara's reaction is the most frequently applied method for the determination of TCA, even in outdoor practice [1, 5]. Another method increasingly being used is that of gas chromatography. For this purpose TCA is changed mostly into lower polarity esters, determined either after extraction into organic solvent [6], or directly in the aqueous phase using head space analysis [7, 8] or electron-capture detection. The simple method with flame ionization detection [9] based on direct injection of urine into the gas chromatograph is very interest-

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ing. Thermal decarboxylation of TCA occurs and results in the formation of chloroform, the peak of which is recorded. This otherwise very rapid procedure is disadvantageous since the column becomes contaminated with urinary components of higher boiling point.

In order to remove the biggest disadvantages of the above methods, i.e. complicated procedure, necessity of electron-capture detection, rapid pollution of the column, etc., we developed a method of our own. In this report we demonstrate the possibility of simple determination of TCA in the urine using gas chromatography. By heating a sample of urine in a closed flask, decarboxylation of TCA to chloroform is attained, immediately determined by head space analysis and evaluated by comparison with an internal standard.

EXPERIMENTAL

Materials

Aqueous solution of TCA p.a., 1000 mg/l, was from Cambrian Chemicals, Beddington, U.K.; *n*-butanol p.a. (Lachema, Brno, Czechoslovakia) was redistilled before use.

Sample bottles were flasks (25.0 ml) with screw cap perforated in the centre, with a 2 mm thick silicone rubber lining covered with aluminium foil.

Procedure

A 5-ml volume of urine was pipetted into a 25-ml flask with 10 μl of *n*-butanol. The flask was closed and placed in a thermostatically controlled water bath. After 75 min at 90°C decarboxylation of TCA to chloroform was attained and the flask was slowly transferred to a water bath maintained at 45°C. After 20 min, 1 ml of gas phase was taken with a heated syringe within 10—15 sec and injected into the gas chromatograph.

Gas chromatography

A gas chromatograph Chrom 42 (Laboratorní Přístroje, Prague, Czechoslovakia) with a flame ionization detector was used. A glass column (2.5 m × 3 mm I.D.) was packed with 10% Carbowax 20 M (Applied Science Labs., State College, PA, U.S.A.) on Chromosorb W AW, 100—120 mesh (Supelco, Bellefonte, PA, U.S.A.). The injector block was maintained at 140°C and the column at 130°C. Flow-rates were 500 ml/min for the air, 30 ml/min for the hydrogen and 70 ml/min for the carrier gas (nitrogen). The sensitivity used was 1:200.

RESULTS

Fig. 1 shows a typical chromatogram obtained from the analysis of a urine sample from a person exposed to trichloroethylene. A small peak with a retention time of about 1 min had no connection with the concentration of TCA and was therefore not studied further.

Calibration was done both by analysing standard aqueous solutions of TCA in the range 0—250 mg/l, and by adding increasing amounts of standard to urine for determining any interference from other substances in the urine. The

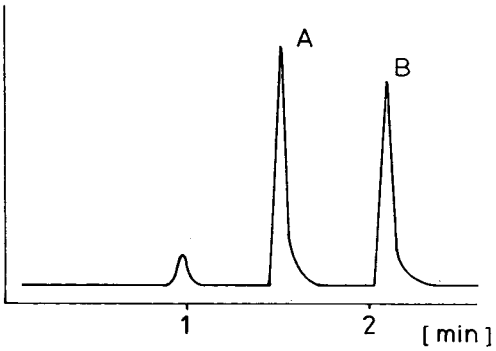

Fig. 1. Typical chromatogram obtained by analysing the urine of a worker exposed to trichloroethylene (urine concentration of TCA 48 mg/l). Peaks: A = chloroform: B = *n*-butanol (internal standard).

TABLE I

PRECISION AND RELATIVE RECOVERY OF URINARY TCA DETERMINATION

$n = 10$.

TCA conc. + standard (mg/l)	Mean (mg/l)	S.D. (mg/l)	C.V. (%)	Relative recovery (%)
9.0 + 0	9.0	0.6	8.5	
9.0 + 125.0	139.5	5.8	7.0	104.3
9.0 + 250.0	246.3	13.0	9.1	95.1

course of the calibration curve was linear in both cases in the indicated range, the lines having the same slope. Thus interference by other substances in the urine was excluded.

Precision and relative recovery of the method were verified by ten-fold repetition of the determination, both in the urine of an exposed worker and in the same urine with increasing additions of standard TCA. For the results see Table I.

The sensitivity of the method was about 2 mg/l.

To control the results, urine samples of persons from three places of work who were exposed to trichloroethylene were determined using both the photometric method and head space analysis. The results are compared in Table II.

DISCUSSION

The time required for the decarboxylation of TCA to chloroform was verified experimentally. During the first 45 min the peak of chloroform is increasing. A maximum is reached within about 60 min and is not changed by further heating. Optimum conditions were taken as 75 min heating at 90° C approximately. The temperature of the bath, 90° C, was chosen on purpose. At this temperature there is no boiling so that a water thermostat can be used, although simply submerging the flask in a vessel of boiling water could obviously also be done. At lower temperatures the period of heating should be prolonged.

TABLE II

COMPARISON OF RESULTS OBTAINED USING THE PHOTOMETRIC METHOD AND HEAD SPACE ANALYSIS

Work place	Subject examined		Concentration of TCA (mg/l)		Comment
	Sex	Age (years)	Photometry	Head space analysis	
Dry cleaning	M	31	23	25	Exposure at admissible level
	F	28	48	47	
	F	28	53	51.5	
	F	40	39	41	
		Mean:	40.75	41.1	
Production of musical instruments (cleaning of brass parts)	M	25	68	72.5	Surpassing the admissible level [10]
	M	32	123	120.5	
	M	44	105.5	108	
	F	27	55	55.5	
	F	28	81	80	
	F	45	115	120	
	F	48	70	76	
		Mean:	88.1	90.3	
Electronics production (cleaning of small parts)	F	28	6	6.5	Low exposure
	F	36	15	14	
	F	50	8.5	8	
	F	33	9	9	
	M	57	12.5	14	
		Mean:	10.2	10.3	

The reason for using another water bath at 45° C is one more of practice than of principle, since it is easier to take a sample of the gas phase from a flask at 45° C than at 90° C. Also this makes the method more reproducible since 45° C is nearer the temperature of the laboratory and thus there is not such a great temperature difference between the syringe and the sample.

The high temperature necessary for the decarboxylation of TCA to chloroform requires paying great attention to the perfect packing of the flasks for head space analysis. We would advise, for instance, to use flasks with ground glass joints covered with a thin layer of silicone oil. The rubber packing should be lined with aluminium foil to prevent undesirable absorption of chloroform into the rubber. In order to exclude the possible production of chloroform from trichloroethanol, the procedure was applied to aqueous solutions of 250 mg/l trichloroethanol (Koch Light, Colnbrook, U.K.). In these conditions no peak for chloroform was recorded.

In comparing the results of the suggested head space analysis (see Table II) with those obtained by the photometric method [1, 5], it was found that in three working places with various exposures to trichloroethylene the methods were equivalent. Thus the long-term practice of applying the photometric

method confirmed the applicability of the suggested procedure for monitoring the exposure of workers. There is, however, a certain disadvantage of the suggested method compared with that of Buchet et al. [9], namely a slightly longer time needed for the analysis of one sample. This is, however, considerably shortened in series analysis. Moreover, if the time necessary for cleaning the column contaminated with volatile urinary constituents is also taken into consideration, the utility of the suggested method in analysing a larger amount of samples is quite evident.

The studies of Breimer et al. [7], Ogata and Saeki [6] and Monster and Boersma [8] report the simultaneous determination of trichloroethanol as well, but in determining TCA alone the procedure is much simpler, less elaborate, and does not require electron-capture detection since flame ionization detection and a relatively low sensitivity are sufficient.

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CHROMBIO. 2334

Note

Simplified gas chromatographic assay of underivatized nitrazepam in plasma

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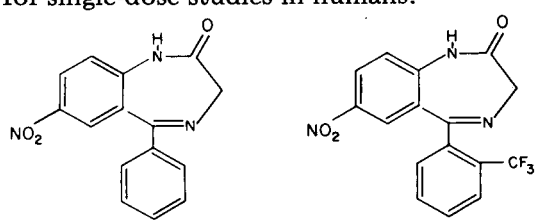
Nitrazepam (Mogadon) (Fig. 1) is a 7-nitro benzodiazepine derivative extensively used as a hypnotic agent [1]. Gas chromatography (GC) with electron-capture detection has been successfully used for quantitation of nitrazepam in plasma [2—5]. However, most prior methods have required either derivatization (alkylation) or hydrolysis of nitrazepam prior to assay [2, 3], or the use of capillary column chromatography [4, 5]. This is because intact nitrazepam has inherently poor chromatographic properties, leading to peak tailing and adsorption due to its amide character [3]. This paper describes a rapid and sensitive GC analysis of intact nitrazepam in plasma, which is suitable for single-dose studies in humans.



NITRAZEPAM (NTZ) TRIFLUOROMETHYL-NITRAZEPAM (TFM-NTZ)

Fig. 1. Structural formulae of nitrazepam (NTZ) and the internal standard, trifluoromethyl-nitrazepam (TFM-NTZ).

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EXPERIMENTAL

Instrumentation

A Hewlett-Packard 5830A or 5840A gas chromatograph equipped with a 15-mCi ^{63}nickel electron-capture detector, data processing module, and automatic sampler was used for analysis. The column was 1.22 m × 4 mm I.D. coiled glass packed with 1% OV-17 on 80—100 mesh Chromosorb W HP (Hewlett-Packard, Avondale, PA, U.S.A.). The chromatographic conditions were as follows: oven temperature, 275°C; injection port temperature, 310°C; detector temperature, 310°C; carrier flow-rate, 50 ml/min. The carrier gas was argon—methane (95:5) obtained from Matheson (Gloucester, MA, U.S.A.).

Reagents and solutions

Stock standards of nitrazepam and the internal standard trifluoromethyl-nitrazepam (TFM-NTZ, Ro5-3590) (Fig. 1), both kindly supplied by Hoffmann-LaRoche (Nutley, NJ, U.S.A.), were prepared by weighing 10 mg of each compound and dissolving it in 100 ml of methanol. The working standards of 1.0 μg/ml nitrazepam and 1.0 μg/ml TFM-NTZ were made by appropriate dilution of each compound with methanol. Both stock and working solutions were stored in amber ground-glass stoppered bottles at 4°C and were stable for at least one year.

A borate buffer was prepared by mixing 500 ml of 0.025 *M* sodium borate (9.53 g/l) with 177 ml of 0.1 *M* hydrochloric acid and adjusting the pH to 8.3.

A priming agent was made by dissolving 20 mg of purified soy phosphatides (asolectin) in 10 ml of benzene. A 2—3 μl aliquot of the agent was injected into the column prior to each day's run.

Method

TFM-NTZ (10 ng) was added to a series of round-bottom 125 × 13 mm screw-top culture tubes. Calibration standards were prepared by addition of 5, 10, 25, 50, 75 and 100 ng of nitrazepam to a series of these tubes. Drug-free blank serum or plasma (0.5 ml) was added to the calibration tubes and 0.5—1.0 ml of unknown serum or plasma added to all other tubes. Borate buffer (1 ml) was added to each tube, and briefly vortexed to mix. All samples were then extracted with 3 ml of benzene—dichloromethane (80:20) by agitation on an automatic vortex-mixer (Kraft Apparatus, Minolea, NY, U.S.A.) in the upright position for 1 min. They were then centrifuged for 15 min at 400 *g*, and the organic layer pipetted into 2-ml Wheaton automatic sampling vials, and evaporated to dryness under conditions of heat (40°C) and mild vacuum. The residue was reconstituted with 200 μl of toluene—isoamyl alcohol—asolectin solution (84:14:3), and the vials capped with aluminium foil. A 6-μl aliquot was injected onto the column using the automatic sampler.

Clinical pharmacokinetic study

A 24-year-old male received a 10-mg oral dose of nitrazepam after giving informed consent. Venous blood samples were drawn into additive-free tubes at 15, 30, 45, 60 min and 1.5, 2.0, 2.5, 3, 4, 6, 8, 12, 24, 36, 48, 60 and 72 h

post dosage. The samples were allowed to clot, and the serum was separated and frozen until the time of assay. Concentrations of nitrazepam in all samples were determined as described above.

Pharmacokinetic parameters of nitrazepam distribution, elimination and clearance were determined by model-independent techniques described in detail previously [6].

RESULTS

Evaluation of method

Using the described chromatographic conditions, nitrazepam and TFM-NTZ gave two well resolved and well defined peaks (Fig. 2). The relation of peak height ratio versus the plasma nitrazepam concentration was linear and passed through the origin. Replicate samples (n = 8) of standards containing 10, 25, 50 and 100 ng/ml nitrazepam yielded within-day coefficients of variation of 5.8%, 4.3%, 6.2% and 5.2%, respectively. The between-day coefficient of variation for the slope of calibration curves run on twelve different days was 8.0%. The limit of sensitivity for nitrazepam is approx. 3—5 ng/ml. Residue analysis indicated greater than 90% recovery.

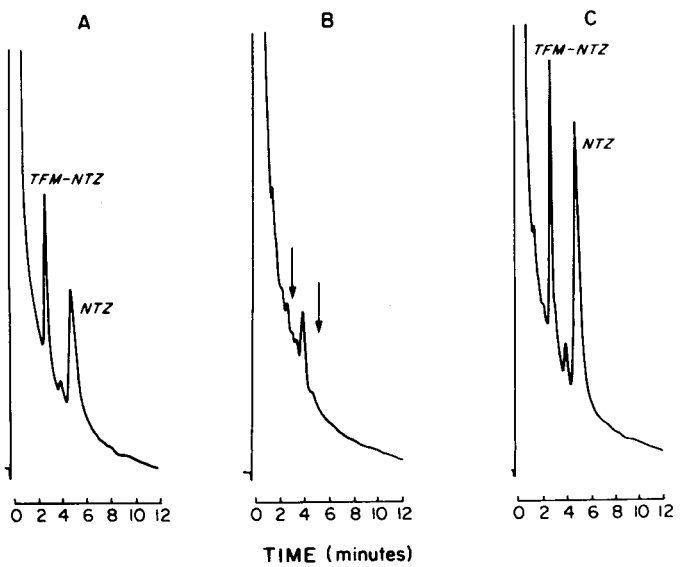

Fig. 2. Chromatograms of (A) a calibration standard containing 10 ng/ml trifluoromethylnitrazepam (TFM-NTZ) and 50 ng/ml nitazepam (NTZ); (B) a drug-free blank serum sample; (C) a serum sample from a subject drawn 12 h after a single 10-mg dose of nitrazepam.

Pharmacokinetic results

Fig. 3. shows serum nitrazepam concentrations in the volunteer subject. Pharmacokinetic parameters for nitrazepam were: elimination half-life, 28 h; total volume of distribution, 297 l; total clearance, 124 ml/min.

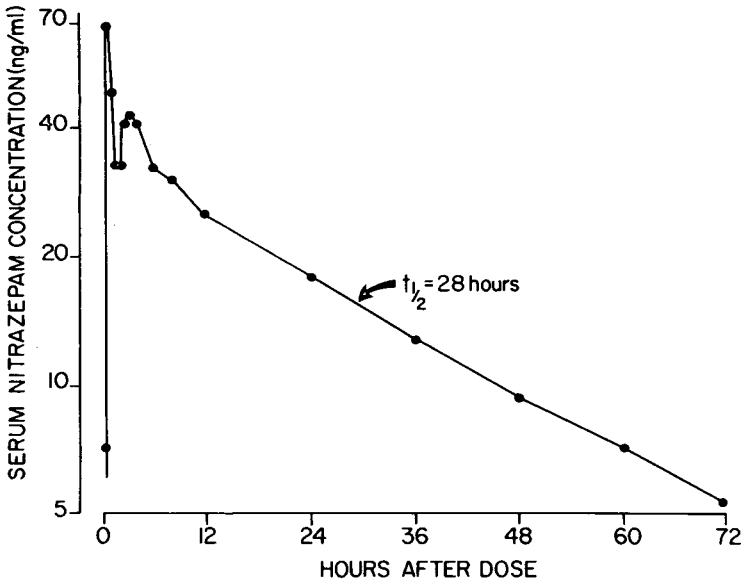

Fig. 3. Serum nitrazepam concentrations in the volunteer subject.

DISCUSSION

The described method allows for the sensitive and reliable quantitation of underivatized nitrazepam in human serum or plasma. After addition of the internal standard having a structurally similar amide configuration, both nitrazepam and the internal standard are extracted into an organic solvent at a slightly alkaline pH. After evaporation and reconstitution of the organic phase, the sample is ready for injection into the gas chromatograph. Although nitrazepam and the internal standard have inherently poor chromatographic properties, use of a low phase load (1%) of column packing, high column temperatures (275° C), and concurrent injection of the lipoidal priming material along with each sample yields narrow peaks and reproducible chromatography of nitrazepam. Drug-free plasma is free of contaminating endogenous substances, thereby eliminating the need for clean-up procedures.

The method is applicable to single-dose pharmacokinetic studies of nitrazepam in humans [7]. Results of the present study in the volunteer subject are consistent with previous studies of nitrazepam pharmacokinetics [8].

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CHROMBIO. 2350

Note

Gas—liquid chromatographic determination of phentermine in human plasma following oral administration to healthy subjects

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(First received June 4th, 1984; revised manuscript received August 23rd, 1984)

Phentermine is a phenylethylamine derivative (Fig. 1). It has appetite depressant properties. When bound to a cationic resin, it is commercially available under the trade name Ionamin®.

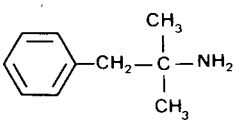

Fig. 1. Chemical structure of phentermine.

Separation and quantitation of biologically active amines by gas chromatographic methods have usually been carried out by derivatization followed by electron-capture [1, 2] or flame-ionization [3—5] detection. Most of these procedures involve chromatography of the trifluoracetamide derivatives of these amines using columns such as 3% OV-17, 3% SE 30, 2% SP-2510-DA and 1% SP-1240-DA [5]. Although these procedures are satisfactory for many amines of biological significance, they have proved unsatisfactory when applied to the assay of phentermine. O'Brien et al. [6] reported that volatilization, adsorption onto glass, as well as incomplete extraction and derivatization combine to produce a decrease in reliability for the determination of plasma phentermine, especially at the low blood concentrations attained when therapeutic doses are administered. These workers introduced procedural modifications to overcome these difficulties. However, in our laboratory reproduction of this method, particularly at low plasma concentrations of phentermine, proved to be difficult.

In this paper we describe a simple, sensitive and selective gas—liquid chro-

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matographic method for the determination of therapeutic levels of phentermine in human blood plasma, using nitrogen—phosphorus detection.

EXPERIMENTAL

Reagents and solvents

Amantadine hydrochloride was obtained from Sigma, 1 *M* sodium hydroxide (concentrated volumetric solution), 0.1 *M* hydrochloric acid (concentrated volumetric solution), diethylamine (AnalaR), and toluene (AnalaR) from BDH and phentermine hydrochloride (pharmaceutical grade) from Loftus-Bryan Chemicals (Ireland).

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

Standards

Phentermine · HCl (12.5 mg, equivalent to 10.00 mg phentermine base) was dissolved in 100 mg deionized water to yield the stock standard solution (100 μg/ml).

A second stock solution was prepared by 1:10 dilution of the 100 μg/ml solution, to yield stock solution A (10 μg/ml). The working standard solutions containing 200, 500, 750, 1000, 1250 and 1500 ng/ml phentermine were obtained by serial dilution of stock solution A with water. Spiked plasma standards were prepared by addition of 100 μl of the working standard solutions to 1 ml of plasma, to provide plasma standards containing 20, 50, 75, 100, 125 and 150 ng/ml phentermine.

Amantadine · HCl (5 mg) was dissolved in 100 ml deionised water to yield an internal standard (I.S.) stock solution (50 μg/ml). This was further diluted to give a solution of 5 μg/ml, of which 100 μl were used to spike both plasma standards and test samples.

Instrumentation

A Perkin-Elmer Sigma 2000 gas chromatograph equipped with a nitrogen—phosphorus detector was used. The column employed was 2 m × 4 mm I.D., glass packed with 10% Apiezon-L + 2% potassium hydroxide on 80—100 mesh, acid-washed, Chromosorb W (Perkin-Elmer).

