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JOURNAL OF CHROMATOGRAPHY

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CONTENTS

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(Contents overleaf)

Contents (continued)

Notes

(Continued overleaf)

Contents (continued)

JOURNAL OF CHROMATOGRAPHY

VOL. 311 (1984)

(Biomedical Applications, Vol. 36)

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CHROMBIO. 2223

ANALYSIS OF MELATONIN, 5-METHOXYTRYPTOPHOL AND 5-METHOXYINDOLEACETIC ACID IN THE PINEAL GLAND AND RETINA OF HAMSTER BY CAPILLARY COLUMN GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A specific capillary column gas chromatographic—mass spectrometric method was used to determine 5-methoxyindoles in the pineal gland and retina of the golden hamster during a light—dark (14:10) cycle. In the pineal gland, the mean levels of melatonin ranged from 0.15 to 2.4 pmol per gland, with a maximum in the dark. The levels of 5-methoxytryptophol and 5-methoxyindoleacetic acid were in the same range, but peaked during light. In the retina the levels of melatonin were about 100 pmol/g, and seemed not to differ between light and dark. The level of 5-methoxyindoleacetic acid were in the same range during light but were below the detection limit during dark.

INTRODUCTION

Interest in 5-methoxyindoles has so far mainly been focussed on melatonin and its function as a pineal hormone [1]. Although another 5-methoxyindole,

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5-methoxytryptophol (5MTOL), has long been known to exert an influence on reproduction [2], it is only recently that the existence and physiological effects of 5-methoxyindoles other than melatonin have gained interest [3, 4]. In addition, several findings have demonstrated that organs other than the pineal gland (e.g. the retina) possess the capability to produce 5-methoxyindoles (see ref. 5).

The aim of the present study was to determine endogenous levels of melatonin, 5MTOL and 5-methoxyindoleacetic acid (5MIAA) in the pineal gland and retina of the golden hamster during a light—dark cycle, by using a specific capillary column gas chromatographic—mass spectrometric (GC—MS) method.

EXPERIMENTAL

Chemicals and biological samples

5MTOL was obtained from Sigma (St. Louis, MO, U.S.A.); melatonin was from Regis (Morton Grove, IL, U.S.A.); 5MIAA was from Aldrich (Beerse, Belgium); pentafluoropropionic anhydride (PFPA) was from Reagenta (Uppsala, Sweden); and 2,2,2-trifluoroethanol (TFE) was from E. Merck (Darmstadt, F.R.G.). 5-Methoxyindole-3-[2-$^2H_2$]acetic acid (5-[$^2H_2$]MIAA) was synthesized according to the procedure of Beck and Bosin [6]. 5-Methoxy-[$\alpha,\alpha,\beta,\beta$-$^2H_4$]tryptophol (5-[$^2H_4$]MTOL) was synthesized by the method of Hesselgren and Beck [7]. N-Acetyl-5-methoxy-[$\alpha,\alpha,\beta,\beta$-$^2H_4$]tryptamine ([$^2H_4$]-melatonin) was prepared by the procedure of Shaw et al. [8]. All other chemicals used were of analytical purity.

Male golden hamsters (*Mesocricetus auratus*) (80—90 g) were obtained from TNO (Zeist, The Netherlands). The animals were maintained under a long photoperiod (light—dark 14:10), with the light on between 4.00 a.m. and 6.00 p.m., at 25°C in constant humidity; they received food and water ad libitum. After decapitation, the pineal gland and retinae were quickly removed. The tissues were frozen in liquid nitrogen and stored at —70°C prior to analysis. During the night the animals were killed in the dark, but dissected in the light.

Preparation of samples

The pineal glands from three animals were pooled and the retinae from each animal were analysed together. The tissue was homogenized in 1.0 ml of ice-cooled 0.15 *M* formic acid containing [$^2H_4$]melatonin (38.7 pmol), 5-[$^2H_4$]-MTOL (74.8 pmol) and 5-[$^2H_2$]MIAA (47.2 pmol), using an Ultra-Turrax homogenizer. The homogenate was centrifuged at 70,000 *g* for 15 min and the supernatant transferred to a clean acid-washed (dichromate—sulphuric acid) 15-ml glass-stoppered tube containing 6 ml of dichloromethane. The tube was shaken and centrifuged at 1000 *g* for 5 min. The organic layer was divided into two equal parts which were transferred to new tubes and evaporated to dryness under a stream of nitrogen. One part of the extract was used for the analysis of melatonin and 5MTOL. The extract was treated with 50 μl of PFPA at 60°C for 1 h, evaporated to dryness under nitrogen and redissolved in 25 μl of ethyl acetate. The other part of the extract was used for the analysis of

5MIAA. This part was treated with 50 μl of a mixture of PFPA and TFE (4:1) at 75° C for 5 min, followed by evaporation to dryness under nitrogen. Thereafter, the residue was treated as described above for the melatonin and 5MTOL fraction.

Gas chromatography—mass spectrometry

Selected ion monitoring was performed using a computer-controlled LKB 2091 gas chromatograph—mass spectrometer. The gas chromatograph and the mass spectrometer were interfaced with a jet separator. The GC separations were achieved using a 25 m × 0.32 mm I.D. WCOT SE-52 capillary column and helium was used as carrier and make-up gas. Splitless injections were carried out using a "moving needle" device. The GC conditions were: injector heater 260° C; column temperature 200° C for 5MTOL and 5MIAA and 230° C for melatonin; column flow-rate ~2 ml/min and make-up gas flow-rate ~12 ml/min. Aliquots of 2 μl of the samples were injected and an initial delay of about 1.5 min in opening the valve was effected to avoid contamination of the ion source. Under these conditions the retention times of the derivatives of the 5-methoxyindole compounds were about 2 min. The MS conditions were: separator temperature 250° C; ion source temperature 240° C; electron energy 70 eV; and trap current 50 μA. The mass numbers monitored for melatonin, 5MTOL and 5MIAA were *m/z* 360:364, 483:487 or 319:322, and 433:435, respectively, where the lower mass number corresponds to the authentic compound and the higher to the deuterated internal standard.

Quantitation

Calibration curves were constructed by plotting the peak height ratios (authentic/internal standard) of the standard samples against the concentration of authentic compound. The levels were then determined from the corresponding peak height ratios of each sample by reference to the calibration curve. The calibration curves showed a linear relationship of the peak height raios to the concentration and always intercepted near the origin.

RESULTS

Identification

The selected-ion monitoring involved recording ion intensities at mass numbers corresponding to the molecular ions and, in the case of 5MTOL, also to a characteristic fragment ion [9—11]. The identification was based on the presence of compounds eluting at the same retention time as authentic compounds and at the correct mass numbers. Evidence for the presence of melatonin, 5MTOL and 5MIAA in the pineal gland (Fig. 1), and of melatonin and 5MIAA in the retina (Fig. 2), was obtained. The reproducibility of the method was better than 10% for all three compounds.

Pineal levels

During the light phase the level of melatonin in the pineal gland was found to be about 0.15 pmol per gland (Fig. 3). The melatonin level was increased about fifteen-fold to 2.4 pmol per gland during the dark. The levels of 5MTOL

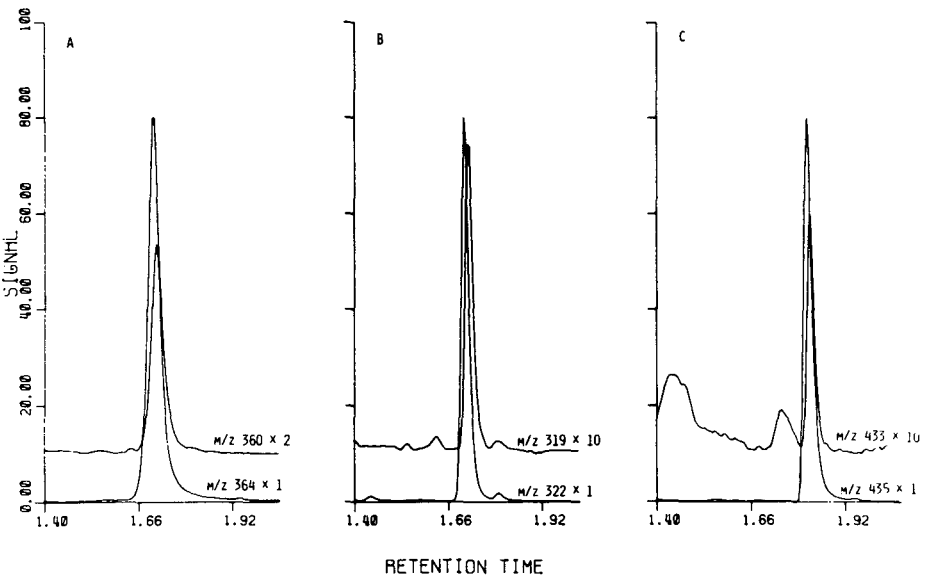

Fig. 1. Ion trace chromatograms obtained from the analysis of (A) melatonin, (B) 5MTOL, and (C) 5MIAA in hamster pineal glands. Mass numbers (m/z) and relative amplification factors are indicated. The retention time is expressed in minutes.

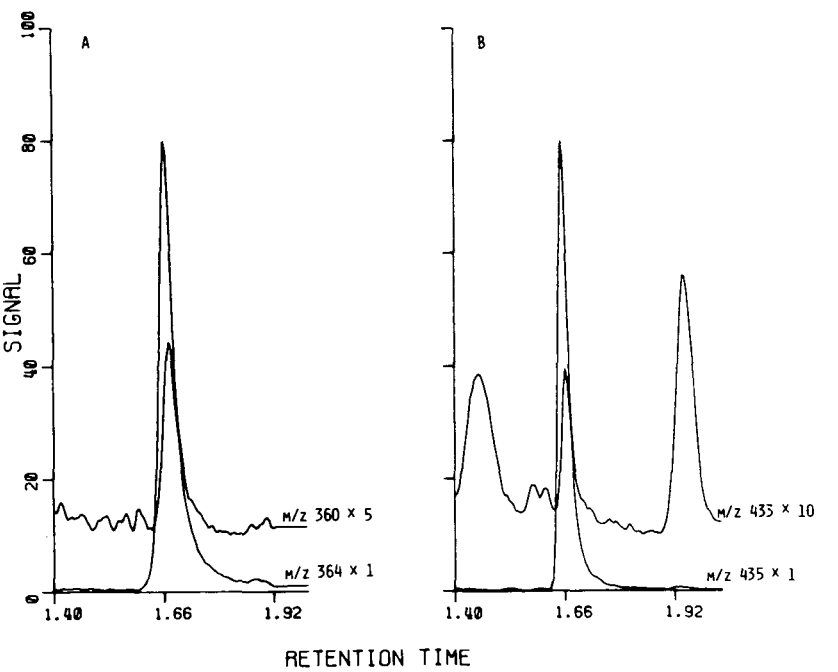

Fig. 2. Ion trace chromatogram obtained from the analysis of (A) melatonin, and (B) 5MIAA in hamster retinae. Mass numbers (m/z) and relative amplification factors are indicated. The retention time is expressed in minutes.

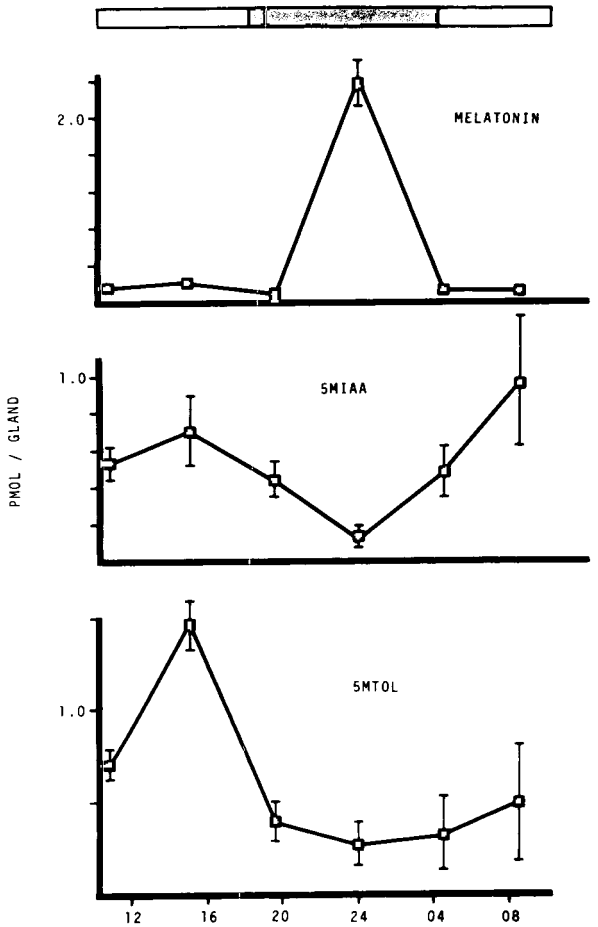

Fig. 3. Concentrations of melatonin, 5MTOL and 5MIAA in the hamster pineal gland during a light—dark cycle (long photoperiod, light—dark 14:10).

and 5MIAA were of a similar magnitude to those of melatonin (Fig. 3). The maximal levels of 5MTOL and 5MIAA occurred, however, during the light phase, and there was less difference than for melatonin between light and dark.

Retina levels

Melatonin was present in the retina at a level of about 100 pmol/g (Fig. 4). No difference in the melatonin level between light and dark phase was evident. 5MIAA was detectable in most samples collected during light and the levels seemed to be at the detection limit during dark (Fig. 4). There was a significant variation in results within the groups. 5MTOL could be detected in a few samples (~10%), with a limit of detection of about 5 pmol/g. There appeared to be a relation between melatonin, 5MIAA and 5MTOL in the retina in that when the melatonin level was high, 5MIAA and 5MTOL also occurred at relatively high levels.

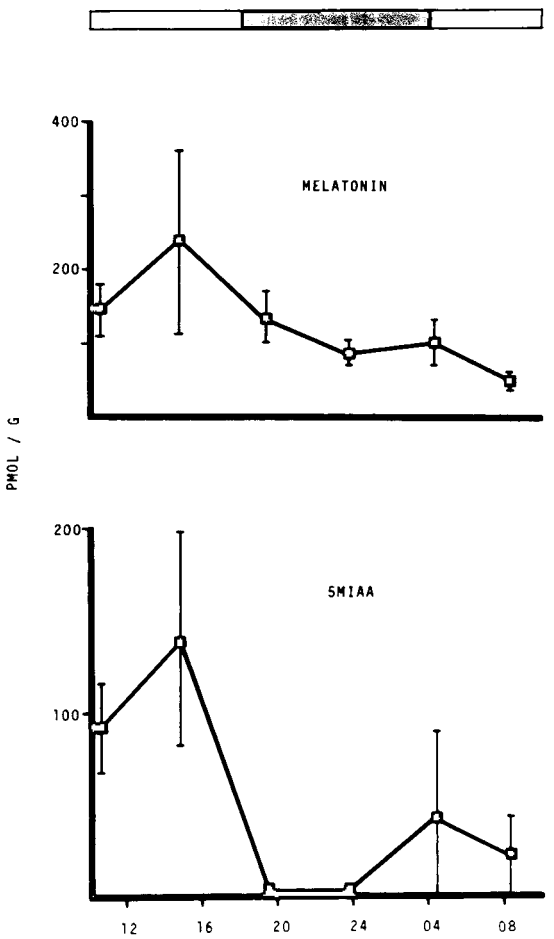

Fig. 4. Concentrations of melatonin and 5MIAA in the hamster retina during a light—dark cycle (long photoperiod, light—dark 14:10).

DISCUSSION

The results of this study, obtained by use of GC—MS, confirm the presence of melatonin in the pineal gland of the male golden hamster, as had previously been found using radioimmunoassay methods [12—14]. The diurnal variation of the melatonin levels in the pineal gland, with a maximum during the dark phase of the light—dark cycle, is also in agreement with the earlier studies. However, some disagreement exists with regard to absolute levels. This study is in accordance with the results of Tamarkin et al. [12] and points to slightly lower levels than reported by others [13, 14].

The presence of melatonin in the retina of both non-mammalian and mammalian species (see ref. 5), including hamster [15], has previously been reported. Our results are in good agreement with those obtained previously in hamster [15]. The absence of a diurnal variation of the melatonin concentration is in agreement with a study of the ground squirrel [16], but in variance

with studies of quail, pigeon, chicken and rat [17—19]. However, our results are in agreement with a study of Pévet et al. [20] in which the capacity of the hamster retina to synthesize melatonin was similar throughout the light—dark cycle.

The presence of 5MTOL and 5MIAA in the pineal gland of hamster is in agreement with earlier studies of other species (see ref. 4). The diurnal variation of the 5MTOL levels, with a maximum during the light phase, is in agreement with an earlier study of the capacity of hamster pineal to synthesize 5MTOL [20]. In rat pineal, however, a maximum in 5MTOL levels during dark has been reported [10, 21].

This study reports for the first time the presence of 5MIAA in retina. The level of 5MIAA appeared to possess a diurnal variation, with high levels during the light phase. However, as for melatonin, there was a significant variation in the results, which may indicate that factors other than light—dark influence its level.

The exact physiological function of the 5-methoxyindoles has yet to be demonstrated. However, both melatonin and 5MTOL possess physiological effects [1, 4]. It is interesting to note that in the pineal gland melatonin and 5MTOL occur at similar concentrations but with maximum levels at different times of the light—dark cycle. In retina, however, melatonin occurs at a substantially higher concentration than 5MTOL. 5MIAA may arise as a metabolite of all the other 5-methoxyindoles [22—24] and as yet no physiological effect has been described for this compound.

In conclusion, the present study has demonstrated that 5-methoxyindoles other than melatonin are present in the pineal gland and retina of hamster, and that their levels possess a diurnal variation. This strengthens the concept that melatonin is not the only 5-methoxyindole that has to be considered when pineal function is studied.

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CHROMBIO. 2222

RELIABLE MEASUREMENT OF NON-ESTERIFIED LONG-CHAIN FATTY ACID PATTERN IN BLOOD PLASMA

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(First received February 8th, 1984; revised manuscript received May 8th, 1984)

SUMMARY

In this reliable assay for determining the non-esterified long-chain fatty acid pattern in plasma, only 100 μl of sample are needed and a single assay can be done within 40 min. The isolation procedure was performed by adsorption of fatty acids from plasma onto graphitized carbon black (Carbopack B) using a column method. After desorption and removal of the eluting phase, fatty acids are methylated by diazomethane and quantified by packed column gas chromatography. Analytical recoveries ranged between 91% and 103%. Within-run precision gave coefficients of variation of 2.3% and 11% for fatty acid concentrations of 58.2 and 0.6 μmol/l, respectively. Studies of plasma samples under various storage conditions indicated that reliable measurement of the non-esterified fatty acid fraction can be obtained even after 60 days if specimens are conserved at —18°C in the presence of a suitable phospholipase inhibitor.

INTRODUCTION

The pattern of non-esterified long-chain fatty acids (NEFA) in blood is of interest in a wide variety of biochemical and clinical investigations. All of the proposed analytical methods involve solvent extraction and gas chromatography of the methyl esters. Most of them are modifications of the procedures of Dole and Meinertz [1] or Folch et al. [2] which make use of time-consuming multiple purification steps to isolate the NEFA fraction from the lipid extract [3—6]. Recently, Mueller and Binz [7] described a rapid method without further manipulations with a chloroform—methanol extract of buffered (pH 6) serum. However, the sophisticated gas chromatographic apparatus proposed may discourage use of the method in other laboratories.

Recently, Carbopack B has been successfully used as an adsorbing medium

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for isolating acidic compounds [8—12] from blood serum and urine. We describe here a reliable procedure which makes use of 100 μl of plasma and employs Carbopack B for sample purification coupled with a conventional packed column for gas chromatography of NEFA methyl esters. The stability of the NEFA composition in plasma, serum and related extracts under different storage conditions was also investigated.

MATERIALS AND METHODS

Reagents

Solvents of analytical grade from various commercial sources were distilled twice in a glass system. Deionized water was distilled in the presence of permanganate. Diazomethane was generated from N-nitrosomethylurea [13] and the diethyl ether solution was stored at −18°C after addition of solid potassium hydroxide. Under these conditions, the solution is stable for at least two months, whereas working aliquots are stable for about one week if conserved at 4°C during the period of use. Benzenemethanesulphonyl fluoride (BMSF) was obtained from Fluka (Buchs, Switzerland) and a solution of 100 g/l in methanol was used. Carbopack B (80—120 mesh) was kindly supplied by Supelco (Bellefonte, PA, U.S.A.).

Standards

Fatty acid standards (puriss, > 99% GC) were from Fluka. The stock standards were 1 g/l in chloroform and were stable for at least six months at −18°C. For studies of recovery at low and high acid concentrations in serum, *n*-eicosanoic and arachidonic acids were dissolved in methanol—water (50:50, v/v) to give a concentration of 0.5 mg/l. This solution was further diluted to give a concentration of 0.1 mg/l. Two internal standard solutions were used. One was prepared by dissolving *n*-heptadecanoic acid in water—methanol (50:50, v/v) to give a concentration of 0.5 mg/l. The second solution was prepared by a five-fold dilution of the *n*-heneicosanoic acid stock standard. The reference standard solution was prepared by evaporating 10 μl of each individual fatty acid stock solution and derivatizing each residue. All standard solutions were stored at 4°C and replaced every two weeks.

Phospholipids were supplied by Supelco.

Glassware preparation

All the glassware was cleaned with hot chromosulphuric acid and rinsed thoroughly with distilled water.

Gas chromatography

A Model 3800 gas chromatograph equipped with a flame-ionization detector from Dani (Monza, Italy) was used. The glass colum, 2 m × 2 mm I.D., packed with GP 5% DEGS-PS on 100—120 mesh Supelcoport was from Supelco. The chromatographic conditions were: oven temperature 185°C; injection port and detector block temperatures 200°C. Nitrogen was used as carrier gas with a dead time of 30 sec.

Samples

Blood was drawn after overnight fast from apparently healthy volunteers by antecubital venipuncture. The blood samples were collected in centrifuge tubes containing ethylenediaminetetraacetic acid as anticoagulant, chilled immediately in an ice bath, stoppered and centrifuged at 4°C for 15 min at 2000 *g*. Plasma was promptly removed, divided into 100-μl aliquots which were collected in 6-ml air-tight screw-capped glass vials containing 3 μl of the BMSF solution and stored at −18°C if not processed within 1 h.

Procedure

Prepare the Carbopack B bed by introducing 0.25 g of the adsorbent into a 15 × 0.6 cm glass column with a small pledget of glass wool in the bottom. Wash the column with 5 ml of chloroform, 3 ml of methanol and 3 ml of distilled water, in sequence. Remove large bubbles by gently pumping distilled water onto the Carbopack B bed with the aid of a Pasteur pipette. Percolate the biological specimen previously diluted with 5 ml of the methanol—water (50:50, v/v) solution containing *n*-heptadecanoic acid as internal standard. Rinse the vial with two portions of 2.5 ml of 3 mmol/l hydrochloric acid solution and pass the rinsings through the column. Wash the column with 1.5 ml of methanol and elute the long-chain fatty acids with chloroform—methanol (70:30, v/v). Collect 3 ml of the eluate from the moment the eluting solution is applied to the column in a cone-shaped glass vial, add 10 μl of the *n*-heneicosanoic acid solution and evaporate the solvent under a stream of nitrogen at 50°C. Place the vial in an ice bath, add 3 μl of methanol, 30 μl of cold ethereal diazomethane and seal. After about 5 min, eliminate the excess reagents under a nitrogen stream at room temperature, dissolve the residue with 15 μl of chloroform and inject 1 μl.

Quantification was performed by peak height measurement since the fatty acid methyl ester peaks are sharp and symmetrical. To calculate the concentration of each fatty acid, the peak height relative to that of the internal standard is compared with that of the reference standard.

RESULTS AND DISCUSSION

Precision

The within-run precision was evaluated by assaying the same plasma sample nine times. The results are shown in Table I.

Recovery

Under the experimental conditions adopted, arachidonic and eicosanoic acids are, respectively, the first and the last compounds to be desorbed from the Carbopack column.

We determined the recovery of fatty acids from plasma by supplementing plasma samples of known NEFA content with known amounts of selected long-chain fatty acids and re-assaying. With respect to the extraction procedure, we evaluated the relative and absolute analytical recoveries by adding two internal standards, i.e. *n*-heptadecanoic acid and *n*-heneicosanoic acid, the first before and the second after the purification process. Results are given in Table II.

TABLE I

WITHIN-RUN PRECISION FOR THE ANALYSIS OF FATTY ACIDS IN PLASMA SPECIMENS (n = 9)

Fatty acid*	Retention time (min)	Mean ± S.D. (μmol/l)	C.V. (%)
$C_{14:0}$	1.8	3.9 ± 0.17	4.3
$C_{16:0}$	2.8	58.2 ± 1.34	2.3
$C_{16:1}$	3.2	4.1 ± 0.25	6.0
$C_{18:0}$	4.8	19.1 ± 0.63	3.3
$C_{18:1}$	5.4	50.0 ± 1.35	2.7
$C_{18:2}$	6.7	19.0 ± 0.80	4.2
$C_{18:3}$	8.8	4.6 ± 0.22	4.8
$C_{20:0}$	8.3	0.6 ± 0.07	11
$C_{20:4}$	15.3	1.7 ± 0.11	6.2

*The fatty acids are denoted by chain length:number of double bonds.

TABLE II

ABSOLUTE AND RELATIVE RECOVERIES OF FATTY ACIDS ADDED TO PLASMA SPECIMENS

Added (μmol/l)	Found (μmol/l)	Recovery (%)	
		Absolute	Relative*
Arachidonic acid			
81.2 (n = 6)	78.8	97	103
16.2	15.6	97	103
n-Eicosanoic			
79.1	67.2	85	91
15.8	13.7	87	93
n-Heptadecanoic			
92.6	86.3	93	

*Relative to n-heptadecanoic acid.

Specificity

Although diazomethane is a toxic compound, we found it could not be replaced as methylating reagent by methanolic acids or alkalies because of the problem of transmethylation by the latter reagents of any phospholipids which may be coeluted with the free fatty acids from the Carbopack column.

Endogenous lipids and substances present in solvents or Carbopack B were considered as possible interfering compounds.

Under the extraction conditions selected, triacylglycerols and cholesteryl esters were not eluted from the Carbopack B purification column, whereas some phospholipids were partly coeluted with free fatty acids. To ascertain the absence of hydrolysis or transesterification reactions occurring during the analysis, a plasma sample was divided into aliquots, one remained unsupplemented and every single other aliquot was supplemented with one of the following phospholipids: diarachidoylphosphatidylcholine, diarachidoylphos-

phatidylethanolamine, egg lysophosphatidylethanolamine, bovine sphingomyelin. These samples were then submitted to analysis in duplicate. In terms of individual concentrations of free fatty acids, we noted no difference between the blank and phospholipid-supplemented plasma samples.

When the chloroform washing of Carbopack B was omitted, the blank showed the presence of some background interfering peaks. Solvents used as supplied gave high blank values especially for palmitic, stearic and oleic acids. These interferences became negligible when the solvents were bidistilled.

Storage

Five fresh plasma samples from different subjects were each divided in two portions, one of which was supplemented with a phospholipase inhibitor such as BMSF [14]. Each portion was further fractionated into aliquots, stored at either −18° C or 4° C and periodically analysed over a period of 60 days. Table III shows that reliable measurements of free fatty acids in plasma can still be made within 60 days, provided the plasma samples are conserved at −18° C wih a phospholipase inhibitor.

TABLE III

EFFECT OF STORAGE ON PLASMA (n = 5) FATTY ACID CONCENTRATIONS

The data are expressed as percentage variation of the initial concentrations of oleic, linoleic and arachidonic acids which are 121 ± 40, 32 ± 12, 2.4 ± 0.7 μmol/l, respectively.

Temperature (° C)	No. of days	ΔC (%)					
		Oleic		Linoleic		Arachidonic	
		Mean	Range	Mean	Range	Mean	Range
+4	1	+7.5	+2.0 to +18	+24	+15 to +40	+33	+25 to +43
−18	1	+0.5	−3.0 to +3.8	+4.1	−0.5 to +8.0	+3.9	+0.4 to +6.8
−18	5	+6.8	+2.7 to +10.1	+11.4	+4.8 to +18.5	+24	+18 to +29
+4	1*	+1.1	−0.5 to +3.8	−1.5	−3.5 to +0.5	−3.3	−5.0 to −2.1
+4	7*	+4.5	−1.0 to +8.9	+17	+9.5 to +21	+39	+21 to +110
−18	60*	+3.6	+0.1 to +11	+3.6	−1.5 to +9.8	+3.1	−2.7 to +10.3

*Plasma samples supplemented with benzenemethanesulphonyl fluoride.

Some fractions collected after purification of plasma aliquots were not processed further but were stored at 4° C. No significant variation in the NEFA concentrations was observed on periodic analysis of the extracts over a period of two months. Moreover, we observed that extracts were stable even when they were left for one week at room temperature.

A mean 15% higher arachidonic acid concentration was measured when serum instead of plasma was submitted to analysis. Serum was obtained by allowing blood to clot for 1 h at room temperature. The higher amount can be explained by the fact that phospholipids can be hydrolysed to a variable extent and that arachidonic acid in its non-esterified form is particularly abundant in phospholipids.

Extraction column variables

A 50-fold dilution of plasma with methanol—water was essential to ensure quantitative recovery of NEFA. Considerable losses in the effluent, especially

of saturated fatty acids, occurred as the dilution ratio was decreased or water was used as diluting agent. This can be accounted for by the existence of a NEFA—protein complex.

Low recovery of fatty acids was observed when the washing step with acidic water was omitted. This can be ascribed to chemical binding between acidic compounds and some chemical heterogeneities present on the Carbopack surface [15]. However, the binding is readily hydrolysed by acidic washing of the purification column.

Methanol washing eliminates water and some potentially interfering compounds [12]. However, loss of arachidonic acid was observed when the volume of methanol exceeded 5 ml.

We evaluated the effect of the composition of the eluting phase on the recovery of fatty acids. When the chloroform/methanol ratio was decreased, saturated fatty acids were partially lost. A chloroform/methanol ratio higher than 70:30 enhanced the recovery of the last acid to be eluted from Carbopack, i.e. eicosanoic acid. However, under such conditions, other compounds were coeluted which caused the appearance on the chromatogram of peaks with high retention times, thus increasing the analysis time.

Under the experimental conditions used, the flow-rate of the solvents percolating through the Carbopack column was about 3 ml/min. As reported elsewhere [12], attempts to increase the flow-rate by exerting an additional pressure provoked loss of analytes in the plasma effluent.

To improve our procedure in terms of economy and speed, we evaluated the reusability of the purification column. After each extraction, the column was restored by washing it sequentially with 3 ml of chloroform, 3 ml of methanol and 3 ml of water. After five such extractions, the NEFA concentration was unchanged within the precision of the method. However, when the restored column was left unused and filled with water, we observed some increase in the unsaturated fatty acids, probably due to the slow hydrolysis of some phospholipids which are strongly retained by the Carbopack column. An analogous effect occurred when the purification process was casually interrupted after the acidic washing step.

TABLE IV

FATTY ACID CONCENTRATIONS IN PLASMA SAMPLES (n = 5) AS MEASURED BY THREE DIFFERENT PROCEDURES

Values are expressed in μmol/l.

	Our method		Mueller and Binz [7]		Hagenfeldt [3]	
	Mean	Range	Mean	Range	Mean	Range
$C_{16:0}$	83.9	58.1—106	79.4	51.3—104	115	71.4—154
$C_{16:1}$	5.6	4.1— 8.2	4.4	2.1— 7.6	15.4	7.0— 21.1
$C_{18:0}$	26.6	19.1— 38.9	24.5	17.6— 38.8	38.0	22.3— 61.6
$C_{18:1}$	99.0	65.3—122	103	64.4—127	132	80.2—191
$C_{18:2}$	30.3	18.0— 39.8	38.1	21.2— 46.9	58.7	37.2— 82.7
$C_{18:3}$	5.7	4.4— 6.4	5.4	3.2— 6.3	6.4	3.6— 9.8
$C_{20:0}$	0.8	0.6— 1.2	1.0	0.8— 1.2	1.3	1.0— 1.8
$C_{20:4}$	2.2	1.2— 4.0	3.9	1.4— 7.8	7.8	4.3— 13.1

We compared results using our method with those using two previously reported methods [3, 7]. Five fresh serum samples from different subjects were each divided into three aliquots, which were analysed in duplicate following the three extraction procedures. In all cases, final samples were injected into the chromatographic column proposed by us. The results are reported in Table IV. As can be seen, we found a fair agreement between our procedure and that described by Mueller and Binz [7], even though higher values for arachidonic acid were occasionally obtained by the latter procedure. However, we found the purification method reported by Mueller and Binz [7] unsuitable for quantification with a conventional-packed column as it appeared to be seriously deteriorated after about ten injections of the extracted and derivatized sample.

Surprisingly, Hagenfeldt's method [3] gave by far higher values for fatty acids, particularly for those that are relatively more abundant in phospholipids, such as linolenic and arachidonic acids. This could be explained by considering that this method includes an acidic extraction step which may provoke hydrolysis of phospholipids. To obtain experimental evidence for this hypothesis, a serum sample was divided into two aliquots; one was spiked with diarachidoylphosphatidylcholine leaving the other one unsupplemented. These samples were then submitted to analysis in duplicate according to the procedure of Hagenfeldt [3]. The large increase of arachidonic acid concentration in the free fatty acid fraction after addition of the phospholipid unequivocally confirmed the hypothesis.

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GAS CHROMATOGRAPHIC ANALYSIS OF ENDOGENOUS CATECHOLAMINES, PHENOLIC AMINES AND DERIVED ISOQUINOLINES USING SHORT GLASS CAPILLARY COLUMNS AND ELECTRON-CAPTURE DETECTION

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SUMMARY

A gas chromatographic method is described for the concomitant separation and analysis of catecholamines, catecholamine or 3,4-dihydroxyphenylethylamine condensation products (tetrahydroisoquinolines), and their isomeric mono-O-methyl (phenolic) metabolites which may be present in neuronal tissues, utilizing short glass capillary columns and electron-capture detection. Isomeric phenolic amines that were not generally separable with conventional-packed gas chromatographic columns were rapidly resolved on the capillary system, and with their catecholamine or catechol isoquinoline precursors, quantitated with high sensitivity (0.25—7.0 pg) and reproducibility. Key steps in the approach with tissues include initial amine isolation with a weak cation-exchange resin (BioRex-70), fluoracyl derivative formation, and brief washing of the derivatives with ammonium phosphate buffer (pH 5.8) just prior to capillary analysis; overall recoveries of amines or alkaloids added to rat brain homogenates ranged from 79% to 89%. Application of the method is demonstrated in an assay of endogenous dopamine in rat corpus striatum and hypothalamus. This new procedure should complement and in some instances may be preferred over liquid chromatographic assays for catecholic and phenolic amines and isoquinolines, and ought to be applicable to mass spectrometric detectors as well.

INTRODUCTION

Gas chromatographic (GC) methodologies for the measurement of catecholamines (CAs) and their amine metabolites in tissues have been available for several years. Such assays generally have required the sensitive electron-capture cell or a mass spectrometer as a detector [1]. One often unacknowledged limiting factor with these similar GC-based assays has been the degree of resolu-

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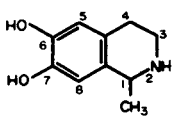
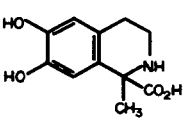
tion of geometric and structural neuroamine isomers in complex mixtures. In GC studies with CAs and their recently appreciated trace carbonyl condensation products, the 1,2,3,4-tetrahydroisoquinolines (TIQs), we frequently experienced difficulties separating certain isomeric O-methylated metabolites on conventional wide-bore (packed) GC columns. Bail et al. [2] first noted this problem with mono-O-methylated derivatives of simple TIQ condensation products of dopamine (DA).

To overcome resolution problems in GC analyses of neuroamines which employ the relatively accessible electron-capture detector [3], we explored the use of short capillary (open tubular) columns. There has been very little application of capillary methodology to assays of endogenous neurochemicals (see Discussion). With their high retention time stabilities and superior efficiencies (3000 plates per m), sufficiently inert capillary columns should be very appropriate for separations of geometric isomers of neuroamines. In this report, separation and quantitation of the CAs, O-methylated CA isomers, and a select number of simple catecholic and phenolic isoquinolines are demonstrated using short glass capillary columns, a state-of-the-art splitter injection mode and an autoinjector. Resolution far exceeded and detection limits easily equaled those obtained with conventional-packed GC columns coupled to an electron-capture detector or a mass spectrometer.

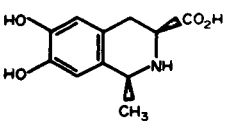
Isoquinolines and β-carbolines (indoleamine condensation products) constitute a relatively new group of potentially endogenous neuroamine derivatives (mammalian alkaloids) that could be of pathophysiological importance in certain disease conditions [4]. This capillary GC method, with its high sensitivity and reproducibility, can aid in determining precisely which alkaloids are in vivo substances. The overall procedure was used earlier [5] to clarify the apparent absence of the TIQs, salsolinol (SAL, Fig. 1) and its 7-O-methylated metabolite (7M-SAL), from corpus striatum of normal untreated rats. For the purpose of this report, an endogenous catecholamine, dopamine, is quantitated



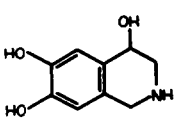
(d,l) Salsolinol (SAL)



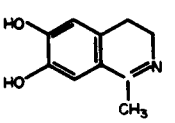
(d,l) SAL-1-Carboxylic Acid
SAL-1-CA



(cis) SAL-3-Carboxylic Acid
(SAL-3-CA)



(d,l)4-Hydroxyl-1-Desmethylsalsolinol
(4-HO-DSAL)



1,2-dehydro-SAL

Fig. 1. 6,7-Dihydroxy (catecholic) isoquinolines used in this study. The ten other isoquinolines in Table I are the respective 6-O-methyl and 7-O-methyl (phenolic) alkaloids related to these five substrates.

in two rat brain regions with the overall capillary GC procedure. In another report we show the applicability of this high-resolution capillary GC method using electron-capture detection (ECD) in studies of TIQ O-methylation patterns in rat brain [6].

MATERIALS AND METHODS

The capillary GC studies with the catecholic and phenolic amines were carried out with a Varian 3700 gas chromatograph equipped with dual ^{63}Ni electron-capture detectors (pulsed mode), a Varian 8000 automatic liquid sampler injector, and a polyphenylmethylsiloxane (OV-17) wall-coated open tubular (WCOT) glass capillary column (10 m × 0.25 mm; Alltech Assoc.). The split ratio was 10:1, and the head pressure of the carrier gas, oxygen-free nitrogen, was 0.8 kg/cm. The temperatures of the injector and detector were 250°C and 350°C, respectively, and column temperatures were as indicated in the figure legends or tables. Conventional columns (1.83 m × 6.35 mm, glass) packed with 3% OV-101, 3% OV-17, 5% SE-30 or 3% SE-54 on Gas Chrom Q (80—100 mesh) were coupled to the other electron-capture detector. Peak areas and retention times were obtained with a Varian CDS-111C computing integrator.

When confirmatory chromatography of the amines before derivatization was needed, an isocratic high-performance liquid chromatographic (HPLC) system consisting of a 25 cm × 4 mm BioSil (Bio-Rad Labs.) 10-μm C_{18} reversed-phase column and a BioAnalytical Systems LC-2A electrochemical detector (0.79 V) was utilized, generally with a mobile phase of 0.1 *M* sodium dihydrogen phosphate (pH range 4—5.5) containing 1 m*M* Na_2EDTA and varying percentages of methanol. A detailed account of the HPLC of simple and complex isoquinolines is in preparation. Thin-layer chromatography (TLC), when required to monitor synthetic reactions, was accomplished on 5 cm × 25 cm silica gel plates (Whatman) in *sec*-butanol—acetic acid—water (4:1:1, v/v/v) or on aluminum oxide plates (Macherey-Nagel) in chloroform—methanol—water (70:50:5, v/v/v). Compounds were visualized in an iodine chamber. The aluminum oxide plate was qualitatively useful for complete separation of catechols, which did not migrate in this case, from phenols, which did.

Chemicals and reagents

All of the isoquinolines used in this study have been characterized and reported in the literature. The TIQs *d,l*-salsolinol (HCl salt), *d,l*-salsolinol-1-carboxylic acid (*d,l*-SAL-1-CA) and *cis*-salsolinol-3-carboxylic acid (*cis*-SAL-3-CA) in Fig. 1, and their respective racemic mono-O-methyl derivatives (6M-SAL, 7M-SAL, 6M-SAL-1-CA, 7M-SAL-1-CA, 6M-SAL-3-CA and 7M-SAL-3-CA), were synthesized in yields ranging from 20% to 75% by aqueous (pH 4—5) condensation of the appropriate open-chain amine or amino acid with a three- to four-fold molar excess of acetaldehyde (Baker, analytical grade) or pyruvic acid (Sigma) at room temperature [7—9]. Elevated temperatures (60—70°C) were necessary to obtain sufficient (but low) yields of the 6-O-methylated TIQ isomers.

The 4-hydroxylated TIQs related to norepinephrine (NE) [*d,l*-4-hydroxyl-1-desmethylsalsolinol (*dl*-4-HO-DSAL) in Fig. 1 and its two mono-O-methyl

derivatives] were available as racemic HCl salts from earlier syntheses [10]. The three dihydroisoquinolines (DIQs) used in this study (1,2-dehydro-SAL in Fig. 1 and its respective 6- and 7-O-methyl analogues) were obtained as HBr salts via Bischler—Napieralski cyclizations of the appropriate mono- or dimethoxylated amide [11—13] followed by demethylation with refluxing 48% HBr where required. 3,4-Dihydroxyphenylethylamine (DOPA), the CAs, and their phenolic metabolites utilized as standards or synthetic reactants were purchased from Sigma, Aldrich or Regis.

Reactions were monitored initially by the TLC and HPLC techniques mentioned. Reaction products that failed to precipitate after several days were isolated by acidification (1 *M* hydrochloric acid), lyophilization and successive recrystallization from ethanol—ethyl acetate. Product purity was assessed by HPLC. Structures were proven by infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy and by melting point comparisons with literature values.

Preparation of fluoracyl derivatives

Fluoracyl derivative preparation for capillary GC—ECD was achieved with the CAs, non-carboxylated TIQs and their mono-O-methylated analogues in a manner similar to a previously reported method [3]. To plastic-lined screw-top scintillation vials containing weighed amounts of the amine or amine salt, or to the lyophilized residues obtained from the ion-exchange column isolation (below) of aqueous extracts of tissue or standards, were added 500 μl of acetonitrile (Pierce) and 50 μl of heptafluorobutyryl anhydride (HFBA, Pierce). In some instances HFBA was replaced by pentafluoropropionyl anhydride (PFPA) or trifluoroacetyl anhydride (TFAA). After 30—40 min at room temperature, reactions were dried in a hood with a nitrogen stream. Samples or dried extracts containing 1- or 3-carboxylated TIQs were first treated with 200 μl of hexafluoroisopropanol (HFIP, Pierce) and 50 μl of fluoracyl agent, usually PFPA, and after 30 min at room temperature, were dried as above. The residue was further treated with PFPA (50 μl) and acetonitrile (500 μl) for 20 min, and blown dry with nitrogen. The final residues from fluoracylation or esterification—fluoracylation were dissolved in 1 ml sequanal-grade toluene (Pierce) and were transferred to glass centrifuge tubes. The toluene solutions were vortexed with 0.5 ml of 1 *M* ammonium phosphate (pH 5.8) for 60 sec and centrifuged briefly (3—4 min) in a clinical centrifuge. The top (organic) phases were carefully transferred to autoinjector vials and injected (1 μl) into the capillary GC system.

Animal experiments

Male Sprague—Dawley rats (100 ± 10 g) were killed by stunning and decapitation. Brains were removed and the cerebellar, corpus striatal and hypothalamic regions were taken according to Holman et al. [14], weighed and frozen on dry ice. Tissues were transferred to 7-ml plastic centrifuge tubes which contained fixed amounts (usually 75 or 150 ng) of 3,4-dihydroxybenzylamine (DHBA), and when required, varying amounts of the CAs, TIQs and/or the phenolic amines. Homogenization was done in ice-cold 75% ethanol (5 ml/g of tissue) utilizing a 20-sec burst of a Techmar Tissuemizer. The homogenizing blade was washed with 1 ml of fresh cold 75% ethanol after each sample, the

washings were added to the homogenates, and the combined fractions were centrifuged at 30,000 *g* (4°C) for 20 min.

In a modification of the weak cation-exchange isolation procedure of Holman et al. [14], supernatants from centrifugations were diluted two-fold with distilled water and applied to small columns (2.5 × 0.6 cm) of BioRex-70 resin (200—400 mesh; Bio-Rad Labs.). The resin was prepared by repeated stirring in distilled water and aspiration of suspended particles, followed by stirring in 3 *M* hydrochloric acid for 1 h, washing continuously with water until pH 5 was achieved, stirring in 3 *M* sodium hydroxide for 1 h and washing again to pH 5. It was then suspended in 0.1 *M* disodium hydrogen phosphate — sodium dihydrogen phosphate (pH 6.5) containing 10% Na_2EDTA. The effluents from the applied supernatants followed by 2 ml phosphate buffer (pH 6.5) and 2 ml distilled water were collected, combined and lyophilized. Assay by HPLC established that the amino acids and carboxylic acid metabolites were contained in these combined fractions. The columns were then treated with 2.5 ml of 1.0 *M* hydrochloric acid to elute amines and the effluent was frozen and lyophilized. The residues were treated as described under *Preparation of fluoracyl derivatives.*

RESULTS

HFB versus other fluoracylated derivatives

Compared to their TFA derivatives, the HFB derivatives of amines and TIQs consistently gave greater ECD responses, more symmetrical peaks and better capillary separations of geometric isomers. The responses and peak symmetries for the PFP derivatives were equivalent to the HFB derivatives, but acceptable separations of the (PFP) O-methylated TIQs or of 3M-DA and 4M-DA were not always obtained. Satisfactory separations of the mono-O-methylated 1- or 3-carboxylated TIQs were achieved, however, when the carboxylated isomers were derivatized with HFIP—PFPA combinations (retention times are listed in Table I).

Effect of ammonium phosphate wash on capillary GC of HFB-derivatized amines

The capillary GC chromatograms of the HFB-derivatized TIQs or open-chain amines, prepared from standards in aqueous solutions or tissue extracts (but not from crystalline standards), showed unacceptably large solvent fronts. A 1-min extraction or wash of the toluene solution of HFB derivatives with 0.5 vol. of 1 *M* ammonium phosphate solution, pH 5.7—5.8, just prior to injection effectively reduced this solvent response (Fig. 2). This step was found necessary for capillary measurements of tissue NE, DA and SAL, or for any assay with DHBA as an internal standard. When HFB-derivatized amines in toluene were washed repeatedly (up to five times) with fresh ammonium phosphate aliquots and immediately injected, peak areas and retention times were not significantly affected. However, extended storage of the washed toluene solutions of the HFB derivatives in contact with the ammonium phosphate solution (pH 5.8) apparently resulted in complete loss of the derivatives. Additionally, when a saturated sodium borate solution or ammonium phosphate of

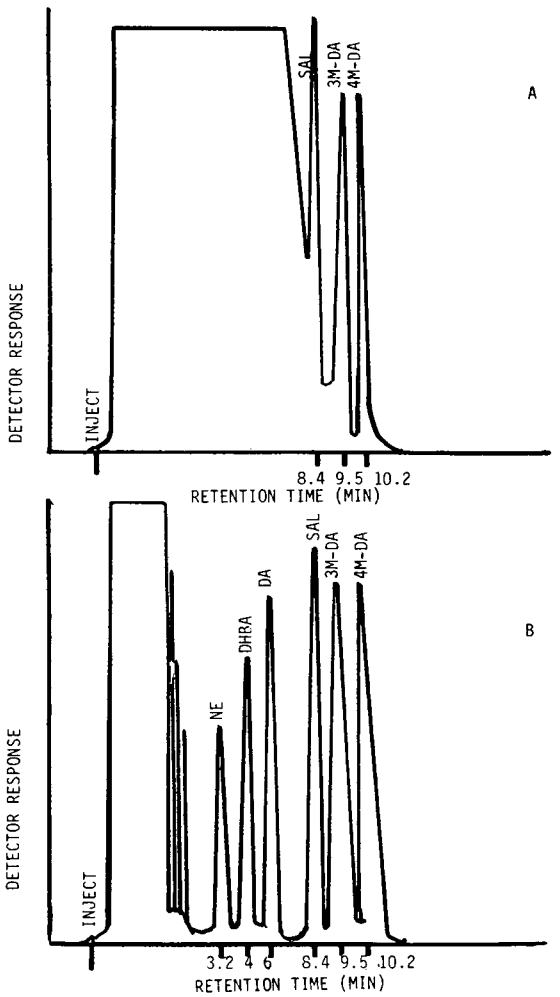


Fig. 2. The effect on the capillary GC—ECD chromatogram of a single ammonium phosphate buffer (pH 5.8) wash of a toluene solution containing a freshly derivatized (HFBA) mixture of NE, DHBA, DA, SAL, 3M-DA and 4M-DA. Chromatograms: (A) before wash; (B) immediately following wash. Procedure and chromatographic conditions are described in the text. Column temperature = 130°C.

pH 6 were employed as washes, recoveries of derivatized CAs or catechol TIQs were reduced to negligible values.

Capillary versus conventional GC separation of standard mixtures of CAs, TIQs and O-methyl derivatives

A comparison of the capillary GC versus conventional (packed) column GC separation of an HFB-derivatized mixture of DHBA, DA, SAL, 3M-DA, 4M-DA, 6M-SAL, and 7M-SAL is shown in Fig. 3. Complete separations of 3M-DA and 4M-DA and the two SAL isomers were readily obtained on the glass capillary column. As shown, a conventional-packed (1.83 m glass, 3% OV-17) column failed to separate the O-methylated DA TIQ isomers. Furthermore,

other packings referred to in Materials and methods in conventional columns were found unsuitable for the separation of the mono-O-methylated SAL isomers.

The 10-m glass capillary columns also were effective in separating mixtures (11—50 pg) of the three principal CAs (epinephrine, NE and DA) concurrent with their three main O-methylated amine metabolites in brain, normetanephrine, metanephrine and 3-O-methyl DA (chromatogram not shown). However, such low amounts of these catecholic and phenolic amines could not be chromatographed on longer (30—50 m) glass capillary columns of the same type and diameter. Fused-silica columns were not examined in this study.

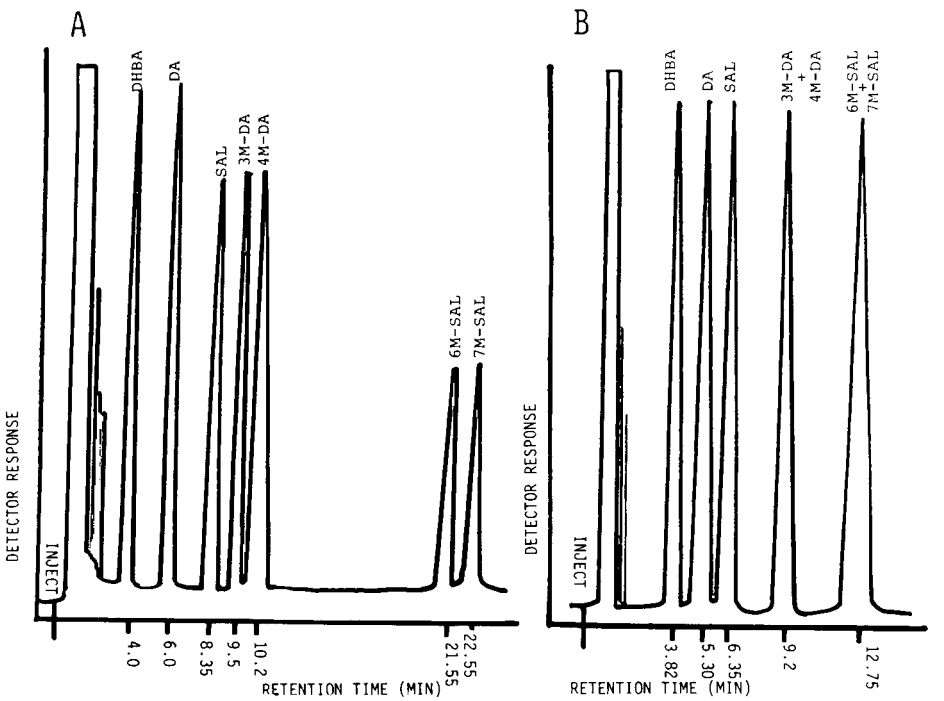


Fig. 3. GC comparison of an HFB-derivatized mixture of DHBA, DA, SAL, 3M-DA, 4M-DA, 6M-SAL and 7M-SAL chromatographed on (A) a 10-m WCOT OV-17 glass capillary column (130°C), versus (B) a conventional 1.83 m × 6.35 mm glass column packed with 3% OV-101 on Porapak Q (160°C).

Summary of capillary GC characteristics and recoveries for DA, representative isoquinolines, and their O-methyl derivatives

Table I is a summary of the relative retention times and minimum detectable quantities for DA, SAL, 4-HO-DSAL, SAL-1-CA, SAL-3-CA, 1,2-dehydro-SAL and their respective isomeric mono-O-methyl analogues following derivatization with either HFBA or PFPA—HFIP and capillary GC—ECD analysis at the indicated column temperatures. The overall recoveries (means of three to four samples) for these catecholic or phenolic compounds following addition to rat brain (cerebellar) tissue, aqueous ethanol extraction, isolation on BioRex-70, appropriate derivatization and capillary analysis are also shown. With this com-

TABLE I

CONDITIONS AND RECOVERIES OF STANDARDS FROM BRAIN TISSUE IN THE CAPILLARY GC ASSAY OF DAs AND TIQs

Chromatographic conditions: 10 m × 0.25 mm OV-17 glass WCOT column, injector temperature, 250°C, detector temperature, 350°C, attenuation range = 10^{-11} a.u.f.s.

Compound	Derivative/ column temperature (°C)	Retention time (min)	Minimum detectable quantity (ng/ml)	Recovery from tissue* (%)
Dopamine (DA)	HFB/130	6.00	0.25	89
3M-DA	HFB/130	9.50	0.45	84
4M-DA	HFB/130	10.20	0.45	85
Salsolinol	HFB/130	8.40	0.25	88
6M-SAL	HFB/130	21.55	0.45	82
7M-SAL	HFB/130	22.55	0.45	82
4-HO-DSAL	HFB/130	12.05	2.00	85
6M-4-HO-DSAL	HFB/130	17.64	5.00	82
7M-4-HO-DSAL	HFB/130	18.80	7.00	82
SAL-1-CA	PFP—HFIP/125	6.45	1.00	83
6M-SAL-1-CA	PFP—HFIP/125	16.20	5.00	80
7M-SAL-1-CA	PFP—HFIP/125	18.16	5.00	80
SAL-3-CA	PFP—HFIP/155	5.40	1.00	85
6M-SAL-3-CA	PFP—HFIP/155	16.70	5.00	82
7M-SAL-3-CA	PFP—HFIP/155	19.25	5.00	82
1,2-Dehydro-SAL	HFB/145	4.55	1.00	79
6M-1,2-Dehydro-SAL	HFB/145	7.10	7.00	82
7M-1,2-Dehydro-SAL	HFB/145	8.50	2.00	84

*Mean of three to four samples of rat cerebellar tissue (100 mg per sample) containing added amines. Standard deviations varied between 3.5% and 7.5%.

plete procedure for the compounds investigated, recoveries varied from 79% to 89% with a standard deviation of less than 8%.

The cation-exchange (BioRex-70) isolation step was found necessary in this overall capillary GC procedure. Attempts to analyze the lyophilized ethanol extracts of TIQ-spiked tissues (after HFBA treatment and the buffer wash) gave unacceptably long (25 min) capillary solvent and reagent peaks. In using the BioRex columns with mixtures of DHBA, SAL, SAL-1-CA and their respective mono-O-methylated derivatives added to cerebellar tissue, 95—97% of the carboxylated TIQs (as determined by HPLC of the eluate on reversed-phase columns) were recovered in the primary elution, the 2 ml phosphate buffer and the 2 ml distilled water, with no detectable elution of CAs, non-carboxylated TIQs or DHBA. The amines (DHBA, SAL and O-methylated SAL isomers) then were eluted completely from the ion-exchange resin by 1 *M* hydrochloric acid, with no evidence of the presence of carboxylated TIQs.

The isolation of amine mixtures was also examined with the relatively strong cation-exchange resin, Dowex-AG50-WX. Removal of bound isoquinolines from columns (2.5 × 0.6 cm) of this resin required relatively stringent acid conditions (4 *M* hydrochloric acid—methanol, 1:1, v/v) and after lyophilization, only 20—40% of the amines was recovered, according to HPLC or capillary GC analysis.

Verification of the precision and linearity of the capillary GC method using SAL and DHBA

The precision of the capillary GC measurement was demonstrated with nine successive autosample injections of a mixture of the two (HFB-derivatized) catechol compounds, SAL (85 pg/ml) and internal standard, DHBA. The retention time and peak area for SAL varied by only 0.07% and 3.7%, respectively, and the mean area ratio (± S.D.) for SAL/DHBA was 0.891 ± 0.01, with a 1.2% relative deviation.

Linearity with standards was shown in experiments in which the area ratios for SAL/DHBA increased linearly from 4 to 83 ng/ml SAL in relation to a fixed (100 ng/ml) concentration of DHBA (internal standard). The correlation for the relationship was 0.9997. Similarly, the linearity of the overall procedure was confirmed in experiments in which SAL (from 10 to 100 ng/ml) and DHBA (100 ng/ml) were added to rat cerebellar tissue and carried through the extraction, derivatization, wash and analysis.

