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MASS FRAGMENTOGRAPHIC IDENTIFICATION OF POLYAMINE METABOLITES IN THE URINE OF NORMAL PERSONS AND CANCER PATIENTS, AND ITS RELEVANCE TO THE USE OF POLYAMINES AS TUMOUR MARKERS

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(First received September 18th, 1984; revised manuscript received November 1st, 1984)

SUMMARY

The mass fragmentographic identification of N-(2-carboxyethyl)-4-amino-*n*-butyric acid, N-(3-aminopropyl)-N[1]-(2-carboxyethyl)-1,4-diaminobutane, N,N[1]-bis(2-carboxyethyl)-1,4-diaminobutane, and δ-aminovaleric acid in acid-hydrolysed urines of a normal person and two cancer patients is described. A previous study, in which the metabolic fate of intraperitoneally injected polyamines in rats was investigated, revealed that these compounds should be considered as non-α-amino acid metabolites of the naturally occurring polyamines. Quantification of polyamines and their non-α-amino acid metabolites by gas chromatography with nitrogen—phosphorus detection showed that, relative to the parent polyamines, humans normally excrete higher quantities of polyamine catabolites in urine than rats, suggesting that humans catabolize polyamines more efficiently. As illustrated by the follow-up of the concentrations of polyamines and their catabolites in the urine of a patient with high-grade non-Hodgkin lymphoma during chemotherapy, the catabolic pressure on polyamines may be considerably increased during neoplastic diseases, since an even higher proportion of oxidized polyamine metabolites was observed. It is therefore suggested that the additional measurement of the circulating concentrations of polyamine-degrading enzymes is of importance for the correct interpretation of polyamine (metabolite) determinations for oncological purposes.

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INTRODUCTION

The polyamines are polybasic compounds that are fundamentally involved in a variety of cellular processes such as stabilization of nucleic acids and membranes, growth, differentiation and protein synthesis [1—3]. Although polyamine biosynthesis has been extensively examined [1, 3, 4], in man relatively little is known about their catabolism [4—6].

The catabolism of polyamines in rats is complex, comprising the action of a multitude of catabolic enzymes, of which the relative activities may be subject to time-dependent variation caused by enzyme induction [7, 8]. One of the degradative pathways for polyamines includes the oxidative deamination of one or both primary amino groups by amine oxidases, followed by intermediate aldehyde oxidation [6—11]. The products of the combined action of these enzymes are the non-α-amino acid metabolites.

In this study the mass fragmentographic identification of N-(2-carboxyethyl)-4-amino-*n*-butyric acid (a metabolite of spermidine), N-(3-aminopropyl)-N^1-(2-carboxyethyl)-1,4-diaminobutane and N,N^1-bis(2-carboxyethyl)-1,4-diaminobutane (both metabolites of spermine), and δ-aminovaleric acid (a metabolite of cadaverine) is described. Using gas chromatographic (GC) quantification a comparison between the concentrations of polyamines and their non-α-amino acid metabolites in the urine of normal humans and rats was made. The significance of isoputreanine, the quantitatively most important urinary non-α-amino acid metabolite, was investigated in a follow-up study of a patient suffering from non-Hodgkin lymphoma and treated with cytostatic drugs. Finally, the role of oxidative catabolism of polyamines in cancer patients is discussed.

MATERIALS AND METHODS

Standards and reagents

δ-Aminovaleric acid was from Aldrich Europe (Beerse, Belgium), Sep-Pak silica cartridges were from Waters Assoc. (Milford, MA, U.S.A.) and 3 ml aromatic sulphonic acid disposable extraction columns from J.T. Baker (Phillipsburg, NJ, U.S.A.); heptafluorobutyric anhydride (HFBA) was from Pierce (Rockford, IL, U.S.A.); all other reagents were from E. Merck (Darmstadt, F.R.G.).

N,N^1-bis(2-Carboxyethyl)-1,4-diaminobutane (spermic acid 2), and N-(2-carboxyethyl)-4-amino-*n*-butyric acid (spermidic acid 2) were prepared as previously described [8, 12]. N-(3-Aminopropyl)-N^1-(2-carboxyethyl)-1,4-diaminobutane (spermic acid 1) was prepared by cyanoethylation [13] of spermidine with acrylonitrile in alkaline solution, followed by acid hydrolysis in 6 *M* hydrochloric acid at 120°C.

Equipment

GC with nitrogen—phosphorus detection was performed with a Hewlett-Packard Model 5880 gas chromatograph equipped with a Model 7672 A automated sampler and interfaced with a Tracor 812 analytical processing data

system. The gas chromatograph was operated under the previously described conditions [12].

Gas chromatography—mass spectrometry was performed using a Varian 3700 gas chromatograph directly coupled to a MAT 212 mass spectrometer, operated under the following conditions: injection temperature 250°C; oven temperature programme 1 min at 50°C, 16°C/min to 250°C, 10 min at 250°C; ion source temperature 200°C; ionization energy 70 eV. The column was a 30 m × 0.32 mm I.D. CP-Sil-5 coated (0.2 μm film thickness), fused-silica capillary from Chrompack (Middelburg, The Netherlands).

Samples

Urine samples (24 h and untimed voidings) from normal persons and patients were collected, acidified to pH 1—2 with hydrochloric acid, and stored at —20°C until analysis.

Prepurification and derivatization

After acid hydrolysis 1,3-diaminopropane, putrescine, cadaverine, isoputreanine, putreanine, spermidine, spermic acid 1, spermic acid 2, and spermine were isolated by silica gel adsorption and converted into their (methyl)heptafluorobutyryl derivatives [12].

From the passage of the silica gel column β-alanine (β-Ala), γ-amino-*n*-butyric acid (GABA), δ-aminovaleric acid, and spermidic acid 2 were isolated by means of cation-exchange chromatography and converted into their isobutylheptafluorobutyryl derivatives [8].

Mass fragmentographic identification

After the on-column injection of derivatized silica gel extracts and cation-exchange extracts, mass fragmentography was performed by monitoring specific electron-impact fragment ions of spermic acid 1 (*m/z* 590, 618, 622, and 650), spermic acid 2 (*m/z* 354, 423, 455, and 483), spermidic acid 2 (*m/z* 368, 410, and 483), and δ-aminovaleric acid (*m/z* 226, 239, 251, 268 and 296). For each compound the peak areas were calculated, using a Finnigan MAT SS-200 data system. Peak areas were expressed as a ratio to the area of the most intense fragment ion.

Identification was performed by comparing peak area data and GC retention times to those of synthetic standards. In some cases the relative peak heights were used.

Quantification of polyamines and their metabolites

Quantification of polyamines and metabolites in untimed voidings of a high-grade non-Hodgkin lymphoma patient were performed by GC with nitrogen—phosphorus detection [12]. Data for the normal 24-h urinary excretion of humans and rats were obtained from previous studies [8, 12].

RESULTS AND DISCUSSION

On the basis of their GC retention times and the peak areas of selected fragment ions spermidic acid 2, spermic acid 1, spermic acid 2 and δ-aminovaleric

acid were identified in the urine of a normal person, a patient with a non-African, Burkitt-type, non-Hodgkin lymphoma, and a patient with metastatic melanoma. In Figs. 1—4 the recordings of the selected ions are depicted together with those obtained from derivatized synthetic standards. The quanti-

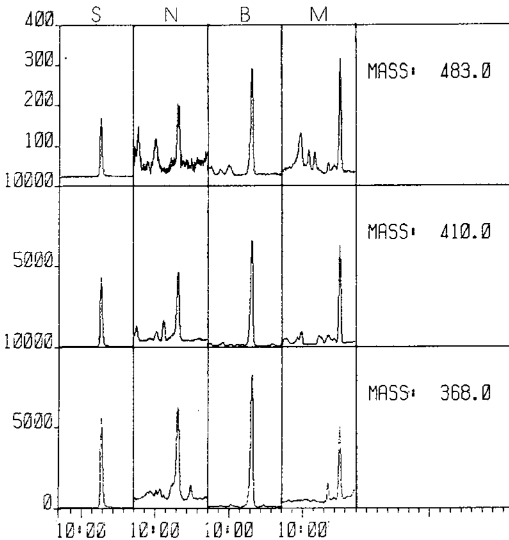

Fig. 1. Mass fragmentograms of spermidic acid 2 in a standard (S) and urine samples of a normal person (N) and patients with non-African, Burkitt-type, non-Hodgkin lymphoma (B) and metastatic melanoma (M). Time scale is in minutes and peak intensity in arbitrary units (counts).

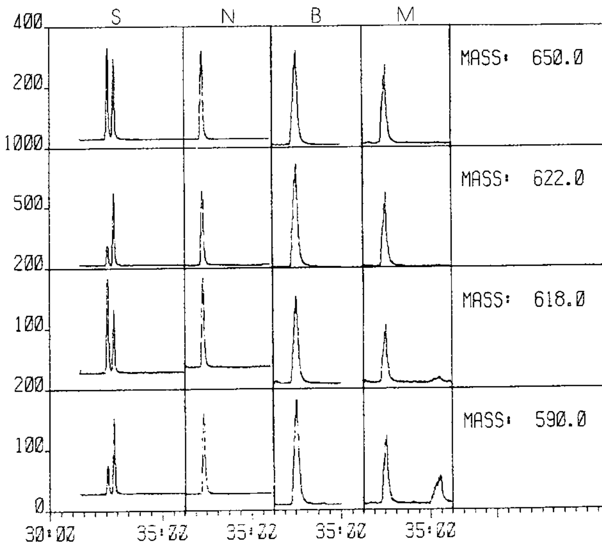

Fig. 2. Mass fragmentograms of spermic acid 1 in a standard (S) and urine samples of a normal person (N) and patients with non-African, Burkitt-type, non-Hodgkin lymphoma (B) and metastatic melanoma (M). Time scale is in minutes and peak intensity in arbitrary units (counts).

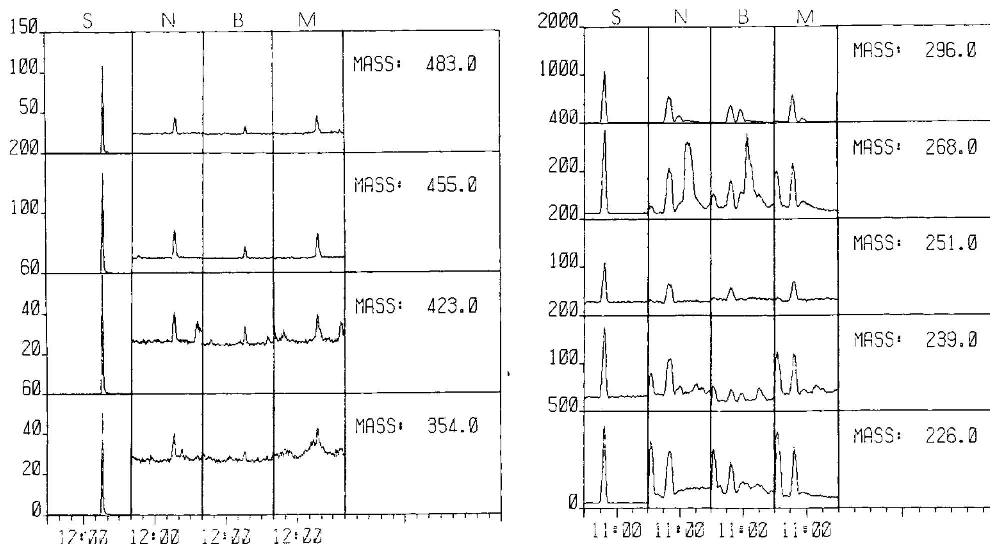

Fig. 3. Mass fragmentograms of spermic acid 2 in a standard (S) and urine samples of a normal person (N) and patients with non-African, Burkitt-type, non-Hodgkin lymphoma (B) and metastatic melanoma (M). Time scale is in minutes and peak intensity in arbitrary units (counts).

Fig. 4. Mass fragmentograms of δ-aminovaleric acid in a standard (S) and urine samples of a normal person (N) and patients with non-African, Burkitt-type, non-Hodgkin lymphoma (B) and metastatic melanoma (M). Time scale is in minutes and peak intensity in arbitrary units (counts).

tative results relevant for the identification are shown in Table I. The intensities of the characteristic ions are presented as a percentage of the most intense fragment ion of each compound. To avoid day-to-day variation in mass spectrometric performance the measurements for each individual metabolite were collected within one day. In Fig. 2 the mass fragmentographic peaks in the standard, with a retention time of 32 min and 35 sec, were caused by N-(3-aminopropyl)-N^1-(3-carboxypropyl)-1,3-diaminopropane. The relative peak areas of this isomer, which was formed in an amount comparable to that of spermic acid 1 during its synthesis, were found to be markedly different from those of spermic acid 1. The relatively high amounts of compounds that were co-extracted during the clean-up procedure led to peak broadening in the analysis of urine samples.

Table II shows a comparison between the levels of polyamines and their non-α-amino acid metabolites in the urine of normal three-month-old rats [8] and human adults [12]. Previous studies in rats [8] revealed that isoputreanine, putreanine and spermidic acid 2 should be considered as unique metabolites of spermidine, while spermic acid 1 and δ-aminovaleric acid are unique metabolites of spermine and cadaverine, respectively. A comparison between the ratios of these unique metabolites and their respective parent polyamines in normal rats and humans shows that, except for δ-aminovaleric acid/cadaverine, all metabolite/parent polyamine ratios are higher in human urine than in rat urine. A similar picture is obtained when GABA/putrescine ratios are

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TABLE I

RELATIVE INTENSITIES AND GAS CHROMATOGRAPHIC RETENTION TIMES FOR SELECTED IONS OF SPERMIDIC ACID 2, SPERMIC ACID 1, SPERMIC ACID 2 AND δ-AMINOVALERIC ACID IN STANDARDS AND URINE SAMPLES

Relative intensities are expressed as a percentage of the most intense fragment ion (m/z = 368 for spermidic acid 2, m/z = 622 for spermic acid 1, m/z = 455 for spermic acid 2 and m/z = 296 for δ-aminovaleric acid). t_R = Gas chromatographic retention time; S = standard; N = urine sample of a normal person; B = urine sample of a patient with non-African, Burkitt-type, non-Hodgkin lymphoma; M = urine sample of a patient with metastatic melanoma.

Compound	S	N	B	M
Spermidic acid 2				
m/z 410	74	74	77	71
m/z 483	3	3	3	3
t_R	10 min 26 sec	10 min 32 sec	10 min 32 sec	10 min 36 sec
Spermic acid 1				
m/z 650	44	45	45	44
m/z 590	19	21	20	19
m/z 618	17	17	16	16
t_R	32 min 51 sec	32 min 53 sec	33 min 01 sec	33 min 01 sec
Spermic acid 2				
m/z 483	56	50	53	54
m/z 423	32	33	38	28
m/z 354	29	28	26	26
t_R	12 min 23 sec	12 min 20 sec	12 min 23 sec	12 min 24 sec
δ-Aminovaleric acid				
m/z 226	39	35	37	36
m/z 268	33	35	32	32
m/z 239	14	14	14	14
m/z 251	8	8	7	7
t_R	10 min 52 sec	10 min 53 sec	10 min 52 sec	10 min 52 sec

considered. However, GABA is not a unique metabolite of putrescine and may also be formed by decarboxylation of glutamic acid [14]. As β-alanine at least partly originates from the catabolism of uracil, and no precursor—metabolite relationship between 1,3-diaminopropane and β-alanine seems to exist in rats [8], this ratio has not been considered. The relatively higher concentrations of oxidized metabolites of polyamines is suggestive for the existence of a higher catabolic pressure on polyamines in humans. Other, less probable, possibilities are that humans salvage polyamines more efficiently, or catabolize the non-α-amino acid metabolites less efficiently. The δ-aminovaleric acid/cadaverine ratio seems to be an exception to the rule, which may be connected with the, contrary to human beings, normal physiological role of cadaverine in rats, such as is established during pregnancy [15].

In rats considerable catabolic shifts were encountered between the first and second intraperitoneal injection of labelled polyamines. These shifts were explained to be indicative of the induction of degradative enzymes by the first

TABLE II

CONCENTRATIONS OF TOTAL POLYAMINES AND METABOLITES IN THE URINE OF RATS OF THE WISTAR STRAIN AND NORMAL HUMAN ADULTS

Concentrations are expressed as mmol/mol of creatinine.

	Rats (n = 5)		Humans (n = 52)	
	Mean	C.V.* (%)	Mean	C.V. (%)
1,3-Diaminopropane	2.10	32.9	0.33	30.5
β-Alanine	9.96	35.5	6.33**	27.3
Putrescine	10.93	19.9	1.44	32.9
γ-Aminobutyric acid	6.12	33.5	3.20**	34.7
Cadaverine	3.04	27.6	0.50	128.1
δ-Aminovaleric acid	10.90	52.3	1.10**	50.5
Spermidine	4.74	19.0	0.58	27.5
Isoputreanine	3.48	30.7	1.31	32.9
Putreanine	0.91	25.3	0.27***	23.8
Spermidic acid 2	1.51	67.5	0.39***	76.9
Spermine	0.45	33.3	0.13	85.2
Spermic acid 1	0.20	25.0	0.10	21.4
Spermic acid 2	1.03	57.3	—	—
γ-Aminobutyric acid/putrescine	0.59	40.7	2.22§	—
δ-Aminovaleric acid/cadaverine	4.21	72.7	2.20§	—
Isoputreanine/spermidine	0.78	47.4	2.34	25.1
Putreanine/spermidine	0.20	35.0	0.54	30.8
Spermidic acid 2/spermidine	0.36	91.7	0.67§	—
Spermic acid 1/spermine	0.47	31.9	1.17	52.5

*C.V. = coefficient of variation.
**Mean of five individuals.
***Population containing 32 individuals.
§Calculated by dividing the respective mean values.

injection [8]. In man, catabolic enzyme induction is perceived when relatively high amounts of polyamines are liberated by spontaneous or therapeutically induced (tumour) cell loss [16]. It has been demonstrated that a gestational age dependent increase in the serum levels of diamine oxidase [17] and polyamine oxidase [18] takes place during normal pregnancy. Increased diamine oxidase levels have been associated with several types of cancer [19].

In the urine samples of an adult patient with non-Hodgkin lymphoma (Fig. 5) we observed a highly significant increase of isoputreanine during successful chemotherapy but not of its parent polyamine, spermidine. The ratio between the areas under the curve of isoputreanine and spermidine was calculated to be 6.02 (normal mean ± S.D. for adults = 2.34 ± 0.59 [12]). These data suggest that at least some cases of cancer are characterized by an increased degradative metabolic pressure on polyamines and endorse the use of simultaneous determination of polyamines and their non-α-amino acid metabolites as general markers for neoplastic diseases. The additional measurements of the circulating concentrations of polyamine-degrading enzymes (e.g. diamine oxidase, polyamine oxidase and possibly monoamine oxidase) may become of use for both the detection and follow-up of cancer, as their increased concentration reflects the body's natural defense against increased polyamine liberation.

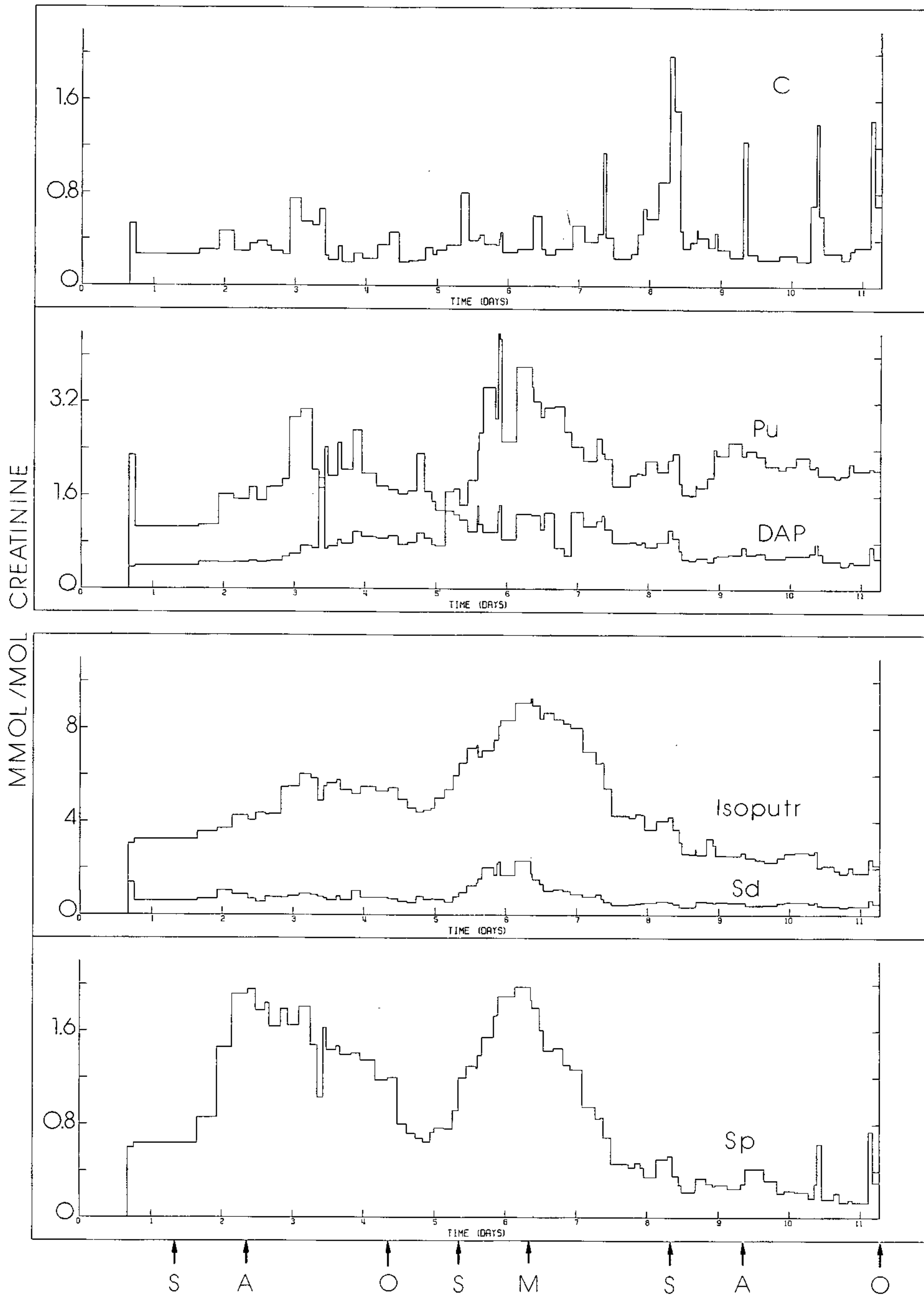

Fig. 5. Follow-up curve of polyamines and metabolite concentrations in untimed urine voidings of a 64-year-old man with stage IV non-Hodgkin lymphoma during treatment with a new chemotherapeutic regimen. S = Solumedrol® (methylprednisolone); A = cytosine arabinoside; O = Oncovin® (vincristine); M = methotrexate; DAP = 1,3-diaminopropane; Pu = putrescine; C = cadaverine; Isoputr = isoputreanine; Sd = spermidine; Sp = spermine. For normal urinary concentrations see Table II.

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DETERMINATION OF PLASMA TESTOSTERONE BY MASS FRAGMENTOGRAPHY USING [3,4-^{13}C]TESTOSTERONE AS AN INTERNAL STANDARD

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SUMMARY

The combination of glass capillary gas chromatography—mass spectrometry is especially suitable for the recognition of compounds. The use of [3,4-^{13}C]testosterone as internal standard, mass fragmentography and isotope ratio measurement have been applied to the quantitative determination of testosterone in plasma. This paper describes the method, using *tert.*-butyldimethylsilylmethoxime and di-heptafluorobutyrate derivatives. The calibration graph in isotopic dilution is examined. The results obtained are compared with the results obtained by radioimmunoassay. The sensitivity of the method is judged from the lower limit of detection: 4.5 pg. The precision, and inter- and intra-assay are calculated.

INTRODUCTION

The need for specific, rapid determinations of steroid hormone levels in the clinical laboratory has led to the development of numerous analytical methods.

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A quantitative method has been developed for the estimation of testosterone in human plasma using the technique of combined gas chromatography—mass spectrometry (GC—MS) and a labelled standard. Sweeley et al. [1] and Hammar et al. [2] demonstrated the applicability of using a labelled analogue as the ideal internal standard to correct for losses of a substance under study in the initial isolation procedures. The usefulness of mass fragmentography in which GC—MS is used, in conjunction with isotopic dilution has been of great interest because of its high specificity and high sensitivity [3—10].

This paper describes a relatively simple method for the determination of plasma testosterone with high specificity and accuracy. After extraction and conversion into methoxime-*tert*.-butyldimethylsilyl (MO-TBDMS) or di-heptafluorobutyrate (DHFB) derivatives, five specific ions have been followed by the multiple ion detector unit of the mass spectrometer.

The sensitivity, precision and accuracy of the procedure were judged. The amounts of unlabelled testosterone in male and female plasma were compared with the results obtained by a radioimmunoassay method.

MATERIALS AND METHODS

Internal standard

[3,4-^{13}C]Testosterone was obtained from the Commissariat â l'Energie Atomique (Molécules Marquées), Saclay, France. The purity and the ^{13}C enrichment of this standard were confirmed by GC—MS and nuclear magnetic resonance (pulsed NMR and ^{13}C-NMR).

Gas chromatography—mass fragmentography

The first instrument used was a Hewlett-Packard 5798 GC coupled with the mass spectrometer V.G. 305.F. The second instrument was a quadrupole mass filter Hewlett-Packard 5970-A coupled with the Hewlett-Packard 5792-A chromatograph. The capillary column was directly connected to the mass spectrometer source, without a silanized glass line. The ionization was realized by electron-impact: 70 eV, ion source temperature 200°C.

The column was a fused-silica capillary column, 25 m × 0.21 mm, with bonded apolar phase (SE-54 type), from Hewlett-Packard. This column was programmed from 210°C to 245°C at 2°C/min, and from 245°C to 290°C at 8°C/min. The first part of the programme allows the chromatographic separation and the high temperature of the second part is concerned with washing the column before a new separation.

Sample preparation

A 2-ml volume of heparinized male plasma or a 4-ml volume of female plasma was added to 80 ng of [3,4-^{13}C]testosterone obtained by evaporation of an ethanolic solution of the labelled standard; 3 ml of distilled water and 0.05 ml of 1 *M* sodium hydroxide were added. After mixing, the diluted plasma was extracted with 20 ml and 10 ml of diethyl ether. After centrifugation, the upper layer was decanted and evaporated to dryness. The derivatives were formed by reacting the residue with reagents as described below. An additional purification of the residue is realizable before this reaction: the residue was

treated with 3 ml of a freshly prepared solution of digitonin (1.7 g in 50 ml of absolute ethanol) and the mixture was allowed to stand at 100°C for 4 min and at 30°C for 30 min. The ethanol was evaporated and the residue was washed with 3 ml of 0.14 *M* sodium chloride. Testosterone was extracted twice with 10 ml of light petroleum. After evaporation of this phase, the residue was treated to form derivatives.

Formation of derivatives

The methoxime-*tert.*-butyldimethylsilyl derivative [8, 11—14] was formed by reacting the residue with 0.3 ml of methoxyamine · HCl (2%) in pyridine (MOX) (Pierce) for 1 h at 60°C. After evaporation, 0.2 ml of a freshly prepared mixture of TBDMS (2.5 mmol TBDMS-trifluoromethanesulphonate (Fluka), 5 mmol imidazole in 25 ml of dimethylformamide) was added. After 1 h at 40°C [11], the MO-TBDMS derivative was washed with 2 ml of water, and was extracted with 0.05 ml of hexane prior to gas chromatography.

Di-heptafluorobutyrate derivative [15] was formed by reacting the extract with 0.2 ml of heptafluorobutyric anhydride for 1 h at 60°C. After evaporation under a stream of nitrogen, the derivative was dissolved in 0.05 ml of hexane.

Radioimmunoassay

Plasma testosterone was determined in duplicate by a radioimmunoassay method used every day in our laboratory. The antibody was directly towards testosterone but reacted also to a small amount of 5α-dihydrotestosterone. The purification of testosterone from plasma was realized by chromatographic separation prior to radioimmunoassay using Celite microcolumns [16].

Isotope dilution—mass fragmentography

The two spectrometers were equipped with a multiple ion detector—peak matcher. The detector was focused on the ions at *m/e* 374 (M − 57), 375, 376, 377 and 378 for MO-TBDMS testosterone [17, 18] and at *m/e* 680 (M), 681, 682, 683 and 684 for DHFB testosterone.

The quantitative analysis and the concentration of the plasma testosterone used the equation of Pickup and McPherson [19]:

$$R_{kl} = \frac{(x/y)\,(P_k/E) + (Q_k/F)}{(x/y)\,(P_l/E) + (Q_l/F)} \quad \text{or} \quad R_{kl} = \frac{(x/y)P_kF + Q_kE}{(x/y)P_lF + Q_lE}$$

where R_{kl} is the ratio between the peaks at (M) and (M + 2) obtained in the recordings, E and F are the molecular masses of natural and labelled material, x is the mass of the natural material and y the mass of labelled compound. P_k, Q_k and P_l, Q_l are the relative intensities of the peaks at (M) and (M + 2) of the natural and the labelled compounds. These four coefficients were calculated using pure natural and labelled testosterones.

RESULTS AND DISCUSSION

Purity of [3,4-^{13}C] testosterone internal standard

The mass spectra of the MO-TBDMS and DHFB derivatives of natural and labelled testosterones are shown in Fig. 1. The presence of non-labelled

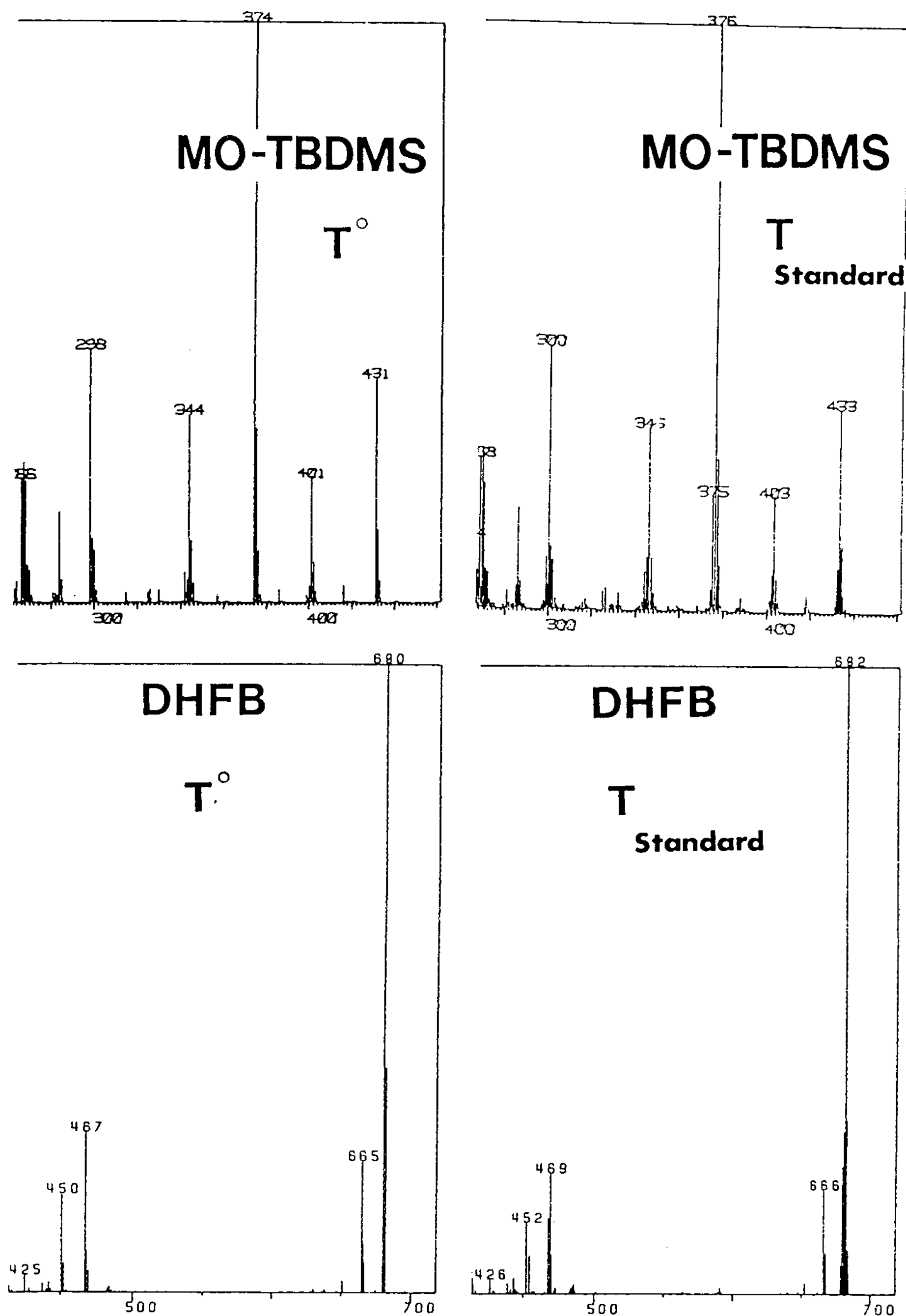

Fig. 1. Mass spectra of the MO-TBDMS and the DHFB derivatives of natural testosterone (T^0) and labelled testosterone ($T_{Standard}$).

testosterone in the standard is verified by calculation using the isotopic molecular ions. [3,4-^{13}C] Testosterone is a mixture of 80% of the bis-^{13}C compound (T**), 17% of the mono-^{13}C compound (T*) and 3% of the non-labelled compound (T^0). This is confirmed by high field NMR.

TABLE I

MEAN VALUES OF THE FOUR COEFFICIENTS FOR THE TWO DERIVATIVES

	MO-TBDMS derivatives		DHFB derivatives	
	Coefficient	C.V. (%)	Coefficient	C.V. (%)
Magnetic spectrometer (n = 22)				
P_k	0.72	0.64	0.71	0.3
Q_k	0.024	8.4	0.029	7.7
P_l	0.056	3.0	0.042	0.6
Q_l	0.62	2.0	0.61	1.1
Quadrupole mass filter (n = 11)				
P_k	Not measured		0.71	1.2
Q_k			0.014	5.5
P_l			0.046	2.8
Q_l			0.62	0.6

The presence of T^0 in the internal standard requires relatively complicated correction techniques for the observed peak height in quantitative analysis. The existence of T^0 in the standard increases Q_k in comparison with Q_l, and the ordinate for null abscissa.

The four coefficients P_k, P_l, Q_k and Q_l were determined using the peak heights of the molecular ions measured with the two spectrometers (Table I).

Standard curves

Standard curves $R_{kl} = f(x/y)$ used for the estimation of testosterone in male and female plasma have a linear part for small amounts of T^0 [4, 19—21]. The ratio R_{kl} between (M) and (M + 2) increases with increasing amounts of T^0.

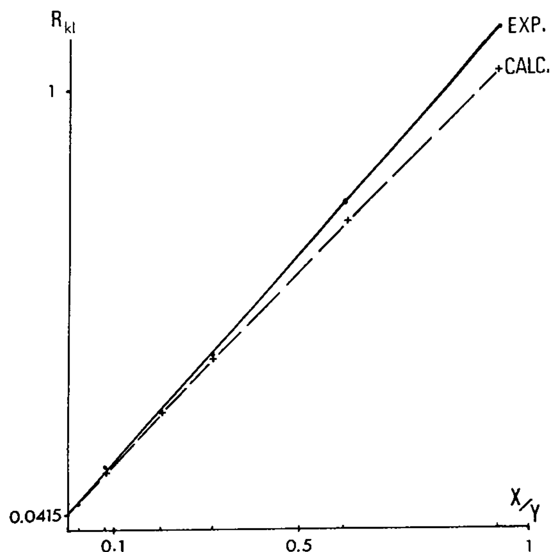

Fig. 2. Standard curves for determination of testosterone in the range of x/y = 0—1.

The Pickup—McPherson equation allows the mathematical approach of isotopic dilution, showing the real nearness of the experimental and the calculated curves (Fig. 2). There is a good agreement between the two curves for small values of x/y.

Nineteen standard curves were realized with the magnetic spectrometer during three years and three standard curves with the quadrupole mass filter. These 22 curves have a relative standard deviation of 4%. The equation of the middle curve for the magnetic spectrometer is

$$R_{kl} = \frac{(x/y)\,(13.47) + 0.48}{(x/y)\ + 11.55}$$

This curve is linear for x/y in the range 0—0.5, and the linear regression equation is $R_{kl} = 1.115\,(x/y) + 0.0447$. For $x/y = 0$, the experimental value of R_{kl} is the same as that of the calculated one.

The quadrupole mass filter gives the standard curve defined by

$$R_{kl} = \frac{(x/y)\,(15.33) + 0.30}{(x/y) + 13.37}$$

The slope of the standard curve has a constant value for the range of x/y = 0—0.5 (Table II). These results show that isotope dilution is obligatorily used

TABLE II

SLOPES OF STANDARD CURVES FOR THE RANGE x/y = 0—0.5

x/y					
0	0.001	0.01	0.1	0.2	0.5
Magnetic spectrometer					
1.163	1.162	1.161	1.143	1.123	1.068
Quadrupole mass filter					
1.145	1.145	1.143	1.128	1.111	1.064

for a good correspondence between the experimental and the calculated standard straight lines. The slope of the curve depends on the four coefficients (Q_k, Q_l, P_k and P_l) and is given by the equation

$$(R_{kl})' = \frac{EF(P_kQ_l - Q_kP_l)}{(P_lF\,x/y + Q_lE)^2}$$

The most important part of the curve is near $x/y = 0$, where the slope is

$$(R_{kl})'_0 = \frac{F}{E} \cdot \frac{(P_kQ_l - Q_kP_l)}{(Q_l)^2}$$

and the ordinate to origin is $R_{kl_0} = Q_k/Q_l$. Consequently, the first stage in developing an isotope dilution assay is the selection of a suitable labelled standard. This standard must be relatively pure, without non-labelled

compound, to give a small coefficient Q_k. Under these conditions, the standard curve is linear near the small x/y values, the ordinate to the origin is minimum, and the slope is then a stable value $(R_{kl})' = P_k F/Q_l E$.

Blanks and accuracy

Water blanks gave no measurable peak with the highest sensitivity of the multiple ion detector. The accuracy of the method was investigated by adding known amounts of natural testosterone to the plasma aliquots from a pool. The regression curves (Table III) indicate that the slopes are not significantly different from unity.

TABLE III

ACCURACY OF THE METHOD

Four series of measurements were performed with different additions of T**.

	Regression line*
14 measurements on 6 points T** = 36 ng (magnetic spectrometer)	$Z = 0.988W + 0.113$ ($r = 0.99$)
16 measurements on 8 points T** = 32 ng (magnetic spectrometer)	$Z = 0.998W + 0.195$ ($r = 0.99$)
10 measurements on 5 points T** = 43 ng (magnetic spectrometer)	$Z = 0.994W + 0.006$ ($r = 0.99$)
10 measurements on 5 points T** = 59 ng (quadrupole mass filter)	$Z = 1.001W + 0.038$ ($r = 0.99$)

*Z = experimental value, W = T** added.

Specificity

The specificity is the most important property of an analytical method. In this instance, it is based on the selective isolation procedure: high resolution of the capillary column, and the highly specific ion detector. The derivatives of possible interfering steroids such as epitestosterone and dehydroepiandrosterone did not interfere in the assay (Fig. 3).

Precision

The precision was calculated from duplicate measurements of 35 plasmas covering the x/y range of 0.03—4. The intra-assay coefficients of variation obtained were near 2.8%. The 22 standard curves have a standard deviation of 4% (inter-assay S.D.).

Limit of detection

The sensitivity of the determination procedures described here is judged on

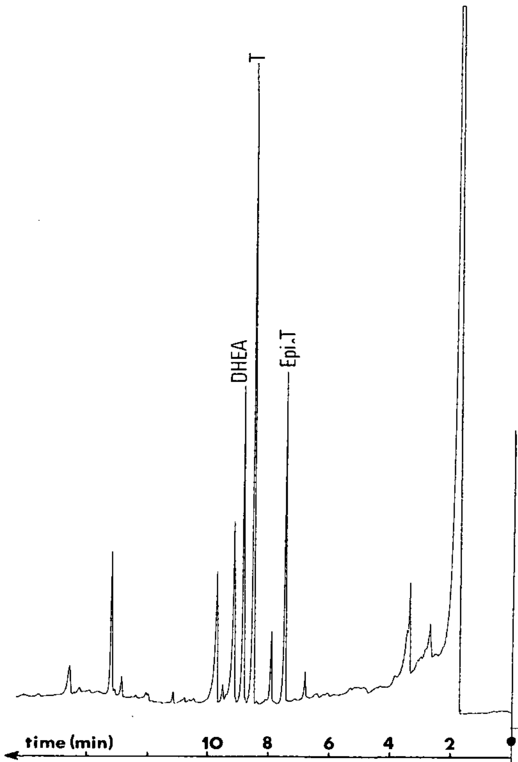

Fig. 3. Gas chromatogram. DHEA did not interfere with testosterone. DHEA = dehydroepiandrosterone, Epi-T = epitestosterone, T = testosterone.

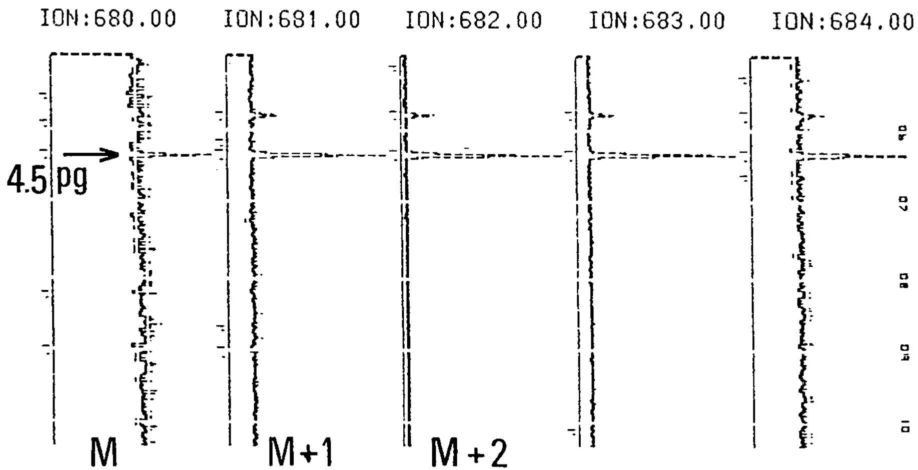

Fig. 4. Gas chromatogram of 4.5 pg of natural testosterone, for 60 pg of labelled standard.

the basis of the signal-to-noise (s/n) ratio. The lower limit of detection is near 4.5 pg of testosterone (Fig. 4) for a peak intensity of s/n = 4.2 [22].

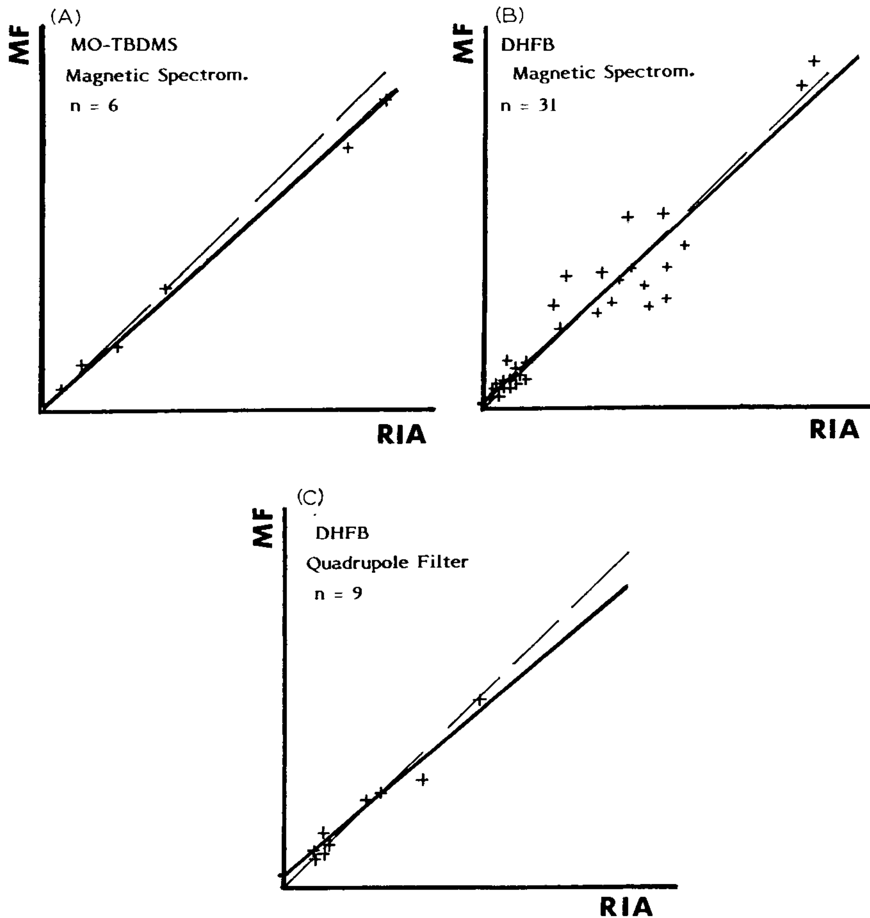

Fig. 5. Comparison between the mass fragmentography (MF) and the radioimmunoassay (RIA) methods: three series of measurements were made using the two spectrometers. (A) $Y = 0.916X - 0.057$ (nmol/l), (B) $Y = 0.961X + 0.519$, (C) $Y = 0.831X + 0.830$.

Comparison of results by isotopic dilution—mass fragmentography (ID—MF) and radioimmunoassay

The amounts of testosterone were measured in duplicate. For ID—MF and radioimmunoassay, three series of measurements were made: (1) six plasmas with MO-TBDMS derivative and (2) 31 samples with DHFB derivative for the two spectrometers, and (3) nine samples (DHFB derivative) for the quadrupole mass filter. The correlations between testosterone concentrations obtained by radioimmunoassay and by ID—MF are shown in Fig. 5. This comparison of results demonstrates that the radioimmunoassay appreciably over-estimates the true value, in accordance with previous reports [23, 24].

In summarize, the ID-MF method described here affords a sensitive and reliable technique to measure plasma testosterone. An internal standard labelled with ^{13}C in the molecular skeleton allows isotopic dilution without losses of it as deuterated standards.

The use of the Pickup—McPherson equation allows a good understanding

of the ID—MF phenomena, especially if the internal standard contains an appreciable amount of non-labelled compound.

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CHROMBIO. 2512

FREQUENCY-PULSED ELECTRON-CAPTURE GAS—LIQUID CHROMATOGRAPHIC STUDIES OF CHEMICAL CHANGES IN SERA OF PATIENTS WITH SCHISTOSOMIASIS

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SUMMARY

Sera from well documented cases of *Schistosoma mansoni* and *S. haematobium* infections as well as controls, were studied by frequency-pulsed electron-capture gas—liquid chromatography (FPEL-GLC) and mass spectrometry for detection of carboxylic acids and amines. Many carboxylic acids and unidentified peaks were detected. In a few serum specimens from infected patients, putrescine and cadaverine were detected. Indications are that in these few patients with high egg counts enough diamines were present to possibly produce amine toxicity. Following the initial investigation, the basic chloroform extractions, which contained amines, were further studied by FPEC-GLC with the aid of splitless injection and a capillary column. Several amines were detected which seemed to be related to schistosomiasis. Mass spectra were obtained on an unidentified schistosamine peak. The possible significance of the data is discussed.

INTRODUCTION

Schistosomiasis is a disease that affects a significant proportion of the world's population, especially in certain developing nations. The disease is endemic and chronic in some countries, and much of the population is infected repeatedly and for long periods of time. Eradication of the disease has relied mainly on snail elimination and other measures which in many instances are cost-inhibitory and slow; and many citizens of these countries live most of their lives infected with the organism. While eradication of the disease is not practical in many instances, improved diagnostic techniques, drug treatment, and investigation of physiologic changes in metabolites that occur in the host during the diseased state are of immediate concern. For example, it is known that people with some type of schistosomiasis have a higher incidence of cancer [1], and it is possible that a metabolite could be responsible. Other physiologic effects have been observed such as an opiate-like effect the infection has on the host. If these physiologic changes were due to metabolites, treatments might be devised that could counteract some of the toxic product build-up that may occur during the course of the disease.

Frequency-pulsed electron-capture gas—liquid chromatography (FPEC-GLC) has been demonstrated to be an effective tool for selective and sensitive detection of chemical changes that occur in body fluids during some diseased states [2—7]. It has been used both to detect metabolic products in vitro and carcinogenic nitrosoamines produced in vivo by *Proteus mirabilis* during urinary tract infections [5, 8, 9]. The method has also been employed as a diagnostic tool to aid in the rapid identification of tuberculous meningitis [3, 6], viral meningitis, cryptococcal meningitis, Rocky Mountain spotted fever [5], and several other diseases [7] by the analysis of serum and cerebrospinal fluid. The purpose of this research was to study sera taken from patients at the stage of the disease when the eggs were detectable, to study derivatized extracts of the sera by FPEC-GLC for changes in these sera, to study reproducibility of the FPEC-GLC patterns from patient to patient, to determine if peaks associated with infection were related to egg count, and to attempt physicochemical identification of some of the important peaks.

MATERIALS* AND METHODS

Serum specimens

A total of 57 serum samples from 19 controls, 34 persons with *Schistosoma haematobium* infection, and 4 persons with *S. mansoni* infection were analyzed by FPEC-GLC. The serum samples were obtained from residents of Kerdasa and Tamooh villages near Cairo, Egypt. The control specimens were obtained from the employees at the Biomedical Research Center for Infectious Diseases in Cairo and from the villages. *S. mansoni* and *S. haematobium* infections were confirmed by fecal and urine egg counts. The 1-ml volume of serum used for FPEC-GLC tests was that remaining after routine laboratory tests had been

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performed. Sera were collected and stored at −20°C without addition of chemicals.

Extraction and derivatization procedures

Each serum specimen was placed in a 50-ml round-bottomed centrifuge tube with a PTFE-lined screw cap; then heptanoic acid (3.15 nmol in 0.1 ml of distilled water, made basic with sodium hydroxide to obtain solubility) and di-*n*-butylamine (1.19 nmol in 0.4 ml of distilled water made acidic to increase solubility) were added to each sample as internal standards. Next, the samples were acidified to about pH 2 with 0.1 ml of 50% sulfuric acid, mixed by shaking, and extracted with 20 ml of nanograde chloroform (Mallinckrodt) by shaking them for 5 min on a Burrell wrist action shaker at a setting of 10. To obtain the amines the residual aqueous phase was made basic (about pH 10) with 0.3 ml of 8 *M* sodium hydroxide and reextracted with 20 ml of chloroform, as described for the acidic extraction. The acidic chloroform extracts were derivatized with trichloroethanol—heptafluorobutyric anhydride (TCE—HFBA) to form TCE esters of carboxylic acids and HFBA esters of alcohols as described [9, 10]. The basic chloroform extracts containing amines were derivatized with HFBA pyridine—ethanol to form amides as described [6, 11]. Selected samples were extracted with chloroform at pH 2, derivatized with HFBA, and analyzed by FPEC-GLC on the same temperature program as the basic extraction to detect compounds that were neutral and to verify the basic extractability of the amines. After the TCE and HFBA derivatives of carboxylic acids, alcohols, and amines were prepared, they were dissolved in 0.1 ml of xylene—ethanol (1:1). A 2-μl injection was used for analysis. The techniques for filling and cleaning the syringe have been described [9].

Apparatus

The derivatives were analyzed on a Perkin-Elmer Model 3920 gas chromatograph equipped with dual 10-mCi ^{63}Ni frequency-pulsed electron-capture detectors. Two glass columns (7.3 m × 0.2 cm I.D.) packed with 3% OV-101 on 80—100 mesh Chromosorb WH P (AW-DMCS-treated) were used under conditions previously described [6, 10].

A Perkin-Elmer programmable processor (PEP-2) equipped with a Modular Software System (MS 16 revision B) accumulated data from the gas chromatograph, analyzed the data according to a stored method, and prepared a report. An internal standard analysis was performed on the data by using heptanoic acid, di-*n*-butylamine, and 2-hydroxyisovaleric acid, which were added as internal standards [6]. The latter part of the study on amines was carried out on a 50-m fused-silica OV-101 capillary column programmed as described below.

Identification of unknowns was made by comparing known standards on OV-101-packed columns as described above, and on a 50-m OV-101 fused-silica capillary column programmed as follows: for carboxylic acids, isothermal at 100°C for 12 min; then 6°C/min to 265°C; for amines, isothermal at 90°C for 8 min then 6°C/min to 265°C. The capillary column was used with a splitless injector. We used a 30-sec solvent vent, and helium as the carrier gas at a flow-rate of 3 ml/min. Analysis of unknowns was performed on a Finnigan

mass spectrometer 4023 (GC—MS—data system) equipped with a 25-m fused-silica OV-101 capillary column. Chemical ionization (positive and negative) and electron impact were used to obtain fragmentation patterns of unknown and known standards. Helium was used as the carrier gas for the GLC, and methane—ammonia was the reagent gas for chemical ionization.

RESULTS

Fig. 1 shows the types of acids detected by splitless injection capillary column (trace) analysis of sera from controls and patients infected with *S. haematobium* and *S. mansoni*. There were reproducible differences detected in the FPEC-GLC patterns in some groups of *S. haematobium* and *S. mansoni* patients' sera as indicated (Fig. 1A and B) by the blackened peaks 7, 8, 9, 10, 11, 16, and 20. However, these differences were not consistent enough

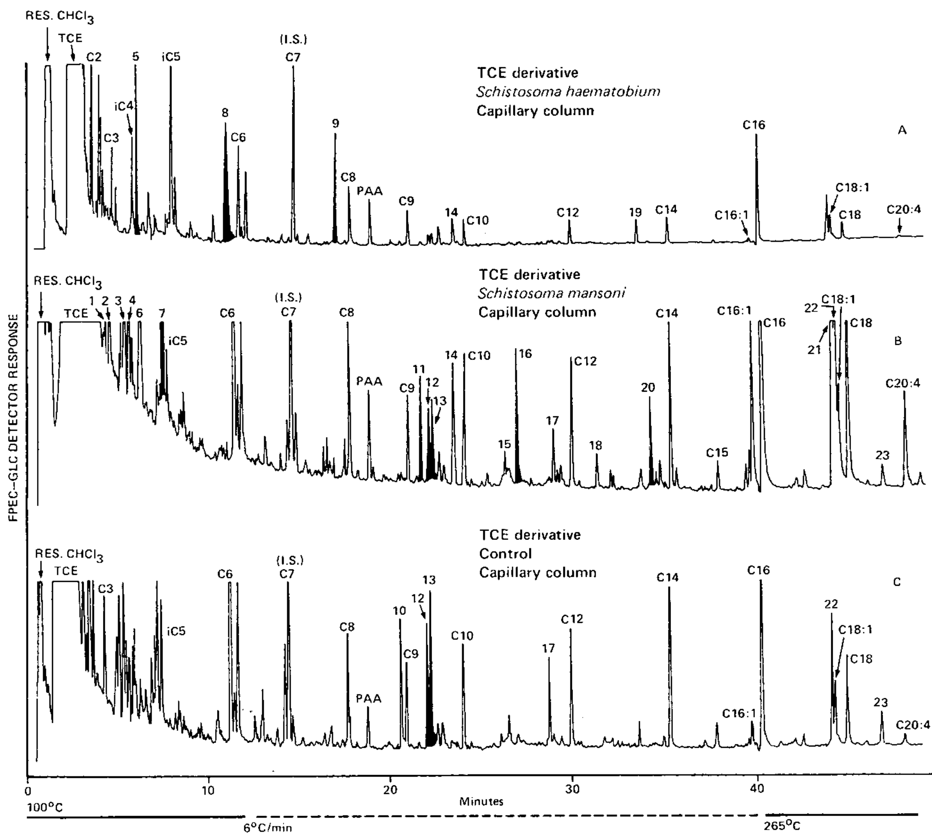

Fig. 1. FPEC-GLC profiles of TCE-derivatized acidic chloroform extracts of sera taken from patients with the indicated disease or control. Analyses were made on an OV-101 fused-silica capillary column. The letter C followed by a number indicates a saturated straight-chain carboxylic acid with the number of carbon atoms indicated by the number. The letter "i" indicates "iso", and the use of a colon between two numbers indicates unsaturation. I.S. is internal standard and PAA is phenylacetic acid. A number, or a number and a letter, over a peak indicates an unidentified peak; TCE is trichloroethanol; RES. $CHCl_3$ is residual chloroform.

throughout the entire study to be used as diagnostic markers to distinguish *S. haematobium* from *S. mansoni*. The FPEC-GLC patterns detected were different from the normal serum pattern and were unlike FPEC-GLC patterns detected in studies of certain types of bacterial, viral, and fungal infections

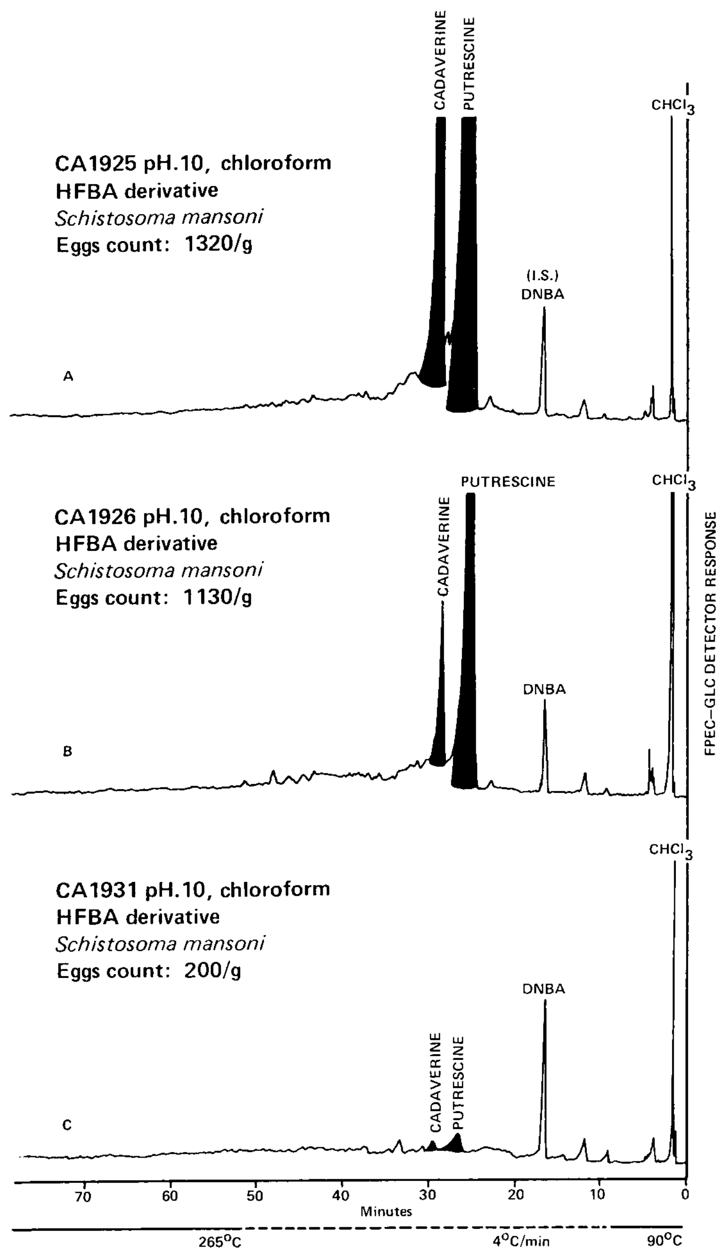

Fig. 2. FPEC-GLC profiles of basic HFBA-derivatized chloroform extracts of sera taken from patients with the indicated disease. Analyses were performed on an OV-101-packed column. I.S. is internal standard, DNBA is di-*n*-butylamine, $CHCl_3$ is residual chloroform, and HFBA is heptafluorobutyric anhydride. For other definitions see legend to Fig. 1.

[2—7]. Variation in certain metabolites found in the FPEC-GLC patterns could have been due to differences in the time of exposure to *Schistosoma*, number of schistosomes infecting the patient, as well as many other factors involved in the very complex life cycle of this organism.

Fig. 2 shows FPEC-GLC profiles obtained from packed-column analysis of the HFBA-derivatized basic extracts of sera. Putrescine and cadaverine were detected in sera from some patients infected with *S. mansoni*. Putrescine and cadaverine identification was verified by a comparison of the mass spectra obtained from the compounds present in the patient serum to known derivatized standards of putrescine and cadaverine. Fig. 2 also shows what appears to be a correlation with egg count. The amount of the diamines detected in these few patients was high. There was about 12.5 μM putrescine and about 6.3 μM cadaverine per ml of serum in the host with the highest egg count. Basic extracts of control sera did not contain putrescine or cadaverine and, except for the presence of the internal standard, were devoid of peaks.

Fig. 3 shows chromatograms obtained by trace analysis with splitless injection of the HFBA-derivatized basic chloroform extracts of sera from schistosomiasis patients and controls. Peaks were detected (Fig. 3A and B) that seem to be related to schistosomal infection. Peak 7 (referred to as "schistosamine") was found in full scale amounts in three patients. Two were infected with *S. haematobium* and one with *S. mansoni*. The three samples were from individuals residing in two different villages. Schistosamine which was found to be strictly basic extractable was further characterized using electron-impact (EI), negative-ion (NICI), and positive-ion chemical-ionization (PICI) mass spectrometry.

Several spectra suggest a molecular weight of 564. Ammonia PICI produced a relatively intense ion at m/z 582. The ion at m/z 582 obtained by PICI is produced as a result of the adduct of the molecular species with NH_4^+ (M + 18). The NICI spectra support the above data in that $(M - H)^-$ is observed at m/z 563. The NICI spectra had fragments at m/z 524, 504, 484, 366, and 197; all of these were from fragmentation of the HFBA derivative and while they showed that derivatization with HFBA occurred, they yielded little information about the structure of peak 7. The EI spectra produced a base peak at m/z 57, several fragments of HFBA, and a high-mass ion at m/z 387. The m/z 387 ion could have resulted from a loss of a neutral 177u. This ion was also observed in the chemical-ionization spectra. The structural significance of either m/z 387 or the neutral loss of 177u is not clear. Several structures are possible for the m/z 57 ion, but considering that the basic extractability of peak 7 indicates an amine, two likely candidates are C_3H_7N and C_2H_3NO. There were several major fragment ions observed in methane PICI. Two major fragments were observed at m/z 107 and 399. Other fragments ranging from 30% to 90% of full scale intensity were observed at m/z 123, 135, 149, 179, 191, and 387. The significance of these fragments in relation to the total structure of the compound is not clear.

As shown in Fig. 3A and B amines other than schistosamine were detected. These amines have not been identified, but they were strictly basic extractable, and even though the FPEC-GLC capillary patterns shown (Fig. 3A and B) differ from each other, in cases where schistosamine was low, as shown in Fig. 3B,

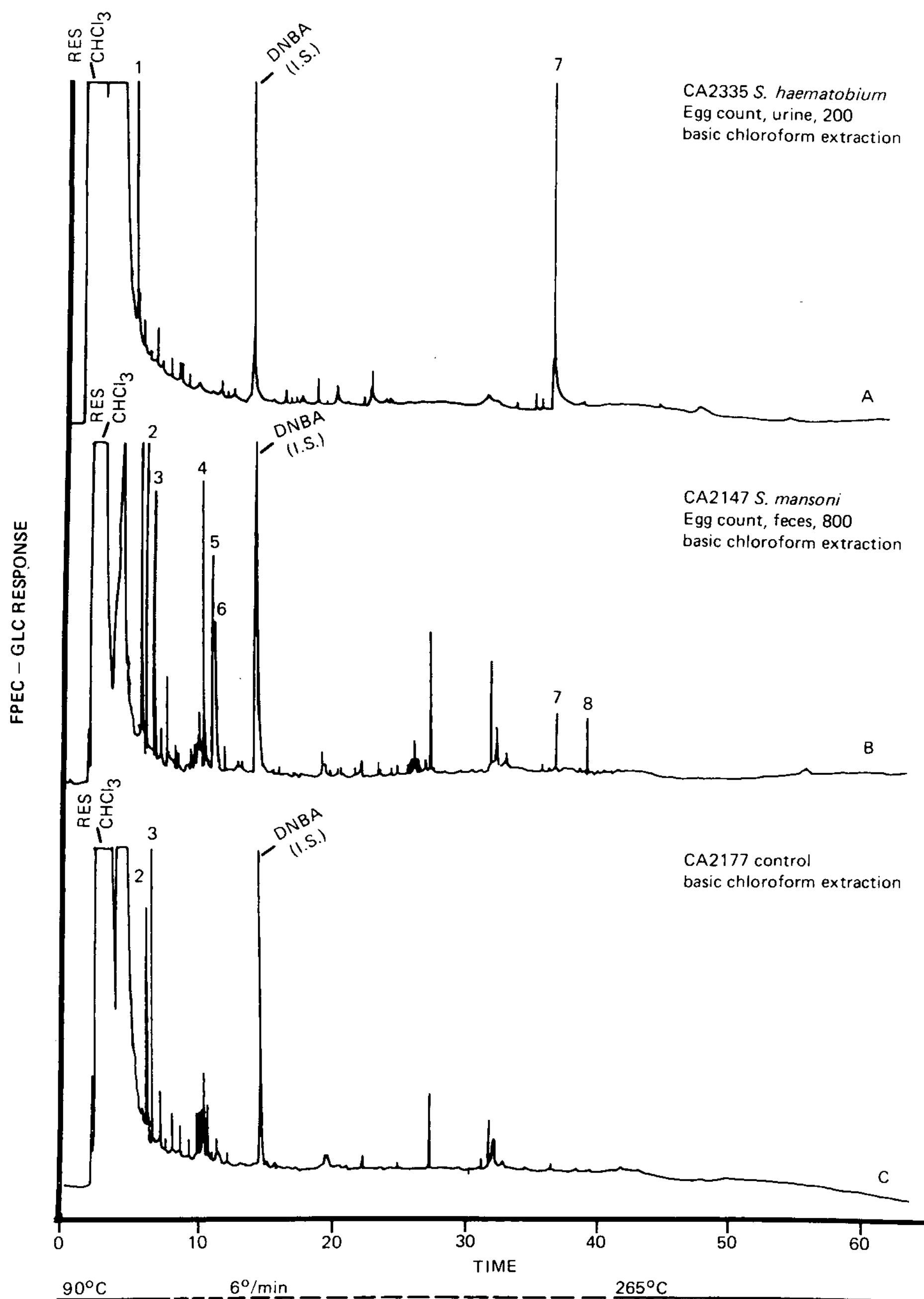

Fig. 3. FPEC-GLC profiles obtained from a 50-m OV-101 fused-silica capillary column. The chromatograms were obtained by analysis of HFBA-derivatized basic extractions of sera taken from patients with the indicated disease or controls. For definitions see legends to Figs. 1 and 2.

peak 7, the FPEC-GLC amine profiles of *S. haematobium* and *S. mansoni* were much alike. Peaks 4, 5, and 6 were generally much higher in the sera from patients with *S. mansoni* infection.