The following operational parameters were found to be optimal: injector temperature 170°C and detector temperature 250°C. The initial oven temperature was 155°C for 7.5 min during which phentermine and amantadine were eluted and the temperature was then increased at the rate of 30°C/min to 200°C and remained at 200°C for 2 min to expedite late-eluting peaks before the system was ready for the next injection. Nitrogen at a flow-rate of 30 ml/min was used as carrier gas. Air and hydrogen were supplied to the detector at a flow-rate of 160 ml/min and 2 ml/min, respectively. Detector output

was set at 10 pA and attenuation was 1 or 2 at range 1. A Perkin-Elmer Model 024 chart recorder was used to monitor the signals, with a chart speed of 0.5 cm/min.

Procedure

Plasma (1 ml) spiked with 100 µl phentermine standard and 100 µl internal standard (5 µg/ml amantadine · HCl) was mixed with 1 ml of 1 *M* sodium hydroxide solution in a glass-stoppered tube. After the addition of 5 ml toluene (containing 0.05%, v/v, diethyl amine) the drugs were extracted by rotating the tubes gently for 15 min on a mechanical rotator, followed by centriguation for 10 min at 700 *g* at 5°C. The organic phase was transferred into a glass culture tube and 1 ml of 0.1 *M* hydrochloric acid was added. The tube was vortexed for 1 min to form the hydrochloride salt and the acidic phase transferred into a small (10 × 75 mm) glass culture tube. Sodium hydroxide (1 *M*, 0.5 ml) was added and the tube again vortexed for 10 sec, after which 100 µl toluene were added and the tube was vortexed again for 45 sec. The toluene layer was removed and 2 µl of this solution were injected into the gas chromatograph.

Plasma samples obtained from volunteers, following ingestion of phentermine, were treated in the same way except that phentermine standards were not added.

Plasma levels of phentermine

The described method has been successfully applied to the measurement of phentermine in plasma samples from a single-dose pharmacokinetic study. Six healthy male volunteers, aged between 18 and 40 years and within 10% of their ideal body weights, participated in this study. Each subject gave written informed consent to participate in the study, the protocol for which was approved by the Institutional Review Board. Subjects were excluded if there was any abnormality in physical or laboratory findings, a history of any previous allergic conditions, a history of medication within two weeks of enrollment or a history of drug abuse. Alcohol was forbidden for the duration of each study period. Subjects received an oral dose of phentermine as a capsule containing 30 mg of the active drug Ionamin, supplied by Pennwalt (Ireland). All doses were administered with a glass of water after an overnight fast. Blood (10 ml) was collected in a lithium heparin vacutainer from an antecubital vein at the following times: pre-dose and 1, 2, 3, 4, 6, 8, 12, 18 and 24 h after dosing. After each blood sampling the plasma was immediately separated and stored at −20°C until required for analysis.

Calibration and calculation

Evaluation of the assay was carried out using five-point calibration standards in duplicate at a concentration range of 20—125 ng/ml phentermine in plasma. The calibration curves were obtained by linear regression of the peak height ratios of phentermine/I.S., versus concentrations of phentermine. These calibration curves were then used to interpolate the concentrations of phentermine in plasma from the measured peak height ratio of phentermine/I.S.

RESULTS

Separation

Fig. 2A and B shows typical chromatograms of phentermine as determined in extracts of human plasma. The mean retention times of phentermine and amantadine were 3.1 and 5.9 min, respectively.

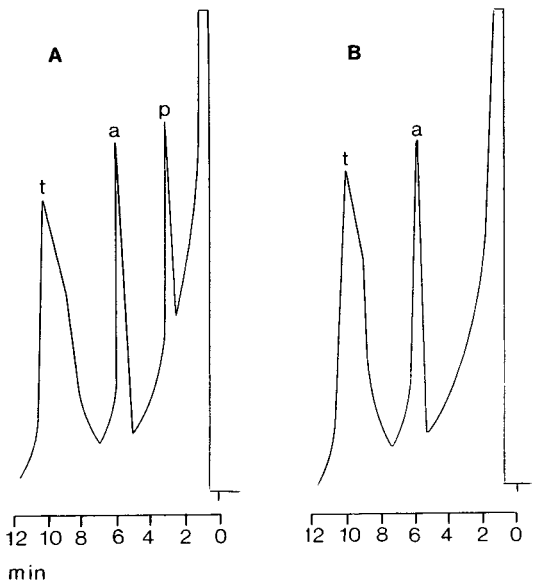

Fig. 2. Chromatograms of (A) a human plasma sample 4 h after a single dose of 30 mg of phentermine; (B) a human pre-dose plasma sample spiked with internal standard, amantadine · HCl. Peaks: p = phentermine; a = amantadine; t = peak due to temperature programming.

Limit of detection and quantitation

Under optimal conditions the limit of detection using a 1-ml plasma sample and 2-μl injection volume was found to be 5 ng/ml where the signal-to-noise ratio was greater than 3:1. The limit of quantitation was taken as 20 ng/ml, where the signal-to-noise ratio was greater than 10:1.

Selectivity

A number of drug-free plasma samples from different human subjects and aliquots of pooled plasma were tested for the presence of interfering endogenous compounds using the outlined extraction procedure. No interference was observed. However, during development of the method an interfering peak was observed which had the same retention time as phentermine (equivalent sometimes to 75 ng/ml phentermine). A systematic study showed that use of plastic pipette tips was responsible for this interference. Subsequently, during all stages of the extraction procedure, glass tubes, pipettes and capillary tubes were used.

Linearity

A measure of linearity is outlined under intra-assay reproducibility (Table I).

TABLE I

METHOD PRECISION AND REPRODUCIBILITY

Concentration added (ng/ml)	Mean concentration found ± S.D. (n = 4, ng/ml)	Coefficient of variation (%)	Difference between added and found concentration (%)
*Intra-assay variation**			
20.0	21.7 ± 0.7	3.36	8.5
50.0	48.6 ± 1.5	3.00	2.8
75.0	75.6 ± 2.6	3.39	0.8
100.0	99.1 ± 2.3	2.36	0.9
125.0	122.5 ± 4.4	3.61	2.0
Inter-assay variation			
20.0	21.8 ± 1.3	5.97	9.0
50.0	52.0 ± 3.1	5.90	4.0
75.0	70.7 ± 1.3	1.89	5.7
100.0	93.7 ± 8.8	9.42	6.3
125.0	130.6 ± 4.0	3.08	4.5

*Regression equation for intra-assay was $Y = 0.024X + 0.039$.

The correlation coefficient was 0.999 and the intercept did not differ significantly from zero.

Precision and reproducibility

The data presented in Table I demonstrate the precision and accuracy of this assay. Intra-assay variability was determined at five concentrations in quadruplicate, at 20, 50, 75, 100 and 125 ng/ml of plasma. Inter-assay variability was determined singly at the same five concentrations in four replicate runs. The precision of the method (mean percent coefficient of variation) and the accuracy (difference between added and found concentrations) for the values of recovered determined standards, when calculated as unknowns against the linear regression line, were acceptable (less than 10% variation from amount added) over the concentration range investigated.

Recovery

Recovery was calculated by comparing the peak height of phentermine after its extraction from plasma with the peak height of a series of unextracted reference standards. In the concentration range 50—150 ng/ml, the mean overall percentage recovery was 94.5 ± 5.7%.

DISCUSSION

This paper describes a method for the measurement of phentermine in human plasma, which has several advantages over previously described procedures [6, 7]. By using a 10% Apiezon L + 2% potassium hydroxide column, the requirement for derivatization of the amine was eliminated. The clean-up and concentration steps, which utilized back-extraction rather than solvent evaporation, improved the overall recovery of phentermine, since losses due to the volatility of the amine were avoided. In addition, the use of

TABLE II

PLASMA PHENTERMINE LEVELS (n = 2)

Time (h)	Plasma phentermine level (ng/ml)						Mean ± S.D.
	Subject						
	1	2	3	4	5	6	
Pre dose	N.D.*	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Post dose							
1	36.0	46.3	36.7	50.0	42.5	32.5	40.7 ± 6.7
2	54.8	71.3	60.0	64.2	101.3	48.8	66.7 ± 18.6
3	72.3	75.0	100.5	160.4	118.8	118.8	107.6 ± 32.8
4	91.8	83.8	105.0	165.8	121.3	185.0	125.5 ± 41.2
6	109.6	102.5	161.7	114.6	122.5	196.3	134.5 ± 36.7
8	113.1	87.5	126.7	162.0	258.8	150.0	149.7 ± 59.7
12	119.9	95.0	123.4	132.2	120.0	111.3	117.0 ± 12.7
18	109.3	82.5	121.7	133.4	110.0	95.0	108.7 ± 18.2
24	128.1	72.8	111.7	236.0	81.3	105.0	122.7 ± 59.0

*N.D. = not detected, below limit of quantitation (< 20 ng/ml).

nitrogen—phosphorus rather than flame-ionization detection significantly improved the sensitivity and selectivity of the gas chromatographic procedure.

O'Brien et al. [6] described a gas chromatographic procedure for the measurement of phentermine in blood at levels to be expected following administration of therapeutic doses of the drug. This method was later employed by Hinsvark et al. [7] who investigated the selective bioavailability of phentermine in man, following oral administration of both resin-bound phentermine and the soluble hydrochloride salt. In this study plasma levels of 70 and 90 ng/ml were observed following administration of 0.375 mg/kg phentermine resinate and hydrochloride, respectively. Following the resinate preparation peak plasma levels were somewhat delayed, occurring at 8—9 h after the oral dose, as compared to 2—3 h following the hydrochloride. The results of the present study (see Table II) confirm these findings, with peak plasma levels being observed 8 h after a 30-mg single oral dose of phentermine resinate (Ionamin).

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Note

One-step extraction procedure for gas chromatographic determination of viloxazine as its acetyl derivative in human plasma

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Viloxazine hydrochloride (ICI-58834) is a morpholine derivative utilized in clinical practice as an antidepressant agent.

Whole blood [1—4] and plasma [5—7] levels of the drug in single-dose studies and in chronic administration have been investigated: concentrations of viloxazine fell after a single therapeutic dose to below 2 μg/ml, while repeated administrations gave plasma levels which largely exceeded this value.

Quantitative determination of the drug has been performed by gas chromatography (GC) with electron-capture detection of the heptafluorobutyryl derivative [8], or flame-ionization detection of its acetyl derivative [5]. ^{14}C-Labelling [3] or high-performance liquid chromatography with fluorescence detection [6] have also been employed for measuring blood viloxazine concentrations. The gas chromatographic methods, even if sensitive and selective, involve several steps before instrumental analysis (extraction, purification, derivatization), so they are time-consuming.

We have developed a simple and rapid GC procedure which allows determination of viloxazine in plasma after a single extraction step and on-column derivatization with acetic anhydride. The internal standard employed, *p*-tolylpiperazine, is derivatized simultaneously with the viloxazine in the chromatographic column.

EXPERIMENTAL

Reagents and standards

Viloxazine · HCl was supplied by ICI Pharma (Milan, Italy), and *p*-tolylpiperazine · 2HCl by the Department of Pharmaceutical Chemistry of Pavia University (Pavia, Italy); benzene, and methylene chloride, both

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pesticide grade, ethanol, acetic anhydride and borate buffer (pH 9.0) were purchased from Carlo Erba (Milan, Italy).

Apparatus and chromatographic conditions

A Carlo Erba Fractovap 2150 gas chromatograph equipped with a nitrogen–phosphorus detector was used. Viloxazine and *p*-tolylpiperazine were separated as their acetyl derivatives in a glass column (2 m × 2 mm I.D.) packed with 3% OV-17 on 100–120 mesh Gas-Chrom P silanized (Carlo Erba). Operating temperatures were: injection port 275°C, detector 275°C, column 242°C. The flow-rates were: nitrogen (carrier gas) 30 ml/min, hydrogen 35 ml/min, air 350 ml/min.

Extraction procedure

p-Tolylpiperazine (internal standard) was added to 1 ml of plasma (from citrated blood) in a centrifuge tube together with 2 ml of borate buffer (pH 9.0) and 6 ml of a mixture of benzene and methylene chloride (9:1). The tube was capped, shaken on a Vortex mixer for 3 min, then centrifuged at 1000 *g* and the supernatant transferred into a conical tube, in which it was concentrated at 50°C in a stream of dry nitrogen to a volume of about 20 μl for the determination of plasma levels of viloxazine in single-dose studies, or about 200 μl for the quantitative determination of the drug after chronic administration. Aliquots (1–3 μl) of these solutions were sampled using a 10-μl syringe previously loaded with 1 μl of acetic anhydride–ethanol (20:80) and injected on to the GC column.

Preparation of calibration curves

Two calibration curves were prepared by adding known amounts of viloxazine · HCl to 1 ml of blank plasma. Concentrations of the drug equivalent to 0.1, 0.5, 1, 1.5 and 2 μg/ml, and 1, 3, 5, 7 and 9 μg/ml as base, were measured, respectively.

An aliquot (100 μl) of internal standard aqueous solution was added to each spiked plasma. The internal standard concentrations were 0.75 μg/ml for the range 0.1–2 μg/ml viloxazine and 2 μg/ml for the range 1–9 μg/ml.

Clinical studies during chronic viloxazine administration

Five depressed patients were examined. Each subject received daily a 500-mg oral amount of viloxazine · HCl in divided doses (200 mg at 7 a.m., 200 mg at noon, 100 mg at 4 p.m.) for 26 days. Blood samples (10 ml), collected in polypropylene tubes containing about 300 mg of sodium citrate, were drawn at 3 p.m. on days 6, 11, 17, 21 and 26. The specimens were centrifuged and the plasma submitted to the GC procedure.

RESULTS AND DISCUSSION

The equations describing the standard curves, determined by linear least-squares regression analysis, were $y = 0.761x - 0.008$ and $y = 0.208x - 0.022$ for the ranges 0.1–2 and 1–9 μg/ml, respectively. The corresponding correlation coefficients (r) were 0.992 and 0.996. Table I shows the precision and accuracy of viloxazine determination in plasma.

TABLE I

ACCURACY AND PRECISON OF METHOD

First calibration curve*			Second calibration curve*		
Amount added (μg/ml)	Amount found (μg/ml, mean ± S.D.)	C.V. (%)	Amount added (μg/ml)	Amount found (μg/ml, mean ± S.D.)	C.V. (%)
0.1	0.11 ± 0.01	3.7	1	1.06 ± 0.07	6.6
0.5	0.51 ± 0.05	9.9	3	2.97 ± 0.10	3.4
1	0.98 ± 0.05	5.6	5	5.01 ± 0.25	5.0
1.5	1.51 ± 0.15	9.7	7	6.80 ± 0.32	4.7
2	2.00 ± 0.08	4.0	9	9.09 ± 0.30	3.3

*Five determinations for each point.

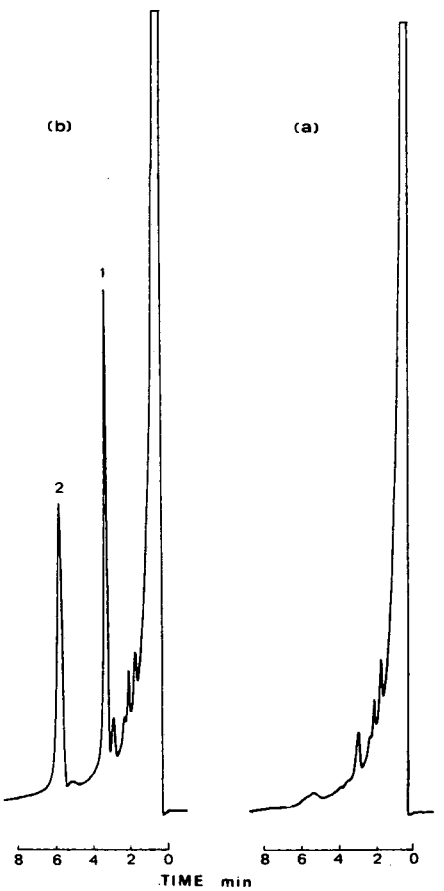

Fig. 1. Chromatograms obtained from a human plasma sample: (a) blank; (b) 2.5 h after oral administration of 50 mg of viloxazine • HCl. Peaks: 1 = internal standard (0.75 μg/ml), 2 = viloxazine (0.82 μg/ml).

The procedure employed gives a detection limit equivalent to 0.1 μg of viloxazine in 1 ml of plasma. Above this concentration, the plasma constituents do not interfere significantly with the internal standard and viloxazine, which were eluted as their acetyl derivatives after 3 min 27 sec and 5 min 47 sec, respectively. Typical chromatograms obtained from human plasma before and after a single oral administration of 50 mg of viloxazine are shown in Fig. 1. The mean (± S.D.) plasma levels found in the five depressed patients undergoing viloxazine therapy were 4.18 (± 1.41), 4.66 (± 2.72), 5.84 (± 2.43), 5.10 (± 1.33) and 5.00 (± 1.10) μg/ml after 6, 11, 17, 21 and 26 days of treatment, respectively.

Derivatization of viloxazine is required to shift the peak of the drug to a region of the chromatogram free from endogenous, interfering compounds. Thus, quantitative determination can be achieved after a single extraction step. Plasma concentration of viloxazine can also be evaluated without derivatization, although purification of the extract is required before GC analysis. In this case, another internal standard (chlorpheniramine) must be employed. The retention time of underivatized viloxazine in the chromatographic system described above (but with the oven temperature at 220° C) was 3 min 27 sec.

Derivatization before injection is not necessary. The acetylation of viloxazine and internal standard in the chromatographic column occurs readily and the speed of analysis is markedly increased. Injection of acetic anhydride causes a negligible influence on the response of the detector and a slight tailing of the solvent peak.

None of the other antidepressant drugs (amitriptyline, nortriptyline, imipramine, norimipramine, chlorimipramine, maprotiline, protriptyline and mianserin), checked using the same derivatization method, interfered with viloxazine and internal standard.

In conclusion, the proposed GC procedure for the quantitative determination of viloxazine in human plasma offers appreciable accuracy, precision and rapidity. The method is easily applicable to the monitoring of plasma concentrations of the drug during chronic treatment and is also sufficiently sensitive for its potential use in pharmacokinetic and bioavailability studies after a single administration.

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Note

Urinary dimethylbenzoic acid excretion as an indicator of occupational exposure to white spirit

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Distillates of crude oil are also used as solvents marketed under names such as Stoddard solvent, white spirit, mineral turpentine or solvent naphtha. Their compositions vary widely depending on the boiling ranges of the fractions. However, aliphatic alkanes dominate in the most versatile mixtures with boiling ranges of 150°C to 200°C. The complex composition of the mixtures — over 200 compounds are frequently encountered — does not allow the identification of the causative agents of the occupational diseases related to the exposure [1]. Our own experimental studies with rats implicate the aliphatic alkanes [2, 3].

Virtually all commercially available white spirit in Finland originates from a single refining company. Its own quality control has shown that the product with a boiling range of 152°C to 182°C has remained very stable in its composition over recent years. Typically, it contains 11% aromatics with 1% trimethylbenzene isomers by weight [2]. When absorbed in the animal body, the

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relative concentrations of individual compounds remain comparable to those in the original liquid without an accumulation. Specifically, the total body burden in experimentally exposed rats is correlated to the inhaled dose [2].

Exogenous lipophilic chemicals are oxidized in the body to render them water-soluble for the excretion in the urine or faeces. The metabolites of aliphatic and alicyclic molecules have been characterized to some extent [4, 5]. Their analysis presents, however, many technical problems while the demonstration of aromatics is relatively simple because of their ultraviolet absorbing properties. Thus, trimethylbenzene-derived metabolites in the rabbit have shown to include dimethylbenzoic acid isomers [6]. Analogous to this, excretion of the same acid isomers by white spirit-exposed rats correlates to the absorbed dose despite the fact that the parent trimethylbenzene isomers represent a minor fraction of the mixture [3].

This communication describes a novel liquid chromatographic method for the quantitation of occupational exposure to white spirit vapour by the analysis of the dimethylbenzoic acid isomers.

SUBJECTS AND METHODS

Ten car washers participated in the study. They were divided into three groups according to their exposure to white spirit. Five people were exposed to 118—150 mg/m^3, three to 152—234 mg/m^3 and two to 420—500 mg/m^3 white spirit vapour calculated as a 6-h time-weighted average concentration in their breathing zone (Niemelä et al. [7]). All were exposed to vapour evaporated from the mist applied by air guns to remove organic stain from the car chassis. The hygienic measurements were carried out on Thursdays, and the urine specimens were collected after the shift in the afternoon of the same days.