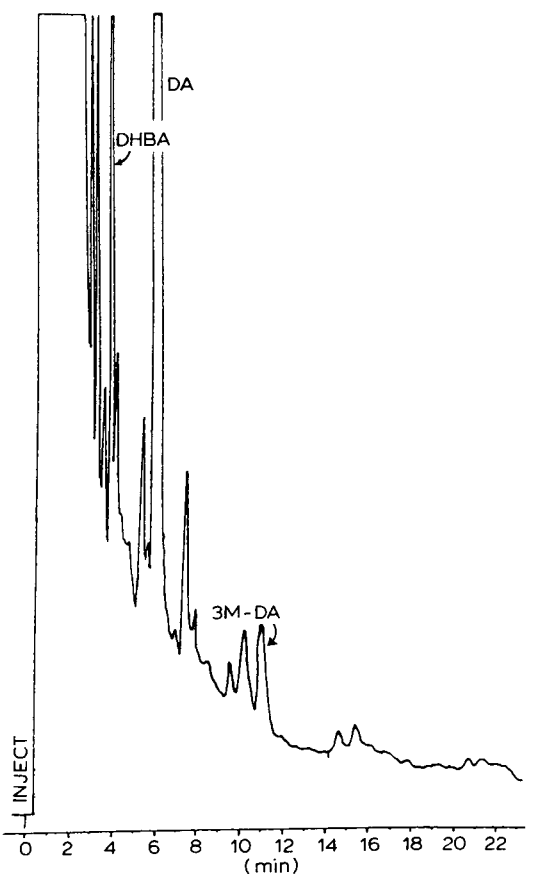


Fig. 4. Capillary GC—ECD chromatogram of HFB-derivatized amines from rat corpus striatum. Areas and retention times of DHBA and DA were obtained by computing integrator. Chromatographic conditions are described in text. Column temperature = 130°C. Attenuation setting of $2 \cdot 10^{-11}$ a.u.f.s. was used to demonstrate the absence of 4M-DA and the presence of 3M-DA, but it resulted in apparent solvent tailing and off-scale deflections of DA and DHBA.

Assay of endogenous dopamine in the rat corpus striatum and hypothalamus by short glass capillary GC—ECD

A representative chromatogram of rat corpus striatum extract (95 mg tissue) is shown in Fig. 4. Control chromatograms showed no interfering substances. The concentration of DA was quantitated with the use of the computing integrator. The striatal and hypothalamic means of twelve rats are shown in Table II, and are compared to reported DA values in the literature.

TABLE II

COMPARISON OF CAPILLARY GC—ECD ASSAY* OF DOPAMINE IN RAT STRIATUM AND HYPOTHALAMUS WITH VALUES IN THE LITERATURE OBTAINED BY HPLC AND FLUORIMETRY

Brain region	Dopamine (μg/g ± S.E.M.)	Method	Reference
Corpus striatum	9.50 ± 0.78 (n=12)	Capillary GC—ECD	This report
	8.80 ± 0.82	HPLC	[29]
	8.78 ± 0.89	Fluorimetry	[14]
Hypothalamus	1.90 ± 0.31	Capillary GC—ECD	This report
	2.10 ± 0.16	Fluorimetry	[14]

*Conditions were as described in the text and in Table I for dopamine.

DISCUSSION

To the best of our knowledge, the use of capillary columns has not been reported previously in GC analyses of endogenous CAs, either alone or simultaneously with their O-methylated metabolites. The TIQ alkaloids also had not been examined by capillary GC prior to this study. However, Martin et al. [15] provided a thorough study of the GC behavior of CAs and their O-methylated derivatives on 30-m fused-silica capillary columns using negative-ion detection, and subsequently determined the concentration of normetanephrine in human cerobrospinal fluid. Several other capillary methods have been reported for selected CA metabolites. LeGatt et al. [16] used capillary GC—ECD for the measurement of rat brain normetanephrine and 3-O-methyl DA. No attempts were made to demonstrate resolution of their potential 4-O-methylated isomers. With capillary GC—mass spectrometry (MS), oxidized CA derivatives were assayed in the urine by Muskiet et al. [17] and in the spinal fluid (homovanillic acid) by Vogt et al. [18]. Recent analytical GC studies have used capillary columns with series of amines which included the catecholamine compound isoproterenol [19, 20]. Overall, however, application of high-resolution capillary procedures in studies of catecholic and phenolic amines has been limited.

Several thorough capillary GC studies are available with indoleamines and particularly their tetrahydro-β-carboline condensation products. Beck et al. [21] used 20-m glass capillary columns for MS studies on the occurrence of β-carbolines in tissues and dietary components. An earlier capillary report from the same laboratory focused on the indoleamine condensation products in urine and spinal fluid [22]. The MS of β-carbolines (following appropriate derivatization) was also investigated in detail on 30-m fused-silica capillary columns by Faull et al. [23]. Serotonin or its O-methylated and/or oxidized

metabolites have also been analyzed on glass capillary columns in GC—MS procedures [17, 24].

Concerning important steps in the methodology of this report, a substantial (and necessary) reduction in the solvent/reagent peak was brought about by the brief ammonium phosphate wash of freshly derivatized (fluoracylated) samples. As noted in Results, repeated washings of toluene solution of HFB-catecholamines with several (up to five were done) fresh ammonium phosphate aliquots, followed by immediate analysis, did not significantly change the peak areas. However, because of the susceptibility of fluoracylated derivatives to hydrolysis, the derivatized sample could not be stored for substantial lengths of time in contact with the aqueous wash. Other researchers using capillary GC—ECD have noted the necessity of a similar wash or clean-up step before injection. Davis et al. [25] washed PFP—HFIP-derivatized hydroxyphenylacetic acids with 1 *M* phosphate, pH 6 (cation not stated) prior to capillary GC—ECD analysis, and suggested that the wash removed excess and possibly hydrolyzed fluorinating agent which would otherwise saturate the electron-capture detector. Sodium borate buffer may have served a similar purpose in the *meta*-O-methyl CA procedure of LeGatt et al. [16] or in a serotonin assay by Calvery et al. [26]. For the derivatized CAs and catechol TIQs, saturated borate was unsuitable as a wash solution, perhaps because it promoted hydrolysis of the fluoracyl derivatives.

Another important consideration is our use of a weak cation-exchange resin such as BioRex-70 rather than a strong amine exchanger such as Dowex, or (for the catechol compounds) a binding material such as aluminum oxide. As indicated, the replacement of BioRex with Dowex required stronger acid eluents and resulted in substantially lower recoveries, probably because of compound degradation. The use of aluminum oxide in place of BioRex also gave lower recoveries of CAs, more contaminated chromatograms, and relatively large solvent fronts that could not be reduced effectively by the phosphate wash. Using the fluoracyl anhydrides as derivatizing agents, best results (maximal responses and essentially single derivatives) were obtained with HFBA, but the HFIP—PFPA combination pioneered by Watson et al. [27] for acid metabolites of CAs was also effective for carboxylated TIQs. Other fluoracylating agents such as fluoracyl imidazoles, found by Barker et al. [28] and Faull et al. [23] to be the best reagents for GC—MS studies with β-carboline condensation products, were not investigated in this study with CAs and TIQs. However, in previous GC—ECD work using conventional columns [3], fluoracyl imidazoles tended to produce multiple derivatives of the TIQs.

Contrary to results of other capillary applications with splitter injector modes, we found that the capillary system with an automatic injector provided a high degree of precision and reproducibility with derivatized mixtures of CAs or TIQs. Equally significant is that the autoinjector in combination with the capillary column permitted very accurate retention time values (0.1% deviation). As an indication of the method's practical applicability, the assay of rat striatal and hypothalamic DA gave values that compared reasonably well with results in the literature from HPLC and fluorimetry (Table II). Due to the extreme sensitivity and good linear range of new models of electron-capture detectors, not only DA but epinephrine or norepinephrine can be readily mea-

sured in the small brain tissue samples that are obtained, for example, by the punch technique.

The capillary GC—ECD procedure described could serve as an important adjunct to reversed-phase HPLC assay, which is now probably the most common means of CA and TIQ estimation. In many situations, when MS is not available or is cost-prohibitive, retention times and quantities of endogenous catecholic and phenolic amines could be confirmed rapidly by this highly specific and yet inexpensive technique. Notwithstanding its complementary role, we suggest that capillary GC would be of great value in determinations of those geometric isomers of mono-O-methylated CAs or TIQs which are not separated by conventional GC (as shown) or cation-exchange HPLC.

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CARBOHYDRATES OF WHOLE DEFATTED CELLS AS A BASIS FOR DIFFERENTIATION BETWEEN *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* AND *HAEMOPHILUS APHROPHILUS*

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SUMMARY

Using gas chromatography and gas chromatography—mass spectrometry, the closely related bacteria *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* were distinguished by means of the sugar content in whole defatted cells. Both species contained rhamnose, fucose, galactose, glucose, galactosamine, glucosamine, and L-glycero-D-manno-heptose. Contrary to *H. aphrophilus*, whole cells of *A. actinomycetemcomitans* contained D-glycero-D-mannoheptose. This sugar may thus serve as a marker for taxonomic differentiation between *A. actinomycetemcomitans* and *H. aphrophilus*.

INTRODUCTION

In Gram-negative bacteria polysaccharides are found as integral parts of the cell wall, in the extracellular capsule, or in both these structures. The cell wall polysaccharides of Gram-negative bacteria are bound to lipid, constituting the somatic lipopolysaccharide (LPS), which represents the endotoxic principle of these organisms. Polysaccharides residing on the bacterial cell surface are involved in phenomena such as cell—cell recognition, binding of bacteriophages, and antigenic expression. Since most microbial polysaccharides cannot be digested by mammalian enzymes, they remain in circulation for a considerable time [1], acting as potent antigens. The fine structure of surface polysaccharides in bacteria may therefore serve as a basis for their serological classification.

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TABLE I

PERCENTAGE CELLULAR SUGAR* COMPOSITION OF METHANOLYSED WHOLE DEFATTED CELLS (S.D. 5%)

Bacteria**	Rhamnose	Fucose	Galactose	Glucose	DD-Heptose	LD-Heptose	Galactosamine + glucosamine
Actinobacillus actinomycetemcomitans							
ATCC 33384 (NCTC 9710***)	5.0	5.7	4.0	10.9	4.1	5.3	2.6
ATCC 29523	6.1	9.3	5.6	13.9	5.9	8.1	4.6
ATCC 29522	11.5	8.8	3.5	10.2	3.6	6.2	6.2
FDC 2112	4.1	7.1	5.7	15.0	5.8	8.1	4.0
FDC 2097	10.9	16.1	7.2	17.9	4.5	6.0	6.6
FDC 2043	10.1	13.3	7.2	22.6	7.1	10.5	4.9
FDC 511	5.0	6.8	5.1	15.9	6.9	10.7	4.4
FDC N 27	4.9	9.3	10.0	10.3	4.4	7.2	3.9
Haemophilus aphrophilus							
ATCC 33389 (NCTC 5906***)	5.2	8.5	8.6	19.0		4.7	2.6
ATCC 19415 (NCTC 5886)	5.3	6.0	6.3	17.8		4.5	2.6

*DD-Heptose = D-glycero-D-mannoheptose; LD-Heptose = L-glycero-D-mannoheptose.
**ATCC, American Type Culture Collection, Rockville, MD, U.S.A.; NCTC, National Collection of Type Cultures, London, U.K.; FDC, Forsyth Dental Center, Boston, MA, U.S.A.
***Type strain of the species.

LPS has also been used as a chemical basis for such classification [2]. This fact prompted us to use carbohydrates of LPS as tools for taxonomic differentiation between the closely related Gram-negative, facultative rods *Actinobacillus actinomycetemcomitans* and *Haemophilus aphrophilus* [3]. Since preparation of LPS is a rather time-consuming procedure, more convenient diagnostic procedures need to be established for the routine laboratory. In the present study, the sugar content of whole cells of *A. actinomycetemcomitans* and *H. aphrophilus* has been used to distinguish between these bacteria.

MATERIAL AND METHODS

Bacteria

The strains of *A. actinomycetemcomitans* and *H. aphrophilus* examined, the sources from which they were obtained, and the procedures for maintenance and cultivation have been described elsewhere [4, 5].

Methanolysis and derivatization

After removal of the free fatty acids with hexane [4, 5], whole lyophilized cells were methanolyzed (2 *M* hydrochloric acid in anhydrous methanol, 24 h, 85°C) [6]. After separating with chloroform (Fluka, Buchs, Switzerland), the organic phase of the methanolysate, which contained the bound fatty acids, from the water phase, the latter was lyophilized and derivatized with acetonitrile (Rathburn, U.K.) and trifluoroacetic anhydride (Fluka) (1:1) [3].

Reference compounds

Sigma (St. Louis, MO, U.S.A.) provided α-D(+)-fucose, D(+)-galactose, α-D(+)-glucose, D(+)-galactosamine, D(+)-glucosamine, D(+)-mannose, and α-L-rhamnose. Natural galactose, glucosamine, L-glycero-D-mannoheptose, mannose, and rhamnose were identified from LPS (Sigma) of *Escherichia coli* [7] and *Salmonella typhimurium* [8]. D-Glycero-D-mannoheptose was determined from *Chromobacterium violaceum* [9]. *Ch. violaceum* and N-glucosaminemyristate were generously provided by Drs. O. Lüderitz and U. Meier, Max-Planck-Institut für Immunbiologie, Freiburg, F.R.G.

Gas chromatography

A Sigma 3 gas chromatograph (Perkin-Elmer, Norwalk, CT, U.S.A.) furnished with an electronic integrator (Sigma 10) was used. The Chrompack (Middelburg, The Netherlands) CP-Sil 5 CB (polydimethylsiloxane) glass capillary column used was 25 m × 0.22 mm I.D. with a film thickness of 0.14 μm and height equivalent of a theoretical plate 0.25 mm. Helium served as carrier gas at a flow-rate of 2 ml/min. The pressure at the inlet of the column was 151.5 kPa. The temperature of the injector and flame-ionization detector was 200°C. Programme: hold 2 min at 90°C, then 90°C to 260°C at 9°C/min with the attenuator of the gas chromatograph set at 8, and the attenuator of the Sigma data 10 system at −1. The paper speed was 10 mm/min. Splitless injection was used. The identity of the methanolysed and derivatized sugars was established by direct cochromatography and by gas chromatography—mass

spectrometry (GC—MS). The sugars were identified tentatively by comparing their retention times with those of authentic standards.

Gas chromatography—mass spectrometry

GC—MS was performed as described previously [3].

RESULTS

The distribution of sugars in whole defatted cells of *A. actinomycetemcomitans* and *H. aphrophilus* is shown in Table I. Both species contained rhamnose, fucose, galactose, glucose, L-glycero-D-mannoheptose, galactosamine, and glucosamine. The most striking feature of the present results was that D-glycero-D-mannoheptose was present in all the strains of *A. actinomycetemcomitans* examined, but in none of the *H. aphrophilus* strains. Ratios between essential sugar markers such as D-glycero- and L-glycero-D-mannoheptose and between glucose and L-glycero-D-mannoheptose are compared in Table II with those of LPS from *A. actinomycetemcomitans* and *H. aphrophilus*. The ratio between D-glycero- and L-glycero-D-mannoheptose was rather constant and similar in whole cells and LPS from *A. actinomycetemcomitans*. The glucose/L-

TABLE II

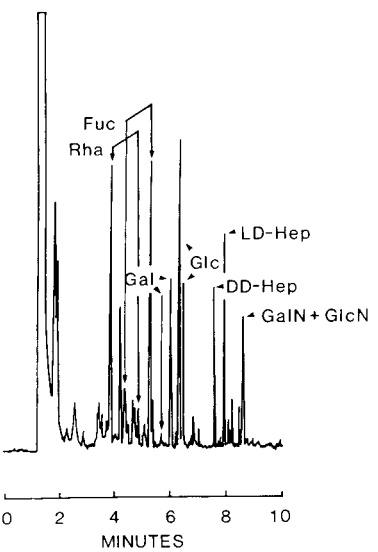
RATIOS BETWEEN SELECTED SUGARS* IN WHOLE DEFATTED CELLS AND LIPOPOLYSACCHARIDE FROM BACTERIA

	Whole defatted cells		Lipopolysaccharide**	
	DD-/LD-Hep	Glc/LD-Hep	DD-/LD-Hep	Glc/LD-Hep
Actinobacillus actinomycetemcomitans				
33384	0.8	2.1	0.8	2.1
29524			0.7	1.9
29523	0.7	1.7		
29522	0.6	1.7	0.6	1.3
2112	0.7	1.9		
2097	0.8	3.0		
2043	0.7	2.2		
511	0.6	1.5	0.6	1.1
HK 435			0.8	1.4
N 27	0.6	1.4	0.6	1.6
Haemophilus aphrophilus				
33389		4.0		1.8
19415		4.0		1.8
655				1.2
654				1.1
626				1.4
621				1.5

*Glc = glucose; DD-Hep = D-glycero-D-mannoheptose; LD-Hep = L-glycero-D-mannoheptose.
**Data from ref. 3.

glycero-D-mannoheptose ratio varied somewhat between the strains of *A. actinomycetemcomitans* but was rather constant within each strain when whole cells and LPS were compared. In whole cells of the *H. aphrophilus* strains examined the glucose/L-glycero-D-mannoheptose ratio was approximately twice as high as in LPS.

Typical gas chromatograms of the sugars in whole defatted cells of the *A. actinomycetemcomitans* and *H. aphrophilus* strains are presented in Figs. 1 and 2.



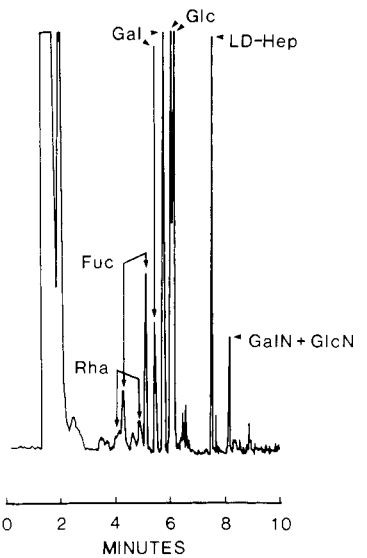


Fig. 1. Typical gas chromatogram of the sugar composition of whole defatted cells of *A. actinomycetemcomitans*, as represented by strain ATCC 33384. Abbreviations: Rha, rhamnose; Fuc, fucose; Gal, galactose; Glc, glucose; DD-Hep, D-glycero-D-mannoheptose; LD-Hep, L-glycero-D-mannoheptose; GalN, galactosamine; GlcN, glucosamine.

Fig. 2. Typical gas chromatogram of the sugar composition of whole defatted cells of *H. aphrophilus*, as represented by strain ATCC 33389. Abbreviations: Rha, rhamnose; Fuc, fucose; Gal, galactose; Glc, glucose; LD-Hep, L-glycero-D-mannoheptose; GalN, galactosamine; GlcN, glucosamine.

The molar response factors of trifluoroacetylated derivatives of the detected sugars were as described previously [3].

The mass spectra of D-glycero- and L-glycero-D-mannoheptose were also consistent with our previous description [3].

DISCUSSION

In the present study, the sugar composition of whole cells was used to differentiate between the closely related bacteria *A. actinomycetemcomitans* and *H. aphrophilus*. Whole cells yielded more complicated gas chromatograms than purified cell wall preparations such as LPS [3]. Trifluoroacetyl derivatized

whole-cell methanolysates from *Neisseriae* and *Moraxellae* also showed complex gas–liquid chromatographic (GLC) profiles with peaks of similar retention in different "finger prints" which were not easily distinguished and identified [10]. Therefore, selective extractions and various other identification techniques had to be performed. For easier interpretation of the present gas chromatograms, defatted cells were analysed, i.e. cells pre-extracted for free and bound fatty acids [4–6].

The most outstanding feature of the present study was that D-glycero-D-mannoheptose could be demonstrated in whole cells of *A. actinomycetemcomitans* but not of *H. aphrophilus*. A similar difference was observed in phenol-extracted LPS from a series of strains, including type strains, other reference strains and laboratory strains of *A. actinomycetemcomitans* and *H. aphrophilus* [3]. Seen together, these observations strongly suggested that D-glycero-D-mannoheptose can serve as a marker for taxonomic differentiation between *A. actinomycetemcomitans* and *H. aphrophilus*. They also supported our previous differentiation between *A. actinomycetemcomitans* and *H. aphrophilus* based on free cellular fatty acids [5], as well as the establishment of *A. actinomycetemcomitans* as a separate species distinct from *H. aphrophilus* in a recent edition of Bergey's Manual of Systematic Bacteriology [11]. The present method of gas chromatography of derivatized methanolysates from whole defatted cells may represent a valuable step towards more accurate identification and classification of *A. actinomycetemcomitans* and *H. aphrophilus*, complementing the few biochemical tests recommended for distinction between these bacteria [12].

The ratio between the percentage of D-glycero- and L-glycero-D-mannoheptose was rather constant and similar to that detected in whole LPS from *A. actinomycetemcomitans*. This suggested that LPS is the principal source of these aldoheptoses in *A. actinomycetemcomitans*. Some bacteria, e.g. *Azotobacter indicum*, have D-glycero-D-mannoheptose in their capsule [13]. Also the glucose/L-glycero-D-mannoheptose ratio was rather constant and similar in whole cells and LPS from *A. actinomycetemcomitans*. This may indicate no other major reservoirs for glucose in *A. actinomycetemcomitans* than LPS. The examined *H. aphrophilus* strains revealed an increase in the glucose/L-glycero-D-mannoheptose ratio in whole cells compared to LPS. This suggested other sources for glucose in these bacteria than LPS. One likely source is their surface exopolymers, i.e. microcapsule, fibrils or slime layer [14], which may be eliminated during purification of LPS with ultracentrifugation [15]. The fact that glucose is a common capsular sugar in bacteria [16, 17] supported this idea.

King and Tatum [18] and Zambon et al. [19] were able to divide *A. actinomycetemcomitans* into serogroups which shared a common antigen with *H. aphrophilus*. The present study on whole defatted cells did not provide any clear chemical basis for creation of groups of strains corresponding to the serogroups suggested in *A. actinomycetemcomitans*. There are many suggestions in the literature (e.g. ref. 20) that exopolysaccharides are highly branched structures, and if so, identical chemotypes may occur within different serogroups. Alternatively, even if the carbohydrate structure is constant, alterations

due to acyl constituents, which are prevalent in exopolysaccharides, are frequently noted [17].

Depolymerization of polysaccharides through methanolysis is preferable to aqueous acid [21]. Methanolysis was used already in 1966 by Ishizuka et al. [22], and has later been modified by a number of authors (for review, see ref. 23). It does not cause significant destruction of sugars [24]. Most glycosidic linkages are quantitatively broken [25] and the reduced monosaccharides stabilized as methyl glycosides during this procedure [10]. After Vilkas et al. [26] reported GLC separation of a number of carbohydrates in 1966, including methyl glycosides of their trifluoroacetates, Tamura and co-workers [27, 28] carried out comprehensive investigations of the conditions necessary for preparation and chromatography of trifluoroacetyl derivatives of glucose, galactose, mannose, glucosaminitol, galactosaminitol, and mannitol, and subsequently adopted GLC of trifluoroacetates of alditols [29] as their standard method for estimation of mono- and disaccharides in biological fluid [30]. The advantage of trifluoroacetates is that they are more volatile than either acetates or trimethylsilyl ethers and therefore chromatographic separation may be carried out more rapidly and at lower temperatures [31, 32]. Trifluoroacetates have thus proved valuable additions to the range of carbohydrate derivatives suitable for GLC, and capillary columns permit the analysis of submicrogram quantities (for review, see ref. 33). It has also been reported that methyl glycosides are stabilised under trifluoroacetylosis and are quantitatively recovered after O-detrifluoroacetylation [34]. Methanolysis can in principle yield four different glycosides for each sugar, i.e. α- and β-anomers of both the methyl pyranosides and the methyl furanosides [32]. The relative portion of the different isomers for a given sugar is a function of the methanolysis conditions and usually is not altered by subsequent derivatization of the methyl glycosides. Quantitation of the sugars in the present chromatograms was usually based on multiple peaks, but can also be made from selected peaks because the ratio between the peaks was stable under the experimental conditions used, which was consistent with the findings of other workers in this field [24, 25, 32]. In some cases the proportion of an isomer of a sugar may be so low that it is negligible. Often, however, two or more isomers may not be resolved under a particular set of chromatographic conditions and yield only a single peak [32]. Thus, galactose yielded only two peaks when analysed as trifluoroacetylated methyl glycoside [32], whereas three peaks were observed when galactose was chromatographed as trimethylsilylated methyl glycoside [35].

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DETERMINATION OF LEUKOTRIENES AND PROSTAGLANDINS IN [^{14}C] ARACHIDONIC ACID LABELLED HUMAN LUNG TISSUE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND RADIOIMMUNOASSAY

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SUMMARY

A liquid chromatographic method for the determination of ^{14}C-labelled prostaglandins, leukotrienes and other lipoxygenase products formed by human lung tissue is described. In this paper we report our problems identifying these substances when ^{3}H- or ^{14}C-labelled compounds are compared with measurements of the mass by absorption or radioimmunoassay. Furthermore, some preliminary results of [^{14}C]arachidonic acid labelled human lung tissue, stimulated by the Ca-ionophore A23187, show that, of the lipoxygenase products, mostly leukotriene B_4 like compounds are formed and less leukotriene C_4, E_4 and D_4. Relatively large amounts of hydroxyeicosatetraenoic acids are present. The main cyclooxygenase products are thromboxane B_2, 6-ketoprostaglandin $F_{1\alpha}$ and prostaglandin D_2.

INTRODUCTION

The biologically active leukotrienes (LTs) are formed from arachidonic acid (AA). Several of these substances induce slow contractions in smooth muscles. The effects of the LTs on peripheral and central airway functions of pulmonary tissue have also been described [1]. Furthermore, it has been reported that the addition of leukotriene C_4 (LTC_4) and D_4 (LTD_4) induces thromboxane A_2 (TxA_2) release from guinea pig isolated perfused lungs [2, 3]. Both the contraction of lung parenchymal strips and the TxA_2 release could be inhibited by the β-adrenoceptor agonist isoprenaline [4]. In previous work, a comparison was made between the contractile activities and TxA_2 release of human, porcine and guinea pig lung parenchymal strips after the application of LTC_4 and LTD_4 [5]. The results indicate that both the contractile activity of LTs on human lung strips and the TxA_2 release were rather low in com-

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parison with the guinea pig lung strip. In cultured endothelial cells from human umbilical vein, however, LTC_4 promotes prostacyclin synthesis [6]. Recently, it has become evident that human alveolar macrophages produce leukotriene B_4 (LTB_4) [7], and it was also shown that human peritoneal macrophages synthesize LTB_4 and LTC_4 [8]. Further, Dahlén et al. [9] showed that allergen challenge of chopped human lung tissue elicits contraction that correlates with the release of both LTC_4, LTD_4 and leukotriene E_4 (LTE_4) and prostaglandins.

In this paper we describe an extraction procedure and high-performance liquid chromatographic (HPLC) techniques for the separation of cyclooxygenase and lipoxygenase products with comparatively high recoveries and discuss some of the problems concerned with the identification of these substances formed from [^{14}C]AA-labelled and Ca-ionophore-triggered chopped human lung tissue.

EXPERIMENTAL

Apparatus

A 1082B high-performance liquid chromatograph (Hewlett-Packard) was used, consisting of double-head pump, temperature-controlled column compartment, variable-volume injector and variable-wavelength detector. The Superrac fraction collector (LKB, Sweden) was connected to this apparatus and used as an automatic sampling system. Radioactivity in the labelled fractions was counted in a 3255 Tricarb liquid scintillation counter (Packard, Brussels, Belgium).

Chemicals

LTB_4, LTC_4 and LTD_4 were gifts of Dr. J. Rokach (Merck Frosst, Canada). Ca-ionophore A23187 was obtained from Hoechst (Calbiochem-Behring, U.S.A.), reduced glutathione from ICN (Cleveland, OH, U.S.A.) and prostaglandins D_2 (PGD_2), E_2 (PGE_2) and $F_{2\alpha}$ ($PGF_{2\alpha}$) from Sigma (U.S.A.). 6-Keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) and thromboxane B_2 (TxB_2) were gifts of Dr. J.B. Smith (Philadelphia, PA, U.S.A.). Siliclad® was obtained from Clay Adams (Becton Dickinson, U.S.A.). Tetrahydrofuran, methanol, acetonitrile, benzene and acetic acid were all of analytical grade from E. Merck (Darmstadt, F.R.G.). Picofluor-15 (Packard) was used as premixed scintillation cocktail.

Radiochemicals

[1-^{14}C]AA, 5-D-[5,6,8,9,11,12,14,15-^{3}H(n)]hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE), 12-L-[5,6,8,9,11,12,14,15-^{3}H(n)]HETE and 15-L-[5,6,8,9,11,12,14,15-^{3}H(n)]HETE were purchased from New England Nuclear. All other radiolabelled compounds mentioned below were obtained from the Radiochemical Centre (Amersham, U.K.): [5,6,8,9,11,12,14,15-^{3}H(n)]LTB_4, [14,15-^{3}H(n)]LTC_4, [14,15-^{3}H(n)]LTD_4, 6-keto-[5,8,9,11,12,14,15-^{3}H(n)]-$PGF_{1\alpha}$, [5,6,8,9,11,12,14,15-^{3}H(n)]TxB_2, [5,6,8,11,12,14,15-^{3}H(n)]PGE_2, [5,6,8,11,12,14,15-^{3}H(n)]$PGF_{2\alpha}$, [5,6,8,9,12,14,15-^{3}H(n)]PGD_2, [1-^{14}C]PGE_2 and [1-^{14}C]$PGF_{2\alpha}$. The purity of the radiochemicals was shown to be greater than 97%.

Materials

Sep-Pak C_{18} and Sep-Pak silica cartridges and HPLC-solvent filters HA (0.45 μm) and FH (0.5 μm) were obtained from Waters Assoc.; prepacked HPLC columns Nucleosil $5C_{18}$ and Zorbax $BPtmC_8$ (each 250 × 4.6 mm) were from Chrompack (Middelburg, The Netherlands).

Antisera

Anti-TxB_2, anti-$PGF_{2\alpha}$, anti-PGE_2 and anti-PGD_2 were obtained from l'Institut Pasteur (Paris, France); anti-6-keto-$PGF_{1\alpha}$ was obtained from Seragen (Boston, MA, U.S.A.). Cross-reactivities are given in Table I.

TABLE I

CROSS-REACTIVITIES OF COMMERCIALLY OBTAINED ANTIBODIES

Data are expressed in per cent at 50% B/B_0 (B = bound).

Compound	Antibody				
	6-Keto-$PGF_{1\alpha}$	TxB_2	$PGF_{2\alpha}$	PGE_2	PGD_2
6-Keto-$PGF_{1\alpha}$	100	—	0.04	<0.01	0.01
$PGF_{1\alpha}$	7.8	—	12.0	0.01	0.01
$PGF_{2\alpha}$	2.2	0.1	100	0.11	0.04
6-Keto-PGE_1	6.8	—	—	0.16	—
PGE_1	0.7	—	0.03	10.7	0.01
PGE_2	0.6	0.1	0.03	100	0.01
PGA_1	<0.01	—	<0.01	0.04	—
PGA_2	<0.01	0.1	<0.01	0.3	—
PGD_2	<0.01	0.2	3.0	<0.01	100
PGD_1	—	—	<0.4	<0.01	78
TxB_2	<0.01	100	<0.01	<0.01	1.2
13,14-Dihydro-15-keto-PGE_2	<0.01	0.1	—	0.6	0.01
13,14-Dihydro-15-keto-$PGF_{2\alpha}$	<0.01	0.1	<0.01	<0.01	0.01
13,14-Dihydro-PGE_2	—	—	—	2.1	—
6-Keto-PGE_2	—	0.1	<0.01	13.2	—

Human lung tissue

Human lung tissues were obtained from adults [5]. Lobectomy was performed on account of tumours. The premedication consisted of 0.25 mg of atropine and 10 mg of Opial®. During the operation, the following agents were administered: Fentanyl® (0.5 mg), pancuronium bromide (Pavulon®, 6 mg), thiopental (Pentotal®, 150 mg) and 3 g of cephalothin (Keflin®). Parenchymal tissue of the outer parts was used in our experiments.

Method

Human lung tissue (10 g) from which the lung membrane had been removed was cut into slices. The slices were chopped in a McIlwain tissue chopper and divided in portions of 0.5 × 0.5 mm. The whole fraction was washed three times with Krebs–Henseleit buffer (20 ml of buffer, 5 min, 400 *g*) in a polypropylene 50 ml tube (Falcon®). Then 20 ml of Krebs–Henseleit buffer were added and the tube was placed in a water-bath of 37°C on a magnetic stirrer (900 rpm). Through a thin pipette, the sample was continuously gassed with a

mixture of 95% O_2 + 5% CO_2. Thereafter, 10 μCi of [1-^{14}C]AA (55 mCi/mmol), glutathione (final concentration 2 m*M*) and 100 μg of Ca-ionophore A23187 (dissolved in 100 μl of ethanol) were added. At the end of the 10-min incubation, [^{3}H]LTs and [^{3}H]PGs were added and the homogenate was centrifuged (10 min, 1400 *g*, 4°C). The pellet was washed once, and the combined supernatant centrifuged (90 min, 30,000 *g* average, 4°C) to separate the cells and small particles. The clear incubation supernatant was then applied to a Sep-Pak C_{18} cartridge and the effluent was placed on a Sep-Pak silica cartridge. (The C_{18} cartridge was prewashed with 10 ml of methanol and 10 ml of distilled water; the silica cartridge was prewashed with 10 ml of methanol and 100 ml of water [10].) The sample was eluted with 2.5 ml of methanol on each column; these eluates were combined and evaporated to dryness with a gentle stream of nitrogen at 40°C. Thereafter, the dried sample was dissolved in 1 ml of solvent A (tetrahydrofuran—methanol—water—acetic acid, 25:30:45:0.1, v/v, adjusted to pH 5.5 with ammonium hydroxide), filtered and kept in a siliconized micro-vial.

Chromatographic system

Reversed-phase HPLC of LTs and other lipoxygenase products was carried out on a Nucleosil 5 C_{18} column, using solvent system A. Mobile phases were filtered by vacuum filtering through a Millipore filter and degassed with helium [11, 12]. The flow-rate was 0.9 ml/min and the absorption was measured at 280 nm. Prior to use, the system was washed with approx. 15 ml of water, thereafter with approx. 30 ml of a 2% (w/v) EDTA solution in water, and rewashed with water [10]. The column was equilibrated with the mobile phase A at an oven temperature of 37°C. Fractions were collected for scintillation counting. After each run (90 min) the column was rinsed for at least 30 min because of contamination with Ca-ionophore, which elutes after approx. 115 min.

Reversed-phase HPLC of PGs was performed on a Zorbax C_8 column. This solvent system (B) contained acetonitrile—benzene—water—acetic acid (24:0.2:0.1:76, v/v). The flow-rate of this eluent was 2.0 ml/min. From the contents of each collected fraction, 50 μl were taken and kept at 4°C for the radioimmunoassay (RIA) of the PGs. The main fraction was immediately used for ^{14}C and ^{3}H counting. The column was rinsed with acetonitrile for 30 min after each sample to elute the lipoxygenase products.

Radioimmunoassay of prostaglandins

Reagents were equilibrated to room temperature before use. After the addition of standards (range 0—500 pg) and diluted samples, [^{3}H]PG and antibody were added. At the end of the incubation (2 h at room temperature and 18 h at 4°C), charcoal suspension was added. The tubes were allowed to stand for 15 min at 4°C and centrifuged for 10 min at 1400 *g*. The supernatants were decanted and mixed with 6 ml of scintillation fluid. The risk of cross-reactions was negligible when RIA was performed after HPLC (cross-reactivities and specifications are given in Tables I and II).

TABLE II

SPECIFICATIONS OF VARIABLE CONDITIONS IN THE RIAs

Immunogen	Amount of added tracer		Bound/total (%)	Non-specific binding (%)
	dpm	pg		
6-Keto-$PGF_{1\alpha}$	16,000	18.4	39.7	9.2
TxB_2	15,000	18.5	57.9	1.9
$PGF_{2\alpha}$	8,500	9.2	17.0	4.5
PGE_2	12,500	13.0	40.1	3.9
PGD_2	8,500	10.7	34.3	7.4

Quantitative evaluation

The settings for double-labelled scintillation counting were such that there was no spillover of radioactivity of ^3H into the ^{14}C channel. Calculations of dpm were carried out using quenched standard sets by a computer (Digital, PDP 11/70). For daily analysis, a plotting system was programmed in order to obtain data of total counts covering the peak areas. Amounts calculated in dpm of both channels were plotted as separate chromatograms.

The data obtained from the RIAs were linearly plotted as the ln (mass) against the negative (−) ln $\{\%B/(100 - \%B)\}$ (where B=bound). The linearity was tested by means of a variance analysis.

RESULTS AND DISCUSSION

Fig. 1 shows a representative chromatogram (one of five experiments) of LTs and other lipoxygenase products, after [^{14}C]AA labelling of the lung tissue. The upper part of the figure represents the mass, measured by absorption at 280 nm. It is evident that substances are present with the same retention times (t_R) as LTs, indicated by C, D and B. The major compound is LTB_4, whereas LTC_4 is hardly detectable because of the strong tailing effect, caused by substances with identical chromatographical properties to some of the phospholipids, running on the front. Based on the retention time, peak 1 is tri-HETE, and peak 2 is 6-*trans*-LTB_4 + 12-*epi*,6-*trans*-LTB_4, according to Verhagen et al. [12].

The lower curve of the HPLC separation gives the plotted ^{14}C-labelled fractions. Peak 1 covers both the above-mentioned tri-HETE and a substance with the same t_R as LTC_4. Prostaglandins, however, nearly cochromatograph with LTC_4, so that a not unimportant part of peak 1 is due to the presence of cyclooxygenase products. The compound indicated by H is most probably HHT (12-OH-5,8,10-heptadecatrienoic acid). The identification of this peak was based upon the following observation: washed rat platelets were labelled with [^{14}C]AA as described before [13], aggregated with collagen, extracted as described above and applied to HPLC for further analysis. Our earlier observations on platelet aggregation indicate that the main compounds formed are, respectively, 12-HETE, HHT and TxB_2. A similar result was obtained by Luderer et al. [14]. As in our system, TxB_2 cochromatographs with LTC_4 and the retention time of 12-HETE appeared to be approx. 60 min; the peak at

26 min is likely to be HHT. In the example shown in Fig. 1, relatively large amounts of HETEs are formed.

Table III lists the recoveries (mean ± S.E.M.) of ^{3}H-labelled standards added to tissue samples. It is remarkable that the overall recoveries of HETEs are low, especially of 5-HETE. The chromatogram given at the bottom of Fig. 1

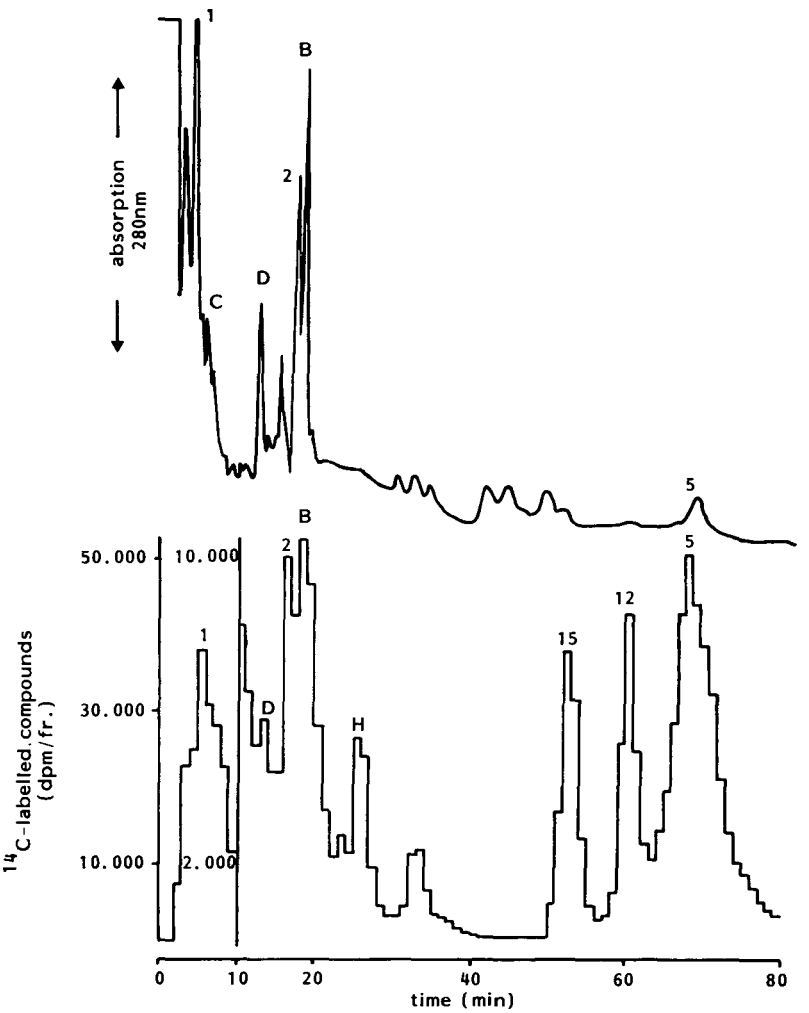

Fig. 1. Chromatograms of LTs and HETEs, synthesized by chopped human lung tissue, after [^{14}C]AA loading, in the presence of glutathione (2 m*M*) and Ca-ionophore A23187 (approx. 10 μM). The incubation medium was processed through Sep-Pak C_{18} and silica cartridges as described in Experimental and the methanol fractions after evaporation to dryness were subjected to HPLC in solvent system A. A Chrompack Nucleosil $5C_{18}$ column (250 × 4.6 mm) was used. Mobile phase: tetrahydrofuran—methanol—water—acetic acid (25:30:45:0.1) adjusted to pH 5.5 with ammonium hydroxide. Flow-rate 0.9 ml/min. The chromatogram at the bottom, representing radioactivity in fractions of one per min, is corrected for delay time between the absorption cell and the fraction collector. Peaks: C = LTC_4, D = LTD_4-like, B = LTB_4, 15 = 15-HETE, 12 = 12-HETE and 5 = 5-HETE. Based on retention times, 1 = tri-HETE, 2 = 6-*trans*-LTB_4 + 12-*epi*,6-*trans*-LTB_4.

TABLE III

RECOVERIES OF TRITIATED LTs, HETEs, AND PGs, MEASURED BY HPLC

Data were obtained after the extraction procedure as described in the methods section. Values are given as the mean ± S.E.M.

	Recovery (%)	*n*
LTC_4	59 ± 5.0	3
LTD_4-like	86 ± 1.5	3
LTB_4	70 ± 5.7	3
15-HETE	34 ± 1.1	3
12-HETE	34 ± 0.9	3
5-HETE	18 ± 0.6	3
6-Keto-$PGF_{1\alpha}$	64 ± 2.9	8
TxB_2	86 ± 2.7	8
PGF_2	44 ± 1.8	8
PGE_2	73 ± 2.9	8
PGD_2	59 ± 2.6	8

is not corrected for recoveries listed in Table III; in this case the plotted quantity of 5-HETE should be three times higher compared to the leukotrienes.

Furthermore, there is a pronounced difference in recovery of certain PGs obtained from HPLC separations as shown in Fig. 2. Fig. 2A shows the internal PG standards (^3H), fig. 2B the ^{14}C-labelled PGs formed from exogenous AA. The small peak 2 shows the immunoreactivity of 6-keto-$PGF_{1\alpha}$, and the wide peak 5 that of TxB_2. However, peak 6 (PGF-immunoreactive), peak 7 (PGE-immunoreactive) and peak 8 (PGD-immunoreactive) do not have the same t_R as the added ^3H standards. The longer the t_R, the greater the delay between ^3H-labelled and unlabelled material.

Determination of the amounts present in the sample by the different RIAs is represented in Fig. 2C. In this case, the immunoreactivities of the PGs mentioned before have the same t_R as the ^{14}C-labelled compounds. Others have noticed that a difference exists between the t_R of PGs labelled with ^3H or ^{14}C [15]. This might be due to the isotope effect. Four double bounds are labelled with ^3H whereas ^{14}C only is labelled on the 1-position. Increase of the mass plays an unimportant role, because spheric occlusion occurs at molecular weights of 2000 and higher (MW_{PG} approx. 350). It would appear that labelling with ^3H makes these substances more hydrophilic. Fig. 3 confirms the findings presented in Fig. 2. Commercially available ^3H- and ^{14}C-labelled PGE_2 and $PGF_{2\alpha}$ have a difference in retention times of approx. 5%. This could be a reason of errors in selecting the wrong fractions for RIA when ^3H-labelled standards are used as markers.

A second observation that needs further explanation is the difference that occurs in the specific activities of the PGs. This can be calculated from the data presented in Fig. 2. The pattern of ^{14}C-labelled and RIA-determined amounts is not identical. In this case, there is even a difference by a factor of 4 between the highest and the lowest specific activity (expressed as dpm/ng, covering the whole peak area). Peaks 3 and 4 in Fig. 2B are unknown. 13,14-Dihydro-15-keto-PGs run after 80 min, so that differences are not caused by these compounds.

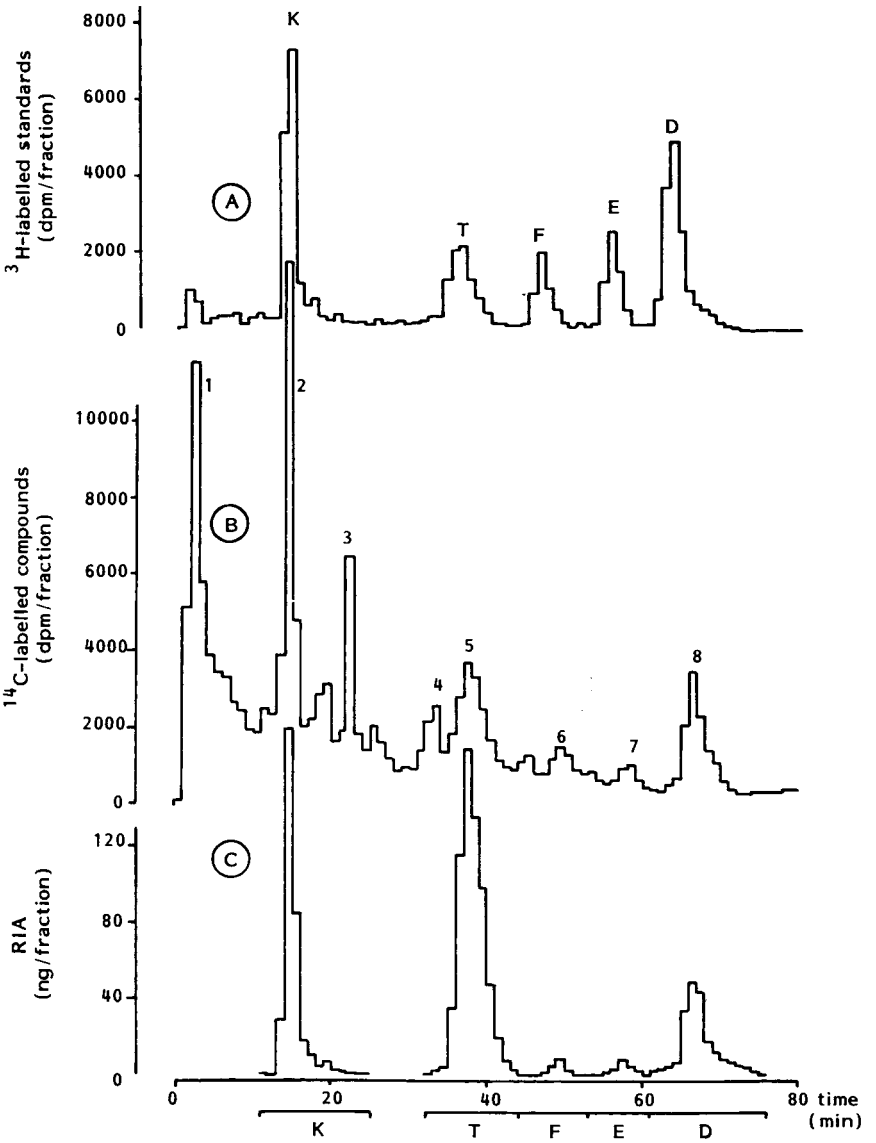

Fig. 2. Reversed-phase HPLC separation of PGs in the same sample as used in Fig. 1. A Chrompack Zorbax BP5 column was used; the solvent system contained acetonitrile—benzene—water—acetic acid (24:0.2:76:0.1); the flow-rate was 2 ml/min. One fraction per min was collected and divided as described in the text. (A) Chromatogram of [^{3}H]PG standards. Peaks: K = 6-keto-$PGF_{1\alpha}$, T = TxB_2, F = $PGF_{2\alpha}$, E = PGE_2 and D = PGD_2. (B) Chromatogram of ^{14}C-labelled compounds. Identification of peaks 1—8 is discussed in the results section. (C) Measurement of the fractions in (A) by RIA.

Fig. 4 shows the chromatograms of an experiment in which ^{3}H-labelled LTs were used as markers, without the addition of [^{14}C]AA to the tissue. Several problems arise when the LTs in the tissue are identified with the use of ^{3}H-labelled substances. This difficulty is due to the fact that in LTC_4 and LTD_4

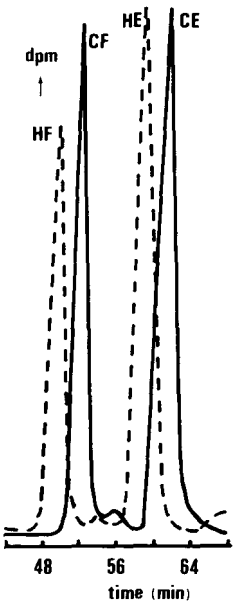

Fig. 3. Chromatogram of collected fractions (two per min) after the injection of ^{3}H- and ^{14}C-labelled $PGF_{2\alpha}$ and PGE_2. The chromatographic conditions were the same as described in Fig. 2. This chromatogram was plotted by means of a computer-programmed XY printer. Peaks: HF = [^{3}H]$PGF_{2\alpha}$, CF = [^{14}C]$PGF_{2\alpha}$, HE = [^{3}H]PGE_2, CE = [^{14}C]PGE_2.

one double bound is ^{3}H-labelled, and in LTB_4 this amount is four. The latter is comparable with the labelled PGs (see section on radiochemicals). In Fig. 4A, peaks 2 and 5 have approximately the same t_R as in Fig. 4B. The delay is only ± 2%. However, the difference is much greater between peak 6 of Fig. 4A and peak 8 of Fig. 4B. Batch 9 of the [^{3}H]LTB_4 used in the experiment as shown in Fig. 4A proved to be a racemic mixture of 6-*trans*-LTB_4 and 12-*epi*,6-*trans*-LTB_4. We thereafter injected both batch 9 and the newly prepared batch 10 directly into the HPLC system. The t_R of the first peak was the same as the one obtained in Fig. 4A after extraction procedures. The second peak (batch 10) had a delay of approx. 1 min. Compared to Fig. 4B, these two peaks were shifted 8% t_R. Peaks 3, 5 and 7+8 were collected in order to compare the biological activity of the so far unknown compound 5. The fractions were evaporated to dryness, dissolved in Krebs' buffer and added to a guinea pig lung parenchymal strip, as described before [5]. Expressed in factors of potency, the biological activities were respectively: peak 3 (LTC_4-like), 20; peak 5, 5; peak 7+8 (LTB_4-like), 1. The unknown peak 5 could be LTE_4 on the basis of its t_R [9, 16] and activity [9, 17, 18].

Finally, we determined the presence of glutamine and glycine in the hydrolysed fraction, compared to standards of LTC_4 (containing cysteine—glycine—glutamine) and LTD_4 (containing cysteine—glycine), following the method as described earlier [19]. In this fraction 5, a relatively small amount of glycine was present. We concluded from these data that this compound may be LTE_4*.

*During the preparation of the manuscript, synthetic LTE_4 was obtained. It shows the same t_R as the compound described here.

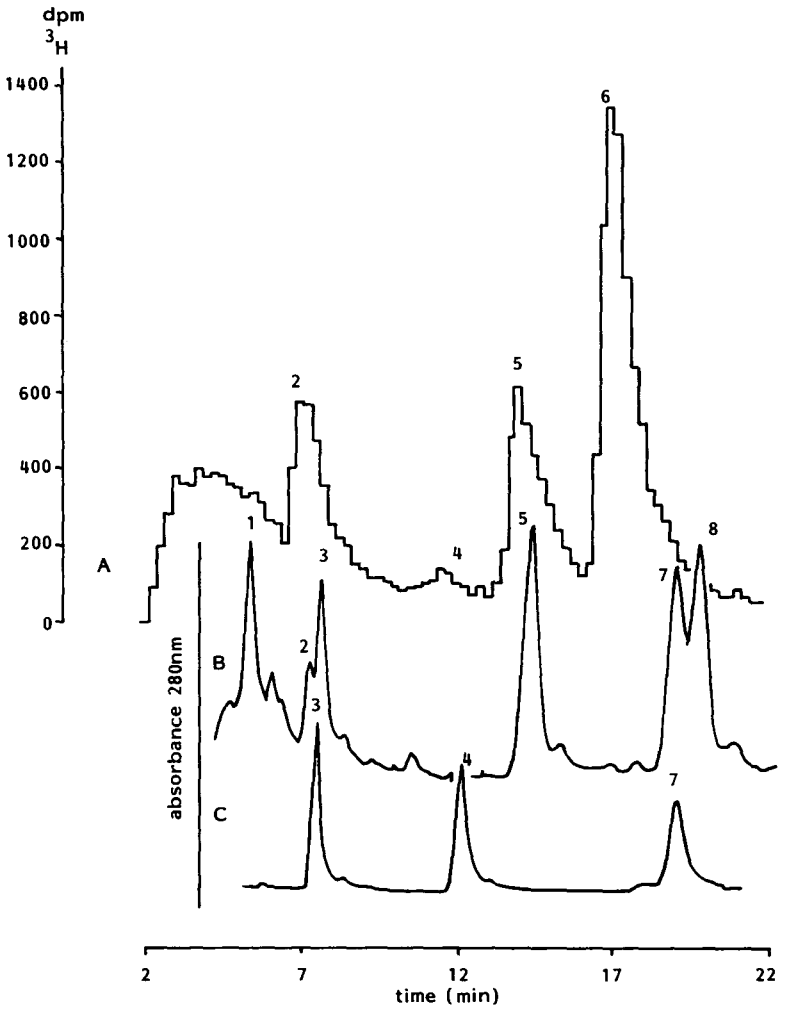

Fig. 4. Reversed-phase HPLC separation of LTC_4, LTD_4 and LTB_4. (A) ^{3}H-Labelled LTs were added to a non-labelled incubation medium of chopped human lung tissue, triggered with Ca-ionophore as described in Experimental. The numbers 2, 5 and 6 indicate the ^{3}H-labelled LTC_4, LTD_4 and LTB_4 (batch 9), respectively. (B) Measurement of the absorbance at 280 nm of the same tissue extract as mentioned in (A). (C) Chromatogram of synthetic LTC_4 (3), LTD_4 (4) and LTB_4 (7, a degradation product?), directly applied to the reversed-phase HPLC system.

When [^{3}H]LTD_4 was injected directly onto the HPLC column, the t_R was the same as peak 4 in Fig. 4A.

CONCLUSIONS

Whole human lung tissue, stimulated with Ca-ionophore A23187, produced under the conditions described, large amounts of LTB_4-like compounds and less LTC_4 and LTD_4. The major PG-like substances were immunoreactive with TxB_2, 6-keto-$PGF_{1\alpha}$ and PGD_2. The role of these different compounds has not yet been established. The formation of comparatively high amounts of TxB_2

and PGD_2 in antigen-challenged human lung tissue has been observed [20]. TxA_2 has bronchoconstrictor activity. The differences observed in the specific activities of the PGs may indicate that these substances are not formed from the same AA pool. Identification of AA metabolites could give problems when tritiated standards are used, due to the decrease of retention times. Whether recently available tritiated LTs are suitable for receptor binding studies and other specific interactions is doubtful as long as it remains uncertain that these compounds are pure LTs and not chemically degraded products.

The conversion of LTC_4 in LTD_4 by γ-glutamyltranspeptidase is inhibited by serine—borate complex [21]. Although in receptor binding studies in guinea pig lung, [^{3}H]LTD_4 was not metabolized to LTE_4 [22], this bioconversion was demonstrated in experiments with guinea pig ileum. In the presence of L-cysteine, the conversion of LTD_4 to LTE_4 was largely inhibited [23]. In future work, the effect of a 5-lipoxygenase inhibitor on the formation of PGs will be investigated, as well as the differences in AA metabolism of lung tissue obtained from asthmatics and non-asthmatics [24].

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CHROMBIO. 2221

DETERMINATION OF FREE 3-METHOXY-4-HYDROXYPHENYLGLYCOL WITH SEVERAL OTHER MONOAMINE METABOLITES IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH AMPEROMETRIC DETECTION

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SUMMARY

A reversed-phase liquid chromatographic method with amperometric detection has been developed for determining free 3-methoxy-4-hydroxyphenylglycol in plasma. The method is based on a simple and rapid extraction procedure employing a small C_{18} column. Vanillyl alcohol was used as an internal standard to obtain a good reproducibility. The 3-methoxy-4-hydroxyphenylglycol concentrations measured with the present method were in reasonable agreement with recently published data using high-performance liquid chromatography with amperometric detection and gas chromatography with mass spectrometry. The additional advantage of the present assay is that it can be performed in parallel with the quantification of other monoamine metabolites in plasma.