DISCUSSION

There have been relatively few studies made on the metabolites present in schistosomal disease [1]. Since these helminths live within large veins of the body, it stands to reason that their metabolic products might be detected in serum by a selective sensitive system such as FPEC-GLC. The early basic extractable peaks that eluted before 15 min seemed to have some relationship to egg count in that they were higher in the specimens with higher egg counts. Although these helminths have a much larger comparative body mass than other infectious agents the number of schistosomes is very small (10—20) in comparison to millions of bacteria or viruses involved in an infection. The organism also has a much longer life span, up to 30 years [1] and a much more complex life cycle. It is therefore not unreasonable to expect some variation in some types of metabolites detected by FPEC-GLC. The source of the acids and amines detected by FPEC-GLC could be a host response to the worms or a combination of both host response and worm metabolites. If the acids and amines detected by FPEC-GLC were a reflection of host response, again one would expect several factors such as duration of infection, number or organisms, and life cycle of the organism to affect the type metabolites detected. Animal model studies combined with human studies which could include chronic and new cases could help point out the source of some of the compounds detected in our study and perhaps find additional metabolites. We cannot rule out the possibility, even though both stool and urine were tested for eggs, that some of the patients we studied may have had dual infection with both *S. mansoni* and *S. haematobium*. Dual infections also could cause the FPEC-GLC profile to be different.

Since many of the peaks detected in the study are unidentified, we cannot determine their physiologic effects on the host. The physiology of diamines has been studied [12], and some, spermine and spermidine, are known growth factors. The affects of putrescine on cultures of *Anacystis nidulan* has been investigated by Guarine and Cohen [13]. They reported that in concentrations of 150 μM there was complete inhibition of protein synthesis. Th ribosomes were affected in an irreversible manner. Putrescine and cadaverine are known to have been produced in vitro by some microorganisms.

Except for the special cases of packed column analysis such as those shown for putrescine and cadaverine (Fig. 2), trace analysis with splitless injection and the OV-101 fused-silica capillary column produced more definitive differences between the schistosomiasis patient specimens and controls. More work is needed, however, to make FPEC-GLC analysis of serum a reliable tool for use in differentiation between *S. mansoni* and *S. haematobium* infection. FPEC-GLC analysis of a larger volume of serum, of feces from patients with *S. mansoni* infection, and of urine from patients with *S. haematobium* infection might improve chances for species differentiation. At the present in some developing nations diagnosis of the type of schistosomiasis is considered by some investigators to be less important than determining host response to schistosomal metabolites to which the host is constantly exposed. Elimination of the disease is very difficult, slow and expensive under present conditions, but counteractants for a toxic substance, once it is identified, is sometimes pharmacologically possible.

ACKNOWLEDGEMENTS

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EFFICIENT CHROMATOGRAPHIC FRACTIONATION OF STEROIDS IN HUMAN SERUM THROUGH REGULATION OF LIQUID—LIQUID DISTRIBUTION RATIOS

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SUMMARY

To improve the clean-up process in the analysis of biological fluid constituents, an efficient liquid—liquid distribution system was developed. Closed-bed columns containing fine diatomaceous earth granules were prepared by slurry packing for the fractionation of steroid hormones in human serum before quantitative assay by liquid chromatography or radioimmunoassay. Four columns were connected to construct the aqueous liquid—liquid chromatography—fractionation system. The first was coated with neutral water for distribution of serum, the second was weakly alkaline with sodium hydrogen carbonate for extrusion of strong acidic components, and the third was strongly alkaline with sodium hydroxide to capture oestrogens. The final column was acidic with sulphuric acid to remove basic components. Optimization of the stepwise gradient solvents was achieved on the basis of the results of a linear relationship between the logarithms of the capacity ratios and solvent composition determined from an analytical run. Neutral steroid hormones added to serum were eluted from the column system by a stepwise gradient elution technique to obtain first very non-polar materials, then progesterone and testosterone, and finally to extract the corticosteroids. Phenolic oestrogens were recovered from the strong alkaline column with a mobile phase solvent after the pH of the stationary phase had been adjusted with a phase transfer neutralizer. The fractional constituents were purified and enriched. This procedure was used to determine Solu-medrol, an acidic corticosteroid drug, in human serum.

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INTRODUCTION

To improve the analytical efficiency and sensitivity of samples consisting of complex matrices such as biological fluids, a clean-up procedure including preliminary fractionation is often ideal. Solvent extraction has been commonly used to fractionate target molecules in a complex mixture. We recently developed a highly efficient aqueous liquid—liquid distribution procedure using closed-bed columns containing fine silica gel or diatomaceous earth granules coated with an aqueous stationary phase [1—3]. The selectivity of this method is based on specific distribution ratios of solutes in organic mobile and aqueous stationary phases; in addition, two phases prepared on the surface of the packing materials participate in the distribution of a high theoretical plate number. A switching technique from the precolumn for aqueous liquid—liquid distribution to the high-performance liquid chromatographic (HPLC) analytical column was applied to the analysis of biological fluids [4, 5]. An on-line extraction— evaporation—injection technique for improving the clean-up of serum for analysis of corticosteroids and certain other drugs has been described in our earlier papers [5, 6].

For greater efficiency of the clean-up process, we present in this paper an improved fractionation procedure employing highly selective aqueous liquid—liquid distribution by which the partition coefficients of the solutes are regulated. If a series of columns are coated with acidic and basic stationary liquids, basic and acidic solutes can be eliminated, and consequently unretained neutral components emerge from the column system so that a pure fraction is finally obtained. The basic and acidic components in a mixture can be recovered by application of a neutralization reagent, adjusting the pH of the stationary phases and solvent extraction. The distribution ratios of the solutes depend on the nature of the organic phase systems; for example, hydrophobic substances are easily distributed in a more lipophilic organic layer and are eluted more quickly than hydrophilic ones. The characteristics of the organic phase can be regulated by mixing two solvents of different polarity. A linear correlation between the logarithms of solvent composition and distribution ratio has been found recently for binary solvent systems using various solute compounds in liquid—liquid chromatography [3, 7]. It is possible to control the distribution ratio of a solute by altering the binary solvent composition in accordance with solute polarity and optimizing the fractionation process in a systematic way.

To carry out the process described above, a trial run was made using steroids in biological fluids as model compounds. Steroids added to human serum were fractionated into oestrogens, corticoids and other neutral steroids, following a time-scheduled sequential operation. An acidic steroid drug, Solu-medrol (6-methylprednisolone 21-hemisuccinate), in human serum was fractionated quantitatively to test the application of this process to clinical assay.

EXPERIMENTAL

Apparatus

The system used in the experiments is illustrated in Fig. 1. Glass tubes 1, 2

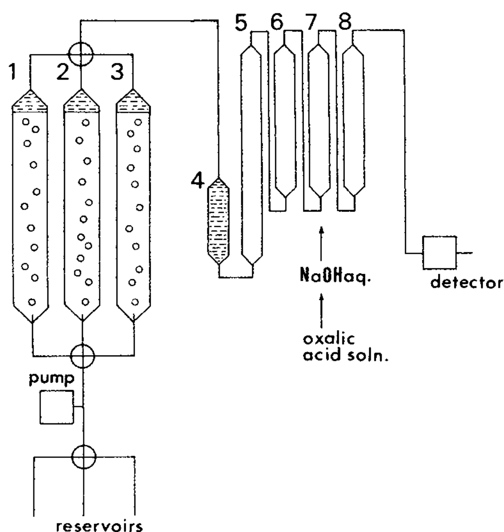


Fig. 1. Schematic diagram of the system. 1—3: Glass tubes containing water; 4: glass tube for injection; 5—8: diatomaceous earth columns coated with water, 5% sodium bicarbonate, 3% sodium hydroxide and 1% sulphuric acid solutions, respectively.

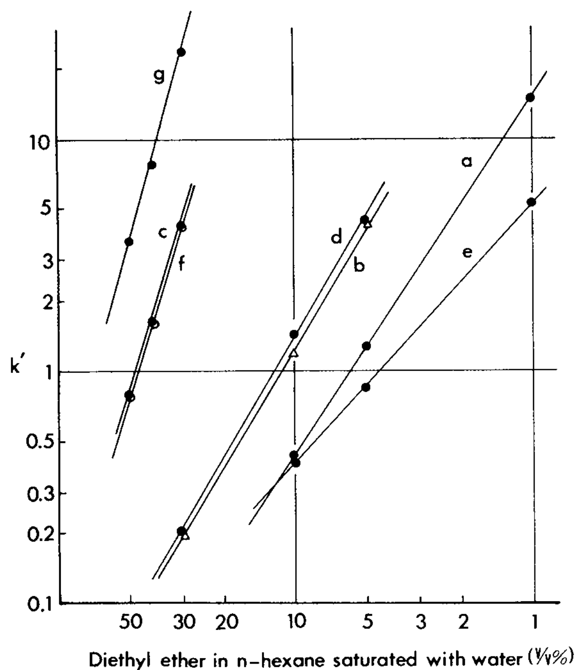


Fig. 2. Correlation between the logarithm of capacity ratio and that of aqueous binary solvent composition in steroid samples. Packing material: diatomaceous earth (AquEx-10, DSD). Samples: a = oestrone, b = oestradiol, c = oestriol, d = testosterone, e = progesterone, f = corticosterone, g = hydrocortisone.

and 3 (30 cm × 2.5 cm I.D.), each containing about 100 ml of water, were used to presaturate the carrier solvent with water as described previously [5, 6]. Glass tube 4 and column 5 were used for the injection and retention measurements of steroid hormones (procedure a). An additional three columns, 6, 7 and 8, were used to fractionate the serum steroids (procedure b). These columns were packed with diatomaceous earth granules using a slurry packing procedure [2, 6]. Tube 4 and columns 5—8 were connected in cascade with PTFE tubes 30 cm × 1 mm I.D. The inlet of tube 4 was connected to glass tubes 1, 2 and 3 and a pump (Twincle, Jasco, Tokyo) via PTFE valves, and the outlet of column 5 (for procedure a) or column 8 (for procedure b) was connected to a UV detector (Uvidec III, Jasco). Selection of tube 1, 2, or 3 in accordance with the carrier solvent was made using a PTFE valve inserted between the pump and tubes 1, 2 and 3. The glass tubes and column tubes (CIG tubes) were obtained from Kusano (Tokyo, Japan). The other mechanical components were the same as described in our earlier papers [5, 6].

Reagents and solvents

The steroid hormones and reagent grade chemicals were supplied from Wako (Osaka, Japan). An authentic sample of Solu-medrol was obtained from Japan Upjohn. Diatomaceous earth for the column extraction, particle size 10 μm, AquEx-10 was obtained from DSD (Tokyo, Japan).

Procedure a: determination of the linear relationship between the logarithm of the capacity ratio and that of the eluent composition

Column 5, 17 cm × 4 mm I.D., containing diatomaceous earth, was washed

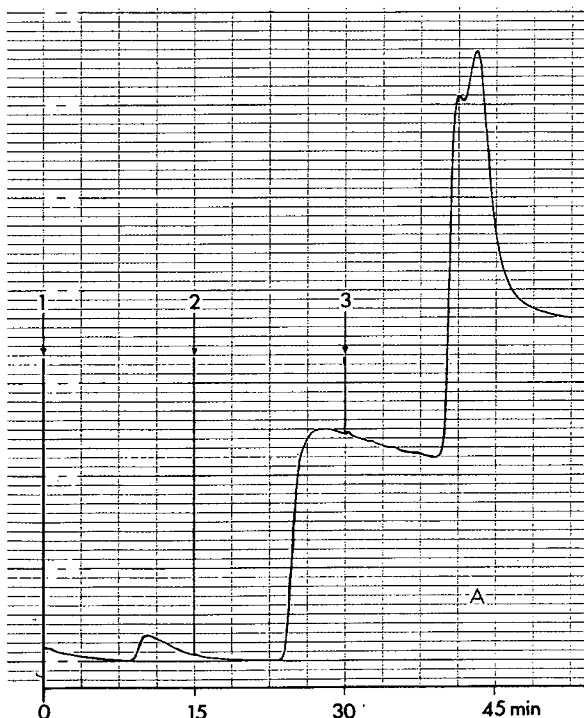
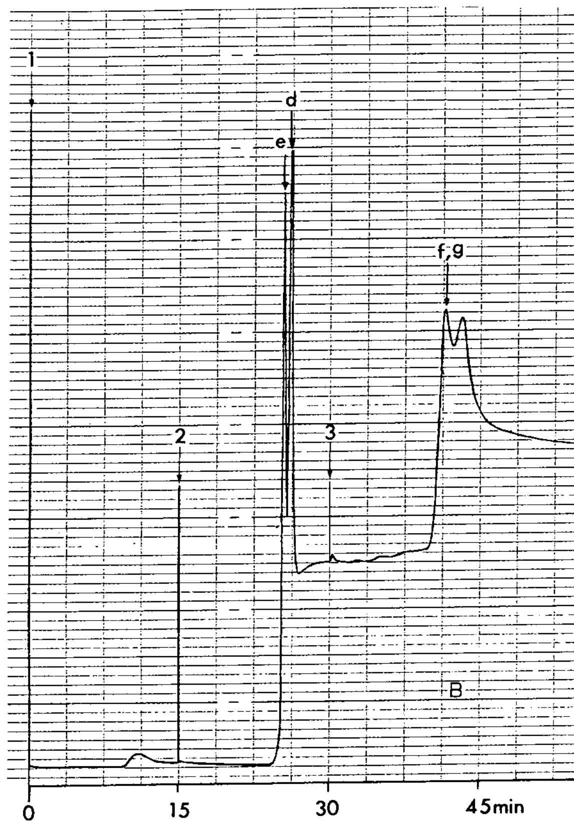


Fig. 3.



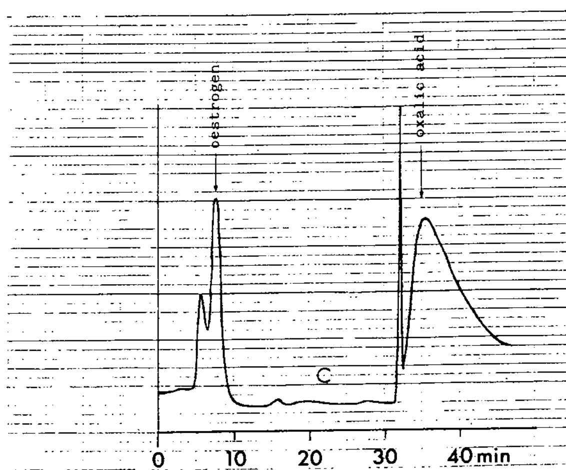


Fig. 3. Chromatograms of steroid fraction obtained by the stepwise elution of serum. (A) Steroid fractions in normal serum (0.6 ml). (B) Neutral steroids added to the serum (0.3 ml). (C) Oestrogens added to the serum (0.3 ml). 1 = Elution by 0.1% diethyl ether in *n*-hexane; 2 = elution by 30% diethyl ether in *n*-hexane; 3 = elution by diethyl ether. Samples d—g as in Fig. 2.

with 6 ml each of methanol and water. The column was washed again with water-saturated diethyl ether applied at a flow-rate of 9.9 ml/min for 5 min to remove any excess aqueous phase. The column contained about 0.1 ml of aqueous phase. The glass tube injector 4 was a CIG tube, 7 cm × 4 mm I.D. The UV detector was set at 240 nm for testosterone, progesterone and corticosteroids, and at 280 nm for oestrogens. The analytical column 5 thus constructed showed theoretical plate numbers ranging from 700 to 1200 for each steroid hormone and a dead volume of about 2.0 ml.

Before the analytical run, an equilibration was made between the aqueous stationary and mobile phases, consisting of a mixture of water and diethyl ether—*n*-hexane made by shaking these components together. The solvent-free injection of the sample was carried out as follows: 10 μl of a 2-propanol solution containing 2 μg of steroid hormones were smeared on a glass rod, 6.8 cm × 3.7 mm O.D. After the solvent had dried, the rod was inserted into the injector and the mobile phase solvent was slowly introduced at a flow-rate of 0.1 ml/min. When the solvent reached the analytical column, the flow-rate was adjusted to 1 ml/min and the eluent was monitored by the UV detector. The mobile phase solvents were *n*-hexane and mixtures of diethyl ether and *n*-hexane with diethyl ether concentrations of 1, 5, 10, 30, 40 and 50 vol.%. The experimental values of the capacity ratios were obtained by the formula $k' = (t_R - t_0)/t_0$, where t_R and t_0 are the retention time and dead time, respectively. The correlation between the logarithm of capacity ratio and that of solvent composition is shown in Fig. 2.

Procedure b: fractionation of the serum steroids

A blood sample (5 ml) was centrifuged for 5 min at 1000 *g*. Tube 4 (7 cm × 4 mm I.D.) was used to inject up to 0.8 ml of serum supernatant. Columns containing diatomaceous earth were 30 cm × 4 mm I.D. (column 5) and 20 cm × 4 mm I.D. (columns 6—8).

Fractional columns 5, 6, 7, and 8 were conditioned with distilled water, 5% sodium bicarbonate solution, distilled water, and 1% sulphuric acid solution, respectively. A 100-μl volume of 3% sodium hydroxide solution was injected into column 7 at its bottom followed by an injection of 0.6 ml of serum into column 4. Columns 5, 6, 7 and 8 thus prepared were neutral, weakly alkaline, strongly alkaline and acidic, respectively. A mixture of 0.1% diethyl ether—*n*-hexane was introduced into column 4 via tube 1 at a flow-rate of 0.8 ml/min. The serum in column 4 was transferred to column 5 where it remained in the aqueous stationary phase. The mobile phase was transferred through the column system and finally entered the detector set at 240 nm. After 15 min the mobile phase solvent was changed to a mixture of 30% diethyl ether—*n*-hexane, and then to diethyl ether after 30 min. The results are shown in Fig. 3A.

This same procedure was carried out with 0.3 ml of serum added along with 2 μg each of progesterone, testosterone, corticosterone, and hydrocortisone, and 4 μg each of oestrone, oestradiol, and oestriol. Eluents within 24—27 min and 40—44 min were collected. The first fraction contained progesterone and testosterone, and the second corticosterone and hydrocortisone. The chromatogram obtained is shown in Fig. 3B.

In the next stage, in order to recover the oestrogens remaining in the

strongly alkaline stationary phase at the bottom of column 7, the column system was rearranged as follows. Columns 4, 5, and 6 were disconnected and column 7 was connected directly to tube 3. After 0.2 ml of saturated oxalic acid solution had been injected into column 7, water-saturated diethyl ether was introduced at a flow-rate of 0.8 ml/min. The eluent from column 8 was monitored by the detector set at 280 nm and within 5—10 min collected so as to obtain the oestrogen fraction. The chromatogram obtained is shown in Fig. 3C.

For extraction of Solu-medrol in the serum, a process similar to that used for the oestrogens was carried out on 0.3 ml of serum supplemented with 10 ng of the steroid drug and 10 μl of 50% hydrochloric acid solution to bring the pH to about 4—5. The Solu-medrol trapped in the weak alkaline stationary phase in column 6 was recovered by injection of a saturated oxalic acid solution and extraction with water-saturated diethyl ether.

Extracts containing the above steroids were applied to the on-line evaporation—injection system described in earlier papers [5, 6]. The steroids were identified by their retention data, using silica gel columns and a normal-phase solvent system containing *n*-hexane—2-propanol for steroid hormones and *n*-hexane—2-propanol—water—acetic acid for Solu-medrol. To check recovery, peak height measurements were made.

RESULTS AND DISCUSSION

From the linearity between the logarithm of capacity ratio and that of solvent composition illustrated in Fig. 2, optimization for selective fractionation was carried out. The retention behaviour in the figure indicates that progesterone and testosterone may possibly be retained to a fair extent and corticosteroids, such as corticosterone and hydrocortisone, strongly retained in the column as a result of using the diethyl ether—*n*-hexane mixture. Thus, two classes of neutral steroids can be accurately fractionated by stepwise elution. The retention behaviour of oestrogens such as oestrone, oestradiol, and oestriol differed so much that their extraction as a single fraction with the diethyl ether—*n*-hexane mixture was not possible. To solve this problem, a column containing an alkaline stationary phase was used to trap the phenolic oestrogens.

After washing out the non-polar materials from the human sera with 0.1% diethyl ether—*n*-hexane (Fig. 3A), progesterone and testosterone were recovered from the columns by a 30% diethyl ether—*n*-hexane mixture. The columns used in this study, which were packed with fine diatomaceous earth granules, have a higher number of theoretical plates in comparison with commercially available cartridge-type columns. As a result, the hormones could be fractionated with only 2—4 ml of the mobile phase solvent, as shown in Fig. 3B. Normal serum components extractable by this solvent were present in rather small amounts so that the fraction obtained was enriched with these steroid hormones. Finally, the mobile phase solvent was changed to diethyl ether and corticosteroids such as corticosterone and hydrocortisone were fractionated.

During the course of this process, the oestrogens continued to remain in the strongly alkaline column 7 as a result of salt formation. Following comple-

tion of the neutral hormone fractionation, the stationary phase in column 7 was neutralized by a phase transfer neutralizer such as an oxalic acid solution to recover the oestrogens in the mobile phase. The results in Fig. 3C indicate a recovery with only 4 ml of the mobile phase solvent. The observed time delay in the oestrogen peak from the hold-up time indicates the time necessary for neutralization of the alkaline stationary phase with oxalic acid. An excess amount of oxalic acid was removed 22 min following completion of the oestrogen elution. Thus, it was quite easy to recover the oestrogens without any contamination from the reagent added for neutralization.

To extend the scope of this method and apply it to clinical assay, an acidic steroid drug, Solu-medrol, was selected for testing. This drug was added to the serum and extracted quantitatively after being trapped in the weak alkaline column and adjusting the pH of the stationary phase.

Steroid calibration curves were made from peak height measurements using

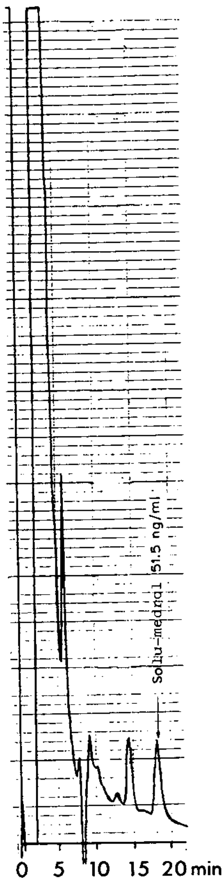


Fig. 4. Chromatogram of serum Solu-medrol obtained using the on-line system for extraction—evaporation—injection into HPLC column 7 using 0.1 ml of serum from a systemic lupus erythematosus patient. The eluent was *n*-hexane—2-propanol—water—acetic acid (72.5:25:2:0.5) for the silica gel column. The flow-rate was 1 ml/min. A detector range of 0.005 a.u.f.s. and recorder range of 10 mV were used.

sample quantities between 10 and 100 ng/ml of serum. The peak heights were reproducible with the coefficient of variation being less than 4%, using authentic samples injected into the analytical column without extraction. These experiments were carried out using the extraction—evaporation—injection system described in our earlier papers [5, 6]. The above method for liquid chromatographic analysis of serum Solu-medrol has found application in clinical assay. Fig. 4 shows a chromatogram obtained from the serum of a patient with a collagen disease administered Solu-medrol. The results of this application will be published in detail.

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RAPID AND ACCURATE REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CONJUGATED BILE ACIDS IN HUMAN BILE FOR ROUTINE CLINICAL APPLICATIONS

THERAPEUTIC CONTROL DURING GALLSTONE DISSOLUTION THERAPY

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SUMMARY

This paper introduces a new method to detect the taurine and glycine conjugates of five different bile acids (cholic acid, deoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid and lithocholic acid) in human bile. Advantages of this method are sufficient separation of compounds within a short period of time and a high rate of reproducibility. Using a mobile phase gradient of acetonitrile and water, modified with tetrabutylammonium hydrogen sulphate (0.0075 mol/l), we were able to maximize the differentiation between ursodeoxycholic acid and lithocholic acid, which is of primary interest during conservative gallstone dissolution therapy. Use of this gradient reduced analysis time to less than 0.5 h. Recovery rates for this modified method ranged from 94% to 100%, and reproducibility was 98%, sufficient for routine clinical applications.

INTRODUCTION

A rapid and simple high-performance liquid chromatographic (HPLC) method is essential for routine clinical evaluation of alterations in conjugated bile acid pattern during gallstone dissolution therapy.

HPLC is capable of analysing glycine and taurine conjugates and producing accurate data within a short period of time, whereas other methods used to detect bile acids have distinct disadvantages. For example, enzyme kits do not differentiate between conjugated bile acids [1, 2], bioluminescence only

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estimates the total amount of 3-α-hydroxy bile acids [3] and gas chromatography requires a substantial amount of time [4, 5].

During conservative gallstone dissolution therapy it is beneficial to monitor ursodeoxycholic acid (UDCA) and chenodeoxycholic acid (CDCA) via conjugated bile acid levels in biological fluids. For this purpose it was necessary to slightly modify previously published HPLC methods [6—9] in order to increase reproducibility and enhance the separation of conjugates within a shorter period of time. Many authors preferred methanol as the main component of the mobile phase [6—8, 10]. However, in the range of 200 nm, where conjugated bile acids are optimally detected, methanol has a high UV cut-off. Therefore we employed an acetonitrile—water gradient, modified with tetrabutylammonium (TBA) hydrogen sulphate. Using this ion-suppressive and ion-pairing HPLC mobile phase we were able to monitor taurine and glycine conjugates, especially UDCA and lithocholic acid (LCA) components, which are of primary interest during conservative gallstone dissolution therapy.

MATERIALS AND METHODS

Instruments

Two Constametric II HPLC pumps combined with a Gradient Master 1601, were equipped with a variable-wavelength UV spectrophotometer (LDC-Milton Roy, Hasselroth, F.R.G.) and a μBondapak C_{18} steel column (10 μm, 300 × 3.9 mm, Waters Assoc., Milford, MA, U.S.A.). A Hewlett-Packard 3380 A integrator graphed and calculated peak areas. Parameter variables were set as follows: a flow-rate of 1.5 ml/min, wavelength detection at 200 nm, within a range of 0.04 a.u.f.s. and an attenuation of 64.

The mobile phase gradient increased exponentially (mixing gradient, $m = 2$) within 30 min from an initial concentration of 10% solvent B, 90% solvent A, to a final concentration of 60% solvent B. Standards were weighed with a Sartorius Scale 2004, precision, $d = 0.01$ mg, and bile acids from human bile were extracted using Sep-Pak C_{18} cartridges (Waters Assoc.). Extracted samples were filtered through a 0.2-μm disposable filter (Gelman Sciences, Ann Arbor, MI, U.S.A.).

Chemicals

Mobile phase solvents were of HPLC grade (Fluka, Buchs, Switzerland; E. Merck, Darmstadt, F.R.G.), further purified by filtration through a 0.45-μm organic filter (Schleicher and Schüll, Düsseldorf, F.R.G.) and degassed under vacuum in an ultrasonic water bath. Solvent A = acetonitrile—water (30:70) plus 0.0075 mol/l TBA; pH adjusted to 2.5. Solvent B = acetonitrile—water (60:40) plus 0.0075 mol/l TBA; pH adjusted to 2.5.

TBA hydrogen sulphate purissimum was purchased from Fluka. Purified conjugated bile acids were purchased from Calbiochem (La Jolla, CA, U.S.A.) and Sigma (St. Louis, MO, U.S.A.).

Patients

Bile from three patients with choledochal calculi was obtained by suction via a nasobiliary tube before and after medical dissolution therapy. Three additional patients with gallbladder calculi were treated with 500 mg of urso-

deoxycholic acid (Ursofalk®) per day and bile was obtained by endoscopic intubation of the choledochus.

Isolation

Prior to extraction a Sep-Pak C_{18} cartridge was rinsed with 3 ml of methanol and washed with 10 ml of water. Then 0.1 ml of hepatic bile, diluted with 5 ml of 0.07 mol/l phosphate buffer (pH 7.0, according to Sjörensen) and 120 μg of dexamethasone, added as an internal standard, were loaded onto the cartridge, followed by 10 ml of water, 3 ml of 10% acetone, and an additional 10 ml of water [11, 12]. Bile acids were slowly eluted from the cartridge with 3 ml of methanol. The filtrate was evaporated under a nitrogen stream and the residue dissolved in 0.3 ml of solvent B. After filtration through a 0.2-μm filter assembly, an injection volume of 20 μl was analysed by the HPLC apparatus.

RESULTS AND DISCUSSION

Selectivity

The separation of a standard mixture consisting of dexamethasone and five different bile acids conjugated with glycine and taurine is shown in Fig. 1. Individual injections of purified bile acids are used in order to correlate the retention time with the corresponding bile acid. Column retention behaviour is

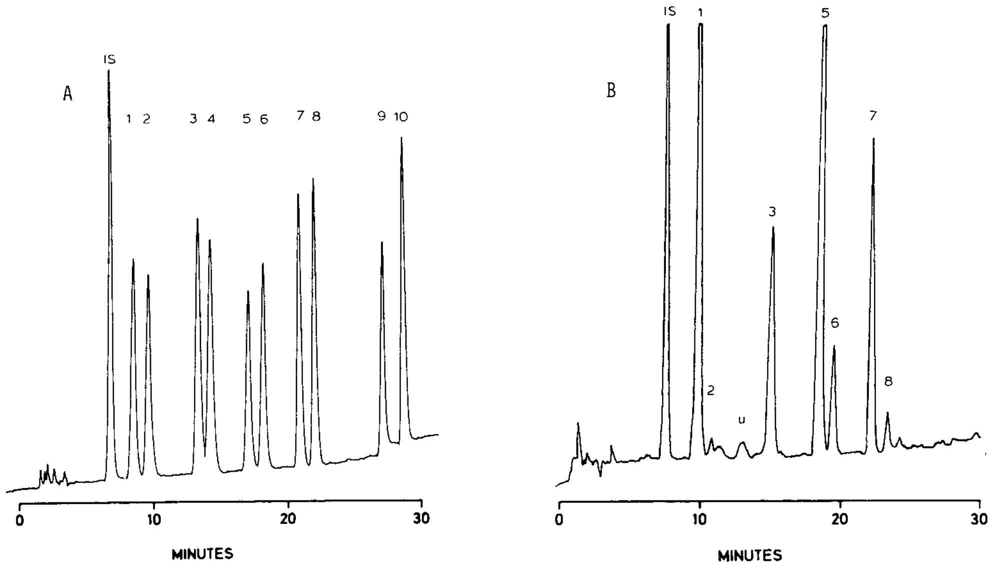

Fig. 1. (A) Chromatogram of a standard mixture of synthetic conjugated bile acids. Peaks: IS = internal standard (dexamethasone); 1 = glycocholic acid (GCA); 2 = glycoursodeoxycholic acid (GUDCA); 3 = taurocholic acid (TCA); 4 = tauroursodeoxycholic acid (TUDCA); 5 = glycochenodeoxycholic acid (GCDCA); 6 = glycodeoxycholic acid (GDCA); 7 = taurochenodeoxycholic acid (TCDCA); 8 = taurodeoxycholic acid (TDCA); 9 = glycolithocholic acid (GLCA); 10 = taurolithocholic acid (TLCA). (B) The distribution of glycine and taurine conjugates in normal human bile obtained by endoscopic choledochal intubation (peaks are labelled as shown in Fig. 1A; u = unknown peak); TUDCA is not detected; GUDCA, GLCA and TLCA are present only in trace amounts.

TABLE I

CAPACITY FACTORS (k', rk') OF TEN CONJUGATED BILE ACIDS AND DEXAMETHASONE IN A STANDARD SAMPLE USING THE PRESENTED HPLC METHOD

Bile acid	k'	rk'
Dexamethasone	4.42	0.35
Glycocholate	5.78	0.46
Glycoursodeoxycholate	6.66	0.53
Taurocholate	9.55	0.76
Tauroursodeoxycholate	10.27	0.82
Glycochenodeoxycholate	12.51	1.00
Glycodeoxycholate	13.37	1.07
Taurochenodeoxycholate	15.43	1.23
Taurodeoxycholate	16.30	1.30
Glycolithocholate	20.32	1.62
Taurolithocholate	21.50	1.72

described by the capacity factor k' and the relative capacity factor rk' (relative to glycochenodeoxycholate) (Table I).

Optimal separation of conjugated bile acids in standard mixtures as well as in biological samples could be obtained using a TBA-modified acetonitrile—water gradient. (TBA is usually applied as a modifier in ion-pair chromatography.)

Da Shi Lu et al. [13] doubt the ion-pair process of TBA for the separation of bile acids at a mobile phase pH lower than 2.85. Our results clearly indicate that an acetonitrile—water gradient modified with TBA at pH 2.5 increases separation as opposed to an acetonitrile—phosphate buffer (0.01 mol/l potassium dihydrogen phosphate) gradient at the same pH, without TBA. Due to the low pK values of taurine conjugates (taurocholic acid: pK = 1.4), TBA may enhance separation at pH 2.5 by ion pairing, while ion suppression may be responsible for separation of conjugates with pK values above 3, such as glycocholic acid (pK = 4.4). Thus, from our experience, we postulate that a combination of ion suppression and TBA ion pairing is responsible for enhancing bile acid conjugate separation. TBA may also interact with free residual silanol groups of the stationary phase, therefore creating an additional factor responsible for optimizing separation [14].

Methanol has an inherently high UV cut-off at 200 nm, where bile acid conjugates are optimally detected. This property causes a high noise-to-signal ratio, which can be significantly reduced by employing acetonitrile.

Dexamethasone proved to be superior to oestrogen [15] and testosterone [16] as internal standard because it does not occur naturally in biological fluids and does not overlap with glycoursodeoxycholic acid (GUDCA) [6] or with glycocholic acid (GCA) (see Fig. 1).

Quantitative analysis

Known amounts of conjugated bile acids (0.5—15.0 μg per injection) were analysed in order to determine the detection response ratio (DRR = response bile acid/response internal standard). Calibration curves were graphed using this

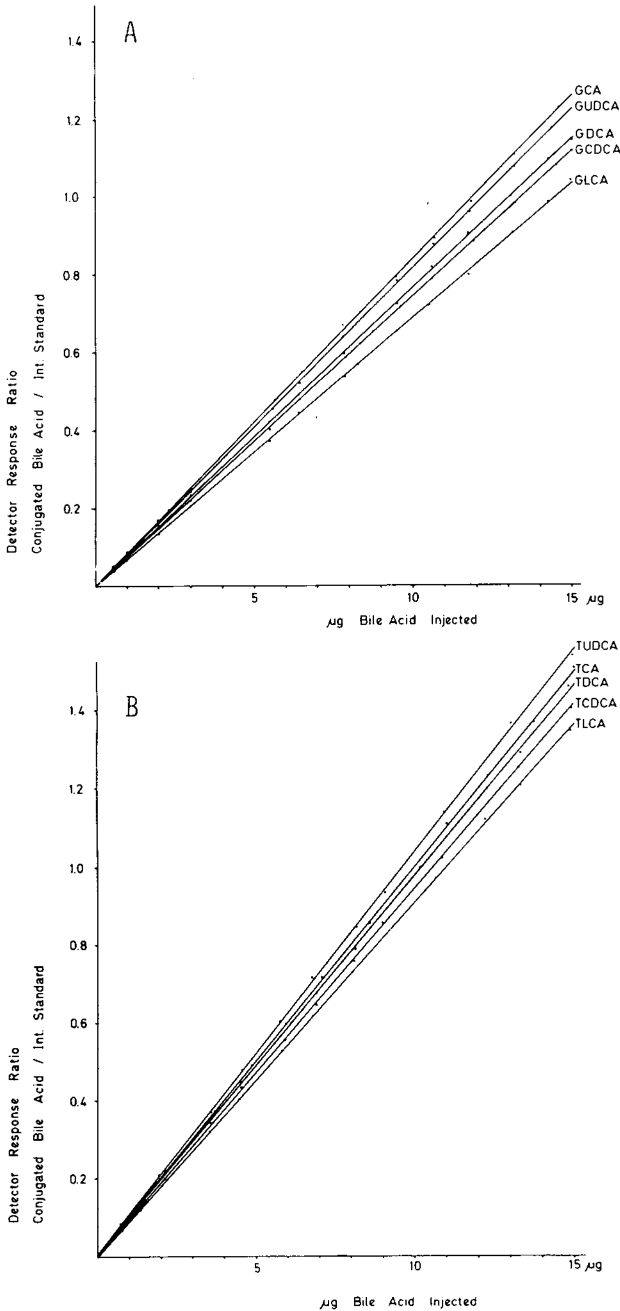

Fig. 2. Calibration curves for glycine (A) and taurine (B) conjugates (see Fig. 1 for abbreviations). The ratio of conjugated bile acid to internal standard (int. standard) was plotted against bile acid concentrations ranging from 0.5 to 15 μg per injection. The linearity of the graph indicates an excellent correlation between peak height ratio and amount injected. Correlation factors are: $r_{GCA} = 0.998$; $r_{TCA} = 0.999$; $r_{GCDCA} = 0.999$; $r_{TCDCA} = 0.999$; $r_{GDCA} = 0.998$; $r_{TDCA} = 0.999$; $r_{GUDCA} = 0.996$; $r_{TUDCA} = 0.999$; $r_{GLCA} = 0.996$; $r_{TLCA} = 0.998$.

information. The accuracy of the presented HPLC method is demonstrated in the graphs by the small deviations of the plotted points from linearity. Correlation factors (r) range from 0.996 to 0.999 (see Fig. 2).

A correlation of quantitative analysis between our HPLC method and gas chromatography was not performed due to the inconsistent conjugate recovery when Sephadex columns are employed for gas chromatography [17].

Recovery

The test samples were prepared by adding known amounts of different synthetic bile acids to human hepatic bile of a UDCA-treated patient. The results are shown in Table II. Our recovery range of 94.6—100% is sufficient for clinical application.

Reproducibility

Reproducibility was tested by analysing 20 μl on ten different days taken from the same stock sample which was isolated from hepatic bile. Results are

TABLE II

RECOVERY OF CONJUGATED BILE ACIDS ADDED TO HUMAN BILE OF A UDCA-TREATED PATIENT

Bile acid	Bile (μg per 100 μl)	Added (μg per 100 μl)	Expected (μg per 100 μl)	Found (μg per 100 μl, mean ± S.D., n = 4)	Recovery (%, mean ± S.D., n = 4)	Recovery (%, mean ± S.D., n = 12)
Glycocholate	12.275	81.90	94.175	92.87 ± 1.56	98.40 ± 1.90	99.00 ± 1.54
		40.95	53.225	52.75 ± 0.77	99.10 ± 1.45	
		13.65	25.925	25.80 ± 0.38	99.51 ± 1.47	
Glycoursodeoxycholate	71.05	96.00	167.05	165.15 ± 1.30	98.86 ± 0.78	99.29 ± 1.05
		48.00	119.05	118.85 ± 1.74	99.83 ± 1.46	
		16.00	87.05	86.77 ± 0.52	99.19 ± 0.83	
Taurocholate	2.59	84.60	87.19	88.26 ± 0.70	101.22 ± 0.79	100.65 ± 1.43
		42.30	44.89	45.06 ± 0.69	100.38 ± 1.54	
		14.30	16.89	16.95 ± 0.33	100.36 ± 1.96	
Tauroursodeoxycholate	4.15	81.90	86.05	87.43 ± 1.01	101.59 ± 1.16	100.39 ± 2.29
		40.95	45.10	45.60 ± 0.64	101.12 ± 1.41	
		13.65	17.80	17.53 ± 0.52	98.47 ± 2.90	
Glycochenodeoxycholate	39.13	75.30	114.43	112.44 ± 2.70	98.25 ± 2.36	98.30 ± 1.52
		37.65	76.78	75.80 ± 0.74	98.72 ± 0.97	
		12.55	51.68	50.61 ± 0.66	97.93 ± 1.27	
Glycodeoxycholate	4.29	75.00	79.29	77.45 ± 0.64	97.68 ± 0.81	97.20 ± 1.22
		37.50	41.79	40.64 ± 0.58	97.24 ± 1.40	
		12.50	16.79	16.26 ± 0.26	96.70 ± 1.50	
Taurochenodeoxycholate	7.4	76.35	83.75	80.92 ± 0.34	96.62 ± 0.41	96.65 ± 1.49
		38.18	45.58	44.34 ± 0.54	97.94 ± 1.51	
		12.73	20.13	19.31 ± 0.44	95.93 ± 2.21	
Taurodeoxycholate	0	76.65	76.65	77.73 ± 0.84	101.40 ± 1.09	100.20 ± 2.13
		38.33	38.33	37.54 ± 0.90	98.61 ± 2.11	
		12.78	12.78	12.88 ± 0.33	100.59 ± 2.35	
Glycolithocholate	0.93	79.95	80.88	76.95 ± 1.04	95.15 ± 1.28	94.61 ± 1.98
		39.98	40.91	38.82 ± 0.98	94.89 ± 2.38	
		13.33	14.26	13.37 ± 0.35	93.79 ± 2.40	
Taurolithocholate	0	78.15	78.15	76.28 ± 1.61	97.60 ± 2.05	95.86 ± 2.77
		39.08	39.08	37.60 ± 0.61	96.22 ± 1.54	
		12.03	12.03	11.28 ± 0.47	93.76 ± 2.39	

TABLE III

REPRODUCIBILITY OF THE CHROMATOGRAPHIC PROCEDURE FOR DETERMINATION OF BILE ACIDS IN HUMAN BILE

The sample was injected ten times on different days.

Bile acid conjugate	Mean ± S.D. (mmol/l) (n = 10)	Percentage deviation of the mean
Glycocholic acid	3.576 ± 0.035	0.98
Glycoursodeoxycholic acid	7.655 ± 0.055	0.72
Taurocholic acid	0.861 ± 0.026	3.02
Tauroursodeoxycholic acid	0.659 ± 0.032	4.86
Glycochenodeoxycholic acid	5.611 ± 0.017	0.30
Glycodeoxycholic acid	5.643 ± 0.014	0.25
Taurochenodeoxycholic acid	1.021 ± 0.020	1.96
Taurodeoxycholic acid	0.841 ± 0.012	1.43
Glycolithocholic acid	0.390 ± 0.028	7.17
Taurolithocholic acid	0.253 ± 0.021	8.30

shown in Table III. The reproducibility ranged from 0.3% for the primary bile acid glycochenodeoxycholic acid (GCDCA) up to 8.3% for the secondary bile acid taurolithocholic acid (TLCA). The larger deviation of secondary bile acids is due to their generally lower concentrations.

Interassay variability

Interassay variability includes repeated Sep-Pak extractions and HPLC analyses of the same bile sample in order to determine the consistency of the entire method. Results are shown in Table IV. In particular, GUDCA and GCDCA showed excellent reproducibility and low inter-assay variability.

TABLE IV

INTERASSAY VARIABILITY OF THE CHROMATOGRAPHIC PROCEDURE INCLUDING THE ISOLATION STEPS FOR DETERMINATION OF BILE ACIDS IN HUMAN BILE

The conjugated bile acids were isolated ten times from an identical bile sample and determined by HPLC

Bile acid conjugate	Mean ± S.D. (mmol/l) (n = 10)	Percentage deviation of the mean
Glycocholic acid	3.551 ± 0.073	2.05
Glycoursodeoxycholic acid	7.368 ± 0.176	2.38
Taurocholic acid	1.198 ± 0.048	4.00
Tauroursodeoxycholic acid	0.598 ± 0.046	7.69
Glycochenodeoxycholic acid	5.460 ± 0.075	1.35
Glycodeoxycholic acid	5.425 ± 0.063	1.16
Taurochenodeoxycholic acid	1.023 ± 0.023	2.25
Taurodeoxycholic acid	0.844 ± 0.021	2.49
Glycolithocholic acid	0.331 ± 0.028	8.45
Taurolithocholic acid	0.220 ± 0.020	9.09

Patient data

The varying amounts of conjugates in human bile are shown in Table V. Patients 1—3 had choledochal calculi; in patient 3 partial obstruction of the bile duct was present. Due to the obstruction, elevated taurine conjugate levels could be observed (see Fig. 3).

Bile was extracted from patients 4—6 by endoscopic choledochal intubation during medical dissolution therapy (500 mg UDCA per day) of gallbladder calculi. During therapy, GUDCA increased substantially, while primary bile acid levels, in particular GCA, decreased (see Fig. 4). This is in accordance with other reports in the literature [18]. A less substantial increase in taurine conjugates, especially UDCA and LCA, was also observed.

In conclusion, the presented method allows the conjugated bile acid pattern in human bile to be monitored in a short period of time. The separation module employed is less laborious than others presented in the literature and guarantees good reproducibility. Using this method concentrations as low as 0.5 μg per injection can be detected, possibly allowing analysis of serum samples.

TABLE V

CONJUGATED BILE ACIDS IN HUMAN BILE IN PATIENTS WITH CHOLEDOCHAL CALCULI (1—3) AND PATIENTS UNDER DISSOLUTION THERAPY OF GALLBLADDER CALCULI (4—6)

Values are expressed in mmol/l.

Patient	GCA	GUDCA	TCA	TUDCA	GCDCA	GDCA	TCDCA	TDCA	GLCA	TLCA
1	10.14	1.69	3.67	0.58	9.25	6.32	3.98	2.735	—	—
2	9.89	0.98	4.63	0.29	8.35	6.59	4.07	1.36	0.27	—
3	7.03	—	9.35	—	3.37	0.83	3.46	0.95	—	—
4	3.55	7.37	1.20	0.60	5.46	5.42	1.02	0.84	0.33	0.22
5	3.82	13.53	1.61	2.14	6.35	3.60	2.29	0.68	—	—
6	2.92	9.86	1.34	1.65	6.13	4.83	1.85	0.73	0.42	—

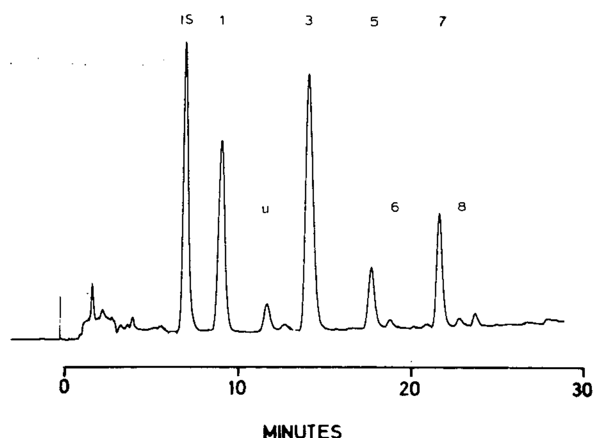

Fig. 3. Chromatogram of a patient with partial bile duct obstruction. In particular, TCA (3) and TCDCA (7) conjugate levels are elevated. LCA is not detected. Complete pattern reversal was observed after endoscopic removal of the obstruction. Peaks are labelled as in Fig. 1; u = unknown peak.

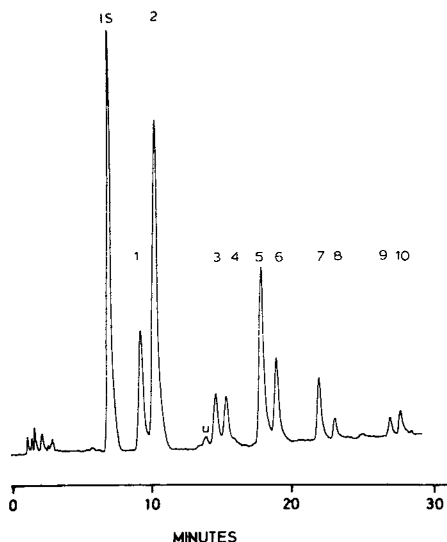

Fig. 4. Bile acid conjugate pattern from a UDCA-treated patient (500 mg per day). An increase in GUDCA (2) and TUDCA (4) peaks is due to therapy. In comparison to Fig. 1B, cholic acid conjugate levels are decreased. Peaks are labelled as in Fig. 1; u = unknown peak.

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SERUM AND PLASMA β-CAROTENE LEVELS MEASURED WITH AN IMPROVED METHOD OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An isocratic high-performance liquid chromatographic method specifically developed to allow simple and rapid determination of β-carotene concentrations in serum and plasma is reported. Using a method modified from a previously published technique, serum and plasma proteins are denatured by exposure to perchloric acid, and β-carotene is subsequently extracted into an organic matrix consisting of ethyl acetate—tetrahydrofuran (1:1); no evaporation step is required. Separation is achieved using isocratic elution from a reversed-phase C_{18} column with UV detection at 436 nm. Recovery of β-carotene from water and plasma was greater than 98.1%; β-carotene was stable in the extraction matrix for at least 4 h. Three anticoagulants (oxalate, citrate, and EDTA) caused losses of β-carotene; perchloric acid and tetrahydrofuran could also destroy β-carotene under certain conditions. Each run required less than 15 min; within-day coefficient of variation for identical samples averaged 2.3%, between-day coefficient of variation was 4.4% and sensitivity was better than 10 ng/ml. Stability of β-carotene in plasma was also examined. This method permits a simple, rapid, sensitive, precise, and accurate determination of β-carotene using 0.5 ml of serum or heparinized plasma.

INTRODUCTION

In the last few years, data from several sources have suggested that β-carotene, a naturally occurring provitamin of retinol (vitamin A), might have anti-cancer properties. The ability of β-carotene to inhibit tumor formation in animals [1] and the association of higher dietary levels of β-carotene with lower rates of cancer such as lung cancer [2], have led several reviewers to suggest that β-carotene might be a clinically important anti-cancer agent [3, 4]. Because of this type of evidence, large prospective clinical trials of β-carotene

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as a cancer chemopreventive agent are now being performed. We are currently conducting such a study at our institution.

It is therefore important to be able to measure blood levels of β-carotene easily and accurately. Measurement of blood levels of β-carotene by high-performance liquid chromatography (HPLC) has been shown to be faster, more accurate, more precise, and more selective than older methods such as open-column techniques, thin-layer chromatography, and conventional spectrophotometric methods [5—10].

One of the earliest HPLC methods published for the determination of β-carotene [5] involved time-consuming saponification and solvent evaporation steps, which would make routine analysis of the large number of samples obtained in large prospective clinical studies considerably more difficult. Two recently reported methods [9, 10] achieved excellent precision, but also required a solvent evaporation step during the sample clarification process.

In 1983, a precise method [6] for measuring β-carotene in human plasma was reported which did not require a solvent evaporation step. However, this method used an internal standard not commercially available and it failed to separate α- from β-carotene. Moreover, the sensitivity (80 ng/ml), although sufficient for normal subjects, would likely not have been satisfactory for subjects with lower dietary intakes and blood levels of β-carotene. Another method reported during the same year [7] used a commercially available internal standard (retinol acetate) and did not require a solvent evaporation step. However, monitoring both the β-carotene peak and the internal standard peak required two different wavelengths, which would raise detector costs considerably. In addition, within-day precision (measured by the coefficient of variation, C.V.) for three carotenoids was between 6.4% and 13.5%, sensitivity of β-carotene detection was not stated and recovery of β-carotene was only 93.6% from rat serum. Recovery from human samples was not evaluated.

In 1983, Peng et al. [8] published a method for β-carotene determination which appeared to be perhaps the most simple, rapid, and sensitive one. However, several questions about this method arose when we attempted to use it in our laboratory. (1) Did the tetrahydrofuran (THF) used in the extraction step and in the mobile phase need to be preserved to reduce the accumulation of peroxides? (2) Was β-carotene labile in the presence of perchloric acid used to denature plasma proteins? (3) How were the external standards prepared? (4) Why was β-carotene unstable in the organic matrix after extraction? (5) What were the within-day and between-day estimates of precision?

In addition to these specific questions, we raised several questions of a more general nature which had not been adequately addressed to in the literature. Is there a difference between plasma and serum levels? If plasma is assayed, does the anticoagulant used have any effect upon β-carotene levels? How quickly must the blood samples be processed and frozen? What are the optimal conditions for long-term storage, and do repetitive cycles of freezing and thawing result in deterioration of β-carotene?

In this paper we demonstrate that a modification of the method of β-carotene determination published by Peng et al. [8] is simple, precise, and sensitive. We address all the questions about their method outlined above.

Finally, we address the general methodologic issues which must be considered by other investigators who plan to collect blood samples and assay them for β-carotene.

MATERIALS AND METHODS

Reagents

Crystalline β-carotene was the highest grade available, and was purchased from Sigma (St. Louis, MO, U.S.A.). This product had only one peak when subjected to HPLC analysis. Butylated hydroxytoluene (BHT) was purchased from the same source. Acetonitrile and methanol were HPLC grade and purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Ethyl acetate and THF preserved with BHT were purchased from Burdick & JacksonLabs. (Muskegon, MI, U.S.A.). Ammonium acetate (HPLC grade), crystalline potassium iodide, concentrated hydrochloric acid, and 70% perchloric acid were purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.). Water used for HPLC mobile phase preparation was house-distilled, then passed through a Milli-Q purification system (Millipore, Bedford, MA, U.S.A.). Absolute ethanol was purchased from U.S. Industrial Chemicals (Tuscola, IL, U.S.A.).

Blood samples

Vacutainer glass tubes were used to collect serum and four types of plasma (anticoagulated with lithium heparin, oxalate, citrate, or EDTA) and were obtained from Becton-Dickinson (Rutherford, NJ, U.S.A.). Serum and plasma samples were protected from direct light (sunlight or fluorescent light) and centrifuged at room temperature within 30 min of drawing unless otherwise stated. After separation, the serum or plasma was transferred by a glass pipet to polypropylene freezer tubes (Nunc brand, A.H. Thomas, Philadelphia, PA, U.S.A.) and analyzed immmediately unless otherwise stated. When stored prior to analysis, the tubes were kept at −35°C. When samples were analyzed after freezing, they were thawed to room temperature, vortexed and centrifuged at 1500 *g* for 5 min to remove any cryoprecipitate present.

Plasma samples for determining between-day precision were obtained from the blood bank. Patients undergoing therapeutic phlebotomy had one unit of whole blood collected in a standard plastic blood bank bag, to which sodium heparin had been previously added. After the phlebotomy was completed, the plasma was separated and stored frozen at −35°C. When needed, the plasma was thawed, mixed thoroughly, centrifuged at 1500 *g* to remove any cryoprecipitate, and transferred to 1-ml polypropylene freezer tubes (Sarstedt, Princeton, NJ, U.S.A.). These individual sample tubes were then re-frozen, and thawed each day when needed.

Peroxide assay

A semi-quantitative assay for the presence of peroxides was utilized. Water (2.5 ml) was placed in a glass test tube, to which were added approximately 250 mg crystalline potassium iodide. Concentrated hydrochloric acid (0.5 ml) was added, and the mixture was mixed with a glass rod. The organic solvent to be assayed was added (1.5 ml) and the tube was vortexed for 30 sec. After 5 min

of incubation, absorbance at 510 nm was measured on a spectrophotometer (Varian Instruments, Springfield, NJ, U.S.A.), using a blank solution prepared as above, but with pure ethanol as the organic solvent.

Standard solutions

Crystalline β-carotene was dissolved in 5 ml benzene to which 15 ml hexane were added. This stock solution demonstrated a loss of β-carotene content of approximately 1% per day when stored at 4°C under nitrogen. Serial dilutions of the stock solution in absolute ethanol were prepared daily to approximate concentrations of 7.0, 3.5, and 1.0 μg/ml. Exact concentrations of these solutions were determined by measuring the absorbance of the 3.5 μg/ml solution at 450 nm, using an extinction coefficient in ethanol ($E^{1\%}_{1\,cm}$) of 2375 [11].

Sample clarification

All procedures were performed in a dark room, illuminated with a 25-W incandescent bulb. To clarify human blood samples, 500 μl plasma or serum were pipetted into a 1.5-ml polypropylene microcentrifuge tube (Fisher Scientific); 50 μl of pure ethanol were added and the tube was capped and vortexed for 15 sec. Protein was denatured by the addition of 100 μl of 5% perchloric acid; after vortexing for 30 sec, 500 μl of the extraction solvent, ethyl acetate—THF preserved with BHT (1:1), were added and the tubes were vortexed for 60 sec. All tubes were centrifuged at 13 000 *g* for 1 min on a Fisher Model 235B microcentrifuge. The organic matrix (top yellow layer) was removed by a glass pipet and transferred to a 500-μl polypropylene microcentrifuge tube (Fisher Scientific), and then centrifuged again at 13 000 *g* for 1 min to remove any remaining microparticulate matter. This final organic matrix was then ready for direct injection into the HPLC system.

To generate the daily standard curve, aqueous solutions were spiked with known concentrations of β-carotene and extracted as above with the following differences: (1) 500 μl water instead of plasma were used; (2) 50 μl of an ethanolic solution of β-carotene instead of 50 μl pure ethanol were added; (3) 100 μl of water instead of 100 μl perchloric acid were added.

High-performance liquid chromatography

The HPLC system consisted of a Waters Model 510 dual-piston pump (Waters Assoc., Milford, MA, U.S.A.), an SSI 0.5-μm in-line filter (Rainin Instruments, Woburn, MA, U.S.A.), a Rheodyne Model 7125 injector with a 100-μl loop (Rainin), a Brownlee precolumn (30 × 4.6 mm) packed with 5 μm diameter RP-18 material (Rainin), an Altex Ultrasphere-ODS column (5-μm spherical packing material, 250 × 4.6 mm, Beckman Instruments, Wakefield, MA, U.S.A.), a Beckman Model 160 UV detector equipped with a 436-nm filter, and a one-channel strip-chart recorder (Model D-5119-1, Houston Instruments, Austin, TX, U.S.A.). A reporter—integrator was also used with equivalent results (Model 3390A, Hewlett-Packard, Avondale, PA, U.S.A.).

Detector sensitivity was set at 0.008 a.u.f.s. The mobile phase consisted of acetonitrile—THF (preserved with BHT)—methanol—1% ammonium acetate

(65:25:6:4). The mobile phase was filtered through 0.45-μm nylon filters (Rainin), degassed under vacuum, and pumped at 2.7 ml/min at ambient temperature, generating a back-pressure of 186 bars. Peak identification was confirmed by comparing plasma peak retention times to those of known standards and by demonstrating coelution of plasma peaks and known standards added to the plasma matrix.

Calculations

The height of the β-carotene peak was measured for each run. A standard curve (β-carotene peak height versus β-carotene concentration) was generated each day from the three spiked aqueous standards (100, 350 or 700 ng of β carotene added to 1 ml water). The best-fit linear regression line was calculated using the method of least squares. The correlation coefficient (r) for each standard curve was also calculated; standard curves were not accepted if the value of r was less than 0.998.

In experiments in which recoveries were tested under different conditions, the significance of differences between means was explored using the one-way analysis of variance. When significant differences were observed, they were further investigated using the Student—Newman—Keuls test [12]. Results of duplicate or triplicate determinations were always expressed as mean ± standard deviation (S.D.). Precision was reported as the C.V. of multiple determinations (C.V. = S.D./mean).

RESULTS AND DISCUSSION

Chromatographic conditions

Our HPLC conditions were similar to those used by Peng et al. [8]. However, we modified the composition of our mobile phase in order to minimize retention volume, while optimizing separation of carotene isomers and peak shape. In addition, we noticed that the peroxide content of the THF employed in both the mobile phase and the organic extraction matrix was critical. THF which was not preserved with BHT, even when new, had measurable amounts of peroxides which caused variable, at times greater than 50%, loss of β-carotene during sample clarification and chromatography. The THF preserved with BHT obtained from Burdick & Jackson Labs. had the lowest peroxide content (several brands were tested) and was used for all of our work. We feel it is important that THF used should always be preserved with BHT; in addition, since lots and sources may vary in peroxide content, each bottle should be assayed prior to use. Finally, the HPLC conditions outlined above are compatible with long analytical column life. We changed our guard column every 400 injections, before any changes were noted in back-pressure or retention time. With this precaution, our analytical column remained satisfactory for over 2200 injections (ten months), before changes in peak symmetry and back-pressure required a new column.

Sensitivity and precision

For spiked aqueous solutions subjected to sample clarification, sensitivity was better than 10 ng/ml using 50-μl injections and a detector sensitivity of

0.008 a.u.f.s. (peak height greater than 5 times baseline noise). This sensitivity is as good or better than that reported in earlier studies [6—10], and is certainly well below the range of β-carotene levels seen in our normal subjects, or reported in other studies [6, 9]. Sensitivity could be increased, if necessary, by either increasing the sensitivity of the UV detector (up to 0.001 a.u.f.s.) or by using an injection volume larger than 50 μl. A typical chromatogram is shown in Fig. 1. This human sample contained 427 ng/ml β-carotene. There is near-baseline separation of the two carotene isomers, and this separation is comparable to or better than that demonstrated in earlier reports [6—10].

Within-day precision was calculated every day, since heparinized plasma standards were run each day in duplicate or triplicate. Over a typical one-month period, within-day precision (C.V.) ranged from 0.0% to 6.7%, with an average value of 2.3 ± 1.7%. Over eleven consecutive days, between-day precision was calculated from the daily mean values of these plasma standards. These eleven values (range 162—186 ng/ml) had a mean value of 173.0 ± 7.6 ng/ml, C.V. = 4.4%. Thus our values of within-day and between-day precision (C.V.) compare favorably to the earlier studies cited above.

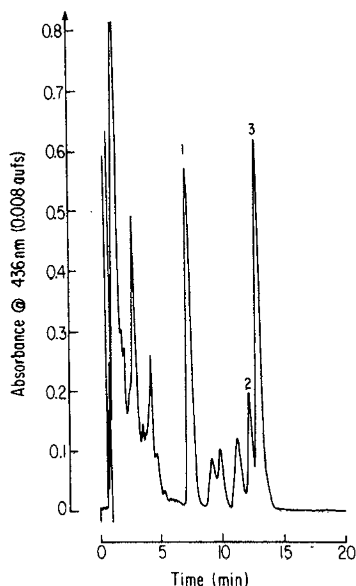

Fig. 1. Chromatogram of a heparanized human plasma sample. HPLC conditions and sample clarification conditions as in text. β-Carotene concentration was calculated to be 427 ng/ml. Peaks: 1 = lycopene; 2 = α-carotene; 3 = β-carotene.

Standard curve

Initially, we prepared our standard curve each day by adding known concentrations of β-carotene to plasma samples and plotting net increase in β-carotene peak height versus amount of β-carotene added. Since it would have been easier to generate daily standard curves from spiked aqueous samples rather than spiked plasma samples, we compared net β-carotene peak heights when water and heparanized plasma samples were spiked with known concentrations of β-carotene. Relative to water (peak height defined as 100%)

recovery of β-carotene from heparanized plasma from two separate subjects was 98.8% and 103.0%, respectively. A full standard curve (y = β-carotene peak height at 0.008 a.u.f.s., x = β-carotene concentration in μg/ml) was prepared from the plasma of a third subject with the best-fit line being $y = 0.489x + 0.006$ ($r = 0.999$). The standard curve prepared using spiked water was $y = 0.493x + 0.002$ ($r = 0.999$).

After demonstrating equal recovery of β-carotene from spiked plasma and aqueous samples, we sought to quantitate absolute recovery of β-carotene from spiked aqueous samples. When β-carotene was added to water to a final concentration of 350 ng/ml, the absorbance of this aqueous solution was 0.260 ± 0.000 in duplicate samples. After both samples were extracted using the usual sample clarification scheme, the residual absorbance in the lower aqueous layer was 0.005 ± 0.000. Thus, at least 98.1% of the β-carotene added to the aqueous layer was removed during the usual extraction process.

We generated a standard curve each day from three aqueous samples spiked with β-carotene (final concentrations 100, 350, and 700 ng/ml) and carried through the entire sample clarification process. This seemed optimal since the recovery of β-carotene from plasma and water was equal; the recovery from water was nearly 100%, and a standard curve so generated would likely alert us to any inter-current difficulties with the entire sample clarification process.

Stability of β-carotene during sample clarification

To 500-μl aqueous samples β-carotene had been added with or without 5% perchloric acid. The mixtures were incubated in the dark at room temperature for 1 or 15 min before the organic matrix was added. Clarification was completed, and the samples were injected immediately into the HPLC system. After injection, some of the organic matrix samples were kept at room temperature in the dark for 3.5 h, and then injected again. The results are summarized in Table I.

With the recovery of β-carotene from water (in the absence of perchloric acid) defined as 100%, it is apparent that incubation for 15 min in the absence of

TABLE I

EFFECTS UPON β-CAROTENE PEAK HEIGHT OF DIFFERENT CONDITIONS DURING SAMPLE CLARIFICATION AND OF PROLONGED STORAGE OF THE ORGANIC MATRIX PRIOR TO INJECTION

Each number is the mean of n determinations and expressed in percent. Each incubation (prior to the addition of the organic matrix) was for either 1 or 15 min. Injection into the HPLC system was either immediately (0 h) or 3.5 h following extraction. Those samples defined as being 100% are underlined.

Sample	1-min Incubation				15-min Incubation	
	0 h	n	3.5 h	n	0 h	n
Water—water	<u>100.0</u>	3	96.3	2	100.0	2
Water—acid	98.9	2	64.5*	2	94.0	2
Plasma—acid	<u>100.0</u>	11	102.2	11	100.4	2

*$p < 0.001$.

perchloric acid or for 1 min in the presence of perchloric acid has no effect upon the recovery of β-carotene. Incubation for 15 min in the presence of perchloric acid caused a small, statistically non-significant, decrease in recovery of β-carotene. When plasma samples without exogenous β-carotene were incubated for 1 min (defined as 100% recovery) or 15 min prior to organic extraction, there was no decrease in recovery of β-carotene. Thus it appeared that β-carotene was stable for 15 min in aqueous solution or for 15 min in plasma treated with perchloric acid. However, β-carotene in aqueous solution was slightly susceptible to 15-min exposure to perchloric acid. In fact, this loss of β-carotene peak height was accompanied by the development of several small, new peaks with retention times less than that of β-carotene and with the development of a new peak occurring on the descending shoulder of the β-carotene peak.

Since our aqueous solutions are extracted promptly in the absence of perchloric acid and since our plasma samples are extracted promptly in the presence of perchloric acid, we felt that there was no demonstration of worrisome acid-induced loss of β-carotene peak height during the clarification process.

Stability of β-carotene in the organic matrix

Although sample clarification can be accomplished promptly without prolonged incubations, we were concerned that the β-carotene extracted into the organic phase might not be stable. Samples were extracted batchwise, and occasionally a sample would have to stand for 15—45 min following extraction before injection onto the HPLC system. Peng et al. [8] reported that samples were stable for 1 h or less after extraction, using a similar extraction scheme. As seen in Table I, when samples were extracted promptly (1-min incubations) but the organic matrix was held in the dark at room temperature for 3.5 h rather than injected immediately, there were variable effects upon β-carotene

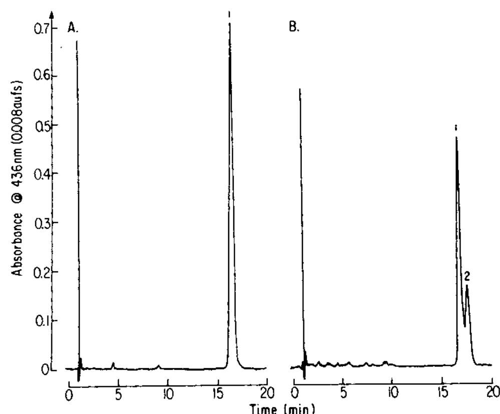

Fig. 2. (A) Chromatogram of β-carotene (1) after extraction from aqueous matrix with exposure to perchloric acid for 1 min, immediate extraction, and immediate injection (peak height 98.9% of control height). (B) Injection of the same organic matrix after 3.5-h incubation in the dark at 22° C. β-Carotene peak height (1) is now 64.5% of control height, possible degradation product (peak 2) is also seen.

recovery. Aqueous samples processed without perchloric acid and plasma samples processed with perchloric acid demonstrated no loss of β-carotene peak height despite the 3.5-h delay prior to injection. However, β-carotene in an aqueous matrix, incubated for 1 min in the presence of perchloric acid, then extracted and held for 3.5 h prior to injection, demonstrated a pronounced loss of β-carotene peak height. This loss of peak height was accompanied by the development of several new peaks on the chromatogram and the development of a large shoulder of the β-carotene peak itself (see Fig. 2).