The urine samples (5 ml) were boiled in stoppered tubes for 1 h after the addition of 5 ml of 50% sodium hydroxide to hydrolyse the dimethylbenzoic acid glycine conjugates [6]. The hydrolysates were neutralized with sulphuric acid and extracted three times with dichloromethane. The extracts were combined, dried with sodium sulphate and evaporated under vacuum. The residue was dissolved in an acetic acid—water—methanol mixture (2 ml, 1:39:60, v/v/v). 2,3-, 2,4-, 2,5-, 3,4- and 3,5-dimethylbenzoic acids served as standards in the liquid chromatographic analysis. The apparatus (Pye Unicam LC 3-XP) was equipped with a 20-μl sample loop and a 15-cm column (5 μm ODS Hypersil, Shandon). An isocratic separation mode was used with the acetic acid—water—methanol (1:39:60, v/v/v) mixture as an eluent. A UV detector (Pye Unicam LC) was empoyed at 238 nm. This procedure yielded a good separation of 2,3- and 3,5-dimethylbenzoic acids while the other isomers migrated as a combined peak (see Fig. 1). The recovery of added isomers (2.5 μg/ml, six experiments) in the extraction and analysis was 95 ± 1%. The coefficient of variation at the same concentration level was 0.11 ± 0.01.

To find out the correlation with the vapour exposure, all isomer concentrations were added together, and the sum was corrected for the creatinine excretion determined by the alkaline picric acid method. The least-squares method was used to find out the mathematical correlation.

RESULTS AND DISCUSSION

No dimethylbenzoic acid was detected in the unexposed controls (Fig. 1) while a good separation of the acid isomers from the solvent front and other aromatic constituents was found in the exposed subjects. The mass spectral identification of the peaks revealed configurations reported for the isomers [6].

The mean excretion rates by the exposed workers were linearly correlated to the total hydrocarbon exposure (Fig. 2). The ranges in the excretion are very likely caused by the biological variables as the accuracy of the method is good. The hygienic correlations were done to the time-weighted average which tends to smooth out the effect of the solvent peaks associated with the spray gun operations. On the other hand, the body functions in a way as an integrator [8].

The biological monitoring method offers several advantages in comparison to the hygienic surveys. The exposure is difficult to characterize because the hydrocarbons evaporate rapidly from the sprayed mist [9] causing sharp peaks in the exposure profile. The urine test is often acceptable to the workers, and it relates better to the individual exposure than hygienic measurements so that the exposure may be more accurately evaluated.

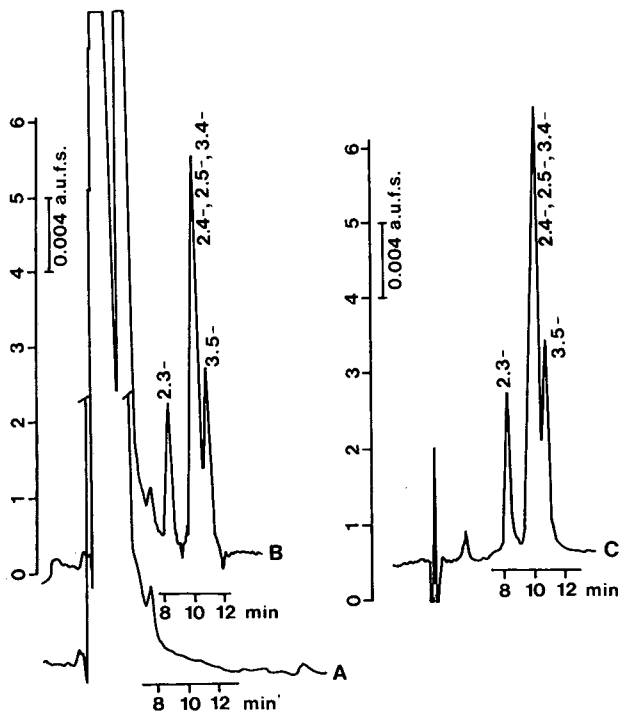

Fig. 1. Liquid chromatographic analysis of dimethylbenzoic acid isomers in urine of a white spirit vapour-exposed worker (B) and of unexposed control (A). 2,3- (2.3 μg/ml) and 3,5-dimethylbenzoic acid (2.2 μg/ml) elute at 8.1 min and 10.5 min, respectively, while 2,4-, 2,5- and 3,4-dimethyl isomers (2.2, 2.4 and 2.0 μg/ml, respectively) at 10.0 min in the standard preparation (C) migrate combined. Note that the control urine does not contain dimethylbenzoic acid.

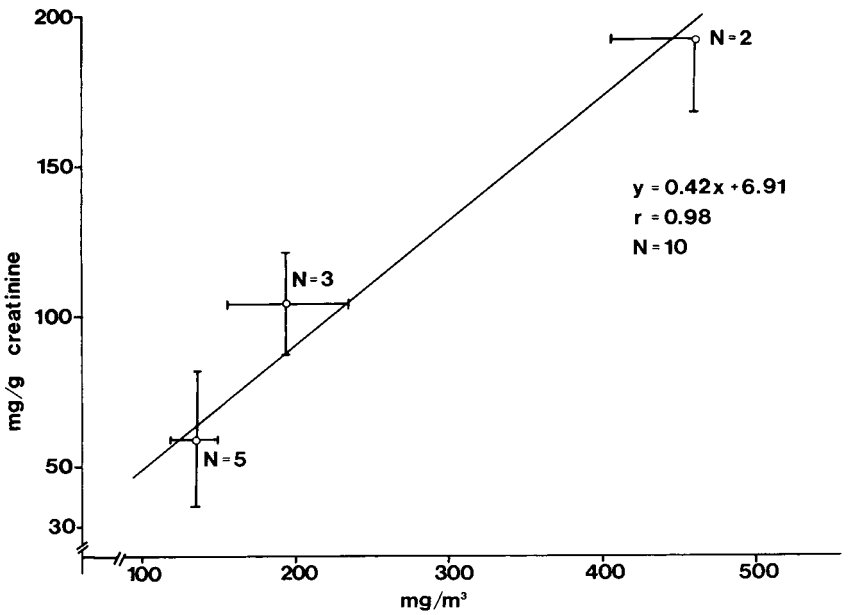

Fig. 2. The white spirit vapour exposure (x) is correlated to the sum concentration of urinary dimethylbenzoic acid isomers (y) in a linear fashion given by the equation. Bars indicate ranges and N the number of subjects. The correlation coefficient to the mean concentration is given by r.

It is likely that the trimethylbenzene isomers in the white spirit mixture have a negligible role in its toxicity while the aliphatic n-nonane may be significant in this respect [3]. The analysis of its metabolites is hampered by the fact that they are in all probability fatty acids and alcohols whose pharmacokinetics and participation in the intermediary metabolism are unknown. Therefore, the dimethylbenzoic acid isomers offer a versatile alternative despite their minor share of the total mixture.

As discussed above, the trimethylbenzene shows similar kinetics to those of the general white spirit as a whole. Therefore, the excretion is dependent on the concentration and composition of the inhaled vapour. If the white spirit originates always from a single producer there is no practical limitation on the reliance on the biological monitoring method. The usage of aromatic-free mixtures, of course, abolishes this possibility. The result is the same when employing products with different boiling ranges possibly originating from different refineries. Therefore, the analysis of excretion of the metabolites with a particular white spirit brand should be carried out before the adoption of the biological exposure test.

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Note

Rapid Extrelut column method for determination of levamisole in milk using high-performance liquid chromatography

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Levamisole (Fig. 1) is a broad-spectrum anthelmintic drug used in cattle, sheep and pigs. Methods have been developed for levamisole quantification in biological fluids, based on polarographic [1], gas—liquid chromatographic (GLC) [2, 3] and high-performance liquid chromatographic (HPLC) [4, 5] procedures. A sensitive GLC method has been reported for the determination of levamisole in milk [6, 7]. However, this method is too time-consuming for determining residues of levamisole in a large number of milk samples.

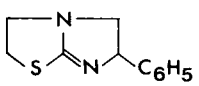

Fig. 1. Chemical structure of levamisole.

The purpose of this study was to develop a rapid, simple, and sensitive method for the determination of levamisole in milk. The method described is based on clean-up on an Extrelut column and quantitation by a HPLC procedure.

EXPERIMENTAL

Chemicals and reagents

Dichloromethane and hexane, both for pesticide residue analysis, were obtained from E. Merck (Darmstadt, F.R.G.), and levamisole hydrochloride from American Cyanamid (Princeton, NJ, U.S.A.). Levoripercol® vet (88.4 mg levamisole hydrochloride per ml) was purchased from AB Leo (Helsingborg, Sweden). All other chemicals were of analytical reagent grade.

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Standards

A stock solution of levamisole (1 mg/ml) in methanol was prepared using the hydrochloride salt. For HPLC calibration, the stock solution was diluted with methanol—0.05 *M* ammonium carbonate solution (65:35, v/v) to obtain standard solutions (1.0, 2.5, 5.0, 10, 20 and 40 μg/ml). For recovery experiments, various standard solutions of levamisole in methanol were prepared. The stock solution and all standard solutions were stored at about 4°C.

Apparatus

The liquid chromatograph consisted of a ConstaMetric III pump module with a SpectroMonitor III variable-wavelength ultraviolet (UV) detector (LDC, Riviera Beach, FL, U.S.A.), and a Valco N60 injector (Valco, Houston, TX, U.S.A.) with a 22-μl loop. The column (250 × 4.6 mm I.D.) was packed with TSK-GEL LS 410 ODS SIL (C_{18} chemically bonded silica gel, particle size 5 μm) (Toyo Soda, Tokyo, Japan).

Chromatographic conditions

The mobile phase was methanol—0.05 *M* ammonium carbonate solution (65:35, v/v), and the flow-rate was 1.0 ml/min. The wavelength of the UV detector was set at 220 nm. The system was operated at room temperature.

Extraction procedure

To 50 ml of milk was added 0.3—0.4 ml of 3 *M* hydrochloric acid to adjust the pH to about 4.6. The suspension was mixed with 15 ml of methanol and heated in a stoppered flask on a water-bath (50°C) for 15 min. The sample was then filtered through a folded filter paper. Of the filtrate 20 ml were applied to the top of an Extrelut 20 prepacked column (E. Merck), and after 15 min the column was washed with 100 ml of hexane. Then the column was eluted with 100 ml of dichloromethane. The eluate was evaporated to dryness in a rotary evaporator, and the residue was dissolved in 1.0 ml of methanol—0.05 *M* ammonium carbonate solution (65:35, v/v). A 22-μl aliquot was injected onto the column.

Calibration curve

The calibration curve was constructed by plotting the peak areas versus the concentrations. The concentrations of unknowns were calculated from peak area ratios by interpolation of the calibration curve.

Recovery study

To measure recovery, various concentrations of levamisole were added to milk and the samples were extracted as described above. The percentage recovery was determined by comparing the peak areas of levamisole extracted from samples with peak areas obtained by direct injection of standard solutions.

RESULTS AND DISCUSSION

The earlier reported GLC method for the determination of levamisole in milk is based on several extraction steps and is rather time-consuming [6, 7]. The use of Extrelut for clean-up, after precipitation of the proteins, was found to be a rapid and simple method for extraction of levamisole in milk samples. The HPLC system used is similar to that earlier reported by Marriner et al. [4] for the determination of levamisole in plasma and gastrointestinal fluids. However, Marriner et al. also use a time-consuming clean-up procedure, which was not suitable for our purpose. Representative chromatograms of milk samples are shown in Fig. 2. In the chromatogram obtained from a blank milk sample (Fig. 2A), no peaks are present which might interfere with the determination of levamisole. Fig. 2B shows a chromatogram of an extract of milk spiked with 0.4 μg/ml levamisole. The retention time of levamisole was about 6—8 min.

The retention time varied slightly from day to day owing to instability of the mobile phase and changes of temperature in the laboratory. The retention time of levamisole was always shifted when a fresh mobile phase was used.

To prevent incorrect identification of levamisole, we constructed a new calibration curve every day. The calibration curve was linear, $y = 2.51x + 0.25$ ($n = 6$) over the range 1.0—40.0 μg/ml, with a correlation coefficient of 0.999. The average recovery of milk samples spiked with 0.2—2.0 μg/ml levamisole (Table I) corresponded to 78 ± 7.1% (mean ± S.D., $n = 11$). The limit for determination of levamisole was found to be between 0.02 and 0.05 μg/ml.

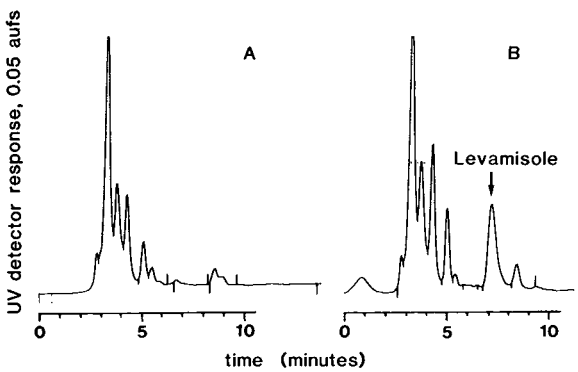

Fig. 2. Chromatograms of an extract of (A) blank milk, (B) milk spiked with levamisole (0.4 μg/ml).

TABLE I

RECOVERY OF LEVAMISOLE FROM MILK

Amount added (μg/ml)	No. of determinations	Recovery (%, mean ± S.D.)	Coefficient of variation (%)
0.2	3	76 ± 10.8	14.2
0.4	4	80 ± 5.9	7.4
2.0	4	77 ± 6.4	8.3
Mean		78 ± 7.1	9.1

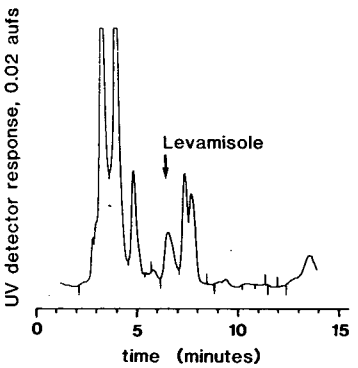

Fig. 3. Chromatogram of an extract of milk from a cow 24 h after administration of 7.1 mg of levamisole hydrochloride per kg body weight.

TABLE II

LEVAMISOLE RESIDUES IN MILK FROM TWO COWS AFTER ADMINISTRATION OF LEVAMISOLE HYDROCHLORIDE (7.1 mg/kg BODY WEIGHT)

Time after treatment (h)	Levamisole conc. (μg/ml)	
	Cow 1*	Cow 2**
0	ND***	ND
0.25	0.02	0.02
8	0.76	0.93
16	0.26	0.15
24	0.06	0.03
48	ND	ND
72	ND	ND
96	ND	ND

*Cow weight = 539 kg.
**Cow weight = 460 kg.
***ND = not detected = less than 0.02 μg/ml.

For routine analysis of a large number of milk samples, the recovery, precision and limit of determination of the described method are satisfactory. The addition of a suitable internal standard prior to extraction would permit a better precision of the method if required. (±)-2,3,5,6-Tetrahydro-6(4-methylphenyl)imidazo[2,1-*b*]thiazole hydrochloride (R-8493) has previously been used as internal standard in two GLC methods [2, 3].

The method has been applied to study levamisole residues in the milk of cows injected with this drug. Levamisole hydrochloride (Levoripercol vet) was administered subcutaneously at a dose of 7.1 mg/kg body weight, the usual therapeutic dose. Milk samples were collected in duplicate before the injection and at intervals up to 96 h after treatment. Fig. 3 shows a chromatogram of an extract of milk from a cow 24 h after administration of 7.1 mg of levamisole hydrochloride per kg body weight. The maximum level of levamisole was found 8 h after the administration and the levamisole level was less than 0.02 μg/ml at 48 h (Table II). In Sweden the recommended withdrawal period for

levamisole is two days for milk. Thus the present method is suitable for the rapid determination of levamisole residues in milk samples to check that this recommendation is followed.

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Note

Liquid chromatographic assay of isoxicam in human plasma and urine

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Isoxicam is a long-action non-steroidal anti-inflammatory agent which has been reported to be effective in the treatment of osteo-arthritis and rheumatoid arthritis [1—3]. It is a member of the oxicam class of anti-inflammatory compounds, a group that differs structurally from other types of non-steroidal anti-inflammatory drugs [4].

A method for estimation of isoxicam in plasma and urine using high-performance liquid chromatography (HPLC) has been reported recently [5]. The present paper describes an alternative reversed-phase liquid chromatographic assay for isoxicam in plasma and urine which can be more rapidly and simply performed. The procedures have been employed successfully in multiple-dose pharmacokinetic studies of isoxicam in man [6].

MATERIALS AND METHODS

Chemicals and solutions

With the exception of acetonitrile and tetrahydrofuran which were HPLC grade (Ajax Chemicals, Melbourne, Australia), all chemicals used were of analytical quality. Water was distilled. Isoxicam was supplied as pure powder by Parke-Davis (Australia). Piroxicam (Pfizer, Australia) and diazepam (Roche Products, Australia) were used as internal standards for the assays.

Stock solutions of isoxicam were prepared in acetonitrile containing 10% tetrahydrofuran, at concentrations ranging from 2 to 100 μg/ml. Stock solutions of the internal standards, piroxicam (100 μg/ml) and diazepam (40 μg/ml) were prepared in acetonitrile. These solutions were stable for at least six months when stored at 4°C.

The following aqueous solutions were used: (a) 0.05 mol/l potassium

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dihydrogen orthophosphate, pH adjusted to 3.0 with orthophosphoric acid; and (b) saturated potassium dihydrogen orthophosphate, pH adjusted to 2.4 with orthophosphoric acid.

Sample preparation

Plasma samples (100 μl) were pipetted into disposable polypropylene Wasserman tubes containing 50 μl of a 100 μg/ml stock solution of piroxicam, the internal standard. After addition of 300 μl acetonitrile, the tubes were vortex-mixed vigorously for 1 min, then subjected to centrifugation at 2000 *g* for 2 min. A 20-μl volume of the supernatant was injected into the chromatograph.

Urine specimens (1.0 ml) containing 50 μl of a 40 μg/ml stock solution of diazepam as the internal standard and 100 μl of saturated phosphate buffer (pH 2.4) were extracted for 2 min with 3 ml dichloromethane using a multi-tube vortex-mixer (Model 2601, Scientific Manufacturing Industries, Emeryville, CA, U.S.A.). After centrifugation at 2000 *g* for 2 min, the organic phase was transferred to a tapered glass tube and evaporated to dryness at 50°C under a gentle stream of nitrogen. The residue was reconstituted with 50 μl of acetonitrile of which 20 μl were injected into the chromatograph.

Assay calibration

The assays were calibrated by addition of stock solutions of isoxicam and the internal standard piroxicam, to pooled blood blank plasma, and by addition of stock solutions of isoxicam and the internal standard, diazepam, to drug-free urine specimens. Calibration curves were established for concentrations of isoxicam up to 100 μg/ml in plasma, and up to 2 μg/ml in urine. Peak area ratios of isoxicam to the appropriate internal standard were used in quantitation of the assays.

Chromatographic analyses

The liquid chromatograph consisted of a Varian Model 5000 high-pressure pumping system (Palo Alto, CA, U.S.A.), a Rheodyne Model 7120 injection valve with a 20-μl loop (Berkeley, CA, U.S.A.), a Varian Vari-chrom ultraviolet absorbance detector and a Hewlett-Packard Model 3380A integrator (Palo Alto, CA, U.S.A.).

Analyses were performed at ambient temperature on an octadecylsilane (ODS) reversed-phase column (Hibar LiChrosorb, 10 μm particle size, RP18, 250 mm × 4 mm, E. Merck, Darmstadt, F.R.G.). For analyses of isoxicam in plasma, the mobile phase was acetonitrile—0.05 mol/l potassium dihydrogen phosphate buffer, pH 3.0 (50:50) delivered at a flow-rate of 2 ml/min. Ultraviolet absorbance at 325 nm was monitored at a sensitivity of 0.05 a.u.f.s. For analyses of isoxicam in urine, the mobile phase was acetonitrile—0.05 mol/l potassium dihydrogen phosphate buffer, pH 3.0 (45:55). All other chromatographic conditions remained the same as for plasma analyses.