INTRODUCTION

Changes in the activity of the brain monoaminergic system have been implicated in a number of pathological states including psychiatric and neurological disorders [1—4]. Free 3-methoxy-4-hydroxyphenylglycol (MHPG) is a principal metabolite of norepinephrine in the human brain [5, 6], and is considered to pass readily out into the blood and cerebrospinal fluid (CSF) [7]. The assessment of metabolite(s) in the CSF is difficult for ethical reasons under usual clinical settings. The measurement of free MHPG concentrations in plasma has been suggested to offer a useful index of central adrenergic activity in human subjects [5, 8], although some controversial problems still remain to be solved [9].

Gas chromatographic methods with mass spectrometry (GC—MS) [10—12],

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which have most commonly been used for the measurement of plasma MHPG concentration, require expensive apparatus and superior technique. High-performance liquid chromatographic methods with amperometric detection (HPLC—ED) have recently been shown to be useful for the determination of MHPG in urine [13, 14], brain [15, 16], and CSF [17—20]. To our knowledge, however, only two recent articles [21, 22] have described the HPLC-ED method for the determination of free MHPG concentrations in plasma. Those experimental procedures [21, 22], however, necessitated a somewhat tedious pre-extraction of MHPG from plasma. In addition, several compounds used as internal standards in the previous HPLC—ED assays [14, 18, 21] do not appear to be readily available.

This article describes an HPLC—ED method for determining free MHPG concentrations in human plasma using vanillyl alcohol as the internal standard. The method necessitates a simple one-step sample purification step on a small C_{18} column. Furthermore, the present assay method, when used in combination with the method recently reported by us [23], permits four major monoamine metabolites in human plasma to be readily profiled.

EXPERIMENTAL

Reagent

4-Hydroxy-3-methxoybenzyl alcohol (vanillyl alcohol, VA) was obtained from Aldrich (Milwaukee, WI, U.S.A.). 3-Methoxy-4-hydroxyphenylglycol (MHPG) hemipiperazine salt and other related compounds were purchased from Sigma (St. Louis, MO, U.S.A.). All other reagents were of analytical reagent grade.

Chromatography

The liquid chromatography—electrochemical detection system consisted of a Yanako Model L-4000 W pump, a 250 × 4.6 mm I.D. 7-μm Yanapak ODS-A reversed-phase column and a Model VMD-501 dual electrochemical detector with glassy carbon electrodes. All of the system was purchased from Yanagimoto (Kyoto, Japan). Applied potential was +0.7 V versus Ag/AgCl reference electrode. The mobile phase, 0.1 *M* potassium phosphate buffer (pH 4.8) containing EDTA · 2Na (10 μM) and methanol (10%), was delivered at a flow-rate of 1.1 ml/min at ambient temperature.

Extraction

Blood was collected in a tube containing 0.1% EDTA · 2Na and 0.1% sodium metabisulphite and put on ice. Plasma was separated by centrifugation at 600 *g* for 7 min at 4° C, and then stored at —80° C until analysed.

Extraction was performed under vacuum using Bond-Elut columns pre-packed with 100 mg of C_{18}-bonded silica (40 μm) in a 1-ml capacity disposable syringe (Analytichem International, Harbor City, CA, U.S.A.). The columns, which were inserted into a vacuum chamber connected with a water aspirator, were prepared by washing with 1 ml of methanol followed by 1 ml of water.

After the addition of 50 μl of a solution of VA (internal standard equivalent

to 5 ng) to 1 ml of plasma, samples were applied to and passed through the columns, followed by 0.75 ml of water to rinse off both the residual samples and easily eluted hydrophilic compounds. The adsorbed materials were eluted with 200 μl of methanol—0.1 *M* phosphate buffer (pH 4.8) mixture (40:60). Usually 20 μl of this solution were injected into the HPLC system.

Calibration curves were generated by processing authentic standard substances through the entire extraction procedure and comparing the peak heights with that of the internal standard.

RESULTS AND DISCUSSION

The principal problems associated with the determination of plasma MHPG concentrations using HPLC—ED have been concerned with the selection of an appropriate extraction procedure prior to injection into the HPLC system [13, 21, 22] and the selection of the compound used as internal standard [14, 21]. To solve the latter problem, various neutral compounds such as 4-hydroxybenzyl alcohol, 3-(*p*-hydroxyphenyl)-1-propanol, and 3-hydroxy-4-methoxybenzyl alcohol (isovanillyl alcohol) were examined in the present study. VA was chosen because of its appropriate chromatographic behaviour and sufficient detectability at the potential of 0.7 V (Fig. 1), which allowed the MHPG analysis to be sensitive and selective.

Typical chromatograms showing the separation of MHPG and VA (internal standard) in standard solution and in an extract of human plasma are shown in Figs. 1 and 2, respectively. Under the present chromatographic conditions, related basic monoamines except for serotonin which was retained longer than MHPG, were eluted within 5 min (Table I). All of the acidic and other neutral metabolites examined did not interfere with the detection of MHPG and VA. Furthermore, the pH of the mobile phase and the column temperature employed in the present assay method substantially contributed to an optimal separation from other possible interferences by endogenous substances in human plasma. Unidentified peaks, which appeared just after MHPG (Fig. 2), overlapped at temperatures above 35°C or with increasing pH. Identities of the peaks were verified by the chromatographic behaviour under varying conditions and electrochemical characteristics determined by a dual-electrode detection system, which enabled the relative magnitudes of current responses of standards and unknowns to be compared at two different potentials.

Effective and rapid extraction was accomplished with the use of a small C_{18} column. Neutral metabolites such as MHPG and VA were readily sorbed on a hydrophobic C_{18} sorbent from plasma and eluted with a methanol—buffer mixture. When the plasma sample was acidified prior to applying it to the extraction column, the bulk of interfering substances was also sorbed, resulting in a disturbance of the subsequent chromatographic analysis. Since MHPG is a fairly hydrophilic compound, the volume of water used for washing had to be set at 0.75 ml in order to remove interference(s) caused by more hydrophilic compound(s) than MHPG and to minimize the possible loss of MHPG. The use of methanol as the eluent in place of methanol—buffer mixture also led to an increase in interfering peaks. Through the entire extraction procedure in the present assay method, the majority of acidic monoamine metabolites and the

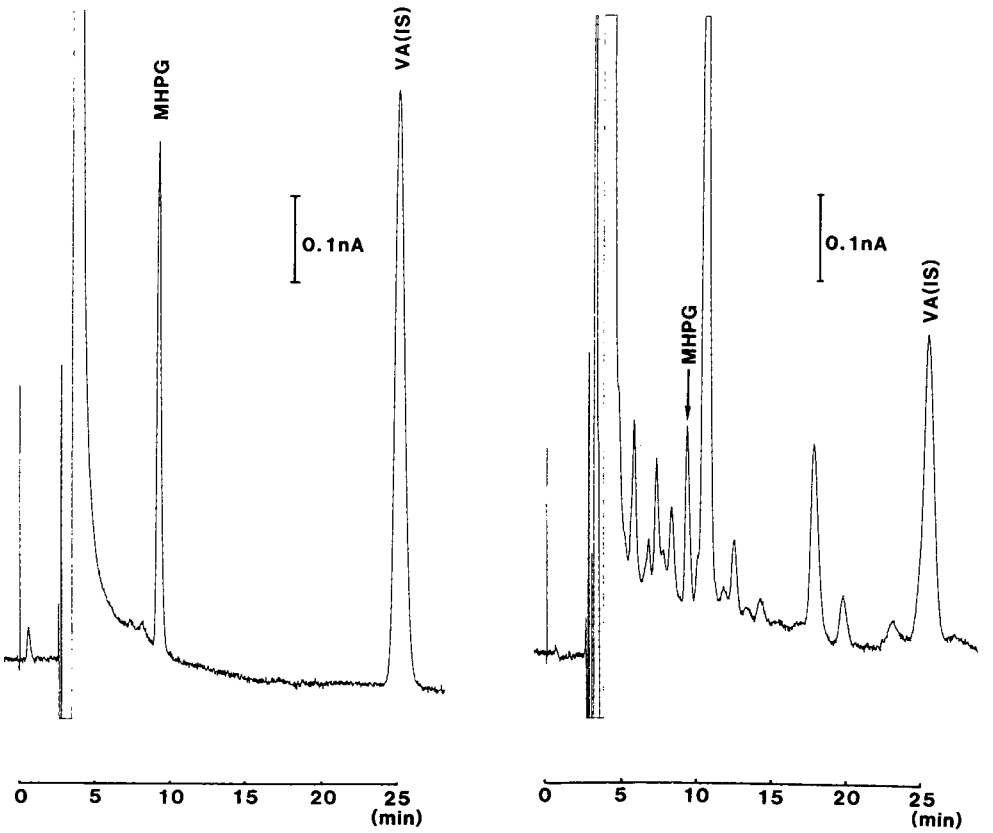

Fig. 1. Chromatogram of authentic MHPG and vanillyl alcohol (VA as internal standard, IS) in standard solution (0.8 ng each).

Fig. 2. Typical chromatogram of MHPG (3.54 ng/ml) and vanillyl alcohol (VA) in an extract from human plasma.

TABLE I

RETENTION TIMES OF SOME MONOAMINE RELATED COMPOUNDS

Compound	Retention time (min)
L-Dopa	3.80
3,4-Dihydroxyphenylglycol	4.65
Dopamine	4.67
Tyrosine	4.73
5-Hydroxytryptophan	8.18
L-Tryptophan	8.23
3,4-Dihydroxyphenylacetic acid (DOPAC)	8.54
3-Methoxy-4-hydroxyphenylglycol (MHPG)	9.24
Serotonin	10.70
5-Hydroxyindoleacetic acid (5-HIAA)	19.65
Homovanillic acid (HVA)	22.74
Vanillyl alcohol (VA)	25.08

relevant amino acids were eliminated together with many unknown interfering compounds.

The recovery rates from plasma throughout the entire procedure were around 60.88% for MHPG and 95.70% for VA (Table II). The highly sufficient reproducibility of the assay was confirmed by the coefficient of variation (C.V.) which ranged from 2.63% to 5.39% (Table II). A standard curve was run on each occasion with known amounts of MHPG added to plasma samples and extracted as described above. The linearity over the range 0.5—20 ng/ml was demonstrated by the plot of the peak height ratios of MHPG to VA (5 ng/ml) against the added amounts of MHPG. The standard curve had an r value of 0.994 and intercepted the origin ($Y = 0.145X + 0.004$, where Y is the peak height ratio and X is the concentration in ng).

TABLE II

PRECISION OF MHPG DETERMINATION FROM POOLED PLASMA AND RECOVERY OF ADDED MHPG AND VA

Compound	Concentration ± S.D. (ng/ml)	C.V. (%)	Amount added (ng)	Recovery ± S.D. (%) (n = 5)
MHPG				
Pool 1	6.48 ± 0.17 (n = 5)	2.63 (within-day)	5	57.55 ± 3.38
Pool 2	4.08 ± 0.22 (n = 9)	5.39 (between-day)	10	60.88 ± 3.53
Pool 3	8.25 ± 0.30 (n = 9)	3.64 (between-day)		
VA (internal standard)	—	—	5	95.70 ± 2.41

TABLE III

DETERMINATION OF PLASMA MHPG FROM HEALTHY HUMAN SUBJECTS

Subject number	MHPG concentration in plasma (ng/ml)
1	6.73
2	6.65
3	3.48
4	6.73
5	5.19
Mean	5.76
±S.D.	±1.28

The values for free MHPG concentrations in plasma from five healthy volunteers are given in Table III, which were found to be in agreement with the recently published data using HPLC—ED [21, 22] and GC—MS [5, 7].

Recently, we described a method for the simultaneous determination of 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), metabolites of dopamine, and 5-hydroxyindoleacetic acid (5-HIAA), a metabolite of serotonin, in human plasma by HPLC—ED [23]. The simultaneous utilization of the present and previous assay methods permits the determination of four major monoamine metabolites — MHPG, DOPAC, HVA and 5-HIAA — in plasma after the parallel extraction of MHPG and the other three metabolites from approximately equal portions of an aliquot of

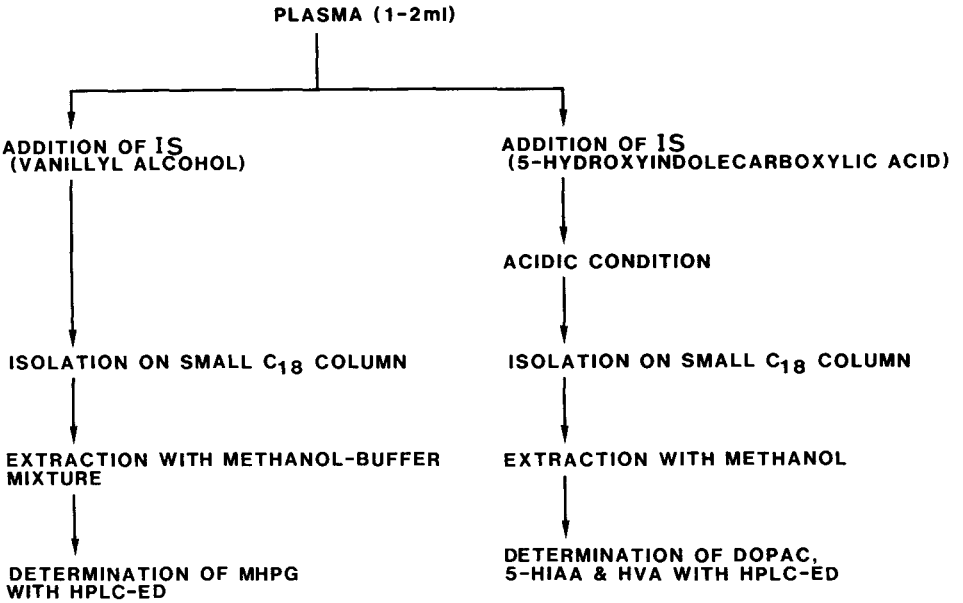

Fig. 3. Combined method for determination of plasma monoamine metabolites. Abbreviations: DOPAC = 3,4-dihydroxyphenylacetic acid; 5-HIAA = 5-hydroxyindoleacetic acid; HVA = homovanillic acid; MHPG = 3-methoxy-4-hydroxyphenylglycol; IS = internal standard.

plasma sample (Fig. 3). The present and previous methods are clinically applicable by employing just 500-μl aliquots of plasma. Such a combined method may further facilitate the clinical applicability of the measurement of monoamine metabolites in plasma at various pathological states [1—4] including psychiatric disorders. By using this combined method, a clinical study is underway in our laboratory; the results will be reported elsewhere.

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CHROMBIO. 2230

ANALYSIS OF DANSYL DERIVATIVES OF DI- AND POLYAMINES IN MOUSE BRAIN, HUMAN SERUM AND DUODENAL BIOPSY SPECIMENS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY ON A STANDARD REVERSED-PHASE COLUMN

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SUMMARY

The concentrations of putrescine, spermine and spermidine were measured in human serum, children's duodenal biopsy specimens and mouse brain homogenates by high-performance liquid chromatography. The chromatographic analysis was performed on dansyl derivatives of the polyamines using a reversed-phase system with an ion-pairing retention mechanism (heptane sulphonate). Capacity factors were determined at different concentrations of acetonitrile. Simple linear gradients were set up for fast (15 min) or routine (25 min) analysis. Three fluorescence detectors were compared for these determinations and their detection limits determined. The minimum detectable amount of polyamines was 25 fmol compared to 500 fmol with standard detectors. While samples prepared from tissues did not require a high sensitivity, a detector of better performance was needed to assay the polyamines in human serum.

INTRODUCTION

Putrescine (Put), spermidine (Spd) and spermine (spm) are polyamines implicated in many processes affecting cell growth and maturation. In animals, experiments have been concentrated on the study of polyamine metabolism in rapidly growing tissues: embryonic growth of different organs, compensatory growth and experimental tumours [1]. The involvement of polyamines in the

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postnatal maturation of the rat small intestinal mucosa has also been demonstrated [2]. Most human studies on polyamines have attempted to relate disturbances of polyamine levels in serum, urine or cerebrospinal fluid with malignant disease, while data on tissue polyamines are scanty [1].

As our laboratory is interested in the study of polyamine metabolism in mice and in humans, we aimed, in the present paper, at developing a method for the measurements of these compounds in biological fluids (serum), and animal (mouse brain) and human (intestine) tissues. Seiler's [3] review of the assay procedures already presented high-performance liquid chromatography (HPLC) as a powerful method [3]. With this technique, these compounds were rapidly separated (20—50 min), and measured in amounts of 10—100 pmol. In addition, the method was shown to be suitable for full automation. The usefulness of HPLC was later apparent for the analysis of unusual polyamines [4], acetylated derivatives [5,6] and in numerous studies on polyamine biosynthesis [7].

As in the case of the amino acid analysis, HPLC approaches are multiple. Separation can be achieved with an ion-exchange column or a reversed-phase column. Post- and pre-column derivatization methods are used to produce UV-absorbing or fluorescent derivatives. Various reagents are available: ninhydrin, *o*-phthalaldehyde, fluorescamine, and dansyl, dabsyl, tosyl or benzoyl chlorides [3—10].

Among these numerous combinations, reversed-phase separation and a pre-column dansylation procedure were selected for the following reasons. The analysis can be performed with a standard liquid chromatograph without additional devices. Using dansyl chloride (Dns-Cl) the derivatization step allows concentration of the sample prior to injection. This well documented derivatization [3,7,11] seems to give the greatest sensitivity in HPLC analysis [10] by formation of di-, tri- and tetradansyl derivatives for Put, Spd and Spm, respectively. The chosen separation mode was a reversed-phase system. The performance of these stationary phases has been recently improved. They are now available in 3-μm particles for high-speed separations or packed in narrow-bore columns to operate at low flow-rates. These methods of low dispersion enable the modern analyst to increase detectability and to reduce sample volume, solvent consumption and separation time down to several minutes, i.e. minimizes the analysis cost.

The present work was derived from the recent paper of Brown et al. [10]. The regulation of retention was investigated to set up linear gradients depending upon specific requirements. The method will be illustrated by the analysis of polyamines in various biological samples: brain homogenate of young mice (10—30 days), children's duodenal biopsy tissue and human serum. As a high detector sensitivity was required for serum analysis, the sensitivity and selectivity of three fluorescence detectors were compared. In a subsequent paper the performances of three types of columns (standard, high-speed and microbore) will be determined as a second alternative for improving the detectability.

MATERIALS AND METHODS

Apparatus

The HPLC equipment was mainly purchased from Altex Scientific (Berkeley, CA, U.S.A.). It was composed of two solvent metering pumps (A and B, Model 110 A) controlled by a microprocessor (Model 421), a high-pressure mixing chamber and a manual injection valve (Model 210) fitted with a 20-μl sample loop. A 5-μm Ultrasphere-ODS column (150 × 4.6 mm) from the same manufacturer was protected by a pre-column (42 × 3.2 mm) dry-packed with Vydac-201 RP (30—44 μm) from Macherey, Nagel & Co. (Düren, F.R.G.) for routine analysis. The performance of this column system was found to be unchanged after one year of use at a rate of two days per week.

Different fluorescence detectors were used. Two filter fluorimeters from Gilson (Middelton, WI, U.S.A.) were compared. The Spectra-Glo model (detector A) was equipped with a 15-μl cell and standard filters for fluorescamine. Model 121 (detector B) had a 9-μl flow cell and similar filters: 310—410 nm and 475—650 nm for excitation and emission, respectively. Detector C was a Model LS-4 fluorescence spectrophotometer from Perkin-Elmer (Norwalk, CT, U.S.A.) with a 3-μl flow cell. With this apparatus optimal wavelengths of 333 and 522 nm were determined by stopping the flow and scanning the excitation and emission spectra.

Electrical signals were processed by a C-R1A Chromatopac recording data calculator from Shimadzu (Kyoto, Japan) or sent to a double-pen recorder (10 mV).

Reagents and solvents

Putrescine, spermidine and spermine were from Sigma (St. Louis, MO, U.S.A.). Dansyl chloride and sodium 1-heptanesulphonate were from Aldrich-Europe (Beerse, Belgium). Carbonate salts, sulphosalicylic acid, acetic and perchloric acids, heptane, methanol and acetone were pro analysi chemicals from E. Merck (Darmstadt, F.R.G.). Acetonitrile-UV was an HPLC-grade solvent from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.).

Procedures

Solvent A for the HPLC analysis was prepared as follows: 20 m*M* sodium 1-heptanesulphonate and 20 m*M* acetic acid solution in water delivered by a Milli-Q apparatus (Millipore, Bedford, MA, U.S.A.). This advantageously replaces the commercial ion-pairing reagent PIC-B7 from Waters Assoc. (Milford, MA, U.S.A.). Solvent B was HPLC-grade pure acetonitrile.

Standard solutions of the polyamines were prepared from 1 m*M* stock solutions in water and stored at −20°C. They were found to be stable for a period of several months.

Mouse brain homogenates were prepared from C57 black deermice. The heads of the decapitated animals were immediately frozen by immersion in liquid nitrogen and stored at −70°C. One hemisphere (100—140 mg) was rapidly treated by the addition of 10 vols. of 0.2 *M* cold perchloric acid and homogenized in a glass Potter. The homogenate was left for 15 min at 0°C and proteins were pelleted by centrifugation at 5000 *g* for 20 min. The pellet

was resuspended in the same volume of acid, homogenized and centrifuged in the same conditions. The pooled supernatants were stored overnight in an ice and water bath. A last centrifugation was performed before derivatization. Aliquots of 200 μl of the clear supernatant were used for derivatization, i.e. 6.8—9.5% of the starting material [10].

Children's duodenal biopsy tissue (2—7 mg) was homogenized in water (1 mg wet tissue per 200 μl) at 0°C using a glass homogenizer. Solid sulphosalicylic acid was added up to a 40 mg/ml final concentration and precipitation was allowed to proceed for 30 min at 0°C. After centrifugation at 2000 *g* for 15 min, aliquots of 200 μl of the supernatant i.e. 14—50% of the original sample, were submitted to the dansylation procedure.

Human serum was deproteinized by sulphosalicylic acid and treated as described for duodenal biopsy material.

RESULTS

Control of retention and selectivity

In the ion-pairing separation of Brown et al. [10], a concave gradient was used to effect the retention using PIC-B7 in 50% acetonitrile as solvent A and pure acetonitrile as solvent B.

Before setting up a simple linear gradient, which is easily reproducible from laboratory to laboratory, we started by reconstituting the pre-mixed PIC-B7 reagent (as described in the experimental part) to study the influence of acetonitrile on retention. The results of these isocratic measurements are presented in a double-logarithmic plot in Fig. 1. This type of presentation was found to be linear, i.e. more useful than the classical plot of log k' as a function of % B. This allows linear regression parameters to be determined which facilitate inter- or extrapolations. High capacity factors are easily obtained by lowering the acetonitrile concentration, which also increases the selectivity. Such high values are required as many peaks were observed before the elution of Put (Figs. 2—4) or in a blank chromatogram (see Fig. 3 of ref. 10). At high detection sensitivity, and depending upon the sample, a large number of non-polyamine fluorescent peaks were visible throughout the whole chromatogram (Fig. 4). Some of these could coelute with polyamines, i.e. the straight lines of Fig. 1 were crossed by those from impurity peaks, coming mainly from all the chemicals involved in the derivatization procedure. Some of these interfering peaks were eliminated by purification of the dansyl reagent as described by Seiler and Demisch [11].

Gradient set-up and peak identification

Two examples of gradient analysis are presented in Fig. 2, differing by a factor of two in time between injections. The amounts of polyamines are similar to those measured in samples from duodenal biopsies of children. Part A of this figure shows an incomplete separation of Put (peak 1) from the first eluting peaks when starting with an initial solvent composition of 70% (v/v). A partial contamination of Spd (peak 2) due to the steepness of the gradient was also observed. This run took only 12 min. A complete resolution of the peaks of polyamines from impurities was obtained by lowering both

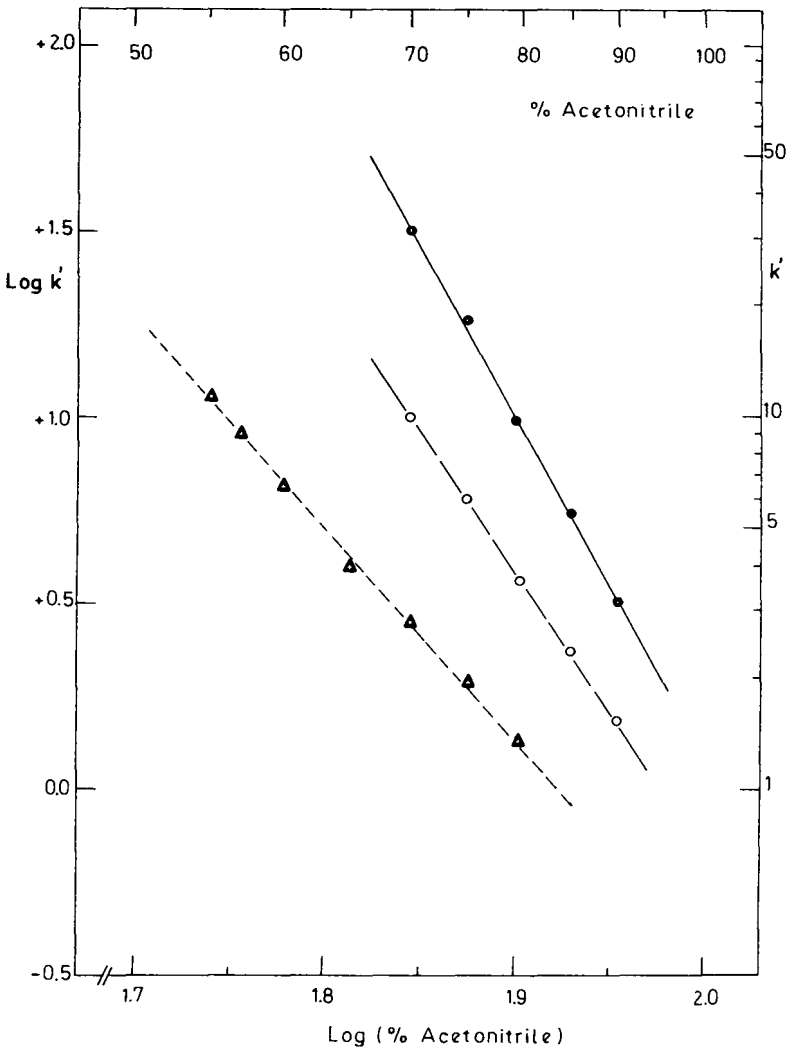

Fig. 1. Dependence of capacity factor mobile phase composition. Sample: 20 μl of dansylated Spm (●), Spd (○) or Put (▲) at 10 nmol/ml. Stationary phase: 5-μm Ultrasphere-ODS, 150 × 4.6 mm. Mobile phase: solvent A = 20 m*M* heptanesulphonate and 20 m*M* acetic acid, in water; solvent B = acetonitrile. Flow-rate: 2 ml/min. Detector B: Model 121 from Gilson with standard filters for fluorescamine. Parameters of the linear regressions: intercepts of 18.55, 14.86 and 10.90 for Spm, Spd and Put, respectively; slopes of −9.24, −7.51 and −5.66 for Spm, Spd and Put, respectively.

initial composition and gradient slope (part B). A complete analytical run was achieved in 25 min with a limited loss of sensitivity. This type of gradient was adopted for routine analysis. Retention times were found to be perfectly constant, i.e. a variation coefficient below 1%, so that peak identification by the data processor was possible.

Quantitative analysis of various biological samples

When the concentrations of the dansylated polyamines were assayed in

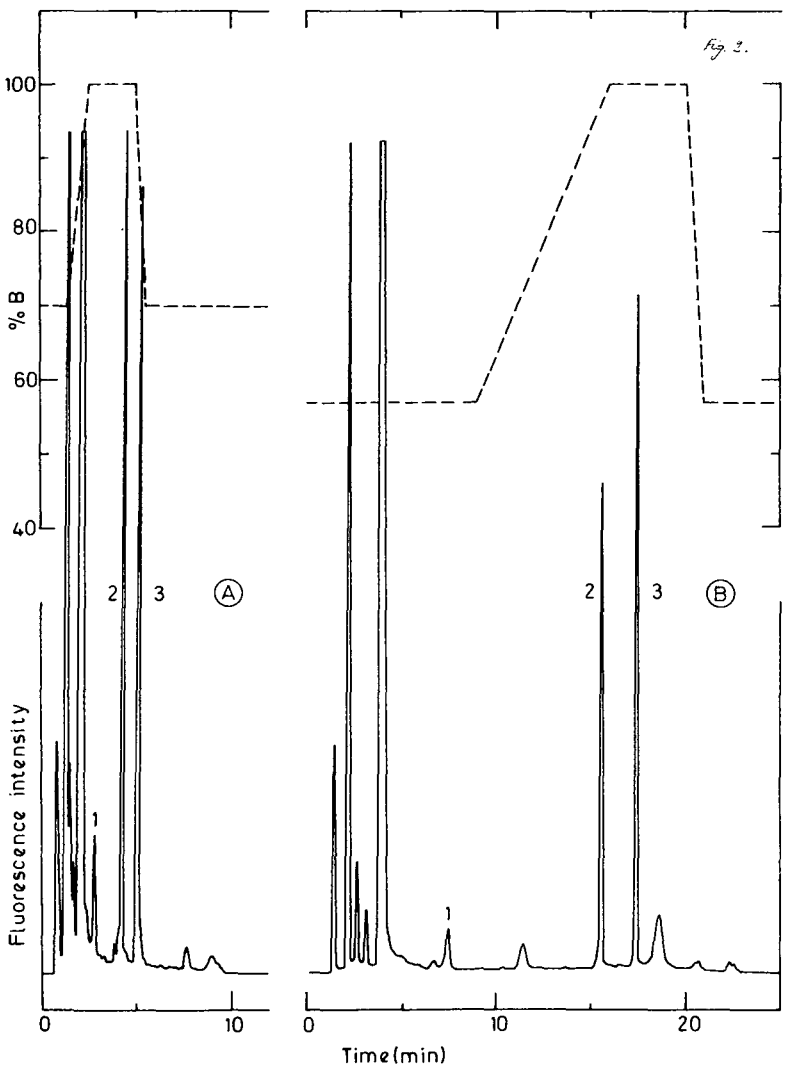

Fig. 2. Linear gradient separation of a diamine (putrescine) and polyamines (spermidine and spermine) on a 5-μm Ultrasphere-ODS column using a filter fluorometer (detector B). Sample: 20 μl of dansylated Put (peak 1, 1 nmol/ml), Spd (peak 2, 4 nmol/ml) and Spm (peak 3, 4 nmol/ml). Full and dotted lines refer to the recorded fluorescence intensities and to the programmed gradient, respectively. Part A (12-min analytical run): the solvent composition is changed after 1.5 min (70% to 100% B in 1 min) and 5 min (100% to 70% B in 0.5 min). Part B (25-min analytical run): the solvent composition is changed after 9 min (57% to 100% B in 7 min) and 20 min (100% to 37% B in 1 min). Maximum operating pressure at 2 ml/min: 18 MPa (2500 p.s.i.).

three types of biological extracts, the response was found to vary over a wide range of sensitivities depending on the amount of starting material available and the content of polyamines in the investigated sample. Samples prepared from mouse brain or human serum were found to contain amounts of polyamines differing by two orders of magnitude, as can be seen from the concentrations of the standard solutions in Table I.

TABLE I

LEVELS OF POLYAMINES IN VARIOUS BIOLOGICAL SAMPLES

The results are expressed as mean values of n measurements ± S.D., nmol/g weight tissue or nmol/ml of serum.

Standards used for calibration (nmol/ml)	Detector type	Sample origin	Type	Putrescine (Put)	Spermidine (Spd)	Spermine (Spm)
5—10	A	Brain from mouse aged 15 days ($n = 5$)	Normal (C_{57} black)	53.6 ± 5.8	402 ± 21	566 ± 38
			Epileptic (DBA/2)	70.0 ± 13.8	470 ± 24	566 ± 38
1—5	B	Children's duodenal biopsy tissue ($n = 7$)	Normal	52 ± 33	402 ± 61	1362 ± 333
0.05—0.1	C	Human serum ($n = 3$)	Normal	1.1 ± 0.3	0.06 ± 0.01	0.05 ± 0.02

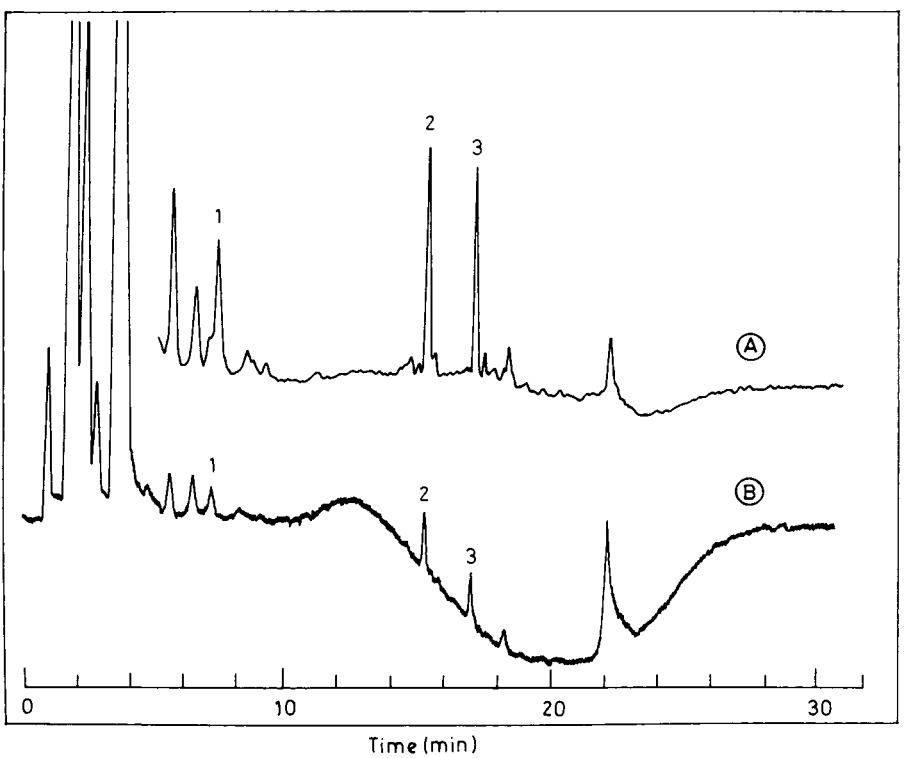

Fig. 3. Comparative response of detectors C (part A) and B (part B) for the injection of 1 pmol of putrescine (peak 1), spermidine (peak 2) and spermine (peak 3) using the 25-min gradient shown in Fig. 2B. Part A (detector C): LS-4 spectrofluorimeter from Perkin-Elmer set at 333 nm (excitation) and 522 nm (emission) using an intermediate scaling factor. Part B: (detector B): Model 121 instrument of Gilson operating at maximum sensitivity.

Mouse brain homogenates and children's intestinal biopsy tissues from normal individuals could be analysed with a low-cost filter fluorimeter. Detector B showed a detection limit of 0.6 pmol for Put and of 0.3 pmol for Spd and Spm, assuming a limiting value of 3 for the signal-to-noise ratio. Detector A was found to be four to five times less sensitive than detector B.

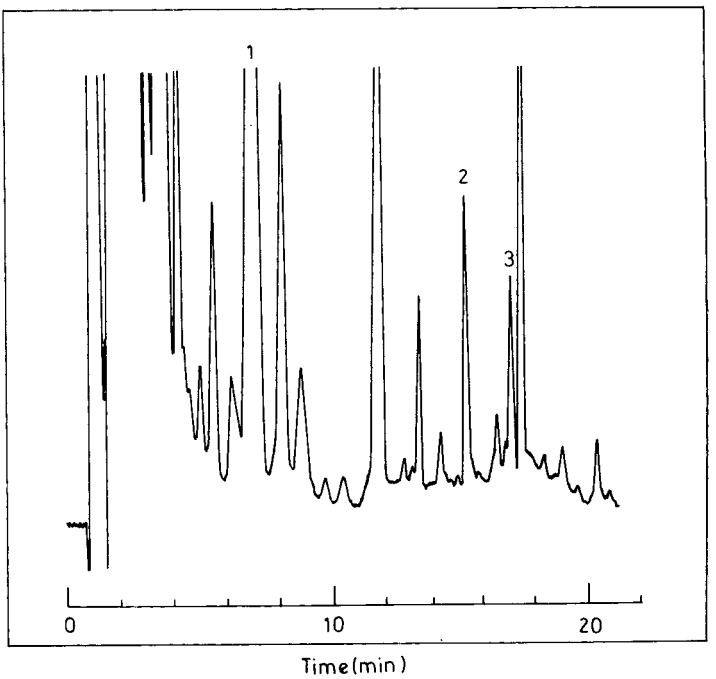

Fig. 4. Analysis of a serum sample using detector C. Peaks as in Fig. 2.

A major drawback of these filter detectors was the significant drift of their baseline when used at high sensitivity in the gradient elution mode. This disadvantage is shown in Fig. 3 where detectors B and C were connected in series for the analysis of a mixture of the three polyamines in equimolar amounts (1 pmol of each dansyl derivative was injected). This effect was proportional to the slope of the gradient and varied from solvent to solvent. It was probably related to changes of refractive index.

The results reported in Table I are in good agreement with those reported in the literature [12,13].

The complete analysis of human serum could not be realized using detector A or B. For this analysis a more sensitive detector was necessary, namely, detector C, which was able to detect less than 0.025 pmol. A typical chromatogram obtained with this detector is shown in Fig. 4. For this type of trace analysis, concentration by peak compression technique would be helpful [14], particularly if a microbore column is used.

CONCLUSIONS

The concentration of polyamines as their fluorescent dansyl derivatives was easily measured by high-performance liquid chromatography in various biological samples. The regulation of retention by heptane sulphonate and acetonitrile as mobile phase components allows high capacity and selectivity factors to be attained. The much higher hydrophobicity of polyamines (spermine and spermidine) as compared to a diamine (putrescine) justified the use of a gradient elution system to shorten the analysis time. Simple linear

gradient led to satisfactory results and a relatively short analysis time. The minimal detectable amount was 25 fmol compared to 500 fmol with standard detectors. This method is well suited for the analysis of polyamines in organ fragments such as those from brain or duodenal biopsies. Preliminary results showed its usefulness for studying the role of polyamines in the mechanism of epilepsy [15] and in the maturation of human intestinal mucosa. The method is presently being extended to the analysis of amines resulting from amino acid decarboxylation, by modification of the elution programme. The application of the method described in the present paper to serum analysis, however, requires a higher degree of sensitivity, which could be reached by the use of a spectrofluorimeter equipped with a small flow cell, instead of using a low-cost filter fluorimeter. Other alternatives for sensitivity enhancement will be considered in a subsequent publication.

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RAPID ION-EXCHANGE METHOD FOR THE DETERMINATION OF 3-METHYLHISTIDINE IN RAT URINE AND SKELETAL MUSCLE

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SUMMARY

A method for the determination of 3-methylhistidine using an automatic amino acid analyser has been developed. A single column system with lithium buffer (pH 3.950, 0.500 mol/l lithium and 0.067 mol/l citrate) was used for elution. The standard amino acid mixture of basic amino acids and some dipeptides usually present in physiological fluids was analysed for the development of the method. 3-Methylhistidine eluted in 46.7 ± 0.049 min and the peak area coefficient of variation for the same sample was 1.07%.

As 3-methylhistidine is completely resolved from the other basic amino acids and some dipeptides (anserine and carnosine), this method is suitable for the analysis of urine and muscle extracts as well as skeletal muscle protein hydrolysates where this amino acid is present in much lower concentrations than other amino acids.

INTRODUCTION

3-Methylhistidine (3-MeHis) is a constituent of skeletal muscle proteins [1]. In other tissues it is present in negligible quantities [2]. As this amino acid is formed by post-translational methylation of peptide-bound histidine [3,4], the rate of its urinary excretion has become a valuable catabolic marker of the skeletal muscle proteins and has increasingly been used for this purpose in recent time [5—8].

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There are many methods for the estimation of 3-MeHis based on ion-exchange chromatography [9—11] or high-performance liquid chromatography (HPLC) [12—15]. Most of them have been developed for urine determinations [9—14]. Some of them have been tested on plasma [14, 19] and muscle [15,17] samples. It seems that there are different problems if various biological samples are analysed. Some workers, using HPLC systems, have employed different ion-pairing reagents for different biological samples [15]. Pyridine elution for muscle protein hydrolysates has been recommended by some authors [16]. Determination of 3-MeHis in the skeletal muscle proteins could be a problem as this amino acid is present in much lower concentrations than the others [17]. If the free form of this amino acid has to be measured in muscle samples, anserine and carnosine could be a problem because of their similar chromatographic characteristics.

This report describes an ion-exchange chromatographic method for the determination of 3-MeHis that overcomes these problems and is suitable for both urine and skeletal muscle samples. It needs neither different analytical conditions for different biological samples nor special samples preparation or derivatization required when other methods are used [12—16]. Besides, it is faster than many methods based on ion-exchange chromatography [18—21], sufficiently sensitive for urine and muscle protein hydrolysate samples and more precise compared to the other methods [9,12,13].

EXPERIMENTAL

Equipment

Analyses were performed using a Model 121 M automatic amino acid analyser (Beckman, Palo Alto, CA, U.S.A.) operated according to the flow diagram presented in Fig. 1. The single-column system using the short column of the instrument packed with ion-exchange resin was employed. Reagent flows were regulated with pumps and the temperature of the chromatographic column maintained by a circulating water bath. Analyser operating conditions are summarized in Table I.

Amino acids eluted from the chromatographic column were detected with a ninhydrin reagent. The absorbance was measured at 570 nm using a flow cuvette and recorded by a two-channel recorder. The channel operating at the highest sensitivity was connected to a calculator; another channel operated at the usual sensitivity of the instrument. The calculator (Beckman, Data 126 System Calculator) was used for the determination of elution times (in minutes), peak area measurement (absorbance converted to the digital signal) as well as for the quantification of separated compounds.

All functions of the analyser were programmer-controlled. The programmer has 42 channels and performs different functions at predetermined times (pumps, transfer and injection of the samples, control buffer selector valves, control of column temperature, etc.). The instrumental analysis sequence is given in Table II and calculator run parameters in Table III.

Chemicals

All chemicals were of analytical grade (Merck). Water for preparing buffers

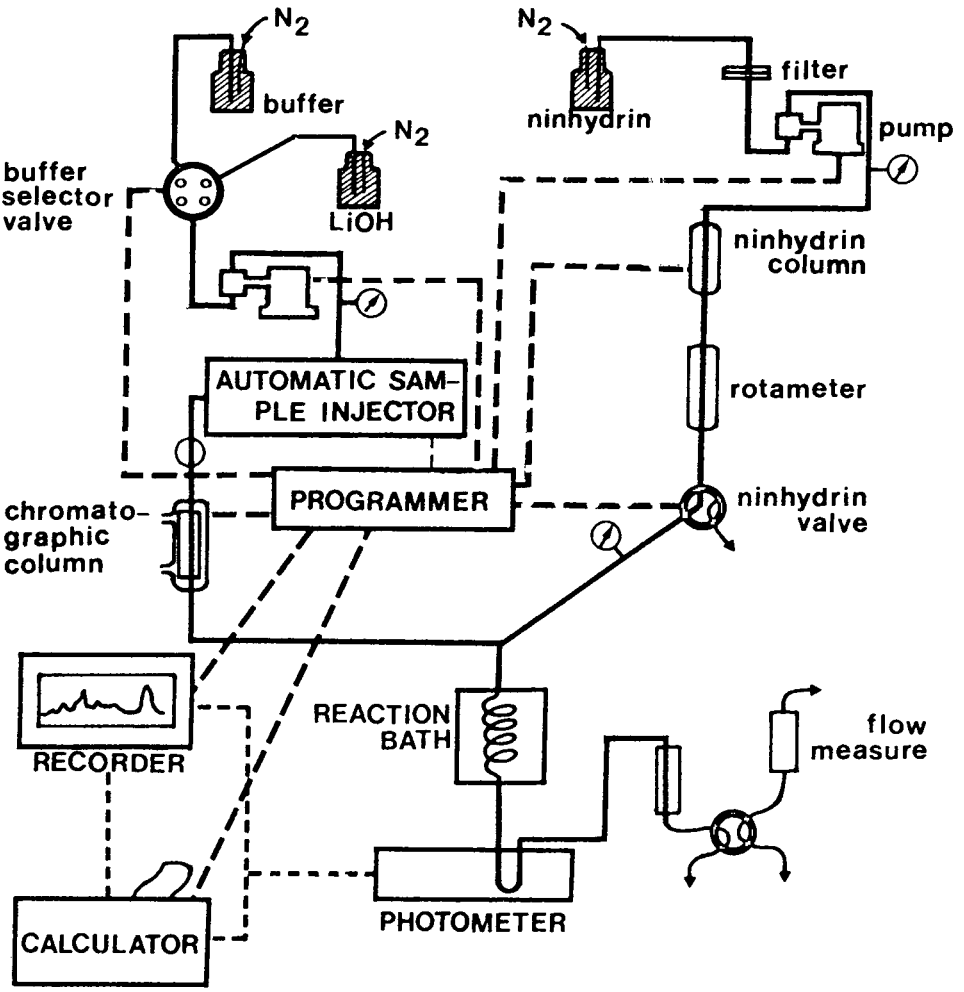

Fig. 1. Flow diagram of analytical system and programmer control.

TABLE I

ANALYSER OPERATING CONDITIONS

Ion-exchanger	AA-10 resin (Beckman)
Column diameter	0.28 cm
Column length	13.0 cm
Resin bed height	4.5 cm
Buffer: pH	3.950 ± 0.010
Lithium	0.500 mol/l
Citrate	0.067 mol/l
Buffer flow-rate	7.5 ml/h
Ninhydrin flow-rate	3.5 ml/h
Column temperature	64°C
Photometer: wavelength	570 nm
absorbance range	0.1 a.u.
Volume of the applied sample on the column	50 μl and 100 μl

TABLE II

INSTRUMENTAL ANALYSIS SEQUENCE

Step No.	Step time (min)	Operation
1	20.0	Equilibration before automatic run start; buffer and ninhydrin to the column
2	0.1	Automatic run start
3	2.0	Sample transfer from the coil on the automatic sample injector plate to the metering loop
4	0.1	Calculator on
5	1.0	Sample injection on the column
6	52.0	Chromatographic analysis
7	2.0	Regeneration of ion-exchange resin; lithium hydroxide to the column
8	15.0	Equilibration of the resin with the buffer
9	5.0	Calculator off, results printing
10	0.1	Programmer chart to step No. 2 for next sample analysis

TABLE III

CALCULATOR RUN PARAMETERS

PW	(peak width)	20
SS	(slope sensitivity)	1000
BL	(baseline test)	5
ML	(maximum baseline level)	50000
MA	(minimum area)	1000
FP	(number of fused peaks)	255

was ion-exchanged and sterile (Milli-Q Water Purification System, Millipore).

Lithium citrate buffer pH 3.950 ± 0.010 (23°C) with 0.500 mol/l lithium and 0.067 mol/l citrate was used. It was prepared by dissolving citric acid monohydrate and lithium hydroxide in water, and was then titrated to the desired pH value with concentrated hydrochloric acid and brought to final volume with water. The buffer was filtered before use (Millipore 0.2 μm mesh). Octanoic acid was used as a preservative (0.1 ml per 1000 ml).

Lithium citrate buffer (0.150 mol/l lithium and 0.050 mol/l citrate) pH 2.2 was used for dilutions. The buffer was filtered (Millipore, 0.2 μm mesh).

AA-10 resin (Beckman, PN 338013) was used. Lithium hydroxide solution (0.3 mol/l) was used for resin regeneration.

Dimethyl sulphoxide—ninhydrin reagent (Beckman, PN 336452) was used for amino acid detection. This reagent was mixed with a premeasured amount of hydrindantin according to the directions given.

The basic amino acid solution (Hamilton, Type P-B, PN 77729) contained 2.5 μmol/ml lysine, arginine, histidine, 1-methylhistidine, 3-methylhistidine, hydroxylysine, γ-aminobutyric acid, ornithine, ethanolamine, 15.0 μmol/ml creatinine, 2.5 μmol/ml carnosine and 1.25 μmol/ml anserine. This solution was diluted 25 times with a sample diluting buffer, pH 2.2 (solution A). Solutions of individual compounds were prepared from standard substances (Serva) by dissolving them in the sample diluting buffer.

Samples

Rat urine was analysed and as the rat excretes 3-MeHis mostly in the acetylated form [4], the urine was hydrolysed in hydrochloric acid (6 mol/l) at 110°C for 20 h, then evaporated and dissolved in the sample diluting buffer.

The rat muscle (m. gastrocnemius) was extracted in sulphosalicylic acid (0.16 mol/l). The deproteinized extract was brought to pH 2.2 with lithium hydroxide and to the final volume with the sample diluting buffer.

Mixed rat muscle protein hydrolysates (m. gastrocnemius) were prepared according to the method of Haverberg et al. [2].

TABLE IV

ELUTION TIMES OF 3-METHYLHISTIDINE AND SOME OTHER COMPOUNDS

Compound	Elution time (min)
Tryptophan	23.9
Hydroxylysine	29.5
Ammonia	33.3
Creatinine	33.6
Ornithine	35.6
Lysine	36.0
Histidine	41.3
1-Methylhistidine	41.6
3-Methylhistidine	46.7
Carnosine	56.9
Arginine	62.5

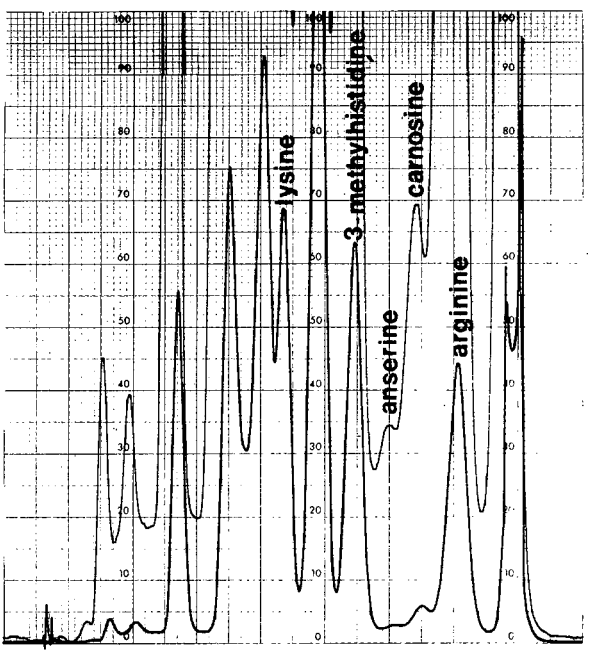

Fig. 2. Chromatogram of standard amino acid mixture (solution A). The mixture was injected into the column in 100 μl of lithium citrate dilution buffer and contained 100 nmol/ml of each compound with the exception of anserine (50 nmol/ml).

RESULTS

Elution times of the compounds examined were determined by their separate injection and elution from the chromatographic column. These results are presented in Table IV.

To test the interaction between compounds in the mixture and to determine the precision of 3-MeHis measurement, a standard calibration mixture (solution A) was analysed. The chromatogram obtained with this mixture is shown in Fig. 2. As can be seen, lysine, 3-MeHis, arginine as well as anserine and carnosine are resolved as separate peaks.

The sensitivity of 3-MeHis determination was also tested. A calibration curve was obtained using solutions with increasing concentrations of this amino acid (from 10 to 300 nmol/ml) and it was found to be linear for concentrations ranging from 10 to 150 nmol/ml.

TABLE V

REPEATABILITY OF ELUTION TIMES AND PEAK AREAS OF SEPARATED COMPOUNDS (n = 5)

Compound	Concentration (nmol/l)	Elution time			Peak area (calculator readings)		
		Mean (min)	S.D. (min)	C.V. (%)	Mean	S.D.	C.V. (%)
3-MeHis	100	46.7	0.049	0.105	9,947,375.4	106,238.0	1.068
Arginine	100	62.5	0.049	0.078	8,668,142.0	55,302.7	0.638
Lysine	100	36.0	0.040	0.111	9,055,923.6	141,906.3	1.567
Anserine	50	52.7	0.040	0.076	137,484.5	6,709.2	4.880
Carnosine	100	56.9	0.075	0.131	615,251.0	12,489.6	2.030

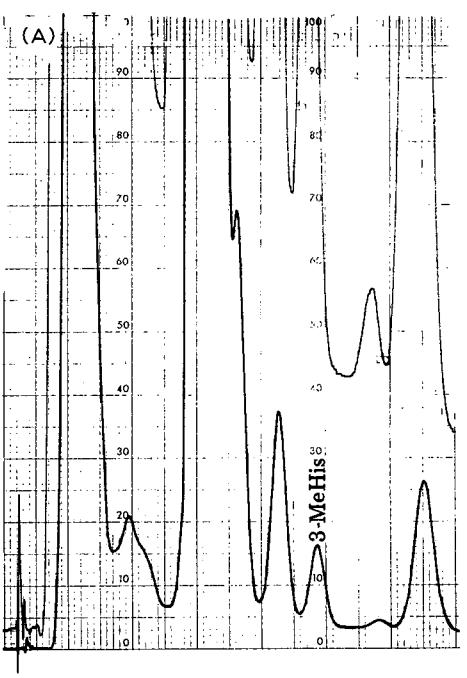

Fig. 3.

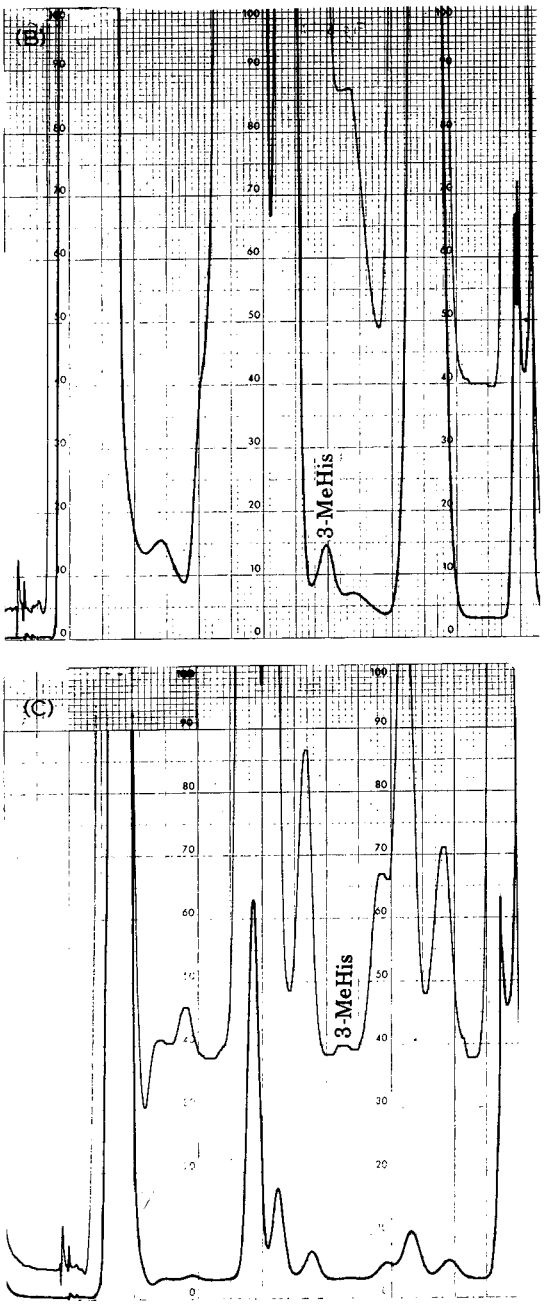

Fig. 3. Chromatograms obtained with rat urine (A), muscle protein hydrolysate (B) and muscle extract (C), illustrating good resolution of 3-MeHis from the other compounds; 50 μl of each sample were applied on the column.

The statistical analysis of the peak areas and elution times obtained for compounds completely separated from the mixture are presented in Table V. There are small variations in elution times and the calculator always identifies

the peaks with good precision. The position of 3-MeHis on the chromatograms ranged from 46.7 to 46.8 min (C.V. = 0.1%) and the peak areas obtained in replicate with the same standard solution had a very low coefficient of variation (C.V. = 1.07%).

Applicability of this method for 3-MeHis estimation in physiological fluids was tested using muscle extract and protein hydrolysates as well as urine samples. Recovery of the added 3-MeHis to muscle extract was 99.4%. In the samples examined this amino acid eluted as a separate peak (see Fig. 3).

DISCUSSION

The separation of 3-MeHis from other basic amino acids was complete under the descirbed analytical conditions. Lysine and arginine as well as anserine and carnosine were resolved (Fig. 2) and could be quantitated by this method. At the same time, 1-MeHis coeluted with histidine (His). The other amino acids eluted together or before ammonia.

As 3-MeHis is separated from the basic amino acids as well as from some dipeptides (anserine and carnosine) present in muscles, this method is suitable for the determination of 3-MeHis in muscle extracts without preliminary removal of peptides (Fig. 3). In addition, since the 3-MeHis peak is sufficiently far from the His/1-MeHis peak, the method can be used with good accuracy for the determination of 3-MeHis in muscle protein hydrolysates where this amino acid is present in much lower concentrations than other amino acids (Fig. 3). It seems that with other methods [16,17] the determination of 3-MeHis in muscle protein hydrolysates could be a problem because the time of its elution from the chromatographic column is too close to histidine. For the determination of 3-MeHis in skeletal muscle protein hydrolysates by the present method, neither special preliminary sample preparation nor pyridine elution, as recommended by some authors [16], is needed.

The method that we have developed for the determination of 3-MeHis is faster than some other methods based on ion-exchange chromatography [18—21]. It is as fast as the method recommended by Long and Geiger [10] but the elution time is achieved with ten times lower flow-rates of buffer and ninhydrin. Although some methods for 3-MeHis based on HPLC are faster [12—14], duration of analysis is rather similar since sample preparation is longer. At the same time, this present method can be used for the determination of lysine, arginine, anserine and carnosine. While this method for 3-MeHis is as sensitive as some other methods [9], its precision is much better compared to those based either on ion-exchange chromatography [9] or on HPLC [12,13]. Repeatabilities of peak location (C.V. = 0.1%) and peak areas (C.V. = 1.07%) permit a precise determination of 3-MeHis concentrations.