We believe that this loss of β-carotene, associated with the development of new peaks, represents degradation or isomerization of the parent molecule. β-Carotene is unstable because of its conjugated system of double bonds; it is destroyed or altered by acids, particularly in the presence of light, and it is easily oxidized, leading to epoxide formation and chain cleavage [13]. In addition, we had previously shown that exposure of retinol to perchloric acid during a plasma clarification process resulted in unpredictable but definite losses of retinol and retinol acetate, which led to sub-optimal precision [14]. Perhaps the reason why we found β-carotene to be relatively more stable during sample clarification and in the organic matrix than did Peng's group [8] may be that we used only THF preserved with BHT and we assayed each lot of THF to make sure that the peroxide content was acceptably low. For example, THF preserved with BHT, when subjected to our peroxide assay, produced a yellow color similar to that obtained when pure ethanol was assayed; the absorbance at 510 nm was approximately 0.015 relative to the blank solution prepared with ethanol. When unpreserved or old THF was assayed, the assay produced a reddish-brown color with an absorbance as high as 1.81.

Stability of β-carotene in plasma

Heparanized plasma from four subjects was analyzed (in duplicate) immediately after venipuncture, and again after the plasma had been exposed to laboratory levels of fluorescent light and room temperature for 4 h. After such treatment, β-carotene peak heights were 104.6 ± 5.6% that of the immediately assayed samples. Therefore, it appeared that no special collection procedures (chilling samples, protecting from light) were required as long as samples were centrifuged and assayed (or frozen) within 4 h of venipuncture. Previous authors [8, 15] have also observed that β-carotene in serum or plasma is stable under these conditions for up to 24 h.

Differences between serum and plasma levels

For each of three subjects, blood was obtained from a single venipuncture and transferred to Vacutainer glass tubes containing no anticoagulant (for serum) or one of four commonly used anticoagulants (heparin, EDTA, oxalate, or citrate). After 1 h (to allow clot retraction to occur), the blood samples were centrifuged and the serum or plasma was separated. Samples were clarified, and injected onto the HPLC system within 30 min. Results are summarized in Table II.

Levels of β-carotene in each subject were highest in serum, with levels in heparanized plasma being slightly (but significantly) less. This may reflect

TABLE II

β-CAROTENE LEVELS IN FOUR TYPES OF PLASMA, RELATIVE TO SERUM LEVELS, IN THREE HEALTHY SUBJECTS

All values (in percent) are expressed as mean ± S.D. of duplicate determinations. For each subject, levels in serum were defined as 100%. p Value reflects significance of difference between mean plasma levels and mean serum level.

Matrix	Subject A	Subject B	Subject C	Group mean	p Value
Serum	100.0 ± 0.6	100.0 ± 5.7	100.0 ± 0.7	100.0	
Heparin	95.7 ± 3.2	98.0 ± 2.0	93.1 ± 0.8	95.6 ± 2.5	<0.05
EDTA	91.8 ± 0.0	92.5 ± 0.2	90.1 ± 0.8	91.5 ± 1.2	<0.01
Oxalate	78.0 ± 0.4	79.2 ± 2.0	76.9 ± 0.3	78.0 ± 1.2	<0.01
Citrate	77.5 ± 0.3	83.4 ± 1.3	75.5 ± 0.8	78.8 ± 4.1	<0.01

the greater protein content of plasma relative to serum. However, such an explanation would not explain the fact that when EDTA, oxalate, or citrate were used as anticoagulants, the loss of β-carotene was even greater. These findings were quite similar to previously reported effects of these anticoagulants upon plasma levels of retinol [14, 16]. While the mechanism of this apparent loss of retinol and β-carotene is not clear, it is possible that EDTA, oxalate, and citrate — all acids — may catalyze reactions resulting in the isomerization or oxidation of retinol and β-carotene. Thus, we suggest that either serum or heparanized plasma be collected when blood levels of β-carotene are to be measured.

Storage conditions for plasma samples

We investigated the effects of multiple freeze—thaw cycles on plasma β-carotene levels by obtaining plasma from one subject, freezing it, storing it at —35°C, and carrying it through sequential freeze—thaw cycles each day for seven days. Plasma levels (all done in duplicate) after 1, 2, 3, 5, 6, and 7 such cycles were 167, 175, 175, 153, 176, and 169 ng/ml. Thus, sample tubes may be thawed and refrozen multiple times without affecting β-carotene levels. This observation supports similar findings previously reported [7].

A related issue concerns optimal long-term storage conditions for blood samples. As shown in Table III, we found that plasma from one subject had

TABLE III

EFFECT OF STORAGE AT DIFFERENT TEMPERATURES UPON β-CAROTENE LEVELS IN HEPARANIZED PLASMA

Baseline levels were measured nine times over one week; levels at six months were measured in duplicate. The p value reflects significance of difference between means at six months relative to the mean value of the baseline sample.

Storage conditions	β-Carotene levels (mean ± S.D., ng/ml)	p Value
Initial sample	167.7 ± 7.3	
Six months at —7°C	154.5 ± 2.1	<0.025
Six months at —35°C	175.0 ± 7.1	>0.20
Six months at —70°C	174.0 ± 0.0	>0.20

stable β-carotene levels over a six-month period whether stored at −35°C or −70°C. Samples stored at −7°C demonstrated a significant loss of β-carotene after six months. Previously reported studies had shown that total carotenoids in plasma stored at −20°C for ten years are reduced by about 97%; in plasma stored at −70°C for one year, total carotenoid values were essentially unchanged [15]. It would seem to be prudent to store serum and plasma at either of the lower temperatures and assay samples for β-carotene as soon as possible; delays of up to six months seem to be acceptable. Optimal storage conditions for periods greater than six months are being investigated.

Normal plasma β-carotene levels

We have analyzed plasma levels of β-carotene from many patients with specific diseases, as well as from seventeen healthy volunteers who were medical school employees, not taking any vitamin supplements containing β-carotene. These volunteers were eleven women and six men, aged 24—55 years. Their range of β-carotene levels was 91—428 ng/ml (mean ± S.D. 274 ± 103 ng/ml).

Two prior studies used HPLC methods [6, 9] to measure serum or plasma levels of β-carotene in normal subjects. One study [6] reported a range of 150—930 ng/ml (mean ± S.D. 330 ± 205 ng/ml) in twenty normal volunteers. A second study [9] reported mean levels of 331 ± 191 ng/ml in eight normal men, and 351 ± 164 ng/ml in eighteen normal women. Both of these studies compared β-carotene levels determined by HPLC with those determined by conventional spectrophotometric methods. In each case, the level determined by HPLC was lower. Thus, the range of plasma β-carotene levels in normal subjects as determined by HPLC methods is beginning to be ascertained, and it appears to be much lower than the reported normal range for total carotenoids.

Further studies using BHT as a preservative

After the above experiments were completed, we sought to slow the rate of loss of β-carotene in the benzene—hexane stock solution. Even though it was stored at 4°C under nitrogen, this solution lost β-carotene at a rate of approximately 1% per day. In an attempt to slow this rate of loss, we added BHT to the hexane, such that the final concentration of BHT in the benzene—hexane solution was 125 μg/ml. Under these conditions, there was no loss of β-carotene over thirty days. This observation, combined with a recent report of BHT being used to stabilize labile compounds during the clarification process [17] stimulated us to investigate the use of BHT during our sample clarification process.

Plasma from ten subjects was analyzed using two methods. The first method was as outlined in Materials and methods. The second method was identical, except that the ethanol added just prior to the addition of perchloric acid contained 1625 μg/ml BHT. The final concentration of BHT in the mixture of plasma, ethanol, and perchloric acid was 125 μg/ml. For each subject, the amount of β-carotene measured using BHT during clarification was defined as 100%. Relative to this, the amount of β-carotene measured without using BHT ranged from 91% to 103%, mean 97.7 ± 4.0% (n = 10). While this difference between group means was not significant (p = 0.10), the use of BHT did make a

clinically important difference in several subjects, increasing the recovery of β-carotene. We therefore decided to use BHT in our sample clarification process for all subjects.

Finally, we also investigated whether the use of BHT affected the results of the clarification of the aqueous standards. Standard solutions were clarified in triplicate, using ethanol with or without BHT, as above. Those samples extracted without BHT yielded β-carotene values which on average were 98.4% of those obtained with BHT. Although this difference was not significant, we continued to use BHT during the processing of the aqueous standard solutions, as well as during the processing of plasma samples.

In conclusion, this report describes a method for determining β-carotene levels in human serum or heparanized plasma which is simple, rapid, precise, and sensitive. The absence of a solvent evaporation step makes this method more attractive for groups analyzing the large number of samples collected during large-population studies. Methodologic problems seen with earlier methods (possible loss of β-carotene due to exposure to perchloric acid, peroxides in THF, or heat during solvent evaporation; differences between β-carotene levels in different types of blood samples) were investigated. Preliminary data on optimal sample handling conditions, storage conditions, and values in normal subject are reported. As more clinical studies are begun which require the determination of blood levels of β-carotene, we anticipate that the observations reported here may be of use to clinical investigators and clinical chemists alike.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF FREE HYDROXYPROLINE AND PROLINE IN BLOOD PLASMA AND OF FREE AND PEPTIDE-BOUND HYDROXYPROLINE IN URINE

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SUMMARY

A rapid, accurate and sensitive method for the determination of free hydroxyproline and proline in plasma and of total hydroxyproline in urine has been developed. Free imino acids and internal standard are extracted from plasma by trichloroacetic acid precipitation of protein and they are selectively derivatized with 4-chloro-7-nitrobenzofurazan, after reaction of the acid extract with *o*-phthalaldehyde. The highly fluorescent adducts of imino acids are separated on a Spherisorb ODS 2 reversed-phase column using acetonitrile—0.1 *M* sodium phosphate buffer, pH 7.2 (9:91, v/v) as mobile phase, followed by fluorometric detection. Total hydroxyproline determination in urine hydrolysates is carried out by reaction of the imino acid with 4-chloro-7-nitrobenzofurazan after clean-up on a Sep-Pak C_{18} cartridge of the *o*-phthalaldehyde-treated sample, high-performance liquid chromatographic separation and fluorometric quantitation of the derivative.

INTRODUCTION

In liquid chromatography post- and pre-column derivatization with fluorogenic reagents is increasingly being used to improve the detection of amino acids in biological material. In this field, the pre-column derivatization of amino acids with *o*-phthalaldehyde (OPA)—mercaptoethanol reagent, followed by high-performance liquid chromatographic (HPLC) separation and

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fluorometric detection of the derivatives, appears at present to be the most reliable method [1—3]. However, this procedure has the disadvantage that proline (Pro) and hydroxyproline (Hyp) do not give a positive reaction with OPA. The selectivity of this reagent toward the primary amino group can be extended to imino acids by using post-column derivatization after chemically changing a secondary amino group into a primary one by treatment with hypochlorite [4—6]. Nevertheless, these procedures are not suitable for the simultaneous analysis of all amino acids since complex apparatus is required and they are so time-consuming that their use is restricted to small series of determinations. Recently, the reagent 4-chloro-7-nitrobenzofurazan (NBD-Cl) has been proposed for the chromatographic determination of primary and secondary amines [7—9]. This reagent has proved to be effective for Hyp and Pro determination in plasma [7] using post-column derivatization after chromatographic separation on a cationic exchange resin. Moreover, Bellon et al. [10] used NBD-Cl for the determination of 4-Hyp and 3-Hyp in urine by thin-layer chromatography of the derivatives. These methods, however, are time-consuming and tedious when compared with the results that can be achieved by HPLC with the pre-column derivatization procedures.

This paper deals with the development of an HPLC assay which allows the easy determination of free Hyp and Pro in blood plasma and of free and peptide-bound Hyp (total Hyp) in urine. The method combines the performance simplicity and reliability of HPLC with the specificity and sensitivity of NBD-Cl pre-column derivatization of imino acids and fluorometric detection of the derivatives.

EXPERIMENTAL

Materials

Materials and their sources were as follows: 4-hydroxy-L-proline, L-proline, 3,4-dehydro-L-proline, NBD-Cl and OPA from Sigma (St. Louis, MO, U.S.A.); reagent-grade sodium dihydrogen phosphate, trichloroacetic acid (TCA) and boric acid from Carlo Erba (Milan, Italy); methanol and acetonitrile (HPLC grade) from Violet (Rome, Italy). Water was demineralized and glass-distilled. Sep-Pak C_{18} cartridges were purchased from Waters Assoc. (Milford, MA, U.S.A.) and were attached to Eppendorf combitips (Eppendorf, Hamburg, F.R.G.) to facilitate sample application and elution.

Biological samples

Blood and urine samples were obtained from healthy volunteers. During three days before sample collection, the donors were subjected to a Hyp-free diet. Urines (24-h) were combined. Blood plasma and urine samples were frozen at —20°C if not immediately processed.

NBD-Cl and OPA reagents

A 25 m*M* NBD-Cl solution was prepared in methanol. OPA reagents were prepared at 150 m*M* concentration in 0.2 *M* borate buffer, pH 9.0 (pH was adjusted with 2 *M* potassium hydroxide), or in methanol.

Standards

Two stock solutions of the analytes in 0.01 *M* hydrochloric acid were prepared. The first contained Hyp and Pro at 2 m*M* and 20 m*M*, respectively. The second contained Hyp at 20 m*M*. A stock solution of the internal standard was obtained by dissolving 3,4-dehydro-L-proline in 0.01 *M* hydrochloric acid up to 4 m*M*. These solutions were stored in a refrigerator (about 4°C) and freshly prepared every two weeks.

Analytical procedure

Plasma samples. A 0.75-ml sample of plasma was pipetted into a plastic 5-ml centrifuge tube and spiked with 200 nmol of 3,4-dehydro-L-proline (50 μl of the internal standard solution). Next, 0.2 ml of 20% (w/v) cold TCA solution was added and the tube was vortexed for 30 sec. After standing on ice for 10 min, the mixture was centrifuged at 15 000 *g* for 10 min at 4°C. The supernatant was decanted and immediately analysed or stored at −20°C for analysis within three days. A 0.1-ml aliquot of the plasma extract was added to a glass tube with a screw cap containing 0.1 ml of 0.4 *M* potassium borate buffer, pH 9.0, and 50 μl of OPA reagent in methanol. After 3 min standing at room temperature, 0.1 ml of NBD-Cl reagent was added to the mixture and the derivatization was carried out at 60°C for 3 min in the dark. The reaction was quenched by the addition of 0.65 ml of cold mobile phase; 50 μl of the solution were injected into the chromatograph.

Urine samples. A urine sample (e.g. 5 ml) was adjusted to pH 4.7—4.8 with 2 *M* hydrochloric acid. The sample was heated in a boiling water bath for 5 min and the precipitated protein removed by centrifugation at 10 000 *g* for 10 min at 4°C. A 0.5-ml aliquot of the supernatant was hydrolysed under vacuum in 6 *M* hydrochloric acid at 105°C for 16 h in a sealed ampoule. The hydrolysate was spiked with 300 nmol of internal standard, diluted 1:4 with water and evaporated to dryness at 40°C on a rotavapor. The residue was dissolved in 5 ml of 0.2 *M* potassium borate buffer, pH 9.0. A 1-ml aliquot of the sample was mixed with 0.5 ml of the OPA reagent in borate buffer. After 3 min at room temperature, 1 ml of the derivatization mixture was passed through a Sep—Pak C_{18} cartridge previously conditioned with 10 ml of methanol and 10 ml of distilled water. The first 0.5 ml of eluate was discarded and the non-derivatized imino acids were eluted with 0.2 *M* potassium borate buffer, pH 9.0, containing 10% methanol, and 2 ml were collected. A 0.2-ml sample of the eluate was added to a screw-capped glass tube containing 0.1 ml of NBD-Cl reagent and 50 μl of methanol. The derivatization was carried out at 60°C for 3 min in the dark and the reaction stopped by adding to the mixture 1.65 ml of cold mobile phase. A 50-μl aliquot of the solution was injected into the column.

Reversed-phase chromatography

The apparatus used consisted of a Violet Model Clar 002 constant-flow pump and of a Shimadzu FC 530 fluorescence spectromonitor (Shimadzu, Kyoto, Japan), equipped with a xenon lamp and a 12-μl quartz flow cell. The NBD derivatives were detected by setting the monochromators at 470 nm for excitation and 530 nm for emission. The detector was connected to a Model 7123A strip chart recorder (Hewlett-Packard, San Diego, CA, U.S.A.). A

Rheodyne 7125 valve-loop injector (Cotati, CA, U.S.A.), fitted with a 100-μl loop, was employed and the separation was performed on a 15 cm × 4 mm I.D. Spherisorb ODS 2, 5 μm particle size, column. A Guard-Pak pre-column module (Waters Assoc.) fitted with a C_{18} cartridge was used as a pre-column. The separation was carried out isocratically, by using a mixture of acetonitrile—0.1 *M* sodium phosphate buffer, pH 7.2 (9:91, v/v) as mobile phase, delivered at 1.3 ml/min at room temperature. Prior to its mixing with acetonitrile, the buffer was filtered through a 0.45-μm Millipore filter and the mobile phase was degassed by ultrasonication prior to use.

Quantitation

The concentrations of the analytes were determined on the basis of their respective calibration curves and quantitation was aided by the addition of internal standard to the samples.

To construct the plasma calibration curve, working standard solutions of 5, 10, 25 and 50 μ*M* Hyp and 50, 100, 250 and 500 μ*M* Pro were prepared by diluting the stock solution of the analytes with a 4% TCA solution. To each working solution was added 3,4-dehydro-L-proline up to a final concentration of 200 μ*M*. Aliquots (0.1 ml) of the solutions were treated according to the derivatization procedure used for the plasma extract.

To construct the urine calibration curve, the stock solution of Hyp was diluted to 50, 100, 250 and 500 μ*M* with 6 *M* hydrochloric acid. To each solution internal standard at 600 μ*M* concentration was added. Aliquots (0.5 ml) were diluted 1:4 with distilled water and evaporated to dryness at 40°C on a rotavapor. The residue was dissolved in 5 ml of 0.2 *M* potassium borate buffer, pH 9.0, and 1 ml of each reconstituted standard solution was processed as the hydrolysed urine sample.

A 50-μl aliquot of each working standard was injected into the column. Attenuation detector sensitivity was set at ×1, except in the plasma calibration analyses when it was turned to ×4 5 min after the injection. To construct the calibration curves, peak heights of derivatized standard/derivatized internal standard versus concentration of standards were plotted. The standard curves were analysed by linear regression analysis to determine linearity.

Precision

To evaluate the precision of the method, within-run and between-run coefficients of variation (C.V.) were calculated from the same sample of plasma acid extract or urine hydrolysate. To determine within-run C.V., the analyte concentrations were calculated from five assays using the corresponding standard curves. To calculate the between-run C.V., five 0.12-ml aliquots of a TCA plasma extract and five 1.2-ml aliquots of a hydrolysed urine sample were frozen for analysis on five subsequent days. Standard curves were constructed for each everyday run. Concentrations were determined in duplicate and mean values of the five runs were used to calculate the between-run C.V.

RESULTS AND DISCUSSION

Chromatographic system

The chromatographic profile shown in Fig. 1A demonstrates a typical

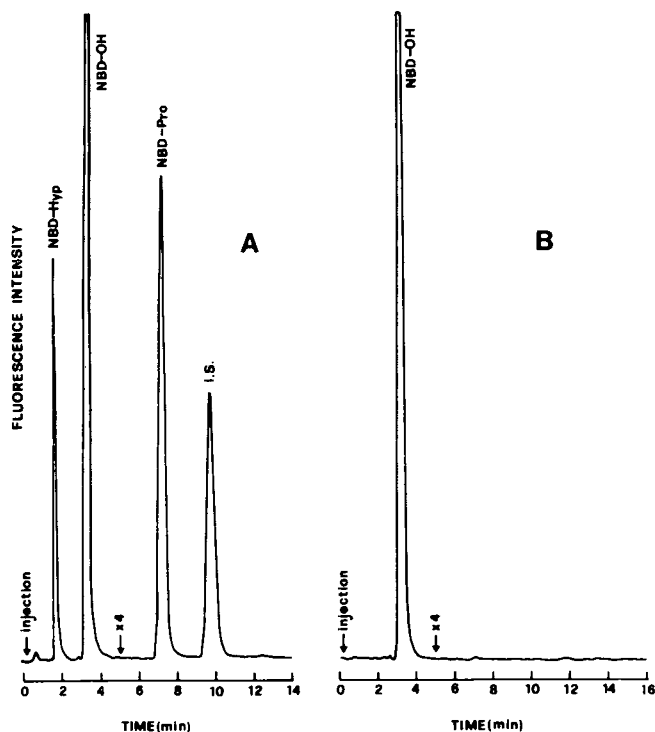

Fig. 1. (A) Chromatogram of standard Hyp, Pro and internal standard (I.S.) derivatized according to the plasma calibration procedure. Analyte peaks correspond to injected amounts of 50 pmol of Hyp and 500 pmol of both Pro and internal standard. (B) Chromatogram of a reagent blank. Chromatographic conditions: 15 cm × 4 mm I.D. column of Spherisorb ODS 2, particle size 5 μm; mobile phase, acetonitrile—0.1 *M* sodium phosphate buffer, pH 7.2, (9:91, v/v); flow-rate, 1.3 ml/min; fluorometer wavelengths, 470/530 nm; 5 min after injection, attenuation detector sensitivity is turned from × 1 to × 4.

separation of the NBD derivatives of Hyp, Pro and internal standard. The retention times of the analytes, eluted as separate symmetrical peaks, were 1.8 min for Hyp, 7.2 min for Pro and 9.6 min for internal standard. Fig. 1B shows that no interfering peaks occurred at these retention times when a reagent blank sample was chromatographed; also it can be seen that, with the derivatization procedure employed, a single not interfering peak due to the side-product 7-nitro-4-benzofurazanol (NBD-OH) appears in the chromatogram. Moreover, in a sample of standard derivatized with NBD-Cl in the absence of OPA, the presence of OPA in the injected sample does not modify the fluorometric detection (not shown).

The mobile phase was chosen after several trials performed at different buffer pH values and different organic modifier concentrations. The optimized conditions allow an efficient separation of the imino acid derivatives, with satisfactory capacity ratios, although the elution was carried out under isocratic conditions and the carboxylic group of the NBD derivatives is dissociated at the pH of the mobile phase.

The linearity of detector response to analyte derivative concentration, checked to ensure the reliability of the procedure in quantitative analysis, was

TABLE I

WITHIN-RUN AND BETWEEN-RUN REPRODUCIBILITY OF REVERSED-PHASE QUANTITATION OF HYDROXYPROLINE AND PROLINE

Figures from five analyses are given in μmol/l for plasma and in μmol/g creatinine for urine.

	Plasma		Urine
	Hyp	Pro	Hyp
Within-run			
Mean	9.2	232.0	122.0
Standard deviation	0.2	6.5	5.2
Coefficient of variation (%)	2.2	2.8	4.2
Between-run			
Mean	10.4	202.7	185.8
Standard deviation	0.4	8.3	9.1
Coefficient of variation (%)	3.8	4.1	4.9

excellent for both Hyp and Pro. Plasma calibration curves of peak ratios against Hyp and Pro concentration showed linearity over ten times the concentration range examined. Correlation coefficients of 0.999 for Hyp and Pro were obtained from linear regression analysis. The corresponding regression equations were: $y = 0.14x + 0.09$ for Hyp and $y = 0.0093x + 0.18$ for Pro. Linear regression analysis of the urine calibration curve for Hyp showed a correlation coefficient of 0.999 and a regression equation equal to $y = 0.0042x - 0.020$.

Precision analysis of Hyp and Pro in five identical aliquots of the same plasma extract and of Hyp in five specimens of the same urine are summarized in Table I. The within-run coefficients of variation for the quantitation of Hyp (2.2%) and Pro (2.8%) in plasma and of Hyp (4.2%) in urine indicate a good reproducibility of the analysis. The between-run coefficients of variation were found to be not higher than 4.9% both for plasma and urine analyses. The detection limits were estimated to be 1 and 5 injected pmol of Hyp and Pro, respectively.

Chromatography of biological samples

HPLC methods using pre-column NBD-Cl derivatization have previously been applied to the determination of Hyp and Pro in standard solutions [11] and of Hyp in purified collagen samples [9]. However, several analytical difficulties arise in the determination of Hyp in biological fluids such as plasma (serum) or urine. The drawbacks occur either because of the very low concentration of the analyte or because of the complexity of the sample matrix. HPLC of a plasma acid extract, derivatized with NBD-Cl without previous reaction with OPA, does not allow Hyp to be quantified because of the presence of several interfering compounds, mostly amino acids, in the chromatographic profile. Moreover, the analytical difficulties related to the matrix complexity are particularly increased when urine samples are analysed. For these reasons the procedure for Hyp (and Pro) determination using pre-column derivatization greatly depends upon the sample matrix, which determines the degree of sample clean-up required prior to the analytical chromatography. Thus, a

satisfactory clean-up of interfering amino acids in plasma samples is obtained by preliminary derivatization with OPA. This treatment of the plasma sample allows the selective derivatization of imino acids with NBD-Cl and the immediate determination of derivatives by HPLC. A further clean-up step of the OPA-derivatized sample on a Sep-Pak C_{18} cartridge is, however, required for total Hyp determination in urine.

Fig. 2 shows the chromatographic profiles of derivatized samples of plasma and urine, processsed as described above. Solute peaks were identified by their capacity ratios from comparison with derivatized reference compounds and by addition of known amounts of standards to the biological samples. Moreover, in order to unequivocally identify the NBD-Hyp peak and to obtain evidence that no other interfering compounds were present, this chromatographic peak from analysis of a 0.1-ml derivatized urine sample was collected and diluted 1:5 with distilled water. The material was adsorbed on a Sep-Pak C_{18} cartridge activated with methanol and washed with distilled water. After washing with 5 ml of distilled water, the material was eluted with 5 ml of methanol. The fraction was dried, resuspended in a minimal volume of methanol and subjected

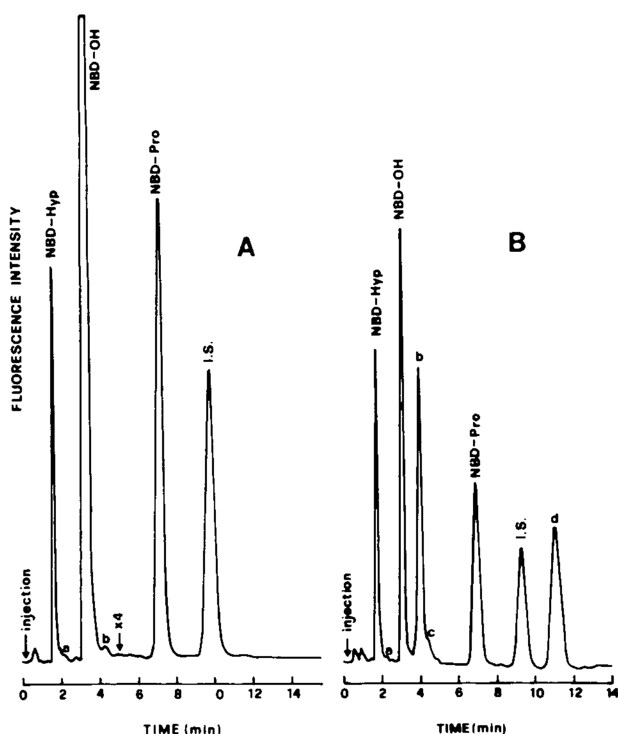

Fig. 2. (A) Representative chromatogram of free Hyp and Pro in a plasma sample, derivatized as described in the text. The calculated concentrations for Hyp and Pro are 13.0 and 138.0 μmol/l, respectively. Chromatographic conditions are as described in Fig. 1. Peaks a and b are unknown. (B) Typical chromatogram of total Hyp in urine. The calculated concentration for Hyp is 152.0 μmol/g creatinine. Chromatographic conditions are as described in Fig. 1, except that attenuation detector sensitivity is maintained at ×1. Peaks a, b, c, and d are unknown.

to thin-layer chromatography (TLC) as described by Bellon et al. [12]. Only one fluorescent spot, with the same R_F as authentic NBD-Hyp, was observed.

The procedure described here was used to assess the concentration range of Hyp and Pro in plasma and of total Hyp in urine from 21 healthy adult volunteers. The amounts of free Hyp and Pro in plasma determined by the present method were 8.5 ± 0.8 μmol/l and 251 ± 36 μmol/l, respectively. These figures agree with those obtained by ion-exchange chromatography using the post-labelling method employing NBD-Cl [7] and OPA—hypochlorite [5]. The level of total Hyp in urine was 158 ± 35 μmol/g creatinine. Similar results were obtained by the fluorometric TLC determination [12], which requires some time-consuming clean-up steps of the urine sample prior to TLC determination. On the other hand, higher figures were obtained by the conventional colorimetric assay with *p*-dimethylaminobenzaldehyde [13, 14].

Finally, due to its accuracy and precision, in addition to its ease of performance, the method may be regarded as a useful biomedical application of liquid chromatography to the determination of Hyp and Pro in both plasma and urine.

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ASSAY OF HUMAN ERYTHROCYTE PYRIMIDINE AND DEOXYPYRIMIDINE 5'-NUCLEOTIDASE BY ISOCRATIC REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

We report a rapid and reproducible assay for activity of human erythrocyte pyrimidine 5'-nucleotidase and deoxypyrimidine 5'-nucleotidase. The nucleotides CMP, UMP, dUMP, dCMP or dTMP are individually incubated 30 min at 37°C with erythrocyte hemolysate and 4 m*M* magnesium chloride in Tris, pH 7.5. Data are provided for standardization of the reaction with each substrate. Individual nucleoside products are assayed in less than 10 min by reversed-phase high-performance liquid chromatography at 280 nm with 0—14% methanol in 0.01 *M* potassium dihydrogen phosphate. This is the first report of a high-performance liquid chromatographic assay system which allows quantitation of the activity of pyrimidine 5'-nucleotidase isozymes using five individual pyrimidine and deoxypyrimidine nucleotides as the substrates.

INTRODUCTION

This report describes the use of reversed-phase high-performance liquid chromatography (HPLC) to quantitate the activity of isozymes of erythrocyte pyrimidine 5'-nucleotidase (P5N). There are at least two isozymes of erythrocyte P5N, which dephosphorylate the 5'-pyrimidine and 5'-deoxypyrimidine mononucleotides [1—4]. P5N deficiency is one of the more common causes of congenital hemolytic anemia but the occurrence of abnormalities of deoxypyrimidine 5'-nucleotidase (dP5N) has not been reported. Assay for activity of both P5N and dP5N can confirm deficiencies of P5N and offers the possibility of exploring abnormalities of the dP5N. Erythrocyte P5N activity is increased in reticulocytosis and anemias characterized by a young population of red cells but decreased in β-thalassemia

trait [5], certain leukemias [6] and in lead poisoning [7]. Activity of dP5N in similar disorders is under investigation.

The method reported has several advantages over current assays. Assays based on the liberation of inorganic phosphorus from a specific mononucleotide such as cytidine monophosphate (CMP) require overnight dialysis of hemolysate to remove endogenous phosphate followed by a 2-h incubation [8]. The radiometric procedure of Ellims [9] requires overnight dialysis; that of Torrance et al. [10] requires approximately 2 h for sample preparation and incubation and with it, we have been unable to achieve acceptable reproducibility. Harley and Berman [11] utilized HPLC separation of labelled products of uridine. Rocco et al. [12], Ericson et al. [13], and Sakai et al. [14] have used HPLC to measure P5N by release of uridine from uridine monophosphate (UMP) but not with other nucleosides including the deoxyribonucleosides which are necessary for quantitation of dP5N activity. Reversed-phase HPLC for analysis of deoxypyrimidine nucleotides and their nucleosides has not been applied to the enzymatic assay of biological material [15, 16]. The method we report requires less than 10 min of HPLC running time for identification of cytidine, deoxycytidine, uridine, deoxyuridine and deoxythymidine allowing rapid quantitation of these products from a methanol—potassium dihydrogen phosphate buffer system.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Model 110A reciprocating pump (Beckman-Altex, St. Louis, MO, U.S.A.), a fixed-wavelength UV Monitor III (Laboratory Data Control, River Beach, FL, U.S.A.) and a syringe-loading injector, Model 7120 (Rheodyne, Berkeley, CA, U.S.A.) with a 10-μl fixed-volume sample loop. Samples were injected by Hamilton syringe with a blunt 22-gauge needle. An Ultrasphere ODS (25 cm $\times$ 4.5 mm I.D., 5 μm particle diameter) column was used in conjunction with a Brownlee guard cartridge RP-18 Spheri 5, particle size 5 μm (Rainin Instrument, Woburn, MA, U.S.A.). Peak heights were recorded with a Series 5000 Recordall (Fisher Scientific, Pittsburgh, PA, U.S.A.). The mobile phase was filtered using a Millipore vacuum system and cellulose membrane filters, 0.5 μm pore size, and degassed by vacuum before mixing.

Materials

HPLC-grade potassium dihydrogen phosphate and methanol for the mobile phase were purchased from Fisher Scientific. The hemoglobin determination kit, Tris buffer, UMP, deoxythymidine monophosphate (dTMP), deoxyuridine monophosphate (dUMP), deoxycytidine monophosphate (dCMP) and their corresponding nucleosides were purchased from Sigma (St. Louis, MO, U.S.A.). CMP was purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Anhydrous magnesium chloride was obtained from Aldrich (Milwaukee, WI, U.S.A.).

HPLC conditions

The isocratic system utilized an aqueous 0.01 *M* potassium dihydrogen

phosphate solution, pH 4.0. Varying amounts (0—14%) of methanol were added, depending on the retention time of the product (see Table I). The mobile phase was pumped at 1.0 ml/min. The chart speed was set at 0.5 cm/min. The UV detector had a fixed wavelength of 280 nm. The a.u.f.s. (range) was set at 0.016, 0.032, or 0.064 depending on the product peak height. The column was maintained at room temperature (approximately 23° C).

Hemolysate enzyme preparation

Heparinized venous blood was washed with 3 vols. of 0.85% saline followed by centrifugation at 4500 *g* and removal of the supernatant after each wash to yield leucocyte-free red blood cells. The erythrocytes were diluted with cold distilled water to four times the original volume of whole blood to initiate hemolysis, then centrifuged at 14 750 *g* for 3 min. Samples were chilled throughout to minimize enyzme degradation. Hemolysate could be stored frozen up to two weeks, but subsequently lost activity. Data reported here were obtained using hemolysate frozen for one to seven days.

Hemolysate can be prepared using as little as 100 μl of whole blood. Data reported here are based on hemolysates prepared from approximately 10 ml of whole blood. The amount of blood used to prepare hemolysate should be consistent, since this variable may affect the amount of product. A 100-μl aliquot of whole blood yields 250—350 μl hemolysate, sufficient for four to five assays, and permits replicate assays of two isozymes.

Hemoglobin determination

A 20-μl aliquot of hemolysate was analysed for hemoglobin by the cyan-methemoglobin procedure, utilizing Drabkin's reagent [17].

Enzyme assay

The assay for enzyme activity is based on the modification of the procedures reported for UMP [12, 14] as substrate. A micromethod was employed which uses minimum amounts of substrate and hemolysate and optimizes magnesium chloride concentration and incubation time. Substrate was prepared in 0.05 *M* Tris, pH 7.5, to deliver the final substrate concentration in 50 μl. Magnesium chloride in 50 μl of 0.05 *M* Tris, pH 7.5, to yield 4 m*M* final concentration, and 50 μl hemolysate were added to substrate in microcentrifuge tubes. The mixture was incubated for 30 min in a 37° C water bath with gentle agitation. The reaction was halted by placing the microtubes in a boiling water bath for 1.5 min. Blanks were prepared by boiling samples of each hemolysate 1.5 min, then adding substrate and magnesium chloride prior to incubation.

Samples were centrifuged (Fisher Model 235 microcentrifuge) at 22 500 *g* for 4 min. Supernatant fractions were removed and recentrifuged before injection onto the HPLC column. We found, unlike Rocco et al. [12], that reaction mixture frozen at —20° C could be rechromatographed without loss of product for up to six weeks.

Standard preparation

Standard solutions of uridine, cytidine, deoxythymidine, deoxyuridine and

deoxycytidine were prepared in 0.05 *M* Tris buffer, pH 7.5, at concentrations of 2.5, 5.0, 10.0, 20.0 and 40.0 μg/ml. Linear regression equations based on standard injections were used to convert peak heights from μg/ml to μmol nucleoside released after 30 min incubation. International units were calculated as μmol nucleoside per h per g hemoglobin.

RESULTS AND DISCUSSION

Retention times of the pyrimidine monophosphates and their nucleosides eluted at varying concentrations of methanol in 0.01 *M* potassium dihydrogen phosphate, pH 4.0 are presented in Table I. Substrate—product separation after the reaction of P5N with CMP and UMP assays were satisfactory at 2—6% methanol; dCMP and dUMP and their deoxynucleosides were separated at 6—9% methanol and the formation of thymidine from dTMP was best measured at 14% methanol. Rocco et al. [12] employed 10% methanol in 0.01 *M* potassium dihydrogen phosphate, pH 5.1, for the assay of rat erythrocyte UMPase but the chromatographic conditions involved other differences with longer retention times for uridine.

Typical chromatograms of the substrate—product pairs obtained at 2% methanol are shown in Fig. 1A and B. Each nucleotide—nucleoside pair was cleanly separated using the specified conditions of Table I. Supernatants from a series of deoxythymidine-producing reactions were combined. Repeated injections from this combination contained 2.41 ± 0.05 mg/dl deoxythymidine ($\bar{x}$ ± S.E.M., n = 10).

The linearity of standards in Tris buffer is illustrated in Fig. 2. Standards were run daily. The relative variability of deoxythymidine was 4.4%, a typical value. Similar linearity was obtained when standards were added to aliquots of inactivated hemolysate instead of Tris buffer assuring that quantitation of product was complete. The recovery of nucleoside standards added to appropriate reactions after incubation averaged 96% (n = 16).

TABLE I

RETENTION TIMES (min) OF PYRIMIDINE MONOPHOSPHATES AND THEIR NUCLEOSIDES

	Methanol concentration (%) in 0.01 *M* potassium dihydrogen phosphate, pH 4.0					
	0	3	6	9	13	14
CMP	4.5	3.3	2.9	2.6		
UMP	4.6	3.4	3.1	2.7		
dCMP	7.2	4.5	3.4	3.1		
dUMP	7.8	4.7	3.5	3.0		
dTMP	16.0	8.1	5.3	4.0	3.7	3.5
Cytidine	12.0	6.4	4.7	3.3		
Uridine	17.0	9.0	6.1	4.5		
Deoxycytidine	23.0	9.5	6.6	4.7		
Deoxyuridine	28.4	13.2	8.4	5.6		
Deoxythymidine	35	34.1	19.5	11.0	8.2	7.0

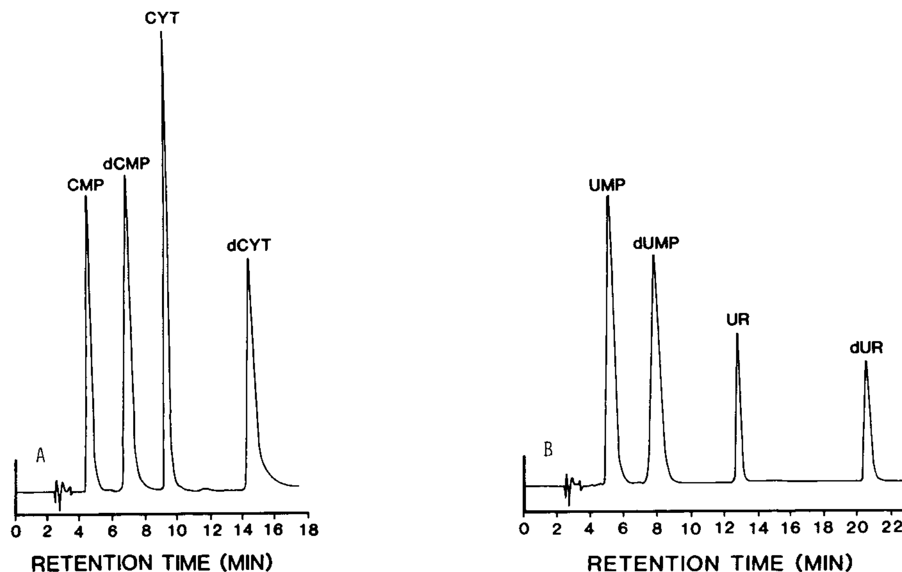

Fig. 1. Separation of the standards for the substrate nucleotides and their nucleoside products by 2% methanol in 0.01 *M* potassium dihydrogen phosphate, pH 4.0. Flow-rate 1.0 ml/min; UV detector wavelength 280 nm; 0.032 a.u.f.s. (A) 0.033 m*M* CMP, 0.033 m*M* dCMP, 0.055 m*M* cytidine (CYT), 0.059 m*M* deoxycytidine (dCYT). (B) 0.06 m*M* UMP, 0.06 m*M* dUMP, 0.04 m*M* uridine (UR), 0.04 m*M* deoxyuridine (dUR).

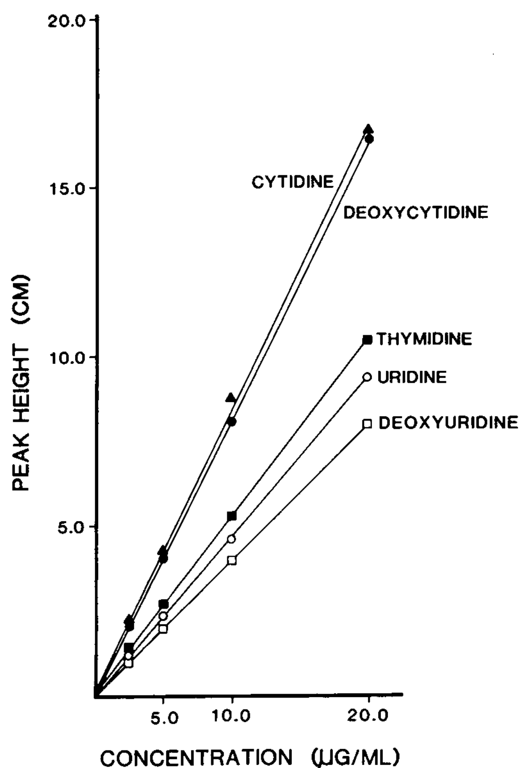

Fig. 2. Peak height (cm) versus concentration (μg/ml) for the nucleosides and deoxynucleosides at 0.032 a.u.f.s.

To investigate the possible interference of extraneous peaks with the enzymatic assay, chromatograms were run of inactivated hemolysate and buffer (Fig. 3A), inactivated hemolysate and substrate (Fig. 3B) and fresh hemolysate plus substrate which permitted product identification (Fig. 3C). These figures are representative of the quantitative product formation after reaction of hemolysate with each of the pyrimidine nucleotides.

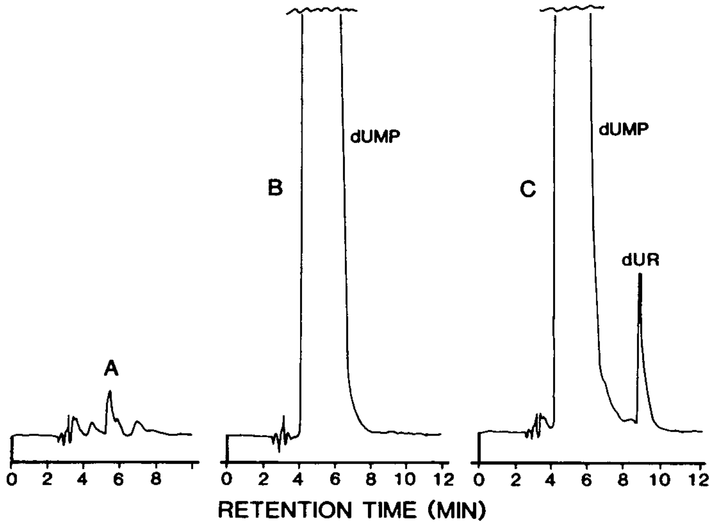

Fig. 3. Chromatographic assay of dP5N. Methanol 6% in 0.01 *M* potassium dihydrogen phosphate, pH 4.0. (A) Erythrocyte hemolysate, boiled, showing baseline peaks. (B) Hemolysate, boiled, and incubated with 2 m*M* dUMP. (C) Hemolysate incubated 30 min with 2 m*M* dUMP showing formation of deoxyuridine (dUR).

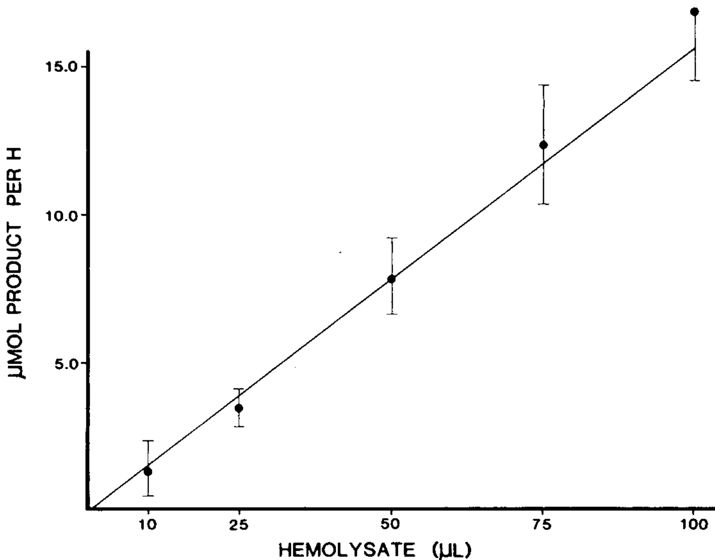

Fig. 4. Enzymatic release of deoxythymidine in µmol/h from 2 m*M* dTMP, pH 4.0, after 30 min incubation, at varying concentrations of hemolysate.

When nucleotide pairs such as CMP and dCMP or UMP and dUMP were incubated with hemolysate, chromatographic separation of the resulting mixture of substrates and products was possible. However, nucleoside yields from simultaneous assays were not equal to yields from the assay of the corresponding individual nucleotides possibly reflecting cross-reactivity of P5N and dP5N with the nucleotides tested. The presence of purines, pyrimidines and nucleosides, especially adenosine, adenine, guanosine, guanine, inosine and cytidine, also inhibits nucleotidase activity [8].

The linearity of enzymatic release of the nucleoside from 2 m*M* dTMP with increasing amounts of hemolysate or hemoglobin is shown in Fig. 4. A 50-μl aliquot, (containing approximately 0.001 g hemoglobin) is on the linear portion of the hemolysate—product relationship.

Fig. 5 illustrates the linearity of deoxythymidine formation per g hemoglobin versus incubation time, employing 50 μl hemolysate with 2 m*M* dTMP. A 30-min incubation produced suitable peak heights for quantitation of all nucleosides.

The release of nucleosides in international units (μmol/h/g hemoglobin) at

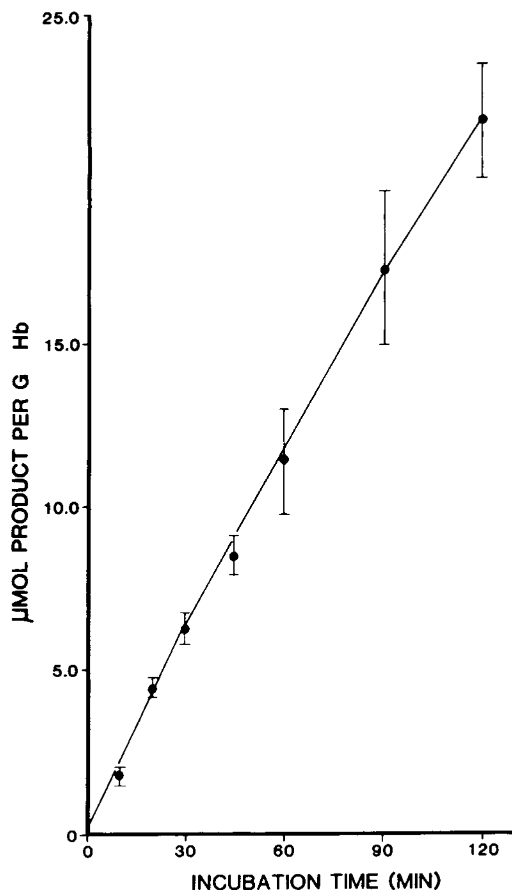

Fig. 5. Enzymatic release of deoxythymidine in μmol/g hemoglobin versus incubation time of 2 m*M* dTMP with 50 μl hemolysate at pH 4.0.

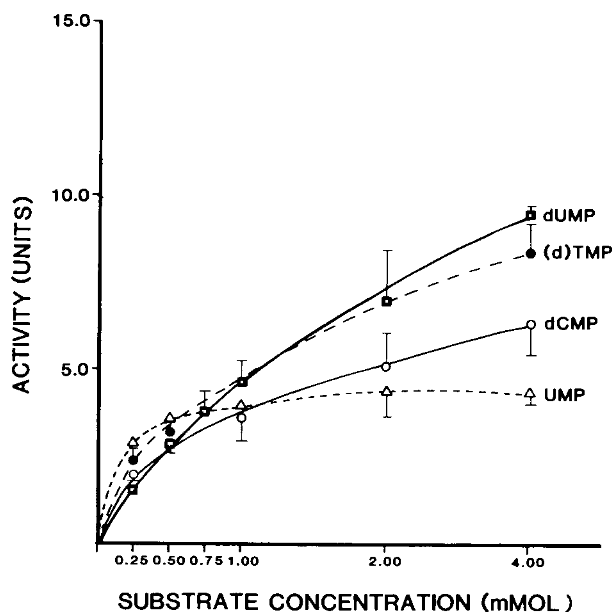

Fig. 6. Nucleoside formation in international units (μmol/h/g hemoglobin) versus concentration of the nucleotide substrates.

varying concentrations of nucleotides is shown in Fig. 6. The reaction appears linear at approximately 0.1—0.5 m*M* concentrations, depending on the nucleotide, with maximum product above 1.0 m*M*. Paglia and Valentine [8], Rocco et al. [12] and Sakai et al. [14] all employed a nucleotide concentration of 2.0—2.3 m*M* while Ericson et al. [13] utilized 0.3 m*M* UMP. Normal erythrocyte enzyme activity in μmol/h/g hemoglobin for 2 m*M* UMP is similar to that of Paglia and Valentine [8] but somewhat lower than that of Sakai et al. [14]. Paglia et al. [4] reported maximal nucleotidase activity for dTMP and dUMP at pH 6.2; activity for dCMP peaked at pH 6.8 but declined more rapidly at pH 7.0 to 8.0 than did activity for UMP and CMP. At pH 7.5, normal erythrocyte nucleotidase activity for all five substrates is the range of 5—10 I.U. An average 9.2 ± 0.8 I.U. ($\bar{x}$ ± S.E.M.) deoxythymidine was produced when ten controls were assayed using 4 m*M* dTMP substrate concentration.

TABLE II

MAGNESIUM CHLORIDE CONCENTRATION AND dP5N ACTIVITY WITH dTMP 1.0 m*M* AS SUBSTRATE

Each value is the mean ± S.D. of six subjects.

$MgCl_2$ (m*M*)	Activity (I.U.)*
0	8.8 ± 0.8
4	12.1 ± 1.0**
8	10.8 ± 1.2
12	9.2 ± 0.8

*International units (I.U.) are μmol/h/g hemoglobin.
**$p < 0.05$ for 4 m*M* magnesium chloride versus zero.

Table II summarizes the effect of varying concentrations of magnesium chloride on the activity of dP5N with 1 m*M* dTMP. The reported assays for P5N and dP5N utilize 0—12 m*M* magnesium chloride [4, 8, 13]. Our data show that magnesium chloride increases the activity of both P5N and dP5N with maximal activity at 4 m*M* magnesium chloride for all five nucleotides tested.

This report of the range of conditions for the HPLC analysis of the nucleoside products from the reaction of human erythrocytes with two pyrimidine nucleotides (CMP, UMP) and three deoxypyrimidine nucleotides (dCMP, dUMP, dTMP) simplifies the assay of both P5N and dP5N. The relative ease of this assay should facilitate biomedical screening for congenital deficiencies of erythrocyte P5N or dP5N, or acquired deficiencies such as seen with increased blood lead concentrations [7]. Standardization of methodology is relevant to investigations of the biological significance of the two or more isozymes of human erythrocyte P5N [4] and to mechanisms of abnormal erythrocyte accumulation of pyrimidine nucleotides and their nucleoside phosphodiesters with enzyme deficiency [18, 19].

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GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC METHOD FOR TRAZODONE AND A DEUTERATED ANALOGUE IN PLASMA

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SUMMARY

A plasma assay method for trazodone and a $^{2}H_{4}$ analogue is described which uses gas chromatography—electron-impact selected-ion monitoring mass spectrometry. Etoperidone is used as an internal standard. The analytes are extracted from basic medium into *n*-butyl chloride, then back extracted into aqueous 0.1 *M* hydrochloric acid. The aqueous layer is made basic and re-extracted with *n*-butyl chloride. The solvent is reduced under nitrogen at 35°C and the residue is redissolved in toluene for gas chromatographic—mass spectrometric

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analysis. The ions monitored are *m/z* 231, 235, and 225 for trazodone, [$^2H_4$]trazodone and etoperidone, respectively. Quantitation is in the range 40—1000 ng/ml with acceptable precision and accuracy. The method is suitable for biopharmaceutical studies.

INTRODUCTION

Trazodone, 2-{3-[4-(3-chlorophenyl)-1-piperazinyl]propyl}-1,2,4-triazolo-[4,3-*a*]pyridin-3(2*H*)-one is widely used for the treatment of major depressive episodes [1]. A procedure was required for the identification and measurement of the drug and its $^2H_4$ analogue in plasma for pharmacokinetic and bioavailability studies. Administration of 50 mg of trazodone in tablet form results in plasma concentrations in the range 40—1000 ng/ml over the following 34 h. A high-performance liquid chromatographic (HPLC) [2] and a gas chromatographic—mass spectrometric (GC—MS) [3] method for quantification of trazodone in plasma have been reported; however, neither method was developed and validated for simultaneous quantification of the unlabeled drug and $^2H_4$ analogue.

A recent review by Vink [4] summarizes the rationale for using GC—MS techniques in biopharmaceutical studies. The patient is administered a solution of stable isotope-labeled analogue simultaneously with the unlabeled drug in the solid dosage form. GC—MS is used to selectively measure the plasma concentrations resulting from the solution and solid dosage forms simultaneously being evaluated. In this way, the power of the study to detect differences between dosage forms is greatly increased over a two-way cross-over design, so that a smaller number of clinical tests are required for the same statistical confidence levels. The increase in power of the test is due primarily to a marked decrease in intra-subject variability [5, 6].

The present paper describes a selected-ion monitoring (SIM) GC—MS method for trazodone, its $^2H_4$ analogue and etoperidone, used as an internal standard. The method covers the concentration range 40—1000 ng/ml and has been validated in two laboratories. It has been successfully used in a bioavailability study which is reported elsewhere [7].

EXPERIMENTAL

Materials

The hydrochlorides of trazodone, [$^2H_4$]trazodone (less than 1% [$^2H_0$]-trazodone content) and etoperidone were provided by Bristol-Myers (Evansville, IN, U.S.A.). [$^2H_4$]Trazodone was prepared by starting with [$^2H_6$]-aminopyridine from KOR (Cambridge, MA, U.S.A.), which was converted to [$^2H_4$]bromopyridine by diazotization and bromination. This was converted to [$^2H_4$]triazolopyridinone with perdeuterated semicarbizide hydrochloride and sulfuric acid in [$^2H_1$]2-(2-ethoxyethoxy)ethanol. This was alkylated with 1-chlorobenzyl-4-chloropropyl piperazine.

Toluene (HPLC grade), *n*-butyl chloride (HPLC grade), methanol (pesticide grade), sodium hydroxide (2.0 *M*) and hydrochloric acid (0.1 *M*) were obtained from Fisher Scientific (Pittsburg, PA, U.S.A.) and were used without further purification.

Standard solutions (1 mg/ml of free base) of trazodone, [$^2H_4$]trazodone, and etoperidone were prepared by dissolving the hydrochloride salts in methanol. These standards were diluted to prepare methanolic standards to be used for the preparation of calibration standards in plasma. Calibration standards at concentrations of 1000, 800, and 400 ng/ml were prepared by adding 100 μl of a trazodone—[$^2H_4$]trazodone standard to control plasma in 50-ml volumetric flasks. Of each of these standards 5 ml were added to 50-ml volumetric flasks and diluted to volume with control plasma to give calibration standards at 100, 80, and 40 ng/ml. Control samples at 600 and 60 ng/ml were prepared in the same manner.

Instrumentation

All analyses were performed on a Finnigan MAT 4023 or a 4510 gas chromatograph—mass spectrometer—data system instrument (San Jose, CA, U.S.A.) operated in the electron-impact (EI) mode. The mass spectrometer was operated at 70 eV electron energy and a source temperature of 190°C. Finnigan MAT 4.0 software was used with the INCOS data system to monitor the selected ions, quantitate peak areas, generate calibration lines and calculate concentration values. The mass spectra in Figs. 1—3 were obtained by injecting about 100 ng of each compound into the GC—MS system and scanning 100—500 a.m.u. in 2 sec. Prior to obtaining these spectra, proper tuning of the mass spectrometer was assured by the method of Budde and Eichelberger [8] using decafluorotriphenyl phosphine. For sample analysis, the following selected ions (m/z) were repetitively scanned over a unit mass window for 0.21 sec each: trazodone, m/z 231; [$^2H_4$]trazodone, m/z 235; and etoperidone, m/z 225.

The chromatographic column was a 4 m × 0.25 mm I.D. DB-1 fused-silica capillary column (0.25 μm film thickness) from J & W Scientific (Rancho Cordova, CA, U.S.A.). The helium carrier gas linear velocity was 200 cm/sec. The split and septum sweep were set at 30 and 3 ml/min, respectively, and were suspended for the first minute of the run for splitless injection. The column oven temperature was 170°C for 1 min, then it was increased at 15°C/min to a final temperature of 255°C. The injector temperature was set at 250°C, and the separator oven temperature at 255°C.

Sample extraction procedure

A 1-ml volume of plasma sample, control, or standard was pipetted into a silanized glass culture tube (125 × 16 mm). A 20-μl aliquot of the internal standard (etoperidone, 20 ng/μl) was added to each sample, standard, and control sample. These were mixed briefly on a vortical mixer and stood for 5 min. The sample was made basic by adding 0.5 ml of 2 *M* sodium hydroxide and brief vortical mixing.

The sample was extracted by adding 9 ml of *n*-butyl chloride, capping the tube with a PTFE-lined cap, and vortical mixing for 10—15 sec. The sample was centrifuged for 10 min at 2000 *g*. The organic layer was transferred to a second culture tube containing 2 ml of 0.1 *M* hydrochloric acid. The tube was capped tightly, vortically mixed for 20 sec, centrifuged, and the organic layer was removed. The aqueous layer was made basic with 0.5 ml of 2 *M* sodium hydroxide, and 9 ml of *n*-butyl chloride were added. The sample was vortically

mixed for 10—15 sec and centrifuged for 10 min at 2000 *g*. The organic layer was transferred to a 15-ml silanized conical centrifuge tube, and the butyl chloride was evaporated at 35°C under a stream of nitrogen. The residue was dissolved in 1 ml of methanol and transferred to a 3-ml Reacti-Vial. The tube was rinsed with an additional 1 ml of methanol and the rinse was added to the Reacti-Vial. The methanol was evaporated under nitrogen. The residue was dissolved in 200 µl of methanol and briefly vortically mixed. The methanol was evaporated under nitrogen, and the residue was dissolved in 20 µl of toluene. Then 2 µl were injected into the GC—MS system for analysis.

Quality control

Quantification was performed by comparing samples to standards prepared from control plasma spiked over the range 40—600 ng/ml and coextracted with samples.

Inter-assay variability was determined by preparing and analyzing five spiked control plasma samples at two concentration levels on five separate days. Intra-assay variability was evaluated by preparing and analyzing five additional identical samples at the two concentration levels on one of these test days for a total of ten replicates for comparison. Blinded control samples were spiked by one person and blindly analyzed by another. They consisted of blanks plus spikes over a range of 40—600 ng/ml and were analyzed on one day. Throughout the bioavailability study [7], which was conducted using this method, quality control (QC) samples were regularly analyzed. These QC samples consisted of spiked control plasma at 60 and 600 ng/ml analyzed at the same time as a set of samples. Nine samples at each of two concentration levels were analyzed.

Clinical pharmacokinetic studies

In order to evaluate the bioequivalence of trazodone and [$^{2}H_{4}$]trazodone, six normal healthy male volunteers were given an oral solution containing 50 mg each of trazodone and [$^{2}H_{4}$]trazodone. Blood samples were collected over a 34-h period and stored at −4°C until analyzed. A detailed description of a bioavailability study performed by coadministering trazodone (in liquid or either of two solid forms) and [$^{2}H_{4}$]trazodone (liquid) is the subject of a separate report [7].

RESULTS AND DISCUSSION

The low resolution 70-eV EI mass spectra of trazodone, [$^{2}H_{4}$] trazodone and etoperidone are presented in Figs. 1—3, respectively. Assignment of the identity of the fragment ions is based on comparison with analogues of the compounds and is not intended to suggest an exhaustive study of fragmentation mechanisms. In some cases ions requiring rearrangements for formation are drawn without special notice for simplicity.

SIM of the base peak of the analytes is not appropriate because trazodone produces a strong response at *m/z* 209 which would interfere with [$^{2}H_{4}$]-trazodone. The *m/z* 231 ion for trazodone and the corresponding *m/z* 235 ion for [$^{2}H_{4}$]trazodone were found to produce sufficient response for the

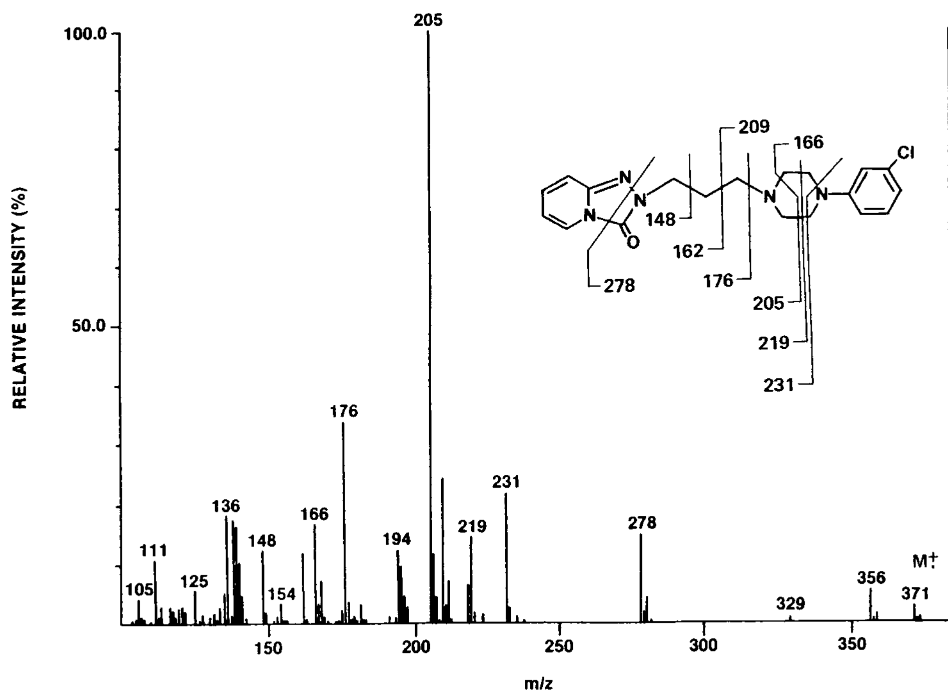

Fig. 1. Electron-impact mass spectrum of trazodone.

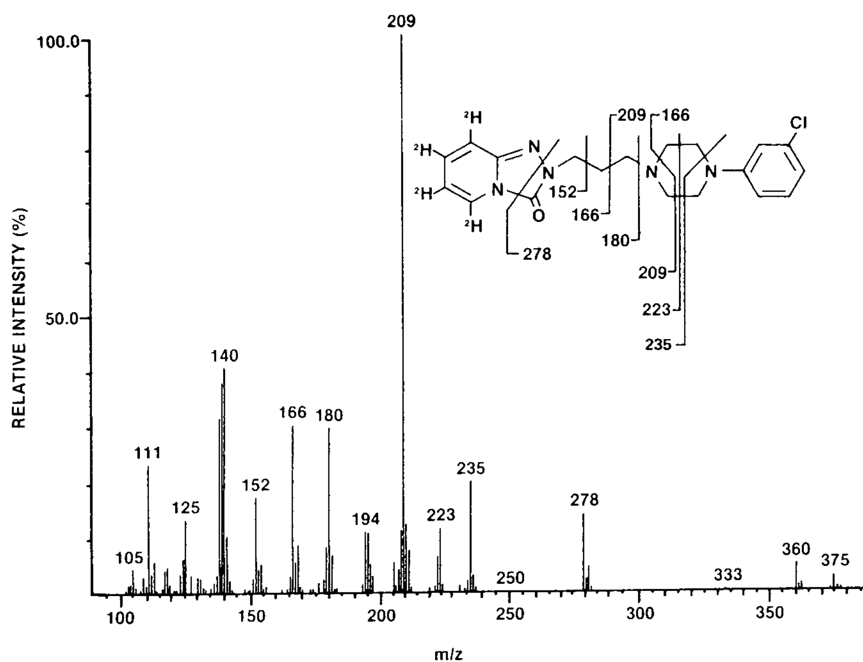

Fig. 2. Electron-impact mass spectrum of [$^{2}H_{4}$]trazodone.

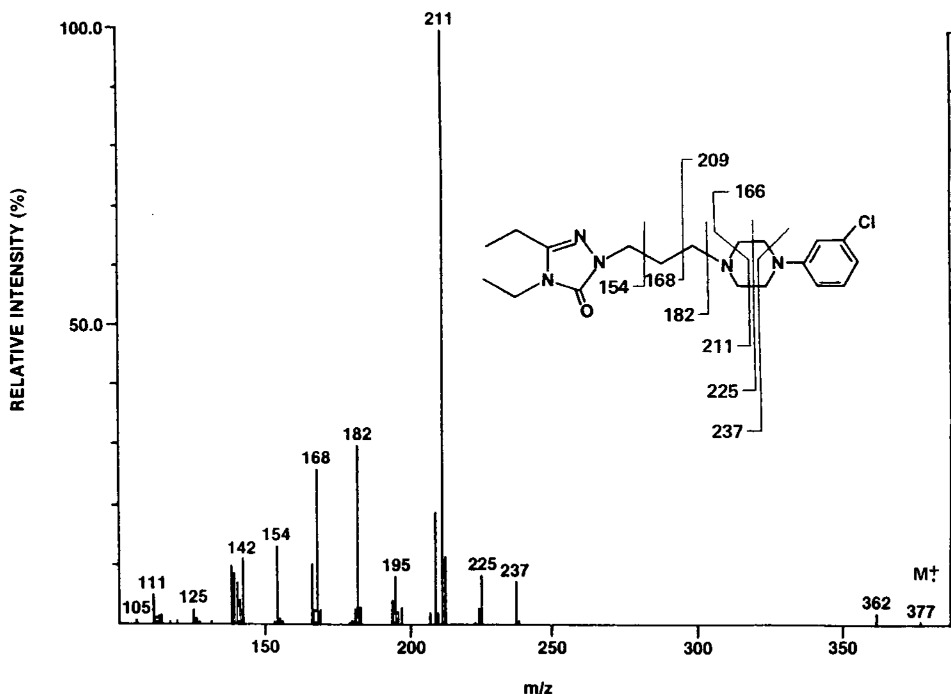

Fig. 3. Electron-impact mass spectrum of etoperidone.

sensitivity requirements of this method. A relatively low intensity ion (*m/z* 225) was also chosen for monitoring etoperidone. The ion resulting from the same fragmentation as chosen for trazodone would have been *m/z* 237 but sample interference was observed at *m/z* 237. Good accuracy and precision were consistently obtained using *m/z* 225.