RESULTS AND DISCUSSION

Typical chromatograms of extracted plasma specimens (Fig. 1) show that control samples are free from interfering peaks. Retention times for isoxicam and the internal standard, piroxicam, were 3.1 and 2.4 min, respectively. Since

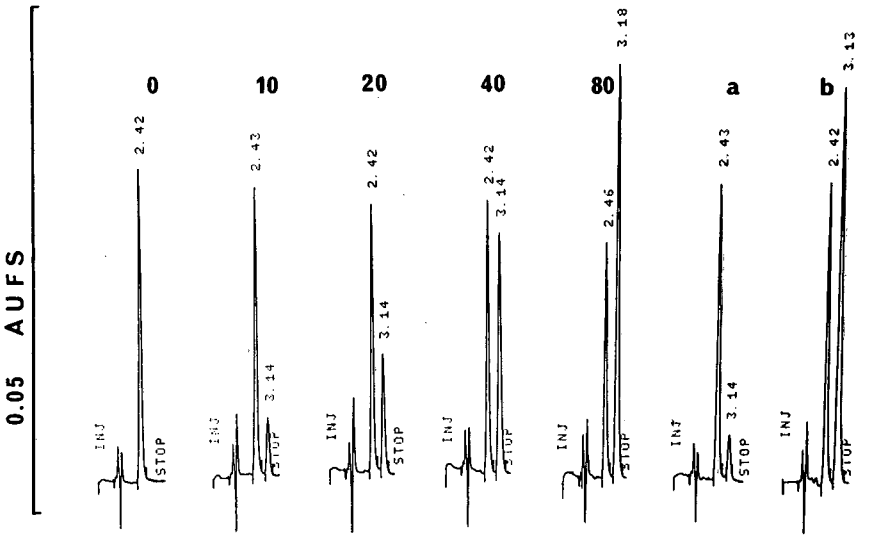

Fig. 1. Chromatograms of human plasma extracts: 0—80 μg/ml isoxicam in pooled blood bank plasma; (a) 6.5 μg/ml and (b) 60.3 μg/ml isoxicam in plasma from a subject who had received medication with isoxicam. Retention times for isoxicam and the internal standard, piroxicam, were 3.1 min and 2.4 min, respectively.

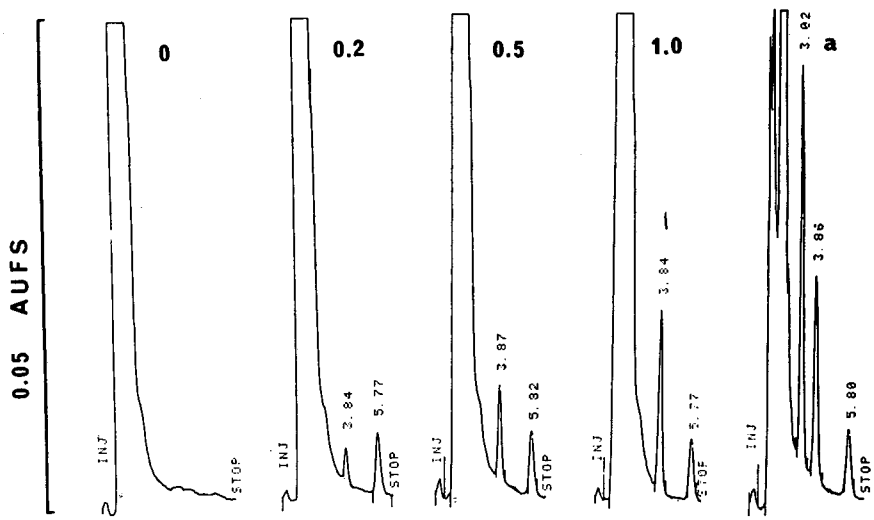

Fig. 2. Chromatograms of human urine extracts: 0—1.0 μg/ml isoxicam in drug-free urine; (a) 0.95 μg/ml isoxicam in urine from a subject who had received medication with isoxicam. Retention times for isoxicam and the internal standard, diazepam, were 3.8 and 5.8 min, respectively.

no endogenous substances with prolonged retention on-column were detected, injection of specimens could be repeated immediately after the elution of isoxicam.

Typical chromatograms of urinary extracts (Fig. 2) show that control samples are free from interfering peaks. Isoxicam eluted in 3.8 min and the internal standard, diazepam, in 5.8 min. Endogenous contaminants remaining in the extracts were eluted before isoxicam. Injections of urinary extracts could

therefore be repeated immediately after elution of the internal standard. Extracts of urine from subjects who had been receiving regular oral doses of isoxicam contained an additional substance which eluted at 3.0 min (Fig. 2). This was assumed to be a metabolite of isoxicam. Since it co-eluted with piroxicam, the latter was unsuitable as an internal standard in the urinary analyses.

For both plasma and urinary assays, calibration curves for isoxicam passed through the origin. They were linear up to 100 μg/ml in plasma and up to 2 μg/ml in urine, the maximum concentrations used. For a signal-to-noise ratio of 4, the minimum detectable concentration of isoxicam in plasma was 0.2 μg/ml and in urine was 0.02 μg/ml.

Recovery of isoxicam from plasma and urine following extraction was assessed by comparison of peak areas from the extracts with those arising from standard solutions of isoxicam in acetonitrile. At a plasma isoxicam concentration of 0.5 μg/ml, recovery was 96%. For isoxicam at plasma concentrations between 10 and 100 μg/ml, mean recovery was 94%. The mean difference beween results of duplicate analyses was 6.1% (n = 60). The inter-assay coefficient of variation was 10.0% (n = 10) at a plasma isoxicam concentration of 0.5 μg/ml, 4.1% (n = 16) at 20 μg/ml, and 2.6% (n = 9) at 80 μg/ml. For isoxicam in urine, recovery was 89% for concentrations between 0.2 and 2 μg/ml. The inter-assay coefficient of variation at an isoxicam concentration in urine of 0.2 μg/ml was 7.1% (n = 9).

The practical effectiveness of the assay was demonstrated by assaying blood and urine specimens obtained from twenty human subjects who had received 200 mg isoxicam daily for up to fifteen days. Steady-state plasma concentrations ranged from 12.2 to 85.9 μg/ml and urinary concentrations ranged from 0.10 to 1.76 μg/ml. No interference with the chromatographic measurement of isoxicam was observed despite concurrent medication of these subjects with a wide variety of drugs including furosemide, chlorothiazide, hydrochlorothiazide, propranolol, metoprolol, prazosin, methyldopa, hydralazine, labetolol, minoxidil, allopurinol, sulphinpyrazone, digoxin, naproxen, doxepin, imipramine, trifluoperazine, cotrimoxazole, cephalexin and mepenzolate.

The HPLC method described here permits selective determination of isoxicam in both plasma and urine. Since sample preparation and chromatographic run times are short, the procedure is suitable for use in clinical research involving large numbers of specimens — 60 plasma or 40 urine specimens may be assayed in duplicate per day. The sensitivity and reproducibility of the assay procedure have proven sufficient for pharmacokinetic studies [6].

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Note

Simultaneous quantitation of verapamil, norverapamil, and N-dealkylated metabolites in human plasma following oral administration

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Verapamil, 5-[(3,4-dimethoxyphenylethyl)methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile (I), a slow channel inhibitor, undergoes extensive hepatic metabolism after an oral dose with some of its metabolites reaching concentrations in plasma equal to or greater than those of verapamil [1]. Major metabolites include the N-demethylated compound, norverapamil (III), and two N-dealkylated compounds, 5-methylamino-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile (IV) and 5-amino-2-(3,4-dimethoxyphenyl)-2-isopropylvaleronitrile (V), shown in Fig. 1. Norverapamil in particular has been reported to have a coronary vasodilator potency of 20% compared to that of verapamil [2].

Six other methods, all with various limitations, have been published addressing the separation of verapamil from its metabolites and their quantitation by high-performance liquid chromatography (HPLC). The original procedure of Harapat and Kates [3] was suitable for the separation of two N-dealkylated metabolites but was inadequate for the quantitation of norverapamil. Jaouni et al. [4] modified this procedure and acetylated norverapamil to prevent its extraction and interference with verapamil. Harapat and Kates [5] also modified their original procedure and thereby were able to measure norverapamil but not the N-dealkylated metabolites. Cole et al. [6] quantitated verapamil, norverapamil and the N-dealkylated metabolites but required potassium bromide, which is corrosive to stainless-steel solvent delivery systems, as a component of the mobile phase. Piotrovski et al. [7] separated the same four compounds but only quantitated verapamil. Kuwada et al. [8] have reported the separation and quantitation of verapamil, five O-demethylated metabolites and the two N-dealkylated metabolites, but did not report the separation of norverapamil.

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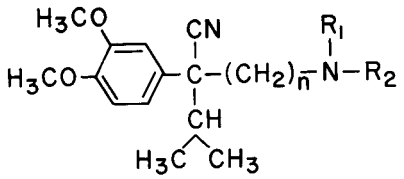


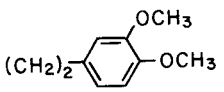
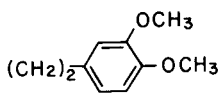
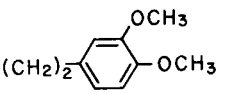
	n	R_1	R_2
I	3	CH_3	
II	2	CH_3	
III	3	H	
IV	3	CH_3	H
V	3	H	H

Fig. 1. Structural formulae of verapamil (I), internal standard (II), norverapamil (III), and two N-dealkylated metabolites (IV and V).

This laboratory has previously reported a rapid, simple, and sensitive method for the extraction of verapamil and its internal standard (II) from plasma with the subsequent quantitative determination of verapamil by HPLC separation and fluorescence detection [9]. The present study confirms the suitability of this method for the simultaneous separation and quantitative determination of verapamil as well as norverapamil, and the N-dealkylated metabolites IV and V, in human plasma following oral administration.

EXPERIMENTAL

Verapamil hydrochloride (I) was obtained from Knoll Pharmaceutical (Whippany, NJ, U.S.A.). Its internal standard, 4-[(3,4-dimethoxyphenylethyl)-methylamino]-2-(3,4-dimethoxyphenyl)-2-isopropylbutyronitrile (II), norverapamil (III), and IV and V, were obtained as the hydrochloride salts from Knoll (Ludwigshafen, F.R.G.). Glass-distilled heptane (Mallinckrodt, St. Louis, MO, U.S.A.) and HPLC-grade acetonitrile (MCB, Cincinnati, OH, U.S.A.) were used. Sodium hydroxide (2 *M*), sulfuric acid (0.1 *M*), and 0.1 *M* phosphate buffer, pH 3.0 were prepared with deionized water.

Other reagents and instruments, as well as the extraction, separation, and detection procedures were identical to our previous report [9]. A liquid chromatograph (Model 33, Altex, Berkeley, CA, U.S.A.) was used with a

fluorescence detector (Model 970, Altex). The column (15 cm × 4.6 mm I.D., particle size 5 μm) (Ultrasphere ODS, Altex) was a C_{18} reversed-phase. The only exception was a change in the proportions of the mobile phase to acetonitrile—0.1 *M* potassium phosphate, pH 3.0 (34:66). The solvent was degassed prior to use by applying a vacuum. The flow-rate was set at 1 ml/min. The detector settings were 203 nm for excitation with a 320-nm emission filter.

To 0.5 ml of plasma were added 50 ng of internal standard, II, 0.2 ml of 2 *M* sodium hydroxide, and 3 ml of heptane. The mixture was shaken mechanically for 15 min and then centrifuged. The organic layer was transferred to a 5-ml conical tube, and 50 μl of 0.1 *M* sulfuric acid were added. The contents were mixed in a vortex mixer for 1 min and centrifuged, and a 10-μl sample was injected into the chromatograph.

RESULTS AND DISCUSSION

An extracted blank plasma sample is shown in Fig. 2A. In Fig. 2B a representative chromatogram is shown of a 0.5-ml plasma sample spiked to the following concentration: I = 94 ng/ml; internal standard, II = 85 ng/ml; III = 92 ng/ml; IV = 75 ng/ml; V = 90 ng/ml. The retention times for I, II, III, IV, and V are 14.8, 12.2, 13.3, 3.9, and 3.3 min, respectively. The concentrations of I, III, IV, and V, as determined from the chromatogram were 95, 95, 73 and 84 ng/ml respectively.

Fig. 3 shows the calibration curves obtained from spiked plasma samples containing verapamil, III, IV and V in concentrations up to 400 ng/ml. Samples that had lower verapamil concentrations also had lower concentrations of the metabolites. Peak height ratios were plotted as a function of the concentration of each compound and were linear within this range ($r \geqslant 0.99$). The

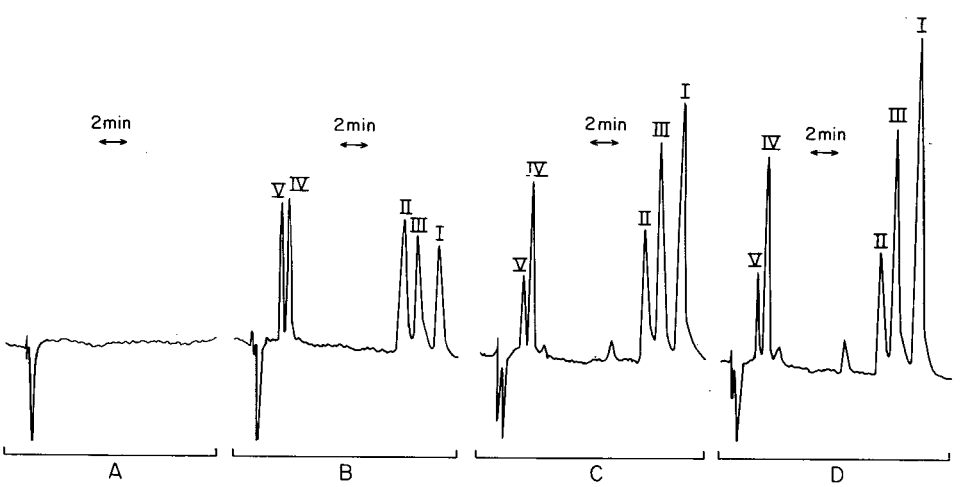


Fig. 2. Chromatograms of the extracts of 0.5-ml plasma samples. A = blank plasma; B = spiked plasma; C = patient plasma after four doses of oral verapamil, 120 mg every 6 h; D = plasma from the same patient as C, after one month of such oral verapamil therapy. Peaks: I = verapamil; II = internal standard; III = norverapamil; IV and V = N-dealkylated metabolites. Concentrations of the compounds are given in the text.

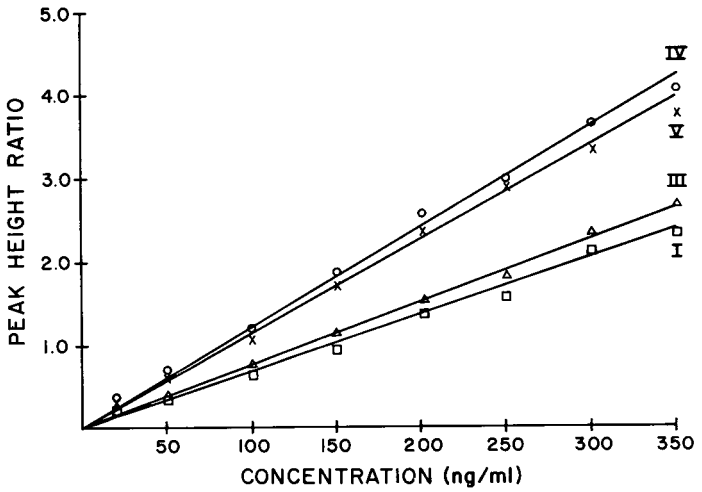


Fig. 3. Calibration curves of verapamil (I, □), norverapamil (III, △), and N-dealkylated metabolites (IV, ○ and V, ×).

coefficients of variation for the normalized peak height ratios of compounds I, III, IV and V over a concentration range of 10—500 ng/ml, assayed on seven different days over a two-week period, were 4.9%, 4.1%, 6.8%, and 6.9%, respectively. To evaluate intra-assay reproducibility, repeated analyses were performed of spiked plasma samples (n = 8) containing 94 ng/ml verapamil, 92 ng/ml III, 75 ng/ml IV, and 90 ng/ml V, to which was added 85 ng/ml internal standard, with coefficients of variation of 3.6%, 4.0%, 4.4% and 5.7%, respectively.

The recoveries of the four compounds were determined by comparing the peak heights of chromatograms obtained from extracted and directly injected samples of each compound at concentrations of 40, 120, and 350 ng/ml. The results are given in Table I, showing the consistency of compound recovery over the concentration range of interest. The minimum detectable concentration for the simultaneous determination of the five compounds was approx. 4 ng/ml of plasma, by requiring a signal-to-noise ratio of greater than 5:1.

Chromatograms of extracted plasma samples from a 46-year-old male patient receiving 120 mg of verapamil orally, every 6 h, for the treatment of

TABLE I

RECOVERY OF VERAPAMIL, INTERNAL STANDARD, AND METABOLITES FROM PLASMA

Concentration in plasma (ng/ml)	n	Percent recovery* (mean ± S.E.)				
		I	II	III	IV	V
40	4	63 ± 1	68 ± 1	65 ± 3	66 ± 1	51 ± 1
120	5	61 ± 3	64 ± 3	62 ± 4	65 ± 2	48 ± 2
350	4	65 ± 2	67 ± 2	67 ± 3	66 ± 2	48 ± 2

*I = verapamil; II = internal standard; III = norverapamil; IV and V = N-dealkylated metabolites.

paroxysmal atrial tachycardia refractory to other modes of therapy are shown in Fig. 2C and D. Fig. 2C is the chromatogram of plasma drawn after the first four doses of oral verapamil therapy. The concentrations of verapamil, III, IV and V after four doses (Fig. 2C) are 220, 175, 76, and 43 ng/ml, and in the one-month sample (Fig. 2D) are 320, 217, 100, and 56 ng/ml, respectively. The patient was taking no other medication of record to explain the peak noted between IV and II in Fig. 2C and D with a retention time of 9.5 min, which may represent a minor metabolite; however, it does not interfere whatsoever with the interpretation of peaks I through V.

The detailed evaluation of verapamil pharmacokinetics with regard to metabolism and disposition after oral administration depends upon a sufficiently sensitive and selective analytical method for the determination not only of verapamil, but of the major metabolites found in significant concentrations in the plasma of patients undergoing oral therapy. The contribution of active metabolites of antiarrhythmic agents to unanticipated drug efficacy or toxicity, as well as their accumulation with abnormalities of renal or hepatic function, has been recently reviewed [10]. Impaired hepatic function has been demonstrated to prolong the elimination phase after intravenous verapamil administration [11], but even in patients with normal hepatic and renal function, the elimination of both verapamil and norverapamil has been found to be prolonged during chronic oral therapy [12]. Measurements of plasma concentrations of verapamil and metabolites during oral therapy, therefore, may help decrease the frequency of adverse drug effects and improve the likelihood of successful therapy.

Toward these ends, the present method has been satisfactorily used to quantitatively determine verapamil as well as three major metabolites in human plasma. Following bolus intravenous verapamil administration, when no metabolites are anticipated, plasma samples can be rapidly analyzed [9]. For samples following long-term or oral administration, it is important to increase the aqueous fraction of the mobile phase during chromatography to provide for adequate separation of verapamil from norverapamil, with the concomitant determination of IV and V. With this modification the total retention time is still only 15 min. The suitability of this method for the assay of verapamil plus the three metabolites in multiple samples daily has the potential to significantly contribute to the understanding of interpatient pharmacokinetic differences and to aid the individualization of dosage and scheduling during oral therapy with verapamil.

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Note

High-performance liquid chromatographic analysis of amodiaquine in human plasma

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The substituted 4-aminoquinoline, amodiaquine, has been available for antimalarial therapy for 40 years [1]. Although more potent both in vitro and in vivo than chloroquine [2], amodiaquine has only recently assumed significance

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as an antimalarial agent consequent to its successful treatment of chloroquine-resistant *Plasmodium falciparum* [3].

In spite of the long standing availability of this compound, there is a dearth of information concerning its pharmacokinetics in man. This is in part due to the lack of suitably selective and sensitive methods of analysis [4, 5] and also until very recently to the limited clinical interest in amodiaquine [3].

This report describes the high-performance liquid chromatographic (HPLC) analysis of amodiaquine in human plasma. In addition. we have applied the method to the measurement of amodiaquine in samples derived from two patients in Thailand undergoing antimalarial therapy with this drug.

EXPERIMENTAL

Reagents

Amodiaquine hydrochloride was supplied by Parke Davis & Co. (Pontypool U.K.) and the internal standard, 6-methoxy-8-aminoquinoline by Aldrich (Gillingham, U.K.). Ammonia solution (specific gravity 0.88), triethylamine and orthophosphoric acid were obtained from BDH (Poole, U.K.). Pronalys AR grade diethyl ether and HPLC-grade methanol were supplied by Fisons (Loughborough, U.K.).