We examined the influence of some working parameters on the 3-MeHis elution rate from the column and on its resolution from other compounds. It is very sensitive to column temperature changes. A temperature decrease produced a slower elution of this amino acid and of the other amino acids from the column. At the same time, lowering of the temperature resulted in an increased distance between anserine and carnosine. At 33°C these peptides eluted very slowly producing wide peaks. At the same temperature 3-MeHis

eluted in 72.7 min, at 41°C in 64.7 min, while at 67°C it eluted in 43.7 min. However, with the temperature higher than 64°C, the peaks were too close and their determination was less precise.

The buffer composition affected the 3-MeHis elution time and its resolution from other amino acids. While the pH of the buffer was unchanged (pH 3.95), change in ionic concentration (1 mol/l lithium and 0.166 mol/l citrate) produced a faster 3-MeHis elution from the column. However, this buffer did not resolve anserine, carnosine and arginine, which eluted together, while the 3-MeHis and His/1-MeHis peaks were too close. A buffer flow faster than recommended produced similar changes in the distance between the 3-MeHis and His/1-MeHis peaks. Therefore, the conditions presented at the beginning of this paper were chosen as optimal.

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RESOLUTION AND QUANTITATION OF APOLIPOPROTEINS A-I AND A-II FROM HUMAN HIGH-DENSITY LIPOPROTEIN BY SIZE EXCLUSION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Apolipoproteins A-I and A-II, extracted from human high-density lipoprotein (HDL), were resolved and quantified by size exclusion high-performance liquid chromatography on TSK 125 and TSK 250 analytical columns connected in series without the use of chemical denaturants or detergents in the eluent buffer. The columns were pre-equilibrated with a solution containing 0.1 *M* sodium phosphate, pH 7.2, 0.2 *M* sodium chloride at a flow-rate of 1 ml/min. Delipidated HDL (1 mg protein per ml) was resolved into two populations of apolipoprotein (apo) A-I: one representing the apo A-I monomer and the other, a self-associated form with a molecular weight of approximately 120,000 daltons. The column eluates were screened for immunoreactivity to apo A peptides, and the identity of each peak was confirmed by sodium dodecyl sulfate—polyacrylamide gel electrophoresis followed by immunoblot analysis. Apo A-I peptides isolated by high-performance liquid chromatography disrupted unilamellar phospholipid vesicles to form smaller phospholipid particles that eluted on gel filtration columns within the size range of HDL. Thus, a rapid method for the isolation and quantitation of non-denatured apolipoproteins from HDL has been developed using size exclusion high-performance liquid chromatography.

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INTRODUCTION

The levels of plasma high-density lipoprotein (HDL) have been correlated inversely with mortality from cardiovascular disease [1, 2], and increased amounts of cholesterol in HDL appeared to protect against development of atherosclerosis [3]. Recent studies indicated that the levels of apolipoprotein (apo)A-I, the major protein component of HDL, may be a better metabolic marker for coronary artery disease than cholesterol levels [4], and for that reason efforts were directed toward the development of a rapid, reliable assay for apolipoprotein A levels.

Size exclusion high-performance liquid chromatography (HPLC) provides a high-recovery system for the detection, isolation and quantitation of proteins and other macromolecules. The size exclusion columns are capable of separating apolipoprotein molecules utilizing denaturants such as urea or guanidinium chloride [5, 6]. However, we have demonstrated that delipidated human HDL (apo-HDL) can be resolved by size exclusion HPLC without the use of these chemicals, thus providing a rapid method for quantification and recovery of native apolipoproteins from human HDL.

EXPERIMENTAL

Materials

Size exclusion protein standards, Biogel A-15 M, and protein assay kit II were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). Human apolipoproteins A-I, A-II and C-III, and antisera to apolipoprotein A were obtained from Calbiochem-Behring (San Diego, CA, U.S.A.). Sodium phosphate, sodium chloride, and HPLC-grade water were obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). Electrophoretic reagents and protein standards were supplied by Bethesda Research Labs. (Bethesda, MD, U.S.A.). Phosphatidylcholine (egg), sphingomyelin, cholesterol, cholesteryl oleate, oleic acid, triolein, and bovine serum albumin were obtained from Sigma (St. Louis, MO, U.S.A.). [^{3}H] Methyl choline dipalmitoyl phosphatidylcholine and [^{125}I] protein A were obtained from New England Nuclear (Boston, MA, U.S.A.).

Preparation of apo-HDL

Plasma was obtained from normolipidemic, fasting donors, and HDL was isolated by the sequential floatation method of Lindgren et al. [7]. In order to isolate very low density lipoprotein (VLDL) and low density lipoprotein (LDL), the plasma was adjusted to a density (d) of 1.063 g/ml by addition of a solution containing 0.96 M sodium chloride, 7.572 M sodium bromide, 1 mM EDTA (d = 1.48 g/ml) and centrifuged at 190,000 g for 24 h at 12°C using a Sorvall (DuPont, Wilmington, DE, U.S.A.) 65.13 rotor in a Sorvall OTB-75B ultracentrifuge. The VLDL and LDL were removed by aspiration, and the infranatant was adjusted to d = 1.21 g/ml using the same salt solution (d = 1.48 g/ml) and centrifuged for 36 h under the same conditions. The HDL fraction was recovered and dialyzed against nitrogen-saturated HPLC-grade water adjusted to 1.0 M sodium chloride. HDL was delipidated by diethyl ether and ethanol extractions according to the method of Shore and Shore [8]. Protein

content was determined with the Bio-Rad protein assay kit using bovine serum albumin as a standard. The protein concentration of the final apo-HDL preparations ranged from 1.0 to 1.5 mg/ml and recoveries of protein averaged 85%. Each HDL and apo-HDL preparation was extracted by the method of Folch et al. [9], and thin-layer chromatography of these extracts was performed on silica gel G 20 × 20 cm plates (Fisher Scientific). The absence of phospholipid in the apo-HDL preparations was confirmed by separation using a solvent mixture of chloroform—methanol—water (65:25:4, v/v/v) [10] as the developing solvent, and the absence of neutral lipid was determined using a solvent system of hexane—diethyl ether—acetic acid (70:30:1, v/v/v) [11]. Phospholipid and neutral lipid in HDL and standards were quantified by a modification [12] of the densitometric procedure of Katz et al. [13]. Each apo-HDL preparation was stored in liquid nitrogen until chromatographed.

Chromatographic system

The HPLC system consisted of one 6000A pump, a 720 system controller, a U6K injector all from Waters Assoc. (Milford, MA, U.S.A.). Column eluate was monitored for ultraviolet absorbance using a Waters 440 detector (280 nm), and relative areas of each peak were determined using a Waters 730 data module. Two size exclusion columns, a TSK 125 connected in series to a TSK 250 (Bio-Rad Labs.) were equilibrated with the eluent buffer consisting of 0.1 *M* sodium phosphate, pH 7.2, 0.2 *M* sodium chloride at a flow-rate of 1 ml/min. The columns were calibrated with molecular weight standards (Bio-Rad Labs.), and no significant variation was observed between column and protein standard lots.

Hybridot screening of column eluates

A 150-μg sample of human apo-HDL was resolved by size exclusion HPLC and collected into 0.3-ml fractions. A 100-μl aliquot of each fraction corresponding to the appropriate peaks was applied to nitrocellulose using a hybridot apparatus (Bethesda Research Labs.). The nitrocellulose sheet was suctioned to dryness and processed with a 1:50 dilution of antisera against apo A peptides (Calbiochem-Behring) according to the method of Burnette [14]. Also, bovine serum albumin, standard apo A-I and A-II injections were chromatographed and collected as described for hybridot analysis.

Polyacrylamide gel electrophoresis

Fractions collected from HPLC resolution of human apo-HDL were dialyzed against 0.1 *M* sodium phosphate, pH 7.4, containing 1% sodium dodecyl sulfate (SDS) in a microdialysis unit from Bethesda Research Labs. and subjected to SDS—polyacrylamide gel electrophoresis (PAGE) on 15% gels using the method of Laemmli [15]. Paired PAGE gels were run using the Bio-Rad Labs. Protean double-slab electrophoresis cell with identical sample volumes applied to corresponding lanes of each gel. Proteins on one gel were visualized by silver staining [16], and the proteins on the other gel were transferred to nitrocellulose and analyzed by the immunoblot procedure of Burnette [14] using a 1:50 dilution of antisera against apo A peptides.

Incubation of vesicles with apo-HDL fractions

Unilamellar phospholipid vesicles were prepared and radiolabeled with tritiated lecithin as previously described [17]. Appropriate fractions from size exclusion HPLC of apo-HDL (167 μg protein per ml) were incubated with egg yolk phosphatidylcholine vesicles (445 μg lecithin per ml) in a total volume of 0.6 ml (2.6 × 30 cm) and chromatographed as described previously [17]. The Biogel A-15M column was calibrated using human HDL, bovine serum albumin and unilamellar vesicles. When incubation mixtures were applied to the Biogel A-15M columns, eluted fractions were analyzed for radioactivity using Aquasol (New England Nuclear) as the scintillation cocktail, counting efficiencies for tritium were approximately 50% and data were expressed as dpm tritium per ml eluted fraction.

RESULTS

Calibration of TSK 125 and TSK 250 size exclusion HPLC columns

When a mixture of molecular weight standards was separated by size exclusion HPLC with serial TSK 125 and TSK 250 columns, a linear relationship was obtained between log molecular weight and retention time (Fig. 1). Thyroglobulin (MW = 670,000), eluting at the void volume of these columns with a retention time of 10.50 min, was excluded from this plot. Therefore, these columns provided an estimate of protein molecular weight from the retention

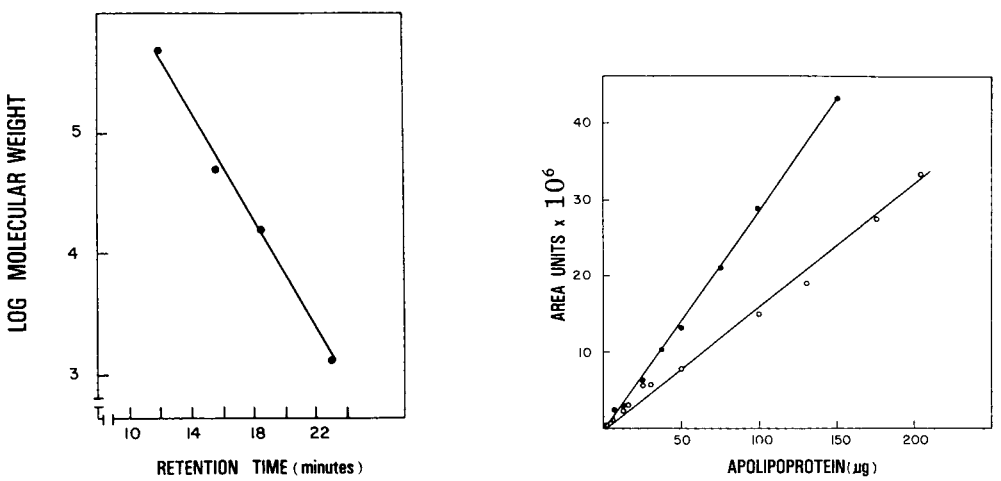

Fig. 1. Plot of log molecular weight versus retention time. A mixture of thyroglobulin, γ-globulin, ovalbumin, myoglobin, and cyanocobalamin was resolved on TSK 125 and 250 size exclusion HPLC columns as described. Thyroglobulin (MW = 670,000) eluted at the void volume of the columns with a retention time of 10.50 min (data not shown). The retention time of each protein was plotted against log molecular weight.

Fig. 2. Plot of apo A-I and apo A-II standard amounts versus area units. Varying amounts of apo A-I (•) and apo A-II (○) were resolved by size exclusion HPLC as previously described. The area of each peak was determined and each peak area was plotted against the amount of protein (μg). Each data point represents the average of at least three separate injections of each apoprotein in a concentration of 0.5 μg/μl eluent buffer. The coefficient of linearity for apo A-I was 0.999, and for apo A-II 0.994.

time values. Varying amounts of apo A-I and apo A-II (Calbiochem-Behring) were chromatographed, and a linear correlation between amount (μg) protein and the area of the resultant peak was shown (Fig. 2). Peak retention times and peak areas of standard apo A-I and apo A-II chromatograms were measured to ascertain levels of apo A-I and apo A-II present. A standard curve was regenerated prior to each analysis and less than 5% variation was observed.

Chromatography of the apo A-I standard (50 μg) in a concentration of 18 μM resulted in an elution profile shown in Fig. 3. Fractions were collected and analyzed for protein content, and approximately 46.4 μg were detected, representing a recovery of 93%. Similar results were obtained for the apo A-II standard and human apo-HDL isolates (data not shown). Therefore, this methodology provided a high-recovery system for the resolution and quantitation of apo A-I and apo-A-II on the basis of a molecular weight separation by HPLC.

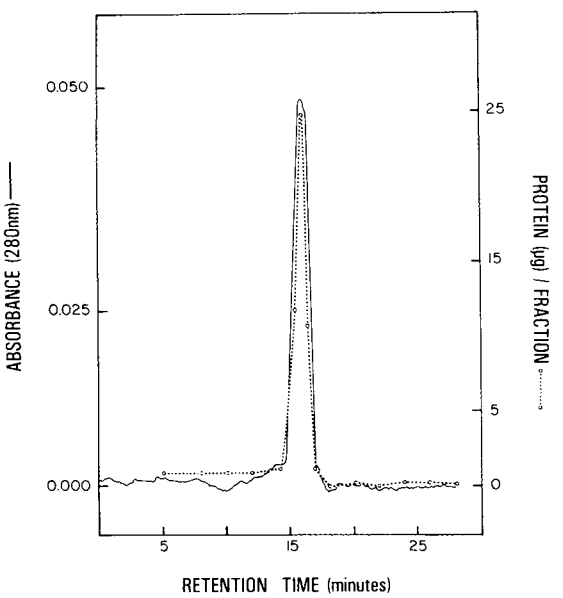

Fig. 3. Elution profile of apo A-I on TSK 250 and TSK 125 columns. Apo A-I, 50 μg in a concentration of 0.5 μg/μl, was resolved by HPLC as described, and column eluates were monitored for protein content at 280 nm (—). Fractions of 0.3 ml volume were collected and analyzed for protein content using the Bio-Rad microassay (o- - -o).

Chromatography of apo-HDL on TSK-125 and TSK-250 HPLC columns

Size exclusion HPLC of human apo-HDL resulted in an elution profile as depicted in Fig. 4B. Human apo-HDL was resolved into three major peaks with retention times of 13.50 min, 15.62 min, and 17.09 min. When a mixture of commercially available apo A-I, A-II, and C-III peptides was resolved by size exclusion HPLC apo A-I eluted at 15.50 min, apo A-II at 17.16 min, and apo C-III at 18.91 min (Fig. 4A). The major difference between these elution profiles was the appearance in the human apo-HDL profile of a major peak with retention time 13.50 min. This peak at 13.50 min represented a protein or

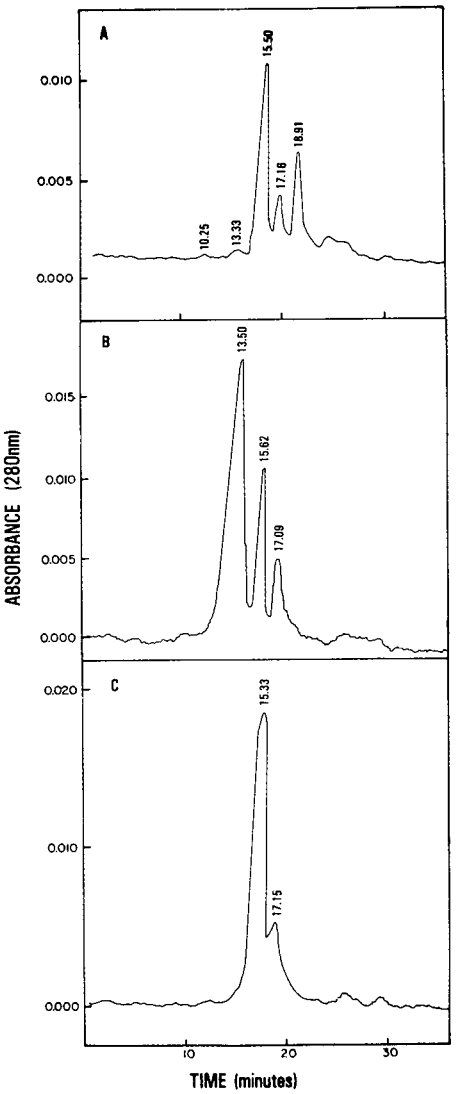

Fig. 4. Size exclusion HPLC of apolipoproteins. (A) A mixture of apo A-I (15 μg), apo A-II (3 μg), and apo C-III (3 μg), each obtained from Calbiochem-Behring, was resolved by size exclusion HPLC under previously described conditions. Retention times for each apolipoprotein were determined by separate injection of each protein (data not shown). Apo A-I, apo A-II, and apo C-III eluted with retention times of 15.50 min, 17.16 min, and 18.91 min, respectively. (B) An aliquot of 25 μl human apo-HDL (1 μg/μl protein) was chromatographed under the same conditions. Three peaks were resolved: peak I, retention time 13.50 min; peak 2, retention time 15.62 min, corresponding to 4.5 μg apo A-I; and peak 3, retention time 17.09, representing 3.8 μg apo A-II. (C) Prior to resolution by HPLC, 30 μl of the apo-HDL preparation was adjusted to 0.1% SDS at 25°C. The aliquot was chromatographed immediately after mixing on size exclusion HPLC columns, and two protein peaks were observed. The peak eluting at 15.33 min corresponded to 24.65 μg apo A-I and the second peak, with retention time 17.15 min to 3.5 μg apo A-II. All protein amounts were determined by comparing integrated areas of each peak to the areas obtained from standard apolipoprotein injections of known concentrations.

protein complex eluting from the TSK 250 and 125 columns with an apparent molecular weight, MW = 120,000. Adjustment of the eluent buffer from 0.2 *M* to 2.0 *M* sodium chloride had no disruptive effect on the peak at 13.50 min. When the respective peaks were collected and injected again, each peak chromatographed with the same retention time, although the peaks were broader and some material (< 20%) eluted at the void volume (data not shown). When apo-HDL was adjusted to 0.1% SDS (w/v) the peak at 13.50 min was abolished and an increase in the 15.33 min peak was observed (Fig. 4C). Also in Fig. 4C, the resolution of apo A-II was obscured somewhat by the descending shoulder of the monomeric A-I peak.

Hybridot analysis of eluates from size exclusion HPLC of apo-HDL

In order to determine the composition of the peaks obtained from size exclusion HPLC of the human apo-HDL isolate, column eluate fractions were analyzed immunologically for the presence of apo-A peptides. Fractions were collected and subjected to hybridot analysis against antisera to apo A peptides (Fig. 5). All three protein peaks resolved by HPLC of apo-HDL displayed immunoreactivity to antisera against apo A peptides. Separate elutions of standard apo A-I, apo A-II, and bovine serum albumin were screened with apo A antisera under described conditions. Apo A-I and apo A-II standards isolated directly from HPLC displayed reactivity (Fig. 5), but bovine serum albumin did not (data not shown).

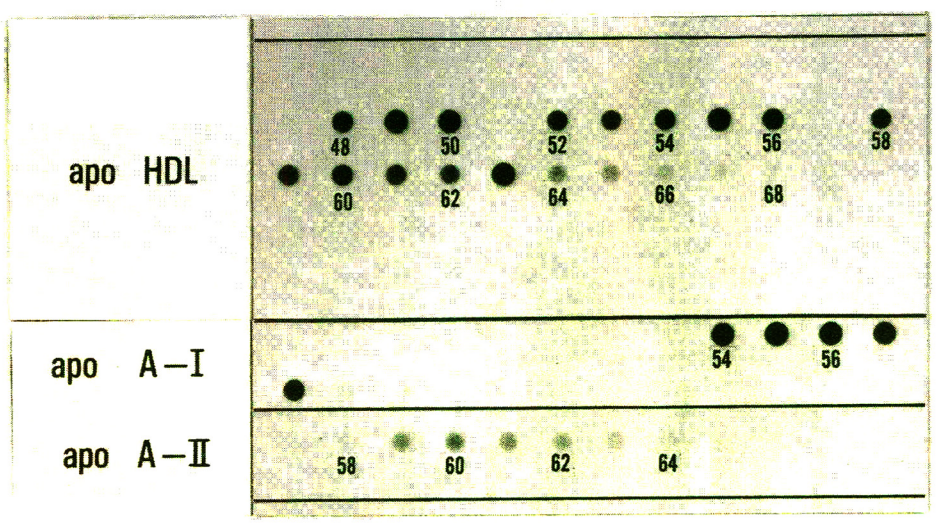

Fig. 5. Autoradiographic screening of HPLC column eluates for reactivity to antisera against apo A peptides. Apo-HDL, apo A-I and apo A-II were chromatographed separately and collected as described. For the human apo-HDL isolate: fractions 48—52 represent peak 1 (t_R 13.50 min); fractions 53—58, peak 2 (t_R 15.62 min); and fractions 59—68, peak 3 (t_R 17.09 min). The apo A-I standard displayed immunoreactivity in fractions 54—58 and the apo A-II standard reacted with antisera in fractions 58—64.

SDS—PAGE

In order to establish the identities of the peaks obtained from HPLC resolution of apo-HDL. fractions corresponding to the appropriate peaks were analyzed by SDS—PAGE (Fig. 6). The total apo-HDL isolate was subjected to SDS—PAGE in lane 5 and appeared as two major bands corresponding in mobility to apo A-I and apo A-II. When fractions collected from HPLC resolution of human apo-HDL were analyzed by SDS—PAGE, both the first peak, t_R = 13.50 min (lane 2), and the second peak, t_R = 15.62 min (lane 3) contained predominately a protein with approximate MW of 24,000. This protein comigrated with apo A-I standard, applied either directly (lane 6) or after HPLC (lane 9). The third peak t_R = 17.09 min, was resolved into two components with MW = 24,000 and 17,500 (lane 4). Although contaminated with apo A-I, this fraction was enriched in apo A-II as shown by co-migration with the apo A-II standard (lane 7).

In order to confirm the identities of the protein bands resolved by SDS—PAGE analysis, parallel slab gel was subjected to immunoblot analysis (Fig. 7). Each apolipoprotein demonstrated immunoreactivity to anti-apo A antisera. Total apo-HDL contained three cross-reacting proteins (lane 5). The

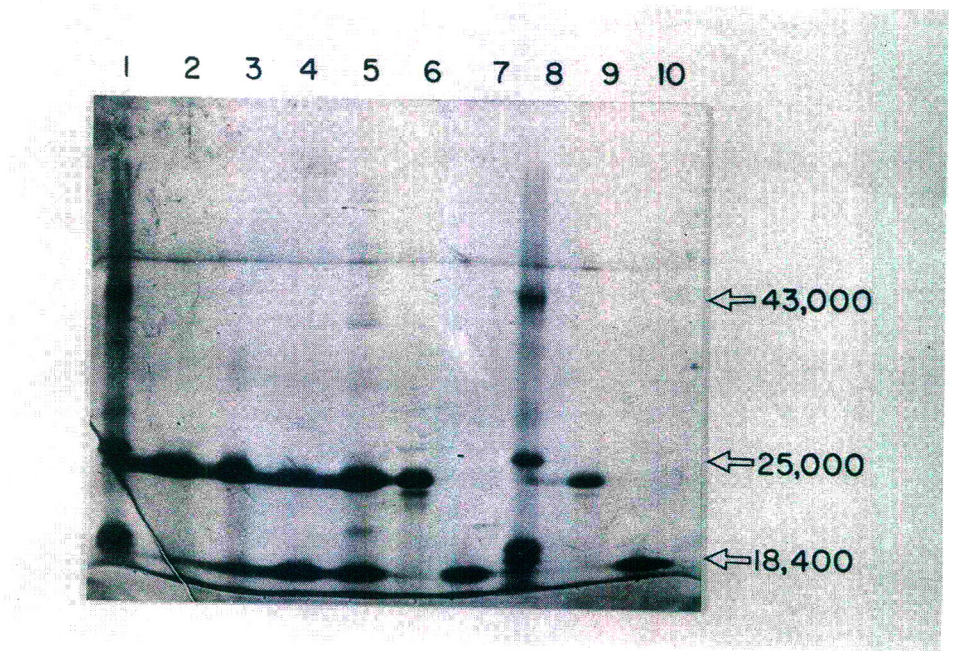

Fig. 6. SDS—PAGE of apolipoprotein standards and apo-HDL before and after resolution by HPLC. Ten injections each of 35 μg apo-HDL were chromatographed, collected, and prepared for electrophoresis as previously described. Lanes 1 and 8 contained as protein molecular weight standards ovalbumin, α-chymotrypsinogen, β-lactoglobulin, lysozyme/cytochrome c. Lanes 2, 3, and 4 contained human apo-HDL isolates from HPLC representing the peaks eluting with retention times of 13.50, 15.62, and 17.09 min, respectively. Lane 5 contained apo-HDL prior to resolution by HPLC. Lanes 6 and 7 contained commercial apo A-I and apo A-II standards prior to injection, respectively, and lanes 9 and 10 contained apo A-I and apo A-II after resolution by HPLC, respectively. Proteins were visualized by silver staining.

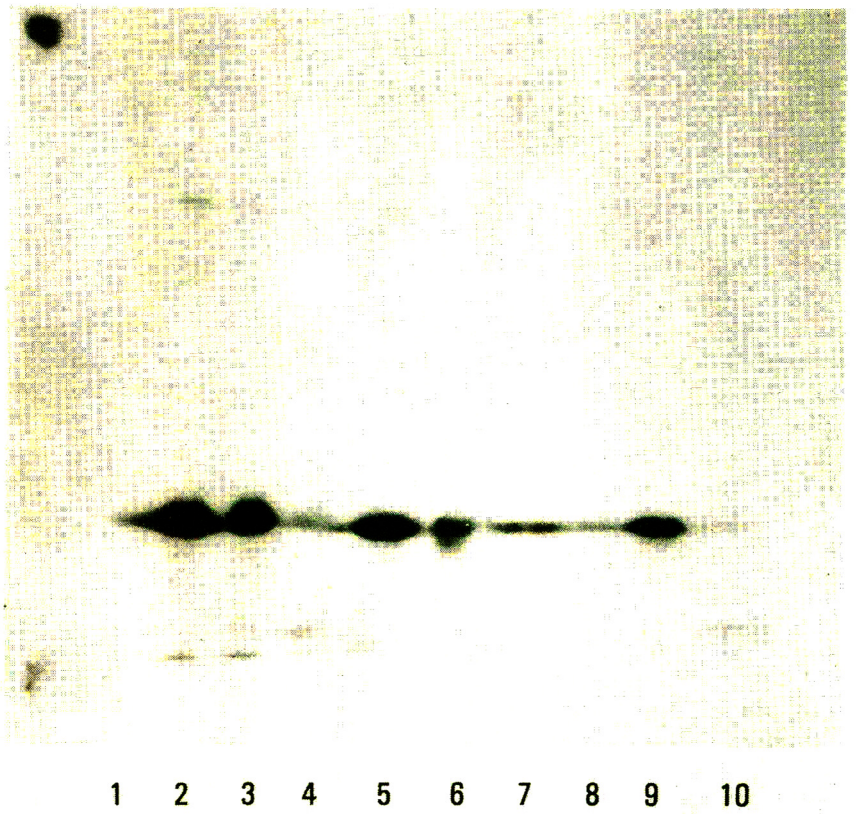

Fig. 7. Immunoblot detection of apolipoproteins resolved by SDS—PAGE. Apolipoproteins were prepared and subjected to SDS—PAGE and transferred to nitrocellulose as previously described. Lanes 10 and 9 represented aliquots of apo A-II and apo A-I isolated from HPLC, lanes 7 and 6 represented apo A-II and apo A-I prior to resolution by HPLC. Lane 5 contained apo-HDL prior to resolution by HPLC. Lanes 2, 3, and 4 correspond to apo-HDL peaks eluting with retention times of 13.50, 15.62, and 17.09 min, respectively.

predominant protein band comigrated with apo A-I standards (lane 6 or 9). Two faster migrating bands in the total apo HDL lane were detected faintly with the antiserum. One of these bands comigrated with the dye front and may represent apo C peptides. The other band, slightly above the dye front, migrated with the apo A-II standards (lane 7 or 10). Lanes 2, 3, and 4 corresponded to peak 1 (13.50 min), peak 2 (15.62 min) and peak 3 (17.09 min), respectively, after fractionation of human apo HDL by HPLC. The peaks at 13.50 min (lane 2) and at 15.62 min (lane 3) contained largely apo A-I. The peak at 17.09 min (lane 4) was identified as apo A-II. The commercial antisera (Calbiochem-Behring) appeared to react predominantly with apo A-I and much less with apo A-II. The relative lack of radioactivity bound at the apo A-I position in the peak 3 fraction (lane 4) indicated much better resolution of apo A-II from apo A-I than indicated by silver-stained gel (Fig. 6, lane 4). Since silver staining occurs predominantly on the gel surface, the apo A-I protein content detected by silver staining may be over-estimated from possible

Incubation of apo-HDL with unilamellar phospholipid vesicles

To determine if apo A-I recovered from HPLC of human apo-HDL retained biological activity, the ability of the apolipoprotein to disrupt unilamellar phospholipid vesicles and form HDL-like phospholipid particles was examined. Apo-HDL was resolved by HPLC and fractions corresponding to both apo A-I peaks were collected and pooled. In other HPLC elutions, the associated form of apo A-I was collected separately from the monomeric apo A-I. Bovine serum albumin was chromatographed also. In separate experiments, monomeric apo A-I, self-associated apo A-I or bovine serum albumin was incubated (167 μg protein/ml) with radiolabeled egg yolk phosphatidylcholine (PC) vesicles (445 μg/ml PC) as described in Experimental. After incubation, aliquots of each mixture were chromatographed on a Biogel A-15 M gel filtration column. Unilamellar vesicles prior to incubation eluted from Biogel A-15M as a symmetrical peak at 104 ml (Fig. 8). When the monomeric apo A-I population isolated from HPLC was incubated with the vesicles, and incubation mixtures were resolved on Biogel A-15M, two peaks of PC radioactivity were detected. One represented unilamellar vesicles (104 ml) and another represented a smaller phospholipid particle eluting within the size range of HDL (Fig. 8). As shown in Table I, incubation of unilamellar vesicles with the apo A-I monomer resulted in the transfer of approximately 58% of the PC from the vesicle to this smaller particle. Similar results were obtained with the self-associated form of apo A-I when incubated under the same conditions (Table I). In addition, vesicle disruption occurred following incubation with a mixture of both apo A-I peaks and with the total apo-HDL preparation prior to resolution by HPLC

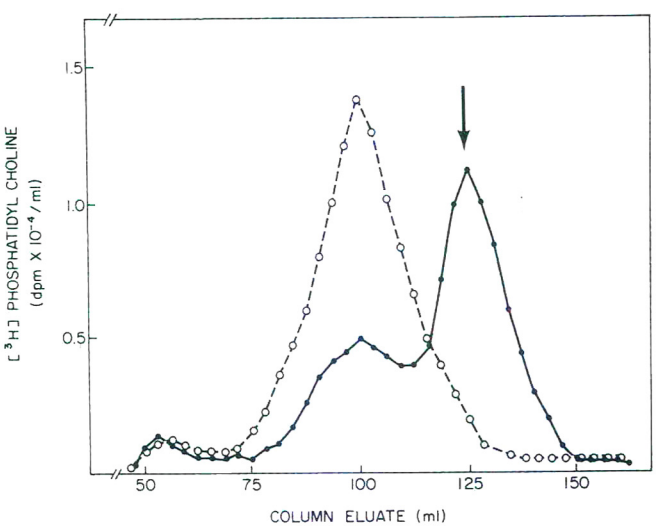

Fig. 8. Effect of apo-A-I isolated by HPLC on unilamellar vesicles. [³H]labeled egg PC vesicles (265 μg) were incubated in a total volume of 600 μl for 18 h at 37° C in the absence (○- - -○) and presence (●—●) of the apo A-I monomer (100 μg) isolated by HPLC. Aliquots of 550 μl were resolved on a Biogel A-15M gel filtration column (2.6 × 30 cm) and eluted fractions were analyzed for radioactivity. Recovery of radioactivity averaged 90%. The void volume of the column occurred at 56 ml, and the internal volume was found at 164 ml. The arrow indicated the elution volume of HDL.

TABLE I

EFFECT OF DIFFERENT PROTEIN FRACTIONS UPON THE DISRUPTION OF PHOSPHOLIPID VESICLES

All incubations included tritium-labeled vesicles (445 μg/ml egg yolk PC) and were conducted for 18 h at 37°C. Aliquots were applied to a Biogel A-15M column for estimation of lecithin disruption. The values shown are the average of at least three separate experiments.

Substance added to vesicles	Percentage labeled phospolipid disrupted
No addition	1
Bovine serum albumin* (167 μg/ml)	1
Apo-HDL** (167 μg/ml)	65
Apo A-I monomer* (167 μg/ml)	58
Apo A-I aggregate* (167 μg/ml)	46
Apo A-I monomer* (84 μg/ml) + apo A-I aggregate* (84 μg/ml)	49
Apo A-I*** (167 μg/ml)	60

*After resolution by HPLC.
**Not resolved by HPLC.
***Calbiochem-Behring, prior to HPLC.

(Table I). No vesicle disruption was detected when bovine serum albumin was incubated with the unilamellar vesicles. Thus, both monomeric and associated apo A-I isolated from size exclusion HPLC can disrupt unilamellar vesicles to form smaller phospholipid particles eluting in the size range of human HDL.

DISCUSSION

A non-denaturing size exclusion HPLC method has been developed for the isolation and quantitation of apo A-I and apo A-II from human HDL. By utilizing the TSK 250 and TSK 125 analytical columns in series, it was possible to obtain reliable estimates of apolipoprotein molecular weight. Also, a linear response was obtained for the amount of apo A-I and apo A-II resolved, thus providing a high-recovery means for determining apolipoprotein concentrations. Human apo-HDL was resolved into three separate peaks in the absence of denaturants. These peaks were identified as a self-associated form of apo A-I, monomeric apo A-I, and apo A-II. Furthermore, apo C peptides were resolved from apo A-I and apo A-II peaks, when a mixture of these proteins was applied to the column. Apo C peptides were not readily detected in apo HDL fractionations because of their relatively low concentrations in apo HDL preparations.

Our data suggested that native apo A-I separated from apo-HDL existed in both polymeric and monomeric forms. The polymeric form observed in the first HPLC peak (t_R = 13.50 min) appears to be a tetrameric complex of apo A-I. Vitello and Scanu [18] have observed the association of human apo A-I into dimers, tetramers, and octomers by resolution on Sephadex G-75 using a non-denaturing buffer. Within the concentration range of $5 \cdot 10^{-7}$ M and $5 \cdot 10^{-4}$ M, bovine apo A-I molecules displayed both tetrameric and

monomeric forms [19]. In our initial solutions of human apo-HDL, apo A-I was present at approximately $2 \cdot 10^{-5}$ *M* and would be within this concentration range. It is of interest to note that apo A-II also has been reported to self-associate in aqueous solution in a concentration dependent manner [20]. In our HPLC system, we observed no apo A-II association, perhaps because the apo A-II levels were too low. The apo A-I standard from Calbiochem-Behring did not form tetramers as readily as the apo A-I molecules isolated from human donors in this laboratory. It is possible that the commercial apo A-I may have been altered by the electrophoretic conditions used for purification such that it no longer readily self-associated within the concentration ranges described by Jonas [19]. Other investigators have resolved apolipoproteins from apo-HDL utilizing conventional gel filtration columns equilibrated with 6—8 *M* urea [19, 21, 22]. Also, apolipoproteins from HDL have been isolated by preparative size-exclusion HPLC using buffered 6 *M* urea or 6 *M* guanidinium chloride in the column eluates [5, 6]. On Sephadex G-150 bovine apo-HDL was resolved into three major peaks [19]. The peak with the highest molecular weight had an estimated molecular weight of 80,000 daltons; the second was 27,000 daltons, and the third approximately 17,000 daltons. Jonas [19] proposed that the first peak represented a tetrameric form of apo A-I and our results appear to confirm this observation with human apo A-I. We have not utilized urea, guanidinium chloride, or other agents known to solubilize apolipoproteins. In our hands, apo-HDL has remained in solution when dialyzed against either 1.0 *M* sodium chloride for ether extractions or 0.2 *M* sodium chloride for ethanol—diethyl ether extractions. The preparation was quickly frozen in liquid nitrogen at a concentration of 1—1.5 mg/ml protein and defrosted within 5 min of its removal from liquid nitrogen. This storage did not alter elution profiles or protein content, and apo-HDL solutions have been stored for as long as six months. Another factor that may have contributed to the successful fractionation without denaturants may have been the rapid resolution of apo-HDL components accomplished by size exclusion HPLC. Within 20 min these components were resolved with minimal dilution of the sample. Since gel filtration columns can take as long as 8 h to elute, it is possible that more column—apolipoprotein interactions may develop necessitating the use of chemical denaturants.

The monomeric and polymeric forms of apo A-I after resolution by HPLC retained the ability to disrupt unilamellar phospholipid vesicles. When spherical HDL was mixed with phospholipid dispersions or vesicles, apo A-I disassociated from HDL to disrupt vesicle bilayers and form discs [23]. It has been proposed that disc-like precursor HDL molecules, composed largely of phospholipid and apo A-I, give rise to mature HDL molecules through the action of lecithin cholesterol acyl transferase [24, 25]. Thus the insertion of apolipoprotein molecules into lipid bilayers is a critical event for the assembly of plasma lipoproteins, and regardless of the origin of HDL molecules, apo A-I is involved in interactions with phospholipids at various stages in HDL metabolism [26]. In aqueous solution apo A-I molecules may self-associate in order to stabilize the amphiphilic apoprotein molecule in the absence of phospholipid. Apo A-I molecules are most stable when associated with a phopsholipid bilayer to form a discoidal particle, followed by association with spherical HDL, and are least

stable in aqueous solution without lipid [23]. In our experiments, both monomeric and polymeric A-I molecules when incubated with phospholipid associated with phospholipid bilayers leading to disruption of vesicles to form smaller HDL discs. Jonas [19] proposed that the bovine tetrameric A-I aggregate may bind preferentially to protein rather than phospholipid based on the more non-polar components of bovine HDL as compared to human HDL composition. Since bovine apo A-I interacts with a larger amount of apolar lipid, it may develop stronger interprotein bonding than human apo A-I [19]. Therefore, our data suggest that both apo A-I fractions isolated here retained biological activity.

Using this method, native apolipoproteins can be isolated directly from HPLC columns and utilized for metabolic studies concerning apolipoprotein interaction with phospholipids and other models of in vivo phenomenon. Furthermore, this system provided a reliable means to determine apo A-I and apo A-II levels in human HDL and can be utilized in clinical studies to determine metabolic markers for coronary artery disease in humans.

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CHROMBIO. 2235

DETECTION OF CHANGES IN RABBIT SERUM PROTEINS AFTER PARTIAL HEPATECTOMY BY MEANS OF TWO-DIMENSIONAL ELECTROPHORESIS UNDER NON-DENATURING CONDITIONS

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SUMMARY

The changes in rabbit serum proteins after partial hepatectomy were examined by means of two-dimensional electrophoresis utilizing isoelectric focusing in a 4% polyacrylamide gel in the first dimension and a 4—30% pore gradient polyacrylamide gel in the second dimension. A rapid increase in seven proteins was observed after partial hepatectomy and a rapid decrease in two proteins. Major serum proteins, including albumin, immunoglobulin G, immunoglobulin M and α_2-macroglobulin, did not change.

The time course of the changes was examined using a densitometer; the maxima of the changes were observed on day 3 after partial hepatectomy.

INTRODUCTION

Since the first report of Higgins and Anderson [1] on the quantitative assessment of liver regeneration after partial hepatectomy, a large number of studies on the regenerating liver cells have been reported. However, what controls the proliferation after partial hepatectomy and how the rapid regenerative growth is stimulated are not yet fully clear. A series of specific substances is supposed to control the proliferation of liver cells, but it is also considered that metabolic changes after partial hepatectomy contribute to the stimulation of these substances. To answer this question, the changes in the serum concentration of various metabolites after partial hepatectomy have been studied [2—5]. The changes in serum proteins after partial hepatectomy, however, are not yet known except for some proteins [2—6] because of the insufficient resolution of the analytical techniques.

On the other hand, Manabe et al. [7] have described a two-dimensional

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electrophoretic technique without denaturing agent, and have shown that human plasma proteins can be resolved into about 250 spots. Since this technique does not use denaturing agents such as sodium dodecyl sulphate or urea during the electrophoretic run, it is suitable for the analysis of a mixture of soluble proteins without loss of their native physicochemical properties [8] or their biological activity [9].

In a previous paper [10] we applied this electrophoretic technique to analysing the changes in rat serum proteins after partial hepatectomy, and could detect changes in more than twenty serum proteins supposed to be closely related to the proliferation of liver cells. In this present paper we describe the changes in rabbit serum proteins after partial hepatectomy.

MATERIALS AND METHODS

Reagents

Ampholine (pH range 3.5—10) was obtained from LKB Produkter (Bromma, Sweden). Acrylamide, N,N′-methylenebisacrylamide, N,N,N′,N′-tetramethylethylenediamine (all special grade for electrophoresis), Tris base, glycine, sucrose and ammonium persulphate were purchased from Wako (Osaka, Japan). Coomassie Brilliant blue R-250 was purchased from Sigma (St. Louis, MO, U.S.A.).

Partial hepatectomy and serum samples

Male rabbits (Japanese White strain) weighing approximately 2 kg were used. Partial hepatectomy (about 60% of liver weight) was performed under light ether anaesthesia. After partial hepatectomy the rabbits were maintained for 1—20 days with food and water ad libitum. Sham operation was done in an identical manner to partial hepatectomy in terms of skin and muscle incision, manipulation of the liver and care of the wound. On days 0, 1, 2, 3, 4, 7, 10 and 20 after partial hepatectomy, blood (about 1 ml) was obtained from an ear vein using a disposable syringe. The blood was left to stand at 4°C for 30 min, and then centrifuged at 3000 *g* for 10 min. Sucrose was added to the serum to give a concentration of 10% (w/v), and the samples were stored at −20°C until use.

Two-dimensional electrophoresis

Two-dimensional electrophoresis was carried out as described in the previous report [10] with some modifications. Isoelectric focusing in the first dimension was performed on a gel column (14.5 cm × 0.5 cm I.D.). A 4% acrylamide (0.2% bisacrylamide) solution containing 2% Ampholine (pH range 3.5—10) and 0.05% ammonium persulphate was poured into the glass tube. After gelling, the bottom of the glass tube was covered with dialysis membrane, and placed in an electrophoresis chamber. Overlay solution (5% sucrose solution containing 2% Ampholine, 50 μl) was layered on top of the gel column and then the serum sample (50 μl) was applied below the overlay solution. The cathode electrode solution was 0.04 *M* sodium hydroxide, and the anode 0.01 *M* phosphoric acid. Electrophoresis was run at a 2-mA constant current per gel column for about 40 min (until the voltage reached 460 V), and then

460 V constant voltage for 20 h at 4°C. After electrophoresis, the gel was pushed out and placed directly on top of the second-dimension slab gel without equilibration.

Gradient polyacrylamide gel electrophoresis in the second dimension was performed with a slab gel apparatus which forms four slab gels of 12 cm long, 16 cm wide, 0.4 cm thick. A 4—30% acrylamide linear gradient (0.2% bisacrylamide) containing a 0—10% sucrose and a 0.05—0.025% ammonium persulphate linear gradient, was poured in about 50 min at 4°C. Gelling occurred in about 2 h in a water-bath at 30°C. The gradient gel buffer was 0.14 *M* Tris—HCl (pH 8.9), and the electrode buffer was 0.05 *M* Tris—0.38 *M* glycine (pH 8.3). Electrophoresis was run at a 36-mA constant current per slab gel for 20 h at 4°C.

Measurement of the pH gradient

The pH gradient was measured as follows. The first-dimension isoelectric focusing gel was duplicated for each sample. One gel was cut into 10-mm sections and these sections were placed in individual test tubes containing 2 ml of distilled water. These test tubes were allowed to stand for 2 h at room temperature, then the pH was measured on a pH meter.

Staining and destaining

Gels were stained for 24 h in 0.025% Coomassie Brilliant blue R-250—7% (v/v) acetic acid—50% (v/v) methanol. Destaining was done in 7% (v/v) acetic acid overnight at room temperature, and then at 80°C for 6 h. During the staining and the destaining the gel container was shaken gently.

Densitometry of Coomassie Brilliant blue stained spots

Densitometric quantitation of Coomassie Brilliant blue stained spots on the slab gel was carried out with a Shimadzu dual-wavelength thin-layer chromatographic scanner CS-900 (Shimadzu, Tokyo, Japan). Sample wavelength was 580 nm (reference wavelength 750 nm) and the Coomassie Brilliant blue stained spots were measured by transmission.

RESULTS

Several serum samples of normal rabbits (Japanese White strain, approximately 2 kg) were subjected to two-dimensional electrophoresis, the protein distributions were compared, and the positions of the serum proteins were reproducible. Fig. 1 shows an example of the two-dimensional electrophoretic pattern of normal rabbit serum. Major serum proteins were located on the gel by comparing the patterns with those of human plasma proteins [11]; some of these are shown in the figure.

The time course of the changes in the two-dimensional patterns of serum proteins after partial hepatectomy was examined. Serum samples obtained on days 1, 2, 3, 4, 7, 10 and 20 after partial hepatectomy were subjected to the two-dimensional electrophoresis and the protein distributions were compared with those of the normal serum pattern. Rapid changes were observed for several proteins; these are shown by arrows in Fig. 1 (proteins in area C are

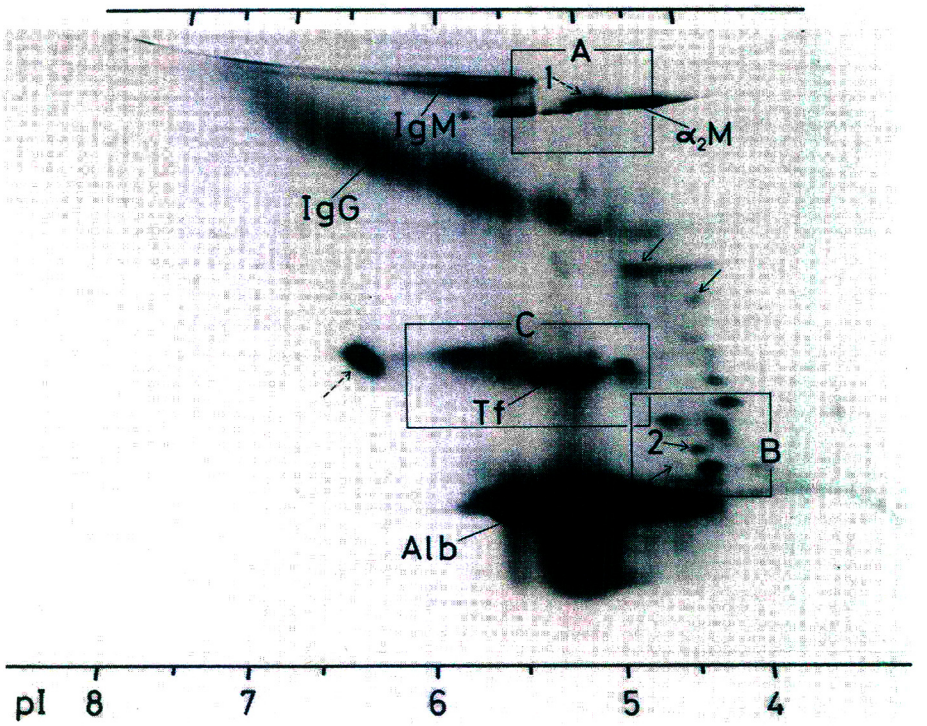

Fig. 1. Two-dimensional electrophoretic pattern of normal rabbit serum proteins. Dotted arrows indicate the decreased proteins and solid arrows indicate the increased proteins after partial hepatectomy. The positions of major serum proteins were tentatively located on the slab gel by comparing the two-dimensional electrophoretic pattern of rabbit serum with those of human plasma proteins [11]. IgM = immunoglobulin M, IgG = immunoglobulin G, α_2M = α_2-macroglobulin, Tf = transferrin, Alb = albumin.

shown in Fig. 2C). Dotted arrows show the proteins that are apparently decreased after partial hepatectomy and the solid arrows show the proteins that are apparently increased. Some major serum proteins, such as albumin, immunoglobulin G, immunoglobulin M and α_2-macroglobulin, did not change after partial hepatectomy.

Fig. 2 shows the time courses of the proteins particularly changed after partial hepatectomy. The proteins and the areas on the acrylamide slab gels are shown in Fig. 1. The protein of spot 1 rapidly decreased after partial hepatectomy, was minimal on day 3, then gradually increased and almost recovered its original level on day 20. The protein of spot 2 rapidly increased after partial hepatectomy, was maximal on day 3, and almost returned to its original level on day 20. Transferrin was observed as several spots over a wide pI range, but after partial hepatectomy distribution of its basic side changed more than that of its acidic side.

Quantitation of proteins on the acrylamide slab gels was carried out with a Shimadzu TLC scanner. Fig. 3 shows the time courses of the changes of protein spot 1 and protein spot 2. The extent of the changes was expressed as relative amount (amount before partial hepatectomy = 100%). It was demon-

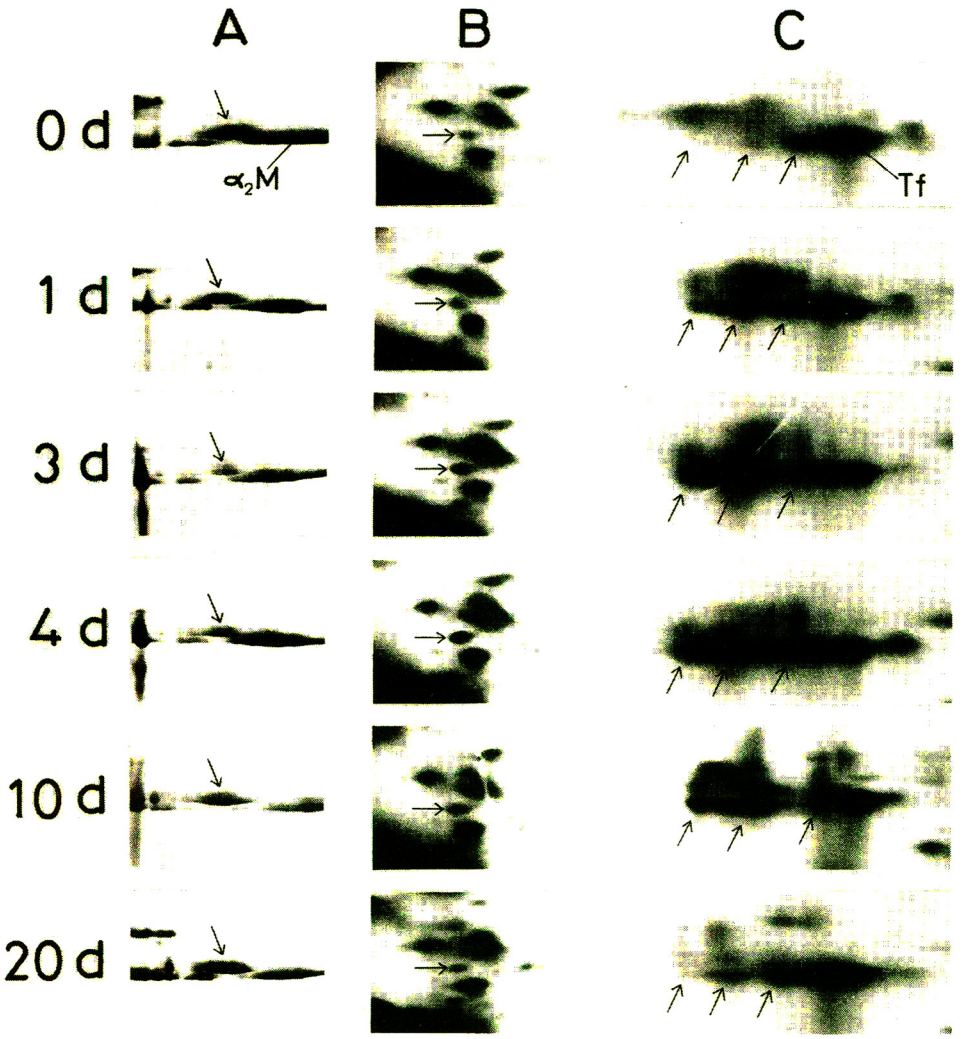

Fig. 2. Time courses of the changes in the two-dimensional pattern of rabbit serum proteins after partial hepatectomy. Serum samples taken on days 0, 1, 2, 3, 4, 7, 10 and 20 after partial hepatectomy were analysed by two-dimensional electrophoresis. Time-dependent changes on the gel sections in areas A, B and C (indicated in Fig. 1) are shown. Protein positions that changed rapidly after partial hepatectomy are indicated by arrows. α_2M = α_2-macroglobulin, Tf = transferrin.

strated that the protein amount of spot 1 decreased below 10% of its initial level on day 3, and the protein amount of spot 2 increased above 300% of its original level on day 3.

DISCUSSION

Changes in serum proteins after partial hepatectomy have been studied by means of one-dimensional electrophoretic techniques, such as cellulose acetate membrane electrophoresis [3] and polyacrylamide gel electrophoresis

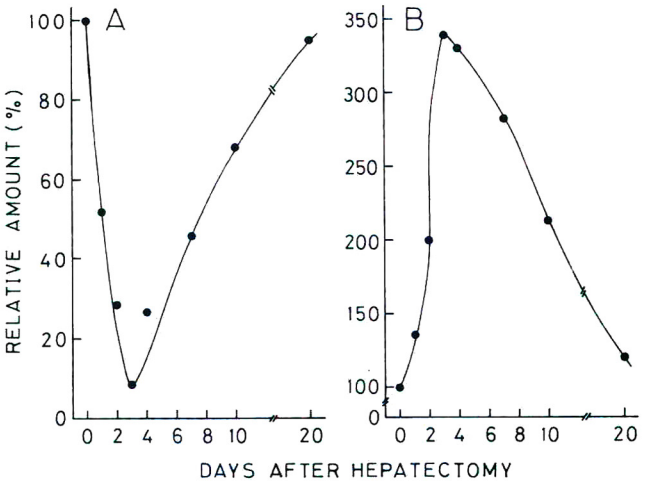

Fig. 3. Densitometric quantitation of proteins on acrylamide slab gels. The quantitation of protein spots 1 and 2 was carried out with a Shimadzu TLC scanner. Time-dependent changes are shown by relative amounts (amount before partial hepatectomy = 100%). (A) protein spot 1; (B) protein spot 2.

[5]. These one-dimensional electrophoretic techniques, however, could offer little information about the changes in the serum proteins supposed to be closely related to the proliferation of liver cells after partial hepatectomy. As shown in Figs. 1 and 2, the two-dimensional electrophoresis described in this paper can detect the changes in serum proteins after partial hepatectomy. Since this technique does not require equilibration of the first-dimension gel, there is no loss of proteins. Therefore, the accurate time-dependent changes in amounts of proteins on acrylamide slab gels can be measured using a thin-layer scanner. The quantitation of rapidly changed proteins will be useful in estimating the restoration of liver cells after partial hepatectomy.

It is also possible that the changes in serum proteins described above are due not only to partial hepatectomy but also to the operation wounds. Therefore, we analysed the serum of sham-operated rabbits by two-dimensional electrophoresis and compared the levels of serum proteins with those after partial hepatectomy. However, no changes were observed except for transferrin, which after sham operation showed changes similar to those shown in Fig. 2C, but the extent was not so much as that after partial hepatectomy. Although the function and role of these changes are still unclear, we suppose these proteins contribute, by themselves or with other substances, to the proliferation of liver cells after partial hepatectomy.

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CHROMBIO. 2252

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PROCEDURE FOR THE DETERMINATION OF DI-(2-ETHYLHEXYL)PHTHALATE IN HUMAN BLOOD SPECIMENS

PROBLEMS OF VARIABLE-EXTRACTION YIELD AND THE USE OF STANDARD ADDITION FOR CALIBRATION

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SUMMARY

A high-performance liquid chromatographic procedure was developed for the determination of di-(2-ethylhexyl)phthalate (DEHP) concentrations in human whole blood samples. The solvent extraction of DEHP was found to be highly variable between samples obtained from different subjects (coefficient of variation of 30.4%). The recovery of DEHP following extraction with ethyl acetate was negatively correlated with serum lipid content, as expressed by the sum of serum cholesterol and triglyceride concentrations ($r = -0.864$). The technique of standard addition of DEHP allowed a single-point calibration of DEHP extractability in individual blood samples, and provided an accurate estimation of DEHP concentration (coefficient of variation of approximately 6% in replicate samples). The potential for intersample variability in the solvent extraction of other highly lipid-soluble compounds should be considered.

INTRODUCTION

Di(2-ethylhexyl)phthalate (DEHP) is a plasticizer commonly used in the production of polyvinyl chloride plastics, comprising up to 40% by weight of

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some medical plastic products such as blood storage bags and flexible tubings [1]. Animal studies have indicated that this compound can induce hepatomegaly and affect a variety of biochemical functions of the liver [2]. A recent long-term toxicity study has also revealed its capacity for producing hepatic tumors in rodents [3]. Concerns over the potential toxicity of DEHP and other plasticizers have led to the problem of assessing the degree of exposure to plasticizers in various hospital patient populations. Several investigations have been directed towards identifying the presence of plasticizers in blood and tissues of those patients likely to be at risk of exposure. For example, significant concentrations of DEHP have been reported in patients with end-stage renal failure requiring maintenance hemodialysis [4—6] and surgical patients who have received blood transfusions [6] or undergone cardiac bypass [7].