When fragment ions are chosen for monitoring in an assay there is a possibility of interference from metabolites of the drug which retain the portion of the molecule that produces the chosen fragment ion. In the case of trazodone the known metabolites were either excluded by the selectivity of the extraction procedure or were easily separated from trazodone by the capillary GC. The major metabolites of trazodone in humans have been reported [9]. These were found to be: (1) the piperazinyl-1 oxide, (2) the 7,8-dihydroxy adduct of the triazolopyridinone, and (3) the 4-hydroxyphenyl compound. Only the third metabolite retains the portion of the trazodone molecule which produces the fragment ion chosen for monitoring. This compound separates from trazodone under the capillary GC conditions used for this method (see Fig. 4).

Fig. 5 depicts typical chromatograms of a plasma extract blank to which etoperidone only has been added prior to extraction and a 40 ng/ml plasma standard extract. A non-interfering matrix background 231 a.m.u. peak is produced at 3 min. A non-interfering 235 a.m.u. peak is produced for etoperidone. No endogenous plasma interferences were observed. The GC column was conditioned at the start of each day with a few injections of standard containing all three analytes to eliminate any GC system absorption effects.

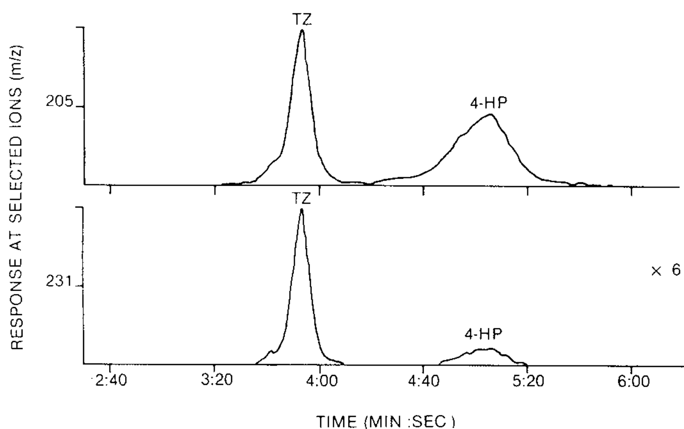

Fig. 4. Chromatographic separation of possible interfering metabolite. Capillary GC conditions were the same as described under *Instrumentation*. Peaks: TZ = trazodone; 4-HP = 3-chloro-4-hydroxyphenyl metabolite (see text).

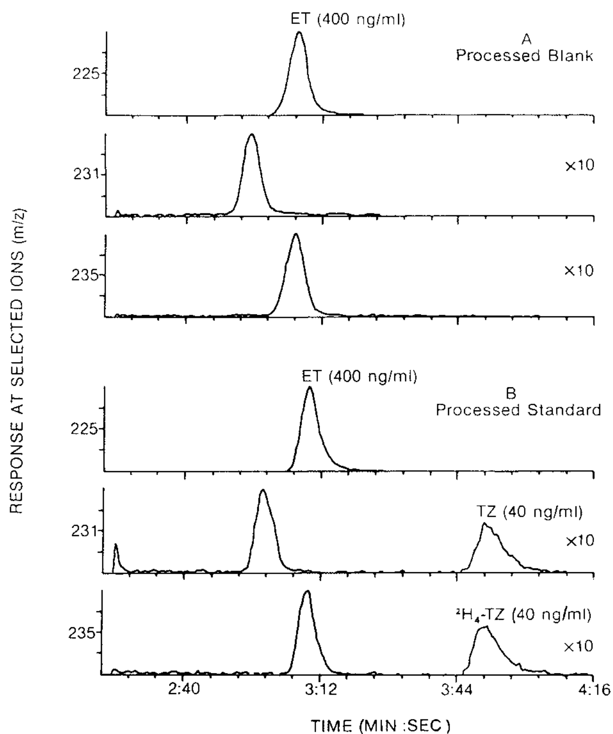

Fig. 5. Selected-ion chromatograms of plasma extracts. (A) Processed blank, spiked with 400 ng/ml etoperidone only; (B) processed standard, spiked with 400 ng/ml etoperidone and 40 ng/ml trazodone and [$^2H_4$]trazodone. Peaks: ET = etoperidone, internal standard; TZ = trazodone; $^2H_4$-TZ = [$^2H_4$]trazodone.

Calibration lines were generated and used by the INCOS data system. An example of the typical linearity of the calibration is presented in Fig. 6. The lower working range of the method is considered to be 40 ng/ml due to the

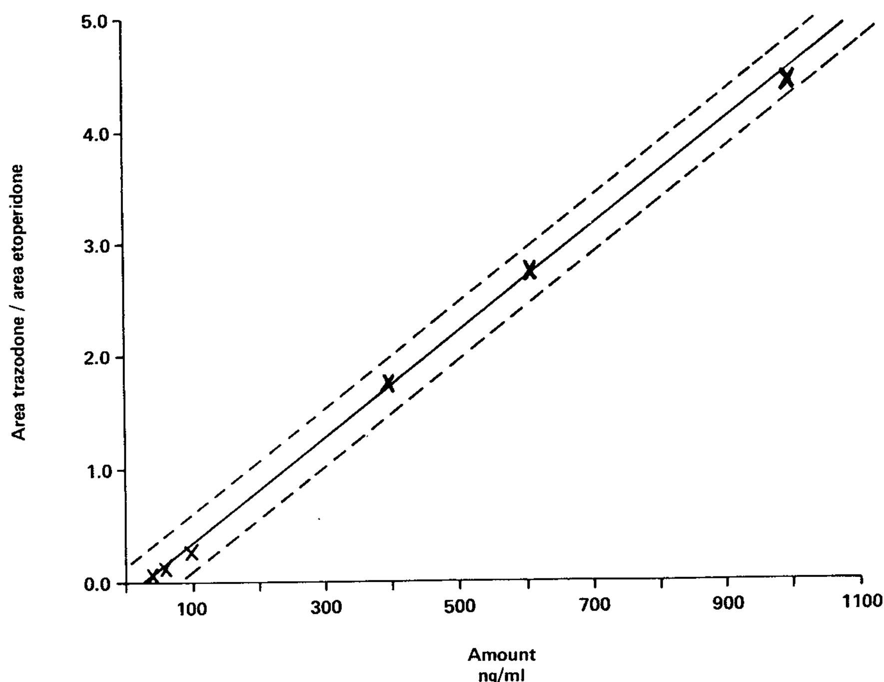

Fig. 6. Trazodone calibration line for a typical day (——); three standard deviations (- - -). Each point represents the mean of two measurements. Correlation coefficient = 0.998.

TABLE I

ACCURACY AND PRECISION OF THE ASSAY

See text for a description of the tests.

Test	Trazodone				$[^2H_4]$Trazodone			
	Lab	Added (ng/ml)	Mean observed (ng/ml)	C.V. (%)	Lab	Added (ng/ml)	Mean observed (ng/ml)	C.V. (%)
Inter-assay	A*	60	60	5.0	A*	60	61	4.9
Inter-assay	A**	600	604	3.1	A**	600	606	4.5
Inter-assay	B	60	66	6.0	B	60	63	4.8
Inter-assay	B	600	594	5.2	B	600	591	5.1
Intra-assay	B	60	59	5.1	B	60	59	4.2
Intra-assay	B	600	616	3.4	B	600	619	3.2
Blinded***	A	40—600	—	16	A	40—600	—	17
Blinded***	B	40—600	—	8.0	B	40—600	—	9.0
QC samples	B	60	63	7.9	B	60	64	9.4
QC samples	B	600	600	4.0	B	600	601	4.7

*One day had three samples.
**Two days had four samples.
***Lab A analyzed twelve samples; Lab B analyzed twenty samples.

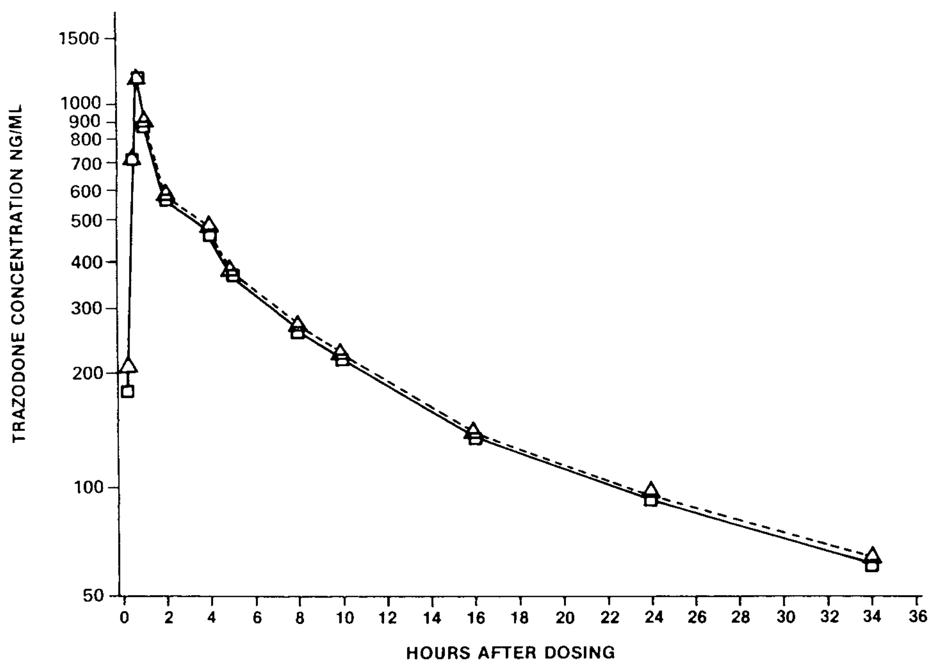

Fig. 7. Plasma concentration versus time profile for trazodone (□), and [$^2H_4$]trazodone (△) orally coadministered in solution to a healthy male volunteer.

low-intensity ions selected. Greater sensitivity could be obtained for trazodone by monitoring its base peak if [$^2H_4$]trazodone was not present in the sample.

Extraction recovery studies over the range 10—4000 ng/ml indicated analyte recoveries ranging from 79% to 96%.

The precision and accuracy of the method was demonstrated in two laboratories. Table I shows the results of this testing. The accuracy (relative difference between observed mean and added concentration) of the method over the range 60—600 ng/ml was 0—10% and the precision (coefficient of variation, C.V.) was 3—17%. The sum of the data in Table I suggests that the method produces acceptable precision and accuracy.

In a test of the bioequivalence of trazodone and [$^2H_4$]trazodone when coadministered as an oral solution, healthy male volunteers were administered a dose of 50 mg and plasma samples were analyzed by the method. Fig. 7 demonstrates for one typical volunteer the equivalent fate of trazodone and [$^2H_4$]trazodone in human plasma. No kinetic isotope effect was observed.

This method has been shown in two laboratories to be accurate and precise in measuring trazodone and [$^2H_4$]trazodone in the range of concentrations resulting from a normal clinical dose. Its utility has been successfully demonstrated in a recent bioavailability study [7]. A preliminary report of this method was previously presented [10].

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CHROMBIO. 2525

DETERMINATION OF ACETYLTRIMOPROSTIL AND ITS METABOLITE TRIMOPROSTIL IN HUMAN OR DOG PLASMA BY GAS CHROMATOGRAPHY—NEGATIVE-ION CHEMICAL IONIZATION MASS SPECTROMETRY

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SUMMARY

A sensitive and specific procedure is described for the determination of the antisecretory prostaglandin acetyltrimoprostil and its metabolite trimoprostil in human or dog plasma using gas chromatography—negative-ion chemical ionization mass spectrometry (GC—NICI-MS). Trideuterated analogues of both compounds are added to plasma as the internal standards. The plasma is extracted at pH 7.3 with benzene—dichloromethane (9:1), and the residue of the organic extract is reacted at room temperature with pentafluorobenzyl bromide in the presence of 18-crown-6-ether and potassium acetate. The derivatives are reconstituted in heptane, and appropriate aliquots are analyzed by GC—NICI-MS with selected-ion monitoring of the intense $(M - C_6F_5CH_2)^-$ fragment ions of acetyltrimoprostil (*m/z* 419), trimoprostil (*m/z* 377), and their respective trideuterated analogues (*m/z* 422 and *m/z* 380, respectively). Quantitation of an experimental plasma sample is based on a comparison of the *m/z* 419 versus *m/z* 422 and *m/z* 377 versus *m/z* 380 ion ratios in each sample to that obtained from the analysis of drug-free plasma fortified with various amounts of both protio compounds, and a fixed amount of each trideuterated internal standard. The limit of quantitation of the assay for human plasma is 0.2 ng ml^{-1} with mean relative standard deviations at this concentration of 15.5% and 9.7% for acetyltrimoprostil and trimoprostil, respectively.

INTRODUCTION

Acetyltrimoprostil (I) is currently being studied for use as a long-acting, orally active inhibitor of gastric acid secretion [1]. Trimoprostil (II), a known metabolite of I, has well established antisecretory activity in dog [2] and in man [3, 4].

The published [5] gas chromatographic—negative-ion chemical ionization

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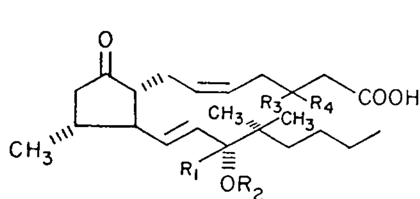

Compound	R_1	R_2	R_3	R_4
I	H	$COCH_3$	H	H
[$^2H_3$]I	^2H	$COCH_3$	^2H	^2H
II	H	H	H	H
[$^2H_3$]II	^2H	H	^2H	^2H

Fig. 1. Chemical structures of acetyltrimoprostil (I), trimoprostil (II) and their trideuterated analogues ([$^2H_3$]I and [$^2H_3$]II, respectively).

mass spectrometric (GC—NICI-MS) assay for II could not be modified to determine I in human plasma because of interferences in the selected-ion current profiles from endogenous compounds. This paper reports a GC—NICI-MS procedure to determine both I and II in the same sample. This assay as the previously reported assay [5] also features the use of both the pentafluorobenzyl (PFB) derivatives of I and II and trideuterated analogues of each compound ($^2H_3$[I] and [$^2H_3$]II, respectively). Unlike the previously reported assay [5], this assay features a packed GC column instead of a support-coated open tubular capillary column, determines II without silylating the hydroxy group, extracts I and II at a neutral rather than an acidic pH, and derivatizes the analytes under conditions where endogenous phenols would be expected to be unreactive. The assay was used to determine I and II in human and dog plasma following oral administration of I.

EXPERIMENTAL

Gas chromatography

A Finnigan (Sunnyvale, CA, U.S.A.) Model 9500 gas chromatograph was equipped with a 1.5 m × 2 mm I.D. U-shaped borosilicate glass column packed with 3% SP-2250 on 80—100 mesh Supelcoport from Supelco (Bellefonte, PA, U.S.A.). The column was conditioned at 320°C overnight with nitrogen as the GC carrier gas. For the assay, methane (2.4 kg cm^{-2}) was used as the GC carrier gas. The injector, column, interface oven and transfer line were operated at 320°C, 315°C, 240°C and 240°C, respectively. The retention times of derivatized I and II under these conditions were 165 and 175 sec, respectively. Prior to use, the GC column was conditioned with several injections of both Silyl-8 (Pierce, Rockford, IL, U.S.A.) and the reconstituted residue from an ethyl acetate extract of drug-free plasma.

Mass spectrometer

The ion source parameters of a Finnigan Model 3200 quadrupole mass spectrometer were set to give the maximum response consistent with reasonable ion peak shape and unit resolution. The modifications to the instrument to permit the detection of negative ions have been described [6]. The continuous-dynode electron multiplier was set to —2.0 kV, and the conversion dynode was set to +2.9 kV. Methane (Liquid Carbonic, 99%, Harrison, NJ, U.S.A.) at an ion source pressure of 68 Pa was used as NICI reagent gas.

Peak monitor

A Finnigan Promim® with a Rikadenki four-channel recorder was used to set the mass spectrometer to monitor *m/z* 419 (I), *m/z* 422 ([$^2H_3$]I), *m/z* 377 (II), and *m/z* 380 ($^2H_3$[II]). All channels were operated at a gain of 10^{-8} A/V, 100-msec dwell time and a filter setting of 0.5 Hz.

Glassware

Culture tubes (Pyrex 9825) of 16 ml provided with Teflon®-lined screw caps were used for plasma extraction. Conical centrifuge tubes (Pyrex 8061) of 5 ml were used for the evaporation of the organic extract. All the tubes were purchased from SGA Scientific (Bloomfield, NJ, U.S.A.). Prior to use, the glassware was treated with Siliclad® (Clay Adams, Parsippany, NJ, U.S.A.) and rinsed with methanol and dichloromethane.

Solvent evaporator

Solvents were removed at 45°C using a nitrogen evaporator (N-Evap®, Organomation Assoc., South Berlin, MA, U.S.A.).

Shaker

Extractions were carried out by shaking (60 strokes min^{-1}) on a variable-speed reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.).

Centrifuge

Centrifugation was carried out on a Damon/IEC Model CRU-5000 refrigerated centrifuge (Needham Heights, MA, U.S.A.) operated at 1600 *g* and 10°C.

Chemicals

Methanol, benzene, methylene chloride and heptane were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). PFB bromide (PFB-Br) and 18-crown-6-ether were obtained from Aldrich (Milwaukee, WI, U.S.A.). Potassium acetate, potassium phosphate (KH_2PO_4) and sodium hydroxide were purchased from Mallinckrodt (St. Louis, MO, U.S.A.), Baker (Phillipsburg, NJ, U.S.A.), and Fisher Scientific (Pittsburgh, PA, U.S.A.), respectively. Compounds I, II, [$^2H_3$]I and [$^2H_3$]II were obtained from Dr. G. Holland, Chemical Research Department, Hoffmann-La Roche (Nutley, NJ, U.S.A.).

Solutions

Separate stock solutions containing 1.00 mg ml^{-1} of compounds I, [$^2H_3$]I, [II] or [$^2H_3$]II were prepared in methanol. An aliquot of each stock solution was diluted with methanol to give a working solution containing 400 ng ml^{-1}. Spiking solutions of I and II were prepared by diluting the appropriate working solutions with methanol to give solutions containing either 0.2, 0.5, 4, 10, 20 or 50 ng of I and II per 50 μl. Spiking solutions containing either 2 ng or 5 ng of both trideuterated internal standards per 50 μl were prepared in methanol from their respective working solutions.

1 M Phosphate buffer (pH 7.3). Monobasic potassium phosphate (136 g)

was disolved in 1 l of distilled water and the solution was adjusted to pH 7.3 with 5 *M* sodium hydroxide.

Benzene—methylene chloride (9:1) solution. Methylene chloride (100 ml) was diluted with 900 ml of benzene.

0.5% PFB-Br solution. A 50-μl volume of neat PFB-Br was added to 10 ml of benzene containing 1.5 mg ml^{-1} of 18-crown-6-ether, and the mixture was rapidly mixed by vortexing.

Clinical samples

Four normal healthy subjects (mean weight ± S.D. = 75 ± 15 kg) were fasted overnight prior to receiving a single oral dose of 9 mg of I. Blood samples (8 ml) were drawn into a glass vacutainer (Becton-Dickinson No. 6527) at 0, 0.25, 0.5, 0.75, 1, 2, 3, 4, 5, 6, 8, 12, 16 and 24 h post-dose. Plasma, isolated after centrifugation, was stored at —20°C.

Dog samples

Five male beagle dogs, with an average body weight of 10 kg, received a single oral dose of 0.16 mg kg^{-1} I. Blood was drawn at 0, 0.5, 1, 2, 4, 6, and 24 h post-dose. The plasma, isolated after centrifugation, was stored at —20°C.

Calibration curve samples

Human plasma. Drug-free human plasma (1 ml) was added to culture tubes containing 20, 10, 4, 0.5, 0.2 or 0 ng of compound I and II, and 2 ng of each trideuterated internal standard. Samples were prepared in duplicate.

Dog plasma. Drug-free dog plasma (1 ml) was added to culture tubes containing 50, 20, 10, 4, 0.5 or 0 ng of compound I and II, and 5 ng of each internal standard. Samples were prepared in duplicate.

Experimental samples

Aliquots (1 ml) of experimental plasma were added to culture tubes containing either 2 ng (human plasma) or 5 ng (dog plasma) of each trideuterated analogue.

Extraction

To each plasma sample was added 1 ml of 1.0 *M* phosphate buffer, pH 7.3. The mixture was rapidly mixed by vortexing, and then was extracted for 20 min with 6 ml of benzene—methylene chloride (9:1). The mixture was centrifuged for 10 min, 5 ml of each extract was transferred to a 5-ml centrifuge tube, and the organic solvent was evaporated to dryness at 45°C under a gentle stream of nitrogen.

Derivatization

The residue was dissolved in 60 μl of 0.5% PFB-Br followed by a few crystals of potassium acetate. The tubes were allowed to stand at room temperature for 60 min, before the benzene and excess PFB-Br were evaporated under nitrogen at room temperature. The residues were dissolved in 50 μl of heptane.

GC—NICI-MS analysis

Aliquots (2—4 μl) from each derivatized extract were injected onto the GC

column. Approximately 30 sec after an injection, the GC divert valve was closed, and 15 sec later the electron filament was turned on.

Calculations

The peak heights in the selected-ion current profile were measured with a ruler, and the ion ratios for *m/z* 419 versus *m/z* 422 and *m/z* 377 versus 380 were calculated. The ion ratio versus concentration data for both I and II were analyzed by weighted ($1/y$) linear regression. Concentrations of both I and [II] in the experimental samples were calculated using the equation: x (ng) = $(R - b)/m$, where R is the experimental *m/z* ion ratio, and b (intercept) and m (slope) were constants generated by the linear regression analysis of the calibration curve data.

RESULTS AND DISCUSSION

The NICI mass spectra of the PFB derivative of I, $[^2H_3]$I, II and $[^2H_3]$II show no molecular ions. Cleavage of the PFB—oxygen bond of the ester function produces an intense fragment ion at *m/z* 419 (approximately 44% of the base peak ion at *m/z* 359) in the mass spectrum of I, and at *m/z* 377 (base peak) in the mass spectrum of II. An additional fragment ion at *m/z* 359 in the

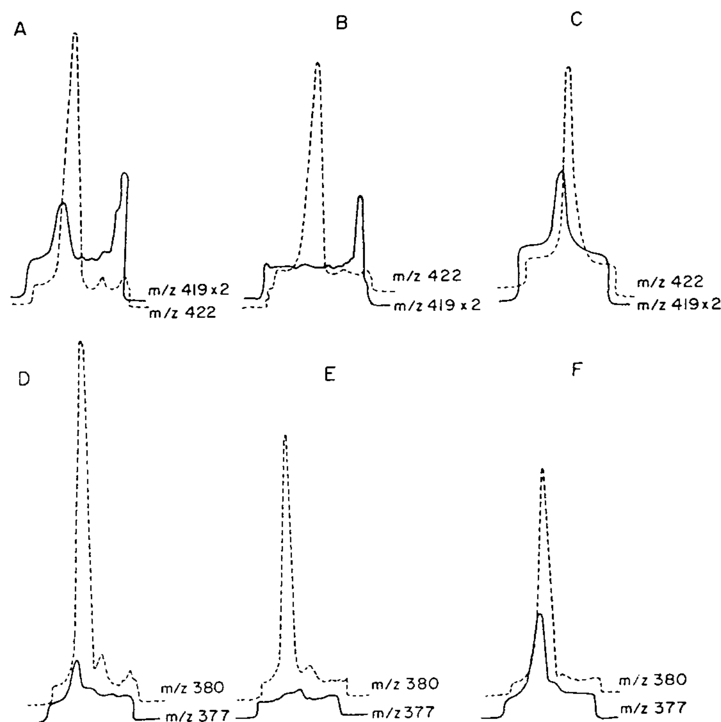

Fig. 2. Selected-ion current profiles from the analysis of either 1 ml of plasma from a dog at either 6 h (C and F) or 0 h (B and E) following an oral dose of 0.16 mg kg^{-1} I, or 1 ml of drug-free plasma fortified with 0.5 ng of both I and II (A and D). All samples were fortified with 5 ng of each trideuterated standard. Concentrations of I and II in the 6-h sample were 1.1 and 0.97 ng ml^{-1}, respectively.

TABLE I

INTER-ASSAY STATISTICS FOR THE ANALYSIS OF I AND II IN HUMAN PLASMA FROM A CONSIDERATION OF THE CALIBRATION CURVE DATA

n = 14 in all cases.

Amount added (ng/ml)	Compound I			Compound II		
	Found (ng/ml)	± S.D.	R.S.D.* (%)	Found (ng/ml)	± S.D.	R.S.D. (%)
0.2	0.18	0.03	15.50	0.18	0.02	9.68
0.5	0.48	0.03	6.35	0.51	0.02	4.81
4.0	4.24	0.17	3.91	4.29	0.33	7.71
10.0	10.41	0.26	2.53	10.42	0.35	3.39
20.0	19.44	0.38	1.95	19.39	0.44	2.27
		Mean =	6.05		Mean =	5.56

*R.S.D. = relative standard deviation.

TABLE II

INTRA-ASSAY STATISTICS FOR THE ANALYSIS OF I AND II IN HUMAN PLASMA FROM A CONSIDERATION OF THE CALIBRATION CURVE DATA

Values are the ratios obtained by dividing the first ion ratio of the duplicate pair by the second ion ratio in the pair. In all cases n = 14.

Calibration curve	0.2 ng/ml	0.5 ng/ml	4.0 ng/ml	10.0 ng/ml	20.0 ng/ml	
Compound I						
1	1.00	0.97	1.03	1.92	1.02	
2	0.94	0.97	1.02	1.01	1.01	
3	0.92	1.00	1.04	1.04	1.01	
4	1.09	1.03	1.05	0.97	1.03	
5	0.75	1.00	0.96	1.02	0.98	
6	0.80	0.93	0.96	1.03	0.98	
7	1.0	1.04	0.94	1.03	1.04	
Mean	0.93	0.99	1.00	1.00	1.02	
S.D.	0.12	0.04	0.05	0.04	0.03	
R.S.D.* (%)	13	4	5	4	3	Mean = 5.8
Compound II						
1	1.20	1.00	0.90	1.10	1.02	
2	1.00	1.10	1.05	0.96	1.05	
3	0.93	1.00	1.00	0.93	1.02	
4	1.00	0.91	0.89	0.95	1.06	
5	0.78	0.91	0.92	1.03	1.03	
6	1.00	1.00	1.04	1.02	1.01	
7	1.00	0.90	0.95	1.01	1.00	
Mean	0.98	0.97	0.96	1.00	1.03	
S.D.	0.12	0.07	0.07	0.06	0.02	
R.S.D. (%)	13	7.4	7.3	6	2	Mean = 7.1

*R.S.D. = relative standard deviation.

spectra of both I and II corresponds to the loss of the elements of acetic acid and water from the [M — $C_6F_5CH_2)^-$ ions of I and II, respectively. The spectra of the trideuterated analogues used in the assay as internal standards are identical to those of the protio compounds, except that the masses are shifted by the appropriate number of daltons.

Fig. 2 shows typical selected-ion current profiles from the analysis of either 1 ml of plasma from a dog at either 6 h (C and F) or 0 h (B and E) following an oral dose of 0.16 mg kg^{-1} I, or 1 ml of drug-free plasma fortified with 0.5 ng of I and II (A and D). All samples were fortified with 5 ng each of $[^2H_3]$I and $[^2H_3]$II. Similar profiles were obtained from the analysis of plasma samples from humans who had received a single oral dose of 9 mg of I. The limit of quantitation of the assay for human plasma samples was 0.2 ng ml^{-1} using 2 ng of each deuterated internal standard. For dog plasma samples the limit of quantitation was 0.5 ng ml^{-1} using 5 ng of each deuterated internal standard. An injection of approximately 4 pg of either derivatized I or II gave a single-ion current profile with a signal-to-noise ratio of approximately 5:1.

Much simpler selected-ion current profiles were observed if the silylation reaction used in the previous assay [5] for II was omitted. In this regard, it was observed that prostaglandins and related analogues not having a gem-dimethyl group adjacent to the hydroxy groups could not be analyzed by GC without first protecting the hydroxy groups. Other modifications increasing assay specificity were the use of the less basic potassium acetate, instead of diisopropylethylamine, to catalyze the esterification reaction, and the extraction of the plasma at pH 7.3 instead of pH 3.0. The former modification

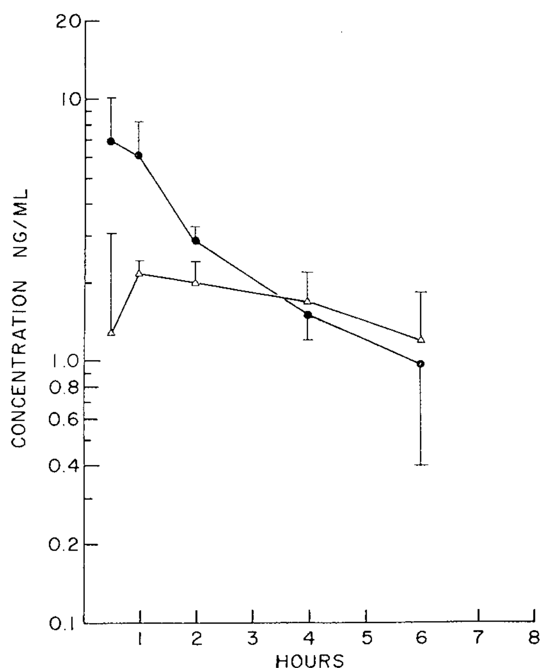

Fig. 3. Mean plasma concentrations (± S.D.) of I (●) and II (△) in five male dogs following an oral dose of 0.16 mg kg^{-1} I.

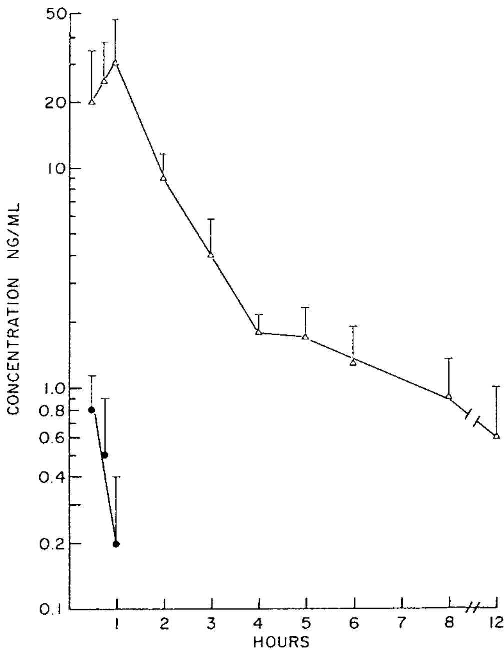

Fig. 4. Mean plasma concentrations (± S.D.) of I (●) and II (△) in four humans (mean weight ± S.D. = 75 ± 15 kg) who received a 9-mg dose of I.

prevents derivatization of endogeneous phenols, while the latter modification decreases the extraction of many endogeneous organic acids.

Inter- and intra-assay precision from a consideration of calibration curve data prepared from human plasma are given in Tables I and II, respectively. The overall inter-assay precision is approximately 6%, while the precision at the limit of quantitation (0.2 ng ml^{-1}) is 15%. Results for dog plasma are similar except the limit of quantitation is 0.5 ng ml^{-1}.

Mean concentrations of I and II in human and dog plasma following oral administration of either 0.16 mg kg^{-1} I to dogs or approximately 0.12 mg kg^{-1} I to man are given in Figs. 3 and 4, respectively. In man, the dose results in relatively high concentrations of II with respect to that of I (which could only be determined for 1 h post-dose). In the dog, the concentrations of I and II are nearly equal and could be determined for up to 6 h postdose.

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CHROMBIO. 2517

IMPROVED GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC ASSAY FOR HALOPERIDOL UTILIZING AMMONIA CHEMICAL IONIZATION AND SELECTED-ION MONITORING

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SUMMARY

An improved method for the analysis of haloperidol in human serum, utilizing gas chromatography—ammonia chemical ionization mass spectrometry is described. A tetradeutero analogue of haloperidol is utilized as the internal standard, while a second drug, thioridazine, is added as a priming compound. The characteristic high sensitivity and selectivity of selected-ion monitoring combined with the added accuracy provided by incorporation of a labeled internal standard provide a reliable method for the quantitation of low levels of haloperidol.

INTRODUCTION

Haloperidol, a neuroleptic butyrophenone, is widely employed in the treatment of neuropsychiatric disorders. However, the relationship between daily dose and steady-state plasma concentration in patients receiving the drug is poor, indicating a need for monitoring the plasma levels if a relationship between drug plasma concentration and pharmacological effect can be found. Several groups of investigators have looked at the relationship between haloperidol plasma or serum concentration and therapeutic outcome in neuropsychiatric patients [1—5]. Studies reporting a positive relationship show large inter-patient variation in the optimal plasma level range [1—3]; other studies were unable to demonstrate a relationship at all [4, 5].

Huntington's disease is a chronic degenerative disease of the central nervous system. Primary treatment consists of the use of dopamine receptor site antagonists, primarily the phenothiazine derivatives and butyrophenones, to pharmacologically alter neurotransmitter inbalances [6]. The most widely

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employed drug of this group in the United States is haloperidol [7]. While patient response is highly variable, the reltionship between clinical response and plasma haloperidol concentration in these patients has not yet been examined.

There are many possible reasons for differences in the relationship between haloperidol plasma concentration and response; one which must be considered is the analytical methodology. Despite the number of gas chromatographic (GC) and GC—mass spectrometric (MS) procedures available [8—14], chemical analysis of plasma concentrations of haloperidol encountered in patients receiving dosages of less than 10 mg per day remains difficult. Butyrophenones, in general, are highly adsorptive compounds; the major problem affecting the accuracy of present GC and GC—MS assays for haloperidol is non-linear adsorption at the nanogram level [12].

This paper describes a highly selective and sensitive method for the analysis of haloperidol utilizing ammonia chemical ionization GC—MS and selected-ion monitoring (SIM). The accuracy of the assay is improved through the use of a deuterated analogue of haloperidol, which has been employed as both the internal standard and a carrier, and the addition a second drug, thioridazine, which added to the injection mixture acts as a chaser and priming compound to further reduce the adsorption of haloperidol in the GC step. The assay was used to determine the serum concentration of haloperidol in 23 patients with Huntington's disease receiving haloperidol therapy.

EXPERIMENTAL

Patient samples

Whole blood samples were drawn by glass syringe from Huntington's disease patients treated orally with haloperidol at several Chicago area medical centers. Samples were transferred to glass centrifuge tubes with PTFE-lined caps and allowed to clot for at least 30 min. They were then centrifuged, the serum was harvested and frozen at −20°C until analysis.

Standards and reagents

High-purity hexane and methanol were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Isobutanol, reagent grade, was purchased from Eastman-Kodak (Rochester, NY, U.S.A.). Certified 1 *N* sodium hydroxide and 0.1 *N* sulfuric acid solutions were purchased from Fisher (Fair Lawn, NJ, U.S.A.). Thioridazine, 10-[2-(1-methyl-2-piperidyl)ethyl]-2-(methylthio)-phenothiazine, was obtained as Mellaril concentrate (30 mg/ml thioridazine hydrochloride in 3% alcohol—water) from Sandoz Pharmaceuticals (East Hanover, NJ, U.S.A.). Haloperidol, 4-[4-(4-chlorophenyl)-4-hydroxy-1 piperidinyl]-1-(4-fluorophenyl)-1-butanone (McN-JR-1625), and the 2,3,5,6-tetradeutero-4-fluorophenyl analogue (*d*4-haloperidol) were supplied by courtesy of McNeil Pharmaceuticals (Spring House, PA, U.S.A.).

Stock solutions

A stock solution A of 1.002 mg/ml haloperidol in methanol was prepared. Dilutions of the stock solution with methanol were made to give haloperidol solutions with the following concentrations: 100.2 μg/ml (B), 10.02 μg/ml

(C), 1.002 μg/ml (D), and 100.2 ng/ml (E). The internal standard solution was prepared by dissolving 40.02 mg of *d*4-haloperidol in 100 ml methanol to give a final concentration of 400.2 ng/ml. A 100-ml volume of the thioridazine spiked solution (2.0 μg/ml) was prepared from a 1:500 dilution of a stock solution containing 1.00 mg of thioridazine · HCl in 10.0 ml methanol. All solutions were stored at −20°C. No decomposition was observed over the course of the study.

Standard curve and quality control standard

Appropriate amounts of haloperidol from the prepared solutions were aliquoted into each of five flasks, the solvent was evaporated and freshly collected, drug-free human serum was added to give the following haloperidol concentrations: 2.00, 5.01, 10.02, 20.04 and 40.08 ng/ml. After equilibration for 30 min, the haloperidol dilutions and remaining blank serum were divided into 2.0-ml aliquots in 10-ml PTFE-lined screw-cap tubes and frozen at −20°C until needed for analysis. A quality control standard was prepared daily by adding 100 μl of haloperidol solution E (100.2 ng/ml) to a 2-ml blank serum aliquot. All standards were processed according to the method described below.

Extraction procedure

The procedure of Hornbeck et al. [14] was followed with minor modification. Serum samples were extracted from 2-ml aliquots in 10-ml PTFE-lined screw-cap glass tubes. Each sample was spiked with 50 μl of the *d*4-haloperidol internal standard solution (400.2 ng/ml) and allowed to equilibrate 30 min after vigorous wrist-action mixing. The samples were made alkaline with 400 μl of 0.5 *M* sodium hydroxide and extracted with 3 ml of 1.5% isobutanol in hexane by agitation on a mechanical shaker for 10 min, followed by centrifugation at 1400 *g* for 10 min. The supernatant was transferred to a second tube containing 1 ml of 0.05 *M* sulfuric acid and extracted into the aqueous phase as previously described. The organic phase was discarded and the aqueous layer was washed with 2 ml of the isobutanol—hexane extraction solvent. After re-alkalinization with 300 μl of 0.5 *M* sodium hydroxide, the aqueous phase was extracted as before. The organic phase was transferred to a 3-ml conical-bottom reaction vial containing 50 μl of the thioridazine solution (100 ng total). The extracts were evaporated to dryness under nitrogen in a 40°C water bath and frozen at −20°C until analysis (overnight). Prior to injection into the GC—MS system, the dry samples were dissolved in 10 μl methanol with vortexing.

Instrumentation

A Finnigan 4510 gas chromatograph—mass spectrometer coupled to an INCOS data system (Finnigan-MAT, Sunnyvale, CA, U.S.A.) was used for this study. The samples were introduced through a 0.61 m × 2 mm I.D. silanized glass column packed with 3% SP-2100 on 80—100 mesh Supelcoport (Supelco, Bellefonte, PA, U.S.A.). The column was initially conditioned using a helium flow-rate of 35 ml/min and the following temperature program: hold at 50°C for 1 h, heat from 50°C to 310°C at 5°C/min, hold at 310°C for 16 h (overnight). The column was then treated with approximately 40 μl of Silyl-8 (Pierce, Rockford, IL, U.S.A.) at 250°C and allowed to run for 30

min at that temperature before connecting to the mass spectrometer inlet. Daily priming of the column consisted of injection of 2 μl methanol twice, 1 μl thioridazine twice and finally 2 μl methanol twice.

The injector and column oven temperatures were 245°C and 235°C, respectively. Zero-grade helium at a flow-rate of 30 ml/min was used as the carrier gas and was admitted into the mass spectrometer through the direct transfer line. The gas chromatograph divert valve was open at injection to minimize solvent flow to the mass spectrometer and closed 45 sec after injection to admit the total sample for analysis. Samples were injected every 5—6 min.

Analysis was carried out in the chemical ionization (CI) mode with ammonia added through the make-up gas inlet to give an inlet pressure reading of 33.3 Pa. (The combined helium carrier and ammonia reagent gas inlet pressure reading was 130.6 Pa.) The GC—MS interface oven and transfer line were maintained at 245°C, the manifold heater at 90°C and the ionizer at 120°C. The ionization current was 0.2 mA with an electron energy of 70 eV; the pre-amp sensitivity setting was $1 \cdot 10^{-8}$ A.

The multiple-ion detection software provided by the instrument manufacturer was used to moniter the ions at m/z 376 and 380, corresponding to the $d0$- and $d4$-haloperidol. A dwell-time bias in favor of the unlabelled haloperidol was used to improve sensitivity of detection. The INCOS target compound analysis (TCA) and quantitation programs provided were modified to correct for ^{13}C-natural abundance contributions and incomplete labeling of the internal standard and for noise across the peak, and were used for computation of the data.

RESULTS

The need to accurately measure serum haloperidol concentrations below 10 ng/ml on reasonable size patient samples led to an examination of alternative CI reagent gases for the GC—MS analysis of this drug. Hornbeck et al. [14] have suggested that methane—ammonia gives a three-fold sensitivity over methane for haloperidol. In our laboratory, ammonia gave less fragmentation and thus a greater ion yield of protonated molecular ions (MH^+) than did methane or isobutane and was used as the reagent gas in this study. The ion yield of suitable ions from methane and isobutane negative chemical ionization (NCI) analysis was also compared with that from positive chemical ionization (PCI); methane NCI proved to be equally sensitive and isobutane NCI less sensitive (NCI production of suitable ions was approximately one fourth that of PCI) for this compound.

To further minimize losses in the extraction process and decrease adsorption on the GC column, a tetradeutero-labeled analogue of haloperidol was used as the internal standard. A stable isotope-labeled analogue is the ideal internal standard as its behavior is identical to the unlabeled haloperidol in the extraction process as well as the analytical process, eliminating the strict requirement for quantitative recovery in these steps. In this instance, the added bulk of the deutero haloperidol also acts as a carrier for the nanogram quantities of drug to be quantitated, thus aiding recovery.

The ammonia CI mass spectra of the unlabeled and $d4$-labeled haloperidol are shown in Fig. 1A and B. The protonated molecular ions are the major ions

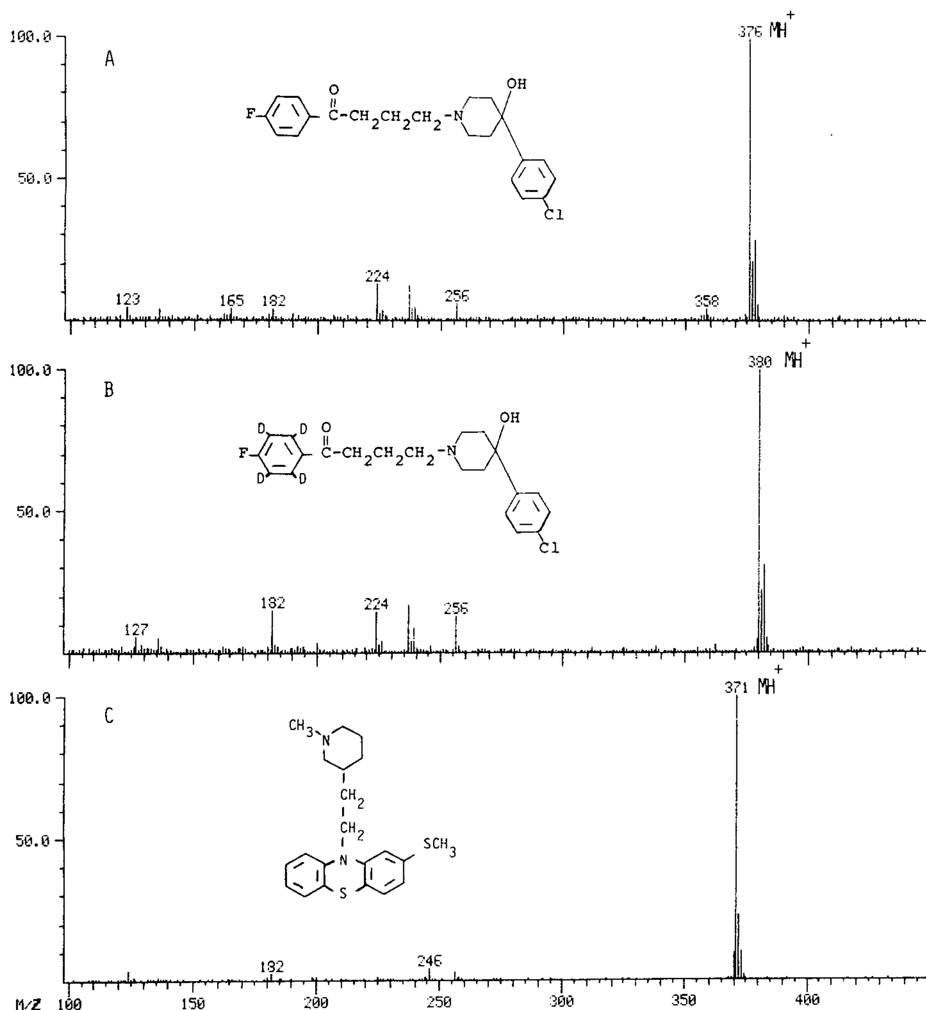

Fig. 1. Ammonia CI mass spectra of haloperidol (A), *d*4-haloperidol (B) and thioridazine (C). Samples were introduced through the gas chromatograph.

in the spectra, with the isotopic molecular ion cluster comprising greater than 70% of the total ion production. Minor ions at *m/z* 224 and *m/z* 237, common in the electron impact (EI) mass spectra, are also observed. MS analysis showed the *d*4-haloperidol to be greater than 98% labeled, minimizing any contribution from residual unlabeled drug in the quantitation.

Before evaporation to dryness, 100 ng of a second drug, thioridazine, was added to each of the extracted serum samples. The presence of thioridazine greatly improves the peak shape and recovery of haloperidol, possibly by displacing it on the GC column. The ammonia CI mass spectrum of thioridazine is shown in Fig. 1C; no interfering ions from the thioridazine can be expected in the MS analysis of *d*0- or *d*4-haloperidol. The compounds separate well on a 0.61-m column packed with 3% SP-2100, the haloperidol eluting with a retention time of approximately 2.0 min, followed by thioridazine at 3.3 min.

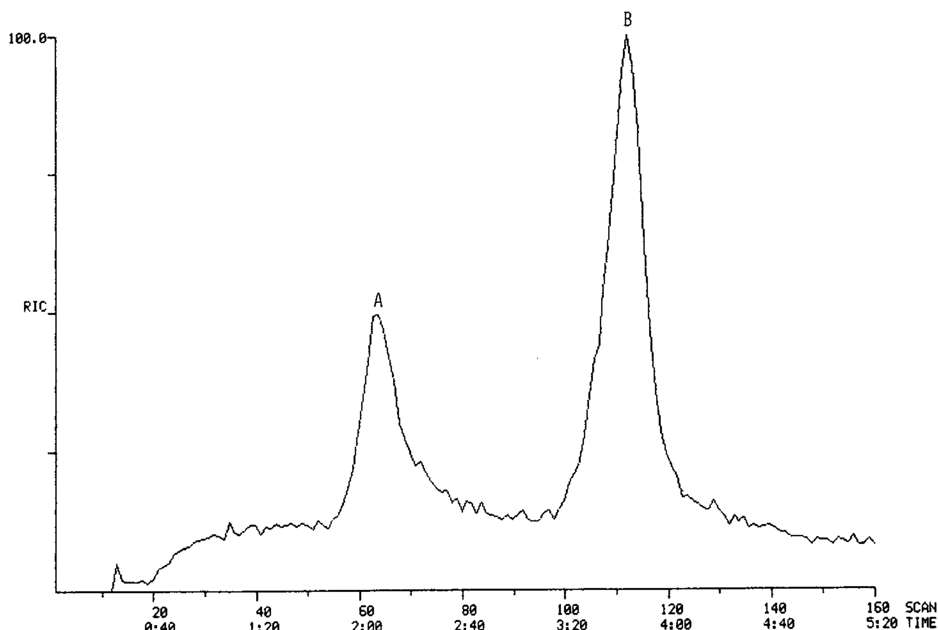

Fig. 2. Total-ion-current chromatogram of a mixture of haloperidol (A) and thioridazine (B) on a 0.61-m column packed with 3% SP-2100.

Fig. 2 shows the total-ion-current chromatogram of a mixture of haloperidol and thioridazine. The addition of thioridazine to the extracted serum samples eliminates memory effects due to the incomplete elution of the drug from the GC column, and permits standard and patients samples to be run in random order without affecting the quantitation. When haloperidol is used to prime the GC column, ghost peaks are commonly seen in subsequent injections. For this reason, thioridazine was also used for daily priming of the GC column; daily treatment with Silyl-8, which necessitates removal of the column in the instrument used, has been eliminated.

Mass chromatograms of the unlabeled and labeled haloperidol obtained from SIM-MS analysis of an actual serum sample spiked with 10 ng/ml of each compound are shown in Fig. 3. The GC conditions chosen give a minimum retention time for haloperidol while still allowing for accurate background calculation before and after the sample peak. The dwell times on the unlabeled drug and the labeled internal standard were 210 and 26 msec, respectively. The multiple-ion detector, set at m/z 376 and m/z 380 ± 0.250 a.m.u., exhibited a drift of less than ± 10 m.m.u. over the period of analysis (approximately 4 h).

Multiple injections of *d*0- and *d*4-labeled haloperidol were made on each day of analysis to determine contributions to the m/z 376 and m/z 380 ion abundances from incomplete labeling and from ^{13}C natural abundance ions. All subsequent samples were corrected for these contributions. A daily calibration curve consisting of five standard serum dilution samples was run with each set of patient samples. Internal standard (*d*4-haloperidol, 10 ng/ml) was added to each sample; the peak area ratio of m/z 376 to m/z 380 versus the concentration of unlabeled haloperidol (in ng/ml of serum) was examined by linear

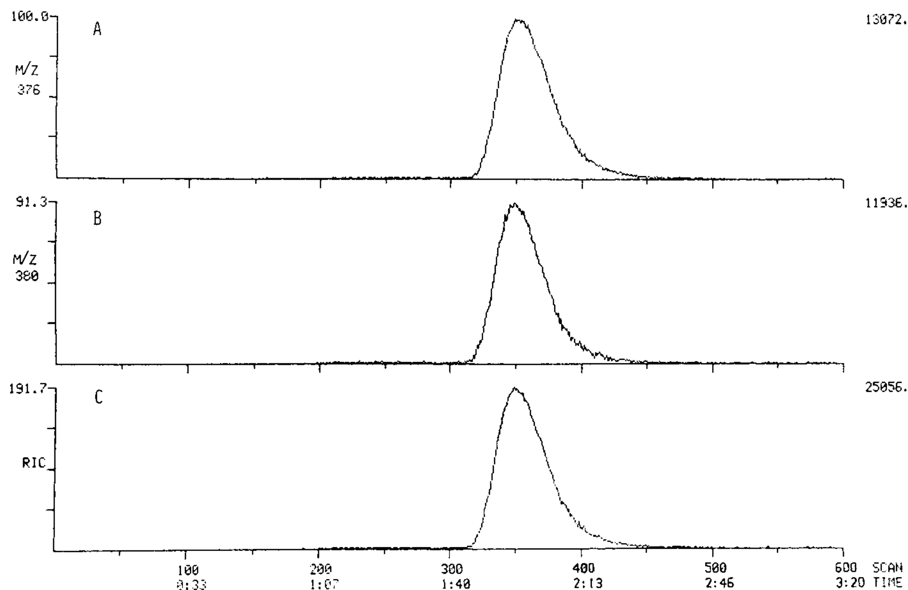

Fig. 3. Multiple-ion detection mass chromatograms of haloperidol (A) and $d4$-haloperidol (B) obtained from analysis of 2 ml of extracted serum spiked with 10 ng/ml of each compound. The displays are normalized to the maximum of each individual peak. The total-ion-current chromatogram is shown in C.

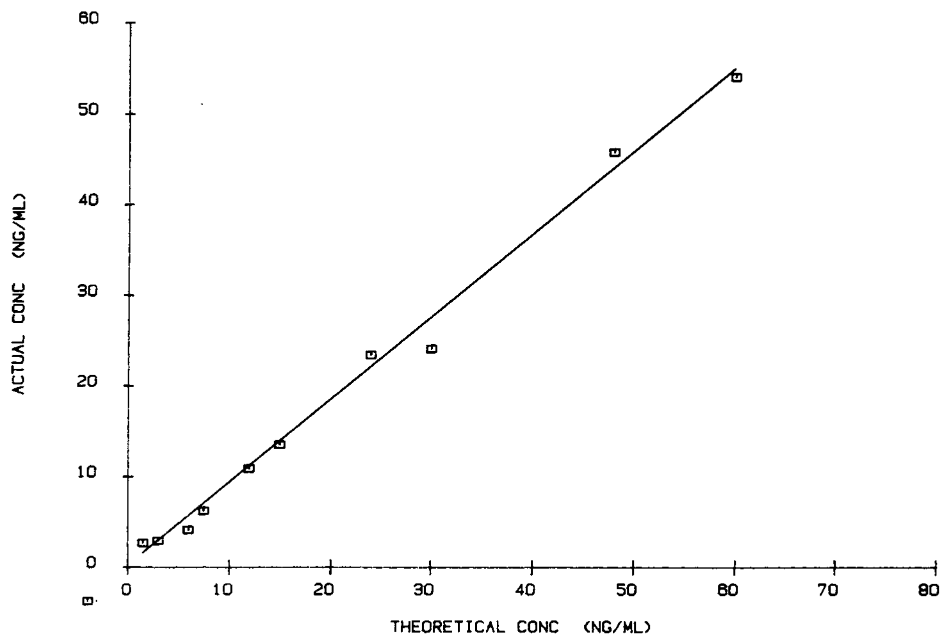

Fig. 4. Plot of the theoretical concentration of haloperidol in ten spiked serum samples versus the measured concentration. A 2-ml volume of extracted serum was used. Linear regression analysis gave the equation values $y = 0.913x - 0.125$; the correlation coefficient was 0.996.

regression analysis. A typical standard curve gave a slope and intercept of 0.110 and 0.074, respectively, and a correlation coefficient of 0.999. The mean linear regression correlation coefficient for seven runs was 0.995 ± 0.007. Four standard serum dilution curves covering the range 1.0—20.0 ng of haloperidol per ml of serum, run prior to analysis of patient samples, gave a linear curve with a mean correlation coefficient of 0.994 ± 0.009.

A plot of the actual concentration of haloperidol measured in ten spiked serum samples is shown in Fig. 4. Linear regression analysis gave a slope of 0.913 and an excellent correlation coefficient (0.996) for the concentration range 1.5—60.0 ng/ml haloperidol. The linear correlation did not hold at concentrations greater than 60.0 ng/ml, and no quantitation above this level was attempted.

The precision and accuracy of multiple measurements made on pooled serum samples spiked with known quantities of unlabeled haloperidol are given in Table I. These samples and the spiked serum curve samples described above were prepared by an outside source. The internal standard was added to each sample prior to extraction. The inter-assay precision for control serum samples spiked with 2—40 ng/ml haloperidol is shown in Table II. Quality control samples prepared fresh with each run by spiking 2 ml of serum with 100 μl of a 100.2 ng/ml standard solution of haloperidol gave a mean value of 4.42 ± 0.32 with a coefficient of variation (C.V.) of 7.3%. These values were not significant-

TABLE I

PRECISION AND ACCURACY OF DETERMINATIONS OF HALOPERIDOL IN HUMAN SERUM (n = 5)

Haloperidol concentration (ng/ml)			C.V.* (%)	Relative accuracy (%)
Theoretical	Observed	S.D.		
7.52	7.56	0.17	2.2	0.5
24.05	25.61	3.12	12.2	6.5
48.10	46.17	4.99	10.8	—4.0

*Coefficient of variation calculated from results for pooled drug-free human serum containing known quantities of unlabeled haloperidol.

TABLE II

INTER-ASSAY PRECISION OF CONTROL SAMPLES

Computations are based on seven runs made over a four-week period on spiked pooled serum.

Haloperidol concentration (ng/ml)			C.V. (%)
Theoretical	Observed	S.D.	
2.00	2.14	0.22	10.1
5.01	4.70	0.46	9.9
10.02	10.22	0.24	2.4
20.04	20.48	0.93	4.6
40.08	39.91	0.35	0.9

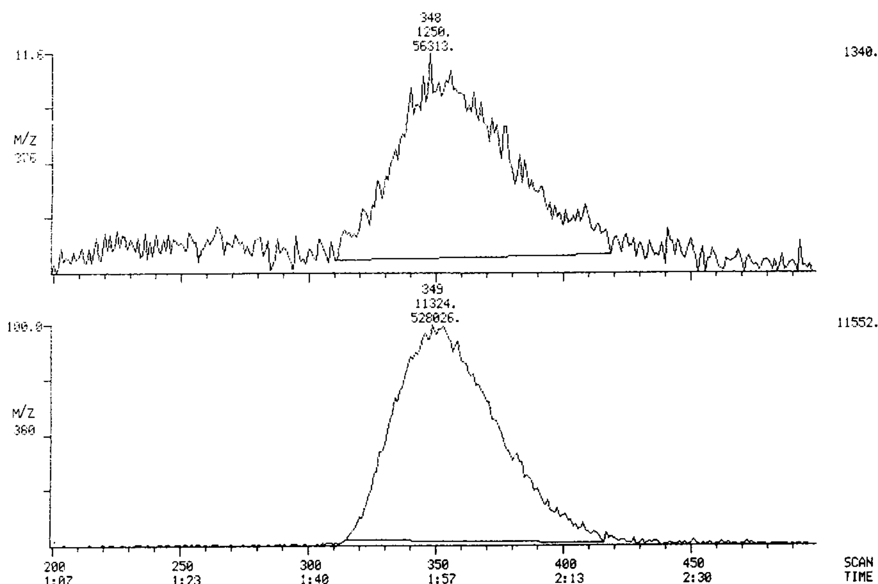

Fig. 5. Multiple-ion detection mass chromatograms showing the quantitation of haloperidol in 2 ml of extracted patient serum. The unlabeled haloperidol (*m/z* 376) and *d*4-haloperidol internal standard (*m/z* 380) peaks contain 280 pg/ml and 10 ng/ml, respectively. Data shown are uncorrected.

ly different from the 5.0 ng/ml frozen control samples above, indicating no deterioration of the haloperidol samples under the storage conditions used.

Serum samples from 22 patients receiving total daily doses of 1.0—40.0 mg (0.01—0.88 mg/kg per day) of haloperidol were analyzed using the method described. The measured serum concentration ranged from 0.3 to 28.7 ng/ml; Fig. 5 shows the quantitation of haloperidol in an actual patient sample using SIM-MS analysis. Dose adjustments were made when they were judged to be clinically necessary, and several patients were analyzed more than once. A minimum of one month was allowed between dosage adjustments to allow patients to reach a new steady state. One sample, from a patient receiving Mellaril only, gave a zero value, as did several pretreatment patient samples. The correlation between clinical response and serum haloperidol concentration in these patients will be reported elsewhere.

DISCUSSION

While the described assay gave satisfactory quantitation of haloperidol in patients receiving total doses of 1.0—40.0 mg per day, measurements in one patient whose daily intake ranged from 80.0 to 180.0 mg proved difficult due to the presence of an interfering peak. The retention time of this compound relative to haloperidol was 1.28; the EI mass spectrum of the compound was consistent with that of hydroxyhaloperidol. In agreement with the previous report by Forsman and Larsson [15], the reduced metabolite was estimated to be present at nearly the same concentration as haloperidol when high doses of the drug were administered. No evidence of the hydroxy metabolite was

observed in patients receiving 40 mg per day or less of haloperidol. Slight modification of the GC conditions and expansion of the standard curve should enable accurate measurement of haloperidol, as well as the hydroxy metabolite, in such patients.

Several investigators have used the chloro-substituted haloperidol (HAC) as their internal standard [10, 12, 13]. Because of the structural similarity of this compound to haloperidol and its greater retention time, one would suspect that the loss of haloperidol by adsorption on the GC column would be reduced with its use. However, HAC contains the same benzylic hydroxyl group probably responsible for the column-adsorption effect as does haloperidol, and reproducible recovery of the internal standard in assays where low nanogram amounts of HAC are used is not guaranteed.

In conclusion, the addition of carrier *d*4-haloperidol in combination with the use of ammonia as the CI reagent gas should theoretically improve the sensitivity of the GC—SIM-MS assay for haloperidol, possibly to the low picogram level. These modifications have not produced the higher sensitivity expected. However, the described method, which incorporates the use of a deuterated analogue of haloperidol as the internal standard, offers the only reliable solution to the adsorption problems encountered in the measurement of low levels of haloperidol. This addition, together with the excellent sensitivity and selectivity provided by ammonia CI in the SIM process and the convenience and improved peak shape gained using thioridazine, contributes to an improved assay for the measurement of haloperidol in patients receiving low doses of this drug.

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GAS CHROMATOGRAPHIC DETERMINATION OF *m*- AND *p*-HYDROXYPHENYTOIN IN THE URINE OF EPILEPTIC PATIENTS

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SUMMARY

An analytical method for determining phenytoin and its metabolites in the urine of epileptic patients is described. The analysis was performed for the *m*- and *p*-isomers of hydroxyphenytoin, the oxidative products of phenytoin, using gas chromatography. As an internal standard, 5-(4-methylphenyl)-5-phenylhydantoin was chosen; the extraction solvent from human urine was ether—chloroform (3:7).

Phenytoin and its hydroxy isomers were satisfactorily determined by the modified on-column methylation technique on a 2% OV-17 column using temperature programming from 180°C to 240°C at 5°C/min.

INTRODUCTION

The high performance liquid chromatographic determination of phenytoin and its hydroxy isomers in serum and human urine was discussed in our first [1] and second [2] reports. The analysis of phenytoin and its metabolites in rat urine was also tried in a previous investigation [3].

Phenytoin is converted to 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (*p*-HDPH) by biological metabolism, about half of the administered dose being excreted in the urine in unchanged form and/or as the conjugated glucuronide. In human urine, the hydantoin ring in the phenytoin molecule is cleaved to produce diphenylhydantoic acid to the extent of 1—5% of the original amount

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of drug, and also to aminodiphenylglycine at 25%. *m*-Hydoxyphenytoin, the isomer of *p*-HDPH, is also present in human urine at a low level [4—6].

The present report is concerned with the determination of human urinary *m*- and *p*-HDPH as well as phenytoin, which was also administered to epileptic patients. Solow et al. [7] reported the determination of phenytoin and phenobarbital in blood by a gas chromatographic (GC) method with an on-column methylation technique. For a simple, rapid and quantitative method, we have attempted to analyse the *m*- and *p*-HDPH isomer using a partially modified on-column methylation technique.

EXPERIMENTAL

Reagents and solutions

Phenytoin (DPH) was recrystallized from one normalized according to the Japanese Pharmacopoeia. *m*-HDPH was from Aldrich (Milwaukee, WI, U.S.A.) and *p*-HDPH was from Sigma (St. Louis, MO, U.S.A.). Diphenylhydantoic acid was prepared from diphenylglycine by the method of Connors et al. [8]. Diphenylglycine was a product from Aldrich. Carbamazepine was one normalized according to the Japanese Pharmacopoeia. Tetramethylammonium hydroxide was used as a 10% solution in methanol, obtained from Tokyo Kasei (Tokyo, Japan). 5-(4-Methylphenyl)-5-phenylhydantoin was purchased from Sigma.

All solvents and other reagents were of analytical grade and used without further purification. As a control urine, Tek-Chek® No. 1 was obtained from Ames (Elkhart, IN, U.S.A.).

DPH and *p*-HDPH (20 mg each) were independently dissolved in 2—6 ml of 0.1 *M* potassium hydroxide and made to a total volume of 100 ml with water. *m*-HDPH solution was obtained by dissolving 10 mg of the substance in 3 ml of 0.1 *M* alcoholic potassium hydroxide and made to 100 ml with water. Diphenylhydantoic acid (5 mg) was dissolved in a small volume of ethanol and made to 100 ml with water. Diphenylglycine (1 mg) was dissolved in a small volume of ethanol and made to 10 ml with water. Carbamazepine solution was prepared by dissolving 2 mg in a small volume of 0.1 *M* alcoholic potassium hydroxide and made to 10 ml with water. As a buffer solution, monobasic sodium phosphate (GR grade, Wako) was dissolved in water to a concentration of 0.3 *M*. As internal standard solution, 0.5 mg of 5-(4-methylphenyl)-5-phenylhydantoin was dissolved in 100 ml of an ether—toluene (3:7) mixture.

Instruments

GC analyses were performed by a JGC-20 KFP (JEOL, Tokyo, Japan) gas chromatograph on a glass column (2 m × 2 mm I.D.) with 2% OV-17 as stationary phase (80—100 mesh, Gas-Chrom Q) coupled with a Hitachi 834 type chromatoprocessor. The injection and flame ionization detector temperature was maintained at 280°C, and the column oven was temperature programmed from 180°C to 240°C at 5°C/min. The carrier gas was nitrogen with a flow-rate at 30 ml/min.

Calibration and recovery

To 1.0 ml of the urine, known amounts of DPH and *m*- and *p*-HDPH solutions were added. Then 1.0 ml of 0.3 *M* phosphate buffer solution (pH 6.8) and 5.0 ml of the internal standard solution were added. The mixture was vortex-mixed for 30 sec and centrifuged at 700 *g* for 30 min. The supernatant was transferred to another centrifuge tube and 0.5 g of anhydrous sodium sulphate was added. The mixture was also vortex-mixed for 30 sec and centrifuged again at 700 *g* for 5 min. To the supernatant thus obtained, 100 μl of tetramethylammonium hydroxide (TMH) were added and this mixture was vortex-mixed for 30 sec, then centrifuged at 1100 *g* for 5 min. After the upper layer was discarded, 50 μl of methanol were added to the TMH (lower) layer and mixed well; 2 μl of the mixture were injected into the gas chromatograph. The calibration and recovery tests were replicated four times for each substance. The recovered amounts of the substances were calculated from the ratio of the peak height of the samples to that of the internal standard.

Analytical procedure

When the analytical procedure was applied to patient's urine, acid hydrolysis was carried out to isolate the conjugated drug and its metabolites. To 1.0 ml of patient's urine, 1 ml of 12 *M* hydrochloric acid was added and heated at 90°C for 2 h. The hydrolysate thus obtained was neutralized with 20% sodium hydroxide, followed by the procedure as described above for calibration and recovery.