Instrumentation

The method was developed on a constant-flow high-performance liquid chromatograph (Spectra Physics, St. Albans, U.K.). This system consisted of a solvent delivery system (Model SP 8700) with an organiser module (Model SP 8750) equipped with a Rheodyne valve injector and was coupled to a fixed-wavelength ultraviolet absorbance detector operating at 340 nm (Waters Assoc., U.K.; Model 441). The reversed-phase plastic column was obtained prepacked (μBondapak Rad-Pak Phenyl, 10-μm particles; 10 cm × 8 mm I.D.; Waters Assoc.) and was housed in a radial compression module (Z module; Waters Assoc.).

Chromatography

The mobile phase consisted of water—methanol (73:27, v/v) containing triethylamine (1%), adjusted to pH 2.8 with orthophosphoric acid. Chromatography was carried out at a flow-rate of 3 ml/min which was associated with a back-pressure of 190 kPa.

Plasma treatment procedure

To samples of plasma (1.0 ml) containing internal standard (200 ng) was added ammonia solution (2 ml). This mixture was extracted twice by mechanical tumbling for 15 min with diethyl ether (5 ml). After centrifugation (1000 *g* for 10 min) and separation, the organic phases were combined and evaporated to dryness under a steady stream of nitrogen at 25° C. The residue was reconstituted in the mobile phase (55 μl) and 50 μl of this were injected on the column.

All glassware was pretreated with dichlorodimethylsilane in toluene (5%, v/v) in order to minimise amodiaquine adsorption. To avoid photodecomposition

of the drug, extraction tubes were wrapped in aluminium foil throughout the sample treatment procedures.

Analytical recovery and assay precision

The analytical recovery of amodiaquine and the internal standard was estimated by comparing the peak height of an extracted plasma sample containing a known amount of the substance with the peak height of an aqueous solution containing the same amount of each compound. The intra- and inter-assay precision were determined by replicate assays of samples from a pool of spiked plasma.

Clinical study

Two Thai patients (24 and 25 years, weighing 47 and 75 kg, respectively) with proven infection with uncomplicated *Plasmodium falciparum* malaria, were admitted to the intensive care ward of the Pra Pikklao Hospital (Chantaburi, Thailand). Each subject received an initial dose of 10 mg/kg amodiaquine followed by 5 mg/kg daily for three days. All doses were given as 4-h intravenous infusions. Venous blood samples (10 ml) were withdrawn prior to the first infusion, and then after this infusion at 0, 10, 30 min and at 2, 6, 12 and 20 h. Blood was collected into plastic, heparinized tubes, centrifuged and the plasma transferred to plain plastic vials and stored at −20°C until assayed.

Calculations

Coefficients of variation were calculated from the ratio of the standard deviation to the mean. The pharmacokinetic parameters, elimination half-life, systemic plasma clearance and volume of distribution were calculated by standard model independent pharmacokinetic formulae [6].

RESULTS AND DISCUSSION

Previous analytical methods for estimation of amodiaquine have been limited to spectrophotometric and fluorometric procedures which lacked both sensitivity and selectivity for this compound [4, 5]. In addition, these methods involved cumbersome sample preparation and non-selective chemical treatment procedures aimed at enhancing either the absorbance or fluorescence of the parent drug. The proposed assay method utilizes selective and sensitive HPLC analysis of plasma extracts obtained by a rapid and simple sample preparation.

The HPLC chromatograms of a stock solution of amodiaquine and internal standard (6-methoxy-8-aminoquinoline) as well as chromatograms of extracts of blank plasma, spiked plasma standard and patient's plasma, are shown in Fig. 1. Amodiaquine (t_R = 3.2 min) and the internal standard (t_R = 4.4 min) were detected as distinct peaks which were chromatographically resolved to baseline. The chromatogram of blank plasma was free of any interfering peaks. An endogenous component eluted with a retention time of 5.5 min, but it was baseline-separated from the internal standard and samples could be injected at 6-min intervals. It was necessary to adjust the mobile phase pH to precisely 2.8 in order to achieve the chromatographic separation between amodiaquine,

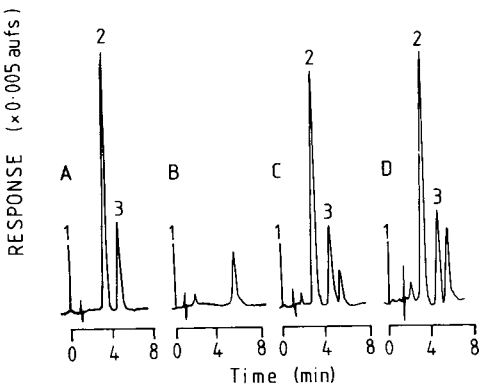

Fig. 1. HPLC chromatograms of (A) stock solution corresponding to 100 ng each of amodiaquine and internal standard; (B) blank plasma extract; (C) standard plasma extract corresponding to 150 ng/ml amodiaquine; and (D) patient's plasma extract corresponding to 142 ng/ml amodiaquine. Peaks: 1 = injection event; 2 = amodiaquine; 3 = internal standard, 6-methoxy-8-aminoquinoline.

internal standard and the endogenous component, as shown in Fig. 1. The chromatography of amodiaquine and the endogenous component was not influenced by varying the pH between 2.5 and 4.0; however, the retention time of the internal standard was very sensitive to changes in pH.

The assay was free of chromatographic interference from the commonly used antimalarial drugs, i.e. chloroquine, primaquine, pyrimethamine, proguanil and cycloguanil.

In the intitial assay development, column effluent was monitored at 229 nm corresponding to the λ_{max} for amodiaquine. This proved to be satisfactory for the analysis of samples derived from volunteer subjects. However, the concurrent administration of antipyretic, antiemetic and sedative agents to malaria patients resulted in a myriad of co-eluted peaks in the chromatogram, which precluded amodiaquine quantitation at this detection wavelength. Therefore an alternative λ_{max} for amodiaquine at 340 nm was preferred. Even though the molar extinction coefficient for this drug was slightly less at 340 nm than at 229 nm, peak detection for amodiaquine was more selective and therefore there was no appreciable loss in analytical sensitivity. At this higher wavelength the minimum detectable concentration of amodiaquine (as the hydrochloride salt), using 1 ml of plasma, was 5 ng/ml, and produced a peak three times the baseline noise on the highest detector sensitivity used (× 0.005 a.u.f.s.).

Calibration curves were linear ($r \geqslant 0.99$) in the range 0—1000 ng/ml and the analytical recoveries from plasma were 60% for amodiaquine and 50% for the internal standard. The coefficient of variation for within-day assays was 5% (100 ng/ml; $n = 6$) and for day-to-day assays over a period of eight weeks 12% (100 ng/ml, $n = 7$).

Amodiaquine was found to be very unstable to light and to adsorb readily to glassware, whereas the internal standard proved to be thermo-labile. Consequently to achieve the analytical accuracy and precision we have reported, it was necessary to protect samples from light, use silylated glassware and avoid temperatures above 25°C during sample preparation.

The assay method was applied to the analysis of samples obtained from two Thai patients undergoing antimalarial therapy with parenteral amodiaquine. There was considerable inter-subject variability in the plasma level—time profiles (Fig. 2) and at the conclusion of the 4-h infusion, maximum plasma concentrations of 1020 and 106 ng/ml, respectively, were achieved. In the two patients, after an initial rapid fall, the plasma levels declined with elimination half-life values of 4.3 and 9.7 h. The systemic plasma clearance of amodiaquine can be estimated in these subjects to be 208 and 674 l/h, and the volume of distribution to be 1291 and 9434 l, respectively. This indicates that amodiaquine, as with the other commonly used 4-aminoquinoline compound chloroquine, undergoes very extensive tissue distribution [7], and also shows that the disposition of amodiaquine appears to differ appreciably between patients.

In summary, this method, which is both sensitive and selective, overcomes the limitations imposed by previous procedures and is applicable to the analysis of plasma samples derived from field studies of the clinical pharmacology of amodiaquine.

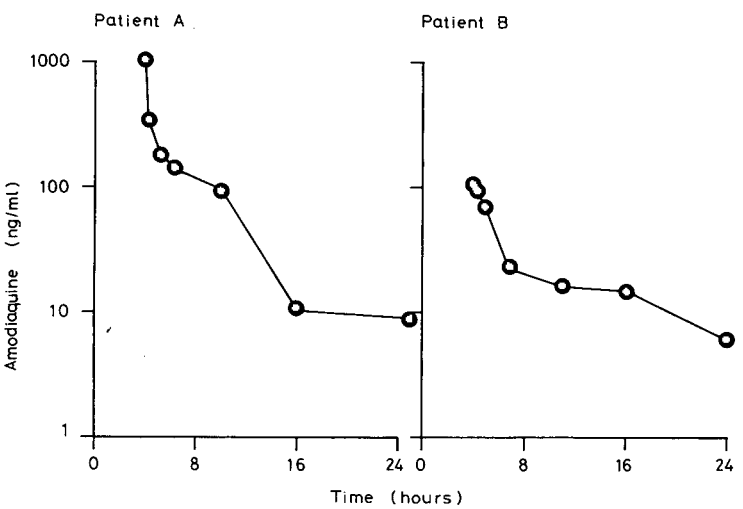

Fig. 2. Semilogarithmic plot of plasma amodiaquine concentrations (expressed as the hydrochloride salt) against time in two malaria-infected patients receiving parenteral amodiaquine, 10 mg/kg, as a 4-h infusion from time 0 to 4 h.

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Note

High-performance liquid chromatographic assay for mexiletine enantiomers in human plasma

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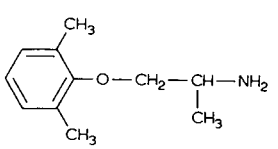
Mexiletine, 1-(2,6-dimethylphenoxy)-2-aminopropane (I, Fig. 1), is an orally effective antiarrhythmic agent possessing the same electrophysiological properties as lidocaine [1, 2]. It has been shown that an important correlation exists between the therapeutic and toxic effects of this agent and its serum concentrations; therapeutic serum concentrations usually fall in the range 0.75—2.0 μg/ml [3, 4]. Mexiletine possesses a chiral centre and is employed clinically as the racemic mixture. To date, neither the pharmacokinetic nor the pharmacological properties of the separate enantiomers have been described. This new antiarrhythmic drug is eliminated mainly by hepatic metabolism, the main metabolites being mexiletine N-glucuronide conjugate, hydroxymethyl-mexiletine and *p*-hydroxymexiletine [5, 6]. The conjugation of mexiletine with glucuronic acid has been found to be stereo-selective with *R*(—)-mexiletine



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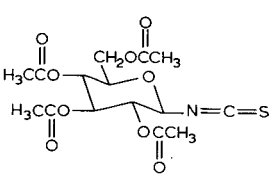

II

Fig. 1. Chemical structures of mexiletine (I) and GITC (II).

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being more extensively glucuronated than the corresponding *S*(+)-enantiomer [6].

Various methods for the separation of enantiomeric mixtures have been reported [7]. These involve the use of chiral eluents [8], of chiral stationary phases [9, 10] or derivatization with chiral reagents [11—13]. A simple method for the simultaneous analysis of mexiletine enantiomers in serum is described herein. The method involves derivatization of each isomer with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC; II, Fig. 1) followed by high-performance liquid chromatographic (HPLC) separation using ultraviolet (UV) detection.

MATERIALS AND METHODS

Chemicals and reagents

Racemic mexiletine hydrochloride as well as corresponding *R*(—)- and *S*(+)-enantiomers were kindly donated by Boehringer Ingelheim Canada (Ontario, Canada); they were used as received. Silver thiocyanate and α-acetobromoglucose were purchased from Ventron (Danvers, MA, U.S.A.) and from Sigma (St. Louis, MO, U.S.A.), respectively. Anhydrous hydrazine was obtained from Matheson Coleman (Lyndhurst, NJ, U.S.A.); phenylbutylamine from Aldrich (Milwaukee, WI, U.S.A.); HPLC-grade methanol and dimethylformamide from Fisher Scientific (Fair Lawn, NJ, U.S.A.). All other chemicals and reagents used were obtained from the usual commercial sources. The chiral reagent GITC was prepared from α-acetobromoglucose and silver thiocyanate as described by Nimura et al. [14]. The yield was 70% and the melting point 109—110°C (reported 113—115°C) [14].

Instrumentation

A Waters chromatographic system consisting of a Model M6000 pump, a U6K injector fitted with a 100-μl loop and a variable-wavelength detector (Model 450) set at 250 nm was used. Separation was performed on a Waters C_{18} μBondapak column (30 cm × 4 mm I.D., 10 μm particle size) using a mobile phase consisting of methanol—10 m*M* phosphate buffer, pH 5.5 (65:35) at a flow-rate of 2 ml/min.

Extraction and derivatization procedure

A 2.0-ml serum or plasma sample was extracted twice with diethyl ether at pH 12—13 after addition of 0.5 ml of an aqueous solution of phenylbutylamine (1.25 μg/ml) as internal reference standard. The combined ethereal extracts were evaporated to dryness in a water bath at 45°C; the residue was dissolved in 50 μl of a freshly prepared solution of GITC in dimethylformamide (0.2%, w/v) on a vortex mixer and the resulting mixture was allowed to stand at room temperature for 10 min. A 10-μl aliquot of a fresh solution of hydrazine in dimethylformamide (0.5%, v/v) was then added, the mixture was again allowed to stand for another 10 min and then injected on to the chromatograph.

Calibration curves

Aqueous solutions of *R*(—)- and *S*(+)-mexiletine hydrochloride and of

phenylbutylamine hydrochloride (0.5 ml) were added to 2.0 ml of blank plasma. The range of concentrations of each enantiomer was 51.9—389.5 ng of free base per ml of plasma and the concentration of phenylbutylamine · HCl was 1.25 μg/ml. All samples were extracted, derivatized and analyzed as above. Calibration curves based on the peak height ratios of each enantiomer to the internal standard were constructed using six different concentrations of each enantiomer analyzed in duplicate. The data were subject to linear regression analysis to give the appropriate calibration factor.

RESULTS AND DISCUSSION

The supplied enantiomers were judged to be pure when the GITC derivative of 2 μg of each enantiomer gave only a single peak using the HPLC conditions developed herein.

Variation of the derivatization time of (*RS*)-mexiletine with GITC in dimethylformamide over the period of 5—240 min showed that derivatization was complete within 10 min. This reaction time is similar to that reported for formation of the GITC derivatives of epinephrine and norepinephrine [14]. Hydrazine is added to the reaction mixture in order to destroy excess reagent [14]; the decomposition products elute at 5.8 min and do not interfere with the assay. GITC has been used for the HPLC resolution of enantiomers of various amino acids [15, 16], of epinephrine and of norepinephrine [14] and of several β-adrenergic antagonists [17]. The isothiocyanate group of GITC reacts rapidly with primary and secondary amino groups to give the corresponding thiourea derivatives [16]. The degree of separation of enantiomers derivatized with a chiral agent depends upon various criteria [13]; amongst these the conformational rigidity around the chiral centres [18], the proximity for a polar or polarizable group to a chiral centre and the distance between the chiral centres of the reagent and the reactant [19, 20] appear to be the most important. The bulky acetylglucosyl moieties of the GITC molecule confer rigidity to the derivative formed and the three-atom distance between the two chiral centres fulfills the distance criteria. HPLC separation of mexiletine can also be affected after derivatization with the chiral agent, N-trifluoroacetyl-*S*-prolyl chloride. However, the derivatives formed do not have the sensitivity required for the UV detection of mexiletine enantiomer serum levels expected after single-dose administration of racemic drug. Thiourea derivatives, on the other hand, are very sensitive to UV detection [15]. This is particularly important in the case of mexiletine which has a low extinction coefficient (ϵ = 255). The minimum measurable amount of underivatized mexiletine using our type of UV detector was 5 μg whilst that of the GITC derivative was 50 ng which is the amount of each enantiomer per ml of serum expected 24 h after administration of a 200-mg dose of racemic mexiletine hydrochloride.

Fig. 2A shows a typical chromatogram obtained after derivatization of an ethereal extract of blank plasma spiked with 311.6 ng of each mexiletine enantiomer and 625 ng of the internal standard, phenylbutylamine, with GITC. Fig. 2B is a chromatogram of a similarly treated blank serum sample. No interference from endogenous products or from mexiletine's major metabolites, *p*-

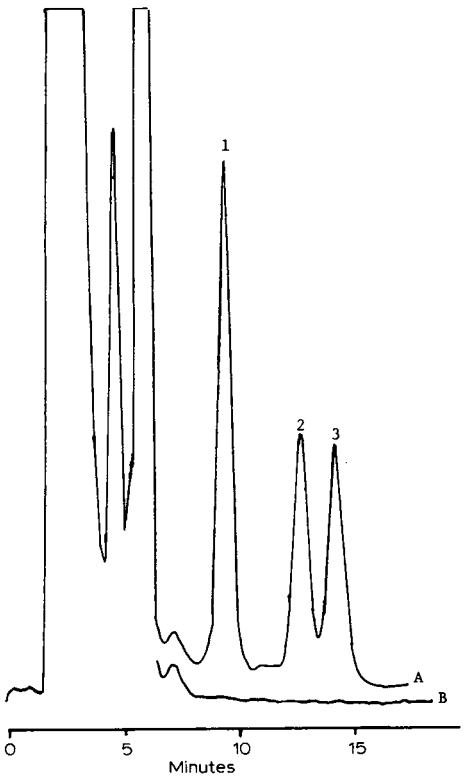

Fig. 2. Representative HPLC chromatograms of (A) serum sample spiked with 311.6 ng each of *R*(—)- and *S*(+)-mexiletine and 625 ng of the internal standard; and (B) blank serum. Peaks: 1 = internal standard, phenylbutylamine; 2 = *R*(—)-mexiletine; 3 = *S*(+)-mexiletine.

hydroxymexiletine and hydroxymethylmexiletine, was observed. The retention times of the GITC derivatives of *R*(—)- and *S*(+)-mexiletine were 12.7 and 14.2 min, respectively, whereas that of phenylbutylamine, the internal reference standard, was 9.4 min. Identification of each enantiomer was made by comparing the retention time with that obtained after separate chromatography of each GITC-enantiomer derivative. Better separation of the enantiomeric pair could be obtained if the methanol concentration in the mobile phase was reduced; for example, when the methanol concentration was 60%, the retention times were increased to 18.2 and 20.7 min, respectively and the peaks were completely separated. However, better sensitivity (narrower and consequently sharper peaks) were preferred to better separation. Methanol was chosen as the organic component of the mobile phase after investigation with the solvents tetrahydrofuran, acetonitrile and ethanol showed that it provided the best separation conditions.

Linear calibration plots were obtained over the range 51.9—389.5 ng/ml of each enantiomer. Typical regression lines were $y = 0.0015x - 0.005$ ($r > 0.99$) for the *R*(—)-enantiomer and $y = 0.0014x - 0.012$ ($r > 0.99$) for the *S*(+)-enantiomer. Intra- and inter-day assay variations for each enantiomer determined at 207, 519 and 779 ng per 2 ml of plasma are shown in Table I. The percentage coefficient of variation (C.V.) for inter- and intra-day analysis was

TABLE I

INTRA- AND INTER-DAY VARIATIONS IN THE SIMULTANEOUS ANALYSIS OF *S*(+)- AND *R*(−)-MEXILETINE IN PLASMA

	Amount of *S*(+)- or *R*(−)-mexiletine (ng) added to 2 ml of plasma		
	207.0	519.0	779.0
Intra-day variation (n = 4)			
S(+)-Mexiletine found ± S.D.	195.9 ± 8.8	520.9 ± 5.7	778.7 ± 15.7
C.V. (%)	4.5	1.1	2.0
R(−)-Mexiletine found ± S.D.	197.8 ± 6.4	525.8 ± 12.6	769.0 ± 17.5
C.V. (%)	3.2	2.4	2.3
Inter-day variation (n = 5)			
S(+)-Mexiletine found ± S.D.	198.7 ± 20.5	509.6 ± 25.1	748.8 ± 27.1
C.V. (%)	10.3	4.5	3.6
R(−)-Mexiletine found ± S.D.	198.8 ± 20.9	524.4 ± 24.1	749.8 ± 30.5
C.V. (%)	10.5	4.6	4.1

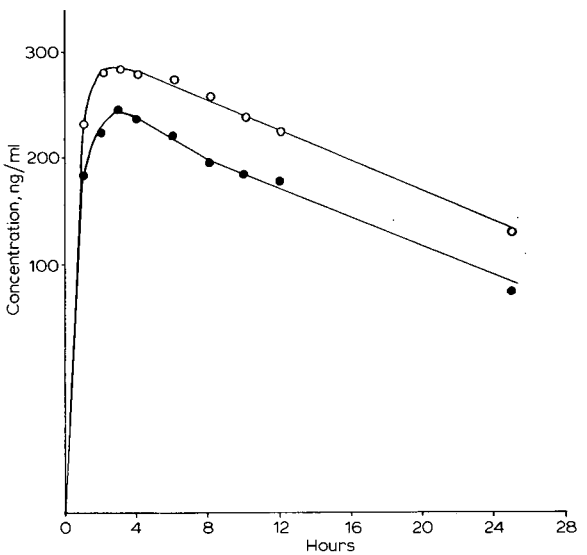

Fig. 3. Plasma concentration—time profile of *S*(+)-mexiletine (○) and *R*(−)-mexiletine (●) obtained after oral administration of 200 mg of *RS*-mexiletine hydrochloride to a healthy young volunteer.

below 5% except for the inter-day variations in the lowest concentration studied which were 10.3 and 10.5% for *S*(+)- and *R*(−)-mexiletine, respectively. Extractibility of (*RS*)-mexiletine with 2 vols. of diethyl ether under alkaline conditions was higher than 90% which agrees with reported values [21, 22].