In order to further characterize the extent of exposure and the disposition kinetics of DEHP, an accurate and reliable method for the measurement of the phthalate ester in various biological fluids or tissue specimens is required. Several chromatographic procedures have been reported for the quantitation of DEHP in blood or plasma [4, 6, 8, 9]. During our attempt to adopt the published assays for DEHP in human blood samples, we encountered a high degree of intersubject variability in the solvent extraction of the plasticizer from whole blood. Although in principal the variability problem can be overcome by preparing calibration standards using each subject's own blank blood, in practice it poses a serious dilemma as completely DEHP-free blood cannot be obtained.

The present study was undertaken to determine whether any specific biochemical factors could be identified which may be responsible for the observed variability in the chemical extraction of DEHP. Furthermore, an assay methodology involving the technique of standard addition was developed for the determination of DEHP concentrations in individual whole blood samples.

EXPERIMENTAL

Chemicals

All solvents were of HPLC grade and were purchased from a commercial source (J.T. Baker, Phillipsburg, NJ, U.S.A.). DEHP (Aldrich, Milwaukee, WI, U.S.A.) and di-*n*-octylphthalate (DOP, Eastman-Kodak, Rochester, NY, U.S.A.) were used without further purification.

Subjects

Blood samples (20 ml) were obtained from thirteen normal volunteers for the purposes of determining the individual variation in DEHP calibration curves and relating the extraction of DEHP from blood to serum lipid and α_1-acid glycoprotein (AAG) concentrations. Blood was drawn through a metal needle into glass syringes. A 10-ml aliquot was set aside and stored in glass vials containing heparin (20 U/ml) at −20° C. The serum fraction was separated from the remaining blood and stored in glass vials at −20° C.

DEHP extraction

Ethyl acetate (5 ml) containing 1 μg/ml of the internal standard DOP was

added to 0.5 ml of heparinized whole blood samples in acid-washed glass culture tubes (16 × 125 mm with PTFE-lined caps, American Scientific Products, McGaw Park, IL, U.S.A.). Preliminary experiments indicated no difference in the extraction of DOP added with the extraction solvent as opposed to added prior to extraction. DOP was subsequently added with the ethyl acetate to facilitate the processing of samples. The tubes were vortexed vigorously (15 sec), shaken (15 min), and centrifuged (1800 *g*, 15 min). The organic layer was transferred to a fresh glass tube, evaporated to dryness at 50° C under a stream of dry nitrogen, and reconstituted with 100 μl methanol.

Chromatographic analysis

Aliquots (10—80 μl) of the reconstituted extracts were analyzed by high-performance liquid chromatography (HPLC). The chromatograph consisted of a Waters Assoc. (Milford, MA, U.S.A.) Model 6000A constant flow-rate pump, a U6K variable-volume injector, and a Model 440 UV absorbance detector. Separation was achieved with a Partisil 5 ODS Rapid Analysis Column, particle size 5 μm (Whatman, Clifton, NJ, U.S.A.). The mobile phase consisted of methanol—water (93:7). The high methanol content was required due to the extremely lipophilic nature of DEHP. The flow-rate was set at 3.0 ml/min with a column back pressure of 7 MPa. Absorbance of the eluent was monitored at 254 nm, and peak area ratios (DEHP to DOP) were determined with a Hewlett-Packard (Avondale, PA, U.S.A.) Model 3390A reporting integrator. Interday variability in instrument response, as assessed by daily injections of a standard methanolic solution containing DEHP and DOP, was 1.8%.

Linearity of DEHP calibration curves

Blood from ten of the thirteen normal volunteers was spiked with DEHP to yield a series of blood standards containing 0, 0.5, 1.0, 2.5, and 5.0 μg/ml plasticizer. A 25-μl aliquot of a methanolic solution of DEHP was added to acid-washed glass centrifuge tubes (16 × 125 mm) and the methanol was gently evaporated under a stream of dry nitrogen. Lysed whole blood was added to each tube, followed by brief vortexing. No DEHP was lost during this preparation. The blood samples were extracted and analyzed by HPLC as described in the preceding sections. Peak area ratios were plotted versus DEHP concentration for each individual set of blood standards, and linear calibration plots were fitted with least-squares regression lines.

Correlation of ethyl acetate extractability with serum biochemistry

The extraction yield of DEHP from blood was assessed with 0.5-ml samples from thirteen normal volunteers to which a known amount (2.5 μg) of DEHP had been added. Since blood from all volunteers contained a measurable amount of DEHP, the peak area ratio due to the spiked DEHP, i.e. $AR(s)$, was calculated:

$$AR(s) = AR(2) - AR(1) \quad (1)$$

where $AR(1)$ and $AR(2)$ are the peak area ratios before and after the addition of 2.5 μg (corresponding to 5 μg/ml) of DEHP. The increase in peak area ratio after the standard addition represents an assay response factor. The only require-

ment with this type of single-point calibration procedure is strict adherence to linearity in response. Extraction yield was calculated by referencing $AR(s)$ to the peak area ratio obtained by direct injection of a methanolic solution containing 5 μg DEHP and 10 μg DOP per ml.

Serum concentrations of triglycerides, total cholesterol, low-density lipoproteins (LDL) and high-density lipoproteins (HDL) were determined in the same samples using standard clinical chemistry procedures [10, 11]. Serum concentrations of AAG were measured with a commercial radial immunodiffusion kit (M-Partigen, Calbiochem-Behring, San Diego, CA, U.S.A.).

Validation of the standard addition method as a calibration procedure

Aliquots of pooled whole blood were spiked with DEHP to yield concentrations of 0, 0.5, 1.0, 2.5, and 5.0 μg/ml. Five 1.0-ml blood samples were prepared at each concentration. Each sample was divided into two 0.5-ml portions. The first portion was extracted directly, as described previously, while the remaining portion was added to a glass tube containing 2.5 μg DEHP (i.e. equivalent to an addition in concentration of 5 μg/ml). The split samples were analyzed as described in the preceding sections. The blood concentration of DEHP was calculated as follows:

$$\text{Sample DEHP concentration } (\mu\text{g/ml}) = AR(1) \times \frac{5\ \mu\text{g/ml}}{AR(2) - AR(1)} \qquad (2)$$

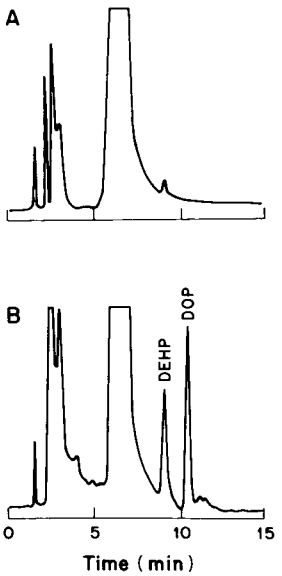
To determine the concentration of added DEHP, a mean peak area ratio was determined in five blank samples of pooled blood. This mean ratio was subtracted from $AR(1)$ and $AR(2)$ in order to eliminate the contribution of endogenous DEHP. The reproducibility of the procedure was assessed with five replicate samples at each concentration of DEHP.

RESULTS

Several organic solvents and solvent mixtures were tested to identify the optimum solvent for recovery of DEHP from pooled human whole blood samples. Hexane, diethyl ether, chloroform, chloroform—methanol (2:1, v/v), ethylene dichloride, methylene chloride, and methylene chloride—pentane (1:1, v/v) were all associated with unacceptably low extraction yields (i.e. $< 50\%$). Only ethyl acetate appeared to be a practical extraction solvent for this compound (mean recovery from pooled blood of 65.2%).

Representative chromatograms of extracts of blank and spiked blood samples are displayed in Fig. 1. DEHP and DOP eluted at 8.8 and 10.4 min, respectively. It should be noted that low concentrations of DEHP were present even in supposedly blank blood samples.

The calibration plots obtained with blood from ten subjects are presented in Fig. 2. Linear response was observed over the entire concentration range in all cases. Correlation coefficients of the regression lines ranged from 0.990 to 0.999. However, significant intersubject differences in the slope of the calibration plots were observed. The slope estimates varied over a two-fold range (0.0200—0.0475) with a coefficient of variation of 30.4%. The large variation in slope value suggested that the extractability of DEHP from whole blood was



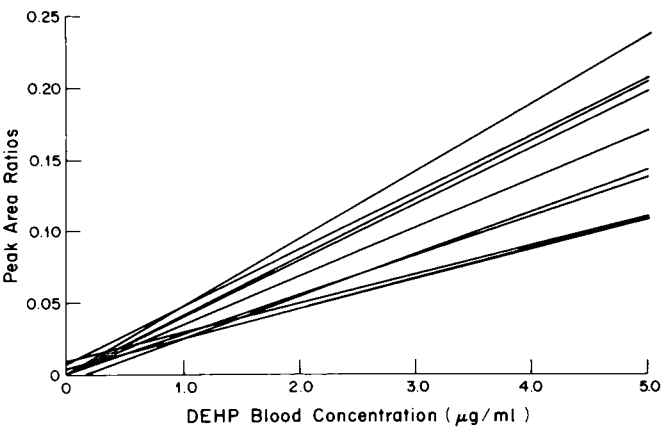


Fig. 1. Representative chromatograms following extraction of a whole blood sample obtained from a normal volunteer (A) and blood spiked with 5 μg/ml DEHP and 10 μg/ml DOP (B). The concentration of DEHP in the unspiked sample was 0.12 μg/ml.

Fig. 2. DEHP calibration plots generated in blood obtained from ten normal volunteers. The lines indicate the least-squares regression of the peak area ratio versus concentration data. Individual points have been omitted for clarity.

highly variable between subjects, while the apparent linearity of the individual calibration curves indicated that the extractability of DEHP was constant for a given sample within the range of DEHP concentrations studied.

A summary of DEHP extraction yield and relevant biochemical data for each subject is presented in Table I. The results of the correlation analyses between DEHP extraction yield and individual biochemical factors and combinations thereof are presented in Table II. Extractability of DEHP was negatively correlated with the concentration of each of the four indices of serum lipid content (viz. LDL, HDL, cholesterol, triglycerides), indicating that increases in serum lipid content result in a decrease in the extractability of DEHP. The strongest correlation was observed between DEHP extraction yield and the sum of serum cholesterol and triglycerides ($r = -0.864$, $p < 0.001$). This relationship is displayed in Fig. 3.

Although DEHP has been found to bind to AAG [12], the serum concentration of this protein did not correlate with the extraction yield of the plasticizer from blood. It is doubtful that intersubject differences in serum albumin concentration would contribute to the intersubject variation in extraction since DEHP does not appear to bind to this protein [13]. It is interesting to note that DEHP extraction appeared to increase with increasing hematocrit, suggesting that the compound is removed from the cellular blood fraction more readily than from the serum fraction. Considering that DEHP is concentrated in red blood cells relative to serum in a ratio of approximately 2:1 [14], extraction of DEHP from whole blood should allow a lower detection limit than extraction from the corresponding serum sample.

TABLE I

DEHP EXTRACTION YIELD AND SERUM BIOCHEMICAL CORRELATES

Subject	Extraction* (%)	Triglycerides (mg/dl)	Cholesterol (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	AAG (mg/dl)	Hematocrit (%)
1	50.4	82	187	58	113	96	42.5
2	69.7	71	165	46	105	100	48.0
3	50.9	115	167	96	48	84	42.8
4	39.3	154	193	56	107	94	42.0
5	60.5	84	211	68	126	92	46.4
6	56.0	82	233	42	175	102	46.4
7	67.8	97	164	49	96	ND**	43.7
8	58.8	66	183	60	110	98	47.3
9	89.0	63	138	38	87	ND	46.3
10	51.3	85	189	36	136	98	44.2
11	68.9	51	167	67	90	86	40.7
12	70.6	78	153	51	86	ND	47.2
13	74.6	66	150	71	66	ND	42.0

*Extraction from whole blood with ethyl acetate.
**ND = not determined.

TABLE II

CORRELATIONS OF EXTRACTION YIELD WITH SERUM BIOCHEMICAL FACTORS

Serum factor	Correlation coefficient	Statistical significance
Cholesterol (mg/dl)	—0.671	<0.02
Triglycerides (mg/dl)	—0.712	<0.01
HDL (mg/dl)	—0.322	>0.2
LDL (mg/dl)	—0.219	>0.4
Hematocrit (%)	+0.471	>0.1
AAG (mg/dl)	+0.547	>0.05
HDL + LDL (mg/dl)	—0.550	>0.05
Cholesterol + triglycerides (mg/dl)	—0.864	<0.001

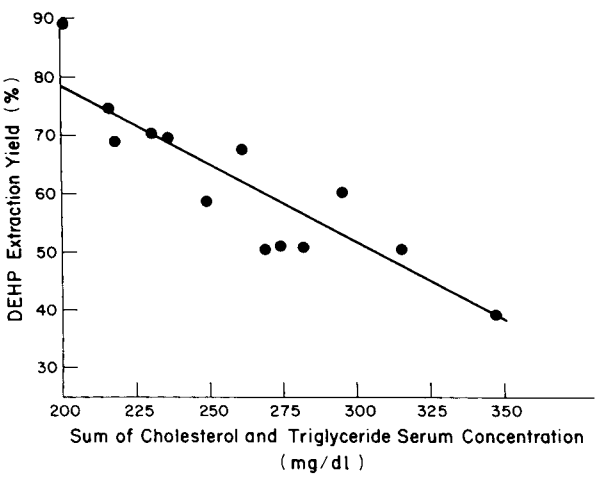


Fig. 3. Relationship between DEHP extraction recovery and the sum serum cholesterol and triglyceride concentrations in blood obtained from thirteen normal volunteers. The line indicates the least-squares regression of the data ($r = -0.864$, $p < 0.001$).

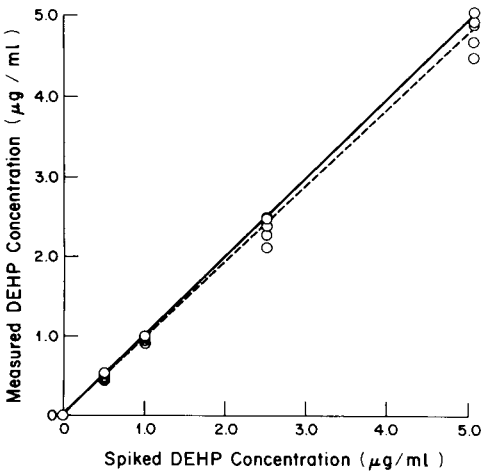


Fig. 4. Comparison of DEHP concentrations estimated using the standard addition procedure with known DEHP concentrations in pooled blood from ten volunteers. Five samples were analyzed at each concentration. The broken line indicates the least-squares regression of the data. The solid line represents a perfect correspondence between the estimated and known concentrations.

Estimates of DEHP concentration in the whole blood standards as determined by the standard addition procedure are compared to the actual concentrations in Fig. 4. The slope of the least-squares regression line between the measured and actual DEHP concentrations was 0.963, which is not significantly different from unity. The regression line also had a negligible intercept. The concentration estimates had an acceptable degree of reproducibility, with a coefficient of variation (n = 5) ranging from 5.16% (5 μg/ml) to 6.78% (2.5 μg/ml). Thus, the method of standard addition should be a reasonable approach to estimating whole blood concentrations of DEHP.

DISCUSSION

Differences in the extractability of a given compound from different biological matrices (e.g. serum, urine, and tissues) or from the same type of specimens from different animal species are well known. However, the potential problem of intersubject variation in extraction from biological samples is often neglected. The usual approach to quantitating a compound in blood, for example, is to develop a calibration curve, usually in pooled blood from several donors. Measurements in unknown samples are subsequently referenced to this standard curve. This traditional calibration procedure becomes invalid given the degree of intersample variability in DEHP extraction observed in the present study. It thus becomes necessary to determine the extraction yield in each sample analyzed.

The physicochemical basis for the intersubject differences in the extractability of DEHP is nor known. The data presented here suggest that increases in serum lipids, particularly as expressed by the sum of serum cholesterol and triglyceride concentrations, decreased the extraction yield. Albro and Corbett [13] have shown that more than 80% of the DEHP present in human plasma

after storage in plastic bags is associated with the lipoprotein fraction. It would appear that a portion of DEHP present in whole blood is tightly associated with the lipoprotein fraction, and that as the lipid content of blood increases the amount of DEHP available for extraction by ethyl acetate decreases. An alternative explanation may be that a fraction of DEHP in blood is trapped in precipitated lipoproteins following the addition of ethyl acetate, leading to incomplete and variable recovery. The latter hypothesis is supported by our observation that no DEHP is detectable in the supernatant after proteins in a serum sample have been precipitated with trichloroacetic acid or ice-cold acetonitrile.

The unusual extraction problem with DEHP is likely due to the high lipid solubility of the plasticizer. It would be anticipated that similar problems may be encountered with other highly lipophilic compounds. The solvent extraction of the plasticizer tris(butoxyethyl)phosphate (TBEP), a constituent of rubber stoppers used in many evacuated blood collection systems, has been found to be highly variable between serum samples obtained from different subjects [15]. The reported variation in the extraction yield of this compound was even greater than that associated with DEHP (i.e. a four-fold variation in calibration slope between subjects). As observed in the present study, the extraction yield of TBEP was found to correlate well ($r = 0.88$) with the serum concentration of cholesterol and triglycerides.

The phenomenon of significant intersample variation in extraction recovery is not restricted to extraction with ethyl acetate. Significant variability in the recovery of DEHP from serum or blood was also observed when using hexane, ethylene dichloride, and chloroform as extraction solvents. Thus, it is likely that the dependence of extraction yield on serum lipid content is a general analytical problem for highly lipid-soluble compounds, and is not restricted to either a single class of compounds or to a specific extraction solvent. The standard addition procedure described herein provides an efficient and acceptably accurate means of estimating blood concentrations of compounds subject to a high degree of intersample variability in extraction.

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DETERMINATION OF BENPERIDOL IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method for the quantitative determination of benperidol in human plasma using haloperidol as internal standard is described. The method involves liquid—liquid extraction, separation of the substances on a reversed-phase column C_{18} followed by ultraviolet detection at 254 nm. The mobile phase consists of 32% acetonitrile in 0.05 *M* potassium dihydrogen phosphate buffer (pH 2.8). The detection limit is 0.5—1.0 ng/ml using 2- or 4-ml plasma samples.

INTRODUCTION

Benperidol (Glianimon®) is an antipsychotic agent of the butyrophenone group. It is one of the most potent neuroleptics, being about eight times more potent than haloperidol, a neuroleptic with related structure (Fig. 1) [1].

No simple and selective method has been described for the determination of benperidol in human plasma, whereas several methods exist for determining haloperidol. The radioimmunoassay developed for haloperidol can be applied to benperidol [2—4]. However, the assay is unspecific, since cross-reaction occurs with other butyrophenones. The radioreceptor assay [5], too, is unspecific, since drugs and metabolites that block dopamine receptors are measured as well.

Gas chromatographic [6—9] and selected-ion monitoring mass spectrometric [10,11] assays have been reported for haloperidol but not for the other butyrophenones. These methods are not suitable for routine monitoring of plasma levels, as they are very time-consuming.

Two methods have been developed for haloperidol using high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [12,13]. Experiments to determine benperidol in human plasma with these methods

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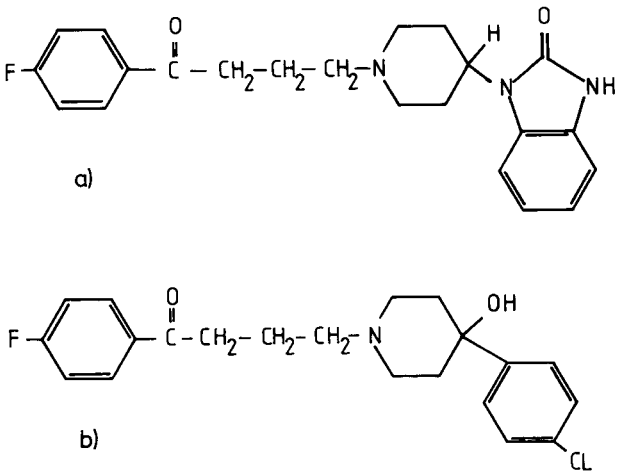

Fig. 1. Chemical structures of (a) benperidol and (b) haloperidol.

have failed in our laboratory. Another HPLC method with electrochemical detection [14] has been reported for haloperidol and reduced haloperidol, not for benperidol.

The method described here, based on HPLC, is selective for benperidol, as well as for haloperidol. It is rapid, and sensitive enough to study the pharmacokinetics of both drugs and to study relationships between plasma concentrations and neuroleptic efficacy.

MATERIALS AND METHODS

Chemicals

Benperidol, 4-[4-(2-oxo-2,3-dihydro-1-benzimidazolyl)-1-piperidinyl]-1-(4-fluorophenyl)-1-butanone, was obtained from Tropon (Cologne, F.R.G.) and haloperidol, 4-[4-(4-chlorophenyl)-4-hydroxy-1-piperidinyl]-1-(4-fluorophenyl)-1-butanone from Janssen-Pharmaceutica (Belgium). *n*-Hexane (Uvasol, Merck), isopropanol (Uvasol, Merck), acetonitrile (LiChrosolv, Merck), sodium bicarbonate (p.a., Merck), phosphoric acid (p.a., Merck) were used without further purification. Aqueous solutions were prepared using deionized water, which had been passed through a Milli-Q water purification system (Millipore).

Standards

Stock solutions were prepared by dissolving 1 mg of benperidol and 1 mg of haloperidol in 1 ml of 0.1 *M* hydrochloric acid. They were stored at 4°C. These solutions are stable for several months. Aliquots of these stock solutions were diluted with 0.05 *M* potassium dihydrogen phosphate buffer (pH 2.8) to a concentration of 1 μg/ml. These solutions served to prepare spiked plasma samples for calibration curves and recovery analyses. Fresh plasma samples were prepared on each day of analysis. Solutions of benperidol in methanol were stable at 4°C for 3—5 weeks only.

Chromatographic system

The chromatographic system consisted of a Waters 6000A solvent delivery

system, a Waters UV detector 440 operating at 254 nm and a Rheodyne 7125 syringe loading sample injector with a 100-μl sample loop. A HP 3390 A integrator was used to determine the concentrations of the neuroleptics by peak area integration. The mobile phase consisted of 32% acetonitrile in 0.05 *M* potassium dihydrogen phosphate buffer (pH 2.8). The buffer solution was passed through a 0.2-μm membrane filter (Schleicher & Schüll) prior to mixing with acetonitrile. The flow-rate was set at 1.0 ml/min. A column (length 250 mm, O.D. 8 mm, I.D. 4 mm) packed with C_{18} Nucleosil (Macherey, Nagel & Co., Düren, F.R.G.; particle size 5 μm) served as stationary phase.

Extraction

The quantity of plasma used for extraction depended on the benperidol concentration. For concentrations >5 ng/ml plasma samples of 1 ml were extracted; for lower concentrations, samples of 2 or 3 ml were used.

Plasma and 50 ng (50 μl) of haloperidol as internal standard were mixed in polypropylene tubes (17.5 ml) with polyethylene stoppers. After adding 1 ml of 2 *M* sodium bicarbonate buffer (pH 10.5) and 5 ml of hexane with 5% or 10% isopropanol (for 1 or 2 ml of plasma, respectively), the samples were shaken for 30 min and centrifuged for 10 min at 1800 *g*. The hexane layer was transferred to another tube containing 100 μl of 0.05 *M* potassium dihydrogen phosphate buffer (pH 2.8). This mixture was shaken again for 30 min and centrifuged for 10 min at 1800 *g*. The hexane layer was aspirated and 90 μl of the aqueous layer were injected into the chromatographic system.

Calibration

Plasma samples of 1 or 2 ml with benperidol concentrations of 1, 3, 5, 10, 20 and 50 ng/ml were prepared. After adding 50 ng/ml haloperidol as internal standard, the samples were treated as described above. Calibration curves were obtained by plotting the peak area ratios of benperidol to haloperidol against the benperidol concentration given.

RESULTS AND DISCUSSION

Extraction

Liquid—liquid extraction of the butyrophenones from plasma in two steps was necessary to separate the drugs from endogenous substances which interfere with the chromatographic analysis. In the first step, the butyrophenones were extracted from alkalized plasma (pH 10.5) with an organic solvent. In the second, they were reextracted with an acidic aqueous solution (pH 2.8). The extractabilities of benperidol and haloperidol were different. The yield of extraction depended on the amount of isopropanol added to hexane (Table I). Plasma samples of 1 and 2 ml, each spiked with 50 ng of benperidol and 50 ng of haloperidol, were extracted with hexane and with hexane + 1%, 5%, 10% or 20% isopropanol.

When the plasma was extracted with pure hexane, a gel was formed in the organic layer; the absolute recoveries were therefore very low and variable. After extraction with hexane + 1% isopropanol the recovery for benperidol was lower than for haloperidol, the ratios being 0.26 for 1 ml of plasma and 0.09

TABLE I

INFLUENCE OF ISOPROPANOL ON THE EXTRACTABILITY OF 50 ng OF BENPERIDOL AND 50 ng OF HALOPERIDOL ADDED TO 1 OR 2 ml OF PLASMA

Values are mean of four extractions.

Plasma volume (ml)	Isopropanol (%)	Extractability (%)		Benperidol/haloperidol
		Benperidol	Haloperidol	
1	0*	8.2 ± 4.5	12.2 ± 5.3	0.67
2	0*	3.2 ± 3.0	26.1 ± 9.3	0.12
1	1	16.5 ± 2.1	63.7 ± 6.1	0.26
2	1	4.8 ± 0.5	56.2 ± 4.6	0.09
1	5	65.3 ± 2.1	75.4 ± 1.6	0.87
2	5	37.4 ± 4.5	73.0 ± 2.8	0.51
1	10	38.8 ± 2.3	29.0 ± 1.9	1.34
2	10	55.2 ± 3.6	68.1 ± 3.2	0.81
1	20**	34.1 ± 3.5	24.0 ± 5.3	1.42
2	20**	19.3 ± 9.8	21.3 ± 9.6	0.91

*Gel formation, extractability very low.
**Peaks very broad and not well separated from other plasma peaks.

for 2 ml of plasma. When 1 ml of plasma was extracted, the best recoveries for both substances were obtained with hexane + 5% isopropanol, with a mean ratio of 0.87. When 2 ml of plasma are extracted, the amount of isopropanol must be increased to 10% in order to obtain comparable recoveries; the ratio was then 0.81. With hexane + 5% isopropanol the recovery for benperidol was too low, the ratio being 0.51.

The addition of isopropanol to hexane is important, especially for the extraction of benperidol; the amount necessary depended on the quantity of plasma used for extraction.

Detection

The absorbances of benperidol and haloperidol at 214 and 254 nm were compared. They were greater at 214 nm than at 254 nm for both drugs.

The ratio of peak area at 214 nm to peak area at 254 nm is 2.0 for benperidol and 1.3 for haloperidol. Nevertheless, the extracted plasma samples were measured at 254 nm, because the signal-to-baseline-noise ratio was better at this wavelength. If there are other drugs in the extracted plasma, the selectivity of analysis can be improved by measuring the absorbances at both wavelengths and comparing the peak area ratios.

Resolution

With the chromatographic conditions described in Materials and methods the retention times are about 6 min for benperidol and about 12 min for haloperidol.

Fig. 2 shows the chromatograms for a blank plasma sample (Fig. 2a), a plasma sample spiked with 5 ng of benperidol and 50 ng of haloperidol (Fig. 2b) and a plasma sample of a patient receiving benperidol 40 mg/day (Fig. 2c). The blank plasma was free of interfering peaks.

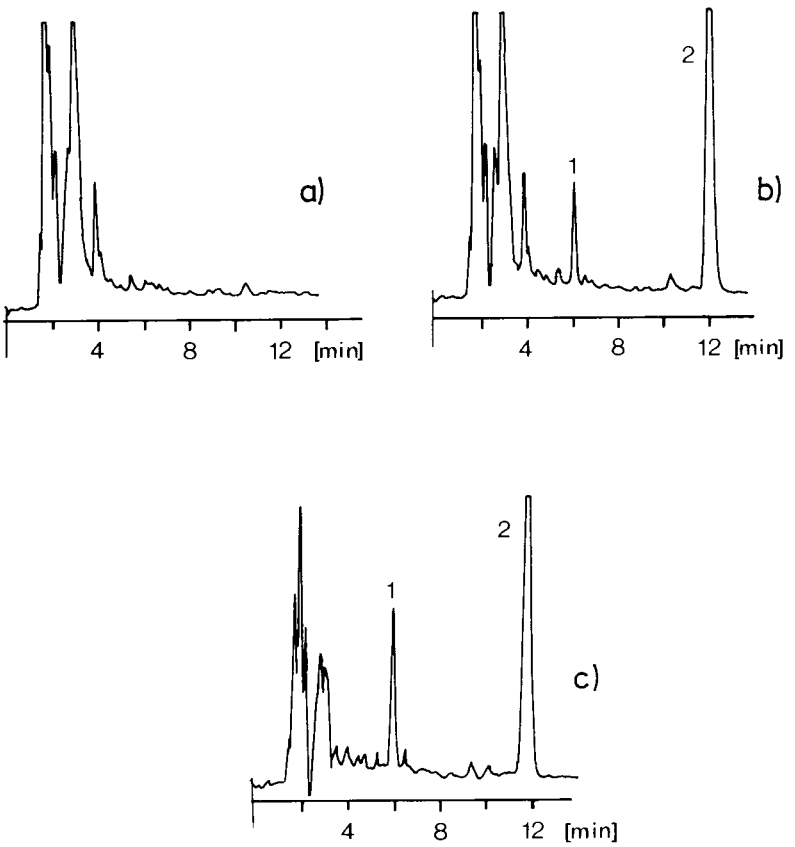

Fig. 2. Chromatograms of blank plasma (a), plasma spiked with 5 ng benperidol + 50 ng haloperidol (b), and plasma taken from a patient treated with 40 mg/day benperidol (c). Peaks: 1 = benperidol; 2 = haloperidol.

The sensitivity of our method was 1 ng/ml if 2 ml of plasma were extracted, and 0.5 ng/ml if 4 ml were extracted.

Calibration and precision

The calibration curves in the range of 1—50 ng/ml benperidol were linear. Mean values of the peak area ratios benperidol to haloperidol with standard deviations and coefficients of variation as well as the regression equation of the line are summarized in Table II. Recovery analyses of spiked plasma samples were done together with each series of patient samples. The results of twelve independent analyses on different days (in the course of two months) are listed in Table III. The coefficients of variation ranged from 3.7% to 6.3%.

Application of the method

Plasma concentrations in patients receiving a high dose of 40 mg of benperidol per day for six or seven days, followed by a low dose of 6 mg/day for fourteen days, were determined. Another group of patients received only a low dose of 6 mg/day for 21 days. The blood samples were collected 14 h

TABLE II

CALIBRATION CURVE DATA

Benperidol concentration (ng/ml)	n	Peak area ratio benperidol/haloperidol (mean ± S.D.)	C.V. (%)
1	6	0.029 ± 0.003	10.6
3	6	0.063 ± 0.006	9.1
5	12	0.106 ± 0.005	4.6
10	12	0.204 ± 0.009	4.4
20	12	0.377 ± 0.016	4.3
50	12	0.922 ± 0.036	3.9

Regression equations

$Y_1 = 0.014 + 0.018X$, $r^2 = 0.9998$ (1—50 ng/ml)

$Y_2 = 0.018 + 0.018X$, $r^2 = 0.9999$ (5—50 ng/ml)

TABLE III

RESULTS FOR TWELVE RECOVERY ANALYSES PERFORMED ON DIFFERENT DAYS

Given (ng/ml)	Found (mean ± S.D.) (ng/ml)	C.V. (%)
5	5.14 ± 0.30	5.8
10	9.97 ± 0.63	6.3
20	18.57 ± 0.78	4.2
50	44.97 ± 1.67	3.7

TABLE IV

BENPERIDOL PLASMA CONCENTRATIONS OF PATIENTS RECEIVING 40 mg OR 6 mg PER DAY

No. of patients	Day of blood sampling	Dose (mg/day)	Plasma concentration (ng/ml, mean ± S.D.)	C.V. (%)
10	4	40	15.1 ± 7.3	48.3
10	14	6	4.1 ± 1.4	34.1
7	4	6	3.1 ± 1.2	40.1
7	14	6	4.2 ± 1.2	28.2

after medication on days 2, 4, 7, 14, 21 in Li-heparin tubes. The samples were centrifuged at 1800 g; the plasma was then separated and frozen at −40°C.

Table IV shows the mean plasma concentrations on day 4 of ten patients receiving 40 mg/day and plasma concentrations of the same patients on day 14 after reducing the dose on day 6 or 7 to 6 mg/day. The relation between dose and plasma concentration is not linear.

The inter-individual variations of the plasma concentrations were wide. The coefficient of variation for a dose of 40 mg/day on day 4 was 48.3%, the mean plasma concentration being 15.1 ± 7.3 ng/ml. On day 14 the mean plasma concentration was 4.1 ± 1.4 ng/ml with a coefficient of variation of

34.1%. The mean values of seven patients receiving a constant dose of 6 mg for at least 14 days are listed in the same table. Plasma concentrations on day 4 were 3.1 ± 1.2 ng/ml with a coefficient of variation of 40.1%. They were somewhat lower than on day 14 with 4.2 ± 1.2 ng/ml and a coefficient of variation of 28.2%.

The present method developed for the determination of benperidol in human plasma is simple and rapid. With our extraction method and chromatographic conditions other butyrophenones like haloperidol, droperidol or trifluperidol can be detected as well. Their retention times and capacity factors are listed in Table V. The detection limit of benperidol and haloperidol is 1.0 ng/ml using 2 ml of plasma per assay and 0.5 ng/ml using 4 ml. The HPLC method for haloperidol with electrochemical detection [14] is as sensitive as our method. The two HPLC methods with UV detection [12,13] are selective for haloperidol; benperidol could not be determined; the detection limit was 2 ng/ml. Our method is somewhat more sensitive and has the advantage that other butyrophenones can be analysed.

TABLE V

RETENTION TIMES AND CAPACITY FACTORS FOR BENPERIDOL, DROPERIDOL, HALOPERIDOL AND TRIFLUPERIDOL

Compound	t_R (min)	k'
Benperidol	5.9	2.3
Droperidol	5.8	2.2
Haloperidol	11.6	5.4
Trifluperidol	18.3	9.2

ACKNOWLEDGEMENT

This work was supported by the Wilhelm Sander-Stiftung.

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CHROMBIO. 2232

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF A CANDIDATE 8-AMINOQUINOLINE ANTILEISHMANIAL DRUG USING OXIDATIVE ELECTROCHEMICAL DETECTION

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SUMMARY

An analytical method was developed for the quantitation of a candidate antileishmanial drug, 6-methoxy-8-(6-diethylaminohexylamino)-4-methylquinoline, dihydrochloride, in canine plasma. The assay utilized internal standard technique with a structurally similar 8-aminoquinoline, 6-methoxy-8-(7-diethylaminoheptylamino)-4-methylquinoline, dihydrochloride, as the internal standard. The method employs a liquid—solid extraction procedure with prepackaged silica gel columns upon which the drug and internal standard are adsorbed, then selectively washed and eluted. Reversed-phase chromatography was then employed to analyze the extracted sample by means of oxidative electrochemical detection at + 0.75 V. Good accuracy and precision were obtained over the range of concentration tested (10—1500 ng/ml plasma). Analyses of plasma samples from human volunteers given the drug demonstrate the method is also suitable for analysis of human plasma samples. The entire procedure is relatively simple and requires only 1 ml of plasma.

INTRODUCTION

The leishmaniases are parasitic diseases affecting perhaps 100,000,000 people in the tropical and semitropical world [1]. The major forms of human disease are papular ulcerative cutaneous disease (cutaneous disease), erosive oral, nasal or pharyngeal disease (mucocutaneous disease), or hepatosplenomegaly (visceral disease). The leishmaniases result from infection of the macrophages of the respective organs by the amastigote form of the parasite. The leishmaniases are presently treated with pentavalent antimonials such as sodium stibogluconate (Pentostam®, Burroughs Wellcome) or N-methylglucamine (Glucantime®, Rhodia) [2—4]. The 10—25% of cases that are antimony-resistant [4] are treated with more or higher doses of antimony, with

pentamidine or with amphotericin B [2—4]. These compounds are parenterally administered and have relatively low therapeutic indices.

The Walter Reed Army Institute of Research has screened more than 3000 compounds in an effort to find an orally administered effective antileishmanial compound. 6-Methoxy-8-(6-diethylaminohexylamino)-4-methylquinoline, dihydrochloride, is presently the most active drug discovered thus far in a screening program for antileishmanial activity in the hamster model of visceral leishmaniasis [5]. In this model, the above compound has consistently shown suppressive activity 400—700 times greater than the reference compound, Glucantime [5]. This compound is being developed for human use and has been tested in a double blind study of safety and tolerance in humans and was well tolerated up to and including a 60-mg single oral dose [6].

To support the development of this drug, the estimation of pharmacokinetic parameters in animals and humans must be determined. Therefore a sensitive and specific assay for this candidate 8-aminoquinoline antileishmanial drug in plasma was needed and is described in this paper.

EXPERIMENTAL

Chemicals

6-Methoxy-8-(6-diethylaminohexylamino)-4-methylquinoline, dihydrochloride* (I) and 6-methoxy-8-(7-diethylaminoheptylamino)-4-methylquinoline dihydrochloride** (II), the internal standard, were synthesized on contract from Starks Assoc. (Buffalo, NY, U.S.A.) and Ash Stevens (Detroit, MI, U.S.A.), respectively (Fig. 1). Compound I and the internal standard were found to be 99.7% [7] and > 99% pure, respectively. 6-Methoxy-8-(6-ethylaminohexylamino)-4-methylquinoline, dihydrochloride hemihydrate***, the salt form of a metabolite of compound I found in both rat and hamster microsomal preparations [8], was synthesized under contract by Starks Assoc. (purity > 99.0%, Fig. 1). Acetonitrile was of HPLC grade and obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). All chemicals used in this study were of reagent grade. Trizma buffer was purchased from Sigma (St. Louis, MO, U.S.A.). Ammonium formate was purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.).

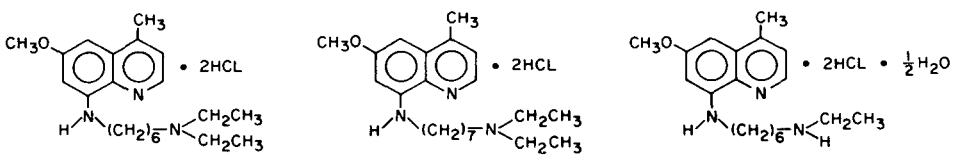

Fig. 1. Chemical structures of compound I (I), the internal standard (II) and the N-deethylated metabolite of compound I (III).

*This compound is an investigational drug and has no generic name. An institutional identification of WR 6026 ◦ 2HCl (Lot AF) is used to identify this compound.

**The internal standard was also obtained from the Walter Reed Inventory and has no generic name. An institutional identification of WR 223,658 ◦ 2HCl (Lot AA) is used.

***This metabolite was synthesized and also obtained from the Walter Reed inventory. The institutional identification for the chemical is WR 211,789 ◦ 2HCl ◦ ½H_2O.

Dehydrated ethanol was purchased from U.S. Industrial Chemical (Tuscola, IL, U.S.A.). Silica gel Bond-Elut® columns (3 ml capacity) were purchased from Analytichem International (Harbor City, CA, U.S.A.). Plasma was prepared from freshly drawn heparinized (20 I.U./ml) canine blood. All water was demineralized, double glass-distilled and stored in glass.

Liquid—solid extraction of compound I from plasma

Canine plasma samples (1 ml) that had been previously spiked with various levels of compound I were thawed at room temperature. The internal standard was dissolved in 0.01 *M* hydrochloric acid and appropriate amounts were added to the thawed samples and vortexed for 10 sec. Acetonitrile (2 ml) was added to the sample and immediately vortexed vigorously for 30 sec. Samples were then centrifuged for 10 min at 500 *g* and 5°C in a Sorvall refrigerated RC-2 centrifuge (DuPont, Newtown, CT, U.S.A.). The supernatant was transferred to another precleaned (tubes rinsed with acetonitrile then methanol) disposable glass test tube and acetonitrile was evaporated to dryness under nitrogen gas (< 35°C) using a N-Evap® analytical evaporator manufactured by Organomation (South Berlin, MA, U.S.A.). The sample was reconstituted with 2.0 ml of water and vortexed vigorously for 15 sec.

The sample was placed onto a wetted silica gel Bond-Elut column previously equilibrated by the passage of 4 ml of ethanol followed by 6 ml of water. The test tube was rinsed with 2.0 ml water and the rinse solution was also put on the column. The sample and test tube rinse were drawn through the column in this and subsequent wash steps by use of a vacuum (200 mmHg) with a Vac-Elute® manifold obtained from Analytichem International. After the sample had been drawn through the column, the volume was washed with 6 ml of water followed by 6 ml of water—ethanol (20:80). Finally, the drug plus internal standard were eluted off the silica gel Bond-Elut column with 8 ml of 0.1 *M* trizma, pH 7.4—ethanol (20:80) into a precleaned glass test tube and evaporated to dryness under nitrogen gas at < 35°C. The dry residue was dissolved in mobile phase for HPLC analysis.

HPLC analysis

Chromatographic system consisted of a Model 6000A pump (Waters Assoc., Milford, MA, U.S.A.), a Model U6K universal injector (Waters Assoc.), an electrochemical detector with a single glassy carbon electrode (Bioanalytical Systems, West Lafayette, IN, U.S.A.), a Model 585 strip-chart recorder (Linear Instruments, Irvine, CA, U.S.A.) and a 250 mm × 4.6 mm I.D. 5-μm particle size Spherisorb-CN® column (Thomson Instrument, Newark, DE, U.S.A.) Column was at ambient temperature. Mobile phase consisted of 0.1 *M* ammonium formate, pH 4.5—acetonitrile (67:33) and the flow-rate was 2.0 ml/min. The pH of the ammonium formate was titrated with concentrated formic acid to pH 4.5 prior to the addition of acetonitrile. Samples were reconstituted in 250 μl of mobile phase and 5—20% of the samples were injected. The electrochemical detector was set in the oxidative mode at an applied potential of +0.75 V using a single glassy carbon electrode at a sensitivity of 2—10 nA full scale. The electrochemical detector signal was filtered at a maximum cut-off frequency of 0.05 Hertz by means of a Model 1021A filter (Spectrum Scientific, Newark, DE, U.S.A.).

Quantitation

Quantitation was achieved using peak height ratios of compound I to internal standard (II). For each analysis, a standard curve was generated by adding known and varying amounts of compound I and a known and constant amount of the internal standard to canine plasma. The ranges of standard curves (8—120 ng or 100—1500 ng) were designed to encompass the range of expected experimental values. Spiked samples were treated as unknowns to evaluate the precision and accuracy of the method. Additionally, blind plasma samples spiked with low concentrations of compound I were prepared by another laboratory and were analyzed to further validate the procedure. Human plasma samples were obtained from volunteers, participating in a safety and tolerance study, 24 h following a single 60-mg oral dose of placebo. The human plasma samples were then frozen (—20°C) for approximately 30 days and then analyzed by this procedure.

Percent recovery was determined by comparison of peak height of compound I for each unknown sample to an external standard curve of ng of compound I on column versus peak height (cm). An external standard curve was constructed for every analysis.

RESULTS

The method was validated by means of spiked canine plasma samples treated as unknowns and blind spiked canine plasma samples prepared in another laboratory. The results are shown in Tables I—III and they demonstrate good precision and accuracy over the concentration range studied. The overall accuracy and precision from 10 to 1000 ng of compound I per ml of plasma were ± 7.2% and 7.2% coefficient of variation (C.V.), respectively. In addition, the overall (10—1000 ng/ml) mean percentage recovery of compound I as determined by external standard technique was 55 ± 13.2% (n = 103).

In order to accurately quantitate plasma levels of compound I over a large concentration range (10—1500 ng/ml) two separate but overlapping standard curves were run. Both low- and high-range standard curves consisted of seven

TABLE I

PRECISION AND ACCURACY DATA FOR ANALYSIS OF LOW CONCENTRATIONS OF COMPOUND I IN PLASMA (n = 16)

Data are a summarization of data of four separate experiments. A standard curve of 8 to 130 ng of compound I per ml of plasma bracketed spiked unknowns. For additional information, see Experimental.

Amount added (ng)	Amount measured (ng, mean ± S.D.)	C.V. (%)	Accuracy |%Δ|*
10.0	9.5 ± 1.32	13.9	11.2
20.0	20.4 ± 1.74	8.5	7.1
50.0	53.5 ± 7.45	13.9	11.7
100.0	99.5 ± 10.38	10.4	8.8

*Represents the mean of individual determinations for the absolute percent difference of amount of drug added to sample versus amount of drug assayed.

TABLE II

PRECISION AND ACCURACY DATA FOR ANALYSIS OF HIGHER CONCENTRATIONS OF COMPOUND I IN PLASMA (n = 12)

Data are a summarization of data of two separate experiments. A standard curve of 100—1500 ng of compound I per ml of plasma bracketed spiked unknowns. For additional information, see Experimental.

Amount added (ng)	Amount measured (ng, mean ± S.D.)	C.V. (%)	Accuracy $\lvert\%\Delta\rvert$*
500.0	520.3 ± 13.96	2.7	4.7
1000.0	1036.6 ± 17.90	1.7	3.6

*Refer to footnote to Table I.

TABLE III

PRECISION AND ACCURACY DATA FOR ANALYSIS OF BLIND SAMPLES (n = 5)

Data represent a single experiment for the analysis of plasma samples spiked with compound I that were supplied by another laboratory. The actual levels of compound I were made known to the analyst only after the blind samples were analyzed. The unknowns were bracketed by a standard curve from 8 to 120 ng of compound I per ml of plasma. For additional information, see Experimental.

Amount added (ng)	Amount measured (ng, mean ± S.D.)	C.V. (%)	Accuracy $\lvert\%\Delta\rvert$*
17.2	16.7 ± 0.69	4.1	3.6
34.3	35.3 ± 1.95	5.5	4.0
85.1	76.9 ± 2.82	3.7	9.7

*Refer to footnote to Table I.

points bracketing the anticipated unknown sample concentrations. Good linearity and a negligible Y-intercept were routinely found. Least-squares linear regression of low- and high-range standard curves yielded a representative equation ($Y = aX + b$) for the line and regression coefficients (r^2) of $Y = 0.0022X + 0.0190$, $r^2 = 0.9991$ and $Y = 0.0231X - 0.0115$, $r^2 = 0.9995$, respectively.

The samples were free of interfering peaks as seen in the blank sample (Fig. 2A). Baseline separation is achieved between compound I and the internal standard and the analysis time is about 11 min (Fig. 2B). Following a single 60-mg oral dose to human volunteers, compound I was detected in human plasma 24 h after administration (Fig. 2C). Analysis of plasma from volunteers receiving a placebo revealed no interfering peak in the region of interest (not shown). Furthermore, Fig. 3 demonstrates that the presence of the N-deethylated metabolite (III) of compound I in plasma would not interfere with the quantitation of compound I. This metabolite has similar chromatographic mobility as that of compound I and is found in in vitro rodent hepatic microsomal metabolism experiments [8]. Therefore, the formation of this metabolite in vivo is likely.

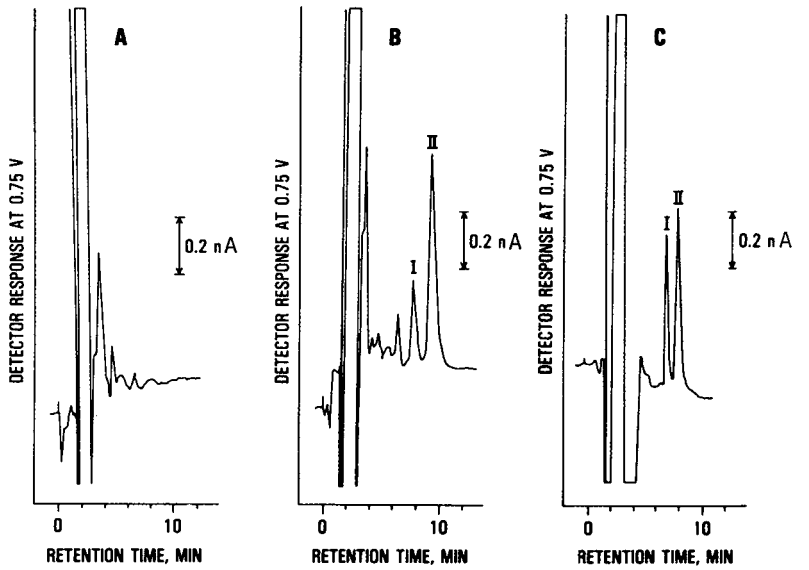

Fig. 2. HPLC chromatograms of extracts from: (A) blank canine plasma; (B) canine plasma (1.0 ml) spiked with 10 ng of compound I (I) and 50 ng of the internal standard (II); (C) patient sample drawn 24 h after oral administration of compound I, spiked with 50 ng of the internal standard (II). A 38-ng amount of compound I (I) per ml of human plasma was detected. In both instances 50 μl of a total 250 μl were injected. For additional information, see Experimental.

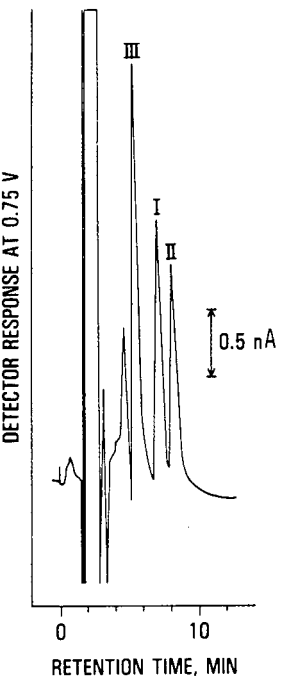

Fig. 3. HPLC chromatogram of extract from canine plasma (1.0 ml) spiked with 500 ng of compound I (I), internal standard (II), and the N-deethylated metabolite of compound I (III). In this instance, 25 μl of a total 250 μl was injected. For additional information, see Experimental.

DISCUSSION

Quantitating this antileishmanial drug by this method has two unique features. First, the liquid—solid extraction of compound I from plasma using disposable prepackaged extraction columns, silica gel Bond-Elut columns, simplifies the extraction by allowing the binding of the drug and internal standard to a solid matrix. This allows the bound sample to be selectively washed to remove unwanted contaminates, and then eluted off the column with the use of aqueous salt and organic mixture. This is less cumbersome, time-consuming and uses less glassware than classical liquid—liquid extraction methods.

The second feature is the use of oxidative electrochemical detection (ED) rather than ultraviolet (UV) detection which provides 50% greater sensitivity and greater selectivity. This observation was determined by the analysis of compound I from plasma and detected simultaneously by both ED and UV at their maximum useable sensitivities (2 and 0.001 nA full scale, respectively) in our laboratory. Oxidative HPLC—ED is an amperometric determination involving the heterogenous electron transfer from the electrode surface to the solute as it passes through the low volume thin layer cell of the electrochemical detector. Therefore the ability to detect a compound by oxidative ED is dependent on its strength as an oxidizing agent which is a more specific process than detection by UV absorption at 254 nm. This selectivity became useful due to the fact that interfering compounds which were detected occasionally by UV were not detected by ED at all, thereby eliminating a major obstacle in the development of this assay.

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CHROMBIO. 2233

DETERMINATION OF ADRIAMYCIN, ADRIAMYCINOL AND THEIR 7-DEOXYAGLYCONES IN HUMAN SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A reversed-phase isocratic high-performance liquid chromatographic assay is described for the measurement of adriamycin, adriamycinol and their 7-deoxyaglycones in human serum. The lower limit of detection in serum is 3 ng/ml for adriamycin and 1 ng/ml for adriamycinol and the 7-deoxyaglycones with coefficients of variation for k' of less than 5% throughout the day.

An extraction technique for serum is described which is capable of an almost equal recovery (> 77%) of adriamycin, metabolites and daunorubicin (the internal standard) without interference from endogenous components of serum. Serum concentrations of metabolites 15 min after intravenous bolus administration of 40 mg/m^2 adriamycin in two different patients were 26.5 and 16.6 ng/ml for adriamycinol; 109.8 and 5.8 ng/ml for the adriamycinol 7-deoxyaglycone and 21.4 and 17.1 ng/ml for the adriamycin 7-deoxyaglycone. A total of six metabolites of adriamycin were detected in the two patients using this methodology.

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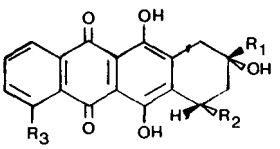
INTRODUCTION

Adriamycin (ADR) is a naturally occurring anthracycline glycosidic antibiotic with a broad spectrum of antitumour activity in human cancer [1]. In man, it was shown to be converted to at least eight different products, which were identified in urine using thin-layer chromatography (TLC) [2]. These were: adriamycinol (AOL), the major metabolite; five aglycones, adriamycin aglycone (ADR-ONE), adriamycin 7-deoxyaglycone (ADR-DONE), adriamycinol aglycone (AOL-ONE), adriamycinol 7-deoxyaglycone (AOL-DONE) and adriamycinol demethyl 7-deoxyaglycone (AOL-DM-DONE); and two conjugates of AOL-DM-DONE (Fig. 1). Pharmacokinetics of several aglycones were followed in fourteen cancer patients using TLC [3].

Recently, normal- and reversed-phase high-performance liquid chromatographic (HPLC) methods have been published for the separation of mixtures of ADR and metabolites [4—11]. Coupled to a variety of extraction techniques the only metabolite of ADR that was detected in patient serum or plasma by



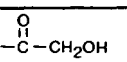
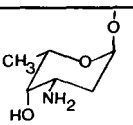
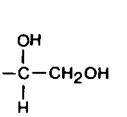
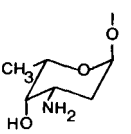
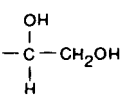
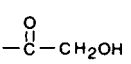
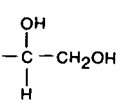
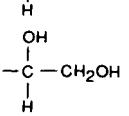
COMPOUND	R_1	R_2	R_3
ADR			
AOL			
ADR-ONE			
AOL-ONE			
ADR-DONE			
AOL-DONE			
AOL-DM-DONE			

Fig. 1. The structure of adriamycin and its metabolites.

HPLC was AOL [4, 7, 9, 12, 13]. The aglycones were suggested to be TLC artefacts caused by the hydrolysis of the glycosides ADR and AOL in situ during prolonged chromatographic development with acidic solvent systems [9, 12]. Whilst chemical decomposition may account for ADR-ONE and AOL-ONE, they can be produced by mild acid hydrolysis [10], it cannot explain the presence of 7-deoxyaglycone metabolites.

ADR and AOL 7-deoxyaglycone are formed by the chemical degradation of semi-quinone free radicals of ADR and AOL [14—16]. Since free radicals have been strongly implicated in the etiology of ADR-induced cardiotoxicity ADR-DONE and AOL-DONE may be important pharmacological markers. We report a new isocratic reversed-phase HPLC method with a simple extraction step which can detect adriamycin, adriamycinol and their 7-deoxy-aglycones in patient serum.

MATERIALS AND METHODS

Apparatus

HPLC was performed throughout using an Altex Model 110A pump and an Altex Model 210 injection port with a 20-μl injection loop (Beckman-RIIC, High Wycombe, U.K.); a Gilson Spectro-glo filter fluorimeter with narrow-band interference filters at 480 nm (excitation) and 560 nm (emission) and a 10-μl quartz micro flow cell (Gilson, Villiers-le-Bel, France); a Shimadzu CR-1B integrator (supplied by Scotlab Instrument Sales, Bellshill, U.K.) or a Rikadenki R-20 pen recorder (Rikadenki Mitsui Electronics, Chessington, U.K.) and μBondapak C_{18} prepacked columns 250 mm × 4.6 mm I.D. (supplied by HPLC Technology, Macclesfield, U.K.).

Reagents and standards

All methanol, acetonitrile, propan-2-ol and chloroform were HPLC reagent grade (Fisons Scientific, Loughborough, U.K.). Orthophosphoric acid and all other solvents and chemicals were of analytical reagent grade (AnalaR, BDH, Poole, U.K.). Water was double-distilled and deionised in a quartz glass still. Pure adriamycin—HCl and adriamycinol—HCl were a gift from Dr. S. Penco (Farmitalia, Milan, Italy). The internal standard, daunorubicin—HCl (DNR), was from May and Baker (Dagenham, U.K.). Adriamycin aglycone and adriamycin 7-deoxyaglycone were from Dr. Penco and were also synthesised. Daunorubicin aglycone (DNR-ONE), adriamycin aglycone and adriamycinol aglycone were all synthesised by mild acid hydrolysis with 0.1 *M* hydrochloric acid at 55°C for 1 h [10]. Adriamycin 7-deoxyaglycone and adriamycinol 7-deoxyaglycone were synthesised by catalytic hydrogenation using a palladium catalyst [2]. Purity of all the aglycones was assessed by TLC using 20 × 10 cm glass plates coated with a 250-μm layer of silica gel G (Analtech uniplates, Scotlab Instruments Sales) and three different ascending solvent systems (S). S 1 was chloroform—methanol—water (80:20:3); S 2 was chloroform—methanol—glacial acetic acid—water (80:20:14:6) and S 3 was ethyl acetate—ethanol—glacial acetic acid—water (80:10:5:5) [2]. The aglycones were visualised as orange spots under ultraviolet light at 254 nm. The chemical identity of methanolic solutions of all the synthesised aglycone

standards was verified by direct probe injection mass spectrometry with a Kratos MS 902S mass spectrometer and a DS 55C data system (Kratos Analytical Instruments, Manchester, U.K.).