RESULTS AND DISCUSSION

Trimethylphenylammonium hydroxide and TMH have been widely utilized as on-column methylation agents for gas—liquid chromatographic (GLC) analysis. The former is useful for methyl esterification of straight-chain fatty acids. On the other hand, the latter has been used in the determination of

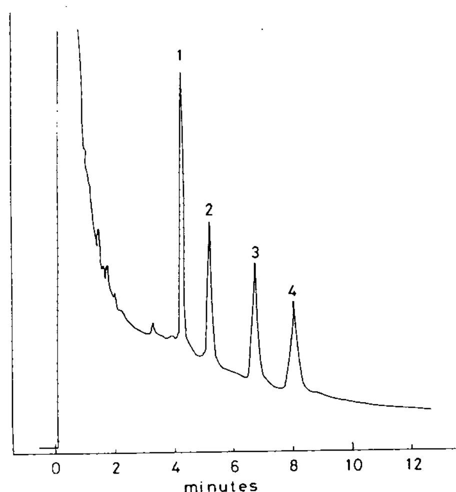

Fig. 1. Gas chromatogram of phenytoin and its hydroxy compounds. Peaks: 1 = phenytoin, 2 = internal standard, 3 = *m*-hydroxyphenytoin, 4 = *p*-hydroxyphenytoin.

barbituric acids [9, 10] or phenytoin [9, 11]. The present report deals with a modification of the latter methylation technique.

An extraction solvent suitable for DPH and *m*- and *p*-HDPH in human urine was sought. Ethyl acetate or ether—chloroform mixed solvents gave undesirable extraction ratios for these substances in urine. Chloroform also makes the layer separation from the TMH layer difficult.

An ether—toluene mixture, which was used by Dorrity and Linnoila [12], was selected as the extraction solvent for DPH and other compounds from urine samples. The extraction was carried out using two solvent ratios: 50% ether and 50% toluene, and 30% ether and 70% toluene. The latter was more effective than the former for the extraction of DPH, and *m*- and *p*-HDPH and internal standard. Consequently, the ether—toluene (3:7) mixture was used as the extraction solvent for DPH and its metabolites; in addition, the internal standard was dissolved in the same solvent system.

The results of the experiment concerning the effect of phosphate buffer concentration on extraction showed that 3 *M* [12] phosphate buffer in 10% methanol was superior to 0.3 *M* [7].

In preliminary experiments using on-column methylation and a constant column temperature of 240°C, the chromatogram showed that DPH was well resolved from methanol used as a solvent for TMH. The improved resolution is required to analyse small amounts of DPH in urine. Solow et al. [7] used the following temperature programme for GLC: 1 h at 250°C, 4 h at 325°C, and overnight at 250°C. Interference from the methanol peak could be avoided by introducing linear temperature programming rising from 180°C to 240°C at a rate of 5°C/min by slightly modifying the conditions of Solow et al. DPH was completely resolved from methanol, as shown in Fig. 1.

The retention times for DPH, internal standard and *m*- and *p*-HDPH were 4.3, 5.2, 6.7 and 8.0 min, respectively. Quantitative measurements were attempted for DPH and *m*- and *p*-HDPH under these experimental conditions. Straight lines which passed through the origin were obtained between detector response and increasing amounts of standards added to the urine samples before extraction. As the detector response, the ratio of the peak height of samples to that of the internal standard, 5-(4-methylphenyl)-5-phenylhydantoin, was adopted. The regression lines between the amount (x) injected into the column and the peak height ratio (y) were $y = 0.06079x + 0.01430$ ($r = 0.997$) in the range 10—50 μg/ml for DPH, $y = 0.02727x - 0.02089$ ($r = 0.998$) in the range 15—50 μg/ml for *m*-HDPH, and $y = 0.02346x - 0.01650$ ($r = 1.000$) in the range 15—200 μg/ml for *p*-HDPH. Thus, it was confirmed that each result showed significant correlations.

Experiments on the recovery of DPH and *m*- and *p*-HDPH added to the control urine and healthy human urine were performed. To 1.0 ml of urine, 20—60 μg of DPH, 20—80 μg of *m*-HDPH, and 10—150 μg of *p*-HDPH were added, and DPH and its hydroxy metabolites were analysed using the on-column methylation technique. The recoveries of DPH were 94.4—99.9% for control urine and 93.9—99.9% for human urine, those of *m*-HDPH were 103.3—109.5% for control and 95.9—106.1% for human urine, and those of *p*-HDPH were 88.4—96.7% for control and 90.8—94.6% for human urine. The recovery percentages were all satisfactorily significant, as shown in Table I.

TABLE I

RECOVERY OF DPH AND *m*- AND *p*-HDPH ADDED TO CONTROL AND HEALTHY HUMAN URINE

	DPH			*m*-HDPH			*p*-HDPH		
	Added (μg/ml)	Recovery* (%)	C.V.** (%)	Added (μg/ml)	Recovery* (%)	C.V. (%)	Added (μg/ml)	Recovery* (%)	C.V. (%)
Control urine***	20	94.4 ± 2.15	2.28	20	103.3 ± 1.15	1.11	10	96.7 ± 3.45	3.57
	40	99.7 ± 0.65	0.65	40	109.5 ± 3.93	3.59	50	93.4 ± 1.05	1.12
	60	99.9 ± 1.73	1.73	80	103.7 ± 2.08	2.01	100	88.4 ± 1.40	1.58
Human urine	40	95.1 ± 1.70	1.79	25	106.1 ± 0.10	0.09	10	90.8 ± 0.65	0.72
	50	93.9 ± 0.85	0.91	50	95.9 ± 2.95	3.08	50	94.6 ± 0.65	0.69
	60	99.9 ± 0.80	0.80	60	103.1 ± 7.68	7.45	150	93.4 ± 0.00	0.00

*Mean ± S.D. (n = 4).
**Coefficient of variation.
***Tek-Chek No. 1 (Ames, Elkhart, IN, U.S.A.) was used as a control urine.

Consequently, DPH and *m*- and *p*-HDPH were quantitatively determined by GC through on-column methylation using TMH as N-methyl derivatization reagent. The partially modified on-column methylation technique allowed the simple and rapid determination of the substances added to the control and the human urine.

Subsequently, the influence of the coexistence of DPH metabolites and another combination drug on the measured value was examined. Carbamazepine, a combination drug, diphenylhydantoic acid and diphenylglycine as DPH metabolites except the hydroxy compound of DPH were selected for this purpose. On the gas chromatogram, carbamazepine, hydantoic acid and diphenylglycine appeared earlier than DPH and also overlapped at the solvent peak. Thus, DPH, and *m*- and *p*-HDPH can be quantitatively determined by GLC without the interference of carbamazepine or DPH metabolites, as shown in Table II.

As an application of the on-column methylation technique using the method described, urinary DPH and *m*- and *p*-HDPH in patients who were hospitalized at the Department of Psychiatry could be determined with a single injection

TABLE II

EFFECT OF CARBAMAZEPINE, HYDANTOIC ACID AND DIPHENYLGLYCINE ON RECOVERY OF DPH

DPH concentration was 20 μg/ml for each experiment.

	Added (μg/ml)	Recovery* (%)	C.V.** (%)
Carbamazepine	24	104.1 ± 1.48	1.4
	36	94.0 ± 1.34	1.4
	240	101.8 ± 1.77	1.4
Hydantoic acid	500	105.2 ± 0.64	0.6
Diphenylglycine	100	100.3 ± 4.3	4.3

*Mean ± S.D. (n = 4).
**Coefficient of variation.

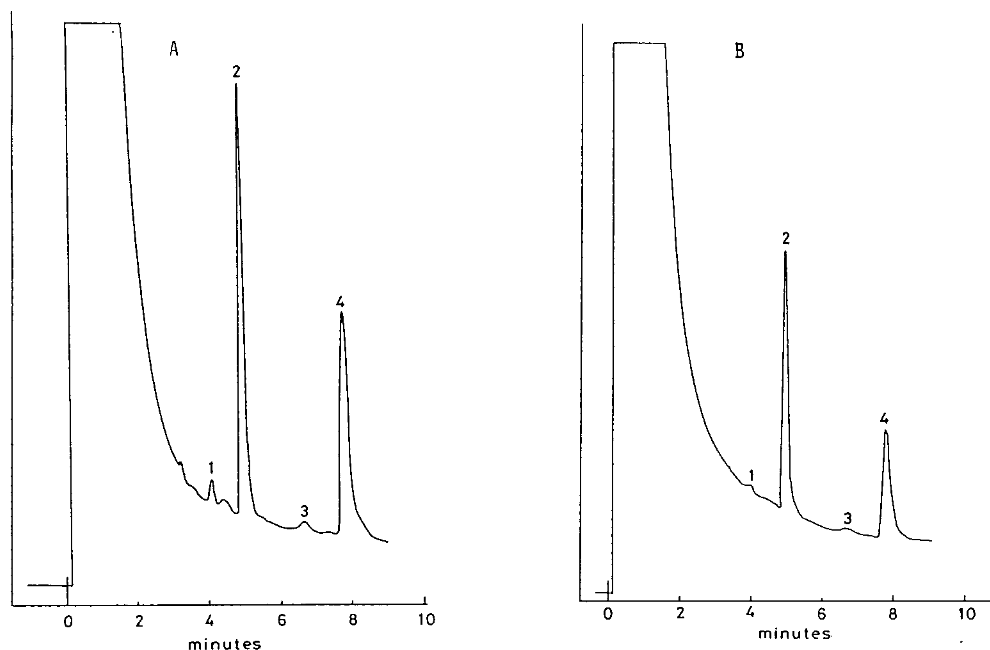

Fig. 2. (A) Gas chromatogram of urine from an epileptic patient after administration of 250 mg of DPH and 300 mg of carbamazepine per day. Peaks: 1 = phenytoin, 2 = internal standard, 3 = *m*-hydroxyphenytoin, 4 = *p*-hydroxyphenytoin. The amounts of peaks 1, 3 and 4 corresponded to 2.23, 5.43 and 74.82 mg per one day's urine, respectively. (B) Gas chromatogram of urine from an epileptic patient after administration of 150 mg of phenytoin per day. Peaks: 1 = phenytoin, 2 = internal standard, 3 = *m*-hydroxyphenytoin, 4 = *p*-hydroxyphenytoin. The amounts of peaks 1, 3 and 4 corresponded to 0.28, 5.30 and 56.48 mg per one day's urine, respectively.

TABLE III

AMOUNT OF DPH AND *m*- AND *p*-HDPH IN PATIENTS' URINE AFTER ADMINISTRATION OF DPH

Name	Age	Sex	Administered (mg)		Found (mg)		
			DPH	Carbamazepine	DPH	*m*-HDPH	*p*-HDPH
R.K.	25	F*	250	300	2.23	5.43	74.82
F.S.	68	F	150	—	0.28	5.30	56.48

*F = female.

of the urine extract. Chromatograms for the extracts of patients' urine are shown in Fig. 2A and B.

Fig. 2A shows a chromatogram from the urine of patients to whom 250 mg of DPH and 300 mg of carbamazepine were administered daily, while Fig. 2B shows that obtained when 250 mg of DPH only were administered to patients. These results are summarized in Table III. From these experimental data it can be seen that only a small amount of DPH, less than 1% of the initial amount, exists as unchanged form, most (30—40%) of the substance being

converted to its oxidative product, *p*-HDPH. Kozelka and Hine [13] reported that DPH is formed through the ring closure of diphenylhydantoic acid by heating with acid. Furthermore, there is an increase in the amount of DPH over that found in untreated urine. These results were all in good agreement with data of Chang et al. [4].

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CHROMBIO. 2507

DETERMINATION OF THE ENANTIOMERS OF INDOPROFEN IN BLOOD PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AFTER RAPID DERIVATIZATION BY MEANS OF ETHYL CHLOROFORMATE

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SUMMARY

A rapid method for the determination of (+)- and (—)-indoprofen in blood plasma has been developed and applied to pharmacokinetic work. By means of ethyl chloroformate, indoprofen is coupled to leucinamide, a reaction which is complete in less than 3 min at room temperature. The diastereoisomeric derivatives are then separated by reversed-phase high-performance liquid chromatography. In two surgical patients given racemic indoprofen intravenously, the pharmacologically active (+)-enantiomer had a lower clearance and a lower distribution volume than the (—)-enantiomer, which gave it a slightly longer half-life.

INTRODUCTION

Indoprofen, 2-[4-(1,3-dihydro-1-oxo-2H-isoindol-2-yl)phenyl] propionic acid, is a non-steroidal anti-inflammatory drug (NSAID) used, in its racemic form, as a general analgetic agent [1]. Several methods have been reported for the determination if indoprofen in body fluids [2—6], and the pharmacokinetics of racemic indoprofen have been investigated [7—9]. Of the enantiomers, only the (*S*)-(+)-isomer is pharmacologically active [10]. It is therefore of interest to study the stereoselective disposition of indoprofen. A gas—liquid chromatographic (GLC) method has been described for the determination of the indoprofen enantiomers in plasma [11]. Separation of the enantiomers is achieved after conversion to acid chlorides with thionyl chloride followed by amide formation with 1-phenylaminoethane (α-methylbenzylamine), a derivatization which takes about 1.5 h. The method has not been employed for any extensive pharmacokinetic work.

Stereospecific assays have also been reported for some other NSAIDs of the 2-arylpropionic acid type. The enantiomers of ibuprofen, 2-(4-isobutylphenyl)-

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propionic acid, were determined in body fluids by GLC as α-methylbenzylamides, the amide bonds being formed by means of 1,1′-carbonyldiimidazole [12]. The enantiomers of benoxaprofen, 2-[2-(4-chlorophenyl)benzoxazol-5-yl]propionic acid [13], and carprofen, 2-(6-chlorocarbazol-2-yl)propionic acid [14], were assayed by high-performance liquid chromatography (HPLC) as α-methylbenzylamides formed by way of acid chloride or by means of 1,1′-carbonyldiimidazole. Also, the enantiomers of naproxen, 2-(6-methoxynaphth-2-yl)propionic acid, could be determined by HPLC after acid-catalysed esterification with (*S*)-(+)-2-octanol [15] or after carbodiimide-mediated amide formation with 1-(4-dimethylaminonaphth-1-yl)ethylamine [16]. Any of these derivatization reactions typically takes 0.5—1.5 h to perform, evaporation steps excluded.

This paper describes the determination of indoprofen enantiomers by HPLC after a derivatization reaction which takes less than 3 min to perform. It also describes the application of this method to determine the indoprofen enantiomers in plasma samples taken from surgical patients after the intravenous administration of racemic indoprofen.

EXPERIMENTAL

Reagents and chemicals

Racemic indoprofen and indobufen, 2-[4-(1,3-dihydro-1-oxo-2H-isoindol-2-yl)phenyl] butyric acid, as well as > 98% pure (*S*)-(+)-indoprofen were supplied by Farmitalia Carlo Erba (Stockholm, Sweden). The compounds were dissolved in 6.7 m*M* phosphate buffer (pH 7.4) and the appropriate stock solutions were then prepared by dilution with distilled water. Ethyl chloroformate, triethylamine and L-leucinamide hydrochloride were purchased from Fluka (Buchs, Switzerland). Diethyl ether (BDH, Poole, U.K.), toluene (8325; E. Merck, Darmstadt, F.R.G.), acetonitrile (30; Merck), methanol (6009; Merck) and ethyl acetate (9623; Merck) were used without further purification. The water was freshly distilled and collected in a stainless-steel vessel. Thin-layer chromatography plates were silica gel with F_{254} fluorescence indicator spread on aluminum foil (Fluka).

Instrumentation and chromatographic conditions

The liquid chromatography system consisted of an LDC/Milton Roy (Riviera Beach, FL, U.S.A.) Constametric III pump, a Rheodyne 7125 loop injector and an LDC Spectro Monitor III variable-wavelength UV detector, at times coupled in series with a Shimadzu (Kyoto, Japan) RF-530 variable-wavelength fluorescence monitor. A LiChroCart RP-18 7-μm column (250 × 4 mm) was used in conjunction with a Hibar precolumn (30 × 4 mm) filled with Perisorb RP-18 (all from Merck). The mobile phase was, unless otherwise stated, a mixture of acetonitrile—10 m*M* phosphate buffer, pH 6.5 (38:62). The flow-rate was 2.0 ml/min and the detection wavelength was 275 nm. For fluorescence detection, the excitation and the emission wavelengths were 275 and 433 nm, respectively.

Direct probe mass spectra were obtained on a Finnigan (Sunnyvale, CA, U.S.A.) 4510 quadrupole instrument operated in the chemical-ionization mode, with methane as the reagent gas.

Samples

Blood samples were drawn from surgical patients participating in a clinical trial of indoprofen in post-operative pain [17]. The study was approved by the ethics committee of the University of Lund and by the Drug Division of the National Social Welfare Board. Informed consent was given by all patients. Racemic indoprofen, 400 mg, was given as an intravenous bolus dose. Blood samples were drawn from a peripheral vein into heparinized Venoject® blood collecting tubes. The plasma was collected on centrifugation of the samples and stored at $-20°$C until analysis.

Work-up and derivatization of plasma samples

To 1.00- or 0.50-ml samples of plasma were added 0.50 ml of internal standard solution (100 μg/ml indobufen in water), 100 μl of 0.6 *M* sulphuric acid and some 0.04 g of sodium chloride. The samples were extracted with 4 ml of diethyl ether on a Hook and Tucker rotamixer and the solvent layers were separated by centrifugation at 1200 *g*. The organic layer was transferred to another tube and the solvent was evaporated on a sand bath ($30 \pm 2°$C) under a stream of dry air. Five drops of toluene were then added and evaporated in the same way to remove traces of water.

The residue was taken up in 200 μl of 50 m*M* triethylamine—acetonitrile solution. To this mixture were added in sequence, at 30 sec intervals, 100 μl each of; (1) a 60 m*M* solution of ethyl chloroformate in acetonitrile and (2) a solution of leucinamide hydrochloride (1 *M*) and triethylamine (1 *M*) in methanol. After 2 min 0.5 ml of 0.25 *M* hydrochloric acid was added and the derivatives were extracted with 4 ml of ethyl acetate. The solvent was evaporated as above, and the residue was taken up in 0.5 ml of methanol; 10 μl of this solution were injected into the chromatograph.

Standard curves and precision

Plasma samples spiked with (racemic) indoprofen to concentrations of 3.13, 6.25, 12.5, 25 and 50 μg/ml (11.1—178 μ*M*) were analysed in duplicate by the above method, and standard curves were drawn using peak heights for quantitation. The precision of the method was assessed by the analysis of eight samples spiked with 6.25 μg/ml and of another eight spiked with 25 μg/ml racemic indoprofen.

Extraction yield

Plasma samples (1.0 ml) were spiked with 12.5 μg of indoprofen and extracted with 4.00 ml of diethyl ether by the standard method. Of the organic phase, 2.00 ml were transferred to another tube, 20 μg of indobufen in 0.50 ml of methanol were added and the solvents were evaporated. The residues were taken up in 0.5 ml of methanol and analysed by HPLC with the mobile phase methanol—0.01 *M* phosphate buffer, pH 3.5 (60:40). In this system, the k' value of indoprofen is 2.7 and that of indobufen 4.4. A mixture of 6.25 μg of indoprofen and 20 μg of indobufen in methanol—water, prepared from the same stock solutions, served as reference.

Derivatization reaction

The two steps in the derivatization reaction, i.e. the formation of a mixed

anhydride of indoprofen and ethyl chloroformate and its conversion to an amide by reaction with leucinamide, were investigated separately.

Step 1. Samples of pure indoprofen (100 μg) were dissolved in 200 μl of 60 m*M* triethylamine in acetonitrile and treated with ethyl chloroformate solution as described above. After 15, 30, 60 or 120 sec, 100 μl of 0.5 *M* ammonia were added. Then, 5 min later the mixture was acidified with 0.5 ml of 0.25 *M* hydrochloric acid and the products were extracted with 4 ml of diethyl ether—hexane (1:1). The residue on evaporation of the organic phase was analysed by HPLC with the mobile phase methanol—0.01 *M* phosphate buffer, pH 3.5 (60:40). The k' value of the reaction product, indoprofenamide, in this system is 1.5, while indoprofen appears at $k' = 2.7$ and the mixed anhydride at $k' = 11$.

Step 2. Samples of pure indoprofen (100 μg) were derivatized by the standard procedure, with the modification that the 0.25 *M* hydrochloric acid, which stops the second reaction step, was added 15, 30, 60, 120 or 240 sec after the leucinamide solution (duplicate samples for each reaction time). Before the extraction with ethyl acetate, a solution of indobufen leucinamide derivative in ethyl acetate (0.20 mg/ml, 0.50 ml) was added as internal standard. Quantitation of the indoprofen derivatives was by the standard HPLC system. Samples of pure indobufen (100 μg) were treated in the same way and indoprofen leucinamide derivative was used as internal standard.

Finally, the overall reaction was checked, after derivatization of pure indoprofen or indobufen samples or of plasma extracts, by thin-layer chromatography (TLC) on silica plates with the mobile phase chloroform—methanol—15% ammonia solution (85:15:2). In this system, the R_F values of the pertinent compounds are: indoprofen and indobufen 0.08, the leucinamide derivatives of (—)-indoprofen 0.46, of (+)-indoprofen 0.51 and of the indobufen enantiomers 0.49 and 0.54.

The possibility of racemization during the reaction was checked by the derivatization and analysis of pure (> 98%) (+)-indoprofen.

RESULTS

Chromatograms

Typical chromatograms of indoprofen and indobufen leucinamide derivatives are shown in Fig. 1. The k' values of the indoprofen derivatives are 3.89 (—) and 4.67 (+), which gives a separation factor, $\alpha = k'_{(+)}/k'_{(-)}$, of 1.20. The corresponding values for the indobufen derivatives are 5.56, 7.56 and 1.36. The assignment of the peaks is unambiguous for the indoprofen derivatives, since the pure (+)-isomer was available. For the indobufen derivatives, it is tentative. There is, however, very little reason to believe that the lengthening of the indoprofen carbon chain by one methylene group to give indobufen would reverse the order of elution of the diastereoisomeric derivatives.

Standard curves and precision

The standard curves of the two indoprofen isomers were calculated as the peak height ratios of (—)-indoprofen to (—)-indobufen and (+)-indoprofen to (+)-indobufen versus indoprofen concentration. The correlation coefficient r was generally > 0.999 for a five-point standard curve.

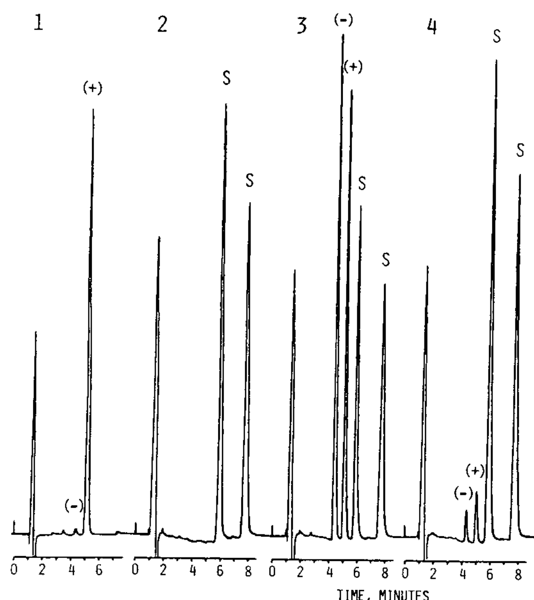

Fig. 1. Chromatograms of derivatized samples. (1) (+)-Indoprofen with 1.4% (—)-enantiomer. (2—4) Plasma samples with internal standard added. (2) A plasma sample taken from a patient before indoprofen administration. (3) A plasma sample taken 15 min after the intravenous administration of indoprofen. The concentrations are (—)-enantiomer 29.8 μg/ml and (+)-enantiomer 31.3 μg/ml. (4) A plasma sample taken 10 h after the administration of indoprofen. The concentrations are (—)-enantiomer 1.3 μg/ml and (+)-enantiomer 2.5 μg/ml. In chromatogram 3, the injected volume was 7 μl, as opposed to the usual 10 μl. Detector: 0.05 a.u.f.s. Recorder: 10 mV, 5 mm/min. Peak designations for the leucinamide derivatives: (—) = (—)-indoprofen, (+) = (+)-indoprofen and S = any of the indobufen enantiomers (internal standard).

The eight samples spiked with 6.25 μg/ml indoprofen showed (mean ± S.D. and relative S.D.): (—)-indoprofen 3.12 ± 0.038 μg/ml (1.2%), (+)-indoprofen 3.14 ± 0.031 μg/ml (1.0%), and those spiked with 25 μg/ml showed (—)-indoprofen 12.6 ± 0.16 μg/ml (1.3%) and (+)-indoprofen 12.6 ± 0.14 μg/ml (1.1%). The sensitivity of the method is limited by small interference peaks from blank plasma, which at the outside correspond to 0.05—0.1 μg/ml of each isomer.

Changing from UV to fluorescence detection did not improve the sensitivity of the analysis.

Extraction yield

The recovery of indoprofen from plasma was 90 ± 2% at a concentration of 12.5 μg/ml.

Derivatization reaction

Step 1. The chromatograms obtained in this experiment showed single peaks due to indoprofenamide regardless of reaction time. It can be concluded that the formation of mixed anhydride is complete within 15 sec.

Step 2. The peak ratios of (—)-indoprofen derivative to added (—)-indobufen derivative and of (+)-indoprofen derivative to added (+)-indobufen derivative

were constant (within ± 2%) at all reaction times from 1 min onwards. With 15-sec reaction time peak ratios had reached 93% of the final values and with 30-sec reaction time 97%. The derivatization of indobufen gave quite similar results. It can be concluded that 2 min is ample time for the completion of the second reaction step.

TLC analysis of the final products showed complete conversion of indoprofen and indobufen to leucinamide derivatives.

Derivatization and HPLC analysis of (+)-indoprofen gave peaks (Fig. 1) corresponding to 1.4% (—)-indoprofen and 98.6% (+)-indoprofen, which is consistent with the certificate of analysis provided by Farmitalia Carlo Erba. There was consequently no detectable racemization during the reaction.

Mass spectrum

The methane-ionization mass spectrum confirmed the structure of the indoprofen leucinamide derivative. The pseudomolecular ion $[M + H]^+$ appeared as expected at *m/z* 394, and it was accompanied by a $[M + C_2H_5]^+$ ion at *m/z* 422. Cleavage of an —OH group ($[M + H]^+ - 17$) and of —COOH ($[M + H]^+ - 45$) from the protonated molecular ion was also observed.

Pharmacokinetics of (+)- and (—)-indoprofen in two patients

Fig. 2 shows the plasma concentration curves of the indoprofen enantiomers after intravenous administration of the racemate to two patients. The main pharmacokinetic parameters, calculated by standard procedures [18], are shown in Table I. There is a consistent large difference in distribution volume and clearance between the two enantiomers which gives the active (+)-isomer a slightly longer half-life.

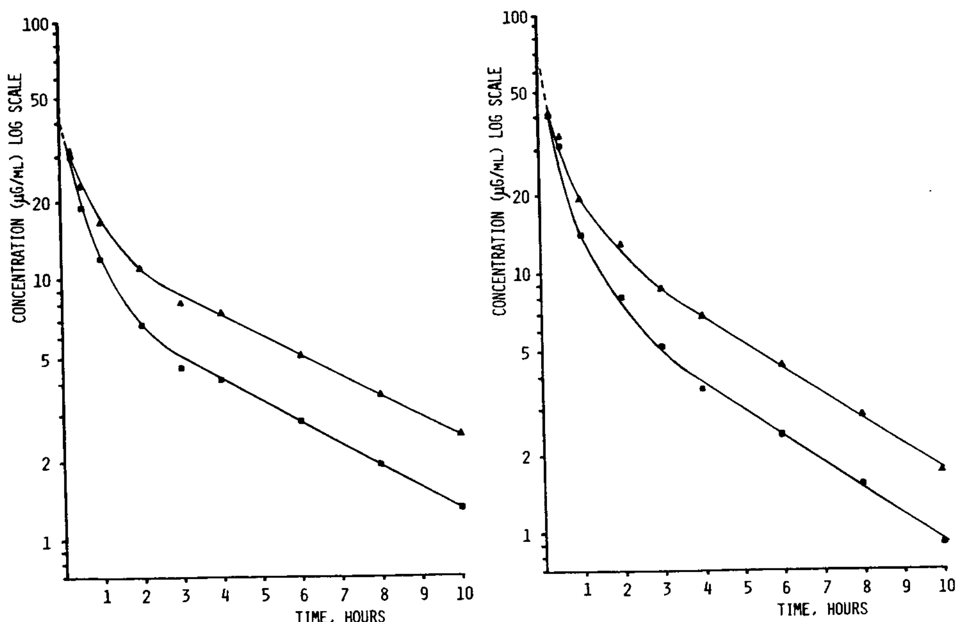

Fig. 2. Plasma concentration—time curves of the indoprofen enantiomers in patients S.A. (female, 70 kg) and K.S. (male, 87 kg) after 400 mg of racemic indoprofen. The upper curves represent the (+)-enantiomer.

TABLE I

PHARMACOKINETIC DATA OF THE INDOPROFEN ENANTIOMERS

	Clearance (ml/min)	Distribution volume (l/kg body weight)	Terminal half-life (min)
Patient S.A. (F, 70 kg)			
(—)-Indoprofen	52.5	0.22	205
(+)-Indoprofen	34.0	0.16	231
Ratio of values	1.54	1.37	0.89
Patient K.S. (M, 87 kg)			
(—)-Indoprofen	48.2	0.14	172
(+)-Indoprofen	34.1	0.10	184
Ratio of values	1.41	1.32	0.93

DISCUSSION

Ethyl chloroformate is a well known reagent for amide formation in peptide synthesis [19]. Apparently, this versatile reagent has not previously been used in this capacity in analytical chemistry. Its main advantage is that the by-products of the reaction are carbon dioxide and ethanol, which, as the reagent itself, are volatile and non-UV-absorbing. It can therefore without difficulty be used in a very large excess to give a rapid reaction.

Non-steroidal anti-inflammatory drugs of the 2-arylpropionic acid type can be analysed stereoselectively by GLC or HPLC. All methods reported so far are based on derivatization, which in GLC is imperative also in order to make the acids volatile. Separation of the diastereoisomeric derivatives is generally no great problem, and the amine component of the amide derivatives can be chosen so as to give good GLC properties [11, 12] or high UV absorption or fluorescence in HPLC [16]. In the present case, volatility was not the object, and since sufficient sensitivity could be attained by UV detection of the indoprofen chromophore, a readily available, non-UV-absorbing amine was chosen. The excess of reagent, ethyl chloroformate, in the first reaction step is approximately ten- to forty-fold, depending on the amount of indoprofen in the sample. On the addition of leucinamide in a seventeen-fold excess over ethyl chloroformate, N^{α}-ethoxycarbonylleucinamide will be formed along with the indoprofen/indobufen derivatives. This by-product is not UV-absorbing at 275 nm and will consequently not be visible in the chromatogram. The excess of leucinamide will, in the final extraction, stay in the acidic aqueous phase.

Several racemic NSAIDs have also been resolved as derivatives with achiral amines on an HPLC column with a chiral stationary phase [20]. Since this analysis also requires a derivatization step, and in addition gives small separation factors, it has no major advantages over the previously discussed methods.

It is also possible to separate the enantiomers of underivatized chiral acids as ion pairs with quinine on straight-phase HPLC [21]. An experiment preliminary to this work showed that the indoprofen enantiomers could be separated with a separation factor of 1.12 on a LiChrosorb DIOL column eluted with 0.5 m*M* quinine acetate in 0.1% pentanol—dichloromethane. The

quinine, however, gave a detector background signal of 12 mV as compared to a 0.8-mV signal due to 0.4 μg of injected (+)-indoprofen. The method was therefore not regarded as feasible for pharmacokinetic work.

In conclusion, a rapid chromatographic method, based on the use of ethyl chloroformate as amide-forming reagent, is described for the quantitation of the indoprofen enantiomers in blood plasma. The sensitivity and precision of the method are satisfactory for pharmacokinetic work. There is also a potential for enhanced sensitivity if a UV-absorbing or fluorescent amine component is chosen.

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CHROMBIO. 2500

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR DETERMINATION OF CIPROFLOXACIN IN BIOLOGICAL FLUIDS

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SUMMARY

A simple and precise high-performance liquid chromatographic procedure has been developed for the determination in biological fluids of ciprofloxacin, a new, with extended antibacterial spectrum, quinoline carboxylic acid. The work-up procedure involves a chemical extraction step followed by isocratic chromatography on a reversed-phase analytical column, with ultraviolet detection. The detection limit for blood levels is 10 ng/ml. The calibration curve is linear from this detection limit to 10 μg/ml. The statistical analysis of the correlation made between this assay and an agar diffusion procedure during a pharmacokinetic study suggests the existence of one or more active metabolites which could be mainly excreted in the bile.

INTRODUCTION

Quinoline carboxylic acids such as nalidixic and pipemidic acids have been available for years. The narrow spectrum of these antibiotics, and the low

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concentrations obtained in serum limit their use essentially against Gram-negative enteric bacilli encountered in urinary tract infections [1].

Enoxacin [2], AT 2266, a new pyridone carboxylic anti-pseudomonal agent [3], ofloxacin [4], norfloxacin [5—8], pefloxacin [9], cinoxacin [10] and ciprofloxacin [11—13] are recently developed quinoline derivatives that are structurally related to nalidixic acid. They show a greater potency and broader antibacterial spectrum which includes Gram-negative and Gram-positive bacteria. Numerous in vitro studies have shown their activity against *Enterobacteriaceae*, *Staphylococcus* spp., *Pseudomonas aeruginosa*, *Haemophilus* spp., *Neisseria* spp., *Bacteroïdes fragilis*, *Acinetobacter calcoaceticum* and *Listeria monocytogenes* [1—13]. Although they have a parallel spectrum of activity, ciprofloxacin seems to be more potent than the other new quinoline derivatives [10, 14—17].

As further pharmacokinetic investigations are needed, particularly on the possibility of treating systemic infections, we have developed a rapid, accurate and specific assay for the measurement of this antibiotic in human serum, bile and urine, by means of reversed-phase high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Chemicals

Ciprofloxacin (Bay 09867) was obtained from Bayer Pharma, France. Stock solutions of 10 mg/ml were prepared in double-distilled water. Acetonitrile, methylene chloride, tetrabutylammonium bromide and phosphoric acid were purchased from E. Merck, Darmstadt, F.R.G. Water was daily double-distilled in quartz.

For the establishment of the calibration curves, serum, urine and bile free of any antibiotic were obtained from patients before any treatment with ciprofloxacin.

Sample treatment

An aliquot (500 μl) of biological fluid (serum, urine diluted 1:20 or bile diluted 1:10) is added to 3.5 ml of methylene chloride in a 6-ml glass screw-capped tube. After mixing for a few seconds on a vortex mixer, the tubes are gently shaken for 10 min by rotation (20 rpm) and then centrifuged for 10 min at 1000 *g*. The upper aqueous layer is discarded by aspiration, and 3 ml of the lower organic phase are transferred to a second glass screw-capped tube. Ciprofloxacin is then back-extracted using 200 μl of phosphoric acid at pH 2 by rotation for 30 min. Centrifugation at 1000 *g* for 10 min, results in phase separation; 20 μl of the upper aqueous phase are then injected into the liquid chromatograph.

Establishment of the standard curves

Sera free of antibiotics were spiked with increasing amounts of ciprofloxacin (10, 20, 50, 100, 200 ng/ml and 0.5, 1, 2, 5, 10 μg/ml). These sera were then submitted to HPLC analysis and peak heights were plotted against the concentrations of drug to check for linearity. The same procedures

were followed for urine and bile, except for the amounts added yielding concentrations ranging from 10 to 200 μg/ml and from 1 to 200 μg/ml, respectively.

Chromatographic conditions

The isocratic liquid chromatograph was constituted from the following units: a 112 solvent delivery module (Beckman, Berkeley, CA, U.S.A.), a 210 sample injection valve equipped with a 20-μl loop (Beckman), a Model 160 selectable-wavelength detector (Beckman) and a Model ICR 1 B recording data processor (Intersmat Instruments, Courtry, France). Separations were performed on a 150 × 4.6 mm, C_{18} reversed-phase analytical column, particle size 5 μm (Ultrasphere, Beckman).

The mobile phase consisted of acetonitrile—0.005 *M* tetrabutylammonium bromide (10:90). Phosphoric acid (14.6 *M*) was then added to adjust the pH to 2. The mobile phase was filtered through a 0.22-μm membrane. The flow-rate was set at 2 ml/min and the eluent monitored at 254 nm. The range setting of the spectrophotometer depended on the concentration of drug measured.

Precision

Serum, urine and bile free of antibiotic were spiked with known amounts of ciprofloxacin for the study of reproducibility.

Within-day reproducibility. Ten aliquots of a serum containing 0.5 μg/ml and ten aliquots of a serum containing 1.5 μg/ml ciprofloxacin were randomly distributed in different series of assays, on the same day, during a pharmacokinetic study. The concentrations of drug tested with bile and urine for the within-day reproducibility were 10 and 20 μg/ml, respectively.

Between-day reproducibility. Ten aliquots of the same biological fluids as those used for the within-day study were assayed one by one during ten days, using each time the calibration curve of the day. For both within-day and between-day reproducibilities, precision was evaluated by calculating the coefficients of variation.

Microbiological assay procedure

The quantitative determination of microbiologically active drug in plasma, bile and urine was made by an agar diffusion assay. The reference strain used was *Escherichia coli* ICB No. 4004 (Bayer, Wuppertal, F.R.G.). Isotonic sensitest agar (Oxoïd) medium was prepared according to the manufacturer's instructions and poured onto 20 cm × 20 cm assay plates (about 80 ml per plate). The final agar thickness was 0.2 cm on the plates. After cooling the agar, 2-mm diameter wells were punched. The arrangement of the punched holes and the positioning of the different samples were randomized. An exactly measured volume of 5 μl of sample was dispended into each well. Serum standard concentration (5, 2.5, 1.2, 0.6, 0.3 μg/ml) are obtained by diluting the stock solution with serum free of any antibiotic. For urine and bile standard concentrations (10, 5, 2.5, 1.2, 0.6 μg/ml) the dilutions were made with buffered saline at pH 7.2. Each sample was run in triplicate. Results were read after an overnight incubation at 37°C. Inhibition zones were measured with the aid of a light projector.

Statistical analysis

The comparison between the two methods was made by orthogonal regression analysis with all of the data for each of the three series of body fluids, i.e. serum, urine and bile. All the samples were obtained during a pharmacokinetic study. For each of these body fluids, the mean difference of all paired values was compared with a difference of zero, using Student's t test for the comparison of two means. An α value of 0.05 was retained as the threshold point for statistical significance.

RESULTS

Typical chromatograms resulting from the analysis of various body fluids are shown in Figs. 1 and 2. Ciprofloxacin appears as a well resolved peak, the retention time of which is 2.4 min. During our pharmacokinetic study, no interference owing to endogenous substances or to co-administered drugs could be detected. These clinical specimens were known to contain one or more of the following drugs: analgesics, salicylate, phenobarbital, carbamazepine,

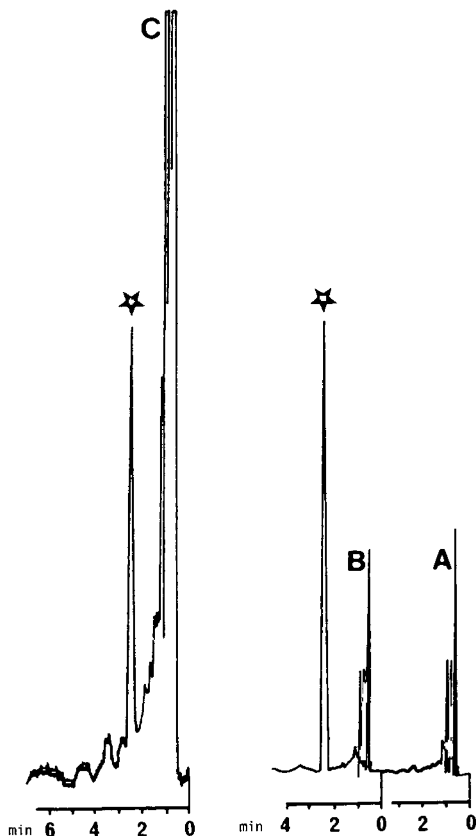

Fig. 1. HPLC profiles of: (A) an extracted human serum before any treatment with ciprofloxacin, at 0.05 a.u.f.s.; (B) an extracted human serum containing 1 μg/ml ciprofloxacin (★) at 0.05 a.u.f.s.; (C) an extracted human serum containing 150 ng/ml ciprofloxacin (★) at 0.005 a.u.f.s. Detector wavelength: 254 nm; chart speed: 0.5 cm/min.

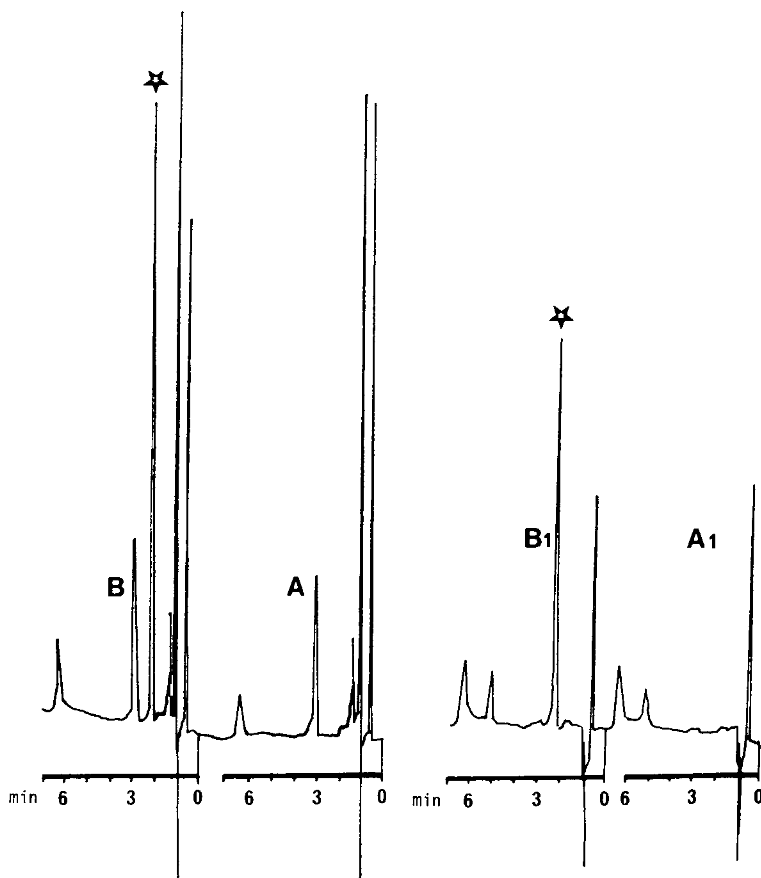

Fig. 2. HPLC profiles of: (A1) human bile before any administration of ciprofloxacin, at 0.05 a.u.f.s.; (B1) human bile from a patient after oral absorption of 500 mg of ciprofloxacin (concentration of this 1:10 diluted bile = 0.9 μg/ml), at 0.05 a.u.f.s. (⋆); (A) human urine before any administration of ciprofloxacin, at 0.05 a.u.f.s.; (B) human urine from a patient after oral absorption of 500 mg of ciprofloxacin (concentration of this 1:20 diluted bile = 1.15 μg/ml), at 0.05 a.u.f.s. (⋆). Detector wavelength: 254 nm; chart-speed: 0.5 cm/min.

phenytoin, primidone, valproic acid, digoxin, quinidine, procainamide, lidocaine, theophylline, digitoxin and furosemide. The limit of detection of our procedure was 10 ng/ml for blood levels, resulting in a signal-to-noise ratio of 4:1. For the urinary and the biliary levels, the detection limits were, respectively, 500 and 200 ng/ml. A profile of drug concentration versus time is depicted in Fig. 3 for two patients after an orally administered dose of 500 mg of ciprofloxacin.

Recovery study

Ten sera spiked with known amounts of ciprofloxacin were chromatographed as described in Materials and methods, and the resulting peak heights were compared with the peaks resulting from aqueous solutions of the same amounts. The double extraction described led to a concentration of ciprofloxacin of 175 ± 6% (n = 10).

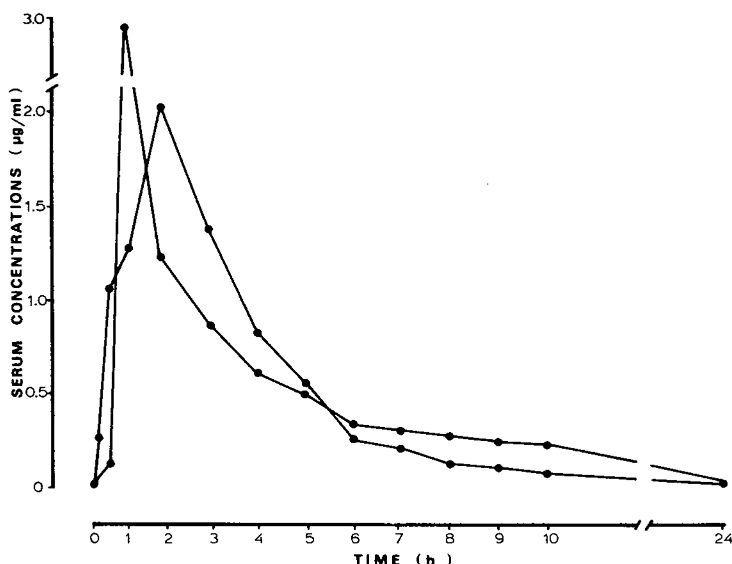

Fig. 3. Profile of drug concentration versus time in two patients after an orally administered dose of 500 mg of ciprofloxacin. Assays were done by HPLC.

Linearity

The study of the linearity was carried out with concentrations ranging from 10 ng/ml to 10 µg/ml. Three detector sensitivity ranges were employed: in the first step, the serum concentrations ranged from 10 to 200 ng/ml and these sera were chromatographed at 0.001 absorbance units full scale (a.u.f.s.) sensitivity range; in the second step, concentrations ranged from 0.2 to 2 µg/ml and the detector was set at 0.01 a.u.f.s.; in the last step, concentrations ranged from 2 to 10 µg/ml and the detector was set at 0.05 a.u.f.s. The analytical procedure looked linear whatever the range setting of the spectrophotometer. The three lines corresponding to the three steps above parallel very well. The sample containing 200 ng/ml has been assayed at 0.001 and 0.01 a.u.f.s., and the sample

TABLE I

PRECISION OF THE CHROMATOGRAPHIC ASSAY

Sample	Concentration (µg/ml)	Coefficient of variation (n = 10) (%)
Within-day reproducibility		
Serum	0.5	5.2
	1.5	4.3
Urine	20	8.2
Bile	10	8.6
Between-day reproducibility		
Serum	0.5	7.2
	1.5	6.5
Urine	20	10.2
Bile	10	10.8

spiked with 2 μg/ml ciprofloxacin has been chromatographed at 0.01 and 0.05 a.u.f.s. In both cases, the product of the peak height and the sensitivity range is constant. The correlation coefficient obtained between the peak heights and the ciprofloxacin concentrations for the three steps together was $r = 0.997$.

Precision

The results of replicate analyses of spiked serum are given in Table I. The coefficients of variation range from 4.3% for serum to 8.6% for bile for the within-day reproducibility, and from 6.5% to 10.8% for the between-day reproducibility.

Accuracy

Our chromatographic procedure has been compared with a reference method, i.e. the microbiological assay as described in Materials and methods. The degree of correlation between both assays should indicate the accuracy of the chromatographic procedure.

Serum. Analysis of the orthogonal linear regression for a series of 135 samples resulted in a correlation coefficient of $r = 0.932$. It resulted in the following equation (Fig. 4): y (= agar diffusion) = $1.02x$ (= HPLC) + 0.05. For the paired values of ciprofloxacin concentrations, the mean difference (agar diffusion − HPLC) compared to a difference of zero did not yield to a statistically significant value: $\epsilon = 0.789$ and $\alpha = 0.43$ (threshold point for significance: $\alpha = 0.05$). Thus, the concentrations as measured by both assays are statistically identical.

Urine. For the urines, the correlation coefficient resulted from the analysis of 35 samples (Fig. 5): $r = 0.979$. The calculated equation is as follows: y (= agar diffusion) = $0.91x$ (= HPLC) + 16.62. The statistical analysis of the paired values (agar diffusion − HPLC) showed no statistically significant difference: $\epsilon = 1.37$ and $\alpha = 0.18$.

Bile. The analysis of the levels of ciprofloxacin measured by both procedures

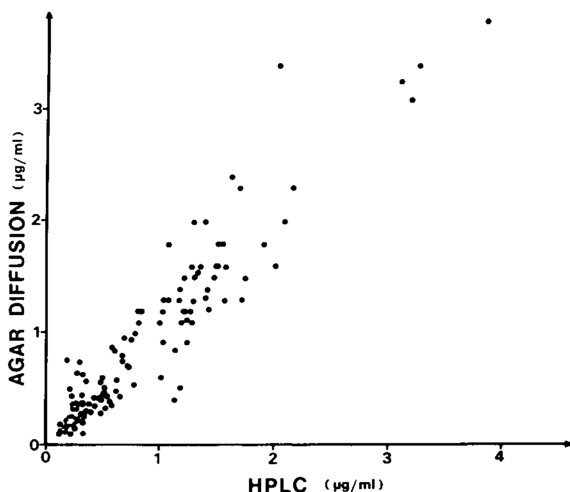

Fig. 4. Correlation of agar diffusion and HPLC results of all the serum samples assayed (n = 135); $r = 0.932$. Orthogonal regression equation: $y = 1.02x + 0.05$.

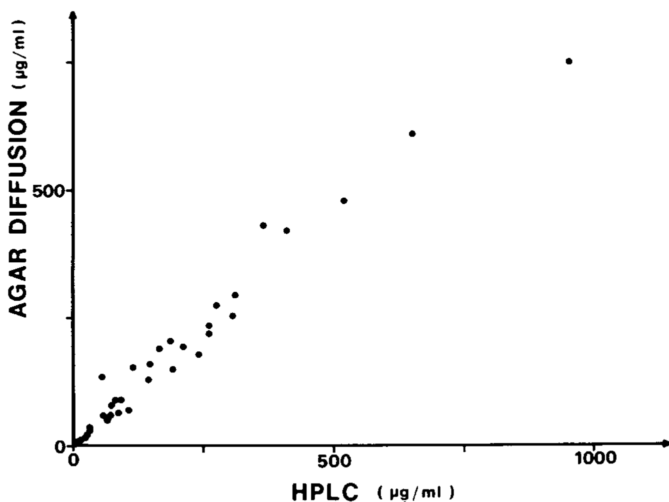

Fig. 5. Correlation of agar diffusion and HPLC results from all the urine samples assayed (n = 35); r = 0.979. Orthogonal regression equation: $y = 0.91x + 16.62$.

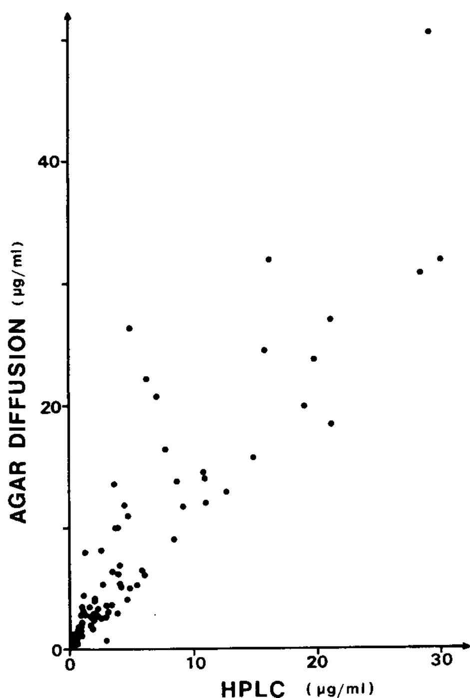

Fig. 6. Correlation of agar diffusion and HPLC results from all the bile samples assayed (n = 86); r = 0.900. Orthogonal regression equation: $y = 1.27x + 1.48$.

in bile yielded different data than those obtained for serum or urine. The coefficient of variation calculated with 86 samples was less good: r = 0.900 (Fig. 6). The following equation is obtained: y (= agar diffusion) = $1.27x$ (= HPLC) + 1.48. Among the 86 samples measured, 69 gave higher values when

measured by the agar diffusion assay compared to HPLC. The comparison of the mean difference for the paired values with a difference of zero yielded a statistically highly significant result: $\epsilon = 5.35$ and $\alpha = 0.000001$. The microbiologically measured levels were higher than those measured chromatographically.

DISCUSSION

High-performance liquid chromatography has already been used for monitoring of quinoline carboxylic acids in biological fluids [9, 18–24]. Up to present, no chromatographic procedure has been described for ciprofloxacin. The assay proposed here is simple, rapid and uses ultraviolet detection for quantitation. Ciprofloxacin shows good absorbance at 254 nm and thus allowed us to avoid using fluorimetric detection which needs more expensive equipment.

Our chromatographic conditions led to chromatograms that appeared "clean" and were similar to those obtained by different authors for nalidixic acid [18], pipemidic acid [21], flumequine, [23], miloxacin [20], rosoxacin [19], norfloxacin [22, 23], and pefloxacin [9]. Among these authors, many used ion-exchange chromatography [18, 20, 22, 24]. Reversed-phase chromatography on a C_{18} bonded silica matrix seemed to us more reproducible [9]. Nevertheless, conventional reversed-phase HPLC never gave us full satisfaction. Indeed, we carried out various assays with "classical" mobile phases generally used in reversed-phase HPLC, e.g. a mixture of water or salt solution with a non-polar organic solvent, and every time it resulted in severe peak tailing of ciprofloxacin, whatever the pH or the ionic strength. Finally, using ion-pairing chromatography with tetrabutylammonium bromide, an excellent resolution of ciprofloxacin was obtained.

Initial attempts to prepare protein-free filtrates by deproteinization with an organic solvent (acetonitrile, methanol) gave good results for many β-lactam antibiotics [25], but caused partial co-precipitation of ciprofloxacin. Cartridge chromatographic techniques (Waters C_{18} Sep-Pak^{TM}) yielded clean chromatograms, but the resulting dilution of the samples lowered the detection limit.

Although no internal standard is used, the coefficients of variation and the linearity are in quite good agreement with those required in hospital routine use or for pharmacokinetic studies, for which the ten times lower detection limit of the HPLC procedure compared to agar diffusion procedure may be very interesting.

Correlation with microbiological assay

Quinoline carboxylic acids are well known to be metabolized in large part [9, 19, 20, 22, 23]. Some of the potential metabolites may present an important antibacterial activity, as is found for norfloxacin, a major metabolite of pefloxacin [9]. When one assays levels in biological fluids using an agar diffusion method, there is a risk of overestimation of drug concentration.

One or more metabolites may exist and could be potentially active. A previous pharmacokinetic study of ciprofloxacin [26] carried out using a microbiological assay revealed that 30% of the dose administered was recovered

as unchanged drug in the urine. This means that metabolism of the drug may occur. We found that the correlation was good between the two assay methods for serum and urine. It is less evident for biliary levels, for which a highly significant difference was found between agar diffusion and HPLC. This difference may be explained by the existence of an active metabolite which could be eliminated in bile. Thus, the agar diffusion test would have assayed the entire antibacterial activity, when HPLC assayed only ciprofloxacin.

To establish the chromatographic condition for assaying the metabolite(s) we need to have them as pure powder. Work is currently being undertaken to obtain these pure substances.

The absence of a significant difference in serum and urine should not exclude the possibility of the existence of a non-active metabolite, or an active metabolite present in these fluids at too low a concentration to be measured by agar diffusion.

Further investigations may be started in the future to determine pharmacokinetics of ciprofloxacin. In this respect, it is important to use a specific and accurate assay technique. The procedure described above may be an interesting alternative.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF CEFONICID IN HUMAN PLASMA AND URINE

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SUMMARY

A high-performance liquid chromatographic assay for determination of cefonicid concentrations in human plasma and urine samples has been developed using cefazolin as an internal standard. For the analysis of plasma samples two calibration curves were utilized covering the cefonicid concentration ranges of 0.05—1.0 μg/ml and 1.0—50.0 μg/ml, respectively. Coefficients of variation of 7.4% or less were obtained for cefonicid concentrations of 0.05—50.0 μg/ml. Mean bias was +6.0% at 0.05 μg/ml cefonicid and between —2.1% and +1.6% for 1.0—50.0 μg/ml cefonicid. Plasma samples containing 30 ng/ml cefonicid could be well distinguished from blank plasma samples. Urine samples were analysed by using a calibration curve for cefonicid concentrations between 1.0 and 50.0 μg/ml. Coefficients of variation ranged from 8.6% at a cefonicid concentration of 1.0 μg/ml to 0.5% at 50.0 μg/ml with a mean bias between —3.0% and +0.3%.

INTRODUCTION

Cefonicid is a new parenterally active cephalosporin which has a prolonged plasma elimination half-life ($t_{1/2}$) of 3.5 h and of 4.8 h after intravenous and intramuscular administration [1], respectively, when compared to cephalothin, cefazolin, cefamandole or cefoxitin with a respective $t_{1/2}$ of 0.5 h, 1.8 h, 50 min and 40 min to 1.0 h [2—4].

The pharmacokinetics of cefonicid was determined by microbiological assay [1, 5—8] for analysis of plasma, urine and tissue samples. However, the microbiological assay has two disadvantages. Firstly, the assay is time consuming as an incubation time of 18 h is required. Secondly, the assay has in principle no inherent potential for detection and quantitation of metabolic derivatives of the parent compound. For further intensive pharmacokinetic investigations of

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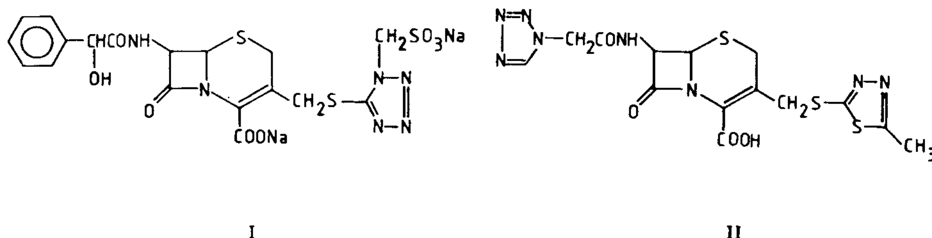
cefonicid a rapid, sensitive and specific assay was needed. Although several high-performance liquid chromatographic (HPLC) assays for various cephalosporins have been developed (ref. 9 and references cited therein), an assay for cefonicid using an HPLC technique has not been published yet. We report here an HPLC assay for plasma and urine samples sensitive enough to detect concentrations of 30 ng/ml.

EXPERIMENTAL

Materials

Cefonicid (I) was synthesized by Smith Kline & French Laboratories (Lot X-1-2X80). Cefazolin (II) was received from Fujisawa Pharmaceutical Co. (Lot 100110G). Methanol HPLC grade was delivered by J.T. Baker (Deventer, The Netherlands), acetonitrile (Chromasolv^{TM}) by Riedel-de-Haen (Seelze, F.R.G.) and triethylamine by Ega Chemie (Steinheim, F.R.G.). Human serum (Humanserum Biotest^{TM}) for preparation of standards was purchased from Biotest Pharma (Dreieich, F.R.G.). For the extraction of cefonicid from plasma or urine 1-ml Bond Elut^{TM} extraction columns (C_{18} bonded phase, part no. 607101) from Analytichem International (Harbor City, U.S.A.) were used.



I II

Apparatus and chromatographic conditions

The chromatographic system consisted of a M 6000 A pump from Waters Assoc. (Eschborn, F.R.G.), an Altex Ultrasphere ODS column (5 µm particle size, 250 × 4.6 mm I.D.) from Beckman Instruments (Munich, F.R.G.), a Spectroflow 773 variable-wavelength UV detector from Kratos (Karlsruhe, F.R.G.), an automatic sample injection system WISP 710 B (Waters Assoc.) and a Data Module integrator (Waters Assoc.). The wavelength of the UV detector was set at 265 nm. Two different mobile phases were used: phase A for the concentration range of 50.0—1.0 µg/ml cefonicid, and phase B for the concentration range 1.0—0.05 µg/ml cefonicid. Mobile phase A was composed of 85% 0.05 *M* phosphate buffer containing 0.01 *M* triethylamine (the pH of the mixture was adjusted to 7.2), 9% acetonitrile and 6% methanol. The range of the UV detector was set at 0.01 a.u.f.s. Mobile phase B consisted of 87% 0.05 *M* phosphate buffer containing 0.01 *M* triethylamine (pH of the mixture was adjusted to 5.0), 11% acetonitrile and 2% methanol. The range of the UV detector was set at 0.002 a.u.f.s.

Preparation of samples

Plasma samples. For conditioning the Bond Elut^{TM} extraction columns, 5 ml

of methanol and then 5 ml of water were passed through within 6 min. A plasma sample (500 μl) containing 50—1.0 μg/ml cefonicid was mixed with 50 μl of a solution of 55 μg/ml cefazolin in 0.05 *M* phosphate buffer pH 6.5 (this solution is stable in the refrigerator for two days). A 500-μl volume of this mixture was pipetted onto the conditioned extraction column and passed through within 1 min under suction. After washing the column with 500 μl of 0.05 *M* phosphate buffer pH 6.5, cefonicid and cefazolin were eluted with 300 μl of methanol of which 15 μl were injected into the HPLC system.

For assay of plasma samples containing 1.0—0.03 μg/ml cefonicid, 500 μl of the sample were mixed with 50 μl of a solution of 5.5 μg/ml cefazolin in 0.05 *M* phosphate buffer pH 6.5; 500 μl of this mixture were worked up as described above. The methanol eluate was then evaporated to dryness under nitrogen at ambient temperature. The residue was dissolved in 100 μl of 0.05 *M* phosphate buffer pH 6.5 and 25 μl of this solution were injected into the HPLC system.

Urine samples. Urine samples were diluted either 1:20 or 1:100 with 1.8 *M* aqueous urea solution containing 0.1 mol/l sodium chloride to result in cefonicid concentrations within the calibration range. A 500-μl volume of the diluted sample was mixed with 50 μl of a solution of 55 μg/ml cefazolin in 0.05 *M* phosphate buffer pH 6.5. Thereafter 500 μl of this mixture were worked up identically to plasma samples containing 50—1.0 μg/ml cefonicid (as described above).

Calibration curves for plasma samples

Standard solutions of 1 mg/ml, 250, 10, 5, and 1 μg/ml cefonicid in 0.05 *M* phosphate buffer pH 6.5 were prepared and stored in the refrigerator (the solutions are stable for two days). Appropriate volumes of these standard solutions were adjusted to 500 μl with human serum (human serum Biotest™ was used instead of pooled plasma as it is homogeneous and of a standardized quality) to result in cefonicid concentrations of 50.0—1.0 μg/ml and 1.0—0.05 μg/ml, respectively. The standards thus obtained were then worked up according to the procedure described for plasma samples and chromatographed. For each concentration range a linear regression line was calculated as a function of the peak area ratios of cefonicid to internal standard versus concentration units of cefonicid. These calibration curves were prepared daily.

Calibration curve for urine samples

Standard solutions of 1 mg/ml, 250 and 10 μg/ml in 0.05 *M* phosphate buffer pH 6.5 were prepared and stored in the referigerator. Appropriate volumes of these standard solutions were adjusted to 500 μl with a 1.8 *M* aqueous urea solution containing 0.1 mol/l sodium chloride, resulting in cefonicid concentrations of 50—1 μg/ml (the aqueous urea solution was taken for preparation of standards instead of water in order to use a matrix similar to urine). These standards were worked up according to the procedure described above and then chromatographed. A regression line was calculated as a function of the peak area ratios of cefonicid to internal standard versus concentration units of cefonicid. This calibration curve was prepared daily.

RESULTS AND DISCUSSION

Fig. 1 shows chromatograms of a blank plasma standard and of a 1.0 μg/ml cefonicid standard. These chromatograms demonstrate that the cefonicid peak is not completely separated to baseline from the preceding broad peak at 4.50 min. However, within the concentration range of 1.0—50.0 μg/ml cefonicid the influence of this interference peak is negligible. The calibration curve of peak area ratio was linear with a correlation coefficient of $r = 0.999$. Mean recoveries from spiked samples were 86% (n = 6, S.D. = ± 7%) at a cefonicid concentration of 1.0 μg/ml and 77% (n = 3, S.D. = ± 4%) at a concentration of 50.0 μg/ml by comparison with chromatograms of cefonicid standard solutions of equivalent concentrations.

The accuracy and precision of the HPLC assay for cefonicid in plasma was determined by adding known concentrations of 0.05, 1.0, 7.5, and 50.0 μg/ml cefonicid to blank human serum. The coefficients of variation ranged from 5.0 to 7.4%. Mean bias ranged from +6.0% at 0.05 μg/ml cefonicid to —2.1% at 7.5 μg/ml cefonicid (Table I).

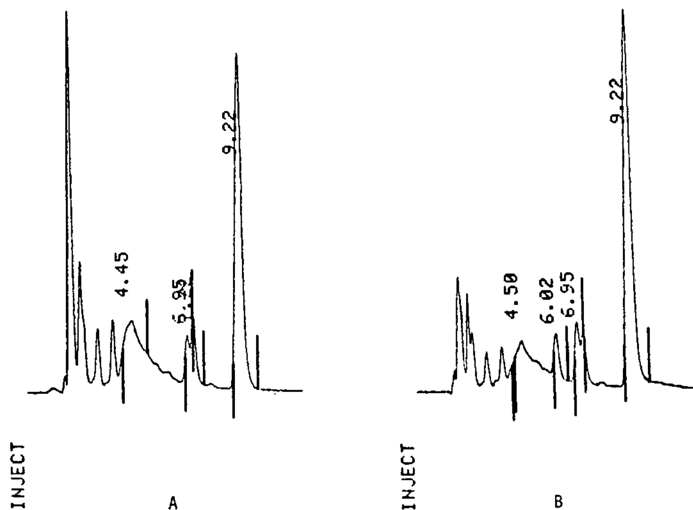


Fig. 1. Chromatograms of a blank plasma standard (A) and of a plasma standard containing 1.0 μg/ml cefonicid (B) with mobile phase A at 0.01 a.u.f.s. Peak at 6.02 min is cefonicid, peak at 9.22 min is internal standard.

TABLE I

ACCURACY AND PRECISION OF THE HPLC ASSAY FOR CEFONICID IN PLASMA

Plasma concentration of cefonicid (μg/ml)	n	Mean concentration found (μg/ml)	Coefficient of variation (%)	Bias (%)
0.05	4	0.053	7.4	+6.0
1.0	5	0.99	6.4	—1.0
7.5	5	7.34	5.0	—2.1
50.0	5	50.81	6.6	+1.6

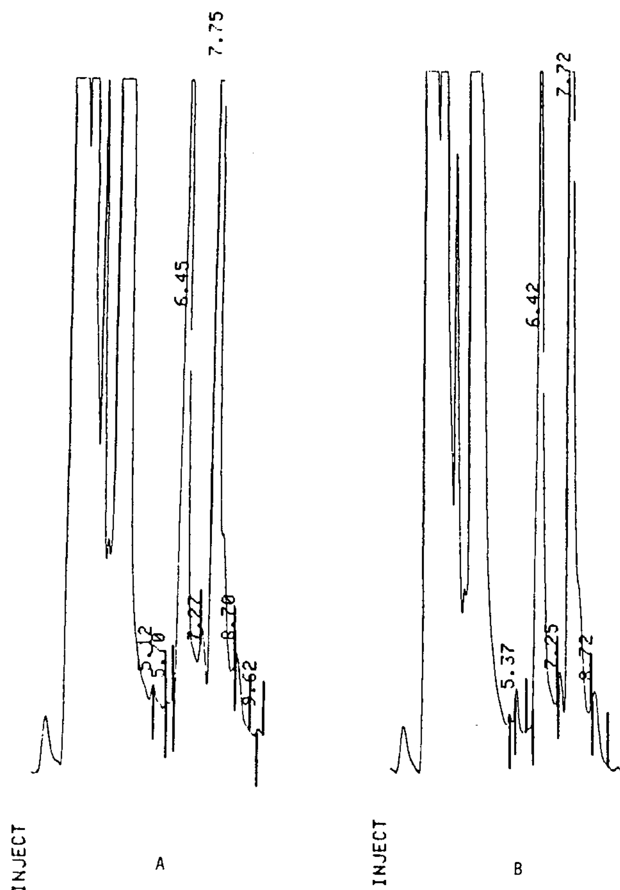


Fig. 2. Chromatograms of a blank plasma standard (A) and of a plasma standard containing 0.03 μg/ml cefonicid (B) with mobile phase B at 0.002 a.u.f.s. Peak at 5.37 min is cefonicid, peaks at 7.75 min and 7.72 min are internal standard.