The method described herein was applied to the determination of the separate enantiomers in the plasma of a healthy volunteer to whom 200 mg of racemic mexiletine hydrochloride had been administered. Fig. 3 shows the concentration—time profile for *S*(+)- and *R*(−)-mexiletine obtained in this

subject. The elimination half-lives for the $S(+)$- and the $R(-)$-enantiomer, as calculated from the slope of the terminal linear portion of the ln serum concentration—time curve, were 13.79 and 12.82 h, respectively.

In conclusion, a stereo-specific method for the simultaneous analysis of mexiletine enantiomers in serum is reported. The method is specific and simple and it can be used to study the serum enantiomeric composition of mexiletine after administration of the racemic drug.

ACKNOWLEDGEMENTS

The authors thank Mrs. Angèle Paquet for withdrawing the blood samples, Mr. Michel Blouin for technical assistance and Miss Carole Murphy for typing the manuscript. J.T. is a recipient of a Medical Research Council of Canada studentship award.

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CHROMBIO. 2361

Note

Antitumour imidazotetrazines

VII. Quantitative analysis of mitozolomide in biological fluids by high-performance liquid chromatography

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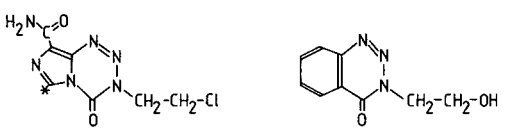
(First received May 21st, 1984; revised manuscript received September 6th, 1984)

The novel agent mitozolomide, 8-carbamoyl-3-(2-chloroethyl)imidazo[5,1-*d*]-1,2,3,5-tetrazin-4(3H)-one, also designated CCRG 81010, M&B 39565 and NSC 35451, see I in Fig. 1, was synthesised in our laboratories [1] as a part of a programme concerned with investigating the chemical and biological properties of molecules containing NNN linkages [2—4]. The compound was found to be active against a wide range of model tumour systems [1, 5] and mechanistic studies [6—8] have indicated that mitozolomide may be a prodrug of the chemically unstable monochloroethyltriazene, 5-[3-(2-chloroethyl)-triazen-1-yl]imidazole-4-carboxamide (MCTIC). Preliminary animal pharmacokinetic data [9] indicated that mitozolomide was well absorbed orally (in mice), and had an elimination half-life of just over 1 h. In order to investigate the drug's in vivo and in vitro kinetics a rapid, sensitive and selective high-performance liquid chromatographic (HPLC) method was developed.



I II

Fig. 1. Structural formulae of mitozolomide (I) and the internal standard (II), 3-(2-hydroxyethyl)-1,2,3-benzotriazin-4(3H)-one. The star denotes the position of ^{14}C in mitozolomide.

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EXPERIMENTAL

Materials

Mitozolomide was kindly supplied by Dr. E. Lunt of May & Baker (Dagenham, U.K.) as was the [^{14}C]mitozolomide (see Fig. 1 for position of label). The internal standard, 3-(2-hydroxyethyl)-1,2,3-benzotriazin-4(3H)-one, was synthesised by G.U. Baig, All chemicals and solvents were of either analytical or chromatographic quality and were used without further purification.

Extraction

Plasma samples were stored at −20°C until required. A 20-μl aliquot of the internal standard solution was added to a 10-ml test tube followed by 0.05 ml of 1 *M* hydrochloric acid and 1 ml of plasma. Ethyl acetate (2.5 ml) was added, the contents mixed by vortexing and the layers separated by centrifugation (10 min at 1500 *g*). A 2-ml aliquot of the organic layer was removed and evaporated to dryness using a stream of dry nitrogen. The residue was dissolved in 0.15 ml of methanol and 0.15 ml of 5% acetic acid in water was immediately added. This solution was transferred to a low volume insert and stored at 4°C until analysed (within seven days).

Calibration curves were constructed over the range of interest by the addition of chloroform solutions of mitozolomide to test tubes and evaporating to dryness using a stream of dry nitrogen. The procedure was then as described above.

Chromatography

A 100 × 5 mm Waters RCM cartridge (10 μm particle size, C_{18}) was used with a C_{18} pre-column. The isocratic mobile phase consisted of methanol—5% acetic acid in water (3:7) and was pumped at a constant flow-rate of 1.5 ml/min. A Waters system was used (Waters Assoc., Northwich, U.K.) which comprised of a WISP Model 720 system controller and M730 data module. The injection volume was 20 μl and detection was at 325 nm using a Waters Lambda-max 480 LC spectrophotometer.

Extraction efficiencies

Aqueous solutions of [^{14}C]mitozolomide were added to control plasma at 1, 5, 10 and 20 mg/l. Following extraction with ethyl acetate both plasma and ethyl acetate samples were counted on a Beckman LS230 liquid scintillation counter. Values (dpm) were calculated using an external standard channels ratio method. The scintillant used was Fisofluor (Fisons, Loughborough, U.K.).

Quantification of mitozolomide

The concentration of mitozolomide was determined from the peak area ratio of mitozolomide to internal standard. Calibration curves were constructed over the range of interest using at least six evenly distributed points and the data analysed by least-squares regression.

RESULTS AND DISCUSSION

Mitozolomide was quantified by HPLC following ethyl acetate extraction of acidified plasma. The extraction efficiencies were determined using ^{14}C-labelled mitozolomide which had been added, at various concentrations, to 1 ml of control plasma. The results indicated that the extraction into 2.5 ml of ethyl acetate was constant, over the range studied, at 76% with 95% of the total label being recovered. The effect of the addition of 0.05 ml of 1 *M* hydrochloric acid on the extraction efficiency was determined and was shown to be negligible. The coefficient of variation for the replicate extractions (n = 6) of plasma samples was 1.8% and for replicate injections of the same sample was 0.5% (n = 6). The detection limit of the assay was 10 ng/ml with a signal-to-noise ratio of greater than 3.0.

The capacity factors for mitozolomide and the internal standard were 1.5 and 2.6, respectively, which gave a total analysis time of 5 min. Typical chromatograms are given in Fig. 2. The chemical stability of mitozolomide in various buffers and physiological fluids was examined and the results are summarised in Table I. The aqueous protein solution was bovine serum albumin at the same concentration as total protein in normal human plasma, 70 mg/ml [10].

Potential difficulties associated with the quantitative analysis of mitozolomide centred on the chemical instability of the compound. However,

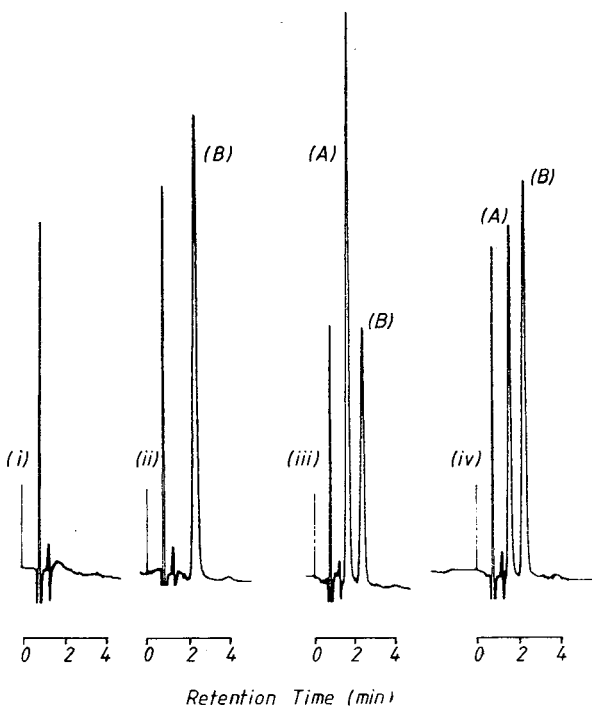

Fig. 2. Chromatograms of mouse plasma extracts. (i) Blank plasma; (ii) blank plasma spiked with internal standard; (iii) blank plasma spiked with 1 μg mitozolomide and internal standard; (iv) plasma from mouse dosed with 20 mg/kg of mitozolomide and extracted with internal standard. Peaks: A = mitozolomide; B = internal standard.

TABLE I

CHEMICAL STABILITY OF MITOZOLOMIDE

Solution	pH	Temperature (°C)	Half-life (h)
Tris buffer (0.1 m*M*)	9.0	22	0.15
Phosphate buffer (0.07 *M*)	7.4	37	0.92
Acetate buffer (0.1 *M*)	4.0	37	240
Bovine serum albumin (70 mg/ml)	7.4	37	0.87
Human plasma	7.4	37	0.48
Human urine	6.2	37	32.8

due to the high stability at low pH (see Table I), it was apparent that, as long as the blood or urine was cooled immediately following collection and that extraction conditions were kept acidic, there were no undue problems in this area. The strong chromophore at 325 nm [1] facilitated interference-free traces to be obtained from control plasma (Fig. 1) and has enabled pharmacokinetic analyses to be performed at doses which correspond to 1/100 of the optimum therapeutic dose for mice innoculated with model tumours [1]. The HPLC assay described above has been used to determine pharmacokinetic parameters of the drug in over 40 patients involved in a phase I trial of mitozolomide. There was no evidence from the pre-dose plasma samples, that other drugs which would commonly be coadministered with this agent (e.g. antiemetics and analgesics) interfere with the assay.

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CHROMBIO. 2343

Note

Modification of a new high-performance liquid chromatographic method for bleomycin to separate epi-, iso-, desamido-, and unmodified analogues

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(Received July 9th, 1984)

This laboratory has recently developed a procedure for the separation and quantitation of many congeners of the antitumor antibiotic bleomycin. The method utilizes a linear water—methanol gradient and pentanesulfonic acid as an ion-pairing reagent [1]. We have since been successful in chemically producing definitive quantities of the epi- and iso-conformers of the common bleomycins. This communication reports a modification of our previous procedure which allows separation, identification, and quantification of epi-, iso-, desamido-, and unmodified bleomycin congeners within the same chromatogram .

MATERIALS AND METHODS

As in the previous study [1], bleomycin (as Blenoxane, lot FOX04, expiration date September, 1981) was obtained through Drs. William Bradner and Stanley Crooke as a gift from Bristol Labs. (Syracuse, NY, U.S.A.). This one lot was used throughout these studies. Milli-Q-purified (Millipore, Bedford, MA, U.S.A.) water was used throughout, as was methanol purchased from Fisher Scientific (Springfield, NJ, U.S.A.). Pentanesulfonic acid, sodium salt, was obtained from Aldrich (Milwaukee, WI, U.S.A.), while the heptanesulfonic acid salt and all other reagents were from Fisher. The structures of all modified and unmodified bleomycins are as previously given [1].

The chromatography system was as described [1]. Solvent A (water) and solvent B (methanol) each contained 5 m*M* of either the sodium salt of pentane- or heptanesulfonic acid and 0.5% glacial acetic acid. The pH of solvent A was adjusted to 4.3 with concentrated ammonium hydroxide, while solvent

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B was used without further additions. All solvents were passed through a 0.2-μm filter and sonicated for 15 min at the beginning of each day. Detection and quantification were, as before, performed using absorbance at 280 nm.

The standard procedure in our previous work [1] was a linear gradient of 28—48% solvent B in solvent A (pentanesulfonic acid) run over 45 min at a flow-rate of 1.5 ml/min through a 300 × 3.9 mm μBondapak C_{18} (Waters) column (10 μm particle size) preceded by a 23 × 3.9 mm Corasil C_{18}-filled guard column (30—38 μm particle size). In this modification, the procedure consists of a 40—50% solvent B in solvent A (heptanesulfonic acid) linear gradient run over 30 min at a flow-rate of 0.7 ml/min through a 150 × 3.9 mm Novapak C_{18} (Waters) column (4 μm particle size) preceded by the same guard column, with a resultant pressure of about 2.2 MPa (3000 p.s.i.).

The desamido-bleomycin analogues were produced using bleomycin hydrolase isolated from mouse liver by the previously described [1] modification of the method of Yoshioka et al. [2]. Epi-bleomycins [3] and iso-conformers [4] were formed exactly as described. Briefly, 100 μl of HPLC-grade water and 200 μl of 2% triethylamine were added to sterile vials (15 units each, or about 9 mg) of Blenoxane lot FOX04. One vial was allowed to react at room temperature for five days to allow carbamoyl migration from the 3-O to the 2-O position of the mannose sugar for iso-conformer formation [4]. To a second vial, 6 μl of 1.0 *M* copper sulfate were added and the vial was heated to 70°C for 6 h to form the epimers [3]. As before, the Cu(II) chelates were always chromatographed.

RESULTS AND DISCUSSION

As can be observed in Fig. 1, we have now been able to form definitive amounts of the epi- and iso-conformers of the bleomycins, but iso-bleomycins could not be separated from their respective parents with any modification of our previously described [1] procedure using pentanesulfonic acid as the ion pairing reagent. In addition, both iso- (Fig. 1B) and desamido- (data not shown) bleomycins eluted just prior to the unmodified drug. We had already performed preliminary experiments to adapt our procedure to the smaller, more efficient and economical Novapak C_{18} column. Using this column, we were also unsuccessful in separating iso- and desamido-congeners with pentanesulfonic acid, although we have been able to achieve excellent separation of the parent congeners, as before, using this salt and a linear 24—48% methanol gradient with a flow-rate of 0.6 ml/min over 35 min. This modification thus allows a saving of 10 min between runs, as well as 60% solvent reduction.

We than attempted to effect separation of these conformers, using the Novapak column and heptanesulfonic acid as the paired ion, additionally hoping to distinguish the desamido-analogues. This resulted in the development of the conditions indicated in Fig. 2. That is, a shallow gradient of 40—50% methanol with 5 m*M* heptanesulfonic acid, pH 4.3, was begun at time zero and run over 30 min. As can be seen in Fig. 2, these conditions afforded separation of the iso- and epi-conformers from their respective unmodified bleomycins. Epimerization (*R*, instead of *S*, at the α-methine carbon of the pyrimidine moiety) has drastically affected retention (Fig. 2C) of all the bleomycin con-

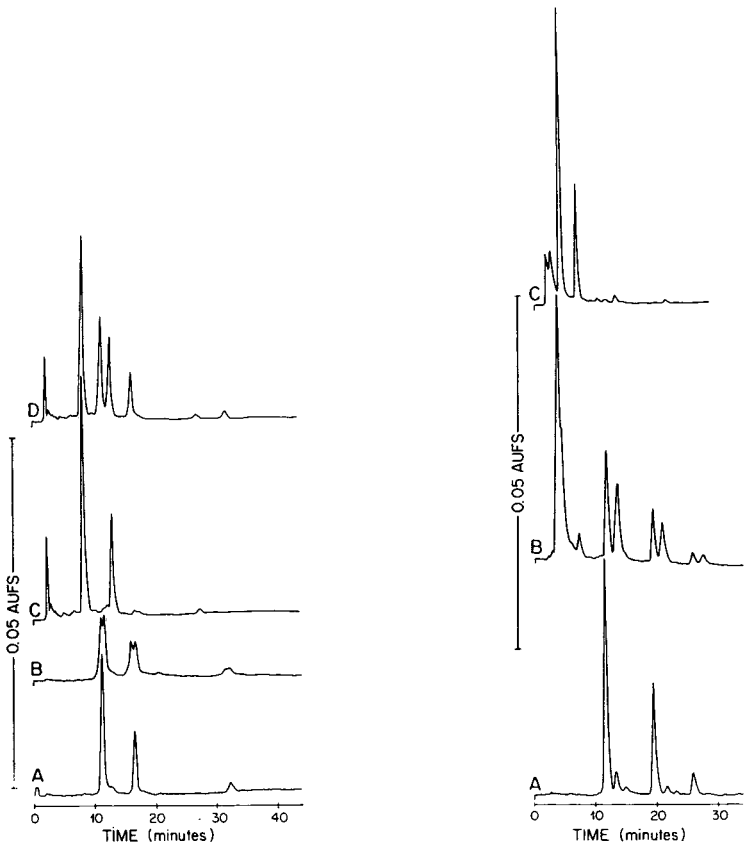

Fig. 1. Standard procedure [1] for the separation of bleomycin congeners using a 300 × 3.9 mm μBondapak C_{18} with 5 m*M* pentanesulfonic acid, as described in Materials and methods. (A) Blenoxane lot FOX04; order of elution: A_2 (about 12 min), B_2 (17 min), and DM-A_2 (33 min); (B) about 50% conversion of FOX04 to the iso-conformer; the first peak in each doublet is the respective iso-bleomycin; (C) essentially 100% conversion to the epi-conformers; order of elution: epi-A_2 (about 8.5 min), epi-B_2 (13.5 min), and epi-DM-A_2 (28 min); (D) a mixture of epi- and unmodified Blenoxane lot FOX04 (70:30 mol ratio). AUFS = absorbance units full scale.

Fig. 2. Modified procedure for detecting and separating epi-, iso-, and unmodified conformers using a 150 × 3.9 mm Novapak C_{18} with 5 m*M* heptanesulfonic acid, as described in Materials and methods. (A) Blenoxane lot FOX04, the order of elution is the same as in Fig. 1; (B) about 50% conversion of this lot to the iso-conformers, which elute after the ummodified congeners (at about 14, 21.5, and 28 min for iso-A_2, B_2 and DM-A_2, respectively); (C) essentially 100% conversion of FOX04 to the epi-conformers (4.5, 7.5, and 22.5 min for epi-A_2, B_2, and DM-A_2, respectively). AUFS = absorbance units full scale.

geners. These epimers are reported to have equal or greater in vitro DNA cleaving ability than the respective parent drug [5], although their antibacterial activity and cytotoxicity are reduced [3, 5]. These phenomena could all be due to more efficient metal binding, although this has not been studied.

On the other hand, iso-bleomycins are retained slightly longer than the umodified drugs (Fig. 2B), induce significantly less DNA strand scission in vitro [5], but retain considerable antimicrobial activity [4, 5]. These data are consis-

tent with a reduced metal affinity in the iso-bleomycins as well as the involvement of the carbamoyl function in metal coordination [6].

Apparently, removing the amide from the β-amino-L-alanine amide N-terminus affects molecular conformation and metal binding similar to epimerization in this region, although to a lesser degree. This can be seen in Fig. 3D, as well as in our previous work [1]. The desamido modification reported greatly reduces the drug's activity with respect to both its DNA cleaving and cytotoxic effects [2].