Stock solutions (100 μg/ml) of AOL, ADR, DNR, AOL-ONE, ADR-ONE and DNR-ONE were prepared in methanol. Stock solutions of 10 μg/ml of ADR-DONE and AOL-DONE were prepared in methanol because of their poor solubility. All further dilutions were made with methanol to give a range of standard solutions of 0.025—10 μg/ml. All standard solutions were stored in PTFE-lined screw-capped bottles at −20°C and were made up fresh every month. Calibration curves were constructed by injecting 20 μl of standard solution to give a range of 0.5—200 ng on the column. All standard curves were linear ($r > 0.99$) and had intercepts at the origin. The limit of detection was set at the 3:1 signal-to-noise ratio.

Chromatographic conditions

The mobile phase consisted of: 5 m*M* (final concentration) orthophosphoric acid, 62.5% in methanol, acetonitrile and propan-2-ol, either 12.5:12.5:12.5 or 15:15:7.5, respectively, pH 3.2. Elution was isocratic at a flow-rate of 2.5 ml/min. Mobile phase was degassed by sonication at 12 μm for 15 min using a MSE sonicator (MSE Instruments, Crawley, U.K.). The column was at ambient room temperature (normally 25°C). Quantitation of sera extracts was by either peak area or peak height and was always by reference to standard calibration curves run on the same day.

Extraction procedure

Blood samples were collected from patients receiving 40 mg/m^2 ADR for the chemotherapy of cancer and were allowed to clot in plain glass tubes for 1 h at 4°C. Heparinised tubes were not used because heparin was shown to interfere with the HPLC assay. Blood was centrifuged at 1000 *g* for 10 min and the sera separated and stored at −20°C. Prior to extraction sera were thawed at room temperature.

To 1 or 2 ml of serum were added 100 ng DNR in 10 μl of methanol as an internal standard. The sera were then mixed with 5 vols. of chloroform—propan-2-ol (2:1) and either whirlimixed for two 1-min periods in 25-ml PTFE-lined screw capped test-tubes or vortexed for 30 min in 35-ml tapered glass centrifuge tubes (28 mm I.D.) with a Buchler vortex evaporator (Gallenkamp, East Kilbride, U.K.). After mixing, the samples were centrifuged at 2000 *g* for 15 min at 4°C to separate two phases. The upper aqueous phase was discarded by aspiration, the lower organic phase was transferred to a clean 35-ml tapered centrifuge tube and was evaporated to dryness in the Buchler vortex evaporator at 40°C and 25 mmHg of vacuum. The residue was redissolved in either 50 or 100 μl of methanol by whirlimixing for 1 min and 20 μl were injected onto the HPLC column.

RESULTS AND DISCUSSION

Physico—chemico properties of ADR and metabolites

An assumption which is commonly made regarding ADR and metabolites is

that they fluoresce all with the same quantum efficiency (Φ) and molar extinction coefficient (ϵ). To establish whether or not this is a valid assumption the fluorescence of each metabolite and DNR which was determined by HPLC was expressed relative to the fluorescence of equal concentrations of ADR (Table I). DNR and ADR were found to fluoresce equally but without exception the relative molar fluorescence (RMF) of the metabolites was greater than ADR. With AOL, ADR-ONE and AOL-ONE it was 1.5 times greater than ADR and with ADR-DONE and AOL-DONE it was 2 times greater than ADR (Table I). Assuming equal fluorescence is, therefore, likely to introduce large errors into quantitation when HPLC is involved [8, 11, 13, 17] and especially when only total fluorescent equivalents are measured [18—20].

TABLE I

PHYSICO—CHEMICO PROPERTIES OF ADRIAMYCIN AND METABOLITES

Compound	MW	Relative molar fluorescence*	Stability**		Percent solubility***		
			4°C	25°C	Water	Methanol	Chloroform
ADR	543.5	1	6.5	9.1	>10	>1	ns
AOL	545.6	1.5	3.6	6.3	>10	>1	ns
ADR-ONE	414.4	1.5	1.1	1.9	ns	>0.5	<0.02
AOL-ONE	416.4	1.5	<1	1.3	ns	>0.1	sparingly
ADR-DONE	398.4	2	1.6	1.3	ns	<0.002	sparingly
AOL-DONE	400.4	2	<1	<1	ns	<0.002	sparingly
DNR	527.5	1	6.1	8.0	>10	>1	ns

*Relative molar fluorescence is defined as the ratio of the integrated peak area of a metabolite and DNR over the integrated peak area of an equimolar concentration of ADR and is an expression of a metabolites fluorescence relative to the fluorescence of ADR.

**Stability data refer to the percentage of chemical decomposition per hour after a day at 25°C and per day after a week at 4°C of 10 μg/ml methanolic solutions.

***Solubility was determined using saturated or near saturated solutions after mixing for 4 h at room temperature; ns refers to not soluble and sparingly to solubility of less than 0.001%.

TLC has been implicated in producing ADR metabolite artefacts [9, 12]. Stability studies of ADR, metabolites and DNR were performed using the HPLC method described in this paper. At 4°C and 25°C the aglycones decomposed at less than 2% per day and per hour, respectively (Table I). ADR, DNR and AOL degraded faster than the aglycones at 3.6—6.5% per day at 4°C and at 6.3—9.1% per hour at 25°C. The glycosides (ADR, DNR and AOL) decomposed into non-detectable, non-fluorescent products and not into aglycones.

The final property studied was solubility. The aglycones and particularly the 7-deoxyaglycones were insoluble in water, methanol and chloroform (Table I). Further solvents were investigated to find one suitable. Acetonitrile, tetrahydrofuran, hexane, light petroleum (80—120°C), benzene and toluene were tried each with equally poor results. The alcohols methanol, ethanol and propan-2-ol gave the best results. A possible explanation for the poor solubility of the aglycones is that strong hydrogen bonding occurs between quinone and hydroxyl groups in the crystals lattice. Alcohols would compete for hydrogen

bonds which may account for their success as solvents. The glycosides were soluble in both water and to a lesser extent methanol but were insoluble in chloroform (Table I).

High-performance liquid chromatography

The separation achieved with the mixture of 12.5:12.5:12.5 methanol, acetonitrile and propan-2-ol is in Fig. 2A with capacity factors (k') and retention times (t_R) in Table II and with the mixture of 15:15:7.5 methanol, acetonitrile and propan-2-ol in Fig. 2B with k' and t_R in Table II. After equilibration with either mobile phase for 1 h k' varied by less than 5% over an 8-h period at 25°C. By reducing the proportion of propan-2-ol, keeping the phosphoric acid constant and increasing the methanol and acetonitrile equally k' can be increased without affecting the separation (compare Fig. 2A and B). Detection limits along with lowest recorded serum level in parenthesis are for AOL, 0.5 ng (2.1 ng/ml); AOL-ONE, 0.5 ng; ADR 1 ng (4.4 ng/ml); ADR-ONE,

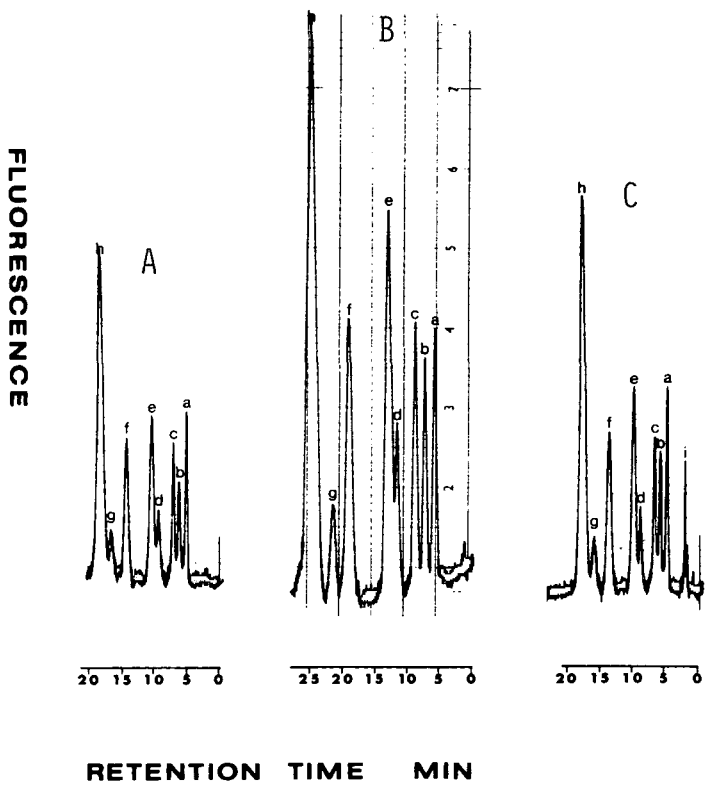


Fig. 2. Separation of a mixture of standards of ADR, metabolites and DNR (internal standard). (A) Chromatographic conditions: 5 m*M* (final concentration) orthophosphoric acid, 62.5% in methanol, acetonitrile and propan-2-ol, 12.5:12.5:12.5, pH 3.2. (B) Chromatographic conditions: 5 m*M* (final concentration) orthophosphoric acid, 62.5% in methanol, acetonitrile and propan-2-ol, 15:15:7.5, pH 3.2. (C) After extraction from human serum. Chromatographic conditions as in Fig. 2A. Elution rate 2.5 ml/min and fluorescence detection at 480 nm (excitation) and 560 nm (emission). Peaks: a = AOL; b = AOL-ONE; c = ADR; d = ADR-ONE; e = AOL-DONE; f = DNR; g = ADR—DONE, h = DNR-ONE; i = serum peaks.

TABLE II

SEPARATION OF ADRIAMYCIN AND METABOLITES BY ISOCRATIC REVERSED-PHASE HPLC USING μBONDAPAK C_{18}

Mobile phase 1 was 5 m*M* (final concentration) orthophosphoric acid, 62.5% in methanol, acetonitrile and propan-2-ol, 12.5:12.5:12.5. Mobile phase 2 was 5 m*M* (final concentration) orthophosphoric acid, 62.5% in methanol, acetonitrile and propan-2-ol, 15:15:7.5. The pH of both mobile phases was 3.2 and the elution rate was 2.5 ml/min. Fluorescence was measured at 480 nm (excitation) and 560 nm (emission).

Compound	Mobile phase 1		Mobile phase 2	
	k'	(t_R min)	k'	(t_R min)
AOL	2.1	(5.0)	2.4	(5.5)
AOL-ONE	2.6	(5.8)	3.3	(6.8)
ADR	3.6	(7.3)	4.1	(8.1)
ADR-ONE	4.2	(8.3)	6.0	(11.2)
AOL-DONE	5.3	(10.0)	6.7	(12.3)
DNR	7.8	(14.1)	10.1	(17.8)
ADR-DONE	8.7	(15.5)	12.8	(22.0)
DNR-ONE	9.4	(16.7)	13.6	(23.3)

1 ng (4.2 ng/ml); AOL-DONE, 0.5 ng (1.4 ng/ml); and ADR-DONE, 1 ng (2.7 ng/ml). Extraction of ADR and metabolites from serum did not introduce interference peaks (Figs. 2C and 3C).

In preliminary studies normal-phase HPLC was investigated for its ability to separate the aglycone metabolites of ADR and the glycoside metabolite AOL. Silica gel particles (5 and 10 μ*M*) (LiChrosorb Si 60, E. Merck, Darmstadt, F.R.G.) and a variety of chloroform-based mobile phases were tried. The aglycones always tended to elute early unresolved.

Negatively charged ions have been employed in the analysis of ADR and metabolites by HPLC [8, 21]. In one study [21] and in our own studies, these agents had little influence on k' of aglycone standards.

Separation of the aglycone metabolites of ADR has only been properly demonstrated using reversed-phase HPLC. In one case gradient elution was used [4], although this method was unable to resolve two of the aglycones, ADR-ONE and AOL-DONE. Another approach employed isocratic elution using one mobile phase to resolve the glycosides and another to resolve the aglycones and two internal standards [10]. A third method used isocratic elution with a single predominately aqueous mobile phase with the consequence that retention times of the later eluting peaks were delayed and poor peak symmetry and decreased sensitivity resulted [7].

The present method achieves a separation of ADR and its major glycoside metabolite AOL as well as four different aglycone metabolites and DNR without requiring gradient elution or more than one mobile phase. Also, a complete analysis can be performed in under 18 min without broadening of later eluting peaks and analysis time can be controlled by changing the proportions of the different components of the mobile phase.

Serum extractions

Over 50 control extractions with blood bank serum were performed. 1 or 2 ml of serum were spiked with 10 μl of a methanolic solution containing 10—1000 ng of standard. Individual extraction efficiencies ± S.D. expressed as a percentage for 10 ng of standard were for AOL, 78.4 ± 12.3%; AOL-ONE, 84.8 ± 9.3%; ADR, 83.4 ± 6.1%; ADR—ONE, 85.8 ± 8.6%; AOL-DONE, 91.0 ± 5.3%; DNR, 77.8 ± 7.4%; and ADR-DONE, 82.1 ± 9.6%. This simple technique did not involve prolonged treatment with ethanolic 0.6 *M* hydrochloric acid [7] or require high-speed centrifugation to separate phases after precipitating proteins with ammonium sulphate [4] or use prepacked mini-columns [12] and was capable of more or less equal recovery of ADR and metabolites along with DNR. A typical blank serum extract is shown in Fig. 3C.

Fig. 3A and B shows chromatograms of blood taken from two different cancer patients 15 min after intravenous bolus administration of 40 mg/m^2 ADR. In the first patient (Fig. 3A) four ADR metabolite peaks were identified

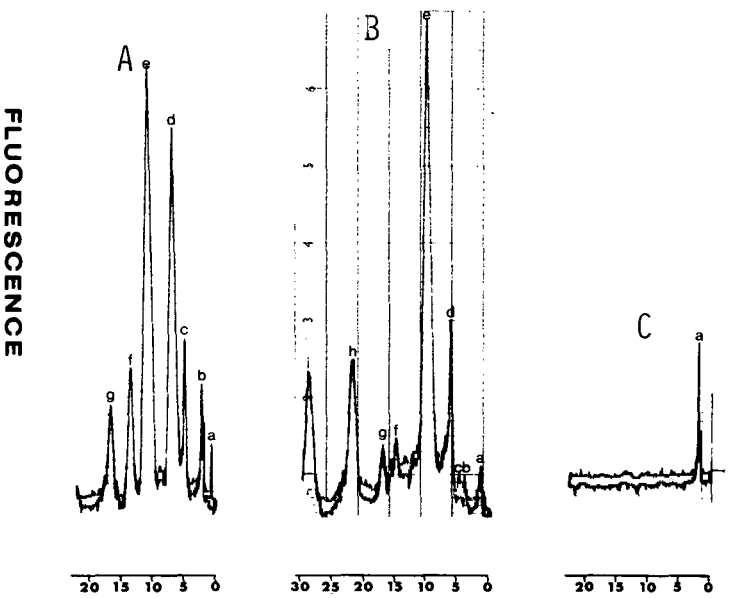


Fig. 3. (A) Serum extract from a patient who received ADR as an intravenous bolus of 40 mg/m^2. Chromatographic conditions as in Fig. 2A. Peaks: a = serum peaks; b = ADR metabolite (k' 1.8); c = AOL (26.5 ng/ml); d = ADR (257.6 ng/ml); e = AOL-DONE (109.8 ng/ml); f = DNR (internal standard); and g = ADR-DONE (21.4 ng/ml). (B) Serum extract from a second patient who received ADR as an intravenous bolus of 40 mg/m^2. Chromatographic conditions: 5 m*M* (final concentration) orthophosphoric acid, 62.5% in methanol, acetonitrile and propan-2-ol, 16.5:16.5:4.5, pH 3.2. Elution rate 2.5 ml/min and detection as in Fig. 2A. Peaks: a = serum peaks; b = ADR metabolite (k' 1.5); c = ADR metabolite (k' 1.8); d = AOL (16.6 ng/ml); e = ADR (260 ng/ml); f = ADR-ONE (4.1 ng/ml); g = AOL-DONE (5.8 ng/ml); h = DNR (internal standard) and i = ADR-DONE (17.1 ng/ml). (C) Blank serum extract. Chromatographic conditions as in Fig. 2A. Peaks: a = serum peaks.

apart from the parent drug and the internal standard. The metabolites present were AOL (26.5 ng/ml), AOL-DONE (109.8 ng/ml), ADR-DONE (21.4 ng/ml) and an early eluting metabolite with k' of 1.8. In the second patient (Fig. 3B) six ADR metabolite peaks were identified apart from the parent drug and the internal standard. The metabolites present were AOL (16.6 ng/ml), AOL-DONE (5.8 ng/ml), ADR-ONE (4.1 ng/ml) and ADR-DONE (17.1 ng/ml) along with two early eluting metabolites with k' of 1.5 and 1.8.

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NEW RAPID METHOD OF ANALYSIS OF CEFOXITIN IN SERUM AND BONE, BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for the extraction and quantification of cefoxitin in blood and bone samples is described in this paper. The procedure, which prepares biological material for reversed-phase high-performance liquid chromatographic analysis is convenient, rapid and reproducible. It also allows for use of cephalothin as an internal standard in measuring serum cefoxitin levels. Conventional extraction procedures, involving use of organic solvents, generally yield drug recoveries of 60—80%. Use of Baker - 10 SPE® disposable extraction columns allowed us to consistently obtain greater than 98% recovery of both cefoxitin and cephalothin. Methods for quantification of the extracted drugs include comparison of peak ratios (for serum) or peak heights (for bone) to first-order equations obtained from regression analyses.

INTRODUCTION

Biological materials such as saliva, serum and urine have been analyzed for cephalosporin content by high-performance liquid chromatographic (HPLC) methods [1—5]. Cefazolin, cephalothin, cefoxitin, cefotaxime, cefamandole, cefuroxime and cefoperazone (T1551) concentrations have been determined in plasma and urine specimen by reversed-phase liquid column chromatography [1]. Cefoxitin has also been measured in serum samples by reversed-phase liquid chromatography [2]. Anion-exchange HPLC has been used to detect cephalothin, cefoxitin and their metabolites in whole urine [3]. Moxalactam and cefazolin levels have been determined in bone and serum samples by HPLC [4]. Microbiological assays have also been used to demonstrate the presence of cephalosporins in samples of blood, skin, soft tissue and bone [6—12].

Procedures for the extraction of cephalosporins from biological materials vary according to methods of analysis and type of sample, with recovery of the

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drugs as low as 60%. In organic solvent extraction, low yields of recovered drug may be partially attributed to unfavorable partition coefficients of the extraction solvent.

In 1981, Dupont and De Jager [5] reported the use of Sep-Pak® cartridges to obtain 97% extraction of cefoperazone from serum samples.

The present communication reports a method which was developed independently in our laboratory for the extraction of cefoxitin from serum and bone of orthopedic patients. This method serves to increase the sensitivity of HPLC and decrease the quantity of drug lost in sample preparation. The procedure includes use of Baker-10 SPE® disposable C_{18} extraction columns and increases the yield of recovered drug 38% over procedures involving extraction with organic solvents. Not only is the method sensitive, but it is rapid and reproducible.

The use of cephalothin as an internal standard in measuring cefoxitin concentration in serum samples is also reported in this paper.

EXPERIMENTAL

Reagents and materials

Sodium cefoxitin was supplied by Merck Sharpe & Dohme (West Point, PA, U.S.A.). Cephalothin was purchased from Sigma. HPLC-grade acetonitrile and methanol (Fisher Scientific) and reagent-grade acetic acid, hydrochloric acid, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, phosphoric acid and potassium hydroxide (J.T. Baker) were used in these studies. Baker - 10 SPE disposable columns containing octadecyl (C_{18}) packing were used throughout these experiments.

Apparatus

A Waters Assoc. (Milford, MA, U.S.A.) HPLC system, which included a Model M6000A solvent delivery system, an U6K injector and a Model 440 fixed-wavelength (254 nm) detector, was utilized. A Hewlett-Packard Model 3390A integrator was used. The chromatograph was equipped with a μBondapak C_{18} (10 μm particle size; 30 cm × 3.9 mm I.D.) column and a Whatman guard column (Clifton, NJ, U.S.A.) packed with Co:Pell ODS.

Filtration of solvents was carried out using a Millipore analytical filtering system (Millipore, Bedford, MA, U.S.A.).

Preliminary treatment of samples

Samples of blood and bone were provided by orthopedic patients, who were undergoing elective arthroplastic surgery.

All samples were obtained from the surgical unit at the University of Mississippi Medical Center immediately upon notification of availability and were processed as follows:

The blood samples were centrifuged. The serum layer was removed and frozen at −60° C until analyzed.

When possible, at least 3 g, wet weight, of tissue were removed from each patient sample of cancellous bone. The bone was then cut into small pieces, approximately 2—3 mm in diameter, using a rongeur. Care was taken to select

tissue free from contamination with blood. Samples were packaged individually and frozen at —60° C until analyzed.

Preparation of standards

For serum, a stock standard solution of cefoxitin (1 mg/ml) was prepared by dissolving 26.37 mg of cefoxitin in 2 ml of 1% phosphate buffer, pH 6.0 [13], and diluting to 25 ml with drug-free serum. Working standards (12.5, 25, 50, 100, 200 and 400 μg/ml) were then made by diluting the stock solution with drug-free serum.

A solution of cephalothin (500 μg/ml), prepared in the 1% phosphate buffer, was used as an internal standard for measuring cefoxitin concentration in serum samples.

For bone, a solution of cefoxitin (1 mg/ml) was prepared in distilled water. Dilutions (50, 100, 250 and 500 μg/ml) were made and a 60-μl aliquot of each solution was added to 3-g portions of cefoxitin-free bone. These samples were then incubated for 30 min in a water bath (38° C). Final cefoxitin concentrations of the standard solution were 1, 2, 5, 10 and 20 μg/g. The use of heat in preparation of bone standards eliminated the possibility of using an internal standard because the loss of cefoxitin from samples during period of incubation with cephalothin was a probability. Since an internal standard was not used, three sets of bone standards were prepared and extracted at separate times.

All standard solutions were either stored at —20° C or kept on ice when not in use.

Assay for serum cefoxitin

Extraction procedure. To 1 ml of serum was added 0.2 ml of the solution of internal standard (cephalothin) and the sample was acidified (pH < 3) by addition of 0.5 ml of 0.4 *M* hydrochloric acid. The sample was then applied to an octadecyl (C_{18}) disposable column that had been conditioned with 2 column vols. of methyl alcohol and 2 column vols. of distilled deionized water. After aspiration of the sample, the column was washed with 1 vol. of 0.1 *M* hydrochloric acid and dried under vacuum for 1 min. The sample was then eluted with 0.5 ml methyl alcohol. A 20-μl aliquot of the methyl alcohol eluent was injected into the HPLC system.

Chromatography. The mobile phase consisted of a mixture of distilled deionized water—acetonitrile—acetic acid (70:29:1). The flow-rate was set at 1.0 ml/min. The detector was set at 0.5 a.u.f.s. All separations were carried out at ambient temperature.

Quantification. First-order regression analysis was performed on data acquired from HPLC analysis of the serum standards. The *X*-coordinate values were represented by the ratio of the peak area of cefoxitin (PACF) versus peak area of cephalothin (PACP) for a known concentration of cefoxitin. The *Y* coordinates were represented by the known concentrations of cefoxitin. Cefoxitin in serum samples was quantified by comparing HPLC data of the ratio of PACF versus PACP to the first-order equation from regression analysis of the serum standards.

Assay for bone cefoxitin

Extraction procedure. After the prepared bone samples were thawed,

approximately 3 g of each sample were weighed into screw-capped conical tubes. The bone was then mixed with water in a ratio of weight of bone (g) to volume of diluent (ml) equivalent to 1:2. The mixture was shaken at 5° C for 5 h. The diluent was then removed and frozen at −20° C overnight. The procedure for extraction of cefoxitin from the diluent was the same as for the serum, but without the addition of internal standard. A 75-μl aliquot of the methyl alcohol eluent was then injected into the chromatographic system.

Chromatography. The mobile phase consisted of a mixture of distilled deionized water—acetonitrile—acetic acid (80:19:1). The flow-rate was set at 1.0 ml/min. The detector was set at 0.02 a.u.f.s. The column was operated at ambient temperature.

Quantification. The chromatograms were quantified by comparing cefoxitin peak height to the first-order equation.

First-order regression analysis on HPLC data of cefoxitin bone standards was done as for serum standards. The X-coordinate values were represented by the averaged peak heights from three different preparations and extractions of the known cefoxitin concentration. The Y coordinates were represented by known concentrations of cefoxitin. Cefoxitin concentration in bone samples was then calculated by comparing the HPLC data of the averaged cefoxitin peak heights to the first-order equation determined by the regression analysis.

RESULTS AND DISCUSSION

Preliminary extraction of cefoxitin bone standards and samples

Due to the lack of available literature on the extraction of cephalosporins from bone, studies were done to determine the period of time necessary to extract cefoxitin from bone with water. Several drug-free bone samples were incubated with 60 μl of 1 mg/ml cefoxitin to obtain 20 μg/g cefoxitin standard. Portions (3 g) of the cefoxitin bone samples were then mixed with 6 ml water and shaken on a mechanical shaker at 5° C for a total of 24 h. Aliquots of 50 μl of the eluent were removed and quantified at 2, 3, 4, 5, 6, 7 and 24 h. Samples analyzed after 5 h showed no increase in cefoxitin concentration, hence, a period of 5 h was used throughout these experiments for extraction of the drug from bone samples.

Standard solutions of cefoxitin (1, 2, 5, 10, and 20 μg/ml) in methyl alcohol were quantified concurrently with the bone extract. Recovery of cefoxitin was calculated at 56 ± 6%. Since the recoveries were low and an internal standard was not used, three different sets of bone standards were prepared, extracted and analyzed. These analyses repeatedly gave recoveries within the 56 ± 6% range. Possibly, adsorption to the bone by cefoxitin can account for this reduction of the recovery. Efforts to improve this recovery were limited by the number of cefoxitin-free bone samples which could be obtained at surgery.

Extraction of cefoxitin from serum and bone

Extraction with C_{18} disposable columns resulted in greater than 98% recovery of cefoxitin from blood and bone standards. Organic solvent extraction, using chloroform—1-pentanol, yielded a recovery of only 60—80%.

Extraction with cyano disposable columns was tried, but since cefoxitin was detected in the eluent of the sample application, the method was rejected.

Analysis of the eluent, after application of sample to the C_{18} disposable column, revealed no loss of either drug from the column; also, analysis of the acid wash eluent, revealed loss of neither cephalothin nor cefoxitin.

The rate of elution of the first methyl alcohol portion (0.5 ml) was regulated in order to maximize the elution of cefoxitin and cephalothin. An elution rate of 1 drop every 2 sec resulted in total recovery of both drugs in the first methyl alcohol elution, as was determined by absence of drug in the second methyl alcohol eluent.

Linear regression analysis

Linear regression analysis performed on serum standards provided a correlation coefficient equal to 0.99998.

Linear regression analysis performed on bone standards provided a correlation coefficient of 0.99886.

HPLC

A typical chromatogram of the sample fraction of serum (A) and bone (B) from a patient are depicted in Fig. 1.

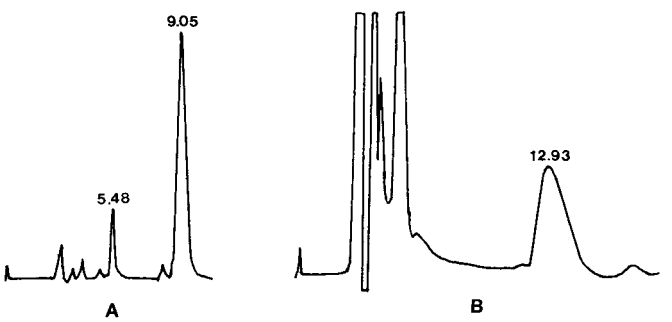

Fig. 1. (A) Chromatogram of sample fraction (0.5 ml) of extracted serum. Blood sample was drawn at time of bone excision. Cefoxitin concentration was 20.4 μg/ml. Retention time of cefoxitin is 5.48 min. Retention time of cephalothin (internal standard) is 9.05 min. λ = 254 nm; 0.5 a.u.f.s.; mobile phase: water—acetonitrile—acetic acid (70:29:1); injection volume; 20 μl. (B) Chromatogram of sample fraction (0.5 ml) of extracted bone. Cefoxitin concentration was 4.2 μg/ml. Retention time of cefoxitin is 12.93 min. λ = 254 nm; 0.02 a.u.f.s.; mobile phase: water—acetonitrile—acetic acid (80:19:1); injection volume: 75 μl.

The mobile phase was altered from water—acetonitrile—acetic acid (70:29:1) in serum cefoxitin determination to water—acetonitrile—acetic acid (80:19:1) in bone cefoxitin determination due to an unidentified peak which occurred in blank bone controls. The occurrence of this peak necessitated delaying the elution of cefoxitin in order to avoid interference. Fig. 2 shows chromatograms of extracted blank serum (A) and extracted blank bone (B) used in preparing standards. In Fig. 2B the peak at 11 min would be separated from any cefoxitin peak which would elute 2 min later, using 80% water in the mobile phase; at 70% water these two peaks are not separated.

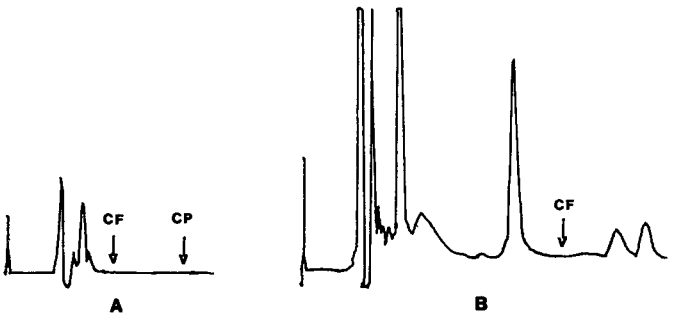

Fig. 2. (A) Chromatogram of blank serum, extracted as for serum standards and samples. HPLC conditions were the same as those in Fig. 1A. (B) Chromatogram of blank bone used in preparing bone standards, extracted as for bone standards and samples. HPLC conditions were the same as those in Fig. 1B. (↓) denotes where cefoxitin (CF) or cephalothin (CP) would elute in serum and where cefoxitin (CF) would elute in bone.

Precision

The 100 μg/ml cefoxitin serum standard was analyzed with each set of samples to determine the precision of the curve. Relative precision of 3.5% for the 100 μg/ml serum standard was obtained during the analysis of the 55 serum samples. Due to the linearity of the serum standard curve, the high rate of recoveries for cefoxitin from serum (> 98%), and the use of an internal standard, no more than one point on the serum standard curve was repeatedly extracted and analyzed.

The relative precision for the bone standard curve was 3% on the lower part of the curve and 10% on the upper portion. To compensate for the variations in precision, three different sets of bone standards were prepared, extracted and analyzed. The averages of their peak heights were used to prepare the standard curve. Bone standards were also analyzed intermittently during HPLC analysis of samples and were within the limits of the standard curve.

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Note

Quantitative analysis of aromatic amines in human urine by gas chromatography—mass spectrometry—selected-ion monitoring

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Epidemiological studies in several countries on workers in the rubber industry point to a high incidence of various types of tumours [1]. Bladder cancer has been associated with exposure to aromatic amines and in particular to 2-naphthylamine. Other types of cancer (lung, stomach, and leukaemia) occurring in excess among rubber workers have not been associated with exposure to specific compounds; but this is explained by the fact that the industrial processes involved use a wide variety of chemicals whose toxicological properties are unknown. Since it is unlikely that toxicological information about these compounds will be available in the near future, a practical approach would be to reduce the exposure of these workers and to measure it.

N-Phenyl-2-naphthylamine (PBNA), N-isopropyl-N′-phenyl-*p*-phenylenediamine (IPPD) and N-(1,3-dimethylbutyl)-N′-phenyl-*p*-phenylenediamine (6PPD) are widely used as antioxidants and antiozonants (generally at levels of 1—2%) in the rubber industry [2] and workers may be exposed to them.

Contrasting results have been reported on the carcinogenicity of PBNA in laboratory animals: oral [3—5], subcutaneous [3,4,6] and aerosol [7] administration to mice resulted in a significantly higher incidence of different tumours; PBNA intragastrically administered for life did not cause neoplastic growth in Sprague—Dawley rats [8] or in Syrian golden hamsters [9]; no bladder tumours were observed in dogs fed orally with PBNA [10]. There are no data in the literature on IPPD and 6PPD carcinogenicity. Hence, it is important to quantify the possible exposure to these chemicals in order to avoid or

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reduce contact with those chemicals for which the toxicological hazard is not yet clear.

One of the simplest and most easily applicable systems is to measure urinary excretion of aromatic amines in exposed individuals. A number of methods have been reported for the determination of aromatic amines in human urine by gas chromatography (GC) [11—13], high-performance liquid chromatography (HPLC) [14] and mass spectrometry (MS) [15]. However, to our knowledge there are no methods to determine PBNA in human urine and only one method reports separation and quantitation of IPPD and 6PPD in this biological fluid [16]; in that method aromatic amines were measured by HPLC with a sensitivity of 50 μg/l.

In order to study the exposure of workers to aromatic amines normally used in the rubber industry, we devised a reliable, specific and highly sensitive analytical method for the determination of trace levels of PBNA, IPPD and 6PPD in human urine using combined gas chromatography—mass spectrometry—selected-ion monitoring (GC—MS—SIM). The application to biological monitoring of rubber industry workers is reported.

EXPERIMENTAL

Chemicals and reagents

PBNA was provided by E. Merck (Darmstadt, F.R.G.); IPPD and 6PPD were obtained from Bayer (Leverkusen, F.R.G.). Reagent-grade sodium hydroxide and ammonium hydroxide were from Farmitalia Carlo Erba (Milan, Italy). Trifluoroacetic anhydride (TFAA) of reagent grade was purchased from Janssen (Beerse, Belgium). Silylation-grade pyridine was obtained from Pierce (Rotterdam, The Netherlands). *n*-Hexane of pesticide analytical grade was supplied by Riedel-de Haen (Hannover, F.R.G.).

Extraction and derivatization of biological samples

In a 40-ml centrifuge tube, 20 ml of human urine were adjusted to pH 11 using 10 *M* sodium hydroxide. The compounds were extracted three times with 10 ml of *n*-hexane by mixing for 10 min on a horizontal reciprocating shaker. After centrifuging at 2000 *g* for 5 min the combined organic extracts were concentrated to 1 ml in a rotating evaporator, transferred to a 10-ml reaction tube and evaporated under vacuum to dryness. The residue was dissolved in 500 μl of *n*-hexane and reacted with 20 μl of TFAA and 20 μl of pyridine at 60°C for 30 min using a thermostated bath. After cooling, 1.5 ml of *n*-hexane and 1 ml of 5% aqueous ammonia solution were added to the sample and the mixture was shaken on a vortex mixer for 1 min. The organic layer was transferred to another 10-ml conical tube and evaporated to dryness under an air stream. The residue was dissolved in 50 μl of *n*-hexane; 4 μl were analysed by GC—MS.

Instrumentation

An LKB 2091 gas chromatograph, low-resolution mass spectrometer, equipped with an LKB 2130 computer data processing system, was used. A 2 m × 2 mm I.D. silanized glass column packed with 3% OV-1 on Gas Chrom

Q (80—100 mesh) (Supelco, Bellefonte, PA, U.S.A.) was used. Column and injector port temperatures were 265°C and 290°C, respectively. Helium was used as carrier gas and the column head pressure was 2.5 bars. The mass spectrometer was operated in the electron-impact (EI) mode with the following conditions: electron energy 70 eV, trap current 50 μA, ion source temperature 250°C. SIM chromatograms were obtained by monitoring the ions at *m/z* 315, 376/418, 376/460 for PBNA, IPPD, 6PPD, respectively.

Calculations

Since early attempts to synthesize deuterium-labelled aromatic amines to be used as internal standard failed, all measurements were made by comparison of the areas of unknown samples with areas of reference mixtures containing known amounts of PBNA, IPPD and 6PPD. Reference mixtures were injected every three samples.

Calibration curves were constructed by derivatizing known amounts (0.1, 0.2, 0.4, 1, 2 ng) of aromatic amines as described above. Extraction and clean-up efficiency were evaluated by adding known amounts (2—80 ng) of standards PBNA, IPPD and 6PPD to 20 ml of blank urine samples which were then processed as described above. For routine analysis, blank and spiked urine samples were processed together with each batch of samples.

Human studies

Urine specimens (200 ml) were collected from 21 workers in a rubber factory, who were likely to have been exposed to aromatic amines by inhalation or skin contact, and kept frozen (−20°C) until analysed.

RESULTS AND DISCUSSION

The mass spectra of the three underivatized amines gave intense peaks which could be used for SIM detection at *m/z* 219 $(M)^+$ for PBNA, *m/z* 211 $(M-15)^+$ and 226 $(M)^+$ for IPPD, *m/z* 211 $(M-57)^+$ and 268 $(M)^+$ for 6PPD as shown in Fig. 1. However, the attempt to use these for SIM analyses failed because aromatic amines gave broad GC peaks because of absorption on the GC columns and interference from urine components.

To improve the GC behaviour of the compounds to be analysed derivatization was decided upon. Trifluoroacetyl (TFA) derivatives were found to be suitable for GC—MS analysis. Derivatization was quantitative: PBNA gave rise to a monoderivative while IPPD and 6PPD reacted with two TFAA molecules. All the derivatives produced sharp and symmetrical GC peaks. The mass spectra of the three derivatives are shown in Fig. 2. The retention times and masses of the most abundant ions in each spectrum are listed in Table I together with their abundance ratios relative to the most intense ion.

Multiple-ion detection was performed by monitoring the ion intensities at *m/z* 315 for PBNA, at *m/z* 376 and 418 for IPPD, at *m/z* 376 and 460 for 6PPD. Typical SIM chromatograms are shown in Fig. 3; there were no interfering peaks from amine-free urine.

Peak areas as a function of the amounts of amines injected were linear in the range 0.1—2 ng as shown by linear regression analysis correlation coefficients of

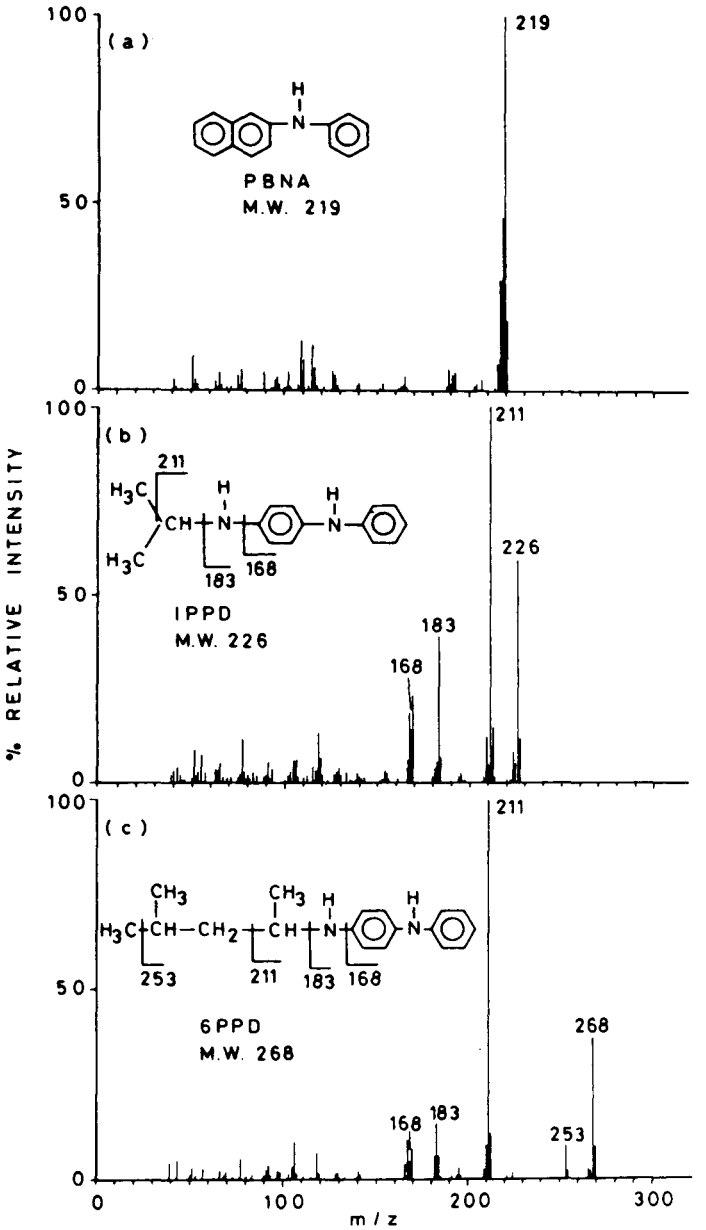

Fig. 1. Mass spectra of PBNA (a), IPPD (b) and 6PPD (c).

0.9999, 0.9991 and 0.9989 for PBNA, IPPD (*m/z* 418) and 6PPD (*m/z* 376), respectively.

The minimum detectable amount of aromatic amines was calculated to be 0.1 μg/l of urine with a signal-to-noise ratio $>3:1$. Mean recovery values and standard errors, in the range of concentrations in urine from 0.1 ng/ml to 4 ng/ml, were 86±3%, 77±5% and 70±5% for PBNA, IPPD and 6PPD, respectively.

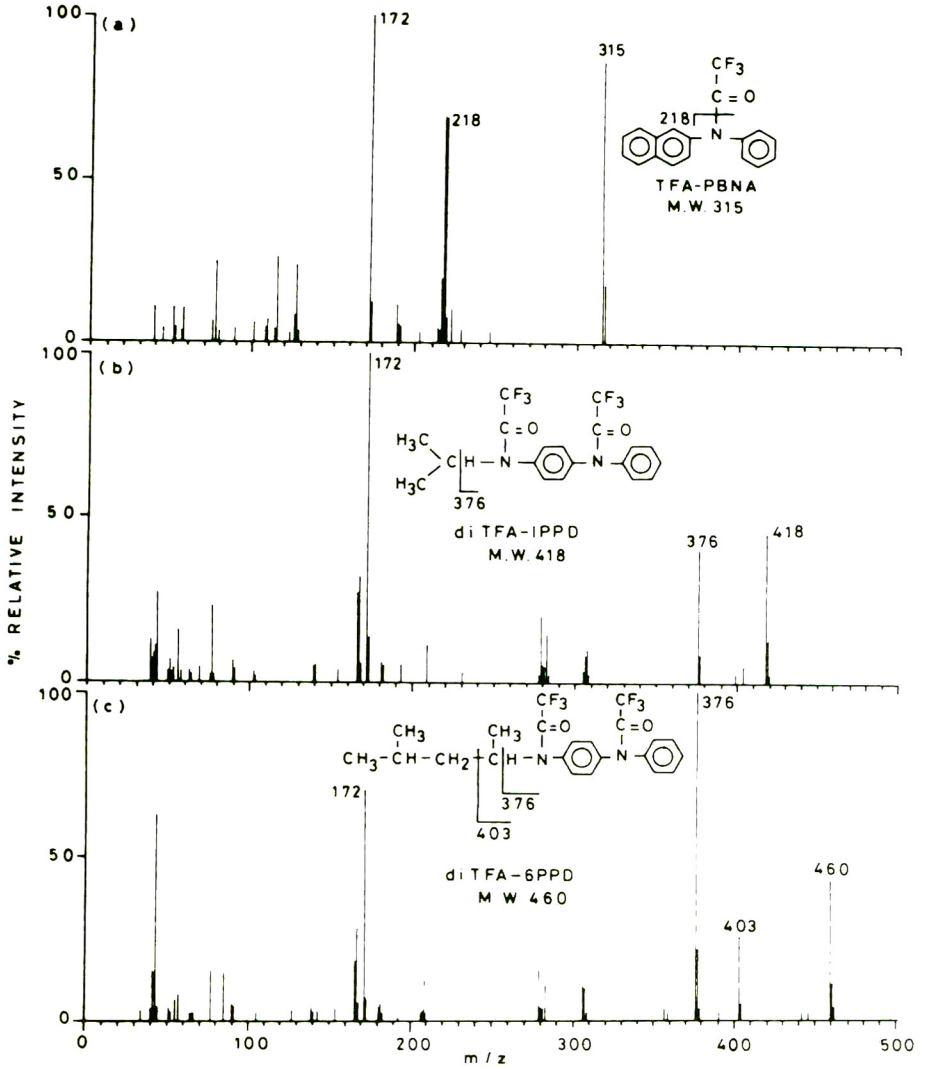

Fig. 2. Mass spectra of TFA derivatives of PBNA (a), IPPD (b) and 6PPD (c).

TABLE I

RETENTION TIME OF TFA AROMATIC AMINE DERIVATIVES, MASSES AND ABUNDANCE RATIOS OF CHARACTERISTIC IONS

Compound	Retention time (min)	Ions, *m/z* (abundance, %)
diTFA-IPPD	1.6	172(100), 418(46), 376(40), 167(32)
TFA-PBNA	2.0	172(100), 315(86), 217(69), 218(68)
diTFA-6PPD	2.4	376(100), 172(70), 43(63), 460(42)

The SIM method was applied to the determination of urine levels of PBNA, IPPD and 6PPD in a group of 21 workers in the rubber industry. Four urine samples were collected during a working week from each worker. About 70%

of the urine samples analysed contained at least one of the aromatic amines studied. As reported in Fig. 4, among the positive samples more than three-quarters presented values ranging from 0.1 to 0.3 μg/l; the highest value detected was 1.3 μg/l and there was no correlation between the presence of the three amines in the same subject. Air samples collected in the work area showed concentrations of 0.01—1 $\mu g/m^3$ for each of the three amines.

In conclusion, the GC—MS—SIM assay described is convenient for detecting and quantifying PBNA, IPPD and 6PPD in human urine with high sensitivity (0.1 μg/l) and lack of interference.

Nevertheless, the information obtained by application of this method is useful at the present time only for qualitative evaluation of workers' exposure to aromatic amines. Studies on laboratory animals [17—19] and humans

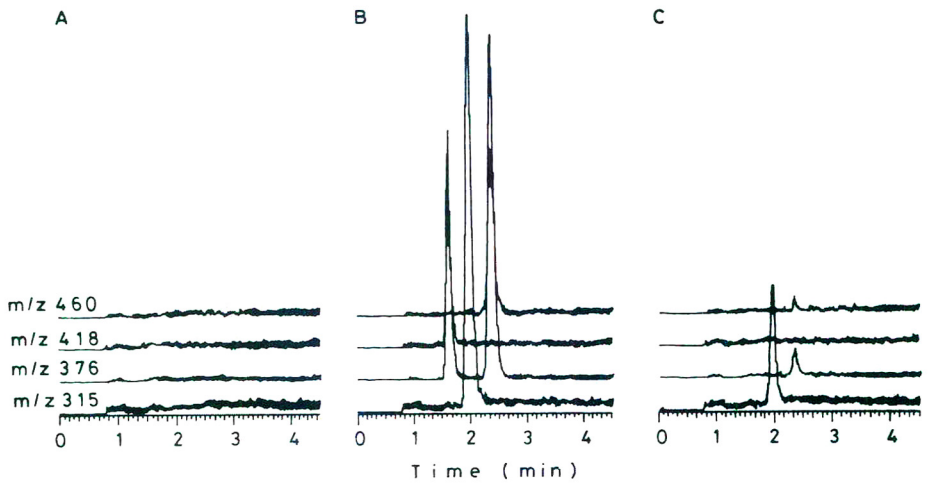

Fig. 3. Mass fragmentograms of selected ions of PBNA (*m/z* 315), IPPD (*m/z* 376, 418) and 6PPD (*m/z* 376, 460). (A) Blank urine sample; (B) standard aromatic amine mixture (2 ng each); (C) urine sample.

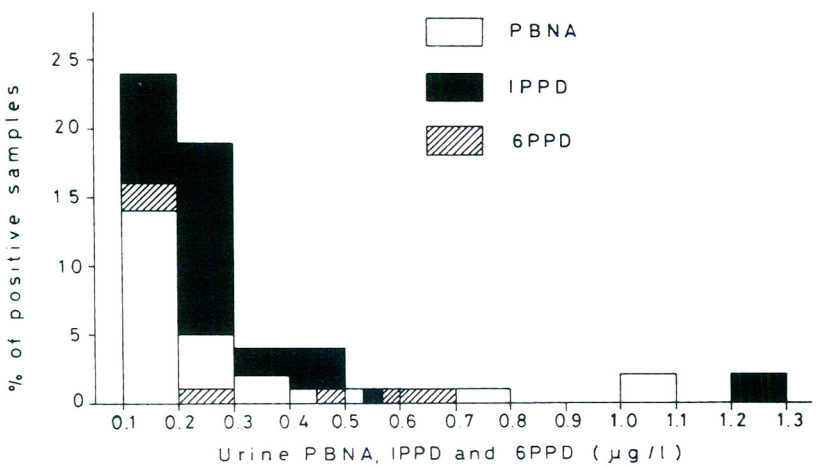

Fig. 4. Distribution of PBNA-, IPPD- and 6PPD-positive samples as a function of urine concentration.

[20,21] have shown that PBNA [18—21] and IPPD [17] are rapidly metabolized; so, in fact, determination of their urinary excretion may not give the best quantitative indicator of exposure. Experiments are in progress to determine whether these compounds are metabolized to products that may better reflect human contamination.

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Note

Dihydrocaffeic acid: a common contaminant in the liquid chromatographic—electrochemical measurement of plasma catecholamines in man

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High-performance liquid chromatography with electrochemical detection (HPLC—ED) after an alumina extraction provides high sensitivtiy and specificity for measuring plasma levels of the endogenous catecholamines norepinephrine (NE), epinephrine (E), and dopamine (DA) [1]. This technique will also detect catechols other than the neurotransmitter catecholamines, because the alumina separation and electrochemical reaction depend on a catechol nucleus and its oxidation potential and not on the presence of an amine residue [2]. This

*The opinions and assertions contained herein are the private ones of the author and are not to be construed as official or reflecting the views of the Department of Defense or the Uniformed Services University of the Health Sciences.

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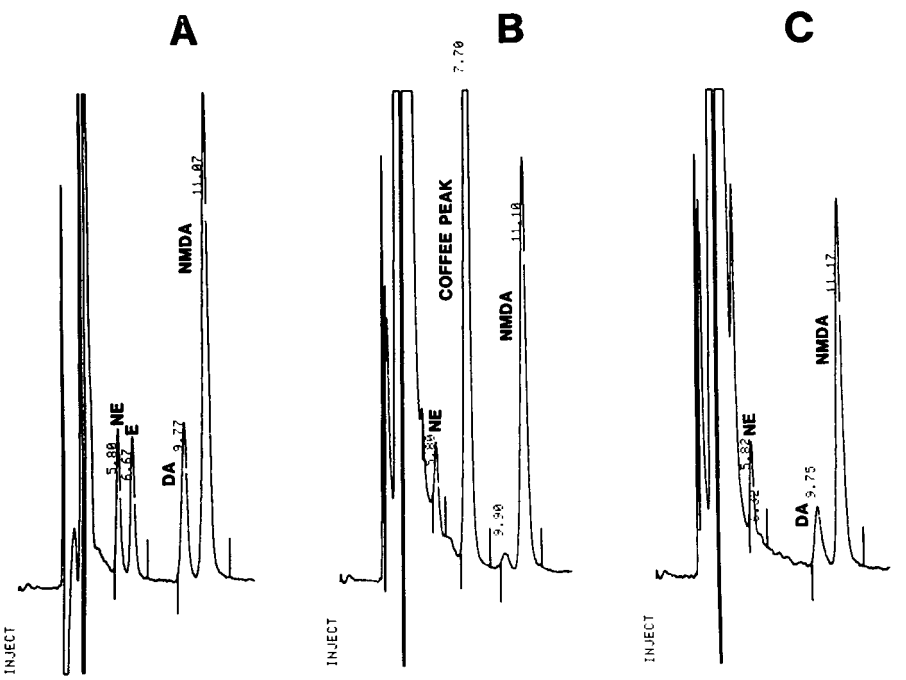

Fig. 1. (A) Chromatogram (BAS system) of 500 pg each of norepinephrine (NE), epinephrine (E), dopamine (DA), and the internal standard, N-methyldopamine (NMDA) after alumina batch extraction. Numbers are retention times in hundredths of a minute. (B) Chromatogram of plasma-derived eluate from a patient with a large coffee peak. (C) Chromatogram from the same patient 18 h after discontinuing coffee. Note complete absence of the coffee peak.

means that in clinical settings where the food and drug intake of the patient is uncontrolled, exogenous catechols may produce contaminating chromatographic peaks by the HPLC—ED technique.

One unidentified substance which we have frequently encountered has been more than a mere annoyance, because it may co-chromatograph with E or DA depending on the chromatographic conditions. In the case shown in Figs. 1 and 2, for instance, this unknown substance co-chromatographed with E in a patient with an adrenal mass and suggested the diagnosis of pheochromocytoma. We sought to identify this substance.

METHODS

We compared the chromatographic characteristics of the unknown substance with those of several standards on different chromatographic systems. The small amount of substance in the unknown peak precluded direct identification by gas chromatography—mass spectrometry (GC—MS) without substantial concentration and isolation efforts.

The HPLC—ED method used was previously validated in this laboratory [1]. In the present study we used two different HPLC—ED systems. One consisted of a Waters 6000A solvent delivery system, U6K injector, μBondapak

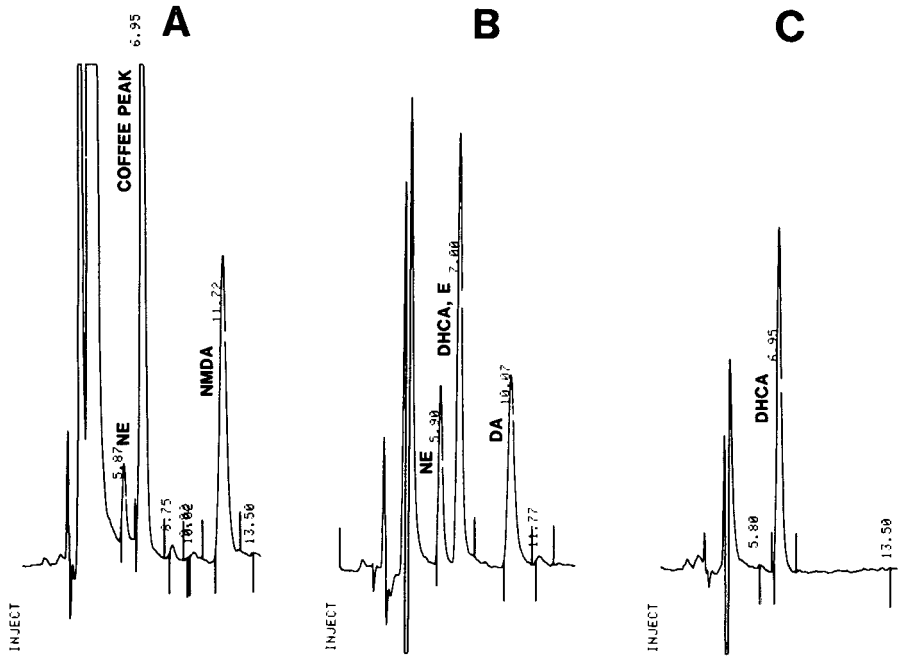

Fig. 2. (A) Co-chromatography of the coffee peak with epinephrine (E) using the same plasma sample as in Fig. 1B but with the plasma-derived eluate injected into a matched system with a somewhat newer column; (B) Co-chromatography of dihydrocaffeic acid (DHCA) with E on this system. Compare with Fig. 1A; (C) Chromatogram of DHCA. Note identical retention time with that of the coffee peak.

C_{18}, 30 cm × 3.9 mm reversed-phase stainless-steel column containing 10-μm irregular particulate packing, and a guard column packed with C_{18} Porasil; a Bioanalytical Systems (BAS) LC4 or LC4A amperometric detector with TL5 glassy carbon electrode; and a Waters Data Module. The mobile phase for this system consisted of 1 l water, 6.8 g sodium acetate, 100 mg EDTA, 1 g heptanesulfonic acid, and 7% acetonitrile at pH 4.8. The mobile phase was pumped at a flow-rate of 1.0 ml/min with the detector set at 1 nA/V at 0.50 V applied potential. The other system was the same, except that an Environmental Sciences Associates (ESA) triple-electrode system was used, with the first electrode in the post-column series set at 0.30 V, the second at 0.15 V, and the third at —0.35 V, so that only reversibly oxidizable species were detected. The ESA mobile phase consisted of 1 l water, 6.8 g sodium acetate, 100 mg EDTA, 1 g heptanesulfonic acid, and 8% acetonitrile at pH 3.5.

After HPLC studies suggested dihydrocaffeic acid as the unknown substance, GC—MS was used to confirm this identification. Aliquots (2 ml) of plasma obtained from subjects without the unknown peak, plasma to which dihydrocaffeic acid (3 ng/ml) had been added, and plasma from a patient who, had a large unknown chromatographic peak were acidified with 1 ml of 1 mol/l hydrochloric acid, and 0.1 ml of 0.114 mol/l ascorbic acid and 0.1 ml of 0.054 mol/l EDTA were added. Each sample was extracted twice with 4 ml of ethyl acetate. The extracts were dried over sodium sulfate, evaporated in vacuo, and transferred with methanol to PTFE-lined screw cap glass tubes. The

samples were evaporated under nitrogen and reacted with 50 μl of pentafluoropropanol and 100 μl of pentafluoropropionic anhydride at 70°C for 15 min, evaporated under nitrogen, and then rereacted with 100 μl of pentafluoropropionic anhydride for 10 min at 70°C. The reacted samples were evaporated to dryness and redissolved in 25 μl of ethyl acetate for GC—MS. A 3% OV-17 (100—120 mesh) column (2 m × 2 mm I.D., glass) was used for analysis. Of each derivatized sample 2—4 μl were injected into a Finnigan Model 3200 electron ionization GC—MS system with a Nermag SADR data acquisition system.

RESULTS

Of 108 people whose plasma was assayed for catecholamine content using HPLC—ED, 36 (33%) had an additional peak larger than that of NE, E, or DA and with a retention time between that of E and DA.