Fig. 1 indicates that in chromatograms of plasma samples containing less than 1.0 μg/ml cefonicid the interference between the cefonicid peak and the preceding peak can not be neglected. For such cases, the mobile phase was slightly altered. Fig. 2 demonstrates that a concentration of 0.03 μg/ml can be well distinguished from a blank sample. The calibration curve of peak area ratio in the concentration range of 0.05—1.0 μg/ml was linear with a correlation coefficient of r = 0.999. However, at plasma concentrations above 1.0 μg/ml cefonicid, interference between the cefonicid peak and the following peak at 6.42 min could not be avoided. Therefore, mobile phase B can only be used for low cefonicid concentrations.

For analysis of cefonicid concentrations in urine a linear calibration curve was obtained within the concentration range of 1.0—50.0 μg/ml with a correlation coefficient of r = 0.999. Accuracy and precision data for cefonicid in urine are summarized in Table II. The coefficient of variation ranged from 0.5 to 8.6%. Mean bias varied from +0.3 to —3.0%.

This assay was used for the analysis of plasma and urine samples from a

TABLE II

ACCURACY AND PRECISION OF THE HPLC ASSAY FOR CEFONICID IN URINE

Urinary concentration of cefonicid (μg/ml)	*n*	Mean concentration found (μg/ml)	Coefficient of variation (%)	Bias (%)
1.0	5	0.97	8.6	−3.0
7.5	5	7.39	3.3	−1.6
50.0	4	50.17	0.5	+0.3

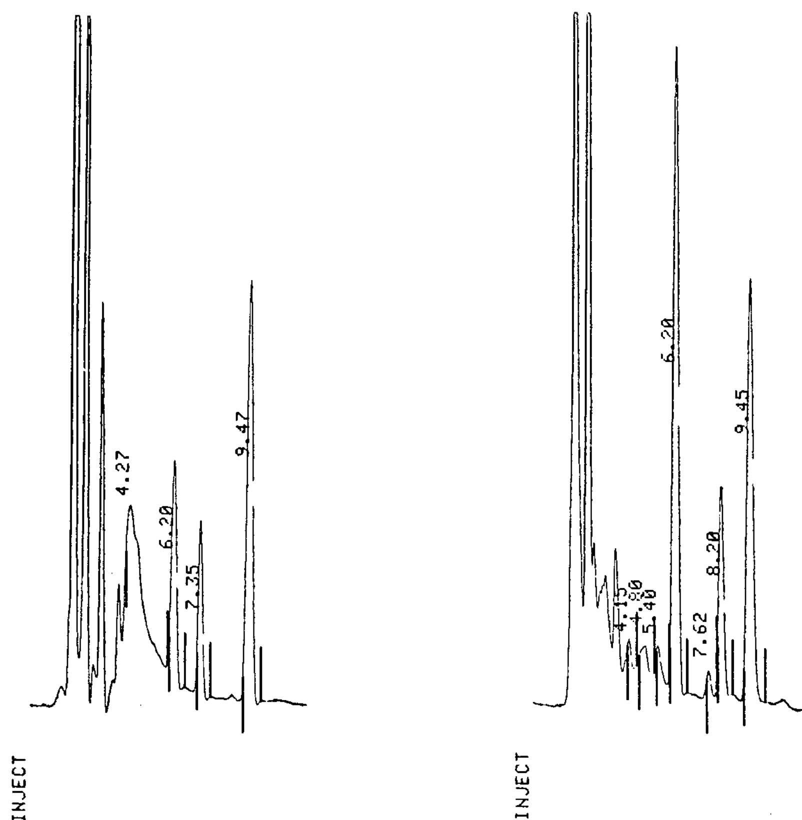


Fig. 3. Typical chromatogram of a plasma sample taken 12 h after intramuscular administration of 20 mg/kg body weight cefonicid to a healthy subject. Mobile phase A at 0.01 a.u.f.s. Peak at 6.20 min is cefonicid, peak at 9.47 min is internal standard. Cefonicid concentration = 4.6 μg/ml.

Fig. 4. Typical chromatogram of a urine sample (diluted 1:20 with 1.8 *M* aqueous urea solution) from the 12—24 h urine fraction after intravenous administration of 20 mg/kg body weight cefonicid. Mobile phase A at 0.01 a.u.f.s. Peak at 6.20 min is cefonicid, peak at 9.45 min is internal standard. Cefonicid concentration = 10.8 μg/ml.

24 h pharmacokinetic study in which either an intravenous or an intramuscular dose of 20 mg/kg body weight cefonicid was administered to healthy subjects. The results of this study are still to be published. Plasma concentrations of cefonicid ranged from about 140 μg/ml to about 1 μg/ml, indicating that in

general the calibration curve from 1.0 to 50.0 μg/ml can be used for most of the samples. Plasma samples containing concentrations of cefonicid higher than 50.0 μg/ml had to be diluted because otherwise the Bond Elut^{TM} columns did not extract cefonicid completely from the sample. Figs. 3 and 4 demonstrate typical chromatograms of a plasma sample and a urine sample, respectively.

The results indicate that the HPLC assay for cefonicid described in this paper is more sensitive than the microbiological assay for which a limit of determination of 0.4 μg/ml is described [7]. This HPLC assay is rapid and provides the possibility of analysing at least 40 samples plus standards during a normal working day. It may therefore be particularly useful for extensive pharmacokinetic studies.

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CHROMBIO. 2499

Note

Plasma amino acid analysis by gas chromatography

Removal of glucose interference

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Until recently it has been a widely held and accepted view, as judged by the number of publications, that amino acid analysis is best accomplished by the classical ion-exchange technique using an automatic amino acid analyser. Despite the high degree of sophistication attained, however, this method has the inherent disadvantages of requiring high initial capital outlay, high running costs, and of being capable of a very limited application, mainly that of amino acid analysis. Furthermore, the actual time for analysis of one sample is approximately 2—2½ h which, when added to the time required for sample preparation, is a major limiting factor in sample throughput.

Several research groups have adopted the alternative procedure based on gas chromatographic (GC) analysis which, in most cases, and in common with the classical ion-exchange technique, requires treatment of the plasma with a protein precipitant. Subsequently, deproteinised plasma must undergo a purification procedure prior to the synthesis of volatile amino acid derivatives necessary for GC. The method of choice for such purification has over the years been that of cation-exchange chromatography. The latter, however, has been used alone by some workers [1, 2] who avoid protein precipitation and directly clean-up a plasma sample pretreated with acetic acid. The limitations and disadvantages of the cation-exchange clean-up step have recently been reviewed [3]. Of particular relevance in this regard is the non-specific nature of this adopted procedure since it removes all anionic and non-charged species present in the plasma irrespective of whether or not they contribute to interference in the subsequent analysis. Furthermore, a wide diversity of protocols

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has been described for this procedure, some of which involve conditions that have been shown to be not only sub-optimal but also detrimental to accurate analysis. Thus, considerable doubt is thrown on the validity of quantitative results obtained from protocols involving this procedure. In this preliminary communication we demonstrate that an alternative specific approach based on the removal of a positively identified interfering compound [4] has been accomplished.

EXPERIMENTAL

Reagents

Pipecolic acid and hexokinase (as an ammonium sulphate suspension) were obtained from Sigma (St. Louis, MO, U.S.A.). ATP (disodium salt) was obtained from Boehringer Mannheim (F.R.G.).

Procedure

To plasma (500 μl) were added aqueous hexokinase solution (200 U in 200

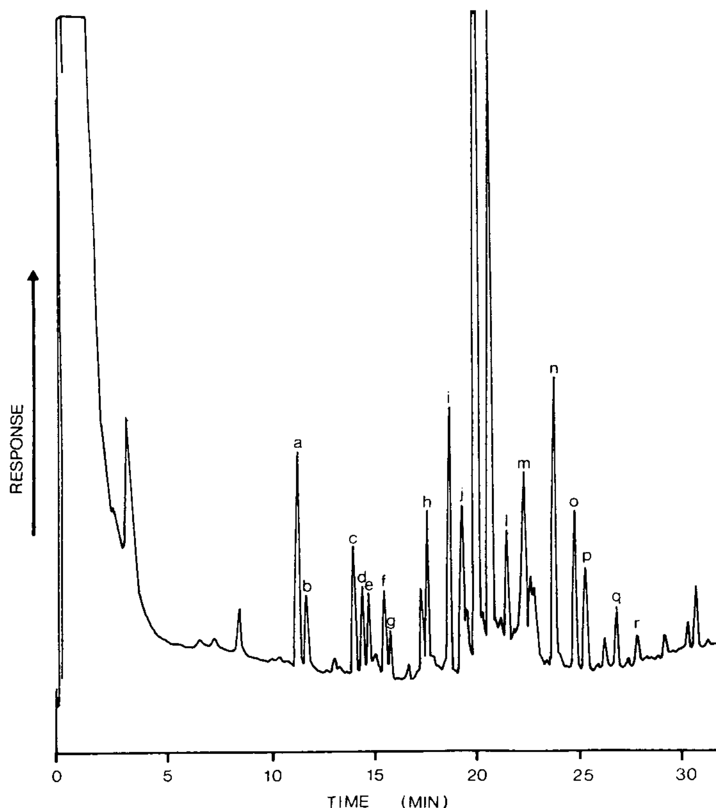

Fig. 1. Chromatogram of plasma amino acid derivatives showing the multiple peaks of glucose interference between hydroxyproline and phenylalanine. Peak identification: a = alanine; b = glycine; c = valine; d = threonine; e = serine; f = leucine; g = isoleucine; h = proline; i = pipecolic acid (internal standard); j = hydroxyproline; k = methionine; l = aspartic acid; m = phenylalanine; n = glutamic acid; o = lysine; p = tyrosine; q = arginine; r = histidine.

μl) and 0.08 *M* ATP solution (40 μl), the pH then being adjusted with 0.5 *M* ammonium hydroxide to 7.4. The mixture was incubated at 30°C for 15 min, deproteinised by addition of 2 ml of methanol—12 *M* hydrochloric acid (4:1) and centrifuged at 1600 *g* for 10 min. The supernatant was taken to dryness in vacuo. Subsequent derivatisation and chromatography of the residual material was carried out as previously described [5].

RESULTS AND DISCUSSION

Our investigations into GC analysis of amino acids in deproteinised plasma have shown that, although most amino acids can be successfully resolved, there is a major component responsible for a number of chromatographic peaks (Fig. 1) which elute over a broad area precluding the resolution of hydroxyproline, methionine, aspartic acid and phenylalanine. We have further demonstrated [4] that this component is glucose. There are two possible mechanisms which may explain the interference caused by glucose, a reducing sugar, during amino acid derivatisation. Firstly, the hydroxyl groups of glucose may react with the acylating reagent (heptafluorobutyric anhydride) [6] forming derivatives which co-elute with hydroxyproline, methionine and aspartic acid, making the quantitation of these amino acids unattainable. Secondly, and in view of the high

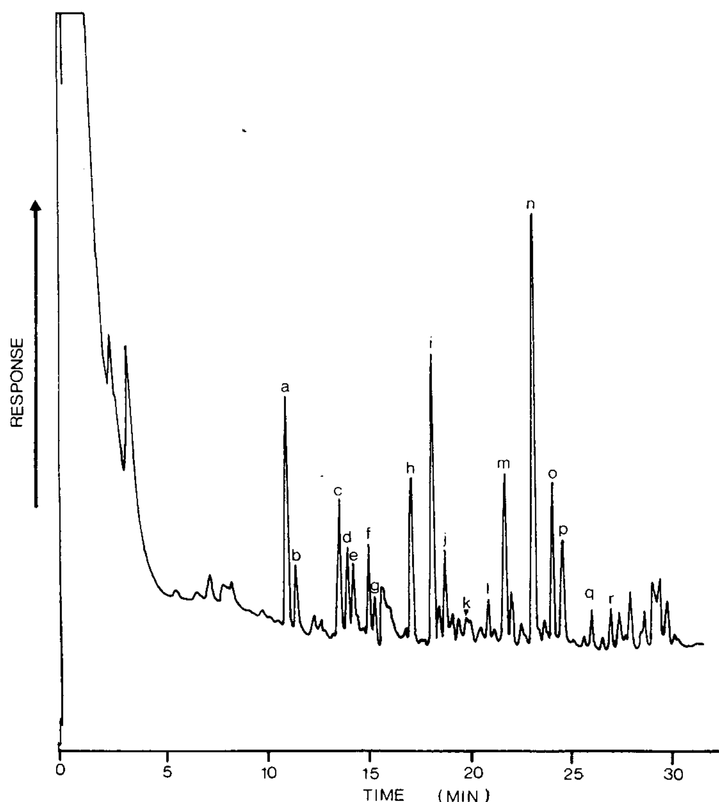

Fig. 2. Chromatogram of plasma amino acid derivatives showing the absence of glucose interference. For peak identification, see legend to Fig. 1.

temperature employed during derivatisation, glucose may react with free amino acids forming the so-called Maillard compounds. These latter compounds may themselves contribute directly to the spurious peaks observed or indirectly, following their esterification and acylation during derivatisation. Under mild conditions of temperature (37°C) lysine will be the main contributor to the Maillard reactions. However, with the increasing severity of temperature conditions (> 90°C), as employed in the derivatisation of amino acids, glycine, arginine, aspartic acid and glutamic acid will also participate in the Maillard reactions [7].

We have successfully overcome the glucose-induced interference by the enzymic conversion of glucose to glucose-6-phosphate. The elimination of glucose interference is shown in Fig. 2. Resolution of peaks is good and identification of amino acids is readily achieved, permitting quantitative results to be obtained. In addition, this novel procedure requires only 15 min in incubation time and obviates the use of the cation-exchange clean-up step. Furthermore, the brevity of our sample preparative procedure compares favourably with that employed in the amino acid analyser technique and thus lends GC to routine laboratory plasma amino acid analysis.

Preliminary data, obtained from a plasma sample spiked with a known quantity of amino acid standards, compared with an unspiked sample of the same plasma, indicate that recovery of amino acids, following deproteinisation, derivatisation and GC, is in general better than previously published data. These results will be reported elsewhere in due course.

CONCLUSION

In seeking a means of removing glucose from plasma, a novel alternative approach to the existing clean-up process has been adopted. By using a technique designed to remove a specific compound (viz. glucose), a more controlled process has been achieved together with the avoidance of a sample purification step which, though widely used, is far less than optimal.

ACKNOWLEDGEMENT

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CHROMBIO. 2523

Note

Electron-capture gas chromatography of taurine as its N-pentafluorobenzoyl di-*n*-butylamide derivative

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(First received October 3rd, 1984; revised manuscript received November 27th, 1984)

Taurine is known to be present in almost all organs of animals, and there is increasing evidence that this compound plays an important role in the body [1].

A variety of methods have been reported for the determination of taurine in biological materials, including colorimetric [2, 3], fluorometric [4—6], radiometric [7], and enzymatic [8] assay methods. However, these methods usually require time-consuming preliminary clean-up of the sample [9]. Chromatographic procedures utilizing an amino acid analyser [9—13] or high-performance liquid chromatography [14—19] have also been used for the assay, but when applying them directly to biological samples there appears to be difficulty in resolving taurine from interfering components [11—13, 17—19].

Recently, we developed a convenient and reliable method for the determination of taurine by gas chromatography with flame ionization detection (GC—FID) in which taurine was analysed as its N-isobutyloxycarbonyl (N-iso-BOC) di-*n*-butylamide derivative (Fig. 1, I), and we demonstrated that complex biological material such as urine could be analysed accurately and precisely by the method without prior clean-up of the sample [20].

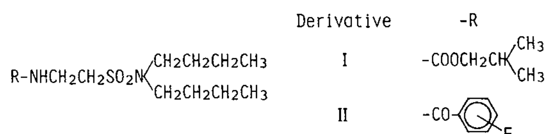

Fig. 1. Chemical structures of the N-isobutyloxycarbonyl di-*n*-butylamide (I) and N-pentafluorobenzoyl di-*n*-butylamide (II) derivatives of taurine.

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The aim of the present investigation was to develop an electron-capture gas chromatographic (GC—ECD) method for microdetermination of taurine based on the preparation of the N-pentafluorobenzoyl (N-PFB) di-*n*-butylamide derivative (Fig. 1, II), an analogue of derivative I, and this was successfully achieved. The GC—ECD method described here is over 500 times more sensitive than the GC—FID method mentioned above, permitting quantitative analysis of taurine in biological materials in the range of pmol/ml.

EXPERIMENTAL

Reagents

Taurine and 3-amino-1-propane sulphonic acid (APS) as an internal standard were purchased from Nakarai (Kyoto, Japan) and Sigma (St. Louis, MO, U.S.A.), respectively, and each was dissolved in water to make a stock solution at the concentration of 1 m*M*. Working standard solutions were prepared by diluting aliquots of the stock solutions with water to give the concentrations 0.1 and 1.0 μM. Pentafluorobenzoyl chloride (PFB-Cl) was purchased from Aldrich (Milwaukee, WI, U.S.A.). Tetrabutylammonium hydrogen sulphate ($TBA^+HSO_4^-$; Tokyo Kasei Kogyo, Tokyo, Japan) was dissolved in 0.2 *M* sodium hydroxide at the concentration of 10%. Thionyl chloride and di-*n*-butylamine (DBA) were purchased from Nakarai and used after distillation. All other chemicals were of analytical grade.

Analytical derivatization procedure

An aliquot of the sample solution (containing 10—500 pmol of taurine) was pipetted into a 10-ml pyrex glass tube with a PTFE-lined screw cap. After addition of 0.2 ml of 1.0 μM APS, 0.1 ml of 0.5 *M* sodium hydroxide was added and then the total reaction volume was made up to 1 ml with distilled water if necessary. Immediately after the addition of 20 μl of PFB-Cl, the mixture was shaken with a shaker set at 300 rpm (up and down) for 2 min at room temperature. The reaction mixture was washed three times with 3 ml of diethyl ether after adjustment to pH 1—2 with 0.5 *M* hydrochloric acid. Subsequently 0.1 ml of 10% $TBA^+HSO_4^-$ solution and 2 ml of methylene chloride were added to the aqueous layer and the tube was shaken for 3 min at room temperature. After centrifugation for 1 min, the organic layer was transferred to another tube and the solvent was evaporated to dryness under a stream of nitrogen. To the residue was added 0.05 ml of thionyl chloride, and the tube was tightly capped and heated at 80°C for 10 min. The excess thionyl chloride was removed at 80°C under a stream of nitrogen. To the residue was added 0.1 ml of 2 *M* DBA in acetonitrile, and the mixture was allowed to stand for 2 min at room temperature after tightly capping. The reaction mixture was acidified with 1 ml of 20% orthophosphoric acid and then extracted twice with 3 ml of *n*-pentane. After the solvent was evaporated to dryness at 60°C, the residue was dissolved in 0.2 ml of ethyl acetate and 2 μl of this solution were injected into the gas chromatograph.

Preparation of reference compound

A reference sample of the N-PFB di-*n*-butylamide derivative of taurine,

m.p. 87—88°C, was prepared from 125 mg of taurine in essentially the same manner as the analytical derivatization procedure. The data for elemental analysis are as follows: calc. for $C_{17}H_{23}N_2O_3SF_5$: C, 47.44; H, 5.39; N, 6.51. Found: C, 47.54; H, 5.73; N, 6.35.

Preparation of samples

Human plasma. To 5 μl of human plasma sample in a small vial, 0.4 ml of 1 μM APS and 1 ml of distilled water were added. The mixture was transferred onto a Centriflo CF-25 filter (Amicon, Lexington, MA, U.S.A.) placed on the top of a centrifuge tube and ultrafiltered by centrifugation (1000 *g*, 5 min). A 1-ml portion of the ultrafiltrate was used for the analysis.

Rat brain sample. A 1—10 mg amount of rat tissue was homogenized with distilled water and the concentration of homogenate was made up to 0.1 mg/ml with distilled water. To 0.2 ml of this homogenate (containing 20 μg of rat brain tissue), 0.4 ml of 1 μM APS and 0.9 ml of distilled water were added and then the mixture was treated in the same manner as the plasma sample.

Gas chromatography

A Shimadzu 4 CM-PFE gas chromatograph equiped with a ^{63}Ni (10 mCi) electron-capture detector and a 1.5 m × 3 mm I.D. glass column packed with 1.0% OV-17—0.2% FFAP on Uniport HP (80—100 mesh) was used. The column packing was prepared by the "solution coating" technique [21]. The column was conditioned with a nitrogen flow-rate of 30 ml/min at 275°C for 24 h. The operating conditions were as follows: column temperature, 240°C; injector and detector temperatures, 260°C; nitrogen flow-rate, 65 ml/min. Peak heights for taurine and the internal standard were measured and the peak height ratios were calculated for the construction of calibration graph.

RESULTS AND DISCUSSION

As expected, the N-PFB di-*n*-butylamide derivative of taurine could be conveniently prepared by a procedure similar to that for the preparation of the N-isoBOC analogue. $TBA^+HSO_4^-$ was used as the most satisfactory counter-ion for the rapid and quantitative ion-pair extraction of the PFB taurine. Chlorination of the sulphonic acid function of PFB taurine with thionyl chloride was accomplished within 10 min at 80°C. The reaction of PFB taurine sulphonyl chloride with DBA proceeded rapidly at room temperature and gave a corresponding sulphonamide derivative. The yield was about 73%, determined with a known amount of pure N-PFB di-*n*-butylamide derivative as reference. Excess PFB-Cl and its degradation products were completely removed at the ether washing step in the derivatization process, and therefore analyses could be carried out without disturbance by these strong electrophores.

The N-PFB di-*n*-butylamide derivative formed showed good GC properties and provided excellent ECD response (Fig. 2). The minimum detectable amount of taurine to give a signal three times as high as the noise under our instrumental conditions was approximately 10 fmol.

The calibration curve for taurine in the range of 10—500 pmol (sample size injected into the GC system accordingly ranged from 0.1 to 5 pmol) was

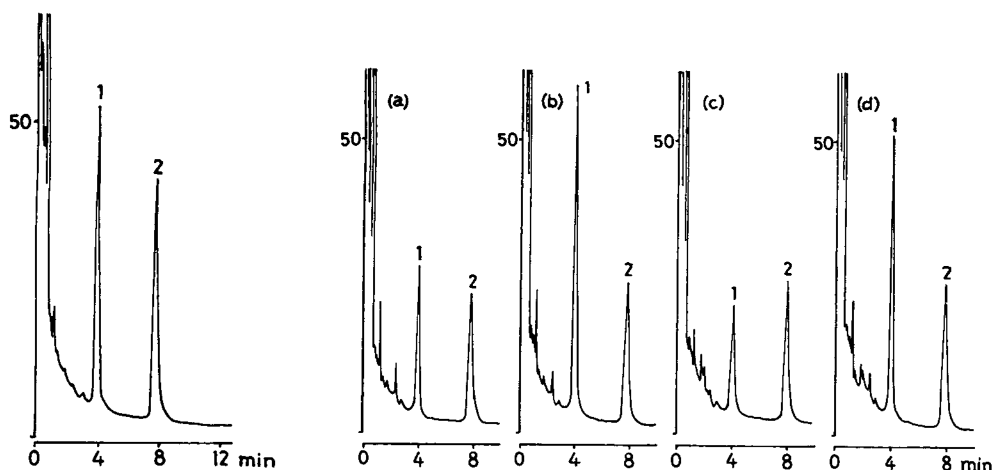

Fig. 2. Gas chromatogram obtained from a standard solution containing 100 and 200 pmol of taurine and APS (internal standard), respectively. GC conditions are given in the experimental section. Peaks: 1 = taurine, 2 = APS.

Fig. 3. Gas chromatograms obtained from biological samples: (a) human plasma (5 μl); (b) human plasma (5 μl) + 200 pmol of taurine; (c) rat brain tissue (20 μg); (d) rat brain tissue (20 μg) + 200 pmol of taurine. The plasma and brain tissue samples originally contained taurine in the concentration of 32.2 nmol/ml and 7.5 μmol/g, respectively. GC conditions are given in the experimental section. Peaks: 1 = taurine, 2 = APS.

constructed by using APS, a synthetic homologue of taurine, as an internal standard. A linear relationship was obtained and the regression line was $y = 0.109x + 0.922$ ($r = 0.999$, $n = 25$) in which y = peak height ratio and x = amount of taurine.

In order to demonstrate the applicability of the method to biological materials, the contents of taurine in human plasma and rat brain tissue were analysed. No preliminary clean-up of the sample was necessary for the analyses except deproteinization by ultrafiltration. Typical chromatograms obtained from these samples are shown in Fig. 3. The sensitivity limit of quantitation per millilitre of human plasma was estimated to be about 100 pmol. The recovery rates of taurine added to 5 μl of plasma and 20 μg of brain tissue at 200 pmol per sample were 102.9 ± 5.6% (n = 5) and 101.1 ± 4.1% (n = 5), respectively.

We believe that this method is valuable as a sensitive specific assay for taurine, especially with small tissue samples and biological fluids.

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CHROMBIO. 2516

Note

Simple and rapid method for the quantitation of plasma phytanic acid by gas chromatography

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(First received August 29th, 1984; revised manuscript received December 16th, 1984)

Phytanic acid accumulates in variable quantities (20—250 mg/l), in plasma, tissues and lipids [1, 2] of humans suffering from a rare neurological disorder, Refsum's disease, while it is absent in normal subjects. Preformed phytanic acid in food seems to be the principal source of diterpenoid acid found in these patients [3]. It has been observed that in Refsum's disease accumulation of phytanic acid in lipids of plasma and tissues might be due to a failure in the oxidation pathway [4, 5]. Phytanic acid accumulation seems at present to be the most characteristic single sign of Refsum's disease.

In this report we describe a simple gas—liquid chromatographic method for the determination of phytanic acid. The usual esterification techniques adopted for detecting plasma lipids [6—8] require a number of manipulative steps which are time-consuming and which may also result in loss or contamination of the sample. A simple esterification technique is desirable. Some authors have developed transesterification methods for methylating triglycerides in fats and in oils, using an organic base catalyst [9, 10]. We decided to apply a similar technique to a small amount of plasma to determine rapidly and conveniently the total phytanic acid in subjects with Refsum's disease.

The free acid, with the other free fatty acids which may be present in the

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sample, is converted to trimethylphenylammonium salts which thermally degrade to methyl esters by the on-column pyrolysis. The transesterification of triglycerides and other lipids containing ester bonds with phytanic acid cannot be demonstrated as at present we do not have any patient suffering from Refsum's disease.

We have compared our final product to the product obtained with a traditional method [11] by mass spectrometry to check methylation. The result was excellent.

MATERIALS AND METHODS

Instruments

A Perkin-Elmer 3920B gas chromatograph equipped with a flame ionization detector and a 2 m × 2 mm I.D. spiral glass column packed with 15% STAB DEGS WAV 80–100 mesh was used for the gas chromatographic analysis. The column was conditioned overnight at 210°C before use. Column, injector and detector temperatures were fixed at 200°C, 250°C, and 250°C, respectively. Nitrogen was used as the carrier gas at a flow-rate of 30 ml/min.

The chemical structure of methyl phytanate obtained by the organic base catalyst was confirmed by using a MAT 44 Varian mass spectrometer coupled to a gas chromatograph.

Reagents

Phytanic acid was obtained from Analabs (North Haven, CT, U.S.A.). Methyl pentadecanoate (internal standard) was obtained from Carlo Erba (Milan, Italy). Trimethylphenylammoniumhydroxide (TMPAH) was obtained from Pierce Eurochemie (Rotterdam, The Netherlands). All organic solvents of analytical grade and methyl pentadecanoate were obtained from Carlo Erba.

A stock solution of the tested substance was prepared in pure chloroform at a concentration of 2.5 mg/ml; the internal standard was made separately in solution at a concentration of 0.4 mg/ml. The two solutions were stored at 4°C. Prior to the assay, aliquots of the stock solutions were diluted to the desired concentrations in blank samples.

Extraction procedure

Aliquots of 0.1 ml of the internal standard (0.4 mg/ml), and 0.1, 0.3, 0.5, 0.8, 1.0, 1.5 ml of phytanic acid solution (0.1 mg/ml) were added to a series of 13-ml glass centrifuge tubes. After evaporating to dryness in a water bath at 60°C, 1.0 ml of human serum or plasma was added to each tube. After mixing for 10 min on a mechanical shaker, 0.1 ml of 12 *M* hydrochloric acid solution was added. The samples were left for 20 min in a water bath at 30°C. After the addition of 8 ml of diethyl ether and mechanical agitation for 30 min, the tubes were centrifuged and 7 ml of the upper layer were transferred to a second series of test tubes; 0.2 ml of 0.2 *M* TMPAH was added to each tube. The tubes were then allowed to stand at room temperature for 30 min. The samples were then evaporated to dryness under nitrogen; the residue was re-dissolved in 0.1 ml of TMPAH; 2 μl of the solution were injected into the gas chromatograph.

RESULTS

Table I shows the within-run precision at two different concentrations (n = 14) for the tested substance. The coefficient of variation did not exceed 8.0%.

Standard curves were constructed by plotting peak area ratios (substance/internal standard) versus substance concentrations (μg/ml). The instrument responses and the concentrations were linearly related over the range 10—150 μg/ml: slope = 19.226, intercept = 1.336, r = 0.999.

The extraction efficiency from serum samples containing known amounts of phytanic acid was 90%.

Fig. 1 depicts a chromatogram of phytanic acid extracted from serum. The retention times for phytanic acid and internal standard were 3 min 59 sec

TABLE I

WITHIN-RUN PRECISION OF THE ANALYSIS OF PHYTANIC ACID

Results are based on fourteen analyses for each of two concentrations of phytanic acid.

Concentration (μg/ml)	Peak area ratio phytanic acid/ internal standard (mean ± S.D.)	Coefficient of variation (%)
50	849.0 ± 68.1	8.0
100	1495.4 ± 81.0	5.4

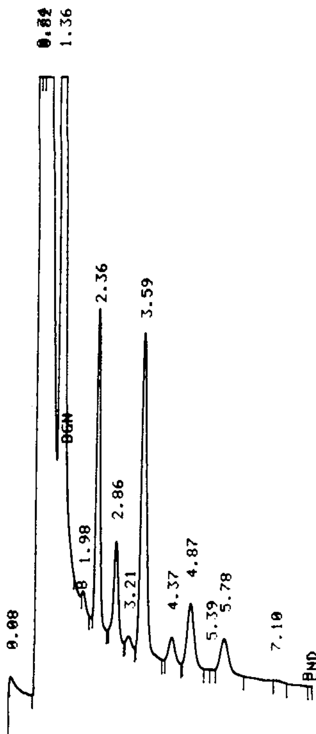

Fig. 1. Gas chromatogram of an extract of 1 ml of plasma pretreated with phytanic acid (retention time 3 min 59 sec) and internal standard (retention time 2 min 36 sec).

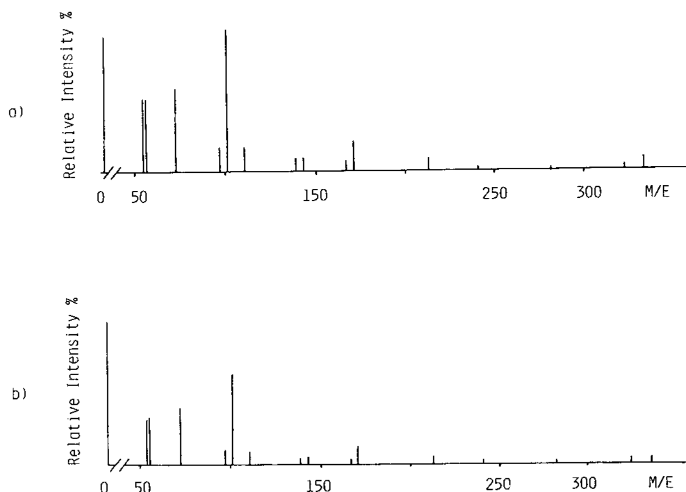

Fig. 2. Mass spectra of phytanic acid obtained by our method (a) and a traditional method (b).

a)

#	MASS	TIME	INTENSITY	
1	101.0	3.5	83.2	100.0
2	213.0	3.5	7.6	9.1
3	171.0	3.5	20.7	24.8
4	326.0	3.5	13.3	15.9

b)

#	MASS	TIME	INTENSITY	
1	101.0	3.5	163.0	100.0
2	213.0	3.5	13.5	8.2
3	171.0	3.5	39.6	24.2
4	326.0	3.5	26.7	16.3.

Fig. 3. Percentage values for the intensity of four peaks of mass spectra obtained by our method (a) and a traditional method (b).

and 2 min 36 sec, respectively. No interference from serum components was observed and 300 μl of 0.2 M TMPAH was sufficient for methylation.

In Fig. 2 the mass spectra of the substances show the formation of methyl phytanate. Fig. 3 shows the ratios of the values of the more significant ions: *m/e* 101, *m/e* 171, *m/e* 213, *m/e* 326.

CONCLUSIONS

The proposed method possesses the important characteristics of precision, sensitivity and selectivity over a wide range of phytanic acid concentrations, in addition to extreme rapidity and simplicity. The retention times for phytanic acid and internal standard are very satisfactory and the two peaks are well separated from the other fatty methyl esters occurring on the chromatogram.

The greatest advantages of this technique are that the number of manipulative steps is kept to a minimum, and loss or contamination of the sample is reduced.

The excess of TMPAH and the high temperature in the pyrolytic methylation often cause isomerization and degradation of unsaturated fatty acids [12], chiefly linoleic acid, but this does not involve phytanic acid, which is a saturated fatty acid.

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Note

High-performance liquid chromatographic method for the analysis of purine and pyrimidine bases, ribonucleosides and deoxyribonucleosides in biological fluids

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Purine and pyrimidine bases, nucleosides and nucleotides have biological importance as components of nucleic acids, coenzymes and mediators of hormone action.

Several studies have revealed disorders in purine and pyrimidine metabolism in inborn errors of metabolism [1—4] and neoplastic diseases [5, 6]. More recently, interest has been focused on the role of purine and especially deoxypurine in hereditary immunodeficiency diseases [7—9] such as adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) deficiency.

Several methods for the analysis of bases and nucleosides by high-performance liquid chromatography (HPLC) have been described in the literature [10—17]. Ion-exchange chromatography has been used to separate bases and nucleosides [16, 17]; however, reversed-phase chromatography is at present the most commonly used liquid chromatographic mode for the separation of bases and nucleosides [10, 11, 13—15].

In this paper is presented a rapid reversed-phase HPLC procedure for the analysis of purine and pyrimidine bases, the corresponding ribonucleosides and major deoxyribonucleosides, with high sensitivity and selectivity.

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Also, ultra-rapid treatment of the blood samples was used to prevent metabolic conversion of purines during the sample preparation. In order to obtain the optimal separation of all compounds, the influence of the pH, the concentration of the organic modifier and the effect of the ionic strength on retention behaviour of the purine and pyrimidine compounds were examined.

This method was applied to the analysis of bases and nucleosides in plasma and cell samples and was used as an analytical tool to determine purine metabolites in ADA deficiency.

EXPERIMENTAL

Reagents

The bases and nucleosides were purchased from Sigma (St. Louis, MO, U.S.A.). Potassium dihydrogen phosphate, perchloric acid, and methanol were obtained from E. Merck (Darmstadt, F.R.G.).

Apparatus

The liquid chromatograph consisted of a Chromatem 800 (Touzart et Matignon, Vitry, France) equipped with a variable-wavelength absorbance detector (Pye Unicam PU 4020; Philips, Bobigny, France) operating at 254 nm and an SP 4100 integrator (Spectra Physics, Orsay les Ulis, France). The column (15 cm × 4.6 mm I.D.) and the precolumn (5 cm × 4.6 mm I.D.) used as a guard column were packed with Hypersil ODS 3 μm (Shandon, Cheshire, U.K.) by the slurry packing technique as described by Coq et al. [18].

Chromatographic conditions

The analyses were performed with the gradient elution mode using 0.02 *M* potassium dihydrogen phosphate, pH 6.0 and 0.02 *M* potassium dihydrogen phosphate, pH 6.0—methanol (40:60) as eluents. The concentration of methanol in the elution solvent was varied from 0% to 12% over a period of 20 min. The gradient proceeded through four steps: 0% for 3 min, 0% to 5% in 2 min, 5% to 12% in 10 min, and 12% for 5 min. The flow-rate was 1.5 ml/min.

Sample collection and treatment

Blood was collected in a heparinized tube. In accordance with our previous findings, this tube was immediately centrifuged to prevent metabolic changes which occur when plasma is left in contact with the erythrocytes [19]. Plasma was decanted and kept in an ice bath. The leucocytes and the upper layer of erythrocytes were removed; 500 μl of the remaining erythrocytes were then rapidly deproteinized by 50 μl of 35% perchloric acid (PCA). Plasma was treated in the same way. The deproteinized samples were centrifuged for 10 min at 1500 *g* at 4°C. After centrifugation, the supernatants were removed and adjusted to pH 6—7 with 5 *M* sodium hydroxide. The extracts were stored at −20°C awaiting analysis.

Lymphocytes were isolated from freshly drawn heparinized blood on a one-step Ficoll—Isopaque gradient [20] by centrifugation at 700 *g* for 35 min. Lymphocytes were washed three times in a sodium chloride medium. The cell

pellet was resuspended ($1 \cdot 10^6$ to $5 \cdot 10^6$ cells per ml) in a sodium-buffered saline medium and an aliquot was removed for cell number determination. The lymphocyte suspension was treated in the same way as plasma and erythrocytes.

In order to minimize the slight dilution caused by PCA deproteinization, an ultrafiltration procedure described by other authors [21, 22] was tested. The plasma samples were filtered through an Amicon Micropartition system MPS 1 (Amicon, Paris, France) at 500 *g* for 20 min. Using this method, we found changes in the metabolite content. On the chromatogram of a plasma spiked with adenosine and deoxyadenosine, we observed an increase of inosine and deoxyinosine peaks and a simultaneous decrease of adenosine and deoxyadenosine peaks. The recoveries of adenosine and deoxyadenosine added to plasma were approximately 96% using PCA deproteinization and only 73% by the ultrafiltration procedure.

RESULTS AND DISCUSSION

Chromatographic separation

In order to optimize the chromatographic separation of sixteen bases and nucleosides, the influence of the pH, the concentration of the organic modifier and the ionic strength of the mobile phase on the capacity ratio (k') were investigated.

Fig. 1 shows the effect of the pH of the mobile phase on the retention of the bases, the ribonucleosides and the deoxyribonucleosides, the pH ranging from 4.5 to 6.5. In the pH range considered, the majority of the compounds are in their neutral form, so they can be analysed by reversed-phase chromato-

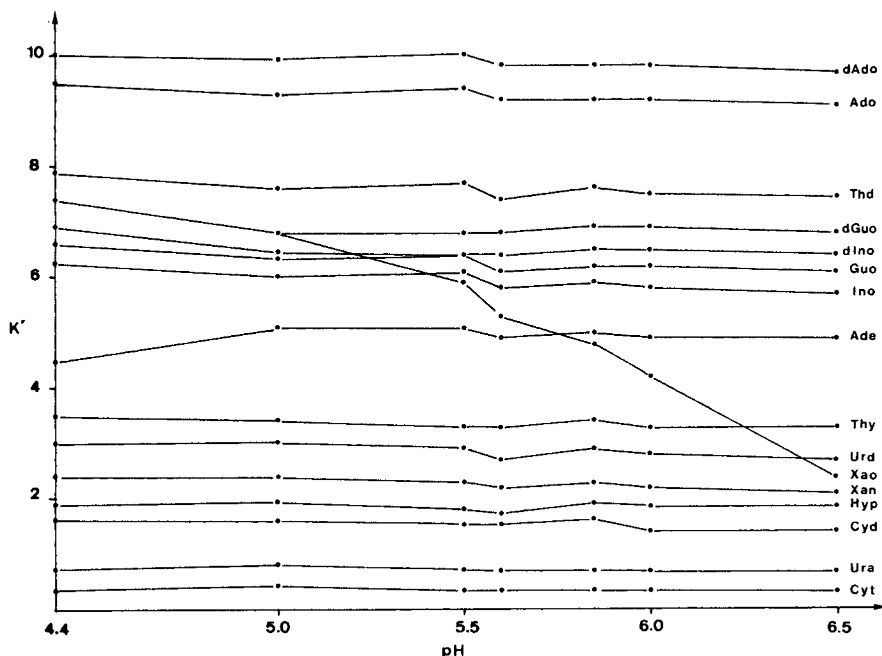

Fig. 1. Effect of the pH of the mobile phase on the retention of bases and nucleosides. Chromatographic conditions are described in Experimental. For abbreviations, see Fig. 3.

graphy. The retention behaviour of the bases and nucleosides is related to their pK values [23]. Xanthosine, which has a pK_a of 5.7, shows important variations of k' when the pH of the eluent increases from 4.5 to 6.5. The retention of the other compounds whose pK_b values are below 4.5 (weak bases) and pK_a values above 6.5 (weak acids) did not change significantly in this pH range. Fig. 1 shows that the separation of all the bases and nucleosides can be attained at pH 6.0.

The influence of the percentage of organic modifier (methanol) in 0.02 M potassium dihydrogen phosphate, pH 6.0 is represented in Fig. 2. The retention of bases and nucleosides decreases as the concentration of methanol is

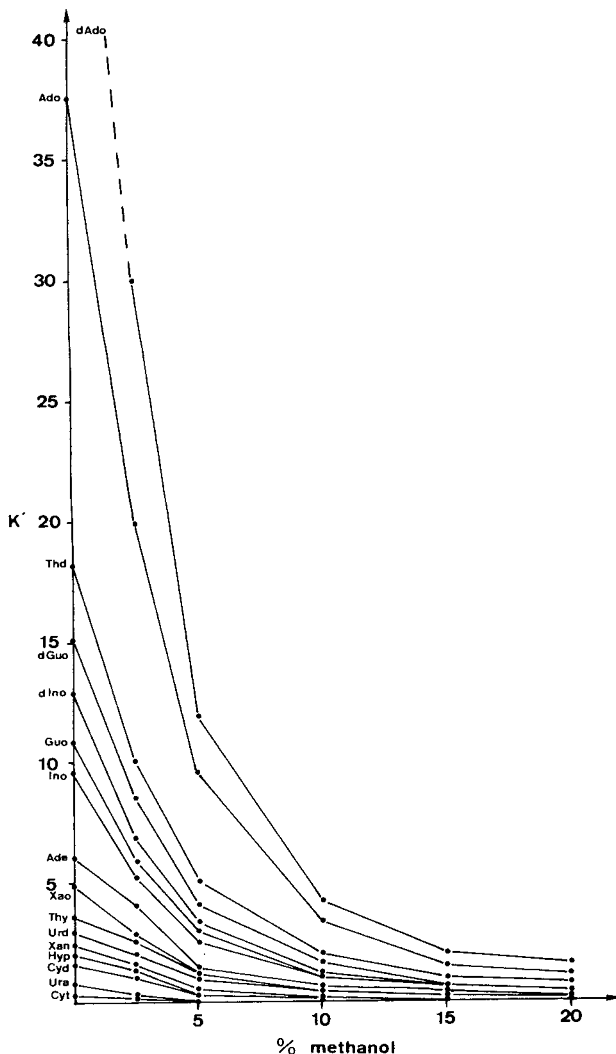

Fig. 2. Effect of the concentration of methanol on the retention of bases and nucleosides. Stationary phase: Hypersil ODS 3 μm. Mobile phase: 0.02 M potassium dihydrogen phosphate pH 6.0 and 0.02 M potassium dihydrogen phosphate—methanol (0—20%). For abbrevations, see Fig. 3.

increased. The influence of the concentration of the organic modifier on the degree of retention of these compounds was used to establish the gradient elution system.

The retention of the compounds was not significantly influenced by the ionic strength of the buffer over the range 0.01—0.10 *M* potassium dihydrogen phosphate.

Using these data, the optimal mobile phase composition for the separation of bases, ribonucleosides and deoxyribonucleosides was determined. The corresponding chromatogram is shown in Fig. 3. The separation of the sixteen compounds can be achieved in 20 min. The chromatogram in Fig. 3 shows that adenine tails in this chromatographic system. Most compounds containing an amino group tail badly on alkyl silica packings [24, 25]. The tailing peak shape of adenine may be related to the interaction of the adenine amino group with residual silica hydroxyls. The data reported by Zakaria and Brown [26], who

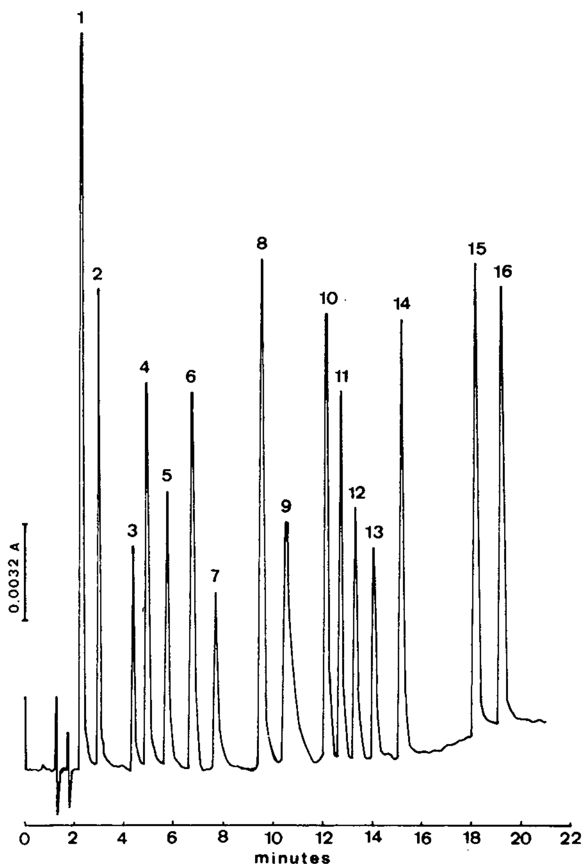

Fig. 3. Chromatographic separation of bases and nucleosides. Chromatographic conditions are described in Experimental. Peaks: 1 = cytosine (Cyt), 2 = uracil (Ura), 3 = cytidine (Cyd), 4 = hypoxanthine (Hyp), 5 = xanthine (Xan), 6 = uridine (Urd), 7 = thymine (Thy), 8 = xanthosine (Xao), 9 = adenine (Ade), 10 = inosine (Ino), 11 = guanosine (Guo), 12 = deoxyinosine (dIno), 13 = deoxyguanosine (dGuo), 14 = thymidine (Thd), 15 = adenosine (Ado), 16 = deoxyadenosine (dAdo).

have found that the 6-aminopurine substituent interacts with residual silanols of the alkyl—silica bonded phase, give support to this hypothesis.

Recovery and detection limit of adenine and purine nucleosides

The recoveries of adenine, inosine, deoxyinosine, guanosine, deoxyguanosine, xanthosine, adenosine and deoxyadenosine added to plasma were 97% ± 4. The relationship between the concentration and the peak area of these compounds was linear over the range 0.25—20 μmol l^{-1}. The minimum quantity detectable was 2.5 pmol for guanosine, deoxyguanosine, adenosine, deoxyadenosine, 3.5 pmol for xanthosine, inosine, deoxyinosine and 5 pmol for adenine.

Applications

This method was applied to the analysis of purine nucleosides and adenine in plasma and cell samples from healthy subjects. Neither xanthosine, guanosine and inosine nor deoxyribonucleosides were detected in plasma and erythrocyte samples. Adenine was found at a concentration of 1.0 ± 0.7 μmol l^{-1} in plasma and at a concentration of 1.9 ± 0.6 μmol l^{-1} in erythrocytes. Inosine was the only nucleoside found in lymphocytes and at a concentration of 1.2 ± 0.7 nmol

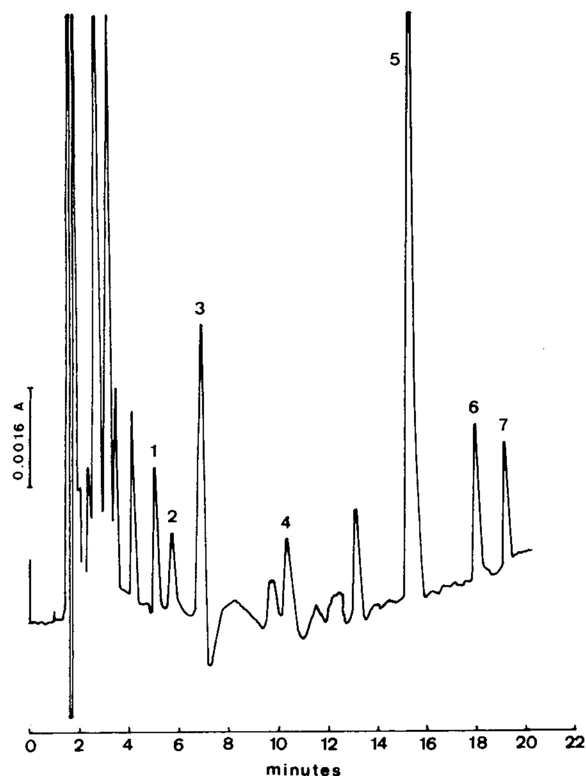

Fig. 4. Chromatogram of plasma sample from a patient with ADA deficiency who had undergone a bone marrow transplant. Chromatographic conditions are described in Experimental. Injection volume: 20 μl. Peaks: 1 = hypoxanthine, 2 = xanthine, 3 = uridine, 4 = adenine, 5 = peak observed when using PCA deproteinization, 6 = adenosine, 7 = deoxyadenosine.

per 10^6 cells (n = 6). Adenosine was not detected in erythrocyte and in lymphocyte cells, whereas in plasma the concentration of this compound was found to be 0.8 ± 0.4 μmol l^{-1} (n = 6). Peak identification of adenosine and inosine was confirmed by the enzymatic reaction with adenosine deaminase and purine nucleoside phosphorylase, respectively.

Also, using this HPLC method, purine nucleosides and adenine were determined in biological fluids and cells of an ADA-deficient patient who had undergone a bone marrow transplant ten years previously. The patient has since recovered normal immunological functions.

Adenosine deaminase deficiency results in a severe combined immmunodeficiency disease [7, 27]. The ADA deficiency leads to an accumulation of adenosine, deoxyadenosine and adenine deoxynucleotides in plasma, erythrocytes and lymphocytes [27].

The chromatogram of a plasma sample from the child who had undergone bone marrow transplant is shown in Fig. 4. In plasma, the adenine and adenosine concentrations were increased (2.2 and 2.3 μmol l^{-1}, respectively). Also, deoxyadenosine was found at a concentration of 1.8 μmol l^{-1} whereas this compound was not detectable in plasma samples from healthy subjects. In erythrocyte samples, adenosine and deoxyadenosine were not detected and adenine was present at a concentration of 1.5 μmol l^{-1}. In lymphocytes, adenosine and deoxyadenosine were undetectable; furthermore, inosine was not detectable whereas this compound was present in lymphocytes from healthy subjects. Our results show that in the patient studied, the bone marrow transplant had not entirely corrected the purine metabolism disorder.

With the rapid reversed-phase procedure described in this paper, the purine and pyrimidine bases, the corresponding ribonucleosides and major deoxyribonucleosides can be separated with high selectivity. A rapid PCA deproteinization of blood samples was used in order to prevent purine metabolic changes during the sample treatment.

The HPLC method described is an accurate analytical tool for the determination of purine metabolite levels in healthy subjects and in patients with purine metabolism deficiency.

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Note

Separation of somatomedins and somatomedin inhibitors by size exclusion high-performance liquid chromatography

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The somatomedins are a family of circulating peptides with structure and biological actions similar to those of insulin [1]. Growing cartilage can be stimulated in vitro by physiologic concentrations of somatomedins, and these factors are thought to mediate the growth-promoting actions of growth hormone [2]. The actions of somatomedins appear to be modulated by other circulating factors, the somatomedin inhibitors [3]. Although both somatomedins and somatomedin inhibitors can be detected in serum after fractionation by conventional gel permeation chromatography [4], these techniques provide poor separation and are not readily applicable to human or animal studies with large numbers of samples. In the present studies, we report the use of size exclusion high-performance liquid chromatography (HPLC) in the separation of somatomedins and inhibitors, and application to the examination of pathophysiology in animal models.

EXPERIMENTAL

Animals and serum

Male CD rats were obtained from Charles River Breeding Labs. and male Sprague—Dawley rats from King Animal Labs. Hypophysectomized male rats were used at 80—100 g. Normal rat serum was used as a source enriched in somatomedins, and was pooled from 90—150 g rats as previously described [4]. Sera from rats with severe diabetes were selected for enrichment in somatomedin inhibitors, and pooled as described [4]. Human serum was obtained from normal males and females, 20—55 years old. For studies of

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pathophysiology, serum was obtained from normal rats (120—160 g), fasted rats (weight loss 33 ± 1 g after three days), and unselected diabetic rats with moderate diabetes (mean serum glucose 441 mg/dl, two days after streptozotocin administration). All samples were kept at −20° C prior to study.

Bioassays

Somatomedin activity was determined as the ability of samples to stimulate sulfate uptake by hypophysectomized rat costal cartilage, as described previously [4]. This tissue responds to all somatomedins identified to date. Rat-to-rat variations require examinations in single animals in order to achieve acceptable precision in the bioassay, and samples from any column can only be compared after assay with cartilage from the same rat. Because of these inherent limitations in assay size (sixteen unknowns per rat), column eluates were usually divided into twelve to sixteen fractions. Stimulation by HPLC fractions was assessed at 0.4%, 2% or 5% (v/v) final concentration. Increases in sulfate uptake were expressed as percentage increase above buffer levels or as a percentage of stimulation provided by whole normal serum (1%, v/v) in the same assay. Somatomedin inhibitory activity was measured by the ability of samples to blunt cartilage stimulation by somatomedins, as described previously [4]. Cartilage incubations were supplemented with whole normal serum at 0.5% or 1% (v/v), and fractions added at 2% or 8%. In dose-response studies, potency was estimated by parallel-line analysis as described previously [5].

High-performance liquid chromatography

Toyo Soda TSK 2000 SW (10-μm modified silica) 100 × 7.5 mm and 600 × 7.5 mm size exclusion guard and separation columns, respectively, were eluted isocratically at 0.7 ml/min using a Beckman 112 pump. A Beckman 210 injector was used for sample application, and absorbance at 280 nm was monitored continuously with a Beckman 160 detector and a Kipp and Zonen BD40 recorder. Samples were eluted with 0.1 *M* ammonium formate, adjusted to pH 3.0 with concentrated formic acid. The eluate was collected in 1.40-min fractions (Pharmacia FRAC-100) beginning with elution of the void volume. Added $Na^{125}I$ appeared in fraction 13. Eluted fractions were lyophilized, relyophilized two to three times after addition of deionized water, reconstituted in 0.5 ml Krebs phosphosaline—amino acid buffer [3], and kept at −20°C prior to bioassay. All chemicals were of analytical-reagent grade. HPLC-grade water was prepared by passing deionized water through a Millipore Norganic cartridge and a Durapore filter, and buffers were filtered through a Durapore membrane (0.22 μm) and degassed before use.

Serum samples were acidified by addition of concentrated formic acid, in a ratio of 240:10, and incubated at either 37° C or room temperature for 1 h. Before application, samples were either Millipore-filtered (0.22 μm), or centrifuged (12 000 *g* for 5 min). Samples (200 μl, approximately 16 mg protein) were injected with a 250-μl Hamilton syringe. Each separation was followed by a 15—20 min rinse with eluting buffer, and the columns were kept in 20% methanol between studies.

RESULTS

Preliminary experiments (not shown) were conducted to determine suitable separation conditions. Porcine insulin and α-chymotrypsinogen-A were used as model proteins of size roughly comparable to that of the somatomedins and somatomedin inhibitors, respectively. Eluted peak separation and contour were not improved by 0.2 *M* buffer, or by decreasing flow-rate to 0.5 ml/min. Pre-acidification was necessary for separation of somatomedins from larger carrier proteins. Under these conditions, elution of protein standards [ovalbumin, molecular weight (MW) 45 000; α-chymotrypsinogen-A, MW 25 700; ribonuclease-A, MW 13 700; insulin, MW 5784; glucagon, MW 3485; and cyanocobalamin, MW 1355) was essentially linear when plotted as log MW versus K_{av} ($r = 0.99$).

Normal human serum was then examined, as shown in Fig. 1. Somatomedins were found in fractions 9 and 10, and somatomedin inhibitors in fractions 4—8. Adjacent fractions (not shown) had no significant stimulatory or inhibitory activity, respectively. Fractions with significant somatomedin activity had apparent MW 5050—16 000, and fractions with somatomedin inhibitory activity had apparent MW 16 000—340 000. Fractions 9 and 10 contained 0.4% of recovered 280-nm absorbing material, and fractions 4—8 contained 30.4% of 280-nm absorbing material.

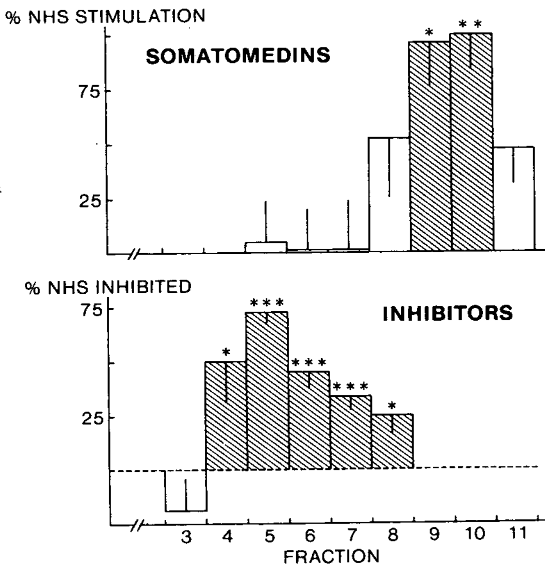

Fig. 1. Isocratic separation of somatomedins and somatomedin inhibitors in four and six samples of normal human serum (NHS), respectively, by HPLC. Whole serum was acidified, centrifuged, and 200 μl were applied to a TSK-2000 column eluted at 0.7 ml/min with 0.1 *M* ammonium formate, pH 3.0, as described in Experimental. Fraction collection began with the excluded volume, as determined with blue dextran. Somatomedin activity was expressed as stimulation of cartilage sulfate uptake relative to that produced by unfractionated NHS, and somatomedin inhibitor activity was expressed as percentage inhibition of cartilage stimulation produced by NHS. Hatching denotes fractions with statistically significant activity. (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$. Mean ± S.E.M.

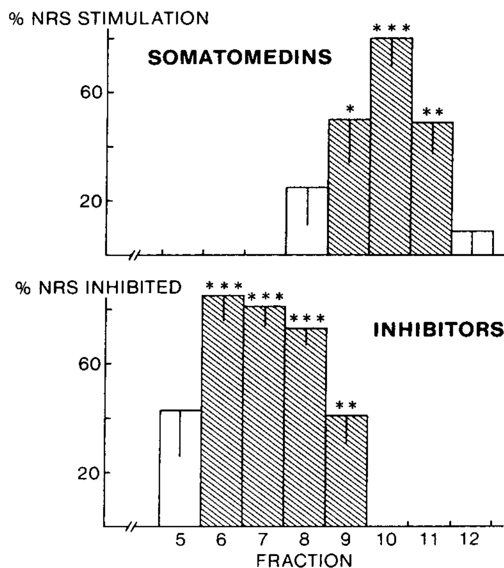

Fig. 2. Separation of somatomedins in normal rat serum (NRS) and somatomedin inhibitors in diabetic rat serum, six samples each, by HPLC. Procedures as in Fig. 1 and Experimental. (*) $p < 0.05$; (**) $p < 0.01$; (***) $p < 0.001$. Mean ± S.E.M.

The profile of somatomedins and somatomedin inhibitors in rat serum was comparable to that of human serum, as shown in Fig. 2. Somatomedins (in normal rat serum) were found in fractions 9—11, and somatomedin inhibitors (in diabetic rat serum) were found in fractions 6—9, slightly retarded as compared to inhibitors in human serum. Of total eluted somatomedins from normal rat serum 28% were found in fraction 9, versus 13% of total eluted somatomedin inhibitors from diabetic rat serum. Somatomedins had apparent MW 2600—16 000, (fractions (9—11) and somatomedin inhibitors apparent MW 9000—98 200. Fractions 9—11 contained 0.3% of recovered 280-nm absorbing material, and fractions 6—9 had 3.7%.

Such HPLC separation permitted rat models of pathophysiology to be tested for alterations in circulating, biologically active somatomedins and somatomedin inhibitors, as shown in Fig. 3. Somatomedin activity was measured in pooled fractions 9—11, and somatomedin inhibitor activity in pooled fractions 6—8 (apparent MW 16 000—98 200). Somatomedins were undetectable in samples from hypophysectomized or diabetic rats (cartilage sulfate uptake at buffer levels), and marginally higher in fasted rats (somatomedin potency 6% compared to normal rats). In contrast, somatomedin inhibitors were present in all groups of animals. Diabetic rats exhibited twenty-fold increases in inhibitor potency compared to normal animals, fasted rats exhibited three-fold increases, and no change was seen in hypophysectomized rats. Inhibitory dose-response lines had slopes which did not differ significantly, indicating qualitative similarity of the factors being measured. Lesser quantities of somatomedin inhibitors were found in pooled fractions 4 and 5 (apparent MW 98 200—340 000), with no differences among animal groups.

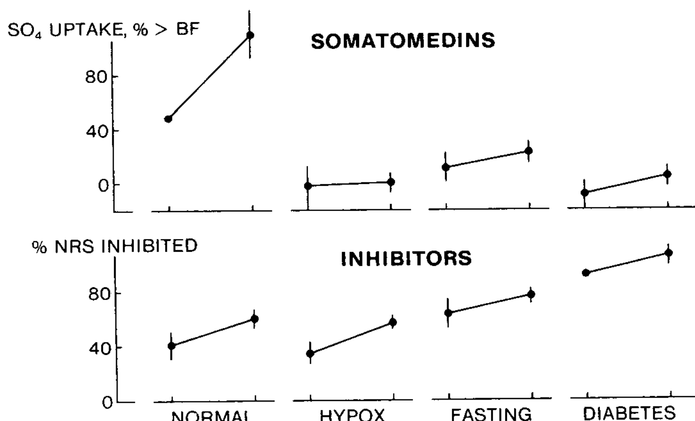

Fig. 3. Somatomedins and somatomedin inhibitors in normal, hypophysectomized (hypox), fasted, and diabetic rats, determined after preacidified serum was subjected to size exclusion HPLC (pH 3.0). Lines connect points denoting bioassay responses to two concentrations of somatomedins (upper panel) and somatomedin inhibitors (lower panel); in each case, the lower concentration is on the left. Somatomedin activity (pooled fractions 9—11) was measured by stimulation of cartilage SO_4 uptake [above buffer level (BF)] at 0.4% and 2% sample, and somatomedin inhibitor activity (pooled fractions 6—8) was measured by the ability of samples (at 2% and 8%) to decrease stimulation by 1% added normal rat serum (NRS). Mean ± S.E.M. for four samples each.

DISCUSSION

The regulation of circulating growth-related factors cannot yet be probed fully by radioligand assay techniques. Although two somatomedins have been isolated and can now be measured by radioimmunoassay [6], recent studies indicate that additional species with somatomedin-like activity are present in the circulation [7, 8]. The somatomedin inhibitors are even less well characterized [9], and at present can be measured only by bioassay. Thus, biological assay systems must be used to quantitate the "total" physiologic contribution of the various somatomedins, and to measure circulating somatomedin inhibitors as well. Such assays require the use of buffer components which either can be removed easily or do not interfere with the biological response, and also require prior separation of somatomedins from somatomedin inhibitors in individual samples.

Since recovery of somatomedins from ion-exchange separations has often been poor, somatomedins and somatomedin inhibitors have largely been separated on the basis of size. Previous reports from our laboratory and other workers [4, 10] have indicated that in samples of serum examined at neutral pH, the somatomedin inhibitors are smaller than the somatomedins (bound to carrier proteins), but at acidic pH the somatomedin inhibitors are larger than non-carrier bound somatomedins. Such differences in size have been exploited in studies of somatomedins and somatomedin inhibitors in samples subjected to conventional gel chromatography [11, 12]. However, these procedures are slow and separation may be relatively poor, with 20—40% of somatomedin activity eluting in the region where 30—60% of somatomedin inhibitory activi-

ty is found [13]. The HPLC approaches employed in the present studies resulted in improved separation, and the rapidity of the procedures facilitated initial physiological examinations. Additional improvements in separation might be obtained by decreasing sample size and the use of buffers of higher ionic strength.

Using animal models, circulating somatomedins and somatomedin inhibitors were measured to examine the applicability of the HPLC separations. Decreases in somatomedins were found in fasted and diabetic as well as hypophysectomized rats. Somatomedin inhibitors were markedly elevated in diabetic animals and modestly elevated with fasting, but unchanged by hypophysectomy. These observations are consistent with the hypothesis of regulation of net circulating somatomedin activity by nutrition and insulin as well as growth hormone [9], and suggest that nutrition and insulin affect both somatomedins and somatomedin inhibitors, whereas growth hormone affects only the somatomedins. In combination, these findings indicate that size exclusion HPLC can be combined successfully with bioassay determinations in the assessment of normal and abnormal physiology.

ACKNOWLEDGEMENTS

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Note

Chromatography of γ-glutamyl transferases from ascites hepatoma AH-66 cells and human primary hepatoma on *Phaseolus vulgaris* erythroagglutinating lectin agarose

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γ-Glutamyl transferase (EC 2.3.2.2) is one of the oncofetal proteins [1] and plays an essential role in the turnover of glutathione in mammalian tissues [2, 3]. The enzyme is highly activated in the livers of rats fed various hepatocarcinogens [4, 5], and a high activity has also been found in tumour tissues [4—6]. Previous studies in our laboratory indicated that the enzymes purified from various tissues such as fetal liver [7], normal adult liver [8], azo-dye-induced hepatoma [6], ascites hepatoma AH-66, as well yolk sac tumour cells [8, 9] are all similar with respect to kinetic, immunochemical and physicochemical properties except that the enzymes from tumour tissues contain more carbohydrate residues. Quite recently we analysed the carbohydrate structure of the enzymes from AH-66 cells and normal liver of rats and found that the enzyme from AH-66 cells contains a complex type of asparagine-linked sugar chains with bisected N-acetylglucosamine residues, while the enzyme from normal rat liver lacks these residues [10].

Phaseolus vulgaris erythroagglutinating lectin has been reported by Irimura et al. [11] to bind specifically to the bisected residues of oligosaccharides. Cummings and Kornfeld [12, 13] also reported that the following bisected

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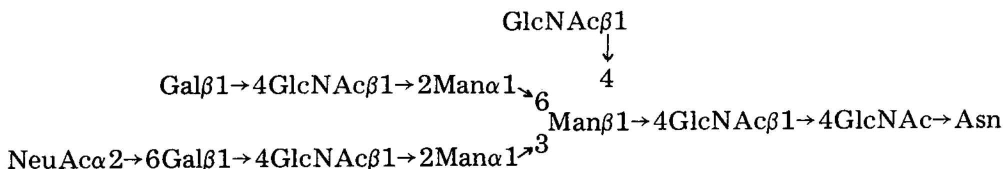
biantennary complex type asparagine-linked oligosaccharides bind to the lectin.