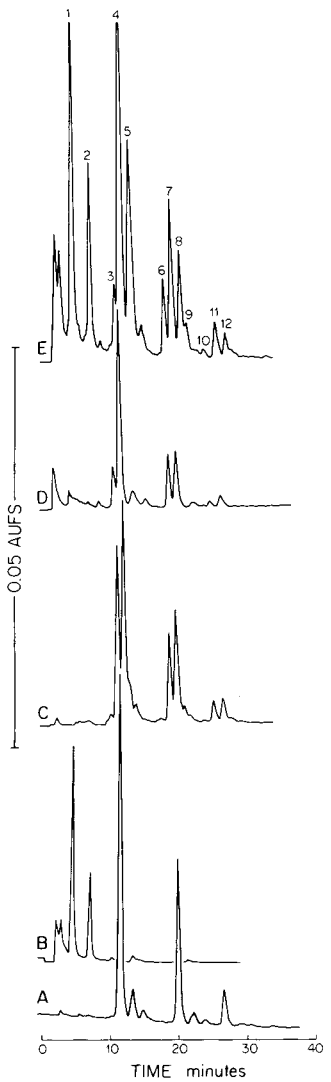

Fig. 3. Modified HPLC procedure as indicated in Fig. 2. (A) Blenoxane lot FOX04; (B) epi-FOX04; (C) 60% iso-FOX04 with unmodified conformers; (D) various proportions of the desamido-analogues eluting just prior to the parent bleomycin; and (E) a mixture of epi-, iso-, desamido-, and unmodified bleomycins identified as follows: (1) epi-A_2, (2) epi-B_2, (3) desamido-A_2, (4) A_2, (5) iso-A_2, (6) desamido-B_2, (7) B_2, (8) iso-B_2, (9) epi-DM-A_2, (10) desamido-DM-A_2, (11) DM-A_2, and (12) iso-DM-A_2. AUFS = absorbance units full scale.

A powerful example of the separation capacity of this technique is shown in Fig. 3E, in which a mixture of epi-, iso-, desamido-, and unmodified drug (Blenoxane lot FOX04) is dramatically separated in a single run. The authors know of no other published HPLC method which has this capability.

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CHROMBIO. 2353

Note

Analysis of aminoglycoside antibiotics as benzoyl derivatives by high-performance liquid chromatography and its application to the quantitation of neomycin in the perilymph

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(First received May 18th, 1984; revised manuscript received August 28th, 1984)

It is well known that application of ototoxic drugs directly to the middle ear often causes severe sensorineural deafness [1]. Severe inner ear pathology or endolymphatic hydrops is sometimes observed in association with otitis media [2, 3]. It is most probable in such instances that the drug or inflammation in the middle ear spreads to the inner ear by way of the round window membrane, and recently the permeable character of the round window membrane has drawn the attention of many investigators.

We also have undertaken investigations on the subject, and have already shown that the round window membrane of the guinea pig is permeable to neomycin, by observing damage to the organ of Corti after topical application of neomycin on the round window membrane [4]. To clarify how much of the neomycin permeates from the middle ear into the inner ear through the round window membrane, it is necessary to determine the concentration of

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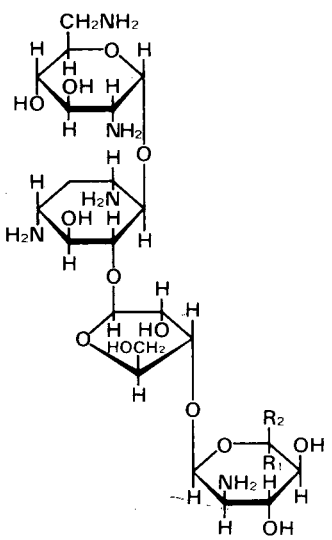
neomycin in the perilymph, which is collected from the inner ear only in an extremely small quantity.

At present, the level of aminoglycoside antibiotics in the perilymph is determined by microbiological assay [5], radioimmunoassay [6] and radioenzymatic assay [7]. However, these methods include rather laborious steps or the use of biohazardous radioactive materials. To overcome these shortcomings, we established a method using high-performance liquid chromatography (HPLC). As is well known, HPLC is very useful for the ultrasensitive and high-speed determination of various biological materials. As for the determination of aminoglycoside antibiotics, derivatization of amino groups in the molecule with *o*-phthalaldehyde [8, 9] or 1-fluoro-2,4-dinitrobenzene [10] has been reported. Instead of these derivatives, we chose benzoylation of both hydroxyl and amino groups in order to increase the sensitivity of detection. This paper describes a convenient method for the analysis of aminoglycoside antibiotics and its application to the determination of neomycin in the perilymph.

EXPERIMENTAL

Materials

Benzoyl chloride, pyridine, *n*-hexane, tetrahydrofuran and sodium carbonate were purchased from Wako, Osaka, Japan. Neomycin, kanamycin, streptomycin and dihydrostreptomycin were obtained from Nippon Kayaku, Tokyo, Takeda, Osaka, Kaken, Tokyo, and Meiji Seika Kaisha, Tokyo, Japan, respectively. Chloroform and methanol were distilled once before use. The structure of neomycin is shown in Fig. 1.



neomycin B : R_1=H R_2=$CH_2 \cdot NH_2$
neomycin C : R_1=CH_2NH_2 R_2=H

Fig. 1. Structure of neomycin. Neomycin is a mixture of two stereoisomers, neomycin B and C.

Benzoylation with benzoyl chloride

Benzoylation of aminoglycoside antibiotics was performed according to the method reported previously [11]. Briefly, about 100 μg of neomycin and other aminoglycoside antibiotics (kanamycin, streptomycin and dihydrostreptomycin) were each dissolved in 90 μl of pyridine, and 10 μl of benzoyl chloride were added. The reaction was allowed to proceed at 80° C for 30 min. After the reaction, pyridine was evaporated under a flow of nitrogen. Then, 1 ml of methanol was added, and the mixture was again heated at 80° C for 10 min to convert excess reagent to volatile methyl benzoate. To the solution were added 50 mg of sodium carbonate powder and 1 ml of methanol which was previously saturated with sodium carbonate. The solution was washed three times with 2 ml of *n*-hexane to remove methyl benzoate, and the hexane layer produced by adding 1 ml of water was further removed. The derivative was then extracted with 3 ml of chloroform. The lower chloroform layer was washed three times with 1 ml of methanol—water (1:1, v/v), and the solution was evaporated to dryness.

High-performance liquid chromatography

The derivative was dissolved in 50 μl of chloroform and 2 μl of the solution were injected into a stainless-steel column (25 cm × 4.6 mm I.D.) packed with surface porosity silica gel (Zorbax SIL, 5—6 μm average particle diameter; DuPont, Wilmington, DE, U.S.A.). For the analysis of less than 10 μg of neomycin, the derivative was dissolved in 15 μl of chloroform, and 5 μl of the solution were injected into the column. The mobile phase was a mixture of *n*-hexane and tetrahydrofuran (1:1, v/v), and a flow-rate of 2 ml/min was maintained with a HPLC system (LC-3A; Shimadzu, Kyoto, Japan). The column effluent was monitored at 230 nm using a variable-wavelength ultraviolet (UV) monitor (SPD-2A; Shimadzu). The peak areas were measured with a computer (C-RIA; Shimadzu) of which the attenuation was set to either 2^7 or 2^4.

Collection of perilymph samples

Three guinea pigs (body weight 250—400 g) were anaesthetized with Nembutal® (Abbot, North Chicago, IL, U.S.A.) and a post-auricular incision was made. The middle ear bulla was opened retroauricularly to visualize the round window membrane. Neomycin (5 mg) was absorbed by a small piece of Gelfoam® (Upjohn, Kalamazoo, MI, U.S.A.) soaked in water, and this piece was placed directly onto the round window membrane of each of the guinea pigs. After the intervals of 15 min, 60 min and 120 min, respectively, the Gelfoam was removed from the round window membrane, and immediately a small hole was drilled on the otic capsule into the scala tympani of the basal turn with an electric motor drill. A sample of 6 μl of the perilymph was collected from each animal with a glass micro-pipette (Drummond Scientific, Broomall, PA, U.S.A.). The perilymph samples were lyophilized, and were directly benzoylated by the method described above.

RESULTS

Benzoylation of aminoglycoside antibiotics

Fig. 2 shows the UV spectrum of benzoylated neomycin. The maximum

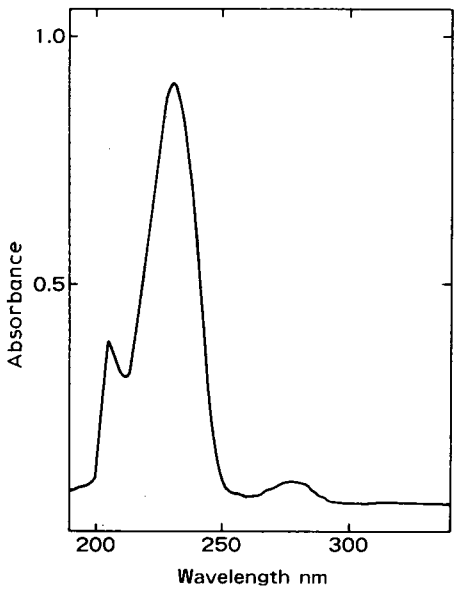


Fig. 2. UV spectrum of benzoylated neomycin. The concentration of derivative was 6.32 μM in ethanol; a cell with a 1-cm light path was used for the analysis.

absorption of the derivative was at about 230 nm, and the molar extinction coefficient at 230 nm was 142,000, indicating that eleven to thirteen benzoyl chromophores are introduced into the molecule. In order to select a suitable mixture of solvents for HPLC analysis, the benzoylated derivatives of several aminoglycoside antibiotics were analysed by thin-layer chromatography (TLC) on silica gel GF plates (0.25 mm thick; E. Merck, Darmstadt, F.R.G.). They were developed with several mixtures of organic solvents, such as *n*-hexane—diethyl ether (7:3, v/v), *n*-hexane—tetrahydrofuran (1:1, v/v), *n*-hexane—dioxane (1:1, v/v) and *n*-hexane—acetonitrile (1:1, v/v). Since detection after HPLC was carried out at 230 nm, the solvents giving a wavelength cut-off shorter than 220 nm were chosen as the developing solvents for TLC. Under UV light, the derivatives were observed as black quenched spots on a fluorescent background. The sharpest bands with significantly high resolution were obtained by development with *n*-hexane—tetrahydrofuran (1:1, v/v), giving the following R_F values: 0.07 for neomycin, 0.11 for kanamycin, 0.19 for streptomycin, and 0.21 for dihydrostreptomycin.

Analysis of benzoylated aminoglycoside antibiotics by HPLC

On the basis of the above results obtained by TLC, *n*-hexane—tetrahydrofuran (1:1, v/v) was selected as the best solvent for HPLC elution and the peaks were monitored at 230 nm, the maximum absorption of the benzoyl derivative. Fig. 3 shows HPLC chromatograms of benzoylated derivatives of neomycin, kanamycin, streptomycin and dihydrostreptomycin. Kanamycin, streptomycin and dihydrostreptomycin were eluted close to each other, whereas neomycin was clearly separated from the other antibiotics. Three peaks of neomycin having the retention times of 8.11, 9.28 and 10.08 min were thought to be

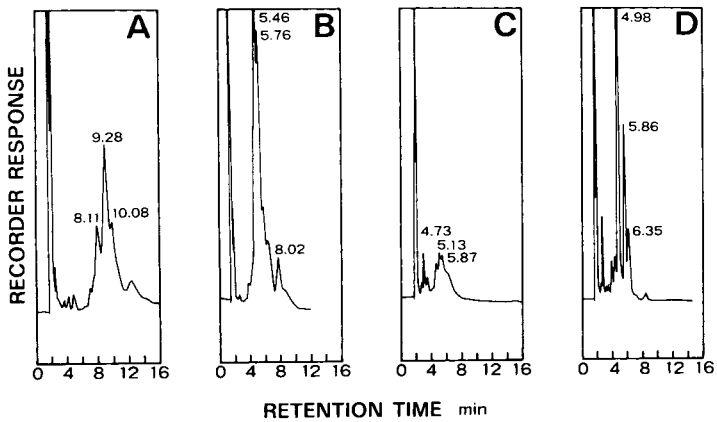


Fig. 3. HPLC chromatograms of various aminoglycoside antibiotics. The elution solvent was *n*-hexane—tetrahydrofuran (1:1, v/v), and the detection was carried out at 230 nm. A = neomycin, B = kanamycin, C = streptomycin, D = dihydrostreptomycin.

structural isomers of neomycin and the relative ratio of the peak areas was constant in different amounts of neomycin.

Quantitation of neomycin by HPLC

Judging from the absorption of benzoylated neomycin eluted from the HPLC column along with the molar extinction coefficient, the recovery of neomycin through derivatization and HPLC analysis was almost quantitative and reproducible (100.0 ± 1.5%). Accordingly, by reacting known amounts of neomycin and injecting the same volume into the HPLC column, a standard curve was made for the quantitation of neomycin. The curve was linear up to 100 μg of neomycin injected, and the lower limit of detection was about 10 ng of neomycin.

Determination of neomycin level in the perilymph

Table I shows the concentration of neomycin in the perilymph at various time intervals after the application of neomycin on the round window membrane. Usually, 6 μl of the perilymph could be collected and the samples were directly benzoylated after lyophilization. The derivatives were dissolved in

TABLE I

CONCENTRATION OF NEOMYCIN IN THE PERILYMPH OF GUINEA PIGS AS MEASURED BY HPLC

After topical application of neomycin on the round window membrane, perilymph samples were collected at the various time intervals indicated, and directly benzoylated as described in the text.

Time (min)	Concentration of neomycin (μg/ml)
15	140
60	350
120	750

15 μl of chloroform and the determination was performed by injecting 5 μl of each solution. The chromatograms thus obtained were essentially the same as that in Fig. 3A, indicating that any material after prewashing did not interfere with the elution of benzoylated neomycin. The results indicate that neomycin can pass through the round window membrane in a short time.

DISCUSSION

Aminoglycoside antibiotics in biological fluids have been determined by a variety of methods [12]. These include microbiological assay which has long been used for measuring antibiotic concentrations in body fluids and tissues, radioimmunoassay which is extremely sensitive, radioenzymatic assay, gas—liquid chromatography, HPLC and others. With the development of flow cells, well controlled surface-porosity column packings and high-pressure pumping systems, HPLC has become a rapid, precise and sensitive method for quantitating a variety of compounds including antimicrobial agents [13]. Determination of aminoglycoside antibiotics by HPLC has been reported by many investigators, for example by Tsuji et al. [10] for neomycin, by Mays et al. [9] for kanamycin, and by Maitra et al. [14] for gentamicin. HPLC has also been applied to the determination of antibiotics other than aminoglycosides [15]. In these methods, antibiotics have been converted to UV-absorbing or fluorescent derivatives by introducing chromophores into amino groups of the molecule.

Since aminoglycoside antibiotics contain multiple hydroxyl and amino groups, we have selected the reaction with benzoyl chloride in pyridine, which introduces the UV-absorbing benzoyl chromophores into hydroxyl and amino groups in a form of ester and acidamide linkages, respectively. As a result, eleven to thirteen chromophores could be introduced into neomycin, and this allowed us to determine a small amount of neomycin in the perilymph. Without any pretreatment of the perilymph, such as deproteinization, the derivatization of neomycin could be successfully performed, and any contamination could be removed in the washing steps of the derivatization procedure. Further protection of the column from contamination was achieved by placing a precolumn (5 cm × 4.6 mm I.D.) packed with reversed-phase packing (C_{18}; DuPont) between the injector and the analytical column. Direct derivatization is quite useful for samples only available in a small quantity, such as perilymph.

The results of the present study clearly showed the permeable property of the round window membrane to neomycin. The level of aminoglycoside antibiotics in the perilymph as a function of time after their systemic administration has already been investigated [6, 16], but analysis of neomycin in the perilymph after its topical application on the round window membrane has not yet been fully undertaken. The application of the procedure to further analysis of the dynamics of neomycin in the perilymph is now in progress in our laboratory. Furthermore, improvement in the accuracy and precision of the procedure is also being investigated by using an internal standard. Kanamycin is one of the candidates for internal standard as shown in Fig. 3.

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CHROMBIO. 2364

Note

New high-performance liquid chromatographic assay for plasma doxorubicin

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Doxorubicin (Adriamycin) is an anthracycline antibiotic employed in the treatment of a wide range of types of cancer. Its effectiveness is limited, however, by potentially severe myelosuppression and by total dose limiting cardiomyopathy [1, 2]. In an attempt to improve the drug's therapeutic index, long-term low-dose continuous infusion is currently being investigated as a method of treatment [3, 4]. This treatment requires an extremely sensitive assay, one capable of measuring doxorubicin in concentrations of 1 ng/ml or less in plasma. While assays utilizing fluorescence detection currently exist which can detect 1—2 ng quantities of doxorubicin, the use of only a fraction of the final processed plasma sample volume results in sensitivities in the range of 5—15 ng/ml of sample [5—7]. In addition, these assays often involve liquid—liquid extraction with organic solvents [5, 6] as well as other time-consuming sample handling techniques. A high-performance liquid chromatographic (HPLC) assay for daunorubicin and its metabolites has been devised using electrochemical detection [8]. This assay utilizes a loop column for sample extraction and has a sensitivity of 2 ng on column. However, the sample volume utilized was only 250 μl, resulting in a detection limit of 8 ng/ml of plasma.

We have developed an assay for doxorubicin that is rapid, simple to use, and highly sensitive; using electrochemical detection the detection limit is 2 ng/ml of plasma; with fluorescence detection 0.5 ng/ml doxorubicin in plasma is easily detectable. With this assay we are able to determine the levels of doxorubicin and doxorubicinol, a major metabolite of doxorubicin, in plasma samples taken from patients participating in a study of long-term low-dose continuous doxorubicin infusion [9]. Here we document the assay and report the levels measured.

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EXPERIMENTAL

Materials

Doxorubicin hydrochloride was obtained from Sigma (St. Louis, MO, U.S.A.). Doxorubicinol was kindly donated by Farmitalia (Milan, Italy), daunorubicin by Adria Labs. (Columbus, OH, U.S.A.). Ultraviolet (UV) grade acetonitrile was obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.), reagent-grade glacial acetic acid from J.T. Baker (Phillipsburg, NJ, U.S.A.). Tritiated water (0.25 mCi/g) and Aquasol liquid scintillation counter cocktail were obtained from New England Nuclear (Boston, MA, U.S.A.). All water used was obtained from a Waters Assoc. (Milford, MA, U.S.A.) Milli-Q water conditioning system.

Chromatography

The HPLC system consisted of an Altex Model 110 solvent metering pump, a Rheodyne Model 7125 sample injector valve equipped with a 400-μl sample loop, a 50 mm × 4 mm I.D. guard column filled with Waters Assoc. Bondapak Phenyl/Corasil packing (37- 50 μm particle size), a 300 mm × 4 mm I.D. Waters Assoc. μBondapak Phenyl column (10 μm particle size), a Beckman Model 157 fluorescence detector and a BioAnalytical Systems Model LC-4 amperometric detector. Outputs from both detectors were recorded on a Scientific Products dual-pen recorder. The fluorescence detector was equipped with a 480-nm excitation filter (filter No. 096480) and a 560-nm emission filter (filter No. 096560). The electrochemical detector was equipped with a TL-5 thin-layer flow cell containing a glassy carbon electrode. The applied voltage was +0.700 V.

The mobile phase was an acetonitrile–acetic acid–water solution (27:1:72) adjusted to pH 4.3 with a 20% (w/v) sodium acetate solution. The solution was filtered with a Nuclepore 0.45-μm filter and degassed prior to use. A small portion of this mobile phase was set aside in a sealed container for use as an extraction buffer.

Flow-rate was 1.2 ml/min at a pressure of 48.3 bars.

Preparation of standards

The stock solution of 5 μg/ml doxorubicin was prepared in 0.1 m*M* hydrochloric acid containing 0.9% sodium chloride, stored in an aluminum foil covered glass container, and kept refrigerated at all times. The concentration of the stock solution was periodically checked by UV absorption measurements made at 233 nm and 253 nm in methanol using known molar extinction coefficients (ϵ_{233} = 38150 M^{-1} cm^{-1}, ϵ_{253} = 25500 M^{-1} cm^{-1}). Dilute solutions (< 50 ng/ml) of doxorubicin in water, methanol or ethanol were found to be unstable; solutions of <10 ng/ml of doxorubicin in hydrochloric acid–sodium chloride were found to be stable for >24 h when kept on ice in a dark environment. Doxorubicin standards in hydrochloric acid–sodium chloride for drug recovery experiments were prepared daily from the stock solution. Doxorubicin standards used for drug recovery experiments or for standard curve determinations were prepared weekly in plasma. Internal standard (daunorubicin) used in the assay was also prepared in plasma as described above.

Recovery data were obtained from a comparison of peak heights of samples of doxorubicin in hydrochloric acid–sodium chloride with peak heights of extracts of normal plasma spiked with equivalent amounts of doxorubicin.

Standard curves of peak height ratio of doxorubicin to daunorubicin versus doxorubicin concentration over the range 0–50 ng/ml were obtained by spiking 1-ml aliquots of normal plasma with varying amounts of doxorubicin and a constant amount of daunorubicin.