When patients with large unknown peaks were interviewed, they all reported being coffee drinkers, with coffee intake varying from 3 to 30 cups per day. Four subjects volunteered to stop drinking all coffee or caffeinated beverages for periods varying from 18 h to 1 week. In all of these subjects, the unknown peak decreased markedly in height or disappeared entirely (Fig. 1C). Two subjects substituted decaffeinated for caffeinated coffee and had blood drawn

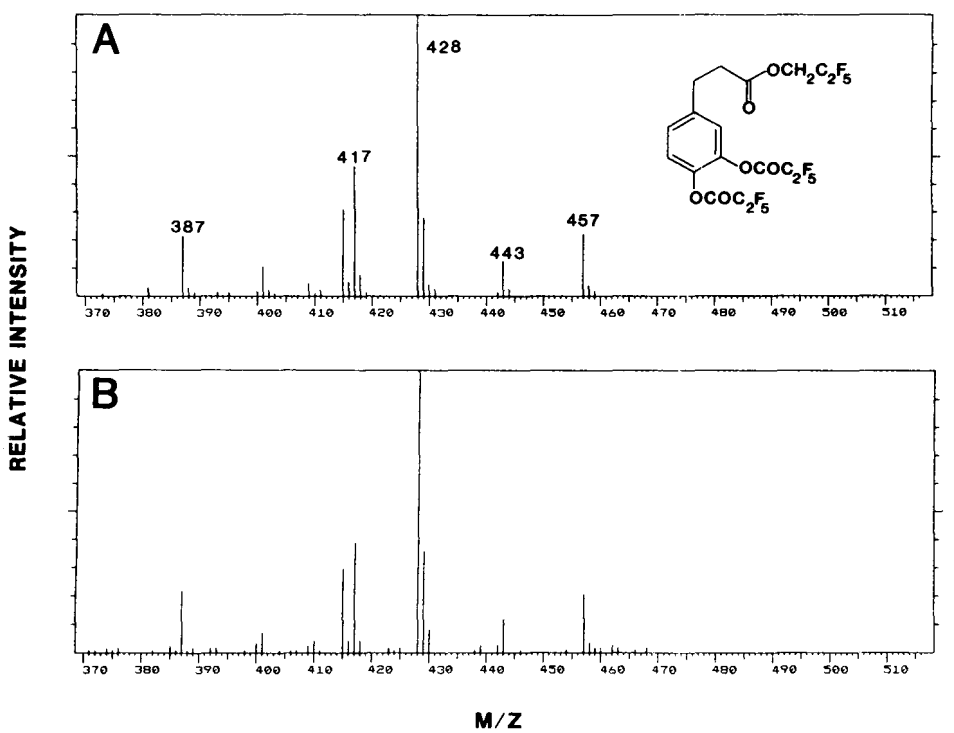

Fig. 3. Electron-ionization spectra for derivatized (A) dihydrocaffeic acid; and (B) plasma extract from a patient with a large unknown peak on HPLC—ED analysis.

1 day or 1 week later, and in both, the unknown peak persisted. These findings, and the fact that neither caffeine nor any of several of its metabolites was detectable by the HPLC—ED procedure, implied that the unknown substance was related to a non-caffeine constituent of coffee. The unknown substance was not detectable in coffee itself after the alumina extraction. Because the unknown substance was retained on alkaline alumina and eluted from acid alumina as part of the HPLC—ED sample preparation, and because it was reversibly oxidizable at low potentials, we reasoned that it was a catechol. None of the known stable catecholamine metabolites or tyramine, synephrine, ascorbic acid, N-methylepinephrine, or dimethylephinephrine co-chromatographed with the unknown substance. We therefore searched for a metabolite of a catechol present in coffee.

Caffeic acid and chlorogenic acid are well known coffee-associated catechols [3]. Caffeic acid was not retained on the HPLC column, and chlorogenic acid did not co-chromatograph with the unknown substance. However, dihydrocaffeic acid (DHCA, 3,4-dihydroxyphenylpropionic acid), a metabolite of caffeic acid, co-chromatographed with the unknown peak on two HPLC systems. DHCA had congruent voltammetry with that of the unknown peak. We therefore concluded that DHCA was likely to be the unknown substance.

Dihydrocaffeic acid was then studied using GC—MS. At a column temperature of 130° C, derivatized DHCA eluted at 2.5 min; the separator and transfer lines were at 200° C; and the ionizing potential was set at 70 eV. Partial mass spectra (*m/z* 370—515) are shown in Fig. 3. Selected-ion recording at *m/z* 428 and 457 (not shown) indicated that these ions were present at the appropriate retention time in the plasma extract which exhibited the unknown peak on HPLC and in the plasma extract to which 3 ng/ml of DHCA had been added but were absent in a plasma sample which did not exhibit the unknown peak on HPLC. Thus, extracted, derivatized plasma without the unknown peak did not contain DHCA, whereas a plasma extract from a subject with a large unknown peak contained a substance with the identical retention characteristics and mass spectra as recorded from authentic DHCA (Fig. 3).

DISCUSSION

In clinical settings where the food and drug intake of the patient is uncontrolled, exogenous catechols may produce contaminating chromatographic peaks by the HPLC—ED technique for catecholamines. One such peak has appeared in about one third of the chromatograms we have obtained, and occasionally this peak has co-chromatographed with epinephrine or dopamine.

On the basis of clinical observations and then HPLC—ED and GC—MS analyses, we identified the unknown substance as dihydrocaffeic acid, a catechol metabolite of caffeic acid. Dihydrocaffeic acid can be produced from caffeic acid by human intestinal bacteria [4].

The presence of detectable DHCA in plasma was not associated with elevations of plasma NE. Thus, despite the fact that acute caffeine ingestion increases plasma catecholamines [5], our apparent marker of coffee drinking was not associated with generalized sympathetic neural activation. When injected intravenously acutely into anesthetized rats and for up to 32 h into

conscious rats, DHCA produced no changes in blood pressure, heart rate, or cardiac output.

We routinely have proscribed drinking caffeinated beverages for 24 h prior to blood sampling in our clinical studies, but we have allowed subjects to drink decaffeinated coffee. Since many subjects have stopped driking coffee in any from prior to blood sampling, the prevalence of the DHCA peak we observed probably underestimated that in the general population.

When HPLC—ED procedures are used to measure plasma catecholamines in people, dietary factors can produce contaminating peaks and potentially lead to erroneous clinical interpretations. One such factor is DHCA, a metabolite of a coffee-containing catechol.

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Note

Determination of DOPA, dopamine, and 5-S-cysteinyl-DOPA in plasma, urine, and tissue samples by high-performance liquid chromatography with electrochemical detection

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There are two basic classes of melanin pigments in animals, i.e. dark eumelanin and reddish pheomelanin [1]. In melanocytes, tyrosinase converts tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and then to a highly reactive dopaquinone. In the absence of sulphydryl compounds, dopaquinone is converted to eumelanin in a complex series of reactions. If dopaquinone encounters cysteine (or glutathione), cysteinyl-DOPA isomers are produced among which 5-S-cysteinyl-DOPA is the major one [2]. Further oxidation of cysteinyl-DOPA gives rise to pheomelanin.

A high level of 5-S-cysteinyl-DOPA was first detected in melanoma tissues [3] and then in urine of melanoma patients [3]. The urinary excretion of this catecholic amino acid was found to reflect the degree of dissemination of melanoma [4] and is now routinely evaluated in many clinical laboratories. Although a fluorimetric method was originally developed for the determination of 5-S-cysteinyl-DOPA, a more sensitive and selective method was then reported using high-performance liquid chromatography (HPLC) with electrochemical detection [5—7].

Commonly, alumina extraction has been applied to extract catechols from biological samples before chromatographic analysis. However, the previously reported methods for 5-S-cysteinyl-DOPA determination use a batch procedure [5] or a column procedure [7] which is tedious and requires large amounts of

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samples, e.g. 6 ml of serum [5]. We have been studying the correlation between the level of DOPA and 5-S-cysteinyl-DOPA, the pigment precursors, and the type of melanogenesis [8,9]. In these studies, we found it convenient to develop a micro-batch procedure for catechol extraction which can be applied to plasma, urine, and tissue samples with minimum variations in the procedure and requires minimum amounts of samples. We describe here an improved method for the determination of DOPA, dopamine, and 5-S-cysteinyl-DOPA in biological samples by HPLC with electrochemical detection. Dopamine was included as an analyte because it is an immediate metabolite of DOPA in the catecholamine pathway.

EXPERIMENTAL

Chemicals

L-DOPA, dopamine hydrochloride, and 2-methyl-3-(3,4-dihydroxyphenyl)-DL-alanine (α-methyl-DOPA) were purchased from Sigma (St. Louis, MO, U.S.A.). Aluminum oxide, from Merck (Darmstadt, F.R.G.), was purified according to the method of Anton and Sayre [10]. 5-S-Cysteinyl-DOPA was prepared as described by us [11]. All other chemicals were of analytical grade and obtained from Wako (Osaka, Japan). Milli-Q system (Millipore, Bedford, MA, U.S.A.) ultrapure water was used throughout this study.

Chromatography

A Yanaco Model L-2000 liquid chromatograph (Yanagimoto, Kyoto, Japan) was used with a Yanaco Model VMD-101 electrochemical detector. The detector was set at +750 mV versus an Ag/AgCl reference electrode. Sensitivity was 4 and 32 nA at full scale. Separation was achieved on a C_{18} reversed-phase column (Yanaco ODS-A, particle size 7 μm, 250 × 4.6 mm) at 40°C. The mobile phase contained 10 g/l phosphoric acid and 7 g/l methanesulphonic acid in water, the pH being adjusted to 2.35 with 5 mol/l sodium hydroxide. To this, Na_2EDTA was added to a final concentration of 0.1 mmol/l. The flow-rate was 0.7 ml/min.

Samples

Blood samples were collected in centrifuge tubes containing Na_2EDTA and plasma was obtained by centrifugation. Urine samples (24-h) were collected at room temperature in bottles containing 50 ml of acetic acid and 1 g of sodium metabisulphite. Tissue samples were extracted with 0.4 mol/l perchloric acid [8]. All samples were stored frozen at −30°C until analysis.

Alumina extraction of DOPA, dopamine, and 5-S-cysteinyl-DOPA

In a conical plastic tube (1.5-ml volume) were placed 50 mg of acid-washed alumina, 100 μl of 2% (w/v) sodium metabisulphite, and 10 μl of 10 μg/ml α-methyl-DOPA in 0.1 mol/l hydrochloric acid as an internal standard. Either 1.0 ml of plasma, 100 μl of urine or tissue extract, or 10 μl of a standard solution containing 1 μg/ml each of DOPA, dopamine, and 5-S-cysteinyl-DOPA in 0.1 mol/l hydrochloric acid were added to the tube. For urine samples, 100 μl of 0.1 mol/l hydrochloric acid were added, and the mixture was extracted twice with

1 ml of ethyl acetate by shaking for 2 min on a JASCO MT-30 multi-tube mixer, and the ethyl acetate layer discarded by aspiration. Catechols were adsorbed onto alumina by adding 1.5 mol/l Tris—HCl buffer containing 2% (w/v) Na_2EDTA (pH 8.6) and immediately shaking for 5 min on the mixer. Volumes of the Tris buffer added were 0.3 ml for plasma and 1.0 ml for urine, tissue extract, and standard. For the analysis of tissue samples except for melanogenic tissues such as melanoma and hair, we used 0.3 ml of extract and 1.0 ml of 1.5 mol/l Tris—HCl buffer containing 2% (w/v) Na_2EDTA (pH 8.8). After centrifugation, alumina was washed twice with about 1.5 ml of water. Catechols were then eluted with 150 μl of 0.4 mol/l perchloric acid by shaking for 2 min. After centrifugation, 50—100 μl of the supernatant were injected into the HPLC system. Catechols were quantified from peak height ratios between catechols and α-methyl-DOPA for sample and standard.

RESULTS

Determination of DOPA, dopamine, and 5-S-cysteinyl-DOPA in biological samples

Fig. 1 shows typical chromatograms of urine samples from a normal subject and from a melanoma patient, and of a plasma sample from a normal subject, and of an extract of a B16 mouse melanoma. DOPA, dopamine, 5-S-cysteinyl-DOPA, and α-methyl-DOPA appeared at about 10, 11, 15.5, and 21 min, respectively. No large peaks appeared after α-methyl-DOPA. Although no interfering peaks were found in plasma and tissue samples after alumina extraction, urine samples contained multiple peaks which interfered with those of the catechols to be analysed. However, most of these peaks were eliminated by the extraction with ethyl acetate.

Method evaluation

The standard curves were linear over the range 0.5—100 ng of DOPA, dopamine, and 5-S-cysteinyl-DOPA after alumina extraction, when compared to 100 ng of the internal standard α-methyl-DOPA.

The absolute recoveries of catechols were estimated by comparison of the alumina-extracted standards with directly injected standards. The results, summarized in Table I, showed modest to good recovery and precision of the extraction procedure. The use of 0.4 mol/l perchloric acid was essential for the good recovery of 5-S-cysteinyl-DOPA; the recovery decreased to 54.3% with 0.2 mol/l perchloric acid. Similar results have been reported by Hansson et al. [6].

The analytical recoveries of the catechols added to normal plasma and urine samples were satisfactory, as indicated in Table II. Since catechols, especially 5-S-cysteinyl-DOPA, are susceptible to autoxidation, great care should be taken to shake the mixture immediately after the addition of Tris—HCl buffer.

Estimation of DOPA and 5-S-cysteinyl-DOPA in plasma

Plasma samples from 31 normal subjects were analysed. Plasama concentrations (mean ± S.D.) of DOPA and 5-S-cysteinyl-DOPA were 0.85 ± 0.38 ng/ml and 0.37 ± 0.30 ng/ml, respectively. These values are much lower than those

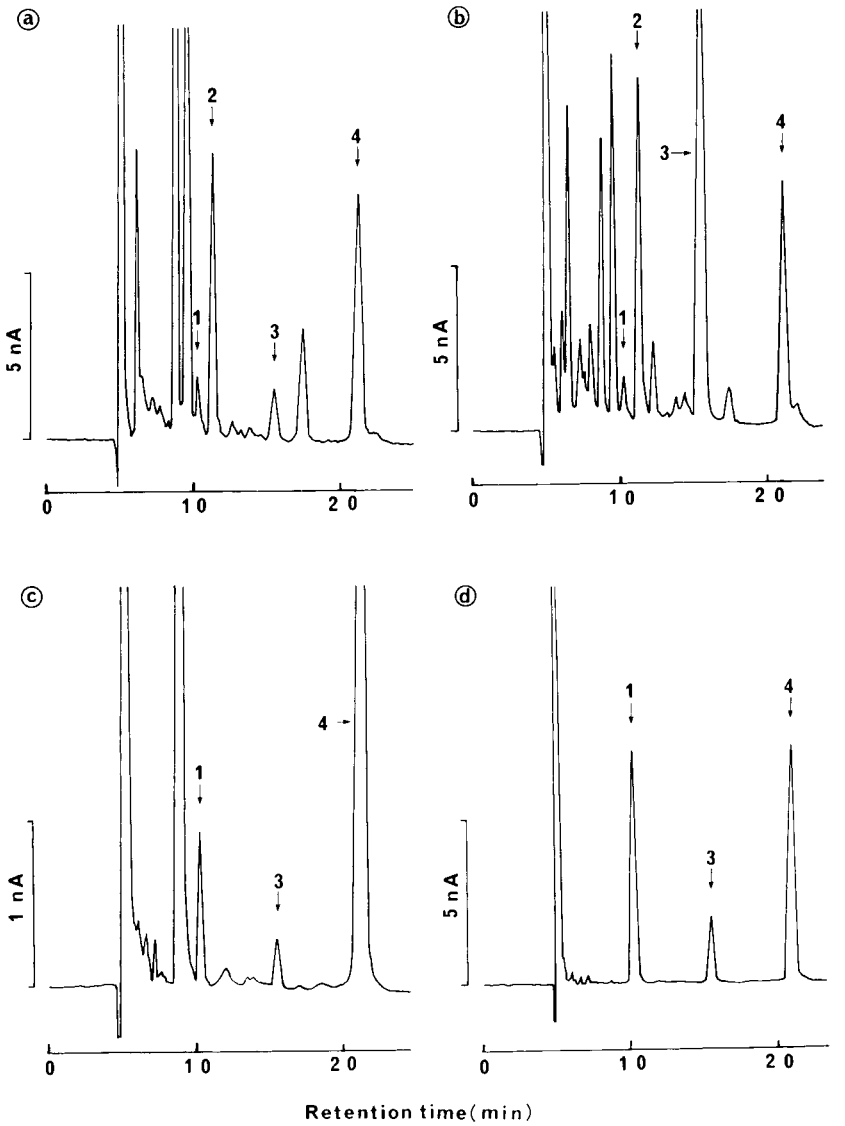

Fig. 1. Chromatograms of (a) a urine sample from a normal subject, (b) a urine sample from a melanoma patient, (c) a plasma sample from a normal subject, and (d) an extract of a B16 mouse melanoma. Chromatographic conditions: column, Yanaco ODS-A (7 μm particle size); mobile phase, 10 g/l phosphoric acid and 7 g/l methanesulphonic acid, pH being adjusted to 2.35 with 5 mol/l sodium hydroxide, Na_2EDTA being added to a final concentration of 0.1 mmol/l; flow-rate, 0.7 ml/min; temperature, 40°C; detector, +750 mV versus Ag/AgCl, 4 and 32 nA at full scale. Peaks: 1 = DOPA, 2 = dopamine, 3 = 5-S-cysteinyl-DOPA, 4 = α-methyl-DOPA.

reported by Hansson et al. [5]. This discrepancy may be ascribed to better clean-up and separation of the present procedure. For plasma samples, the detection limits at a signal-to-noise ratio of 2 were about 0.02 ng/ml for DOPA and dopamine and about 0.05 ng/ml for 5-S-cysteinyl-DOPA (injection volume 100 μl). Dopamine was below the detection limit.

TABLE I

ABSOLUTE RECOVERIES OF DOPA, DOPAMINE, AND 5-S-CYSTEINYL-DOPA AFTER ALUMINA EXTRACTION

Mean ± S.D. for 5 determinations.

Compound	Amount (ng)	Recovery (%)	C.V. (%)
DOPA	10	76.4 ± 1.4	1.8
Dopamine	10	75.4 ± 1.3	1.7
5-S-Cysteinyl-DOPA	10	69.6 ± 3.7	5.3
α-Methyl-DOPA	100	74.9 ± 1.5	2.0

TABLE II

ANALYTICAL RECOVERIES OF DOPA, DOPAMINE AND 5-S-CYSTEINYL-DOPA ADDED TO NORMAL PLASMA AND URINE SAMPLES

Mean ± S.D. for 5 determinations.

Sample	Amount added (ng)	Recovery (%)		
		DOPA	Dopamine	5-S-Cysteinyl-DOPA
Plasma	10	94.7 ± 4.2	104.1 ± 4.9	92.2 ± 2.3
Plasma	100	93.7 ± 0.5	105.9 ± 1.1	94.0 ± 0.9
Urine	100	99.7 ± 7.3	102.3 ± 6.7	99.5 ± 8.3

DISCUSSION

Previous studies by Rorsman and his associates [3,4,12,13], by us [8,9], and by others have shown that the determination of DOPA and 5-S-cysteinyl-DOPA in urine, blood, and tissue samples has a biochemical and clinical significance. HPLC with electrochemical detection has been adopted for the determination of these catecholic amino acids as for catecholamines.

Commonly, urinary 5-S-cysteinyl-DOPA is determined after alumina extraction. Kågedal and Pettersson [7] found that the alumina extraction alone was not enough to remove many interfering compounds and they recommended a combination of ion-exchange chromatography with alumina extraction for the clean-up. We replaced ion-exchange chromatography with ethyl acetate extraction to simplify the procedure.

DOPA and 5-S-cysteinyl-DOPA in serum have also been determined by HPLC with electrochemical detection after alumina extraction [5,6,13]. However, as far as we know, the reported method requires 6 ml of serum, which seems to be a burden to patients. Our improved method requires only 1 ml of plasma, and, moreover, involves only minimum variations in the procedure for plasma, urine, and tissue samples. This enabled us to analyse different types of samples at the same time.

Hansson et al. [6] described a good separation of DOPA, cysteinyl-DOPA isomers, and catecholamines using a mobile phase of pH 1.75. We also obtained similar results with a pH 1.75 buffer, but found a rapid deterioration of the column due to the low pH. At pH values higher than 2.50, uric acid, which

appears just in front of DOPA, comes too close to DOPA. Thus, we chose pH 2.35 for the mobile phase.

Advanced methodology has recently been applied to the determination of urinary 5-S-cysteinyl-DOPA. First, the use of a diastereomer of naturally occurring 5-S-L-cysteinyl-L-DOPA as an internal standard should improve the precision of the analysis because of the close similarity in chemical properties [14,15]. Secondly, phenylboronate affinity gel can be used to purify 5-S-cysteinyl-DOPA with a higher selectivity [15]. If only 5-S-cysteinyl-DOPA is to be analysed, the combined use of these two methodologies would be advantageous over the present method. However, our method can analyse not only 5-S-cysteinyl-DOPA but also DOPA and dopamine. Furthermore, our method is sufficiently simple, sensitive, precise, and accurate for routine use.

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Note

Chromatographie en phase liquide automatique de la dopamine urinaire

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Dans l'organisme la dopamine est un neurotransmetteur à la fois central et périphérique, ainsi que le précurseur de la biosynthèse de deux autres catécholamines, la noradrénaline et l'adrénaline. La dopamine excrétée dans l'urine humaine provient indistinctement de territoires systémiques et/ou des régions centrales riches en dopamine, encore que la forte teneur des urines — comparée à celle du plasma — ait fait évoquer une production locale rénale par décarboxylation de la dopamine [1].

Néanmoins, on a signalé une majoration de l'excrétion urinaire de la dopamine dans les neuroblastomes [2], dans certains phéochromocytomes où cette majoration concerne aussi la noradrénaline, dans les stress [3] et dans les états d'hypertension artérielle [4].

Au niveau central on a observé une diminution de l'excrétion urinaire de dopamine, concomitante de l'akinésie, au cours de la maladie de Parkinson. Par ailleurs une application intéressante du dosage de la dopamine urinaire pourrait consister en l'estimation de l'activité de la dopamine-β-hydroxylase [5] qui représente un marqueur de l'activité sympathique.

L'analyse des catécholamines dans les milieux biologiques fait classiquement appel à une ou deux étapes d'isolement, précédant une séparation chromatographique en phase liquide. Ces deux étapes sont très généralement une fixation des catécholamine sur résine échangeuse d'ions et/ou une fixation en milieu basique sur alumine convenablement activée [6]. Ces opérations sont menées soit sur de petites colonnes chromatographiques (résine) soit par simple équilibre (alumine). Après cette étape préliminaire le type de séparation le plus souvent utilisé est la chromatographie de partage à haute performance, à polarité de phases inversée avec formation de paires d'ions, comme le montre une revue récente [7]. Le système de détection utilise soit l'oxydation électro-

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chimique [8] soit la fluorescence naturelle des catécholamines [9] ou bien la fluorescence de dérivés formés en sortie de colonne chromatographique [10, 11].

Le choix d'une ou deux étapes d'isolement dépend essentiellement de la complexité du milieu biologique considéré. Le choix de la méthode de détection dépend surtout des concentrations à doser, la détection électrochimique est plus sensible mais en général plus délicate à employer en analyse de routine.

En ce qui concerne le dosage de l'adrénaline, noradrénaline et dopamine dans l'urine, les concentrations assez élevées permettent l'utilisation d'une détection fluorimétrique des molécules elles-mêmes. Pour l'adrénaline et la noradrénaline l'utilisation de deux étapes préalables d'isolement (résine + alumine) est généralement pratiquée compte tenu de l'interférence de certains composés endogènes. En revanche, pour la dopamine (présente à plus forte concentration) une seule étape de fixation sur alumine paraît suffisante.

Nous avons recherché à automatiser l'analyse de la dopamine urinaire de façon simple en employant la fixation sur une précollone d'alumine suivie d'une séparation par chromatographie de paires d'ions et détection spectrofluorimétrique.

Un systéme de précolonne, garnie d'une phase stationnaire de même nature que celle de la colonne analytique [12] ou d'un support différent [13], a déjà été utilisé pour des analyses de substances médicamenteuses dans les prélèvements biologiques.

L'utilisation de microcolonnes d'alumine a été proposée pour l'analyse des catécholamines mais dans le cadre d'un système assez compliqué [14].

Nous avons appliqué le principe d'une précolonne utilisée en "back flush" en mettant en oeuvre un matériel limité (et en particulier une seule pompe chromatographique) présent dans beaucoup de laboratoires.

MATÉRIEL ET MÉTHODE

Réactifs

Sel disodique de l'acide éthylènediaminetétracétique (EDTA disodique), Fluka (Buchs, Suisse). Tris(hydroxymethyl)aminométhane (Tris), acide ascorbique, octylsulfate de sodium, alumine pour chromatographie LiChrosorb Alox T (granulométrie 10 μm), E. Merck (Darmstadt, R.F.A.). Dihydrogénophosphate de potassium, acide citrique, méthanol, Prolabo (Paris, France). Gel de silice greffée alkyle Nucleosil C_{18} (granulométrie 5 μm), Macherey, Nagel & Co. (Düren, R.F.A.). Tous ces réactifs sont de qualité pour analyse. La dopamine sous forme de chlorhydrate et la 3,4-dihydroxybenzylamine sous forme de bromhydrate (DHBA) sont des produits Sigma (St. Louis, MI, E.U.).

Des filtres GF/F Whatman (Springfield Mill, Kent, Grande-Bretagne) sont utilisés pour filtrer les phases mobiles, et des filtres Millex HA adaptables sur seringue (Millipore, Bedford, MA, É.U.) permettent de filtrer les échantillons urinaires.

Appareillage

Le schéma du système utilisé est représenté Fig. 1. Il comprend une pompe chromatographique Altex 110 A (Berkeley, CA, É.U.), alimentée par l'inter-

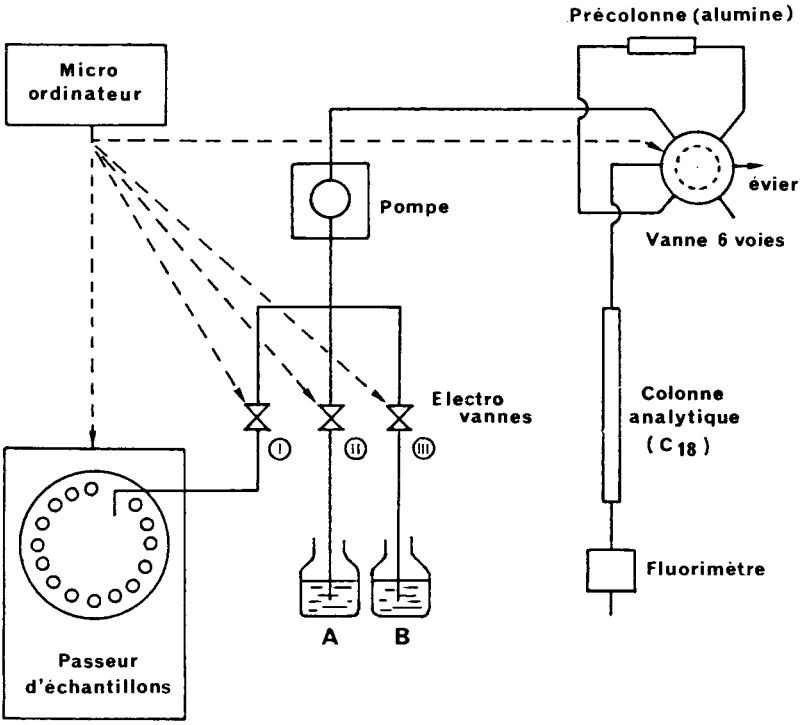

Fig. 1. Schéma de principe du système chromatographique. (A) Tampon pH 8.6 (Tris 0.1 *M*, EDTA disodique 0.002 *M*, acide ascorbique 0.001 *M*). (B) Phase mobile [octylsulfate 0.003 *M*, dihydrogénophosphate de potassium 0.035 *M*, acide citrique 0.03 *M*, acide ascorbique 0.001 *M* et 14% (v/v) de méthanol]. Sur le passeur d'échantillons un godet sur deux contient un liquide de rinçage (EDTA disodique 0.002 *M*, acide ascorbique 0.001 *M* ajusté à pH 7).

médiaire de trois électrovannes (General Valve, Fairfield, NJ, É.U.). Une précolonne Merck Hibar, modifiée par nos soins pour en réduire la longueur (1 × 0.3 cm I.D.) et une colonne analytique (15 × 0.48 cm I.D.) sont reliées à une vanne six voies Rhéodyne 7010 (Cotati, CA, É.U.), à commande électrique (Touzart et Matignon, Vitry, France) selon le schéma de la Fig. 2. La précolonne est remplie à sec (alumine), la colonne analytique est remplie classiquement par voie humide (silice greffée alkyle). Le détecteur est un spectrofluorimètre JY 3 (Jobin et Yvon, Longjumeau, France) (excitation 282 nm, fente 10 nm, émission 315 nm, fente 10 nm). Un passeur d'échantillon Sampler IV (Technicon, Tarrytown, NY, É.U.) est utilisé. Les trois électrovannes, la vanne six voies, l'avancement du passeur d'échantillons sont commandés par un microordinateur Zx 81 Sinclair (Oxford, Grande-Bretagne). Ce dernier est équipé d'une mémoire additionnelle 64 K Memopak de Memotech (Oxford, Grande-Bretagne) et d'une interface "entrées—sorties" permettant la commande de huit relais (cinq sont utilisés). La programmation en temps utilise la fonction "pause" dans le programme écrit en basic.

Prélèvement

Au moment du recueil des urines est ajouté 1% d'EDTA disodique et 0.1% d'acide ascorbique, le prélèvement est ensuite congelé. Lors du dosage, à 10 ml

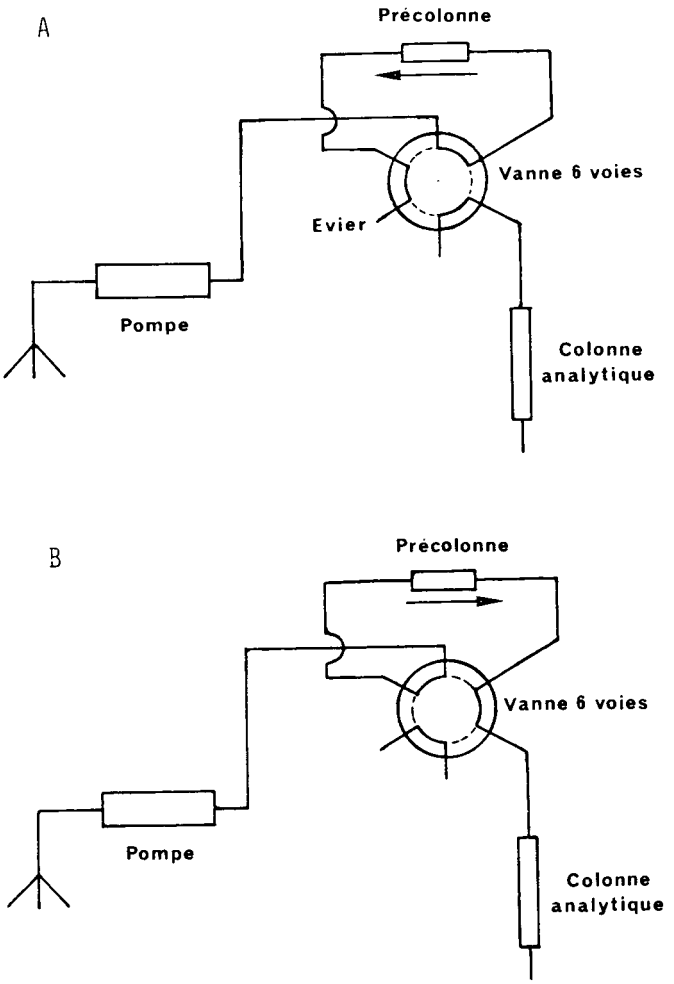

Fig. 2. Schéma de branchement de la vanne six voie. (A) Fixation sur précolonne d'alumine en présence de tampon pH 8.6 (la phase mobile ne circule pas dans la colonne analytique). (B) Élution chromatographique par la phase mobile acide.

d'urines ainsi traitées est ajouté 0.1 ml d'une solution à 5 mg l^{-1} de DHBA comme étalon interne, l'échantillon est filtré sur filtre Millex HA.

Étalon

Solution-mère de dopamine à 100 mg l^{-1} en base dans l'acide chlorhydrique 0.1 *M*, conservée au congélateur. Solution-fille à 5 mg l^{-1} dans l'acide chlorhydrique 0.1 *M*. Solution de DHBA à 5 mg l^{-1} dans l'acide chlorhydrique 0.1 *M*. Une gamme de dopamine de 0.05 à 0.3 mg l^{-1} (0.32 à 1.95 μmol l^{-1}) contenant 0.05 mg l^{-1} de DHBA est préparée en présence de 1% d'EDTA disodique et 0.1% d'acide ascorbique.

Principe de la méthode

Le principe consiste à fixer la dopamine à pH 8.6 sur la précolonne

d'alumine, le tampon basique ne circulant pas sur la colonne analytique qui serait rapidement dégradée en raison de la solubilité du gel de silice à ce pH. Puis après élimination du tampon présent dans le volume poreux de la précolonne, par passage d'une solution neutre, l'élution de la dopamine est obtenue par circulation d'une phase mobile acide, en sens inverse de celui utilisé pour la fixation.

Le prélèvement n'est amené à pH basique par mélange avec le tampon pH 8.6 qu'au moment de la fixation sur précolonne d'alumine (par mélange en amont de la pompe) pour éviter la dégradation de la dopamine instable à ce pH.

Chromatographie

Le débit de la pompe est 2 ml min^{-1}.

Fixation sur la précolonne. La vanne six voies étant en position A représentée sur la Fig. 2, la vanne II (Fig. 1) est ouverte pendant 3 min, la précolonne est alors conditionnée par passage de 6 ml de tampon basique (Tris 0.1 *M*, EDTA disodique 0.002 *M*, acide ascorbique 0.001 *M*, ajusté à pH 8.6), qui est ensuite rejeté à l'évier. Le passeur d'échantillon étant en position de prélèvement les vannes I et II sont ouvertes alternativement pendant 3 min (toutes les 6 sec), pour amener le prélèvement à pH 8.6 par mélange avec la solution tampon basique. Le volume de liquide circulant pendant cette période est de 6 ml dont 3 ml d'échantillon et 3 ml de tampon basique. Sur le passeur un godet de prélèvement et un godet de liquide de rinçage (EDTA disodique 0.002 *M*, acide ascorbique 0.001 *M*, ajusté à pH 7 par de la soude 1 *M*) alternent. La vanne II restant seule ouverte le passeur change de godet, la vanne I s'ouvre, la vanne II se ferme. Pendant 3 min les canalisations depuis l'aiguille de prélèvement jusqu'à la précolonne d'alumine, sont rincées par la solution neutre prélevée dans le godet de rinçage.

Elution chromatographique. La vanne III est ouverte, en même temps que la vanne I se ferme et que la vanne six voies vient en position B représentée sur la Fig. 2. L'élution chromatographique des solutés préalablement fixés sur l'alumine en milieu basique a lieu grâce à une phase mobile acide de chromatographie de paires d'ions décrite par Krstulovic et al. [15] [octylsulfate 0.003 *M*, dihydrogénophosphate de potassium 0.035 *M*, acide citrique 0.03 *M*, et 14% (v/v) de méthanol] à laquelle nous rajoutons de l'acide ascorbique (0.001 *M*) pour éviter les risques d'oxydation de la dopamine.

RÉSULTATS ET DISCUSSION

Les chromatogrammes obtenus à partir d'une solution étalon et d'une urine humaine normale sont représentés Fig. 3. Dans le cas de l'urine les pics de dopamine et de DHBA apparaissent bien séparés des composés endogènes.

La présence de la précolonne nuit peu à l'efficacité du système chromatographique (finesse des pics). En effet, la comparaison de chromatogrammes enregistrés après injection directe en tête de colonne ou par élution à partir de la précolonne après fixation sur cette dernière, a montré que l'efficacité du système colonne/précolonne reste supérieure à 80% de celle de la colonne chromatographique utilisée seule (en nombre de plateaux théoriques). Ceci

montre que la libération de la dopamine et du DHBA fixés sur l'alumine est rapide en présence de la phase mobile chromatographique acide. L'inversion du sens de circulation de la phase liquide entre la fixation et l'élution contribue à ce bon résultat.

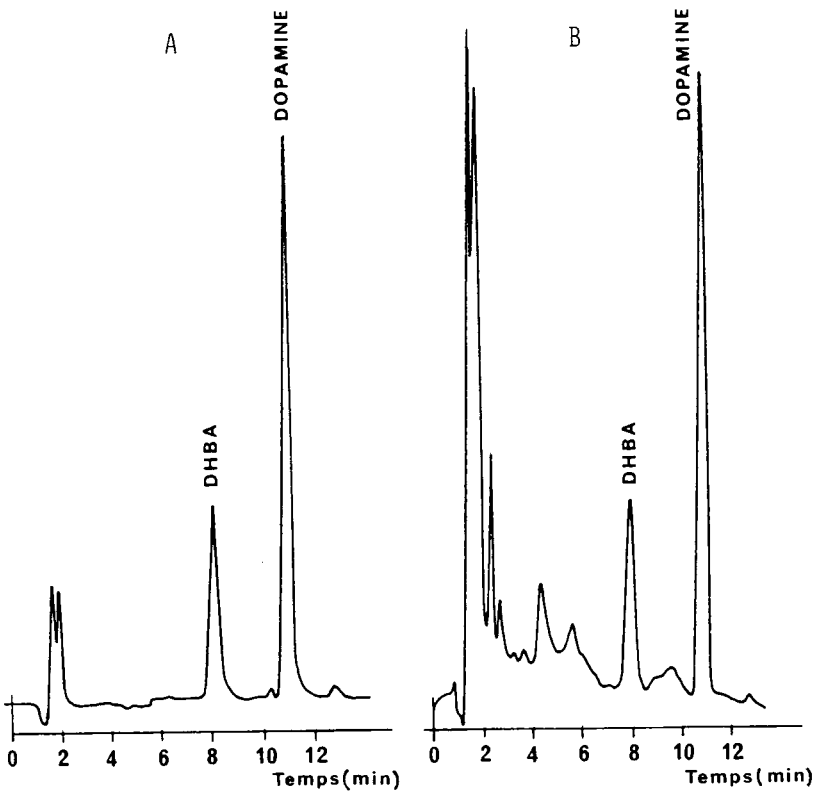

Fig. 3. (A) Chromatogramme correspondant à une solution étalon de dopamine (étalon interne = DHBA) après fixation sur précolonne d'alumine (1 × 0.3 cm) et séparation par formation de paires d'ions. Colonne: 15 × 0.48 cm; phase stationnaire: Nucleosil C_{18}, 5 μm; Phase mobile: selon Fig. 1; débit: 2 ml min^{-1}; température: 20°C; concentration de dopamine dans l'échantillon: 0.2 mg l^{-1} (1.3 μmol l^{-1}). (B) Prélèvement urinaire dans les mêmes conditions. Concentration de dopamine déterminée: 0.23 mg l^{-1} (1.5 μmol l^{-1}).

La courbe de calibration pour la dopamine est linéaire au-delà de 1 μg injecté; c'est-à-dire, en considérant un prélèvement de 3 ml, au-delà de 0.33 mg l^{-1} (2.15 μmol l^{-1}) dans l'urine. Ceci est bien adapté aux valeurs fréquentes de concentration de dopamine dans l'urine humaine normale (pour une diurèse moyenne de 1.5 l): 0.04—0.26 mg l^{-1} (0.28—1.7 μmol l^{-1}).

Le coefficient de variation pour le dosage repété de la dopamine sur un même échantillon d'urine a été 2.7% (n = 10). Le rendement d'extraction, de la dopamine rajoutée à l'urine, par l'alumine de la précolonne était 98.2 ± 3.2% (moyenne ± déviation standard, n = 10). L'utilisation prolongée d'un même échantillon d'alumine ne semble pas poser de problème (jusqu'à 100 analyses successives). La sensibilité, la reproductibilité, la précision et le rendement d'extraction sont suffisants pour l'analyse de la dopamine urinaire. Si ce

dispositif automatique d'analyse qui n'utilise qu'une seule pompe fonctionne sans arrêt, environ 70 échantillons peuvent être traités par jour.

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CHROMBIO. 2242

Note

Ascorbic acid oxidase speeds up analysis for catecholamines, indoleamines and their metabolites in brain tissue using high-performance liquid chromatography with electrochemical detection

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We report a simple procedure to speed up high-performance liquid chromatographic—electrochemical detection (HPLC—ED) of catecholamines, indoleamines and their metabolites in brain samples using commonly available HPLC equipment and minimal sample preparation. We analyze for norepinephrine (NE), epinephrine (E), dopamine (DA), 5-hydroxytryptamine (5-HT) and the metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindole-3-acetic acid (5-HIAA), and homovanillic acid (HVA) in 30 min per sample by direct injection of supernatant from sonicated brain tissue [1]. The first component eluted, NE, is frequently overlapped by a large solvent front. Alternative mobile phases using increased ion-pairing agent or decreased organic solvent in order to resolve NE result in untimely appearance of 5-HT. There is a trade-off necessary between using a method that allows short enough sample time for routine processing of large numbers of samples and resolution of the NE peak. Processing samples in 0.1 *M* perchloric acid, which is expected to lower protein concentration of the supernatant, did not decrease the solvent front. The primary cause of this large solvent front in brain samples is ascorbic acid. A shorter sample time can be obtained by a simple enzyme addition (ascorbic acid oxidase, AAO) which reduces the solvent front by 80%. The small quantity of AAO is added during sample preparation, and NE, the first peak of interest, is readily detected. The alternative way to increase the rate at which samples are processed is to change to 3-μm particle columns with the added equipment expense and more stringent sample handling procedures [2].

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MATERIALS AND METHODS

Chromatographic analysis was carried out using an Altex Ultrasphere ODS reversed-phase separation column (5 μm particle size, 250 × 4.6 mm) attached to a glassy carbon electrode (Bioanalytical Systems, TL5) and a BAS-LC4A amperometric controller. A Milton-Roy single piston pump (No. 396-57) and a Universal Scientific repackable guard column (C_{18}, 10 μm particle size; 46 × 2.0 mm) were used.

A methanol—water (10:90) eluent, pH 4.0, containing 8.2 g sodium acetate (0.1 *M*), 14.0 g citric acid (0.07 *M*), 100 mg octylsulfonic acid (sodium salt) ($5 \cdot 10^{-4}$ *M*), and 25 mg EDTA flowed at a rate of 1 ml/min. E_{app} was 0.61 V. Temperature was controlled via a water-jacketed column at 32° C. (A shorter sample time of 18 min on a similar column was possible using 15% methanol and running at room temperature.)

Ascorbic acid oxidase solution was made from 1.0 mg of ascorbic acid oxidase (E.C. 1.10.3.3) from Boehringer-Mannheim into 1.0 ml of acetate buffer, pH 5. Refrigerated enzyme solution stays active for at least seven days. The pH 5 buffer used for sample homogenization is composed of 0.1 *M* Ac^-, $3.5 \cdot 10^{-2}$ *M* in glacial acetic acid; the pH was adjusted to 5.0 with sodium hydroxide.

Samples of human brain tissue (weight ≈ 30 mg) are sonicated on ice in 450 μl of pH 5 buffer, 50 μl of $1 \cdot 10^{-6}$ *M* dihydroxybenzylamine (DHBA), internal standard solution. Of the ascorbic acid oxidase solution 10 μl are added to each sample, which is then centrifuged at 0° C at 40,000 *g* for 20 min. The action of the AAO is immediate. The supernatant is injected directly into the chromatographic system.

RESULTS

Fig. 1 shows the comparison of human brain sample chromatograms

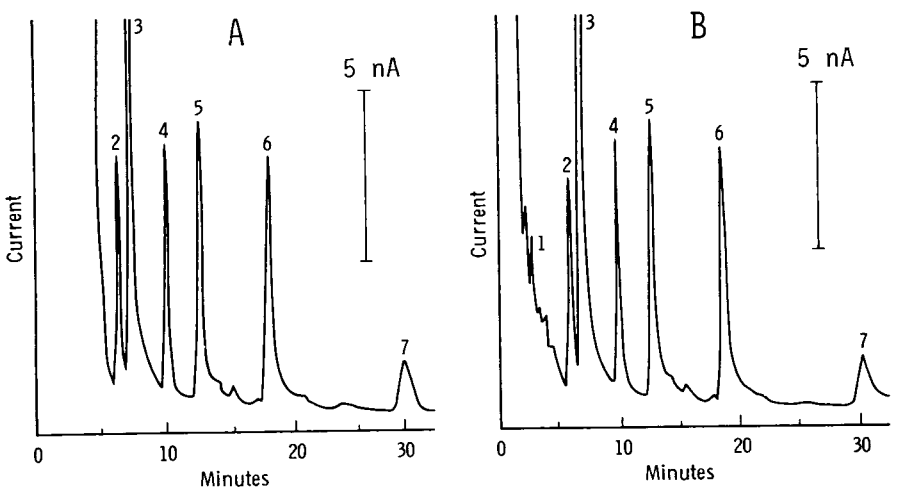

Fig. 1. HPLC—ED chromatograms of human brain tissue (septal region) without (A) and with (B) the addition of ascorbic acid oxidase to brain tissue supernatant. Peaks: 1 = norepinephrine; 2 = DHBA; 3 = DOPAC; 4 = dopamine; 5 = 5-HIAA; 6 = HVA; 7 = 5-HT.

without and with the addition of the ascorbic acid oxidase. With this method we are able to detect many biogenic amines oxidizable at 0.61 V in 30—35 min sample time with minimal sample preparation. AAO-treated human brain supernatant spiked with known quantities of NE, DA and 5-HT gave linear calibration curves. Catechol and indole stability was not dependent on the presence of ascorbic acid. We have found after injection of 100 samples (700 mg of tissue supernatant) the precolumn packing must be changed, but the separation column has resolved more than 1800 samples and retained its separation ability after a year of use.

ACKNOWLEDGEMENT

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CHROMBIO. 2237

Note

Determination of non-protein-bound N-acetylcysteine in plasma by high-performance liquid chromatography

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N-Acetylcysteine has been identified as a normal constituent in urine [1] and its excretion has been determined by both gas—liquid chromatography (GLC) [2] and high-performance liquid chromatography (HPLC) [3]. The compound has not been detected in plasma except after drug intake. As a mucolytic agent it has been used for inhalation treatment for a long time [4], and it has also been documented as an effective oral drug in chronic bronchitis [5,6]. The compound is a precursor for the in vivo synthesis of glutathione, and it therefore exerts a beneficial effect in paracetamol (acetaminophen) poisoning [7].

If only disulphide interchange is considered, N-acetylcysteine may be found as a free thiol, as a low-molecular-weight disulphide, and as a disulphide with SH-containing proteins. The present paper describes a HPLC method for the determination of non-protein-bound N-acetylcysteine in plasma suitable for pharmacokinetic studies. After precipitation of plasma proteins, low-molecular-weight disulphides in the supernatant are reduced, and liberated N-acetylcysteine is purified using an organomercurial adsorbent and a cation exchanger. Derivatization is then done with N-(7-dimethylamino-4-methyl-3-coumarinyl)-maleimide (DACM), and quantitation is accomplished by fluorometric detection after separation of the N-acetylcysteine derivative from other acid thiol derivatives by reversed-phase ion-pair HPLC.

EXPERIMENTAL

Materials

N-Acetylcysteine was obtained from Sigma (St. Louis, MO, U.S.A.) and N-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide (DACM) was from Fluka (Buchs, Switzerland). Stock solutions of these compounds were prepared

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as described previously [3]. Thiopropyl-Sepharose 6B (TPS) was from Pharmacia (Uppsala, Sweden). It was used in its free thiol form and contained 20 μmol of thiol per ml of gel suspension. The organomercurial adsorbent *p*-acetoxymercurianiline-Sepharose 4B (PAMAS) was prepared according to the description of Sluyterman and Wijdenes [8], and the strong cation exchanger AG 50W-X8, 100—200 mesh, was from Bio-Rad Labs. (Richmond, CA, U.S.A.).

High-performance liquid chromatography

HPLC of the fluorescent DACM derivatives of N-acetylcysteine was performed with tetramethylammonium as ion-pairing reagent as previously described [3]. We employed a Supelcosil LC-8 (250 × 4.6 mm, particle size 5 μm) column from Supelco (Bellefonte, PA, U.S.A.), and as detector we used a Fluoromonitor III filter fluorometer from LDC (Riviera Beach, FL, U.S.A.). The detector was operated with a mercury lamp, a 360-nm excitation filter and a 418—700 nm emission filter. An RP-8 (particle size 10 μm) column, 30 × 4.6 mm (RP-GU) from Brownlee Labs. (Santa Clara, CA, U.S.A.) was connected to the injector instead of the sample loop. Once the N-acetylcysteine derivative had passed this loop column, the injector was switched to the load position again. Late peaks were thereby trapped in the loop column and could be removed by backward flushing with the same mobile phase as in the analytical column. Backward flushing was accomplished with a mini HPLC pump (Eldex E-120-S-2, Eldex Labs., Menlo Park, CA, U.S.A.).

Procedure

Venous blood was taken in vacuum tubes containing K_3EDTA (Vacutainer), and plasma was obtained by centrifugation. A 1.0-ml aliquot of each plasma sample was precipitated with 0.2 ml of trichloroacetic acid, 200 g/l. After vortex mixing and standing for 15 min the samples were centrifuged, and 0.6-ml aliquots of the clear supernatants were transferred to new tubes and mixed with 0.4 ml of 20 mmol/l Na_2EDTA and 1.0 ml of sodium borate buffer, pH 10.0 (prepared from boric acid, 0.24 mol/l final volume). Then 1.0 ml of TPS suspension was added and the tubes were placed in an end-over-end mixer for 30 min. Then 1.0 ml of 4.0 mol/l acetic acid was added to the tubes. After centrifugation, 3.0 ml of the supernatant were transferred to a 1.3 × 0.7 cm column of PAMAS which retains the thiols. Washing was done with 2 × 1 ml of water and elution was effected by 3.0 ml of 10 mmol/l cysteine hydrochloride. The eluate was transferred to a 2.5 × 0.5 cm AG 50W-X8 (H^+) column, which retains the cysteine and other positively charged thiols. The effluent was collected in a tube containing 0.2 ml of 0.18 mol/l Na_2EDTA together with a further 1-ml washing with 10 mmol/l hydrochloric acid. From the effluent, 2.0 ml were transferred to a new tube, 0.2 ml of 0.1 mol/l sodium hydroxide was added for neutralization, and then 3 ml of 50 mmol/l carbonate buffer containing 10 mmol/l Na_2EDTA pH 9.0 were added. Finally, 0.5 ml of 20 μmol/l DACM in acetone, was added. After mixing, the tubes were placed in a water bath 37°C, usually overnight. Standard was derivatized by mixing 50 μl of N-acetylcysteine working solution (10 μmol/l) with 5.0 ml of the carbonate buffer used in the derivatization of the plasma samples. Then 0.5 ml of 20 μmol/l DACM was added, and after mixing the tubes were placed in the water bath.

Plasma concentrations after oral intake

Seven healthy subjects aged 21 to 40 years received 400 mg of N-acetylcysteine (Fabrol®, Ciba-Geigy, V. Frölunda, Sweden). The drug was dissolved in about 100 ml of water and was given orally to the subjects after overnight fasting. Venous blood samples were taken from a catheter placed in a cubital vein, at the times shown.

RESULTS

Derivatization

In the previous studies on urinary thiols [3] the derivatization was carried out by incubation at 37°C for 20 h, but preliminary experiments indicated that it should be possible to shorten this time. Fig. 1 shows the effect of pH on the development of the N-acetylcysteine derivative with DACM. It can be seen that the fluorescence developed most rapidly at high pH (9.7) and was completed within less than half an hour. At lower pH ($\leqslant 7.6$) the fluorescent product developed more slowly. Maximal fluorescence was obtained at pH 9.0, and this pH was therefore chosen in the final method.

Reduction of low-molecular-weight disulphides

Preliminary experiments showed that protein-free extracts of plasma taken at about 1 h after N-acetylcysteine ingestion contained N-acetylcysteine mainly as disulphide. The N-acetylcysteine could be liberated by reduction with thiopropyl-Sepharose 6B in alkaline medium. We found that transferring the extracts to a borate buffer pH 10 was more suitable than adjusting the pH with strong ammonia [2,3]. We also found higher N-acetylcysteine values when reduction with the thiopropyl-Sepharose 6B was performed in this buffer. Fig. 2 shows that with the chosen amount of thiopropyl-Sepharose 6B, the reduction was completed in 20—30 min. A reduction time of 30 min was therefore chosen in the final method.

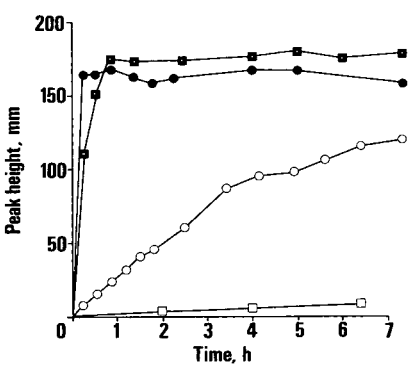

Fig. 1. Effect of pH on the development of the fluorescent DACM derivative of N-acetylcysteine. Temperature 37°C. □, pH 6.0; ○, pH 7.6; ■, pH 9.0; ●, pH 9.7.

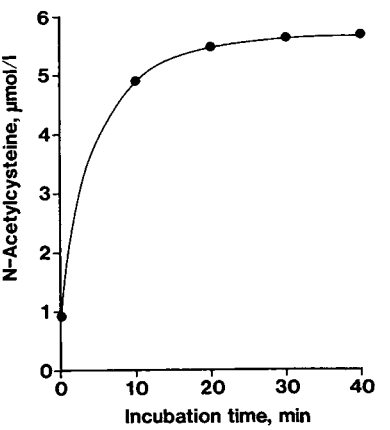

Fig. 2. Liberation of free N-acetylcysteine from supernatant of plasma by reduction with thiopropyl-Sepharose 6B.

High-performance liquid chromatography

Ion-pair HPLC [3] was adopted for DACM derivatives of plasma thiols. In addition to the peaks from the N-acetylcysteine derivatives there were also other peaks, some of them with long retention times. Fig. 3 shows that, by use of the column switching technique described, late peaks could be eliminated by trapping in the loop column. This substantially increased the possible number of injections that could be done in a working day.

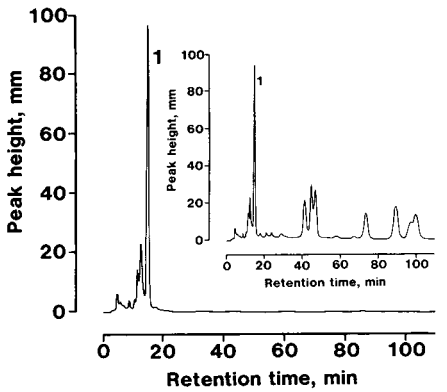

Fig. 3. Chromatogram of acid plasma thiols by HPLC, before and after introduction of the column-switching technique. The N-acetylcysteine peak is designated 1.

Precision

Plasma samples obtained at different times after intake of N-acetylcysteine were used for precision studies. From duplicate determinations the imprecision (coefficient of variation) was calculated as 5.7—6.5% for different ranges of measurement (Table I). From the standard deviation (S.D.) obtained for the lower range the detection limit (2 S.D.) was calculated as 0.14 μmol/l, which was quite satisfactory.

TABLE I

PRECISION OF THE METHOD CALCULATED FROM DUPLICATE DETERMINATIONS

No. of duplicates	Range (μmol/l)	Mean (μmol/l)	S.D. (μmol/l)	C.V. (%)
28	0.2—2.0	1.09	0.07	6.5
42	2.0—10	4.49	0.25	5.7

Recovery

We added N-acetylcysteine to plasma samples to increase the concentration by 1.0 μmol/l (Table II). The increase found by analysis before and after addition showed a quantitative recovery.

Application to pharmacokinetic studies

Fig. 4 shows the plasma concentrations obtained in seven subjects after ingestion of 400 mg of N-acetylcysteine. As can be seen, the maximum value, ranging from 2.6 to 18.4 μmol/l (mean 7.4 μmol/l), was obtained at 25—45

TABLE II

ANALYTICAL RECOVERY OF N-ACETYLCYSTEINE ADDED TO PLASMA SAMPLES, CORRESPONDING TO AN INCREASE IN CONCENTRATION OF 1.0 μmol/l

Initially present (μmol/l)	After addition (μmol/l)	Increase found (μmol/l)	Recovery (%)
0.22	1.34	1.12	112
0.25	1.19	0.94	94
0.28	1.27	0.99	99
0.30	1.27	0.97	97
		Mean ± S.D.	101 ± 8

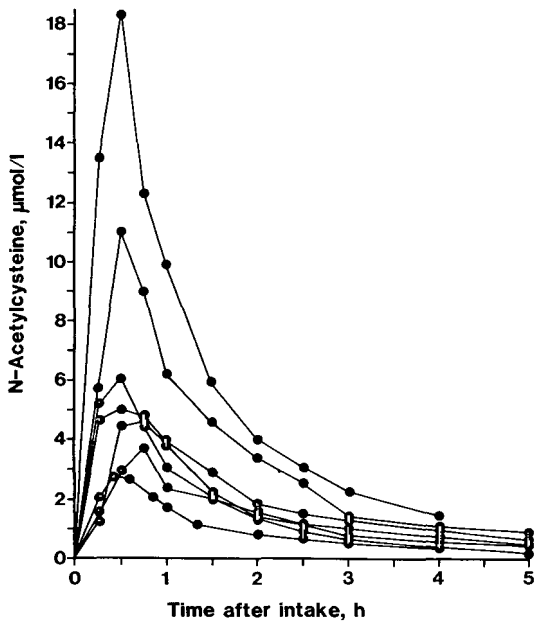

Fig. 4. Non-protein-bound plasma N-acetylcysteine concentration after oral intake of 400 mg of N-acetylcysteine (Fabrol).

min after intake. The half-life of elimination ($t_{½}$) for N-acetylcysteine was calculated from the 1.5—5-hour segment of the log plasma concentration versus time curve, and was found to vary between 1.1 and 2.3 h, with a mean of 1.7 h.