This was further confirmed by Yamashita et al. [14], who reported a detailed structural requirement for binding to the lectin.

In the present study we found that γ-glutamyl transferase from AH-66 cells bound to the lectin column, while the enzyme from normal rat liver did not. We also found that the neuraminidase-treated human hepatoma enzyme bound to the column, while the normal human liver enzyme did not. This is the first report to show that highly purified γ-glutamyl transferase has an affinity for E-PHA agarose. Affinity chromatography on E-PHA agarose is of value for the isolation and identification of glycoproteins with bisected N-acetylglucosamine residues.

MATERIALS AND METHODS

Affinity chromatography on E-PHA agarose

E-PHA agarose (*Phaseolus vulgaris* erythroagglutinating lectin agarose) was purchased from E-Y Labs. San Mateo. A column (10 × 1 cm) was equilibrated with 50 m*M* Tris—HCl buffer, pH 7.5 containing 0.01% sodium azide and 0.15 *M* sodium chloride. The untreated or neuraminidase-treated sample (100 μl) was loaded on the column, and 0.2-ml fractions were collected at room temperature. The flow-rate was 5 ml/h.

Purification of γ-glutamyl transferase

γ-Glutamyl transferase was purified from AH-66, spontaneous hepatoma and normal rat liver according to the method described previously [1, 9, 10]. The purified enzymes from AH-66, spontaneous hepatoma and normal rat liver had specific activities of 428, 450 and 440 U/mg of protein, respectively. The purification procedure for the enzyme from normal human liver and hepatoma was essentially the same as that for normal rat liver enzyme except that the antibody used for the immunoaffinity column was goat antibody against human kidney γ-glutamyl transferase which was purified according to the method of Tate and Meister [15]. The purified enzymes from normal human liver and hepatoma had specific activities of 456 and 480 U/mg of protein, respectively. All these enzymes were found to be homogeneous and composed of two non-identical subunits by polyacrylamide gel electrophoresis in the presence of 1% sodium dodecyl sulphate.

Assay of γ-glutamyl transferase

γ-Glutamyl transferase activity was assayed according to the method described previously [7] using a Gilford Model 240 recording spectrophotometer at 37° C.

Neuraminidase treatment of the purified enzymes

Neuraminidase treatment was done in the presence of 0.1 U of neuraminidase (*Arthrobacter ureafaciens*, Marukin Shoyu Japan) at pH 6.8 for 36 h as previously described [8].

RESULTS AND DISCUSSION

γ-Glutamyl transferase from normal rat liver, which lacks the bisected N-acetylglucosamine residue, was loaded on the column. However, almost all of the enzyme was washed through the column (Fig. 1). On the other hand, the enzyme from AH-66 cells, which has bisected N-acetylglucosamine residues, was retarded on the column as shown in Fig. 2. The recovery of the enzyme activity after this procedure was over 85%. These results indicate that the E-PHA column binds bisected N-acetylglucosamine residues found in the γ-glutamyl transferase from AH-66 cells [10]. We also found that the spontaneous hepatoma enzyme of the rat bound to the E-PHA column (Fig. 3). Therefore it seems that the enzyme from the spontaneous hepatoma has a bisected N-acetylglucosamine structure similar to that of the enzyme from AH-66 cells.

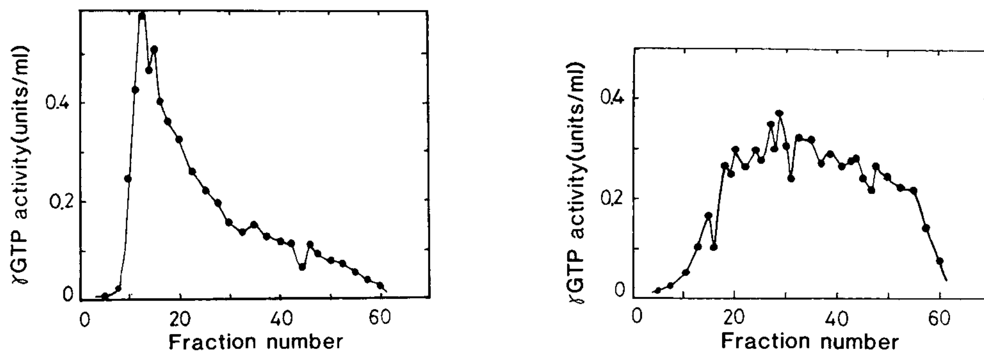


Fig. 1. Chromatography of AH-66 γ-glutamyl transferase on E-PHA agarose. Purified γ-glutamyl transferase (2 μg) from AH-66 cells was loaded on the column of E-PHA agarose, which had been equilibrated with 0.05 *M* Tris—HCl buffer, pH 7.5 containing 0.01% sodium azide and 0.15 *M* sodium chloride. γ-Glutamyl transferase activity of each fraction was assayed as described in Materials and methods. γGTP indicates γ-glutamyl transferase.

Fig. 2. Chromatography of normal rat liver γ-glutamyl transferase on E-PHA agarose. Purified γ-glutamyl transferase (2 μg) from normal rat liver was loaded on the column according to the method described in Materials and methods. γGTP indicates γ-glutamyl transferase.

The enzymes purified from normal human liver and hepatoma were also loaded on the column of E-PHA agarose. As shown in Fig. 4 (upper panel), the enzyme from human hepatoma partially bound to the column. However, the affinity for the column was not as strong as that of the AH-66 enzyme or the spontaneous hepatoma enzyme as described above. We also found that the enzyme from azo-dye-induced hepatoma of rats did not bind to the column so well as those from AH-66 and spontaneous hepatoma, while its desialylated form bound to the column very well (data not shown).

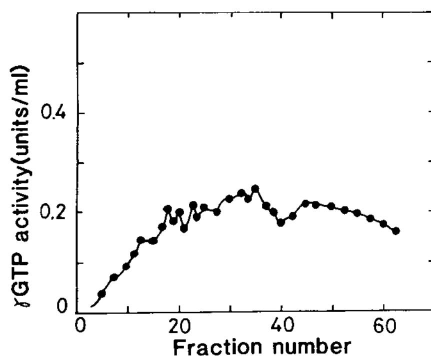


Fig. 3. Chromatography of spontaneous hepatoma of rats on E-PHA agarose. Purified γ-glutamyl transferase from spontaneous hepatoma (2 μg) was loaded on the column according to the method described in Materials and methods. γGTP indicates γ-glutamyl transferase.

Previous studies [16] in our laboratory on the affinity of γ-glutamyl transferase from hepatoma patients for E-PHA agarose indicated that the enzyme from hepatoma patients' sera was retarded on the column, while that from sera of patients with other hepatic diseases was not. Quite recently Hitoi et al. [17]

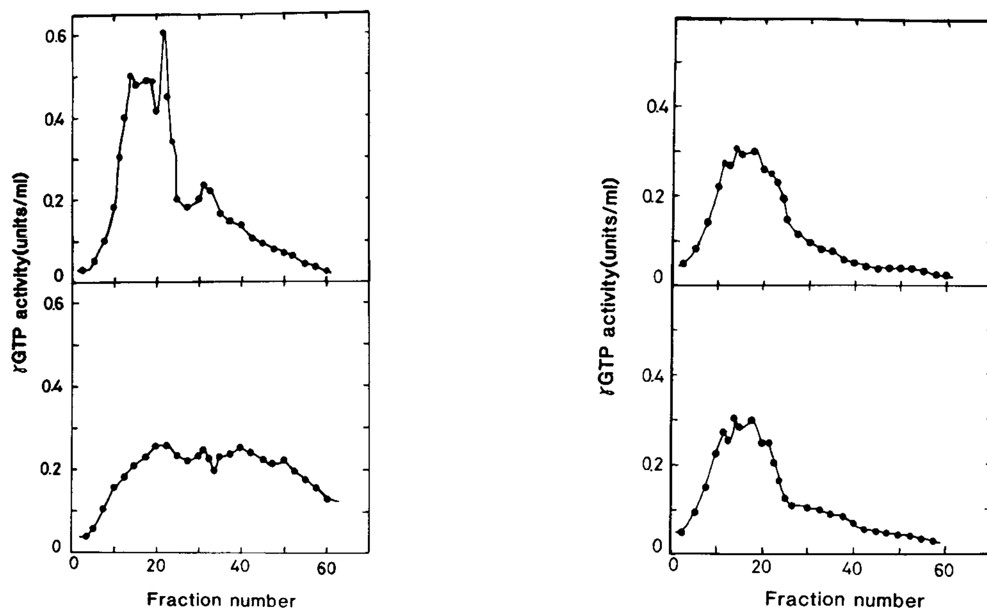


Fig. 4. Chromatography of human hepatoma γ-glutamyl transferase on E-PHA agarose. Purified γ-glutamyl transferase (2 μg) was loaded on the column according to the method described in Materials and methods. Upper panel: untreated enzyme; lower panel: enzyme treated with 0.1 U of neuraminidase at pH 6.8 for 36 h according to the method described in Materials and methods. γ-GTP indicates γ-glutamyl transferase.

Fig. 5. Chromatography of human normal liver γ-glutamyl transferase on E-PHA agarose. Purified enzyme (2 μg) from normal human liver was loaded on the column of E-PHA according to the method described in Fig. 1. Upper panel: untreated enzyme; lower panel: enzyme treated with 0.1 U of neuraminidase at pH 6.8 for 36 h according to the method described in Materials and methods. γGTP indicates γ-glutamyl transferase.

reported that the sialylated enzyme from human sera did not bind to the column, while the desialylated form bound to the column. These reports suggest that this affinity column may be of clinical value for the diagnosis of primary hepatoma.

The enzyme from human hepatoma was treated with neuraminidase and then loaded on the column. As shown in Fig. 4 (lower panel) the neuraminidase-treated enzyme was retarded on the column. On the other hand, neither neuraminidase-treated nor untreated enzyme from normal human liver was retarded on the column (Fig. 5, upper and lower panels). From the above results we could conclude that the bisected N-acetylglucosamine residues of γ-glutamyl transferase from ascites hepatoma AH-66 cells have an affinity for E-PHA agarose, and human hepatoma enzyme may have a sialylated complex type of carbohydrate chain with bisected residues. The treatment of the enzyme from normal rat liver with neuraminidase did not change the pattern or E-PHA agarose chromatography. This indicates that the normal rat liver enzyme does not contain a sialylated carbohydrate chain with bisected N-acetylglucosamine residues. E-PHA chromatography will be useful for obtaining structural information about bisected N-acetylglucosamine residues.

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Note

Rapid purification of human placental angiotensin I converting enzyme by captopril affinity chromatography

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(First received September 18th, 1984; revised manuscript received December 18th, 1984)

Angiotensin I converting enzyme (ACE, peptidyldipeptide hydrolase, E.C. 3.4.15.1) converts angiotensin I to angiotensin II by releasing the C-terminal sequence residue (His-Leu) of the decapeptide, and also degrades the vasodepressor peptide bradykinin [1].

Captopril, D-(3-mercapto-2-methylpropanoyl)-L-proline, is a potent inhibitor of ACE with a k_i value of 5.7 n*M* [2]. Since Gavaras et al. [3] demonstrated that captopril reduces blood pressure in severely hypertensive patients, treatment of hypertension by regulating the angiotensin concentration by inhibitors of ACE has been under investigation. We have attempted to use this drug as an affinity ligand for the purification of ACE from human placenta.

MATERIALS AND METHODS

Hip-His-Leu (hippurylhistidylleucine) was purchased from the Peptide Institute, Osaka, Japan. Captopril was kindly supplied by Sankyo, Tokyo, Japan.

Preparation of the affinity column

Captopril (48 mg) was esterified with 160 mg of N-hydroxysuccinimide in the presence of 160 mg of N-ethyl-N′-carbodiimide hydrochloride at 4°C for 16 h. AH-Sepharose 4B (4 g suction-dry weight) [4], after repeated washing with 0.5 *M* sodium chloride and distilled water, was added to a solution of the activated captopril, and coupling of the peptide to the gel was carried out at

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room temperature for 22 h with gentle stirring under exclusion of atmospheric moisture. The gel was washed with distilled water and then with 20 m*M* potassium phosphate buffer (pH 7.8); 1 ml of the wet gel was found to contain 13.9 μmol of covalently bound captopril.

Purification of ACE from human placenta

Fresh human placenta (200 g) after normal delivery was chopped into small pieces and suspended in 400 ml of 20 m*M* potassium phosphate buffer (pH 7.8) containing 0.25 *M* sucrose. The suspension was homogenized for 30 min with a Polytron homogenizer at setting 7. The resulting homogenate was centrifuged at 700 *g* for 30 min. The supernatant was adjusted to pH 5.2 with 0.1 *M* acetic acid, stirred for 5 min and centrifuged at 15 000 *g* for 30 min. The pellet was suspended with 50 ml of 20 m*M* potassium phosphate buffer (pH 7.8) and adjusted to pH 7.8 with 1 *M* sodium hydroxide. This solution was incubated with a solution of trypsin (1 mg per 500 mg of protein) containing 1 m*M* calcium chloride for 120 min at 37°C and then centrifuged at 15 000 *g*. The supernatant was adjusted to pH 7.8 with 1 *M* sodium hydroxide, dialysed overnight against 2000 ml of 20 m*M* potassium phosphate buffer (pH 7.8) and applied to the captopril affinity gel (1.6 × 16 cm) previously equilibrated with 20 m*M* potassium phosphate buffer (pH 7.8). The column was washed with the same buffer and eluted with a 200-ml linear gradient of sodium chloride (0—0.5 *M*).

Enzyme assay

ACE activity was measured by high-performance liquid chromatography according to the method of Horiuchi et al. [5] using Hip-His-Leu as a substrate. One unit of activity is defined as the amount of enzyme catalysing the release of 1 nmol of hippuric acid from Hip-His-Leu per min at 37°C. Protein was determined according to the method of Lowry et al. [6] with bovine serum albumin as a standard. Polyacrylamide slab gel electrophoresis was performed by the method of Davis [7].

TABLE I

PURIFICATION OF HUMAN PLACENTAL ANGIOTENSIN I CONVERTING ENZYME

Purification step	Total activity (nmol/min)	Total protein (mg)	Specific activity (nmol/min/mg)	Purification (*n*-fold)	Activity recovered (%)
The supernatant of homogenate centrifuged at 700 *g*	2120	3320	0.63	1	100
Acidification, the pellet centrifuged at 15 000 *g*	310	760	0.40	0.6	15
Trypsin treatment	475	135	3.52	5.6	22
Affinity chromatography	305	$108 \cdot 10^{-3}$	2824	4482	14

RESULTS AND DISCUSSION

As summarized in Table I, a 4482-fold purification was achieved to obtain a purified preparation of ACE from the human placenta with an overall yield of 14%. An approximately 800-fold purification achieved by the captopril affinity chromatography facilitated the present purification work (Fig. 1). The major ACE peak fractions obtained by this chromatography gave a single band on electrophoresis in polyacrylamide gel (Fig. 2).

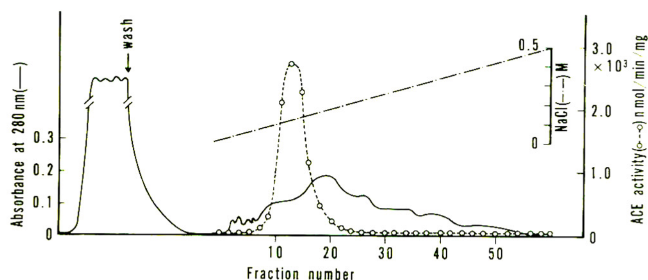

Fig. 1. Purification of angiotensin I converting enzyme on captopril affinity chromatography. (——) Distribution of proteins monitored by UV absorbance at 280 nm; (- - -) distribution of angiotensin I converting enzyme monitored by enzymatic activity. The flow-rate was 0.3 ml/min and the fraction volume was 3.25 ml.

Although affinity gels using the immunoadsorption technique [8] and potent inhibitors such as D- and L-cypteinyl-L-proline [9] have been used to purify human serum ACE successfully, relatively harsh elution conditions, such as 2 *M* magnesium chloride or 3 *M* urea, may have resulted in preparations with lower specific activity. In the present study, such conditions were avoided by using a low concentration of sodium chloride (0.5 *M*) to elute the enzyme from the affinity gel (Fig. 1). Another type of affinity column was recently developed by El-Dorry et al. [10], utilizing a potent inhibitor of ACE, N-α-[1-(*S*)-carboxy-3-phenylpropyl]-L-lysyl-L-proline, as affinity ligand.

ACE was first isolated from horse plasma [11]. Since the work of Ng and Vane [12] implicating the pulmonary vasculature as a major site of conversion of angiotensin I to angiotensin II, attention had been focused on the properties of the lung enzyme [13, 14]. We could purify ACE from human placenta by affinity chromatography using captopril, the orally active antihypertensive agent and potent inhibitor of ACE.

The physiological role of the ACE in various human tissues is not yet well understood. In the field of obstetrics and perinatal medicine, abundant evidence points to the importance of the renin—angiotensin and kallikrein—kinin systems in the physiological adjustment of circulation of a fetus in the feto-placental unit [15, 16]. The purified enzyme from human placenta by the present method could be used in the elucidation of the physiological significance of human ACE.

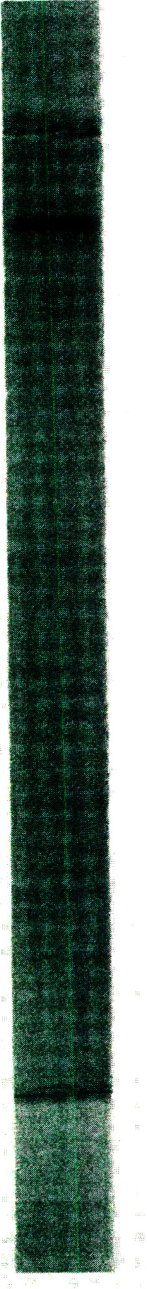

Fig. 2. Slab gel electrophoresis of the human placental angiotensin I converting enzyme in the major peak fractions of Fig. 1. The gel was stained for protein with Coomassie Brilliant Blue.

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Note

Simultaneous determination of metoprolol and deuterium-labelled metoprolol in human plasma by gas chromatography—negative-ion mass spectrometry

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The simultaneous administration of a drug and the same molecule labelled with a stable isotope is used in pharmacokinetic studies [1—3] to avoid most of the pitfalls caused by intra-individual variability. Both the unlabelled and the labelled compound must be assayed in the same sample. This assay is performed by gas chromatography—mass spectrometry (GC—MS).

Since the appearance of the first assay method for metoprolol using gas chromatography with electron-capture detection (ECD) by Ervik [4], several other methods have been published [5—8]. A GC—MS assay of metoprolol and its main metabolites in plasma has also been reported [9].

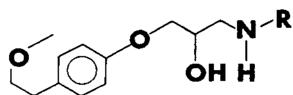
The present paper describes the simultaneous determination of metoprolol and [$^{2}H_{6}$]metoprolol by GC—MS in the negative-ion mode using [$^{13}C_{3}$]-metoprolol as internal standard.

EXPERIMENTAL

Chemical and reagents

[$^{2}H_{6}$]Metoprolol hydrochloride (Fig. 1) was synthesized in our laboratories according to the method of Chaudhuri and Ball [10] from [$^{2}H_{6}$]isopropyl-amine. The latter was obtained by the method of Colombini et al. [11] from [$^{2}H_{6}$]acetone (CEA, Saclay, France). [$^{13}C_{3}$]Metoprolol was prepared similarly from [$^{13}C_{3}$]acetone (Prochem, London, U.K.).

A buffer solution (1 l) was prepared by dissolving 168 g of potassium hydroxide (Merck 5033) and 360 g of tripotassium phosphate (Merck 5013) in distilled water. Heptafluorobutyric anhydride (HFBA) was purchased from Ventron (Ref. PCR, 1300-3; Ventron, Karlsruhe, F.R.G.).



R	Compound
$-CH(CH_3)_2$	Metoprolol
$-CH(C^2H_3)_2$	[$^2H_6$]Metoprolol
$-^{13}CH(^{13}CH_3)_2$	[$^{13}C_3$] Metoprolol

Fig. 1. Chemical structures of the labelled compounds.

Equipment

The glassware was washed using Extran (Merck) as detergent, silanized by immersion in a toluene bath containing hexamethyldisilazane (HMDS), chlorotrimethylsilane and pyridine at a concentration of 1% each for 15 min. It was rinsed twice with methanol, immersed in an ultrasonic bath of methanol for 15 min and dried in an oven at 100°C.

The GC—MS instrument was a Ribermag Model 10-10 equipped for negative-ion detection. The software of the on-line PDP8/A computer was only used for the measurement of the chromatographic peaks. Further calculations were made on a Wang MVP computer.

The column was a 2 m × 2 mm I.D. glass tube. The tube, treated at room temperature with a 5% HMDS solution in toluene for about 1 h, was packed with 3% OV-1 on 100—120 mesh Gas-Chrom Q. The column was operated with a helium flow-rate of 20 ml/min. Before use it was silanized at 80°C (by injecting HMDS) and conditioned at 300°C. The injection port, the column and the interface temperatures were 250°C, 230°C and 300°C, respectively. The retention time of metoprolol was about 1.4 min and the total run time about 2 min.

The mass spectrometer was operated in the negative-ion chemical-ionization mode with nitrous oxide as reagent gas (3 ml/min). The masurements were performed at *m/e* values 488, 494 and 491 for metoprolol, [$^2H_6$]- and [$^{13}C_3$]-metoprolol, respectively.

Extraction, derivatization and detection

A 1 ml aliquot of plasma spiked with 50 μl of the internal standard solution

(16 μmol/l aqueous solution), 1 ml of the buffer solution and 4 ml of dichloromethane—diethyl ether 1:4 were shaken mechanically at 300 rpm for 15 min. After centrifugation, the organic phase was removed and evaporated to dryness under a nitrogen flow at 40°C. Then 1 ml of a mixture of hexane—pyridine (97:3) was added. The tube was shaken for about 15 sec; 10 μl of HFBA were added and the tube was shaken again for 15 sec. The derivatization was allowed to proceed at room temperature for 15 min, then 1 ml of a saturated aqueous solution of potassium dihydrogen phosphate was added. The mixture was shaken for about 15 sec, and 1—2 μl of the upper phase were injected.

Correction of the data for isotopic contributions

Due to isotopic impurities, the measurements at *m/e* values 488, 494 and 491 were not specific for metoprolol, [$^{2}H_{6}$]- and [$^{13}C_{3}$]metoprolol, respectively. As shown by Fig. 2, chromatographic peaks were recorded at the various *m/e* values after separate injection of each of the three compounds.

For the estimation of the isotopic contributions, three aqueous solutions containing either metoprolol, [$^{2}H_{6}$]- or [$^{13}C_{3}$]metoprolol at a concentration around 1.5 μmol/l were processed as described for the plasma samples except for the addition of the internal standard which was omitted. After separate injection, the recorded areas at *m/e* 488, 494 and 491 were used for the estimation of the isotopic contributions and correction of the measured peak areas as described [12].

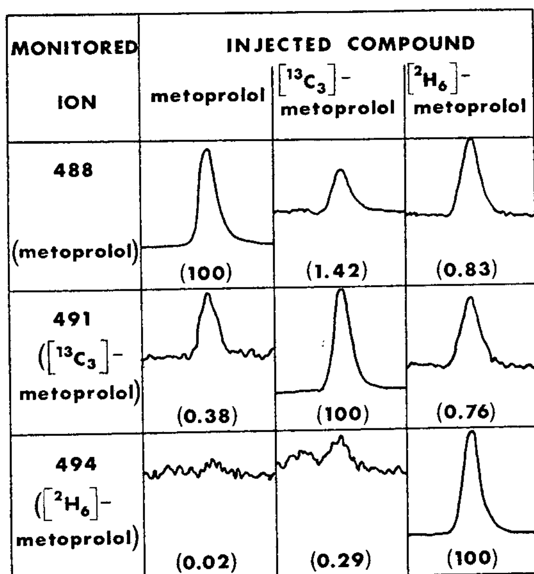


Fig. 2. Interferences for each substance from the other two substances. Unlabelled, [$^{2}H_{6}$]- and [$^{13}C_{3}$]metoprolol derivatives were measured at *m/e* values of 488, 491 and 494, respectively. The peaks and the related areas (arbitrary units) after separate injection of each substance are shown.

Calibration

Solutions of equal concentrations of metoprolol and [$^{2}H_{6}$]metoprolol in

the concentration range 0.25—40 μmol/l were prepared using the internal standard solution as solvent. Blank human plasma aliquots (1 ml) were spiked with 50 μl of each of these solutions resulting in final concentrations in the range 12.5—2000 nmol/l. These calibration samples were processed as described above. The calibration curves were fitted to the results as the weighted linear least-squares regression using the inverse of the squared concentration as the weighting factor [13].

RESULTS AND DISCUSSION

The fragmentation of the heptafluorobutyryl derivatives in the conditions used for the analysis resulted in a base peak ($C_3F_7COO^-$, m/e = 213), common to all three derivatives. The monitored ion, whose probable structure is displayed in Fig. 3, is specific for the side-chain of metoprolol with the isopropylamino group. The back-extraction into an aqueous phase required by GC—ECD methods could be omitted. This, and a reaction time for the derivatization reduced to 15 min, resulted in a fast analytical technique.

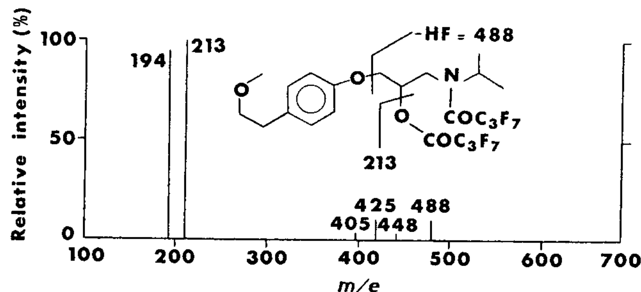


Fig. 3. Mass spectrum of metoprolol as its heptafluorobutyryl derivative (molecular weight = 659) using negative-ion detection and nitrous oxide as reagent gas.

TABLE I

MEAN PERCENTAGE RECOVERY OF METOPROLOL IN PLASMA SAMPLES SIMULTANEOUSLY SPIKED WITH METOPROLOL AND $^2H_6$-LABELLED METOPROLOL

$[^2H_6]$Metoprolol added (nmol/l)	Metoprolol added (nmol/l)				
	20	50	250	750	1500
0		97.9	101.9	103.0	100.8
20	99.1		101.4		
50		100.8	102.7	102.4	101.0
250	109.3	104.7	103.8	103.3	100.7
750		94.6	101.7	101.7	100.6
1500		99.3	102.4	102.0	101.0
Mean*	102.5	99.7	102.4	102.4	100.8
S.D.*	5.9	3.6	1.0	1.2	0.53

Mean overall recovery ± S.D.: 101.5 ± 2.78%

*Calculated on actual individual values.

TABLE II

MEAN PERCENTAGE RECOVERY $^{2}H_6$-LABELLED METOPROLOL IN PLASMA SAMPLES SIMULTANEOUSLY SPIKED WITH METOPROLOL AND $^{2}H_6$-LABELLED METOPROLOL

Metoprolol added (nmol/l)	$[^{2}H_6]$Metoprolol added (nmol/l)				
	20	50	250	750	1500
0		102.4	99.6	101.7	100.6
20	107.4		102.8		
50		102.7	100.9	101.8	101.7
250	117.3	104.4	101.9	101.9	101.4
750		106.0	102.3	101.2	101.9
1500		104.0	101.5	102.3	100.3
Mean*	110.7	103.9	101.5	101.7	101.2
S.D.*	5.9	1.8	1.4	0.52	1.1

Mean overall recovery ± S.D.: 103.1 ± 3.79%

*Calculated on actual individual values.

Plasma samples simultaneously spiked with variable amounts of metoprolol and $[^{2}H_6]$metoprolol in the concentration range 20—1500 nmol/l were used for the validation of the method. The mean recovery (Tables I and II) was in the range 99.7—103.9% for both compounds at all the investigated concentrations except for $[^{2}H_6]$metoprolol at the level of 20 nmol/l (110.7%). The good precision, as indicated by the low values of the standard deviation, was probably due to the use of $[^{13}C_3]$metoprolol as the internal standard which provided an efficient compensation for the loss of metoprolol and $[^{2}H_6]$metoprolol. The slope of the regression straight line of recovered amounts plotted versus the theoretical was 1.01 for both compounds (coefficients of correlation higher than 0.9998). The value of the intercept was 2.12 and 1.14 nmol/l for metoprolol and $[^{2}H_6]$metoprolol, respectively. The method described by Ervik [4] requires 2—4 ml of plasma and the internal standard is added just before injection. The other methods [5—8] have a sensitivity limit in the range 3—30 nmol/l. A sensitivity limit of 1 nmol/l was claimed for the GC—MS method [9] but without experimental evidence.

The sensitivity limit of the present technique is 20 nmol/l, but since its objective is essentially the simultaneous determination of metoprolol and $[^{2}H_6]$metoprolol, it cannot be compared to previously published methods.

CONCLUSION

The GC—MS method described here permits the simultaneous determination of metoprolol and $[^{2}H_6]$metoprolol present in the same plasma sample at concentrations in the range 20—1500 nmol/l (7 ng/ml of the tartrate salts). It is suitable for the performance of pharmacokinetic studies in which both compounds are administered simultaneously to the same individual by two different routes and/or as two different preparations.

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CHROMBIO. 2496

Note

Gas chromatographic determination of pentacaine in rat serum

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(First received August 28th, 1984; revised manuscript received November 6th, 1984)

Pentacaine, *trans*-2-(1-pyrrolidino)cyclohexyl-3-pentyloxycarbanilate chloride (Fig. 1), is a local anaesthetic of alkoxycarbanilate type [1] that has recently been undergoing clinical studies. It has been determined in biological fluids only as [^{3}H]pentacaine by selective ion-pair extraction into an organic solvent at low pH [2].

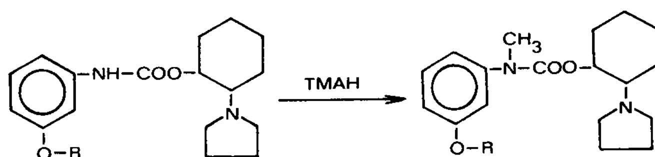

Fig. 1. Molecular structures of pentacaine, the internal standard and their N-methyl derivatives.

In this paper we describe a gas chromatographic (GC) method based on previous findings of Štefek et al. [3] concerning the possibility of stabilizing the thermally labile carbamate bond in alkoxycarbanilates by its reaction with trimethylanilinium hydroxide (TMAH) in the GC injector (Fig. 1).

For the separation of pentacaine from serum, the method described by Šoltés and co-workers [4, 5] was used to isolate the drug from biological material rapidly and selectively, via its adsorption—desorption properties on C_{18}-silanized silica (Sep-Pak C_{18}, Silipor C_{18}).

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EXPERIMENTAL

Standards and reagents

Pentacaine and *trans*-2-(1-pyrrolidino)cyclohexyl-3-hexyloxycarbanilate chloride (Fig. 1), used as internal standard, were kindly supplied by the Faculty of Pharmacy, Comenius University, Bratislava, Czechoslovakia. Trimethylanilinium hydroxide (0.1 mol l^{-1} in methanol) was purchased from Serva (Heidelberg, F.R.G.). Irregularly shaped Silipor C_{18} silica gel [16.3% (w/w) C], particle size 125—160 μm, was obtained from the Research Institute of Pure Chemicals, Lachema, Brno, Czechoslovakia. The organic solvents (acetonitrile, methanol and benzene, all supplied from Lachema, Brno, Czechoslovakia) were distilled before use as also was the water used for reagents.

Glassware

All glassware was cleaned in hydrochloric acid, then silanized with a 5% solution of Surfasil (Pierce, Rotterdam, The Netherlands) in benzene.

Gas chromatographic conditions

GC separations were performed under isothermal conditions on a Perkin-Elmer 900 gas chromatograph equipped with a flame ionization detector. The glass column (2 m × 2 mm I.D.) was packed with Chromosorb W AW DMCS (80—100 mesh) coated with 2% OV-17. The column temperature was 260°C, injection port temperature 280°C. Nitrogen was used as carrier gas with a flow-rate 40 ml min^{-1}.

Sample preparation

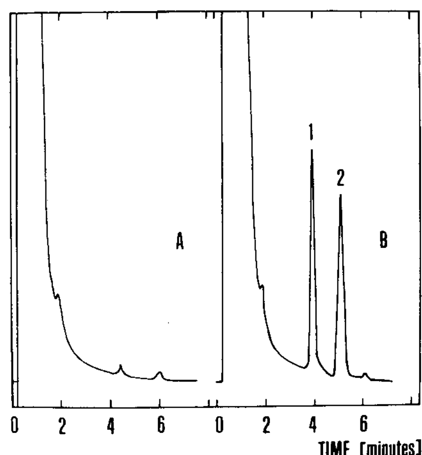
A serum sample (1 ml) was placed in a glass-stoppered tube and 0.1 ml of internal standard solution (10 μg ml^{-1} in water) was added. Silipor C_{18} (100 mg), fixed in a 2-ml plastic syringe barrel [5], was conditioned before use by washing with acetonitrile (1 ml) and water (1 ml), then the serum sample was applied. After the sample had passed through the syringe barrel the sorbent layer was washed with 1 ml of water and then 1 ml of acetonitrile. Pentacaine retained by the sorbent was eluted with 2.5 ml of methanol. The methanol was evaporated to dryness in a water bath at 60°C under a mild stream of nitrogen; 10 μl of TMAH in methanol were added to the dry residue and 1 μl of the resulting solution was injected into the gas chromatograph.

Standard curves

Varying quantitites of pentacaine (0.1—10 μg) were added to 1 ml of rat control serum. After standing overnight, the samples were carried through the analytical procedure. The ratio of the peak height of pentacaine to that of the internal standard was plotted against the concentration of pentacaine.

RESULTS AND DISCUSSION

Typical chromatograms obtained with blank serum before and after spiking with pentacaine and the internal standard are shown in Fig. 2. The peaks of the two compounds were well resolved (R = 2.6) and no interference was observed



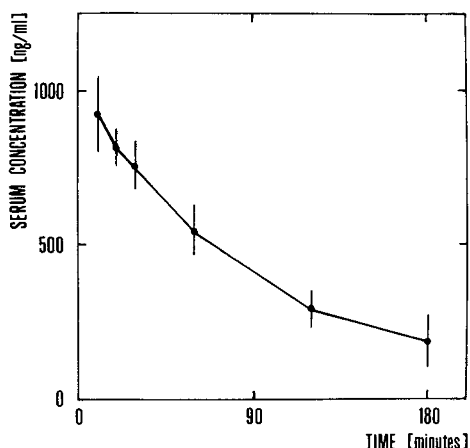


Fig. 2. Gas chromatograms of serum extracts. (A) Drug-free serum; (B) serum spiked with pentacaine (1) and the internal standard (2), both in a concentration of 1 μg/ml.

Fig. 3. Serum concentration—time curve of pentacaine after intravenous administration of 4 mg/kg to rats (n = 6).

TABLE I

REPEATABILITY AND RECOVERY OF THE METHOD AS APPLIED TO SPIKED RAT SERUM SAMPLES

Amount added (μg ml^{-1})	Average of six assays ± S.D. (μg ml^{-1})	R.S.D. (%)
0.1	0.11 ± 0.01	9.1
0.5	0.52 ± 0.03	5.8
1.0	0.96 ± 0.08	8.3
5.0	5.18 ± 0.10	1.9
10.0	9.92 ± 0.11	1.1

from endogenous substances. Interference from metabolites was not anticipated because of their different chromatographic behaviour [6].

The absolute recovery of the extraction procedure determined with ^{3}H-labelled pentacaine was found to be 83.4%. A standard calibration curve obtained after extraction of pentacaine from serum was linear in the concentration range studied (y = 0.741x + 0.021; r = 0.9995). The repeatability and relative recovery of the method are reported in Table I.

The absolute sensitivity of the flame ionization detector for pentacaine (10 ng) allowed application of the method for the determination of serum levels of pentacaine in rats after a single intravenous administration of the drug. Fig. 3 illustrates these levels up to 3 h post dose.

The serum levels determined by the described GC method are in good agreement with the results of Bezek et al. [7], which were obtained with ^{3}H-labelled pentacaine.

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CHROMBIO. 2522

Note

Determination of cyclobenzaprine in plasma and urine using capillary gas chromatography with nitrogen-selective detection

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(First received September 13th, 1984; revised manuscript received December 5th, 1984)

Cyclobenzaprine, a tricyclic muscle relaxant, has been analyzed by thin-layer chromatography [1], and packed-column gas chromatography (GC) with either flame ionization [2] or nitrogen-selective detection [3]. With the nitrogen-specific detector, the intra-day coefficient of variation (C.V.) was 11% when 5 ng cyclobenzaprine in 2 ml plasma was quantified. In addition, numerous extractions were required as part of the sample preparation procedure to quantify the drug in the therapeutic range of drug concentrations. In support of pharmacokinetic studies, it was desirable to increase the sample throughput by reducing the sample preparation time and to lower the limit of reliable quantification for a fuller mapping of the plasma concentration—time courses. A capillary GC method with nitrogen-selective detection was developed which employs a single extraction of the biological specimen. The intra-day coefficient of variation at 1 ng/ml was less than 10%.

EXPERIMENTAL

Materials and reagents

Normal hexane (nanograde, Mallinckrodt, Paris, KY, U.S.A.), triethylamine (TEA, sequanal grade, Pierce, Rockford, IL, U.S.A.), methanol, and ethyl acetate (HPLC grade, Burdick & Jackson Labs., Muskegon, MI, U.S.A.), anhydrous sodium carbonate and sodium bicarbonate (reagent grade, Merck, Rahway, NJ, U.S.A.), sodium hydroxide (reagent grade), hydrochloric acid (concentrated, reagent grade), monobasic sodium phosphate, dibasic sodium phosphate (Fisher Scientific, Fair Lawn, NJ, U.S.A.), β-glucuronidase (bovine liver, P.L. Biochemicals, Milwaukee, WI, U.S.A.) and LR101 concentration tubes (Laboratory Research, Los Angeles, CA, U.S.A.) were purchased from

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their respective suppliers. The internal standard, 5-(2-dimethylamino-ethylidene)dibenzo[*a,e*] cyclohepatriene was obtained from Merck.

Assay procedures

Plasma (1.0 ml), internal standard (10 ng in 0.1 ml), carbonate buffer (1 ml, 0.2 *M*, pH 9.8), and hexane (5 ml) were transferred to a glass centrifuge tube (13 ml). The tube was stoppered and the contents shaken and then centrifuged (5 min at 2 *g*). The organic layer was transferred to a LR101 concentration tube and the hexane evaporated to dryness (50°C) under a stream of nitrogen. The residue was reconsituted in TEA—hexane (0.01% TEA, 20 μl) and an aliquot (5 μl) of the TEA—hexane mixture injected onto the GC column.

Urine (1.0 ml), internal standard (500 ng in 0.1 ml), and β-glucuronidase (1 ml = 5000 Fishman units prepared in 0.2 *M* phosphate buffer, pH 6.5) were transferred to a glass centrifuge tube (13 ml). The tube was stoppered and placed in a Teacam Driblock at 37°C for 18 h. At the end of this time period, sodium hydroxide (0.5 ml, 0.5 *M*) and hexane (5 ml) were added. The tube was stoppered and the contents shaken and then centrifuged (5 min at 2 *g*). The hexane was transferred to another tube and drug and internal standard were back-extracted into acid (0.2 ml, 0.1 *M* hydrochloric acid). After removal of the hexane, chromatographic contaminants remaining in the aqueous phase were extracted with additional hexane (3 ml). The hexane layer was discarded and the aqueous layer was made basic by the addition of sodium hydroxide (0.1 ml, 0.5 *M*). Cyclobenzaprine and internal standard were extracted from the basic milieu with ethyl acetate (0.5 ml) and an aliquot (2 μl) of the ethyl acetate was injected onto the chromatographic column.

Calculations

A standard curve of cyclobenzaprine in plasma or in urine was run daily with the clinical specimens. The calibration curves for plasma and urine were linear from 1 to 30 ng/ml and 2 to 8 μg/ml, respectively. The equation for the resulting line was $y = 0.0890x + 0.05309$ (with $r^2 = 0.99939$) for plasma and $y = 0.73663x - 0.04853$ (with $r^2 = 0.99947$) for urine. The peak ratio of the drug to internal standard from the unknown sample was employed to calculate their concentrations from the standard curve.

Instrumentation

Analyses were performed on a Varian 6000 gas chromatograph equipped with a nitrogen—phosphorus detector (thermal specific detector). For plasma and urine, respectively, a 40- and 20-m length standard bore, 0.25-μm film thickness, DB-5, J & W. Scientific capillary column was used. A fritted glass liner with the top portion packed with 1% OV-17 on Gas-Crhom Q (80—100 mesh), which has been conditioned previously, was installed in the injection port to act as a guard column for plasma analysis. A fritted glass liner without packing was installed in the injection port for urine analysis. The splitter was off and the septum purge was capped.

Instrumental conditions

The injection port temperature and detector temperature were set at 300°C. The oven temperature programme for plasma was (a) 140°C initial temperature, 0 min hold; (b) 50°C/min to 230°C; (c) 4°C/min to 245°C; (d) 50°C/min to 300°C, 3 min hold. The oven temperature programme for urine was (a) 140°C initial temperature, 0 min hold; (b) 50°C/min to 230°C; (c) 4°C/min to 245°C, 2 min hold. Flow-rates of the hydrogen, air, and helium (make-up) gases were 4.5, 175 and 20 ml/min, respectively. The helium (carrier) gas had a column head pressure of 2.068 bars.

RESULTS AND DISCUSSION

The intra-day and inter-day precision of the methods are presented in Table I. Typical chromatograms are presented in Figs. 1 and 2.

With the thermionic bead operating at 80% maximum current, a minimum of 10 pg of drug could be detected easily (signal-to-noise ratio > 10). In the presence of endogenous interferences, 1 ng/ml of plasma could be quantified reliably as indicated by Table I. Since one fourth of the prepared plasma sample was injected, approximately 250 pg of the drug were being quantified in the precence of the endogenous interferences with absolute recovery of 90%.

Following cyclobenzaprine administration in man, unchanged cyclobenzaprine constitutes only a minor fraction in urine, the major fraction is a glucuronide-like conjugate of cyclobenzaprine [4]. After conversion of the glucuronide back to the parental form reliable quantification of 0.2 μg of cyclobenzaprine per ml of urine can be achieved with recoveries of 95%.

A clean-up procedure which was more efficient in reducing the endogenous interferences relative to the drug would, in principle, yield an even more sensitive method. However, as with the structurally similar tricylic antidepressants, the clean-up procedures can lead to losses of the drug due to adsorptive phenomena [5]. To minimize these losses, a single extraction with hexane was employed to maintain high drug recovery. High-efficiency capillary

TABLE I

INTRA- AND INTER-DAY PRECISION OF THE METHOD

Plasma			Urine		
ng/ml	C.V. (%)	*n*	μg/ml	C.V. (%)	*n*
Intra-day precision					
1	8.1	6	0.2	5.1	6
10	3.6	6	0.5	5.0	6
30	3.6	6	1.0	2.6	6
			2.0	2.7	6
			4.0	2.6	6
			8.0	2.6	6
Inter-day precision					
2.0	10.0	16	0.80	6.6	20
20.0	10.9	16			

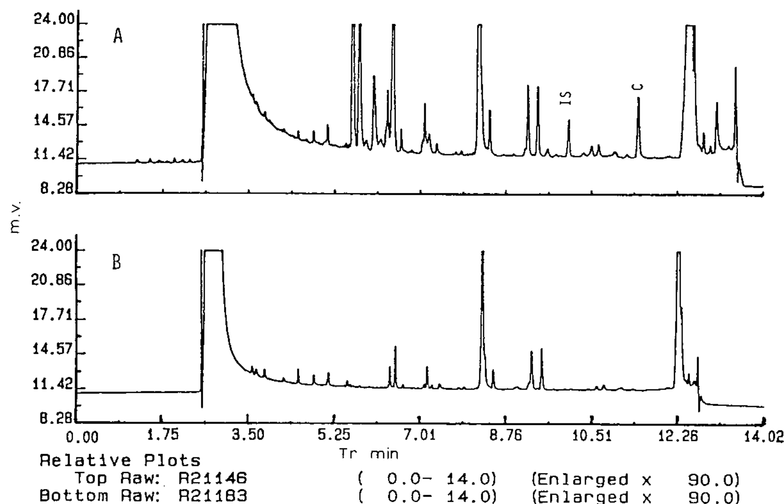

Fig. 1. Representative chromatograms of cyclobenzaprine (C) and internal standard (IS) in plasma: (A) 20.0 ng/ml cyclobenzaprine and internal standard; (B) blank plasma.

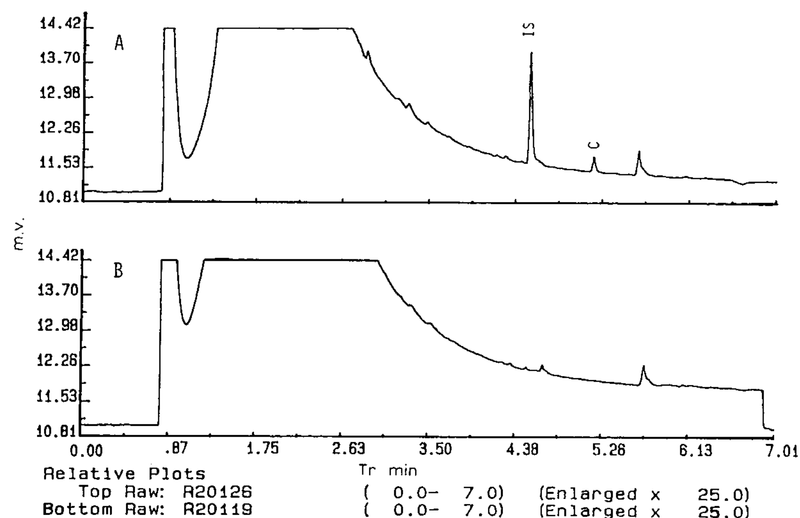

Fig. 2. Representative chromatograms of cyclobenzaprine (C) and internal standard (IS) in urine: (A) 0.2 μg/ml cyclobenzaprine and internal standard; (B) blank urine.

GC was then employed to resolve the drug from the endogenous interferences. In order to insure efficient reconstitution of the prepared samples containing nanogram quantities of the drug, it was necessary to add small amounts of triethylamine to the hexane. The triethylamine minimized adsorptive losses. In fact triethylamine in hexane has previously been used as an extracting solvent in a packed-column method for amitriptyline using a nitrogen-selective detector [6]. For cyclobenzaprine there was no advantage in using triethylamine in hexane compared to using hexane alone for the extracting solvent. However, as mentioned above it was a requisite for the reconstituting solvent. Neither

in the newly developed method for cyclobenzaprine nor in the previously reported method [6] for amitriptyline did the disturbance of the nitrogen detector by triethylamine prevent the quantification of the drug. The triethylamine eluted much earlier than the drug of interest.

This present method has been employed for the routine analysis of 600 plasma and 500 urine samples.

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Note

High-performance liquid chromatography of etoposide in plasma and urine

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Etoposide (VP16-213) is a semi-synthetic podophyllotoxin with activity against a variety of solid tumours and haematological malignancies [1—3]. It is one of the most active agents against small-cell lung carcinoma [1, 2, 4] and germ cell tumours [5]. Over the past few years several high-performance liquid chromatographic (HPLC) assays have been developed for the measurement of etoposide in biological fluids [6—12] and most of these employ the closely related epipodophyllotoxin teniposide (VM26) as internal standard. However, the large capacity factor and poor efficiency of teniposide combine to give extended run times (Fig. 1). To shorten analysis time an assay using diphenylhydantoin (DPH) as internal standard has been developed. Methylphenytoin (MPPH) was used in patients receiving DPH as an anticonvulsant.

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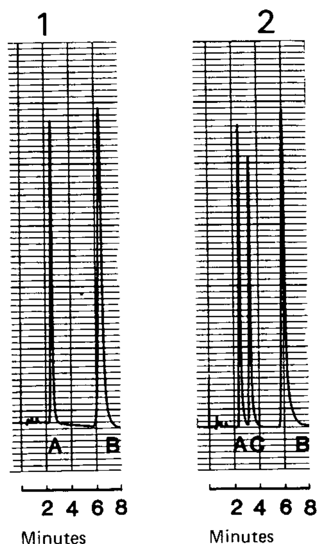

Fig. 1. (1) Chromatogram of etoposide (A) with teniposide (B) as internal standard. (2) Chromatogram of etoposide (A) and teniposide (B) with DPH (C) as internal standard.

MATERIALS AND METHODS

Materials

Etoposide was provided by Bristol-Myers U.K. Chloroform (AnalaR grade) and methanol (liquid chromatography grade) were obtained from BDH, and DPH and MPPH from Sigma. The HPLC separation was carried out using an Applied Chromatography Systems Series 300 pump with a Rheodyne 7125 injector and a Laboratory Data Control 1204D variable-wavelength UV detector. Separation was achieved with an isocratic solvent mixture using an ODS Hypersil column (see below).

Methods

Standards and control samples. Standards and control samples were prepared from a stock solution of 1000 μg/ml etoposide in methanol—water (51:49) which was added to drug-free pooled plasma or urine to give final concentrations of 0, 0.5, 1.0, 5.0, 10.0, 15.0 and 25.0 μg/ml of plasma and 0, 10.0, 25.0, 50.0, 75.0 and 100.0 μg/ml of urine. Quality-control samples to monitor within-run and between-run imprecision were prepared in the same way but from a different stock standard. Standards and controls were aliquoted into 1.5-ml capped tubes and stored at −20°C prior to use.

Sample extraction. A 50-μl volume of internal standard (200 μg/ml DPH, or MPPH in those patients receiving therapeutic DPH) was added to 1 ml plasma or 200 μl urine (urine was buffered with 1 ml phosphate-buffered saline, pH 7.3). Following the addition of 5 ml chloroform the tubes were mixed by rotation and centrifuged at 400 *g*, each for 10 min. The organic layer was then filtered into a clean glass tube, evaporated to dryness at 50°C and reconstituted in 200 μl mobile phase. Extraction efficiency was determined by addition of internal standard after extraction.

Chromatography. Chromatography was carried out using a 5-μm ODS Hypersil column (100 × 5 mm) with methanol—water (51:49) solvent at a flow-rate of 2 ml/min. Sample introduction was by means of a 7125 Rheodyne injection valve with a 50-μl sample loop and detection was by UV absorbance at 229 nm. This system typically gave retention times of 2.1 min for etoposide, 2.9 min for DPH and 4.5 min for MPPH. Flow-rate and mobile phase methanol content were adjusted as necessary to optimise resolution and retention on different columns. Calibration was achieved by running standards samples and using peak height ratios of etoposide to internal standard.

RESULTS

Chromatographic separation of etoposide and internal standards is shown in Fig. 2. Extraction efficiency of etoposide in plasma was > 80% at levels of 1.0, 5.0 and 15.0 μg/ml and in urine was > 90% at levels of 25.0 and 50.0 μg/ml. Extraction efficiency of DPH and MPPH determined using etoposide as internal standard was > 75% for both plasma and urine. Linear regression of peak height ratios against etoposide concentrations typically gave correlation coefficients of > 0.99. Within-run imprecision was < 4% in plasma at levels of 0.80, 4.71 and 15.9 μg/ml (n = 10) and < 3% in urine at a level of 50.0 μg/ml (n = 9).

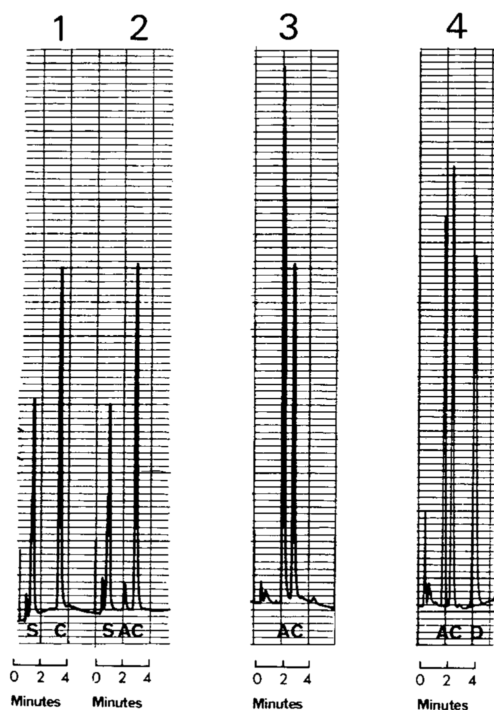

Fig. 2. Chromatograms of human plasma extracts (0.2 a.u.f.s. throughout). (1) Human plasma extract before etoposide administration showing solvent front (S) with DPH (C) as internal standard. (2) Etoposide peak (A) equivalent to 0.55 μg/ml. (3) Etoposide peak (A) equivalent to 10.27 μg/ml. (4) Patient sample containing both etoposide (A) and DPH (C) with MPPH (D) as internal standard.

Between-run imprecision found by running control samples twice daily was < 6.7% in plasma at levels of 0.82, 4.49 and 16.02 μg/ml (n = 24), and < 5% in urine at levels of 10.0, 25.0 and 50.0 μg/ml (n = 12).

There was no interference from metabolites of the drug or other UV-absorbing substances. The major metabolite, the picrohydroxy acid, is chloroform-insoluble [7] and another metabolite [8], the picro isomer, was not found. Drugs tested for possible interference included cytotoxics (cyclophosphamide, adriamycin, vincristine, methotrexate and procarbazine) and other commonly co-administered drugs (aspirin, paracetamol, dextropropoxyphene, dihydrocodeine, diamorphine, morphine, prednisone, metoclopramide, prochlorperazine and phenobarbitone). None of these compounds interfered in the assay either in simple solution or in the plasma and urine of patients to whom they had been administered.

DISCUSSION

The method described allows for the rapid measurement of etoposide in multiple samples with a run time of less than 3.5 min. This is a considerable improvement over the run times encountered with teniposide as internal standard and avoids handling a second cytotoxic agent on a regular basis. In patients taking DPH for therapeutic reasons MPPH was used as internal standard. Despite the longer retention time of 4.5 min this was still an improvement over teniposide. The limit of detection in this assay of 100 ng/ml is adequate for sample measurement up to 24 h following doses within the clinical range commonly used (> 100 mg orally or > 50 mg intravenously) but may not be sufficiently sensitive to monitor drug levels over more prolonged periods. Under these conditions alternative means of detection such as fluorescence [7, 13] or electrochemical activity [11] may be required.

There was no interference from the major metabolite, the picrohydroxy acid, which is chloroform-insoluble [7]. The picro isomer of etoposide was poorly resolved from the parent drug in this system, but was still detectable. Although this metabolite has been found in some children [8] it was not present in any of the patient samples. It has been suggested that co-administered drugs may interfere with the UV detection of etoposide [13] but this was not noted for a variety of drugs given with etoposide, including cytotoxics, analgesics and anti-emetics.

The method can also be used for the determination of teniposide and may be particularly useful if etoposide and teniposide are given concurrently, as has been suggested by Allen et al. [14].

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Note

Determination of 4-amino-3-(*p*-chlorophenyl)butyric acid (baclofen) in plasma by high-performance liquid chromatography

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(First received August 7th, 1984; revised manuscript received November 27th, 1984)

Baclofen, a derivative of γ-aminobutyric acid (GABA), is used to relieve symptoms of spasticity in patients suffering from multiple sclerosis and other spinal lesions [1, 2]. Existing assay methods based on either gas–liquid chromatography [1, 2] or gas chromatography–mass spectrometry [3, 4] require chemical derivatization steps prior to assay. This paper describes a rapid, simple high-performance liquid chromatographic (HPLC) method for assaying baclofen in plasma without derivatization.

EXPERIMENTAL

Materials and reagents

Baclofen was supplied by Ciba-Geigy (Sydney, Australia). Acetonitrile (HPLC grade) was obtained from Waters Assoc. (Sydney, Australia) and all other reagents were of analytical grade. C_{18} Bond-Elut™ columns (1 ml capacity) and a Vac-Elut™ manifold (Analytichem, Harbor City, CA, U.S.A.) were purchased from FSE Scientific (Melbourne, Australia).

The HPLC system consisted of a Constametric III pump and a SpectroMonitor III variable-wavelength UV detector (LDC, Riviera Beach, FL, U.S.A.), a Model 7125 injection valve with a 50-μl loop (Rheodyne, Berkeley, CA, U.S.A.) and an Omniscribe recorder (Houston Instruments, Austin, TX, U.S.A.). The chromatography was carried out on a 25 cm × 0.3 cm I.D. stainless-steel glass-lined column packed with 5-μm Spherisorb ODS silica (SGE Scientific, Ringwood, Victoria, Australia).

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Bond-Elut extraction procedure

Baclofen was extracted from plasma using 1-ml Bond-Elut columns packed with ODS silica (C_{18} columns). The columns were placed in luer fittings in the top of the Vac-Elut chamber which has the capacity for ten columns. A vacuum of 25—50 cmHg was applied to the manifold to effect the various stages of the extraction. Prior to use the columns were activated by washing with 2 × 1 ml acetonitrile followed by 2 × 1 ml of 0.1% orthophosphoric acid solution.

To extract baclofen from plasma, 1 ml of plasma (sample or standard) was passed through the activated C_{18} Bond-Elut column which was then washed with 0.1% solution of orthophosphoric acid (0.5 ml) followed by 0.5 ml of 0.1% orthophosphoric acid—acetonitrile (80:20). The vacuum was then released and the stainless-steel needles of the Vac-Elut chamber wiped. Appropriately labelled tubes were placed under the columns, which were then eluted with 0.3 ml of 0.05 *M* sodium dihydrogen orthophosphate—acetonitrile (75:25). An aliquot (50 µl) of these extracts was then injected onto the HPLC column.

Chromatography

In order to separate baclofen from other endogenous components of plasma the HPLC column was eluted with a mobile phase of 0.05 *M* sodium dihydrogen orthophosphate—acetonitrile (95:5) at a flow-rate of 1 ml/min. Baclofen was detected at a wavelength of 220 nm and a sensitivity of 0.01 a.u.f.s.

Preparation of standards

Stock solutions of baclofen (1 mg/ml) were prepared in distilled water and stored at 4°C. Plasma containing known concentrations of baclofen were prepared by appropriately diluting the stock solution with drug-free plasma. These plasma standards were stored frozen at −20°C and made fresh each week. The plasma standards were then used to standardize the extraction procedure and to calibrate the HPLC determination. The concentration of baclofen in plasma samples was determined by measuring peak height and using a calibration plot determined with the plasma standards.

Extraction recoveries

The recovery of baclofen extracted from plasma with the Bond-Elut columns was estimated by comparing the results obtained with a non-extracted standard at the same concentration in the phosphate—acetonitrile (75:25) buffer used to elute the baclofen from the Bond-Elut columns. The recoveries were determined from the mean of six replicates.

Screening for interfering drugs

A range of drugs that are commonly co-administered with baclofen were screened for their possible interference in the assay. Plasma from patients known to be taking a particular drug was processed by the method and the resultant HPLC profile checked for interfering peaks at the retention time of baclofen. This process eliminated any potential interference from the parent

drug and its metabolites at levels likely to be encountered in the clinical situation.

Baclofen plasma levels

Blood samples were taken from multiple sclerosis patients on chronic baclofen therapy by venipuncture at hourly intervals (for 5 h) starting 1 h after the morning dose. The blood samples were placed in heparinised tubes and centrifuged immediately to obtain the plasma which was stored frozen at −20°C until assayed.

RESULTS AND DISCUSSION

Fig. 1 shows the chromatograms obtained from plasma spiked with a known amount of baclofen (A), drug-free plasma (B), and plasma from a patient after a normal oral dose of baclofen (C). Under the HPLC conditions used baclofen had a retention time of 9.4 min. No interference was observed from any endogenous plasma component, although the chromatogram obtained with the drug-free plasma contained several peaks. It was not possible to remove these endogenous plasma components from the Bond-Elut column without suffering further losses of baclofen. The recovery of baclofen from plasma was found to be 38.7 ± 1.4% (n = 6) at 50 ng/ml and 46.0 ± 0.9% (n = 6) at 1000 ng/ml. These recoveries are comparable to those reported for the gas chromatographic methods [2]. No baclofen could be detected in plasma collected after passing through the Bond-Elut column and reextracted. Therefore, the relatively poor recoveries are a result of the clean-up washes of the Bond-Elut column rather than poor initial extraction of baclofen by the Bond-Elut column.

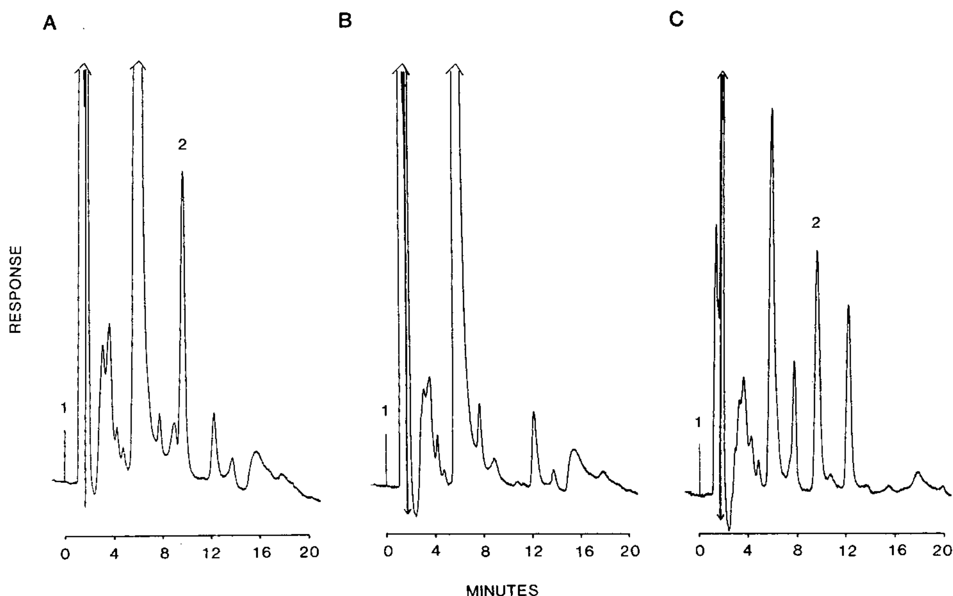

Fig. 1. HPLC profiles of (A) drug-free plasma spiked with 800 ng/ml baclofen, (B) drug-free plasma, (C) patient plasma sample (725 ng/ml baclofen) collected 1 h after a dose of 90 mg baclofen. 1 = Injection site; 2 = baclofen.

The intra-assay precision of the method was determined from replicate assays (n = 6) of drug-free plasma spiked with known concentrations of baclofen. A coefficient of variation (C.V.) of 2.0% at 1000 ng/ml and 3.7% at 50 ng/ml was obtained. This is an improvement over C.V. values of up to 10% obtained with previously published methods [1—4]. The relatively low C.V. obtained for the method reported here makes the use of an internal

TABLE I

DRUGS SHOWING NO INTERFERENCE WITH BACLOFEN DETERMINATION

Anticonvulsants	*Tranquilisers*	*Antibiotics*
Phenytoin	Phenobarbitone	Amikacin
Carbamazepine	Oxazepam	Doxycycline
Primidone	Chlordiazepoxide	Rifampicin
Valproate	Haloperidol	Penicillin
Clonazepam	Thioridazine	Septrin
	Amylobarbitone	Gentamicin
		Mandelamine
Antidepressants	*Sedatives*	*Analgesics*
Doxepin	Nitrazepam	Doloxene
Amitriptyline	Temazepam	Aspirin
Nortriptyline	Chloral hydrate	Paracetamol
Imipramine	Lorazepam	Naprosyn
Antispasticity	*Antinauseants*	*Anticholinergics*
Diazepam	Prochlorperazine	Propantheline
Orphenedrine	Metoclopramide	

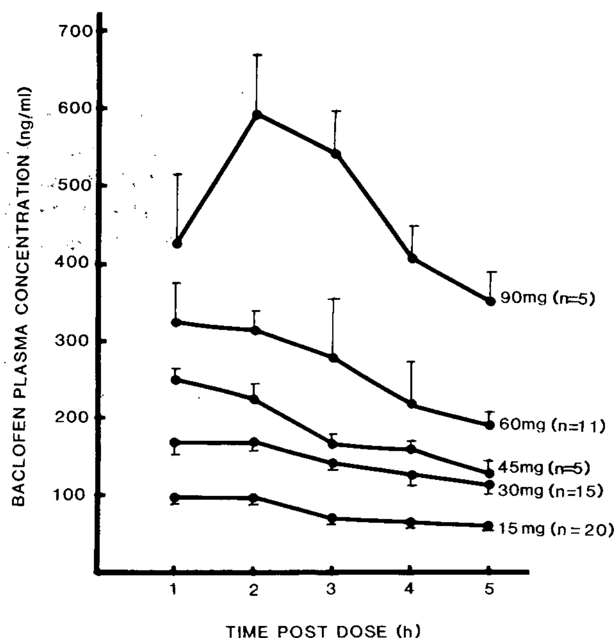

Fig. 2. Mean (± S.E.M.) baclofen plasma levels in multiple sclerosis patients on chronic baclofen therapy.

standard unnecessary despite the low recoveries obtained. This is perhaps fortunate as it would be difficult to find a suitable compound with similar properties to baclofen and not suffer any interference from endogenous plasma components.

The assay was linear over the plasma concentration range 0.05—1.0 μg/ml ($y = 0.187x$, $r = 0.997$, $n = 7$) which spans the accepted therapeutic range [5]. No interference was observed with plasma from patients known to be taking drugs that are commonly co-administered with baclofen (Table I). Possible interference may be observed with flucloxacillin and amoxycillin.

As an example of the use of the method Fig. 2 shows the mean baclofen plasma levels in multiple sclerosis patients on chronic therapy at a range of doses. The difficult and previously lengthy methods for the determination of baclofen has meant that this information in so many patients has not been available before.

In summary, the method described here offers quick and straightforward sample preparation giving a total assay time of 15 min compared to up to 2 h for the extraction alone as in a previously published method [2]. The use of commonly available HPLC equipment rather than the less common gas chromatograph—mass spectrometer overcomes the need for derivatisation and makes the method widely accessible.

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Note

Simple and rapid analysis of atenolol and metoprolol in plasma using solid-phase extraction and high-performance liquid chromatography

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Metoprolol and atenolol are chemically diverse cardioselective β-adrenoceptor antagonists, with atenolol relatively hydrophilic and metoprolol relatively lipophilic. As a consequence of widespread use of these and other β-adrenoceptor antagonists in the treatment of hypertension and angina pectoris [1, 2], there are many published methods for their determination in plasma and urine. Early methods employed gas—liquid chromatography using either electron-capture [3—14] or mass spectrometric [11] detection. These methods, while specific and sensitive, involved a lengthy derivatization step. Recent methods based on high-performance liquid chromatography (HPLC) [15—23] have been shown to be selective and sensitive; however, extraction procedures used (differential, pH-dependent solvent extraction followed by evaporation and reconstitution with mobile phase) tend to be laborious, taking from 30 min to 1 h or more.