Cartridge preparation

The extraction cartridge consisted of a Rainin 200-μl disposable pipette tip filled with 50 mg of Waters Assoc. Bondapak Phenyl packing sandwiched between two glass wool plugs. The cartridge was attached to a Becton, Dickinson and Co. 10-ml plastic syringe.

Prior to use, the cartridge was activated by rinsing with 2 ml of extraction buffer (HPLC mobile phase), followed by a 4-ml water rinse.

Sample extraction

To each 1-ml sample to be analyzed, 5 ng of daunorubicin (internal standard, in 100 μl normal plasma) were added before extraction. The sample was then transferred to the syringe–cartridge combination and pushed through with the syringe plunger at a flow-rate of 0.3–0.5 ml/min, with the sample being collected in its original container. The sample was passed through the cartridge a second time at the same flow-rate, then discarded. The cartridge was washed once with 4 ml of water, after which 20 ml of air were pushed through the cartridge to remove as much of the water remaining in the cartridge as possible. Mobile phase buffer (300 μl) containing 30% acetonitrile (v/v) was then pushed through the cartridge at a flow-rate of 0.3–0.5 ml/min and collected in a polypropylene conical test tube. All of this extraction buffer was then immediately injected onto the column.

Extraction efficiency

To determine the percent recovery of the 300 μl of buffer used in the extraction procedure and the percent of the total applied to the HPLC column, 0.05 μCi of ^{3}H-labelled water was introduced into the extraction buffer, and aliquots were taken at several points in the extraction/injection process and analyzed for radioactivity. Samples were taken at the following points: (1) before doxorubicin was eluted from the cartridge; (2) after the eluate was collected; and (3) immediately prior to injection of the sample onto the column. The samples were collected in 10 ml of Aquasol liquid scintillation counter cocktail and analyzed on a Beckman Instruments Model LS 8100 liquid scintillation counter.

RESULTS AND DISCUSSION

Chromatography

Under the conditions used the elution order of compounds of interest was doxorubicinol, doxorubicin, and daunorubicin. The retention times were 6.6, 10.3, and 20.1 min, respectively. Fig. 1 shows a chromatogram of doxo-

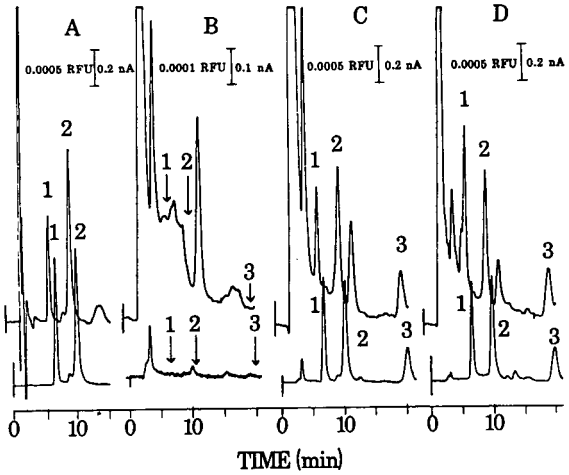

Fig. 1. Chromatograms of doxorubicinol, doxorubicin, and internal standard using electrochemical (top) and fluorescence (bottom) detection. (A) Doxorubicinol (10 ng) and doxorubicin (10 ng) standards; (B) drug-free human plasma; (C) human plasma spiked with 10 ng of doxorubicin, 10 ng of doxorubicinol, and 5 ng of internal standard; (D) plasma sample of patient 3 h after intravenous infusion of doxorubicin. Peaks: 1 = doxorubicinol, 2 = doxorubicin and 3 = daunorubicin, internal standard.

rubicinol and doxorubicin standards diluted in hydrochloric acid–sodium chloride and chromatograms of three plasma sample extracts; blank plasma, blank plasma spiked with doxorubicinol, doxorubicin, and internal standard; and plasma taken from a patient 3 h after an intravenous infusion of 70 mg of doxorubicin.

Extraction efficiency

In order to establish the efficiency of the extraction process, it is necessary to know the maximum attainable recovery (MAR) as well as the actual recovery. To determine the MAR, samples of the extraction buffer were analyzed at several points in the extraction/injection process for any concentration and/or volume changes that might affect the recovery of the drug. The results of the analyses show that: (1) the extraction buffer undergoes a 2.0 ± 0.4% dilution during the doxorubicin elution process; (2) only 90 ± 2% of the extraction buffer can be recovered from the cartridge; and (3) only 88 ± 7% of the cartridge eluate can be injected onto the column. If one assumes that the 2% dilution has only a negligible effect on the elution process and that the cartridge extracts 100% of the doxorubicin present in the plasma, then the MAR is 79%.

The percent of doxorubicin recovered by the extraction process was established by determining the recovery of varying amounts of doxorubicin from a 1-ml plasma sample and the recovery of a fixed amount of doxorubicin (10 ng) from various-sized samples. Table I lists these results, which show that the percent of doxorubicin recovered varies from 66.1 ± 0.9% to 70.0 ± 1.4% depending on sample volume, while recovery from 1 ml of plasma is essentially independent of doxorubicin concentration at 69.7 ± 0.3%. The extraction process therefore has an efficiency of 0.88.

TABLE I

MEAN PERCENT RECOVERY OF DOXORUBICIN FROM PLASMA (n = 3)

Amount of doxorubicin (ng)	Sample volume (ml)	Percent recovery (± S.D.)
1	1.0	69.6 ± 0.7
10	1.0	70.0 ± 0.4
100	1.0	69.6 ± 0.6
10	0.5	67.4 ± 1.0
10	2.0	67.6 ± 1.5
10	3.0	66.1 ± 0.9

Recovery of doxorubicin from 1 ml of plasma using only a single sample pass through the cartridge, as well as using three sample passes through the cartridge, was briefly examined. For a 1-ml plasma sample containing 10 ng of doxorubicin it was found that using a single sample pass through the cartridge reduced the recovery to 52.7 ± 3.0% while increasing the number of sample passes to three did not significantly increase the recovery. Moreover, the cartridge exhibited a tendency to clog during the third pass. Plasma samples larger than 3 ml also tended to clog the cartridge when extracted with only two sample passes.

Recovery of doxorubicin from 1 ml of plasma as a function of amount of packing used was also examined. Recoveries from 25 mg of packing were 9.1 ± 0.8% less than with 50 mg; recoveries with 100 ng were 8.6 ± 1.3% greater than with 50 mg. However, at 100 mg, clogging of the cartridge occurred quite frequently even on the first pass of a 1-ml sample.

Quantitation of standards

A plot of doxorubicin added to plasma versus doxorubicin measured in plasma was prepared from standard curve data gathered over several weeks for each method of detection. For fluorescence detection the slope was 1.004, the y-intercept was 0.02, and the correlation coefficient was 1.00. For electrochemical detection the slope was 1.01, the y-intercept was 0.03, and the correlation coefficient was 1.00. Sensitivity with the electrochemical detector has a lower limit of 2 ng per sample; with the fluorescence detector 0.5 ng per sample levels are easily determined.

Doxorubicinol

Quantitation of doxorubicinol, one of the major metabolites of doxorubicin, was also examined. The recovery and fluorescence detection limits of doxorubicinol are similar to those of doxorubicin. The electrochemical detector is approximately 40% more sensitive to doxorubicin than to doxorubicinol, resulting in a proportionately higher detection limit for doxorubicinol.

Application

We have used the HPLC assay described here to analyze serial plasma samples from a patient with metastatic adenoid cystic carcinoma of the parotid. This patient was part of a phase I study to examine the safety of long-term low-dose continuous doxorubicin infusion therapy using an implanted Medtronic pump.

Details of the study are published elsewhere [9]. Briefly, infusion of doxorubicin was initiated at a dose of 1.5 mg/m^2/day, and in the absence of severe symptoms of toxicity, the dose was escalated by 0.5—0.75 mg/m^2/day every two weeks. Plasma samples were taken every two weeks prior to dose escalation. The samples were collected in heparinized tubes, centrifuged, and frozen at —20°C for two to four weeks prior to the analysis for doxorubicin and doxorubicinol using fluorescence detection. Results are listed in Table II. Doxorubicin and doxorubicinol plasma concentrations increased in a dose-dependent manner. On four out of five days the ratio of doxorubicin to doxorubicinol concentration appeared independent of dose, remaining at approximately 2.

TABLE II

DOXORUBICIN AND DOXORUBICINOL PLASMA CONCENTRATIONS IN A PATIENT RECEIVING LOW-DOSE CONTINUOUS DOXORUBICIN INFUSION

Sample date	Dose* (mg/day)	Doxorubicin concentration (ng/ml)	Doxorubicinol concentration (ng/ml)
14	2.59	2.0	0.8
28	3.21	1.4	1.3
56	5.64	7.2	3.5
68	6.25	8.4	4.4
82	6.21	9.9	5.2

*Dose listed is for the two-week period prior to sample date; dose administered was based on patient's body surface area (1.58 m^2).

The assay described here is rapid, highly sensitive, and easy to use. It replaces the widely used multiple liquid—liquid extractions with a single solid—liquid extraction, and results in a concentration of sample in a final volume small enough to be totally analyzed. This procedure eliminates the need for concentrating the final sample by evaporation, or analysis of only a small fraction of the final sample volume. Utilization of the entire final sample volume yields sub-nanogram sensitivity, which allows the method to be used to measure heretofore undetectable plasma levels of doxorubicin and doxorubicinol in patients undergoing low-dose continuous doxorubicin infusion, as well as allowing its use with patients undergoing standard treatment.

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CHROMBIO. 2359

Letter to the Editor

High-performance liquid chromatographic method for the simultaneous determination of myocardial creatine phosphate and adenosine nucleotides*

Sir,

In their recent note on the simultaneous analysis of myocardial creatine phosphate and adenine nucleotides, Bedford and Chiong [1] refer to our methodology "before publication". We would like to point out that our work, which is quite similar, has already been published, in 1982 in this journal [2], a fact ignored by the above authors. Our high-performance liquid chromatographic method did not work in their hands, which could be due to ultraviolet-absorbing impurities in the potassium dihydrogen phosphate, which vary from batch to batch. When we analyse small heart biopsies, we routinely purify this salt as described by Karkas et al. [3], using a Chelex-100 column (Bio-Rad Labs., Richmond, CA, U.S.A.). Alternatively, we substitute ammonium dihydrogen phosphate (Merck, Darmstadt, F.R.G.) for the potassium dihydrogen phosphate in buffer B, and use ammonium hydroxide (Merck) instead of potassium hydroxide for the preparation of buffer A [2]. For the analysis of freeze-clamped rat hearts, however, this purification or substitution was never necessary.

Bedford and Chiong [1] replaced half of the potassium dihydrogen phosphate by potassium chloride to avoid salt precipitation in the chromatographic system. We clean our chromatographs twice a month with water to avoid this problem (which is likely to occur also with potassium chloride). Besides, solutions containing halide salts should be avoided as mobile phases, since components made from stainless steel are subject to their attack [4].

The separation with the modified system [1] is hardly an improvement. Only half of the creatine phosphate peak disappears with the enzyme shift method (cf. Fig. 1B and D in ref. 1), indicating that this compound coelutes with substantial amounts of an unknown one.

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*For reasons of consistency in indexing and abstracting systems, the title conforms to the original publication by Bedford and Chiong [1]. The authors want to point out, however, that they consider "adenosine nucleotide" incorrect, and that they strongly prefer the term "adenine nucleotide".

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In conclusion, the modification proposed by Bedford and Chiong [1] for the analysis of myocardial creatine phosphate and adenine nucleotides does not seem to offer advantages compared to our original method [2].

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(Received August 3rd, 1984)

Journal of Chromatography, 337 (1985) 203
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2360

Letter to the Editor

High-performance liquid chromatographic method for the simultaneous determination of myocardial creatine phosphate and adenosine nucleotides

Reply to E. Harmsen et al.

Sir,

We would like to comment on Drs. Harmsen, De Tombe and De Jong's Letter to the Editor regarding our note "High-performance liquid chromatographic method for the simultaneous determination of myocardial creatine phosphate and adenosine nucleotides" [1].

First, we gave proper credit to Dr. Harmsen et al. for an abstract [2] and for a personal communication, when their publication in this Journal [3] was not available to us. We were also thankful for their kindness in letting us use their method before publication, as stated in our note. As far as we are concerned, there is no question of priority and we do not claim one.

Secondly, our high-performance liquid chromatographic (HPLC) system is different from that used by Dr. Harmsen et al., and salt precipitation was a real problem within a few days of use.

Thirdly, we never claimed that our method is better than that described by Dr. Harmsen et al. [3]. Our method is a variation adapted to our different HPLC system and works well for us. This point was also made quite clear in our Discussion.

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M.A. CHIONG*

1 G.K. Bedford and M.A. Chiong, J. Chromatogr., 305 (1984) 183.
2 E. Harmsen, P.P. de Tombe and J.W. de Jong, J. Mol. Cell. Cardiol., 13 (Suppl. 1) (1980) 38.
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Vol. 337, No. 1.

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

MEETINGS

9th INTERNATIONAL SYMPOSIUM ON BIOMEDICAL APPLICATIONS OF CHROMATOGRAPHY, ROSTOCK, G.D.R., SEPTEMBER 25–27, 1985

The Society for Clinical Chemistry and Laboratory Diagnostics of the German Democratic Republic and the Chromatography Group of the Czechoslovak Chemical Society will organize their 9th international symposium in Rostock, G.D.R.

The scientific programme will include invited plenary lectures, selected original contributions, posters and discussion. The scope of this symposium will cover all kinds of chromatographic techniques with special emphasis on (1) new developments in chromatographic techniques; (2) analysis of biopolymers; (3) analysis of endogenous compounds; (4) use of chromatography for diagnostic purposes; and (5) analysis of exogenous compounds (including drug monitoring).

Further information can be obtained from Dr. Joachim Wagner, Medical Clinic of the K. Marx University, Johannisallee 32, DDR-7010 Leipzig, G.D.R.

2nd SYMPOSIUM ON HANDLING OF ENVIRONMENTAL AND BIOLOGICAL SAMPLES IN CHROMATOGRAPHY, FREIBURG, F.R.G., OCTOBER 24–25, 1985

The organisation of this second event – the first was held in Lausanne, Switzerland in November 1983 – is in the hands of the International Association of Environmental Analytical Chemistry and sponsored by national bodies. A strong industrial participation is planned. It is the intention to bring together specialists in this field who can give a good account of the state-of-art in their respective specialty and to present first-hand experience in sample handling. Continuous flow extraction techniques, solid surface sample handling with pre-column technology (on-line and off-line), pre-chromatographic use of derivatization techniques, column-switching methodology for handling of complex samples are some of the topics that will be treated and extensively discussed. Special emphasis will be placed on techniques with automation potential and actually automated procedures suitable for routine handling of larger series of samples. Much of this methodology and "philosophy" can be applied to different types of matrices and problem solving but it is the intention to concentrate on applications to biological (urine, blood, tissue, plant material) and environmental samples (water, waste water, air) with drugs (pharmaceuticals) and priority pollutants as the analytes.

Further information from: Workshop Office IAEAC, M. Frei-Hausler, Postfach 46, CH-4123 Allschwil 2, Switzerland.

PRIZE

PRIZE BIOCHEMICAL ANALYSIS 1986

The German Society for Clinical Chemistry awards the prize Biochemical Analysis every two years at the conference "Biochemische Analytik" in Munich, F.R.G.

The prize of DM 10,000.00 is donated by Boehringer Mannheim for outstanding and novel work in the field of biochemical analysis or biochemical instrumentation or for significant contributions to the advancement in experimental biology especially relating to clinical biochemistry.

Competitors for the prize 1986 (conference June 3–6, 1986) should submit papers concerning one theme, either published or accepted for publication between October 1st, 1983 and September 30th, 1985, before November 15th, 1985, to: Prof. Dr. H. Feldmann, Secretary of the prize Biochemical Analysis, Institut für Physiologische Chemie der Universität, Goethestrasse 33, D-8000 Munich 2, F.R.G. If several authors are involved in this work, please, indicate the name(s) of the candidate(s).

CALENDAR OF FORTHCOMING EVENTS

Feb. 25–March 1, 1985 New Orleans, LA, U.S.A.	**36th Pittsburgh Conference and Exposition on Analytical Chemistry and Applied Spectroscopy** Contact: Linda Briggs, Pittsburgh Conference, 437 Donald Road, Dept. J-005, Pittsburgh, PA 15235, U.S.A. (Further details published in Vol. 312.)
March 25–29, 1985 Loughborough, U.K.	**Separations for Biotechnology and Biochemistry** Contact: Miss C.D. Newton, Department of Chemistry, Loughborough University of Technology, Loughborough, Leics. LE11 3TU, U.K. Tel.: (0509) 263171 ext. 351.
March 26–June 7, 1985 Uppsala, Sweden	**Biochemical Separation Methods** Contact: Eva Linder, secretary, Institute of Biochemistry, University of Uppsala, Biomedical Center, P.O. Box 576, S-751 23 Uppsala, Sweden. (Further details published in Vol. 312.)
April 14–19, 1985 Limassol, Cyprus	**2nd Cyprus Conference on New Methods in Drug Research** Contact: 2nd Cyprus Conference on New Methods in Drug Research, c/o Reso Congress Service, S-105 24 Stockholm, Sweden. Tel.: 08-14 49 10. Telex: 103 23 Reso S
April 28–May 3, 1985 Miami Beach, FL, U.S.A.	**189th National Meeting of the American Chemical Society** Contact: Meetings Department, American Chemical Society, 1155 Sixteenth Street, NW, Washington, DC 20036, U.S.A.
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 17–21, 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.
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.)
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. 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. 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.
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.
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

Contemporary practice of chromatography, by C.F. Poole and S.A. Schuette, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, X + 708 pp., price Dfl. 159.00, US $61.25, ISBN 0-444-42410-5.

Chromatography of alkaloids, Part B, Gas–liquid chromatography and high-performance liquid chromatography (*Journal of Chromatography Library,* Vol. 23B), by R. Verpoorte and A. Baerheim Svendsen, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, ca. 450 pp., price Dfl. 245.00, US$ 94.25, ISBN 0-444-42265-x.

Analysis of neuropeptides by liquid chromatography and mass spectrometry (*Techniques and Instrumentation in Analytical Chemistry,* Vol. 6), by D.M. Desiderio, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, XVIII + 236 pp., price Dfl. 165.00, US$ 63.50, ISBN 0-444-42418-0.

Microprocessor programming and applications for scientists and engineers. (*Data Handling in Science and Technology,* Vol. 1), by R.R. Smardzweski, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, XIV + 354 pp., price Dfl. 98.00, US$ 37.75, ISBN 0-444-42407-5.

Current practice of clinical electromyography, edited by S.L.H. Notermans, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, 568 pp., price Dfl. 350.00, US$ 134.50, ISBN 0-444-805-2.

Clinical pharmacology of antihypertensive drugs (*Handbook of Hypertension,* Vol. 5), edited by A.E. Doyle, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, 428 pp., price Dfl. 260.00, US$ 100.00, ISBN 0-444-90354-2.

Methods of protein analysis, edited by I. Kerese, Ellis Horwood (Wiley), Chichester/ Halsted (Wiley), New York, 1984, 371 pp., price £ 39.50, ISBN 0-85312-176-1 (Ellis Horwood), 0-470-27497-2 (Halsted).

A guidebook to lipoprotein technique (*Laboratory Techniques in Biochemistry and Molecular Biology,* Vol. 14), edited by G.L. Mills, P.A. Lane and P.K. Weech, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, XIV + 512 pp., price Dfl. 250.00, US$ 96.25 (hardback), Dfl. 80.00, US$ 30.75 (paperback), ISBN 0-444-80591-5 (hardback), 0-444-80574-5 (paperback).

Biomedical and clinical aspects of coenzyme Q, Vol. 4, edited by K. Folkers and Y. Yamamura, Elsevier, Amsterdam, Oxford, New York, Tokyo, 1984, XVI + 432 pp., price Dfl. 224.00, US$ 86.25, ISBN 0-444-80380-7.

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Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	N 1984	D 1984	J 1985	F	M	
Journal of Chromatography	312 314	315 316 317	318/1 318/2 319/1	319/2 319/3 320/1	320/2	The publication schedule for further issues will be published later
Chromatographic Reviews		313				
Bibliography Section				335/1		
Biomedical Applications		336/1 336/2	337/1	337/2 338/1		

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.)

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