The procedure reported here for the extraction of metoprolol and atenolol from plasma is based on solid-phase extraction media used in Bond-Elut™ columns. The extraction is versatile, efficient, rapid (1 min for extraction) and avoids exposure to alkaline conditions. Extracts can be immediately subjected to sensitive and selective assay using HPLC with fluorometric detection. While a sample is being chromatographed the next can be extracted, or up to ten samples can be extracted at any one time. We are only aware of one previous report of this extraction technique being used [24], application being limited to the hydrophilic compound, atenolol.

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EXPERIMENTAL

Materials and reagents

Metoprolol was supplied by Ciba-Geigy (Switzerland) and atenolol by ICI (Australia). HPLC-grade acetonitrile was obtained from Waters Assoc. (Sydney, Australia), all other reagents were of analytical grade. Bond-Elut CN and C_{18} columns (1 ml capacity) and the Vac-Elut™ manifold (Analytichem, Harbor City, CA, U.S.A.) were purchased from FSE Scientific (Melbourne, Australia).

The HPLC system consisted of a Constametric III pump (LDC, Riviera Beach, FL, U.S.A.), a Model 7010/7011 injection valve with a 20-μl loop for metoprolol and a 50-μl loop for atenolol (Rheodyne, Berkeley, CA, U.S.A.), a Model FS970 fluorescence detector (Schoeffel Instrument, Westwood, NJ, U.S.A.) and an Omniscribe recorder (Houston Instruments, Austin, TX, U.S.A.). For metoprolol determination a 30 cm $\times$ 0.46 cm I.D. stainless-steel column packed with C_{18} 10-μm μBondapak (Waters Assoc.) was used. For the atenolol determination a 25 cm $\times$ 0.3 cm I.D. stainless-steel glass-lined column packed with spherisorb 5-μm nitrile silica (SGE Scientific, Ringwood, Australia) was used.

Bond-Elut extraction procedure

Atenolol was extracted using a Bond-Elut column containing silica modified with covalently bound cyanopropyl groups (CN column) and metoprolol was extracted using a Bond-Elut column containing ODS-modified silica (C_{18} column). The Bond-Elut columns were placed in luer fittings in the top of the Vac-Elut cover, which has the capacity for ten columns. A vacuum of 25—50 cmHg was applied to the manifold to effect the various stages of the extraction. Both types of column were activated before use by washing with 2 $\times$ 1 ml of acetonitrile followed by 2 $\times$ 1 ml of distilled water.

To extract metoprolol from plasma, 1 ml of plasma was passed through the activated C_{18} Bond-Elut column which was then washed twice with 0.5-ml aliquots of distilled water—acetonitrile (90:10). The vacuum was released from the Vac-Elut and the stainless-steel needles of the Vac-Elut cover were wiped. Appropriately labelled tubes were placed under the column, which was then eluted with 0.5 ml of actonitrile—0.1 *M* hydrochloric acid (50:50), with the vacuum re-applied. The collected extract was then ready for injection onto the HPLC column.

A similar process was used to extract atenolol from plasma. Plasma (1 ml) was passed through an activated CN Bond-Elut column which was then successively washed with 0.5 ml distilled water and 0.5 ml acetonitrile. To elute the atenolol from the column two 0.25-ml aliquots of 0.05 *M* sodium dihydrogen orthophosphate—acetonitrile (70:30) containing 4 m*M* triethylamine adjusted to pH 4 with orthophosphoric acid were used.

Preparation of standards

Stock solutions of metoprolol and atenolol at a concentration of 1 mg/ml were made in distilled water. Appropriate dilution of this solution with drug-free plasma gave a range of standards which could be used to standardize the extraction procedure and calibrate the HPLC determination. The amount of

drug in plasma samples was then determined from peak heights and a calibration line obtained with the standards.

Chromatography

For metoprolol determination the C_{18} HPLC column was eluted with acetonitrile—0.1% orthophosphoric acid (23:77) at a flow-rate of 1.0 ml/min. For atenolol determination the CN HPLC column was eluted with acetonitrile—0.05 *M* phosphate buffer (10:90) with pH adjusted to 7.0 with orthophosphoric acid at a flow-rate of 1.2 ml/min. For both assays the detector excitation wavelength was set at 193 nm with no emission filter. Sensitivity was set at 0.2 μA for metoprolol and 0.1 μA for atenolol.

Extraction recoveries

Recovery with these extraction procedures was estimated by comparing peak heights obtained by direct injection of solutions containing atenolol or metoprolol in the appropriate solvent with those obtained by extraction of plasma containing an equal concentration, an appropriate allowance being applied for the volume of the extract.

$$\text{Percentage recovery} = \frac{\text{peak height of extracted plasma sample} \times \text{extract volume} \times 100}{\text{peak height of non-extracted sample}}$$

Recoveries were determined from the mean of eight replicates taken for each drug.

Screening for interfering drugs

A range of drugs that are commonly co-administered with atenolol or metoprolol were screened for their possible interference in the assays. Plasma from a patient known to be taking the particular drugs was processed by the two methods and the HPLC chromatogram checked for any interfering peaks at the retention time for atenolol and metoprolol. This process eliminated any potential interference from the parent drug and its metabolites at the normal levels likely to be encountered in the clinical situation.

RESULTS

Representative chromatograms for atenolol and metoprolol are shown in Figs. 1 and 2, respectively. Each figure shows plasma spiked with a known amount of β-blocker (A), drug-free plasma (B), and plasma from a patient after a normal oral dose of β-blocker (atenolol or metoprolol, respectively) (C). The assay procedure was linear over the range 0—500 ng/ml for atenolol ($y = 0.078x$, $r = 0.993$) and 0—1000 ng/ml for metoprolol ($y = 0.161x$, $r = 1.000$). At atenolol concentrations above 500 ng/ml, the extraction capacity of the 1-ml CN Bond-Elut column appeared to decrease slightly causing a slight curvature in the calibration line. The recovery of atenolol at concentrations of 20 and 200 ng/ml was 74.5 ± 2.0% ($n = 8$) and 61.7 ± 1.5% ($n = 8$), respectively, while for metoprolol at concentrations of 400 ng/ml and 50 ng/ml it was 95.4 ± 2.7% ($n = 8$) and 99.8 ± 9.8% ($n = 8$), respectively. The sensitivity limit (three times baseline) for the atenolol assay was 10 ng/ml and for metoprolol 2 ng/ml.

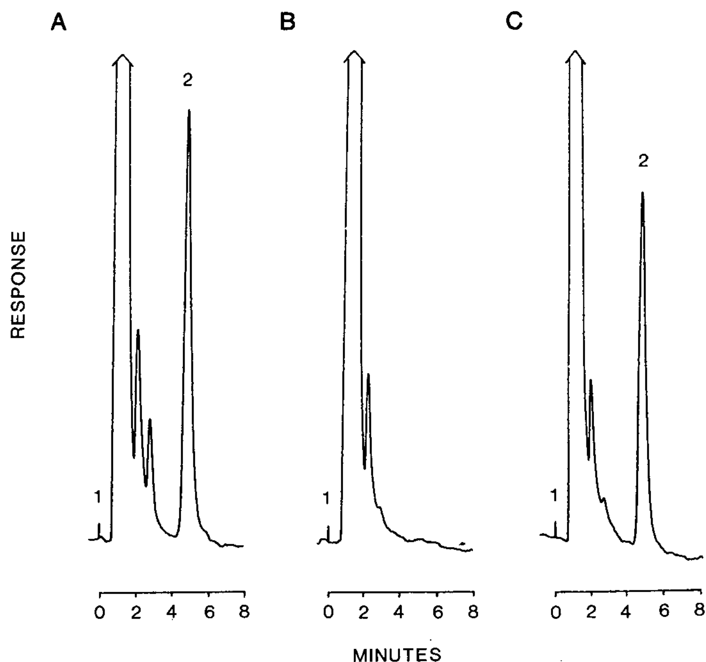

Fig. 1. HPLC profiles of (A) drug-free plasma spiked with 400 ng/ml atenolol, (B) drug-free plasma, (C) patient plasma sample collected 3 h after a dose of 50 mg atenolol. 1 = Injection site; 2 = atenolol.

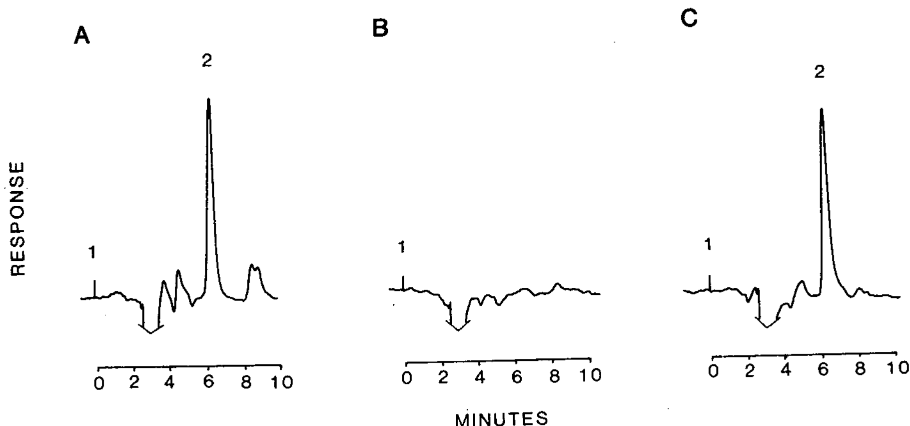

Fig. 2. HPLC profiles of (A) drug-free plasma spiked with 200 ng/ml metoprolol, (B) drug-free plasma, (C) patient plasma sample collected 2½ h after a dose of 100 mg metoprolol. 1 = Injection site; 2 = metoprolol.

The intra- and inter-day precision of the methods were determined by the assay of eight samples of drug-free plasma containing known concentrations of atenolol or metoprolol. The coefficient of variation of atenolol was 2.67% at 20 ng/ml and 3.06% at 400 ng/ml, while for metoprolol it was 6.89% at 20 ng/ml and 2.87% at 500 ng/ml.

Drugs which have been eliminated as causing potential interference in both assays are: chlorothiazide, prazosin, hydralazine, α-methyl-DOPA, verapamil,

frusemide, disopyramine and lignocaine. There was also chromatographic separation of other commonly used β-blockers, i.e. pindolol, propranolol, alprenolol, oxprenolol, practolol and timolol.

DISCUSSION

The determination of atenolol and metoprolol using solid-phase extraction techniques together with HPLC has proven to be simple, rapid, sensitive and specific. The assays are adequate to determine atenolol or metoprolol in plasma after normal oral doses of either drug as shown in Fig. 1C for atenolol and Fig. 2C for metoprolol.

A further advantage of solid-phase extraction systems is that they avoid the strongly alkaline conditions of the previous solvent extraction methods. This is important in the case of drugs which are subject to oxidation under such conditions or can bind to glassware (i.e. β-adrenoceptor antagonists).

The methods reported here do not suffer from interference from the drugs commonly co-administered with either atenolol or metoprolol and therefore they would be suitable for use in routine drug monitoring or pharmacokinetic studies.

In summary and conclusion we present a novel extraction procedure generally applicable to both hydrophilic and lipophilic β-blockers, illustrated in this communication with particular reference to metoprolol and atenolol. This method offers a major advance in ease of execution and speed without sacrifice of precision, sensitivity or selectivity.

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CHROMBIO. 2508

Note

High-performance liquid chromatographic analysis of melphalan in plasma, brain and peripheral tissue by *o*-phthalaldehyde derivatization and fluorescence detection

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Melphalan, 4-[bis(2-chloroethyl)amino]-L-phenylalanine, is used to treat multiple myeloma, ovarian carcinoma and in adjuvant therapy of breast cancer [1—4]. Although it has been in clinical use for some twenty years, it has only been with the relatively recent development of sensitive and specific high-performance liquid chromatographic (HPLC) assays that its pharmacokinetics have been analysed [5—8].

Several HPLC assays have been developed to quantify therapeutic concentrations of melphalan in blood; most have relied on detection of melphalan by UV absorbance [5—8]. Of these, all have been limited to the analysis of the drug in plasma and serum [6—8] or have lacked sufficient sensitivity for the analysis of other tissues [5]. Our attempts to adapt these assays to determine melphalan in other tissues have proved unsuccessful, due to the presence of interfering endogenous substances, particularly in brain where melphalan concentrations are low. Egan et al. [9] utilised the natural fluorescence of melphalan to determine its concentration. However, up to 3 ml of plasma or tissue homogenate were required. We therefore developed a new rapid and sensitive method for analysing melphalan in plasma and tissue samples by employing *o*-phthalaldehyde derivatization and fluorescence detection which yielded a product whose fluorescence was significantly greater than that of melphalan alone. In this paper we describe the technique.

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MATERIALS AND METHODS

Reagents

Melphalan was a gift from Burroughs Wellcome (Research Triangle Park, NC, U.S.A.). Methanol and acetonitrile were HPLC grade and were used as supplied by Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Sodium chloride, sodium monophosphate and sodium diphosphate, all reagent grade, were from Fisher (Fairlawn, NJ, U.S.A.). Ethanethiol, sodium acetate and acetic acid, all reagent grade, were from Kodak Chemical (Rochester, NY, U.S.A.). *o*-Phthalaldehyde was supplied by Pierce (Rockford, IL, U.S.A.).

Apparatus

HPLC was performed on a Waters Assoc. (Milford, MA, U.S.A.) system. This consisted of a Model 6000A solvent delivery pump, a Model 720 system controller, a WISP 710 B automatic injector and a Model 720 data module. The detector was a Kratos Model FS-950 Fluoromat fluorometer (Ramsey, NJ, U.S.A.) with a mercury lamp, an excitation filter of 365 nm and an emission UV cutoff filter of 426 nm. Separation was performed on a 10-μm Partisil 10 ODS 3 column (Whatman, Clifton, NJ, U.S.A.) with a guard column packed with pellicular C_{18} material.

Instrument conditions

Samples were eluted in a mobile phase of acetonitrile—15 m*M* phosphate buffer, pH 7.0 (36:64, v/v). The phosphate buffer was prepared from deionised, distilled water and was mixed with the acetonitrile. The mixture was degassed and filtered through a 0.22 μm pore diameter Millipore filter (Bedford, MA, U.S.A.) and allowed to equilibrate to room temperature. All columns were equilibrated to new solvents for at least 30 min. The flow-rate was maintained at 3 ml/min, which resulted in a column pressure of 170 bars.

Tissue preparation

Adult male Fischer 344 rats (Charles River, Wilmington, MA, U.S.A.), approximately 250 g weight, were anaesthetised (sodium pentobarbital 40 mg/kg, intraperitoneally) and injected intravenously (4 ml/kg) with melphalan (10 mg/kg) in 5% ethanol, 95% isotonic saline [0.9% (w/v) sodium chloride, pH 7.2]. At different times following melphalan administration, the animals were killed, blood was taken by cardiac puncture and the brain removed. The blood was placed in a heparinised tube and centrifuged at 7000 *g* for 1 min. The plasma was removed and together with the brain stored on dry ice. The brain was later thawed, dissected into regions and placed in preweighed vials, reweighed, and stored at $-70°$C.

Extractions

Plasma samples were thawed and placed in an ice bath. Then 200 μl of plasma were added to 400 μl of methanol. The mixture was vortexed for 20 sec and centrifuged at 7000 *g* for 1 min. A 200-μl aliquot of the supernatant was removed and stored at $-70°$C prior to analysis.

Tissue samples were similarly thawed and placed in an ice bath. A 5-μl

aliquot of methanol was added to every 1.5 mg of tissue. Each sample weighed approximately 150 mg. The tissue was sonicated (Ultrasonics, Plainview, NY, U.S.A.) for 45 sec and maintained at 0°C with an ice bath. The resulting suspension was centrifuged at 7000 *g* for 15 min at 4°C. A 200-µl aliquot of the supernatant was removed and stored at −70°C prior to analysis.

Derivatization

The methanolic solutions of extracted plasma and tissue were brought to room temperature immediately prior to HPLC analysis. To each 200-µl sample were added 50 µl of 0.5 *M* acetate buffer, pH 5.15, and 100 µl of methanol containing 20 mg/ml *o*-phthalaldehyde and 20 µl/ml ethanethiol. The mixture was vortexed for 20 sec, allowed to stand for 2 h at room temperature and injected onto the column.

Calibration and quantitation

Melphalan plasma and tissue concentrations were quantified from calibration curves. Standard curves of six points, two samples per point, were run daily and intermixed with the unknown samples. Drug-free plasma (200 µl) or drug-free tissue (150 mg) was spiked with known and increasing amounts of freshly prepared melphalan. A least-squares linear regression between concentration and peak height was calculated for each preparation. The mean correlation coefficient of six curves run over a month period for plasma was 0.998 while that for brain was 0.999. The intercept was negligible for both. The plasma standard curves were linear over the range 10 ng to 1 µg and 1 µg to 50 µg. The brain standard curves were linear over the range 25 ng to 10 µg. Melphalan stability, tested by running identical samples at the beginning and end of each day, was maintained by keeping the samples at −70°C prior to derivatization.

RESULTS

The in vivo and in vitro breakdown of melphalan proceeds by hydrolysis to the monohydroxymelphalan and dihydroxymelphalan. We prepared these compounds by the method of Furner et al. [5] and obtained peaks at 1.24 min for dihydroxymelphalan, at 1.64 min for monohydroxymelphalan and 4.69 min for melphalan. Neither the mono- nor dihydroxy products, however, could be quantitated in biological samples because they co-eluted with the solvent front.

Fig. 1 shows the chromatograms of a blank plasma sample (A), and a plasma sample taken 30 min after the administration of melphalan (10 mg/kg, intravenously) (B) to a three-month-old male Fischer 344 rat. Fig. 2 shows chromatograms of a blank brain (A) and a brain sample taken at 30 min from the same animal as in Fig. 1B (B). Calculations against standards gave melphalan concentrations of 5.16 µg/ml and 0.82 µg/g for plasma and brain, respectively.

Sample stability was assessed by routinely analyzing, over a month period, single reference preparations of both plasma and brain stored at −70°C. No melphalan breakdown occurred in either case. Assay reproducibility was assessed by analyzing reference melphalan samples from six separate preparations on six different days over a period of one month. The coefficient of

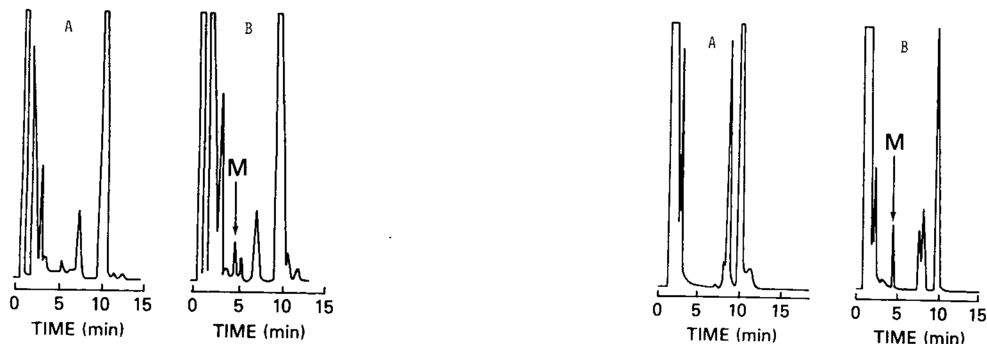

Fig. 1. Chromatograms of a 25-μl injection of (A) a blank plasma sample and (B) a plasma sample containing 5.16 μg/ml melphalan (M) taken at 30 min from a rat injected intravenously with 10 mg/kg melphalan.

Fig. 2. Chromatograms of a 25-μl injection of (A) a blank brain sample and (B) a brain sample containing 0.82 μg/g melphalan (M) taken at 30 min from a rat injected intravenously with 10 mg/kg melphalan.

variation for plasma (1 μg/ml) was 5.9% of the mean, and for brain (100 ng per 150 mg) was 5.3% of the mean.

DISCUSSION

HPLC with fluorescence detection, utilizing both pre- and post-column derivatization, has been widely used for the sensitive analysis of biogenic amines in body tissues. In particular, the fluorogenic reaction of *o*-phthalaldehyde with ethanethiol has been successfully employed for the separation and quantitation of amino acids and proteins in plasma and brain [10, 11]. Because melphalan is a primary amine and the nitrogen mustard derivative of the amino acid phenylalanine, it was thought that it also could be analysed by this technique.

For the analysis of amino acids, the reaction between *o*-phthalaldehyde, ethanethiol and the amines normally proceeds at a pH between 9 and 10.5. Attempts to derivatize melphalan at pH 10.4, however, caused its rapid hydrolysis to mono- and dihydroxy products. Buffering the reaction at pH 7.1 also caused hydrolysis but at a less rapid rate. An acetate buffer, pH 5.15, which was used eventually for the reaction, eliminated hydrolysis; however, it may also have decreased the fluorescence intensity and slowed the reaction rate [12]. Despite this the fluorescence was found to be approximately three times greater than that of the natural fluorescence of melphalan alone.

Fig. 3 relates the fluorescence intensity of the reaction to time up to 24 h, for a single sample of melphalan in brain and in plasma. In both tissues the reaction took 1 h to reach maximum intensity and then decayed at a rate that did not exceed 4% of the maximum intensity after 2 h. Samples therefore were injected onto the column 2 h after derivatization. At this time, fluorescence intensity was approximately 97% of maximum. The decay curve for melphalan was pseudo first-order and had a half-life of 20 h in plasma and 25 h in brain. Since no monohydroxymelphalan or dihydroxymelphalan could be detected

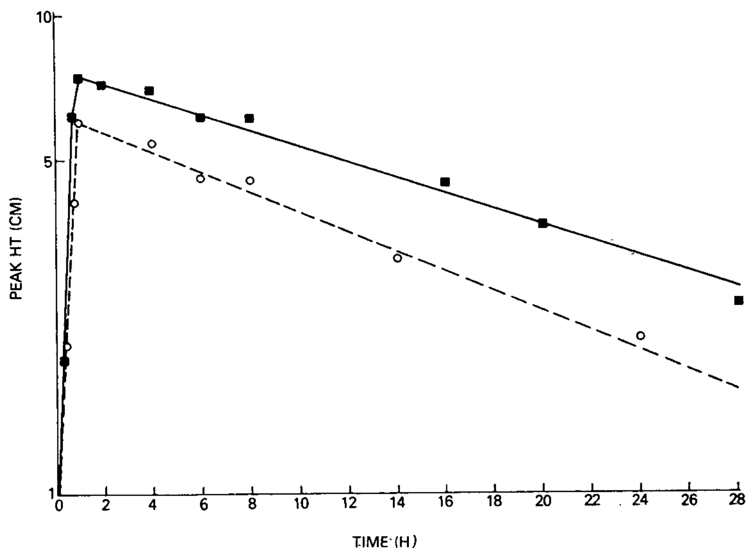

Fig. 3. Relationship between chromatogram peak height and time for a single sample of melphalan in plasma (■) and in brain (○).

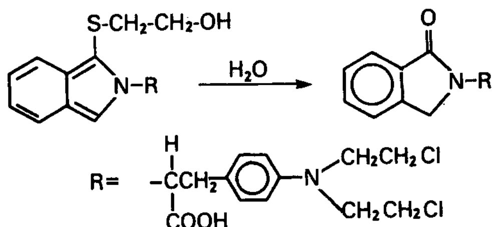

Fig. 4. Hydrolysis of the iso-indole product of the derivatization of melphalan (5 μg/ml) to the cyclic amide.

TABLE I

COMPARISON OF THE FLUORESCENCE ASSAY AND UV ABSORBANCE ASSAY FOR MELPHALAN IN PLASMA

UV absorbance assay: Chang et al. [6]; fluorescence detection assay: present paper. No significant difference between melphalan concentrations determined by either technique (Student's t-test: $p < 0.05$).

Plasma sample	Melphalan concentration (mean ± S.E.M., n = 6) (μg/ml)	
	UV detection technique	Fluorescence detection technique
1	0.953 ± 0.06	0.953 ± 0.04
2	0.546 ± 0.03	0.520 ± 0.03

in non-biological samples up to 24 h, it can be assumed that this fluorescence decay resulted from the hydrolysis of the iso-indole product to the non-fluorescent cyclic amide [13] (Fig. 4).

The fluorescence assay was compared to the UV absorbance melphalan assay

of Chang et al. [6] for the measurement of unknown plasma samples. There was no significant difference between the concentration determined by either technique (Table I). In addition, the fluorometric assay allows for the analysis of as little as 100 μl of plasma containing as little as 10 ng/ml melphalan. Further, for the first time, it provides a rapid and accurate analysis of as little as 25 ng/g melphalan in as little as 50 mg of tissue.

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Note

High-performance liquid chromatographic assay for trilostane and its major metabolite, 17-ketotrilostane, in human plasma

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Trilostane [(4α,5α,17β)-4,5-epoxy-3,17-dihydroxyandrost-2-ene-2-carbonitrile] is a novel steroid [1] which is effective in inhibiting adrenal hormone production in experimental animals [2]. In humans, the drug is an effective adjunct in the treatment of Cushing's syndrome [3].

This report describes a high-performance liquid chromatographic (HPLC) method for the determination of trilostane (Fig. 1, I) and 17-ketotrilostane [(4α,5α)-4,5-epoxy-3-hydroxy-17-oxoandrost-2-ene-2-carbonitrile, Fig. 1, II], its major metabolite [4], in human plasma. The assay was used to quantitate trilostane and 17-ketotrilostane in the plasma of a subject who had received trilostane by oral administration.

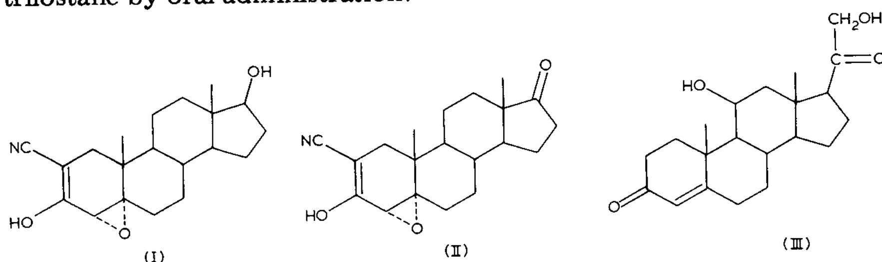

Fig. 1. The chemical structures of trilostane (I) 17-ketotrilostane (II) and the internal standard (III).

EXPERIMENTAL

Materials

Trilostane and 17-ketotrilostane were synthesized at the Sterling-Winthrop

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Research Institute. The internal standard corticosterone (4-pregnene-11β,21-diol-3,20-dione, Fig. 1, III) was obtained commercially from Sigma. Other chemicals were obtained commercially (reagent grade) and used without further purification.

Preparation of plasma standards and samples

Plasma standards were prepared by supplementing 1.0 ml of normal human plasma with a methanolic solution of trilostane and 17-ketotrilostane to achieve concentrations of 0 (methanol only), 0.050, 0.10, 0.25, 0.50, and 1.0 μg/ml. Duplicate standards at each concentration were prepared.

Two sets of randomized and coded samples were prepared for analysis under single-blind conditions, as above. Each plasma set contained triplicate samples at final concentrations of 0.12, 0.30 and 0.60 μg/ml. One set of plasma samples was analyzed immediately after preparation. The other set was stored in the laboratory freezer for five days before analysis.

Assay method

To a tube containing 1.0 ml of plasma (containing potassium oxalate as the anticoagulant) were added 50 μl of internal standard solution (0.065 mg/ml in methanol), 0.50 ml of 1 *M* phosphate buffer (pH 6.6) and 5.0 ml of diethyl ether. The samples were agitated on a rotary shaker for 10 min and centrifuged at 900 *g* for 10 min. The organic phase was transferred to a clean silanized 15-ml centrifuge tube. The aqueous phase was again extracted with 5.0 ml of diethyl ether as above. The organic phases were pooled, placed in a 60°C heating block, and were evaporated to dryness with the aid of a stream of nitrogen. The residue was dissolved in 300 μl of the HPLC mobile phase and 100 μl were injected into the HPLC system for analysis.

Chromatography

The HPLC system was operated isocratically at ambient temperature. The system consisted of an automatic injector (WISP, Waters Assoc., Milford, MA, U.S.A.), a pump (Model 45, Waters Assoc.), a 10 cm × 9.4 mm I.D. Partisil 5 CCS/C_8 RAC column (rapid analysis chromatography column, 5 μm particle size, Whatman, Clifton, NJ, U.S.A.), with a 37–50 μm particle size Phenyl/Corasil precolumn (Waters Assoc.), and a UV detector with a 280-nm filter (Model 440, Waters Assoc.). The mobile phase consisted of 0.1 *M* sodium acetate (adjusted to pH 5.0 with 0.1 *M* acetic acid)–methanol (40:60). The flow-rate was 2.0 ml/min.

The output of the detector was interfaced with a Model 3356 Laboratory Automation System (Hewlett-Packard, Palo Alto, CA, U.S.A.) computer for data acquisition and handling. Peak height ratios (trilostane:internal standard, 17-ketotrilostane:internal standard) for each standard and sample were calculated and least-squares regression analysis was performed on the peak height ratios versus nominal concentration. The concentrations of trilostane and 17-ketotrilostane in each sample were determined by inverse prediction from the linear regression [5]. The minimum quantifiable level (MQL) of the assay was estimated as that concentration at which the lower 80% confidence interval just encompassed zero concentration [6].

The observed concentrations for the prepared, spiked samples were expressed as percent differences from the nominal values. The range of these percent differences was used to define the accuracy of the assay. Precision was estimated from the standard deviation derived from the analysis of variance on the percent differences.

The percent recoveries of the extraction procedure for trilostane, 17-keto-trilostane and the internal standard were determined by comparing the peak heights of trilostane, 17-ketotrilostane and the internal standard with those obtained by direct injection.

Analysis of clinical samples

Plasma samples from one subject who had received two 60-mg capsules of trilostane were analyzed by the above procedure. Blood samples were taken at specified intervals by venipuncture. The plasma was separated by centrifugation and stored in the laboratory freezer until analyzed as described above.

RESULTS AND DISCUSSION

Analytical method

Representative chromatograms of an extracted plasma blank and an extracted plasma standard are shown in Fig. 2A and B, respectively. The results of the analysis of prepared plasma samples are summarized in Table I. A plot of peak height ratios (trilostane:internal standard, 17-ketotrilostane:internal standard) versus concentration for the plasma standards was linear over the range of 0—1.0 μg/ml of plasma, as determined by linear regression analysis ($y = 3.14x + 0.002$, $r = 0.999$ for trilostane and $y = 3.78x + 0.04$, $r = 0.999$ for 17-ketotrilostane).

An overall estimate of the assay precision, based on the derived standard

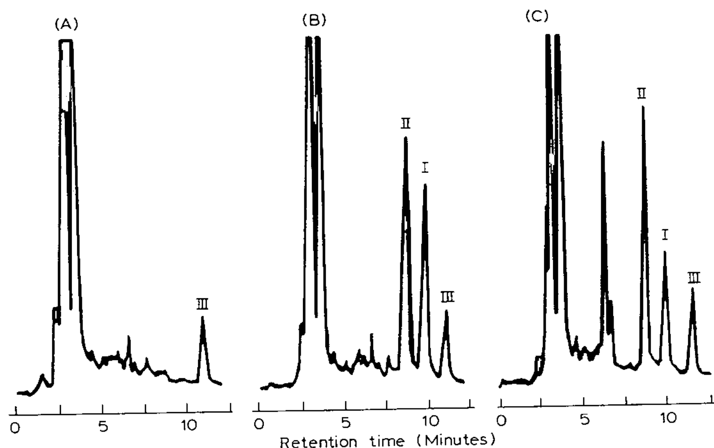

Fig. 2. Representative chromatograms of (A) an extracted plasma blank with internal standard (III) only; (B) an extracted plasma standard containing 1.0 μg/ml 17-ketotrilostane (II), 1.0 μg/ml trilostane (I), and internal standard (III); and (C) an extracted plasma sample from a human volunteer (taken 1 h after a 120-mg dose) containing 0.48 μg/ml trilostane (I), 1.3 μg/ml 17-ketotrilostane (II), and internal standard (III).

TABLE I

RESULTS OF THE ANALYSIS OF PREPARED PLASMA SAMPLES (n = 6)

Concentration added (μg/ml)	Mean concentration found (μg/ml)	Percent S.E.M.	Mean percent difference
Trilostane			
0.12	0.12	1.1	−1.2
0.30	0.30	0.6	1.4
0.60	0.60	0.5	−0.8
17-Ketotrilostane			
0.12	0.12	0.7	1.5
0.30	0.30	0.9	0.6
0.60	0.60	0.6	−0.1

deviation, was equal to 3.20% for trilostane and 3.89% for 17-ketotrilostane. The accuracy of the assay, defined by the range of the mean percent differences from the expected values, varied from −2.00% to 4.33% for trilostane, and from −0.44% to 2.22% for 17-ketotrilostane. The mean (± S.E.M.) MQLs of the two sets were 0.026 (± 0.006) μg/ml for trilostane and 0.028 (± 0.004) μg/ml for 17-ketotrilostane.

The fresh and frozen sample sets were compared to determine whether freezing affected the sample concentrations. The observed concentrations for the triplicate determinations were expressed as percent differences from the nominal values and analyzed in a two-way analysis of variance with replication to test for a concentration effect, a time (fresh versus frozen) effect, and a concentration-by-time interaction. No significant differences ($p > 0.05$) were observed for trilostane or 17-ketotrilostane.

The extraction efficiency studies in plasma indicated mean recoveries of 88% for trilostane, 84% for 17-ketotrilostane and 102% for the internal standard.

TABLE II

PLASMA CONCENTRATIONS OF TRILOSTANE AND 17-KETOTRILOSTANE IN CLINICAL SAMPLES FROM ONE SUBJECT RECEIVING 120 mg OF TRILOSTANE

Time after administration (h)	Trilostane concentration (μg/ml)	17-Ketotrilostane concentration (μg/ml)
0	<MQL*	<MQL**
0.5	0.08	0.09
1.0	0.48	0.83
2.0	0.35	1.33
3.0	0.45	1.33
4.0	0.24	0.98
6.0	0.04	0.31

*Less than the minimum quantifiable level of 0.02 μg/ml.
**Less than the minimum quantifiable level of 0.02 μg/ml.

The results of the analysis of the clinical plasma samples from one subject receiving 120 mg of trilostane are given in Table II. The maximum concentration found for trilostane was 0.48 μg/ml in the 1.0-h sample and the maximum level for 17-ketotrilostane was 1.3 μg/ml in the 2.0- and 3.0-h samples. Fig. 2C is a chromatogram of the 1.0-h sample.

CONCLUSION

In summary, an accurate, selective, reproducible and precise HPLC assay has been developed for the determination of trilostane and 17-ketotrilostane concentrations in human plasma. This method has proven useful for analysis of specimens obtained during clinical trials; details of these results will be reported elsewhere.

ACKNOWLEDGEMENT

The authors wish to thank Dr. Jerome Edelson, Senior Director for Drug Safety Evaluation for his continuing interest and support.

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CHROMBIO. 2511

Note

High-performance liquid chromatographic determination of 2-[3′-(methoxycarbonylamino)-phenyl]-3-phenyl-6-methoxycarbonylamino-4-(3H)-quinazolone (NSC-251635) in human serum

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(First received May 22nd, 1984; revised manuscript received December 13th, 1984)

2-[3′-(Methoxycarbonylamino)-phenyl]-3-phenyl-6-methoxycarbonylamino-4-(3H)-quinazolone (NSC-251635, I) is a water-insoluble antimitotic compound (Fig. 1).

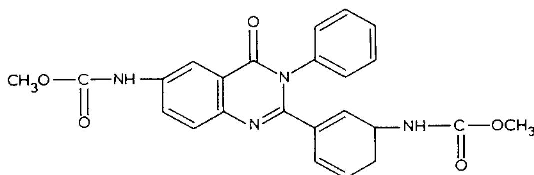

Fig. 1. Chemical structure of I.

Recently, we investigated the use of liposomes (phospholipid vesicles) as carriers for water-insoluble drugs [1, 2] and demonstrated that I was active on L1210 murine leukaemia only after entrapment in liposomes [3]. These results prompted us to determine whether large amounts of liposomes containing I could be infused into man; preliminary results demonstrated that liposome volumes as large as 400 ml could be infused into man by the intravenous route without major side-effects [4]. In order to investigate the main pharmacological parameters of the liposome-entrapped drug, a selective, sensitive and quantitative method of I determination was needed.

This report describes the application of reversed-phase high-performance liquid chromatography (HPLC) to the determination of the concentration of I in human serum samples previously submitted to a quantitative extraction procedure.

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EXPERIMENTAL

High-performance liquid chromatography

A Waters chromatograph was equipped with a Model 6000A solvent delivery system, a Model 710B sample processor and a Model 480 Lambda-Max spectrophotometer operating at 297 nm; peak areas were integrated by a Waters Data Module M 730. A radial compression separation system (module Z) equipped with a Radial-Pak C_{18} cartridge (mean particle size 10 μm) was also supplied by Waters Assoc. (Milford, MA, U.S.A.). The column was preceded by an on-line stainless-steel precolumn (5 cm × 3.9 mm I.D.) packed with Vydaç™ 201 RP (particle size 30—44 μm; Macherey, Nagel & Co., Düren, F.R.G.).

Reagents and standards

I was provided by Dr. R. Bierling (Bayer, Wuppertal, F.R.G.), HPLC-grade methanol was purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

A stock solution of I (0.5 mg/ml) was prepared in methanol and working standard solutions (0.05—250.0 μg/ml) were prepared by dilution of the stock solution with methanol.

TABLE I

CHROMATOGRAPHIC CONDITIONS

Parameter	Conditions
Column	μBondapak C_{18} (10 μm)
Mobile phase	Methanol—water (625:375)
Wavelength	297 nm
Flow-rate	3.5 ml/min
Temperature	Ambient
Chromatography time	5 min
Chart speed	0.5 cm/min
Sample size	50 μl

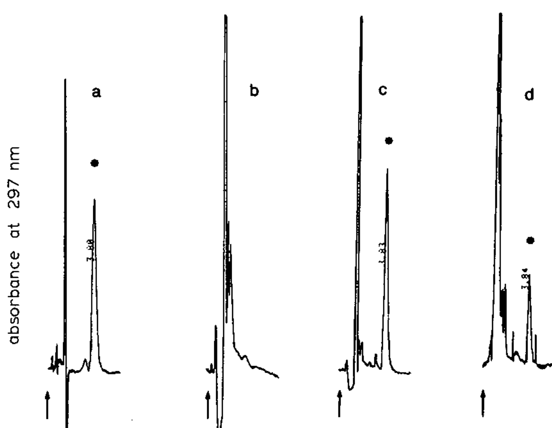

Fig. 2. High-performance liquid chromatograms of I (*): (a) standard (0.005 a.u.f.s.); (b) blank human serum (0.005 a.u.f.s.); (c) control sample (0.02 a.u.f.s.); (d) patient serum sample (0.005 a.u.f.s.). Arrow indicates start of injection.

Control samples were prepared by adding I to pooled drug-free sera to obtain concentrations of I ranging from 0.05 to 250.0 μg/ml.

Blood samples obtained from patients infused with a liposome preparation containing I were centrifuged at 1500 *g* for 10 min and the serum supernatants stored at −20°C until analysis.

Extraction procedure and conditions of analysis

The sample to be tested (1 ml) was mixed vigorously on a vortex mixer with 4 ml of methanol, left at 6–8°C for 30 min, and centrifuged at 1500 *g* for 5 min at 4°C. The clear supernatant was decanted and injected into the liquid chromatograph.

Table I lists the chromatographic conditions used in the analysis. Typical chromatograms for a standard, a serum blank, a control sample and a patient serum sample are shown in Fig. 2.

RESULTS AND DISCUSSION

Analytical variables

Among the different extraction procedures tested, the best drug recovery is obtained using the methanolic extraction.

Fig. 3 shows the absorbance scan of I in the ultraviolet–visible spectrum. The absorption maximum is at 297 nm. At shorter wavelengths more methanol-extractable material is detectable in extracts of biological specimens.

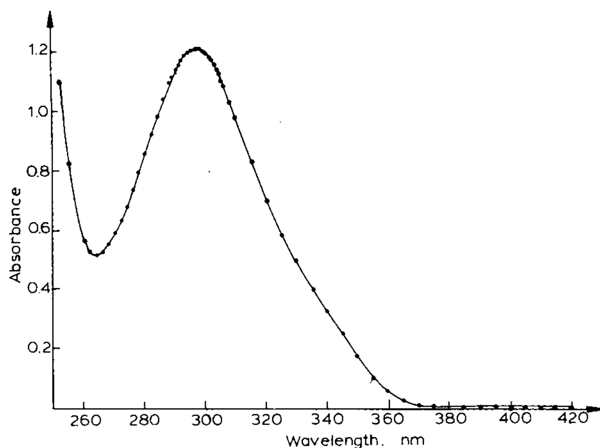

Fig. 3. Absorbance scan of I (25 μg/ml) in methanol.

As shown in Fig. 2, in the chromatographic system the elution profiles of both standard (a) and extracted serum samples (c and d) are similar. In serum, the drug peak is well separated from other detectable compounds and the extract of serum blank (b) shows no interference peak at the retention time of I.

All samples are extracted and chromatographed in duplicate, and the drug concentration is calculated from the peak area of I.

Linearity

There is a linear relation between the concentrations of I in the standard ($r = 0.9999$) and control serum samples ($r = 0.9998$) and the recorded peak areas. This linear relation extends to concentrations of at least 500 μg/ml.

Recovery

Known amounts of I were added to drug-free serum to provide concentrations ranging from 0.05 to 250 μg/ml. After duplicate extraction and chromatography of ten samples of each concentration, the peak areas obtained for I were compared to the peak areas obtained by direct injection of corresponding working standard concentrations. Recovery was calculated as

$$\frac{\text{Amount of drug measured}}{\text{Amount of drug added}} (\mu\text{g/ml}) \times 100$$

The results summarized in Table II show that a 98.0—100.4% recovery of I is obtained within the concentration range 0.05—250.0 μg/ml of serum.

Repeated assays of serum containing I stored at —20°C for one to twelve months, yielded concentration values identical to those obtained for the fresh serum sample; thus there is no indication of loss of I during this storage period.

TABLE II

RECOVERY STUDY

Concentration of I added (μg/ml)	Concentration of I measured* (μg/ml)	Recovery (%)
0.05	0.049	98.0
0.5	0.502	100.4
1.0	0.992	99.2
2.0	2.002	100.1
5.0	4.931	98.6
10.0	10.027	100.3
20.0	19.965	99.8
100.0	99.703	99.7
250.0	249.750	99.9

*Values represent the mean of ten separate determinations.

TABLE III

BETWEEN-DAY PRECISION

Concentration of I (μg/ml)	Coefficient of variation ($n = 12$) (%)
0.5	3.4
1.0	2.9
2.0	2.5
5.0	2.0
10.0	2.8
20.0	2.4

Sensitivity

The sensitivity of the assay (i.e. peak height corresponding to twice the baseline noise) was found to be 25 ng/ml.

Precision

The between-day precision was calculated for six concentrations of I in serum. Six samples of each concentration were extracted and chromatographed each day for twelve working days. Table III shows that the method has a satisfactory precision with a coefficient of variation equal to or less than 3.4% for I serum concentrations in the range 0.5—20 μg/ml.

Clinical application

The method described in this paper provides a rapid, selective and sensitive assay for measurement of concentrations of I in human serum. The sensitivity of this method makes it very useful for pharmacokinetic studies of I entrapped in liposomes and administered intravenously to man. The results of such a pharmacokinetic study is shown in Fig. 4. A 225-ml volume of liposomes containing 158 mg of I was infused into a patient over a 2-h period. The peak value of the concentration of I reached after 2 h is followed by a marked decrease until the sixth hour after the start of the infusion; during the following 14 h a very slow decrease in the serum concentration of I occurs, but some drug is still present in the blood 48 h after the start of the infusion.

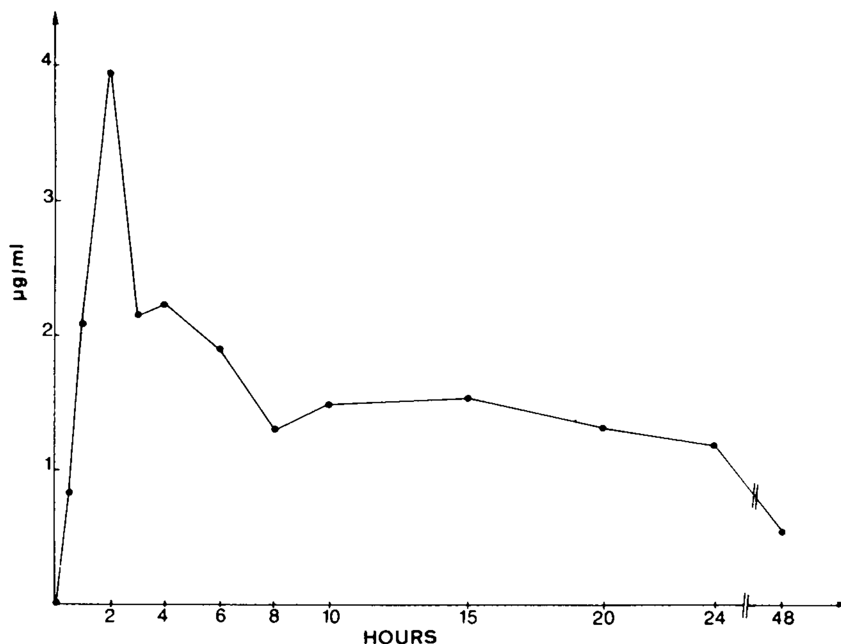

Fig. 4. Blood concentration of I as a function of time after intravenous infusion to man of liposomes containing I.

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CHROMBIO. 2514

Note

Quantification of ciclopirox by high-performance liquid chromatography after pre-column derivatization

An example of efficient clean-up using silica-bonded cyano phases

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(First received August 29th, 1984; revised manuscript received December 14th, 1984)

6-Cyclohexyl-1-hydroxy-4-methyl-2(1H)-pyridone, 2-aminoethanol salt (ciclopirox olamine, HOE 296, Batrafen®) (A, Fig. 1) is a broad-spectrum antimycotic drug [1]. Application is either dermal or vaginal. After absorption of ciclopirox olamine its free acid (ciclopirox) is rapidly conjugated with glucuronic acid. The concentration of unconjugated ciclopirox in serum is about 1—3% of the total concentration. Other metabolites were only observed in urine in negligible quantities [1].

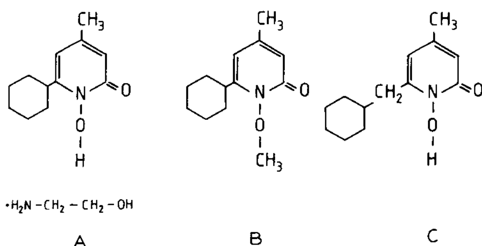

Fig. 1. Structural formulae of (A) ciclopirox olamine, (B) ciclopirox methyl derivative, and (C) internal standard [6-cyclohexylmethyl-1-hydroxy-4-methyl-2-(1H)-pyridone].

To determine the rate of systemic absorption, sensitive assay methods were required for the drug in plasma. Therefore, we tried to measure ciclopirox using high-performance thin-layer chromatography (HPTLC) and high-performance liquid chromatography (HPLC) both on normal and reversed phases.

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It was not possible to quantify it in trace amounts in situ on TLC plates. Neither the free acid nor its salt with ethanolamine, zinc(II) and iron(II, III) migrate as uniform spots. Severe tailing was always observed. Similar phenomena were observed with HPLC. Small quantities of 1-hydroxy-2(1H)-pyridone were irreversibly retained on the column, and the injection of large amounts of 1-hydroxy-2(1H)-pyridone resulted in several tailing peaks.

This paper describes a derivatization reaction for the drug to form a derivative showing regular chromatographic behaviour and a method for the quantification of ciclopirox olamine in human plasma by means of this derivatization reaction.

Formation of derivatives

The problems mentioned above presumably arise from the complexing properties of the compound. Obviously, the chelating function of the N-hydroxypyridone group (Fig. 2) interacts strongly with silica-gel-based stationary phases. This functional group has thus to be blocked prior to chromatography which is accomplished by methylating the weak acidic N-hydroxyl group ($pK_a \approx 7$) of the 1-hydroxy-2(1H)-pyridones with dimethyl sulphate. The resulting 1-methoxypyridones (Fig. 2) show a normal chromatographic behaviour even on silica.

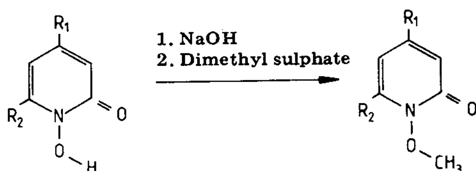

Fig. 2. 1-Hydroxy-2-(1H)-pyridone: derivatization of the chelating function to a 1-methoxy-2-(1H)-pyridone.

The optimal condition for derivatization of 1-hydroxypyridones found is described in the experimental section. Removal of excess dimethyl sulphate is indispensable for obtaining reliable analytical results. This can be achieved by reaction with triethylamine, the result of which is a non-extractable reaction product not interfering with the assay.

EXPERIMENTAL

Materials

The following solvents and reagents were used without special purification: dimethyl sulphate, *n*-hexane AR, methanol AR, toluene AR, triethylamine (Riedel-de Haën, Seelze-Hannover, F.R.G.), acetonitrile HPLC-grade S (Rathburn, Walkerburn, U.K.), 1/15 *M* phosphate buffer solution (pH 5), β-glucuronidase solution from *Escherichia coli* (Boehringer, Mannheim, F.R.G.; Cat. No. 127680). The bonded-phase cartridges containing 100 mg of cyanopropyl packing (Bond-Elut™ CN cartridges, Cat. No. 6131101) were obtained from ICT-Handelsgesellschaft (Frankfurt, F.R.G.).

Compounds A, B and C (the internal standard) (for structures see Fig. 1) were synthesized by Dr. Lohaus (Hoechst, Frankfurt, F.R.G.).

Chromatographic conditions

The chromatograph consisted of a Waters M-45 solvent pump with a Rheodyne 7100 injection port (100-μl sample loop). We used a 125 mm × 4.6 mm I.D. column (Bischoff, Leonberg, F.R.G.) packed with Nucleosil 5 C_{18}, particle size 5 μm (Macherey, Nagel & Co., Düren, F.R.G.). The mobile phase was acetonitrile—water (40:60). The chromatogram was monitored by a Biotronic BT 3030 spectrophotometer at 300 nm. The chromatograph was operated at ambient temperature with a flow-rate of 2.0 ml/min. Under these conditions the retention time was approximately 5 min for the ciclopirox derivative and 8.5 min for the derivative of the internal standard. Quantification was based on the peak height ratio of ciclopirox derivative/internal standard derivative.

Sample preparation

Plasma or serum (1 ml) was incubated with 1 ml of 1/15 *M* phosphate buffer (pH 5) and 10 μl of β-glucuronidase solution at 37°C for 24 h; 40 μl of the internal standard solution (10 μg/ml in water) were then added. For derivatization, 0.5 ml of 2 *M* sodium hydroxide solution and 200 μl of dimethyl sulphate were added, the mixture was vortexed for a short time and kept at 37°C for 15 min. Subsequently, 200 μl of triethylamine were added and the test tube was vortexed again. The mixture was extracted for 20 min with 5 ml of *n*-hexane using a Rotary mixer. The phases were separated by centrifugation, and 4 ml of the organic phase were applied to a 1-ml Bond-Elut CN cartridge which had previously been conditioned twice with 1 ml of acetonitrile.

The columns were rinsed with 1 ml of toluene and aspirated to dryness under reduced pressure for 3 min. Following this, 300 μl of mobile phase (acetonitrile—water, 40:60) were applied onto the column and eluted in a conical glass tube by centrifugation for 3 min; 100 μl of this eluate were injected.

RESULTS

Ciclopirox olamine was admixed to blank plasma in six concentrations between 20 and 1000 ng/ml. Each sample was split into eight portions forming eight identical series of samples. Each series was then analysed on consecutive days so that a total of eight independent analytical results was obtained for each concentration.

Linearity, accuracy and precision

Linearity, accuracy and precision of the method were assessed over a concentration range of 20—1000 ng/ml of serum (Table I).

Extraction and reaction yield

The reaction yield of the methylation and the extraction yield of the methyl derivative together are 75% over all steps of the sample preparation.

Sensitivity

The limit of quantification of ciclopirox olamine in plasma, defined as three

TABLE I

ACCURACY AND PRECISION OF THE ASSAY OF CICLOPIROX OLAMINE IN SPIKED HUMAN PLASMA SAMPLES (n = 8)

Amount added (ng/ml)	Amount measured (mean ± S.D., ng/ml)	Coefficient of variation (%)
20	18.5 ± 1.9	10.3
50	47.9 ± 2.6	5.5
100	97.0 ± 4.0	4.1
200	195 ± 5	2.6
500	483 ± 17	3.5
1000	999 ± 21	2.1

times the standard deviation at the lowest amount measured, is 6 ng/ml. Most of the patients investigated showed no detectable blank values; in a few cases (cf. Fig. 4), blank values up to 15 ng/ml were observed.

Plasma concentration profile in man

Single doses of 5 g of Vaginal Cream P containing 1% ciclopirox olamine were administered by disposable vaginal applicator to patients suffering from vaginal candidiasis. Plasma concentrations of ciclopirox were monitored using the assay method described. In topical administration, absorption of the drug should be minimal. A typical concentration profile (in ciclopirox olamine equivalents) is shown in Fig. 3. (We thank Dr. P.U. Witte (Hoechst AG) for permission to publish these data.)

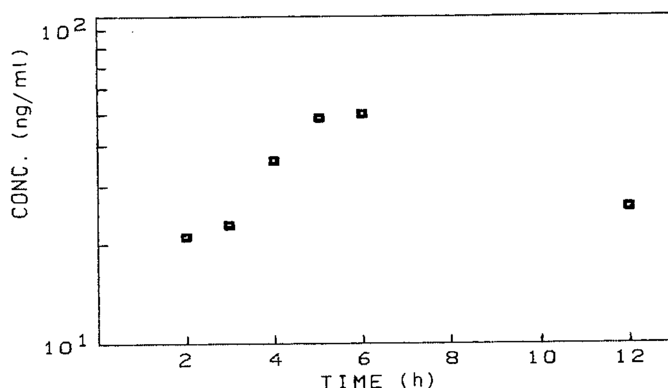

Fig. 3. Profile of plasma concentration (ng/ml) in a patient following vaginal administration of 50 mg of ciclopirox olamine.

DISCUSSION

Alkylation of ciclopirox was shown to produce a single non-tailing peak. To obtain a reproducible extraction yield, the method of alkylating ciclopirox in plasma first and then extracting the methyl derivative is given preference to the inverse procedure in which the free ciclopirox acid is first extracted and then

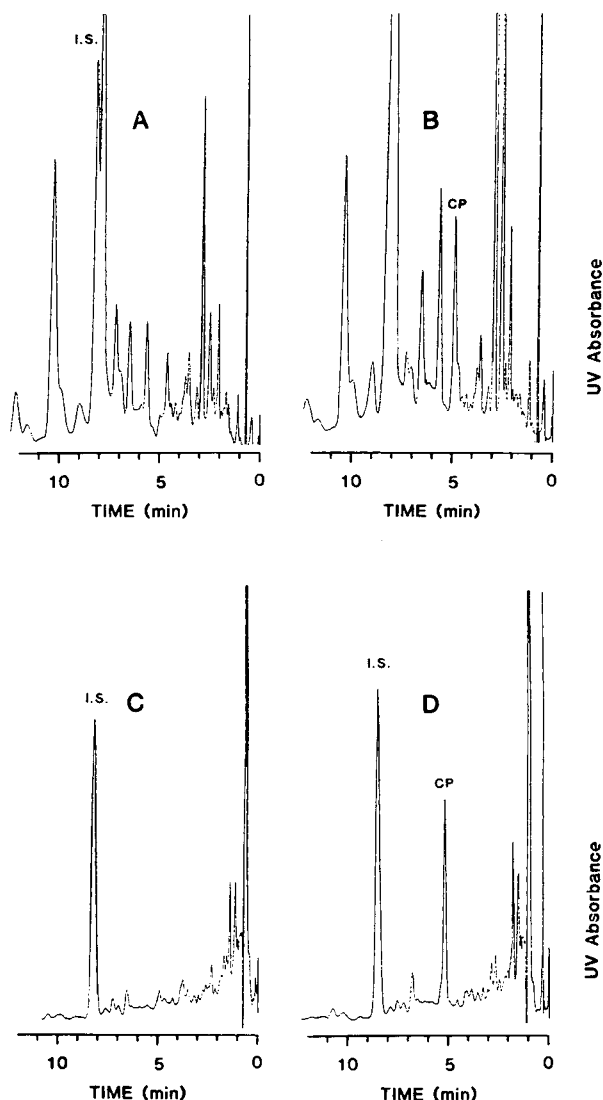

Fig. 4. Chromatograms of plasma samples without clean-up (A, B) and after clean-up (C, D) from patients without (A, C) and with (B, D) ciclopirox olamine treatment. Samples B and D each contain 184 ng/ml. Peaks: I.S. = internal standard derivative. CP = ciclopirox olamine derivative.

alkylated. However, as the methylation in plasma transforms many compounds into hexane-soluble methyl derivatives, it is necessary to introduce a further clean-up after extraction. We have found that this is done very efficiently by using cartridges filled with polar material such as cyanopropyl-modified silica (CN phases). In Fig. 4, the chromatograms of patient sera with a high proportion of endogenous interfering substances are shown before and after clean-up with the CN cartridges.

By this means, a selectivity is obtained which combines two separation principles, namely the interaction with polar groups on CN phases and Van der

Waals interactions on the RP phases. A further advantage of the method described is that there is no need to evaporate the organic phases used for extraction. The ciclopirox methyl derivative is eluted from the polar clean-up cartridge with a volume of the mobile phase small enough to allow direct injection onto the analytical column.

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Note

High-performance liquid chromatographic method for quantification of cyproheptadine in serum or plasma

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Cyproheptadine, 1-methyl-4-(5H-dibenzo[*a,d*]-cycloheptenylidine) piperidine, is a potent serotonin and histamine antagonist [1] and has a variety of possible therapeutic uses. It has been used as an antipruritic [2], an appetite stimulant [3], an antiabortifacient [4] and is reported to be useful in treating post-gastrectomy dumping syndrome [5].

Cyproheptadine has been analyzed by colorimetric procedures [6, 7], gas chromatography (GC) with flame ionization detection [8, 9], and GC with nitrogen–phosphorus detection (NPD) [10]. This report describes an analytical

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method for low nanogram per milliliter concentrations of cyproheptadine using high-performance liquid chromatography (HPLC) and a variable-wavelength ultraviolet detector. This method is applicable for microliter amounts of plasma or serum.

MATERIALS AND METHODS

Apparatus

The analytical HPLC equipment consisted of a Model 6000A delivery system, a WISP 710B automatic injector, a Model 720 system controller, a Model 730 data module, and a Model 450 variable-wavelength detector (Waters Assoc.). A 10-μm C_{18} reversed-phase column (Waters Assoc., μBondapak) 30 cm × 3.9 mm was used for separations.

Standards and reagents

Cyproheptadine · HCl (Merck Sharpe and Dohme, West Point, PA, U.S.A.) was dissolved in a solution consisting of methanol—0.1 *M* hydrochloric acid (50:50) to produce a concentration of 1 mg/ml cyproheptadine. Four working standards containing 60 ng/ml (A), 100 ng/ml (B), 200 ng/ml (C) and 400 ng/ml (D) were prepared by appropriate dilution of the above stock solution with 0.1 *M* hydrochloric acid. The internal standard was desmethyldoxepin · HCl (Pfizer, Groton, CT, U.S.A.) which was dissolved in 0.1 *M* hydrochloric acid to yield a solution of 1 μg/ml. All standard solutions were stored at 4—8°C. The mobile phase consisted of methanol—acetonitrile—0.1 *M* phosphate buffer and 5 m*M* pentanesulfonic acid at pH 4.7 (41 : 15 : 44).

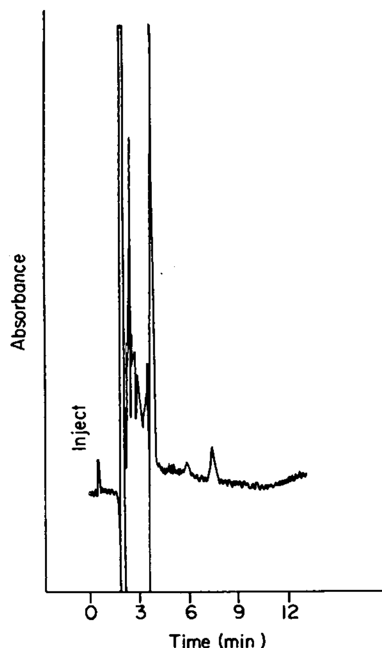
Procedure

A 50-μl aliquot of desmethyldoxepin standard solution and 50 μl of working standards A, B, C or D, were added to four 15-ml conical centrifuge tubes containing 100 μl of plasma. The four tubes contained 3, 5, 10, and 20 ng of cyproheptadine, respectively. To each tube were added 100 μl of 1.5 *M* sodium hydroxide and 3 ml of hexane—isoamyl alcohol (99:1). The extraction was carried out by mixing the solution for 2 min on a vortex mixer, then freezing and thawing to break up the emulsions, and centrifugation for 5 min at 2000 *g* (3000 rpm). The organic layers were transferred to four clean 15-ml conical centrifuge tubes and the aqueous layer was extracted a second time. The organic layers of the second extraction were combined with those of the first and 100 μl of 0.05 *M* sulfuric acid were added to each tube. Cyproheptadine was back-extracted into the acid by vortexing for 2 min. The aqueous solution was separated by centrifugation and the organic layer discarded.

Of the aqueous solution 90 μl were injected onto the HPLC column. The flow-rate of the mobile phase was 1.5 ml/min, the detector was set at 228 nm and the sensitivity was 0.01 a.u.f.s. Using 2 ml of plasma there is sufficient absorbance at 254 nm to run the analysis.

RESULTS AND DISCUSSION

Fig. 1 shows the HPLC curve for the extraction of 200 μl of plasma blank.



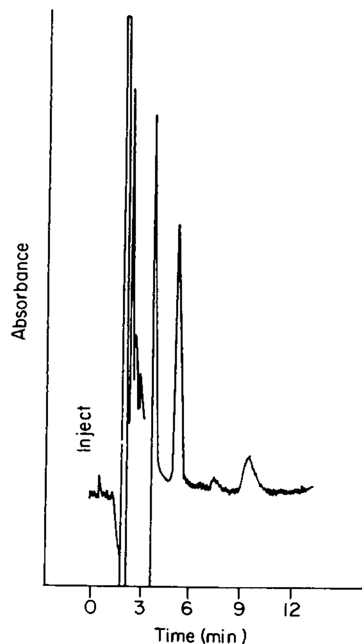


Fig. 1. Chromatogram of plasma blank extracts.

Fig. 2. Chromatogram of plasma extract containing 50 ng of desmethyldoxipin (5.19 min) and 7.84 ng of cyproheptadine (9.40 min).

Peaks at retention times of 5.85 and 7.36 min represent some endogenous compounds in the plasma. Fig. 2 represents the HPLC curve for the extraction of 100 μl of plasma to which were added 50 ng of desmethyldoxipin and 7.84 ng of cyproheptadine. The retention time for desmethyldoxepin is 5.19 min and for cyproheptadine 9.40 min. Although there is some overlap between desmethyldoxepin and the peak at 5.85 min, the error in measurement from this interference is less than 1%. A sensitive GC—NPC method [10] was unable to separate the desmethylcypropheptadine, a possible metabolite from the internal standard, 1-ethyl-4-(5H-dibenzo[*a,d*]-cycloheptenylidene)piperidine. In a separate analysis it was determined that under the conditions described here desmethylcyproheptadine had a retention time of 7.84 min. These HPLC conditions are suitable for determinations of cyproheptadine and its metabolite.

TABLE I

RECOVERY OF CYPROHEPTADINE ADDED TO PLASMA (n = 11)

Added (ng)	Recovered (ng)			R.S.D. (%)	Percentage error
	Mean	Range	S.D.		
3	3.02	3.36— 2.68	0.23	7.5	+0.67
5	4.87	5.34— 4.52	0.29	6.0	—2.60
10	10.16	10.70— 9.62	0.36	3.6	+1.60
20	19.95	22.30—17.40	1.52	7.6	—0.25

Quantification of the data was accomplished by measuring peak heights and establishing a ratio of cyproheptadine to desmethyldoxepin. Eleven separate determinations of the cyproheptadine/desmethyldoxepin ratios were obtained over a two-month period. The results were linear from 3 to 20 ng. The equation for the resulting straight line was $y = 0.016x + 0.005$ and the Pearson Product Moment correlation coefficient was 0.999. Calculation of the recoveries is presented in Table I.

The day-to-day variation in the precision and accuracy ranged from 3.6% to 7.6% and from −2.60% to 1.60% error, respectively. The within-day variations were determined on ten 100-μl plasma samples spiked with 7.84 ng of cyproheptadine. The average of recovered cyproheptadine was 7.57 ng ± 3.9%. The analytical recovery was 96.6%.

Serum levels were measured in two groups of Sprague–Dawley rats (weighing 150–250 g) that were given 10 and 20 mg/kg cyproheptadine per day via osmotic pumps (Alza, Palo Alto, CA, U.S.A.) which delivered at a constant rate for four weeks. A control group received saline alone. Steady-

TABLE II

DOSE VERSUS SERUM LEVEL (n = 11)

Dose (mg/kg per 24 h)	Serum concentration (ng/ml)
10	28.3 ± 14.9
20	42.0 ± 23.1

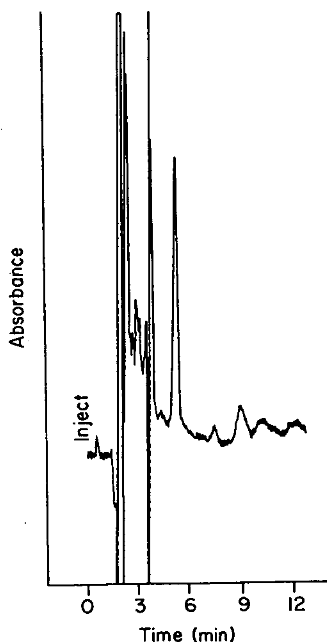


Fig. 3. Chromatogram of serum extract from a rat that received 20 mg/kg cyproheptadine per day. The serum cyproheptadine concentration is 37.9 ng/ml.

state serum concentrations were determined from tail bloods (100—200 μl serum) on the fourth week of treatment (Table II).

A typical chromatogram of serum analysis after dosing the rats with cyproheptadine is presented in Fig. 3.

CONCLUSIONS

In summary, the present method was shown to be sensitive and reliable for low nanogram level determinations of cyproheptadine. The use of desmethyldoxepin as an internal standard serves to minimize error in quantitation resulting from sample preparation. This method is more sensitive than previous colorimetric [6, 7] and flame ionization GC methods [8, 9]. A similar sensitivity for cyproheptadine was reported using GC—NP [10], but the internal standard used in that assay had the same retention time as the desmethylcyproheptadine metabolite. In the present method, there is no interference with this metabolite.

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Noordwijkerhout, The Netherlands 22 - 24 April, 1986

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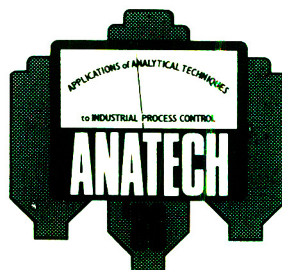

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