DISCUSSION

In an earlier paper we described an HPLC system which was capable of resolving N-acetylcysteine and mercaptoacetate as their derivatives with N-(1-pyrene)maleimide (PM) and DACM. The applicability of the method was shown for the determination of urinary thiols using PM. However, the sensitivity for the thiols was about ten-fold higher with DACM than with PM. With DACM we have now obtained a highly sensitive method for the determination of N-acetylcysteine in plasma, both for study of the high concentrations after intake and for study of its lower concentration during elimination.

Pharmacokinetic data for N-acetylcysteine after peroral intake are scanty. Rodenstein et al. [9] gave 100 mg of ^{35}S-labelled N-acetylcysteine to five patients with heart and pulmonary diseases. Calculated from the radioactivity determinations given in the paper, a maximal plasma concentration of 21—39 μmol/l was obtained after 2—3 h. A substantial amount of radioactivity still remained in the plasma after 24 h. The free N-acetylcysteine concentration in plasma after oral intake of 400 mg of N-acetylcysteine was estimated by Maddock [10], who found maximal plasma concentrations of 0—3.6 μmol/l. No data were given regarding the method used, and therefore the results are difficult to evaluate. Recently, Morgan et al. [11] published a GLC method for plasma using the principles developed by Hannestad and Sörbo [2]. No protein precipitation was performed before reduction, and therefore their method should estimate the total concentration in plasma. They gave 250 mg of N-acetylcysteine in gelatin capsules per m^2 body surface to five subjects with bronchogenic carcinomas. Assuming a standard body surface of 1.73 m^2 for their patients this corresponds to a mean dose of 433 mg which was close to what we gave. They obtained a mean maximal N-acetylcysteine concentration of 10.7 μmol/l, i.e. a mean value only slightly higher than our mean value for non-protein-bound N-acetylcysteine. Their maximum values were obtained at a mean of 0.72 h after intake, and the mean $t_{½}$ was 2.1 h, again values similar to ours. This in comparison with our data may indicate that the protein binding of N-acetylcysteine by disulphide bridges is low. However, direct measurements must be done in order to estimate the protein binding of N-acetylcysteine in plasma.

ACKNOWLEDGEMENTS

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Note

Determination of α-tocopherol in human plasma by high-performance liquid chromatography with electrochemical detection

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High-performance liquid chromatography (HPLC) has been employed successfully to determine the naturally occurring vitamin E analogues, α-, β- and γ-tocopherol (TP) in human plasma (Fig. 1). Most recently published procedures make use of ultraviolet (UV) or fluorimetric detection and of reversed-phase methods by which the prevalent form, α-TP, can be separated from its minor co-eluting β- and γ-homologues [1—5].

In this communication we wish to report a rapid, sensitive and reproducible HPLC method with electrochemical detection for routine measurement of α-tocopherol in human plasma. We have selected electrochemical detection, which was first described by Ikenoya et al. [6], for the analysis of tocopherols on the basis of its sensitivity and specificity. Since δ-tocol, which has been employed as internal standard in most HPLC assays based on UV, fluorimetric or electrochemical detection [1,5,6,7], can no longer be commercially obtained, we have evaluated the use of δ-tocopherol. The homologue, δ-TP, which has been demonstrated in some plant oils, e.g. in soybean oil [8], has the advantage that it is a natural readily available form of vitamin E. δ-Tocopherol (Fig. 1) contains a free hydroxyl group at the C-6 position which is responsible

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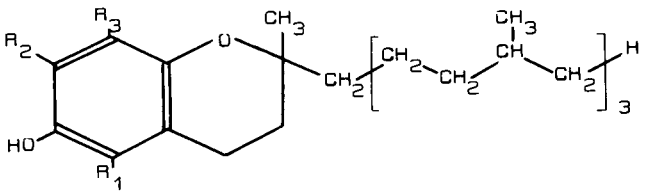

α-TP: $R_1 = R_2 = R_3 = CH_3$

β-TP: $R_1 = R_3 = CH_3$; $R_2 = H$

γ-TP: $R_2 = R_3 = CH_3$; $R_1 = H$

δ-TP: $R_3 = CH_3$; $R_1 = R_2 = H$

Fig. 1. Chemical structures of α-, β-, γ- and δ-tocopherol.

for its electrochemical reactivity and, furthermore, meets the other criteria of an internal standard: (1) it is quantitatively of minor importance in human blood; (2) it is structurally closely related to the compound to be determined so that the use of this homologue can compensate for losses during the sample work-up procedures; and (3) it can be separated from α-TP and its minor β- and γ-homologues during reversed-phase HPLC. The experimental conditions for suitable electrochemical detection of both α- and δ-TP have also been investigated because of the known different reactivity toward singlet oxygen, which correlates with the antioxidant vitamin E activity, α-TP being about ten times more potent than δ-TP [9].

MATERIALS AND METHODS

Chemicals

The solvents and reagents used were obtained from the following companies: ethanol, *n*-hexane, isopropanol and sodium perchlorate from Fluka (Buchs, Switzerland); methanol from Ferak (West-Berlin, F.R.G.); and pyridine from E. Merck (Darmstadt, F.R.G.). All solvents were analytical grade and were used without further purification.

The vitamin E standards, α-, γ-, and δ-TP were purchased from Supelco (Bellefonte, PA, U.S.A.).

High-performance liquid chromatography

The HPLC analyses were performed with a M45 solvent delivery system (Waters Assoc., Milford, MA, U.S.A.), equipped with a U6K universal sample injector (Waters Assoc.) and an electrochemical detector (Model LC5, BioAnalytical Systems, West Lafayette, IN, U.S.A.) with a glassy carbon working electrode. The detector was operated at a potential setting of +0.7 V versus the Ag/AgCl reference electrode. The chromatographic trace was registered by a potentiometer recorder. A reversed-phase LiChrosorb RP-18 column was used in all experiments and was obtained from Chrompack (Middelburg, The Netherlands) (250 × 4.6 mm; 5-μm particles). The eluent consisted of methanol containing 0.1% pyridine and 0.05 *M* sodium perchlorate

as supporting electrolyte, as described by Ikenoya et al. [6] for reversed-phase analysis of tocopherols in combination with electrochemical detection. The eluent was filtered through a Millipore type FH 0.5-μm filter and was degassed before use. The solvent flow-rate was 1 ml/min with a corresponding pressure of 7.9 MPa.

Sample preparation

Blood samples were collected in Li-heparin vacutainers (Becton Dickinson, France) and plasma was separated and frozen until analysed. The plasma was extracted following the procedures described by De Leenheer et al. [5] with minor modifications. In a centrifuge tube, 40 μl of a δ-TP solution in isopropanol (100 μg/ml) and 100 μl of ethanol were added to a plasma sample of 100 μl. After mixing, the sample was extracted with 1.2 ml of *n*-hexane by thoroughly mixing using a vortex-type mixer for 1 min. After centrifugation (5 min, 700 *g*) the organic layer was transferred to a brown conical tube and evaporated under a nitrogen stream. The residue was redissolved in 500 μl of isopropanol and an aliquot of 30 μl was used for HPLC analysis.

Quantification

Known amounts of α-TP, covering the range of 5.80—46.45 μmol/l were added to samples of a plasma pool. The regression curve was made by plotting peak height ratios (α-TP/δ-TP) against the α-TP concentration.

The exact concentration of the α-TP standard solution was determined by measuring the UV absorbance at λ_{max} = 292 nm and by using an ε-value of 3500 l/mol cm [5].

RESULTS AND DISCUSSION

Typical chromatograms of extracts from human plasma, with and without addition of the internal standard δ-TP, are illustrated in Fig. 2. The retention characteristics of the vitamin E homologues are given in Table I. The total elution of the vitamin E homologues takes about 13 min. Because δ-TP is a natural vitamin E homologue, the plasma of 40 healthy individuals was checked for the possible occurrence of δ-TP. In only 8 out of 40 samples could traces of endogenous δ-TP be detected (0.82 ± 0.65 μmol/l) at the lower detection limit of δ-TP (signal-to-noise ratio = 3). The finding that δ-TP is quantitatively of minor importance in human plasma justifies its use as internal standard and is in agreement with data published by Kato et al. [7], who reported the total absence of δ-TP in human serum for a group of 19 individuals. In order to minimize the possible error in the determination of peak height ratios (α-TP/δ-TP) due to the contribution of traces of endogenous δ-TP, a 120-fold excess of the expected δ-TP amount was added as internal standard.

In a series of experiments we have also investigated the experimental conditions for suitable electrochemical oxidation of both α- and δ-TP. α- and δ-TP have been demonstrated to display a different reactivity toward singlet oxygen, which correlates with the antioxidant vitamin E activity, α-TP being about ten times more potent than δ-TP [9]. Consequently, different half-wave potentials for the electrochemical oxidation of α-TP ($E_{1/2}$ = 0.45 V versus Ag/AgCl) and of

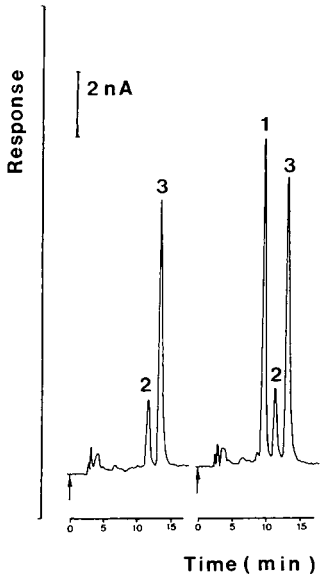

Fig. 2. High-performance liquid chromatogram of a plasma extract without and with addition of the internal standard, δ-TP. Column: 25 cm × 4.6 mm I.D., packed with 5-μm LiChrosorb RP-18. Eluent: methanol containing 0.1% pyridine and 0.05 *M* sodium perchlorate; flow-rate 1 ml/min. Detection: electrochemical oxidation at a potential of +0.7 V. 1 = δ-TP; 2 = β- + γ-TP; 3 = α-TP.

TABLE I

RETENTION CHARACTERISTICS OF TOCOPHEROLS

	Capacity ratio (k')	Retention time (min)
δ-TP	2.8	9.2
β-TP/γ-TP	3.5	11.0
α-TP	4.2	12.6

δ-TP ($E_{1/2}$ = 0.62 V) have been found [6]. Mixtures of α- and δ-TP, containing 5.8 and 7.5 ng, respectively, were subjected to HPLC analysis and the oxidizing potential of the glassy carbon working electrode was varied from +0.50 to +0.90 V versus the Ag/AgCl reference electrode in order to select a reasonably low oxidizing potential for δ-TP in its plateau region. Potentials higher than +0.90 V were not applied because of serious baseline instability, probably due to impurities in the eluent which started to oxidize. Fig. 3 shows the relationship between the peak response (in nA) and the applied potential. As can be seen from Fig. 3, a plateau value is reached for α-TP at approximately +0.80 V but not for δ-TP at the highest potential evaluated, i.e. +0.90 V. Because of baseline instability, which already started to occur at +0.80 V, and because sensitivity was not a limiting factor (detection limit 0.6 ng; signal-to-noise ratio = 10), we finally decided to use an oxidizing potential of +0.70 V for routine determinations of α-TP. A linear relationship between peak height ratios (α-TP/δ-TP) and the α-TP concentration was found (Y = 0.7005X + 0.7866; r = 0.99985; n = 6).

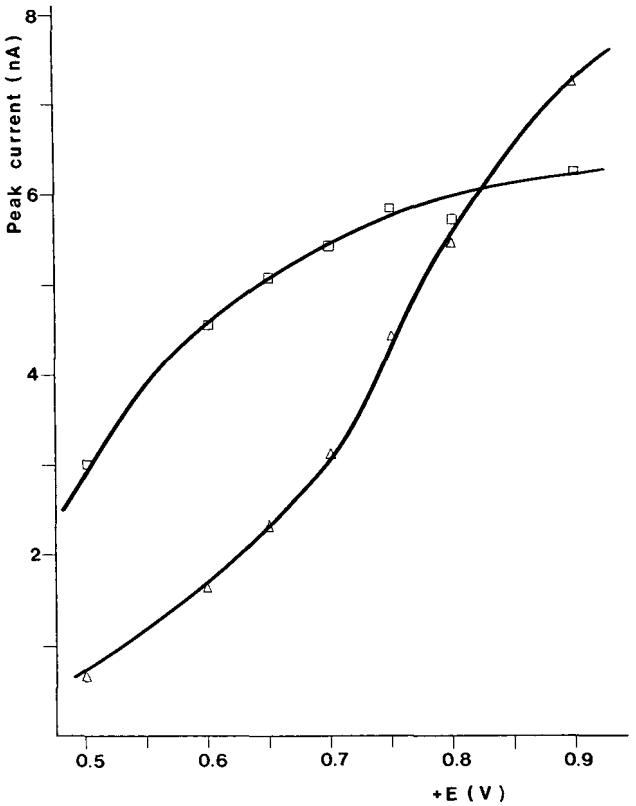

Fig. 3. Voltammograms for α-TP (□) and δ-TP (△) obtained under the conditions of Fig. 2. Injected amounts: 5.8 ng of α-TP; 7.5 ng of δ-TP.

For the estimation of the unknown concentrations, the procedure described by De Leenheer et al. [5] is applied. Briefly, the calibration curve is used after subtraction of the intercept, which represents the endogenous level of α-TP in the plasma pool, and the concentration in unknown plasma samples is determined after calculation of the peak height ratios. Analysis of nine samples of a plasma pool revealed a within-day precision (coefficient of variation, C.V.) of 2.5% (mean ± S.D. = 18.05 ± 0.46 μmol/l). The day-to-day precision (C.V.) as measured over a period of ten days was 4.3% at the concentration level of 26.89 mol/l (S.D. = 1.16 μmol/l). All these values are within an acceptable 5% limit. The detection limit of the procedure was established using aqueous standards and was shown to be 0.23 μmol/l (signal-to-noise ratio = 10), which corresponds to concentrations about 100 times lower than those normally found in human plasma.

As a test of the applicability of the method, plasma from 25 human donors was analysed. Data are given in Table II and are in good agreement with literature data [5,10].

The HPLC method in combination with electrochemical detection for the estimation of α-TP is as simple and as short (total analysis time approximately 30 min) as the UV method described by De Leenheer et al. [5] and has a comparable precision: the C.V. for the UV method was 3% against 4% for our

TABLE II

PLASMA α-TOCOPHEROL CONCENTRATIONS FOR A GROUP OF ADULT HUMANS

Age (years)	Sex*	α-TP concentration (μmol/l)
20	M	15.79
20	F	23.46
22	F	21.83
22	M	22.53
25	M	16.49
25	F	24.15
26	F	23.46
27	F	26.70
28	M	25.08
29	M	33.44
30	F	31.12
30	F	21.60
31	M	50.39
31	M	29.96
31	M	22.76
32	F	27.64
32	F	25.78
36	F	34.60
36	F	26.70
36	M	20.44
39	F	22.29
51	F	58.52
62	M	36.00
63	F	22.53
66	M	36.23

Mean ± S.D.: 28.05 ± 9.48 μmol/l
Range: 15.79—58.52 μmol/l

*M = male; F = female.

method. The very clean chromatograms, which are totally free from interferences, indicate that the specificity of electrochemical detection, in which a relatively low oxidizing potential of +0.70 V is employed, is high. This is in contrast with methods based on direct fluorimetry and UV spectrophotometry, in which co-extracted compounds interfere in the analysis [3,11]. The HPLC method proposed is extremely sensitive as only 100 μl of plasma are utilized and only 1/16 of the final extract is employed for HPLC analysis. The method may therefore have a particular value in cases where the sample size is a limiting factor, e.g. in white blood cell analysis [1].

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Note

Quantitative analysis of cystathionine and perhydro-1,4-thiazepine-3,5-dicarboxylic acid in the urine of a patient with cystathioninuria using isotachophoresis

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We have reported in previous papers [1—3] that the following unusual sulphur-containing amino acids have been excreted in the urine of a cystathioninuric patient: S-(3-hydroxy-3-carboxy-*n*-propyl)cysteine, S-(β-carboxymethyl)homocysteine, S-(2-hydroxy-2-carboxyethyl)homocysteine, perhydro-1,4-thiazepine-3,5-dicarboxylic acid, cystathionine sulphoxide and N-acetylcystathionine. After that, it was reported that cystathionine was oxidized with snake venom L-amino acid oxidase [4]. But the biosynthesis and physiological roles of these compounds in vivo are as yet not entirely understood.

All sulphur-containing amino acids described above, except for perhydro-1,4-thiazepine-3,5-dicarboxylic acid, could be easily determined with an amino acid analyser, but the extinction coefficient of perhydro-1,4-thiazepine-3,5-dicarboxylic acid in the ninhydrin reaction, because it only has an imino group in its structure, was too low for this compound to be determined by an amino acid analyser.

Therefore, a new method for determining urinary perhydro-1,4-thiazepine-3,5-dicarboxylic acid was devised using isotachophoresis [5—10]. The determination of cystathionine in rat tissue using isotachophoresis has been reported in a previous paper [11]. The determination of cystathionine in the urine of a cystathioninuric patient has also been achieved using this method.

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MATERIALS AND METHODS

Cystathionine was obtained from Sigma. Authentic perhydro-1,4-thiazepine-3,5-dicarboxylic acid was synthesized as reported in previous papers [4, 12]. All other chemicals used were analytical grade. The urine samples from normal humans were obtained from laboratory personnel. The sample from a patient with cystathioninuria was obtained from an elder sister reported in a previous paper [1].

A 2-ml volume of each urine sample was applied to a column containing 5 ml of Diaion SK-1 (H^+ cation exchanger, mesh 100; Mitsubishi Kasei, Tokyo, Japan), washed with 50 ml of water and eluted with 30 ml of 2 *M* ammonium hydroxide. The eluate was evaporated to dryness under reduced pressure. An aliquot of the residue was hydrolysed in 6 *M* hydrochloric acid, and the hydrolysate was evaporated to dryness under reduced pressure. The two residues of non-hydrolysate and hydrolysate were analysed by isotachophoresis for the determination of cystathionine.

The determination of cystathionine using an amino acid analyser was carried out by directly analysing the urine of a cystathioninuric patient. The effluent plus 50 ml of water from the Diaion SK-1 (H^+) column described for the analysis of cystathionine was adjusted to pH 9.0 with 2 *M* ammonium hydroxide and applied to a column containing 5 ml of Diaion SA (OH^- anion exchanger, mesh 100; Mitsubishi Kasei) washed with 30 ml of 2 *M* acetic acid and eluted with 30 ml of 0.5 *M* hydrochloric acid. The eluate was evaporated to dryness under reduced pressure. The identification of perhydro-1,4-thiazepine-3,5-dicarboxylic acid in the residue was carried out by comparing its chromatographic behaviour with that of an authentic sample. An aliquot of the residue was analysed by an isotachophoretic analyser.

Instrumentation

Assay conditions for perhydro-1,4-thiazepine-3,5-dicarboxylic acid. The capillary apparatus used was a Shimadzu IP-1B isotachophoretic analyser (Shimadzu Seisakusho, Kyoto, Japan). The separations were carried out in a capillary tube, 20 cm × 0.5 mm I.D., maintained at 20°C. The migration current was 100 μA. The detector cell was 0.05 cm × 0.5 mm I.D., the chart speed was 10 mm/min. The leading electrolyte consisted of 0.01 *M* hydrochloric acid and β-alanine (pH 3.1). The terminating electrolyte was 0.01 *M* glutamic acid.

Assay conditions for cystathionine. The leading electrolyte consisted of 0.01 *M* hydrochloric acid and 2-amino-2-methyl-1,3-propanediol (amediol) containing 5% polyvinyl alcohol (pH 8.9). The terminal electrolyte was 0.01 *M* γ-aminobutyric acid and barium hydroxide (pH 10.9). The other conditions were the same as for the analysis of perhydro-1,4-thiazepine-3,5-dicarboxylic acid.

RESULTS AND DISCUSSION

Determination of cystathionine

The described procedure was used to determine cystathionine in the urine of a cystathioninuric patient. Authentic cystathionine gave a sharp zone under the

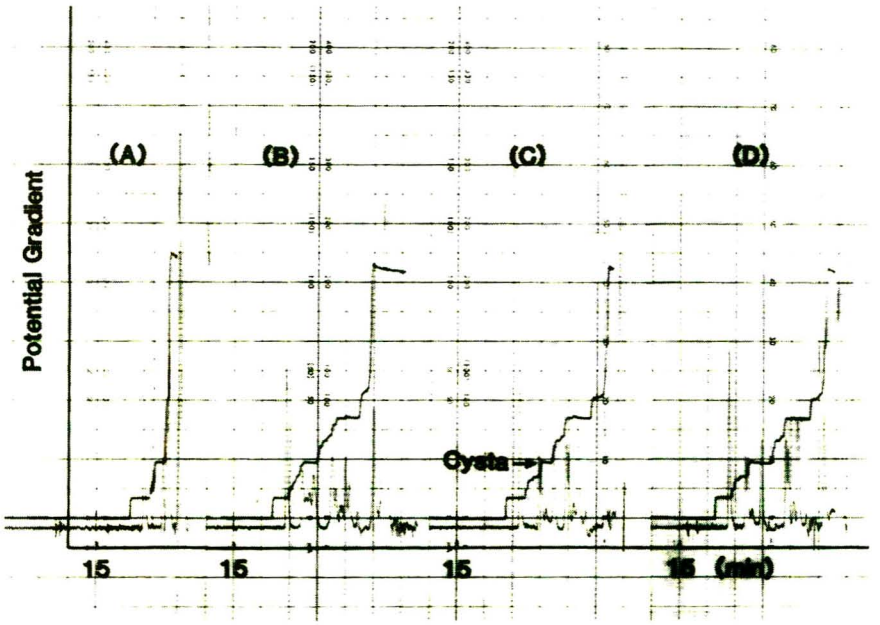

Fig. 1. Isotachophoretic runs of (A) authentic cystathionine, (B) non-hydrolysed urine and (C) hydrolysed urine of a cystathioninuric patient, and (D) hydrolysed urine as in C plus authentic cystathionine. Analytical conditions are described under Materials and methods.

described analytical conditions (Fig. 1A). A zone that had the same potential gradient as authentic cystathionine was not detected in normal human urine, but it was detected in the urine sample of a cystathioninuric patient (Fig. 1B). The sample of Fig. 1B was hydrolysed in 6 *M* hydrochloric acid for 24 h. An aliquot of the hydrolysate (Fig. 1C) was analysed by the isotachophoretic analyser just at the point where the zone of cystathionine in the hydrolysate was shorter than the zone of cystathionine in the non-hydrolysate as shown in Fig. 1C. The zone of cystathionine in Fig. 1C and that of authentic cysta-

TABLE I

COMPARISON OF CYSTATHIONINE CONCENTRATION IN THE URINE OF A CYSTATHIONINURIC PATIENT AS DETERMINED BY ISOTACHOPHORETIC AND AMINO ACID ANALYSERS

Values are expressed in μmol/ml.

Sample	Isotachophoretic analyser		Amino acid analyser
	Non-hydrolysed	Hydrolysed	
1	4.26	2.84	2.89
2	4.33	2.84	2.91
3	4.25	2.41	2.86
4	4.31	2.84	2.93
5	4.26	2.83	2.86
Mean ± S.E.	4.28 ± 0.032	2.75 ± 0.17	2.89 ± 0.028

thionine were made to overlap by adding authentic cystathionine to the urine sample; this resulted in an elongation of the cystathionine zone in the urine sample as shown in Fig. 1D. The recovery of authentic cystathionine during hydrolysis was 93—96% (n = 5).

The results for the determination of cystathionine in the urine of the cystathioninuric patient using an isotachophoretic analyser and an amino acid analyser are compared in Table I. The values of cystathionine in non-hydrolysed urine determined using isotachophoresis were higher than the values obtained using the amino acid analyser. On the other hand, the values of cystathionine in the hydrolysed urine agreed well with the values obtained with the amino acid analyser.

These results indicate that the zone with the same potential gradient as authentic cystathionine in non-hydrolysed urine samples comprises several zones, but the cystathionine zone in the hydrolysed urine is a single zone. Therefore, the method presented here is also applicable for the determination of cystathionine in urine as reported previously [11].

Determination of perhydro-1,4-thiazepine-3,5-dicarboxylic acid

Isotachophoretic runs of authentic perhydro-1,4-thiazepine-3,5-dicarboxylic acid, normal human urine, and perhydro-1,4-thiazepine-3,5-dicarboxylic acid added to normal urine are shown in Fig. 2A—C, respectively. The results indicate that perhydro-1,4-thiazepine-3,5-dicarboxylic acid is absent in normal human urine (Fig. 2B and Table II).

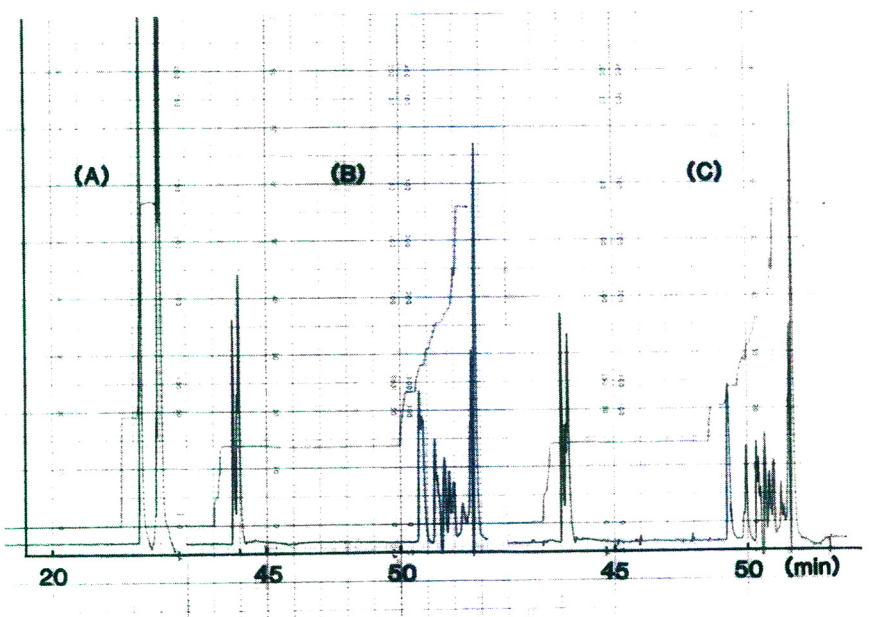

Fig. 2. Isotachophoretic runs of (A) authentic perhydro-1,4-thiazepine-3,5-dicarboxylic acid, (B) normal urine, and (C) normal urine plus authentic perhydro-1,4-thiazepine-3,5-dicarboxylic acid.

TABLE II

DETERMINATION OF PERHYDRO-1,4-THIAZEPINE-3,5-DICARBOXYLIC ACID IN THE URINE OF A CYSTATHIONINURIC PATIENT AND IN NORMAL HUMAN URINE

Values are expressed in μmol/ml.

Sample	Cystathioninuric patient	Normal human urine
1	0.97	N.D.*
2	0.91	N.D.
3	0.94	N.D.
4	0.93	N.D.
5	0.95	N.D.
Mean ± S.E.	0.94 ± 0.02	

*N.D., not detectable.

Isotachophoretic runs of urine samples of a cystathioninuric patient are shown in Fig. 3. The zone that had the same potential gradient as the zone of authentic perhydro-1,4-thiazepine-3,5-dicarboxylic acid was made to overlap by adding authentic perhydro-1,4-thiazepine-3,5-dicarboxylic acid to the urine sample, resulting in an elongation of the zone of perhydro-1,4-thiazepine-3,5-dicarboxylic acid in the urine sample, as shown in Fig. 3.

The urine sample described above contained a compound with the same R_F value as authentic perhydro-1,4-thiazepine-3,5-dicarboxylic acid on paper chromatography using *n*-butanol—acetic acid—water as mobile phase. The compound moved with the same mobility as synthetic perhydro-1,4-thiazepine-3,5-dicarboxylic acid on high-voltage paper electrophoresis, and gave a positive

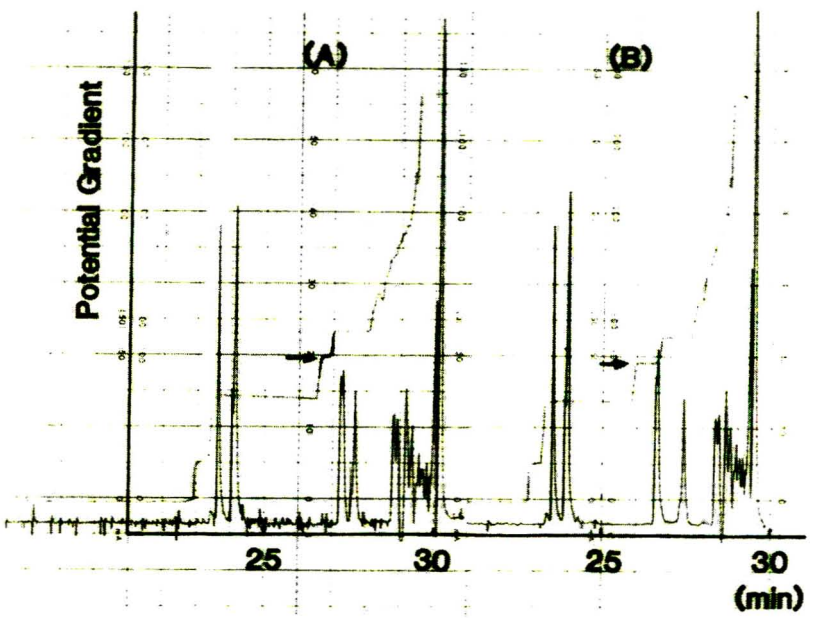

Fig. 3. Isotachophoretic runs of (A) urine of a cystathioninuric patient, and (B) the same urine plus authentic perhydro-1,4-thiazepine-3,5-dicarboxylic acid.

sulphur test [3]. These results indicate that the zone has the same potential gradient as the zone of authentic sample shown in Fig. 3A is perhydro-1,4-thiazepine-3,5-dicarboxylic acid.

The slope of a standard curve drawn by plotting zone length against concentration of authentic perhydro-1,4-thiazepine-3,5-dicarboxylic acid using isotachophoresis was linear from 0 to 50 nmol. It was possible to detect 1 nmol of perhydro-1,4-thiazepine-3,5-dicarboxylic acid using isotachophoresis.

The recovery of perhydro-1,4-thiazepine-3,5-dicarboxylic acid added to normal urine using the column chromatographic procedure described under Materials and methods was 92.3—103.1% (n = 5) and 92.6—95.3% (n = 5) in the urine of a cystathioninuric patient. Analyses of authentic perhydro-1,4-thiazepine-3,5-dicarboxylic acid and aspartic acid were also carried out simultaneously by using the amino acid analyser. In this case, 0.5 nmol of aspartic acid could easily be detected, but not even 200 nmol of perhydro-1,4-thiazepine-3,5-dicarboxylic acid could be detected because of the lower colour value of the respective ninhydrin solution. When 500 nmol of this amino acid were analysed, it finally appeared as a small peak on the chart.

However, when the urine sample was analysed with the amino acid analyser, several compounds overlapped with perhydro-1,4-thiazepine-3,5-dicarboxylic acid and interfered with its analysis. It was thus impossible to determine this compound after the same column treatment as used in isotachophoretic analysis when using the amino acid analyser.

The isotachophoretic assay presented here was more simple and sensitive than that using the amino acid analyser. The results indicate that the method should be very useful, and can be adequately utilized for the quantitative estimation of perhydro-1,4-thiazepine-3,5-dicarboxylic acid in the urine of cystathioninuric patients.

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Note

Gas—liquid chromatographic analysis with electron-capture detection of diclofensine in human plasma following derivatization

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(First received March 19th, 1984; revised manuscript received May 31st, 1984)

Diclofensine (Fig. 1) is a newly synthesised isoquinoline antidepressant. The only commercially available antidepressant with the same basic isoquinoline moiety is nomifensine [1] (Fig. 1). Rapid and high-resolution gas chromatographic (GC) analyses for nomifensine have been developed [2, 3]. Electron-capture detection of a metabolite of nomifensine, 4′-hydroxynomifensine, in biological samples has been studied utilising pentafluoropropionic anhydride as a derivatizing agent [4]. Heptafluorobutyric anhydride was used to derivatize nomifensine in an earlier GC study [5] and very recently in a comparative study of high-performance liquid and gas—liquid chromatography (GLC) [6].

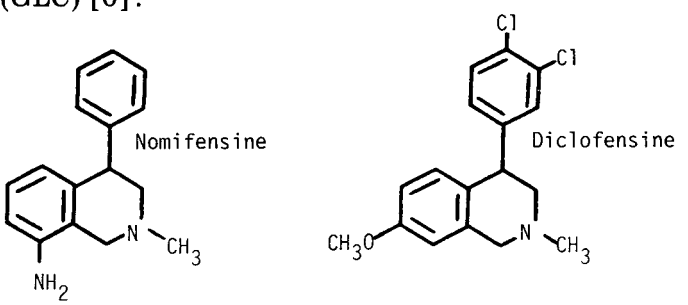

Fig. 1. Chemical structures of nomifensine and diclofensine.

To date no analytical method for the determination of diclofensine has been described. Previous work has concentrated on the antidepressant effect of diclofensine [7—9]. Preliminary investigations in our laboratory have indicated

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that diclofensine does not exhibit sufficient electron-capturing ability to be determined directly. In this paper, we describe a GLC method for diclofensine in plasma which is based on solvent extraction and the formation of a fluorinated derivative of the amine.

MATERIALS AND METHODS

Reagents

Diclofensine [RAC-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-7-methoxy-2-methylisoquinoline hydrochloride] was kindly supplied by its manufacturers (Hoffmann-La Roche, Basle, Switzerland). The internal standard imipramine hydrochloride (purity 99%) was obtained from Sigma (St. Louis, MO, U.S.A.). Heptafluorobutyric anhydride, HFBA (Pierce, Rockford, IL, U.S.A.) was used directly as the derivatizing agent. Methyl chloroformate was obtained from Aldrich (Gillingham, U.K.). Diethyl ether, *n*-heptane and triethylamine were of analytical-reagent grade (Hopkins and Williams, Essex, U.K.). Plasma was prepared by adding triply distilled water to dry plasma (Blood Transfusion Service Board, Dublin, Ireland).

Preparation of standards

Diclofensine hydrochloride (11.12 mg equivalent to 10 mg diclofensine) was dissolved in 100 ml triply distilled water (working standards). Plasma standards were freshly prepared each day by spiking the blank plasma with working standards to yield diclofensine concentrations ranging from 100 to 700 ng/ml in plasma. A stock solution of the internal standard was prepared by dissolving 11.28 mg imipramine hydrochloride (equivalent to 10 mg imipramine) in 100 ml of distilled water to give an imipramine concentration of 100 μg/ml. This solution was further diluted to give a 10 μg/ml solution of imipramine.

Extraction and derivatization procedures

To 1-ml aliquots of plasma were added 100 μl of the working standards together with 100 μl of imipramine hydrochloride, from an internal standard solution containing 10 μg/ml. The plasma was made alkaline (pH 9) with 0.2 ml 1 *M* sodium hydroxide followed by the addition of 1.5 ml diethyl ether. The mixtures were shaken for 30 sec on a vortex mixer and then centrifuged for 5 min at 700 *g*. Aliquots (1.10 ml) of the supernatant were removed and evaporated to dryness under nitrogen in glass tubes. Following reconstitution in 0.5 ml *n*-heptane, sodium carbonate (10 mg) and methylchloroformate (200 μl) were added to each tube. The tubes were then attached to an air condenser and heated at 100°C for 30 min in an oil bath. Following this, the samples were evaporated to dryness under a gentle flow of nitrogen and 0.5-ml quantities of 30% hydrogen bromide in glacial acetic acid added. The tubes were then heated for a further 10 min at 100°C and cooled. Concentrated ammonia (1 ml) was added to each tube. The contents of each tube were shaken with 300 μl of *n*-heptane and 250-μl aliquots of the supernatant were separated out. To each 250-μl aliquot were added 50 μl triethylamine and 5 μl HFBA in a dry glass tube. After 1 h at room temperture, the samples were washed with 2 ml of 0.1 *M* sodium hydroxide and the organic layer separated for analysis by GLC.

Gas—liquid chromatography

A Sigma 4 Perkin-Elmer gas chromatograph with a Ni^{63} electron-capture detector was used, with a 2 m × 2 mm glass column packed with 3% OV-17 on Chromosorb W HP (80—100 mesh). The oven temperature was 265°C with the detector and injection port temperatures maintained at 300°C. The flow-rate was 45 ml/min (oxygen-free nitrogen) with the make up carrier flow-rate at 75 ml/min. The column was conditioned each day by increasing the oven temperature from 100°C to 265°C at a rate of 1°C/min. A Hewlett-Packard 3390 A reporting integrator was used to record and measure the peak height values.

RESULTS AND DISCUSSION

Diclofensine (pK_a = 7) can be extracted from plasma at pH 9 with a number of organic solvents. The efficiency of extraction was determined using flame-ionisation detection by comparing peak heights obtained with a variety of extracting solvents with those obtained by direct injection of a standard diclofensine solution in methanol. The extraction efficiency was found to be 80% with diethyl ether, 50% with chloroform, 72% with toluene and 75% with hexane. The derivatization procedure itself was used for imipramine, amitriptyline and a series of diphenylmethane alkyl tertiary amines [10] and is adapted here for use in the analysis of diclofensine with imipramine conveniently as the internal standard.

Amines are generally difficult to analyse using GC. Although nomifensine can be gas chromatographed as the free base, the chromatographic properties of these compounds are considerably improved by derivative formation. Diclofensine is first demethylated using methyl chloroformate followed by hydrolysis of the urethane to N-desmethyldiclofensine (Fig. 2). The final step involves the derivatization reaction with heptafluorobutyric anhydride in the presence of triethylamine.

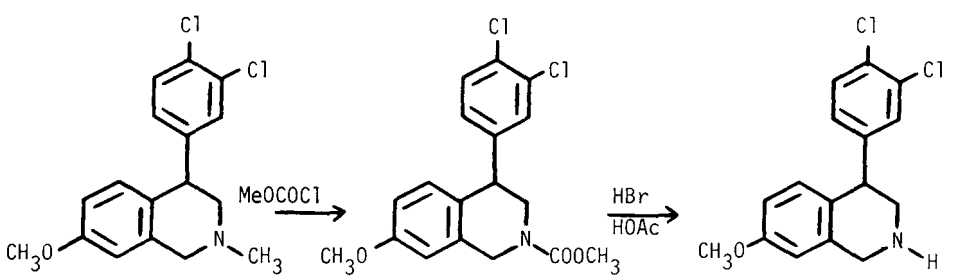


Fig. 2. Demethylation of diclofensine followed by hydrolysis of the urethane to N-desmethyldiclofensine.

A typical chromatogram of diclofensine and the internal standard imipramine is shown in Fig. 3 together with appropriate blank chromatograms. The retention times are 2.78 min for diclofensine and 1.96 min for the internal standard. A plot of peak height ratios of diclofensine to those of the internal standard against diclofensine concentration in plasma is linear over the concentration range 100—700 ng/ml with a correlation coefficient > 0.99.

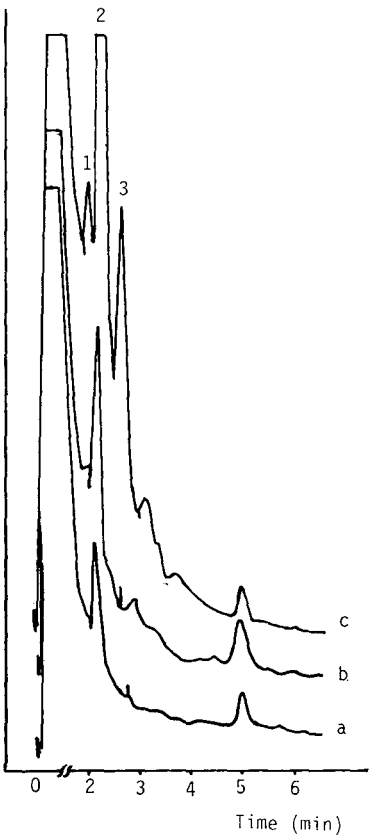
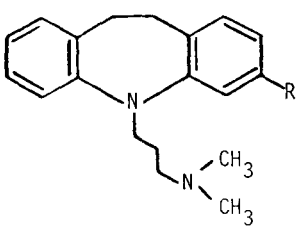


Fig. 3. Chromatograms of (a) derivatized extract of drug-free plasma; (b) underivatized extract of spiked plasma; (c) derivatized extract of spiked plasma containing 600 ng/ml diclofensine. Peaks: internal standard, imipramine (1), endogeneous (2), diclofensine (3).

Selectivity of the method

With respect to potential interferences in multiple drug therapy, some information on the selectivity of the method is obtained from a comparison of retention volumes. Apart from the internal standard, the tricyclic antidepressant cianopramine (Fig. 4) was also studied by this method. The retention volumes for the antidepressants were considerably different: diclofensine 125 ml, imipramine 88 ml, cianopramine 167 ml. The results indicate that a high selectivity is achieved.



	R
Imipramine	H
Cianopramine	CN

Fig. 4. Structural formulae of imipramine and cianopramine.

Precision of the method

Intra-assay variability was determined in the 100—700 ng/ml concentration range studied, yielding a mean coefficient of variation of 4.8% for the method. The coefficient of variation was 3.3% at 700 ng/ml and 6.2% at 100 ng/ml (n = 4).

Limit of detection

Using the conditions outlined, the limit of detection with 1 ml plasma and 1-μl injection was 70 ng/ml where the signal-to-noise ratio was just greater than 3:1.

CONCLUSION

The derivatization and electron-capture detection of diclofensine is a sensitive and accurate method for the determination of the drug in plasma. Although the levels determined did not reach the expected therapeutic plasma level, the method offers a means of measuring trace amounts of diclofensine. The extraction, separation and determination of spiked plasma samples indicates the potential use of the method in clinical applications. At the time of this study the expected metabolites of this compound were not available. N-Desmethyldiclofensine, a possible metabolite, is an intermediate in the preparation of extracted diclofensine for derivatization. However, such a metabolite could be distinguished by immediate derivatization with heptafluorobutyric anhydride and triethylamine.

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Note

Rapid gas chromatographic determination of ifosfamide in biological fluids

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Ifosfamide [IF, 3-(2-chloroethyl)-2-(2-chloroethylamino)tetrahydro-1,3,2-oxazophosphorine 2-oxide] is a structural analogue of cyclophosphamide in which one functional chloroethyl group is transferred from the extracyclic to the endocyclic nitrogen. IF has efficacy in testicular tumours including teratomas, osteosarcoma, lymphoma, pancreatic carcinoma and small cell bronchial carcinoma [1—4]. Like cyclophosphamide, IF requires metabolic activation to alkylating metabolites which have anti-mitotic actions. These latter compounds, only some of which are presently known, cannot at the moment be satisfactorily or easily estimated in biological fluids. Therefore pharmacokinetic analysis of the disposition of IF and its relationship to efficacy and dosage regimes must largely rest upon the estimation of IF and alkylating activity in plasma.

Methods for IF estimation by gas chromatography (GC) have previously been published. The earliest published method was for underivatised IF and separated IF from other compounds on a 3.8% W-982 column followed by flame-ionisation detection. The technique required, however, 2 ml plasma and a complicated extraction procedure to yield a sensitivity of only 1 μg/ml [5]. Greater sensitivity was achieved by conversion to the trifluoroacetyl (TFA) derivative and chromatography using electron-capture (ECD) or nitrogen—phosphorus detection [6—8] which allowed considerably better selectivity and sensitivity. The estimation of underivatised oxazophosphorines is complicated by the occurrence of intramolecular alkylation. This can be prevented by derivatisation [6—8] or by careful selection of assay conditions. Such conditions have been determined for cyclophosphamide and, by implication, for IF [9]. Recently an ECD—GC method for IF determination has been described [10] which requires derivatisation using heptafluorobutyric

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anhydride. The present report describes a practical assay for underivatised IF in biological fluids and compares it with a more complex assay which requires prior trifluoroacetylation of IF. The use of the underivatised technique for IF estimation is exemplified by a study of IF disposition in a dog.

EXPERIMENTAL

Chemicals

IF was a gift from WB Pharmaceuticals (Bracknell, U.K.); trophosphamide was donated by Prof. Dr. N. Brock, Asta-Werke (Bielefeld, F.R.G.). Other chemicals were purchased from BDH (Poole, U.K.).

Instrumentation

The chromatograph was a Pye 104 model fitted with a glass column (0.9 m × 2 mm I.D.) packed with 5% SE 30 on 80—100 mesh Chromosorb W (acid-washed, DMCS-treated). It was operated at 200°C with a nitrogen (carrier) flow-rate of 40 ml/min. The nitrogen detector was maintained at 350°C. The flow-rates were 65 ml/min and 635 ml/min for hydrogen and air, respectively.

Analytical methods

For direct assay of ifosfamide, the internal standard (5 μg trofosfamide) and 0.2 ml of 0.1 *M* sodium hydroxide were added to 1 ml of plasma containing IF and briefly mixed. This mixture was extracted with 3 ml ethyl acetate by mixing for 1 min on a vibration mixer (Whirlimixer; Luckhams, Burgess Hill, U.K.). After centrifugation for 10 min at 1000 *g*, 1 ml of the supernatant was transferred to a clean dry tapered glass tube (15 ml) and evaporated to dryness in a gentle stream of air in a 50°C waterbath. The dried residue was redissolved in 100 μl ethyl acetate and 2 μl of this were injected into the gas chromatograph. The volumes given above are suitable for clinical samples; adjustment of volumes will allow greater sensitivity for other circumstances.

Assay of TFA-derivatised IF was by a previously published method [6].

The 4-(4-nitrobenzypyridine) pyridine (NBP) alkylating activity was measured spectrophotometrically [7].

RESULTS

Typical chromatograms from the assay of underivatised IF are shown in Fig. 1. The retention times were 0.6 min for IF and 1.3 min for the internal standard. Identification was made by retention time in comparison with standards and peak superimposition. No interference was noted from endogenous plasma constituents. Standard solutions of IF in blank human plasma gave excellent linearity with respect to ratio of peak heights of IF:internal standard over a range of plasma concentrations from 1 to 50 μl/ml ($r = 0.999$; $n = 22$; $P < 0.001$). The relative standard deviation of between-assay replicates over this range of concentrations varied from 0.06 to 0.11. The minimum detection level (signal-to-noise ratio 2:1) was 100 ng/ml for plasma (Fig. 1).

The accuracy in assay of known IF concentrations in spiked plasma samples

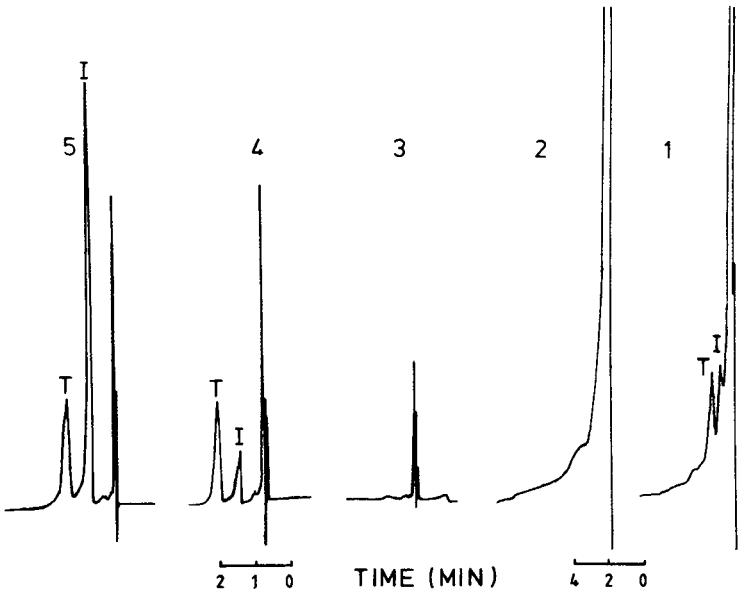

Fig. 1. Representative chromatograms from plasma. Injection artifact is first peak on right and all chromatograms are read from right to left. Chromatograms: (1) blank plasma containing 100 ng/ml ifosfamide; (2) the corresponding blank plasma; (3) blank plasma recorded at the attenuation appropriate to demonstrate the peaks due to 2 μg/ml ifosfamide (4); and 10 μg/ml ifosfamide (5). Peaks: T, trophosphamide (internal standard); and I, ifosfamide.

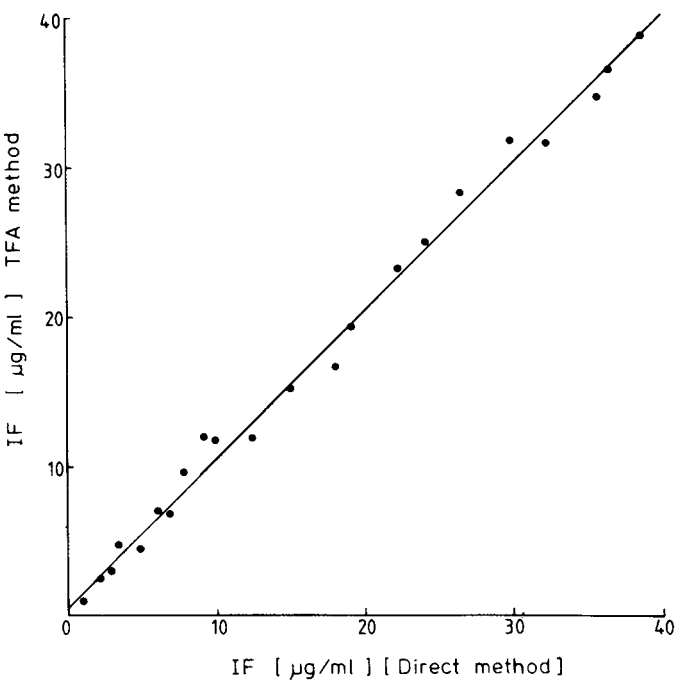

Fig. 2. Relationship between 22 estimates for IF concentrations assayed from the same sample by the direct and TFA-derivatisation methods.

was excellent. The correlation coefficient between known and estimated IF concentrations was 0.999 with a slope of 0.989 (n = 22). The intraclass correlation coefficient R_i was 0.997 indicating similarity of slope and intercept of the regression [11].

To compare the direct assay of IF with its assay as the trifluoroacetylated derivative, 22 plasma samples containing IF concentrations between 1 and 40 μg/ml were assayed by both techniques. Fig. 2 shows the results of this study. The least-squares regression line shown in Fig. 2 is derived from the equation [TFA method result] = 0.988 [direct method result] + 0.61. The 95% confidence limits of the intercept are 0.38—0.84 and of the slope 0.949—1.028. It will be noted that the latter interval spans unity which would be the slope for perfect parallelism. For this regression, r = 0.996 ($P < 0.001$). The intraclass correlation coefficient R_i tests for concordance and was 0.984. A residual plot indicated no significant bias and this was confirmed by analysis of variance.

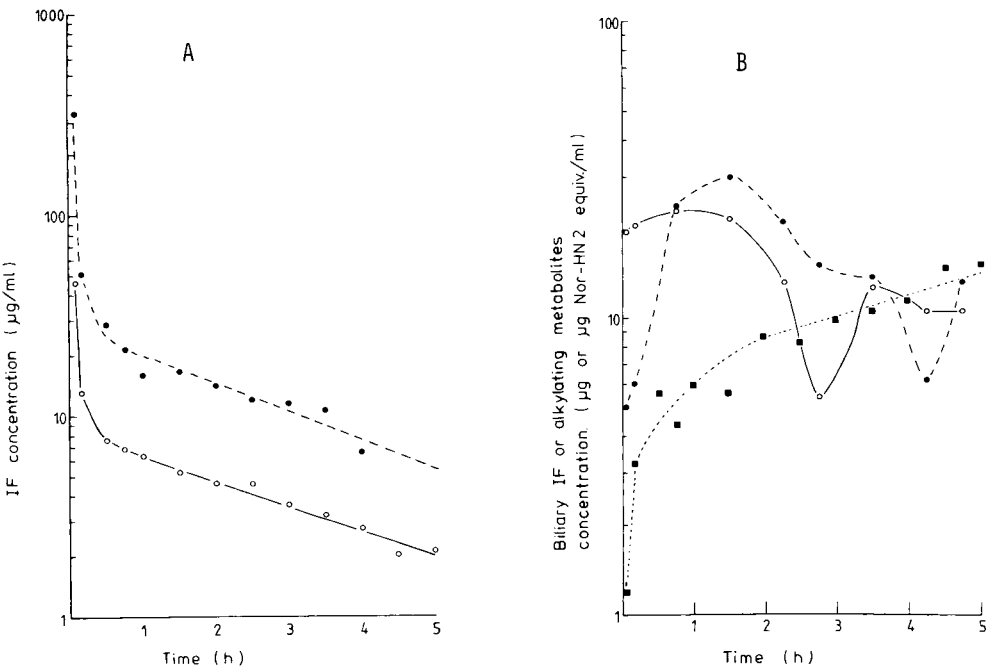

Fig. 3. (A) Plasma concentration—time profiles of unchanged IF in the femoral (•) and hepatic portal (○) veins of a dog following intravenous injection of 20 mg/kg IF. (B) Total NBP alkylating activity in the same dog (expressed as μg nornitrogen mustard equivalents per ml) determined in femoral vein (▪) and bile (•) compared with elimination of unchanged IF in bile (○).

Fig. 3 shows the plasma concentrations of unchanged IF in the femoral artery, hepatic portal vein and bile of a male greyhound (27.5 kg) given IF 20 mg/kg as an intravenous bolus following cannulation under general anaesthesia of the appropriate vessels. The half-life in the β-phase of IF in both femoral arterial and hepatic portal venous plasma was 2.3 h, the apparent volume of distribution in the β-phase of the central compartment (V_β) 1.0 l/kg, and the systemic clearance 0.3 ml/kg/h. IF elimination in bile showed a discontinuous pattern and this was mirrored by elimination of NBP-alkylating

activity. There was only moderate correlation between biliary and femoral artery IF concentrations ($r = 0.78$; $n = 6$).

IF may also be estimated in urine from patients undergoing treatment for sarcomas using this underivatised method [12].

DISCUSSION

The assay for estimation of underivatised IF was found to be as accurate as that involving the more complex requirements of derivatisation with trifluoroacetic acid. The extraction time is rapid and chromatography on a relatively narrow-bore column gives a short retention time so that some 50 samples can easily be assayed in a working day. A previous report suggested decompositon of underivatised IF on an SE 30 column [8]; this was not noted in the present study. Under the present assay conditions, cyclophosphamide shows formation of double peaks and has a brief retention time which made it unsuitable as an internal standard. An assay for underivatised cyclophosphamide has previously been published [9].

In our hands this rapid assay has proved suitable for pharmacokinetic studies of IF in animals and man using samples of saliva, plasma, bile and urine. The assay is suitable for pharmacokinetic studies as illustrated by a study of IF kinetics in bile and plasma following intravenous administration to a dog. The elimination of IF and its NBP alkylating metabolites in bile has not been previously reported but is consonant with analogous observations on cyclophosphamide made in man [13]. This route of elimination may explain the finding of IF in faecal samples from patients receiving IF by the intravenous route [14]. Further studies will be required to elucidate the kinetics of this in the bile but a quantitative study of cyclophosphamide in man [11] suggests by analogy that the fraction of the drug dose eliminated by this route is likely to be small.

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Note

Simple method for routine determination of betaxolol in blood and urine by automated high-performance liquid chromatography with fluorimetric detection

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Betaxolol, (±)-1-{4-[2-(cyclopropylmethoxy)ethyl]phenoxy}-3-isopropylamino-2-propanol hydrochloride (Kerlon®), is a new adrenoceptor blocking agent, approved for the treatment of hypertension in many European countries. In two previously published reports, the quantification of betaxolol in biological fluids with gas—liquid chromatography (GLC) and electron-capture detection was described [1,2]. These methods are time-consuming because several extraction steps are necessary in order to obtain clean chromatograms. Moreover, derivatization with heptafluorobutyric anhydride is needed to allow the quantification of concentrations as low as 0.5 ng/ml betaxolol. These GLC methods are specially suitable for pharmacokinetic studies. Betaxolol has a long terminal half-life (16—22 h), and following oral administration of a 20-mg dose the peak blood concentration ranges between 30 and 60 ng/ml. Hence, it is absolutely necessary to have a method sensitive enough to follow betaxolol blood concentrations down to 1—2 ng/ml with good precision in order to measure elimination half-lives.

For therapeutic drug monitoring the emphasis is not on sensitivity; what is required is a fast, simple and reliable method. If compliance is good, betaxolol steady-state concentrations are never below 10 ng/ml with a dose of 10 mg/day. The present report describes a high-performance liquid chromatographic (HPLC) method with fluorimetric detection and automatic injection for the determination of betaxolol in clinical setting, and gives some examples of drug monitoring in a group of hypertensive patients treated for at least one year with the drug.

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EXPERIMENTAL

Standard and reagents

Betaxolol hydrochloride was synthesized in the Chemistry Department of Synthélabo (Bagneux, France); metoprolol hydrochloride was kindly supplied by Dr. Cavero from the Cardiovascular Department of Synthélabo (Paris, France).

Diethyl ether, acetic acid and sodium hydroxide, all analytical reagent grade, were obtained from Carlo Erba (Milan, Italy). Acetonitrile (HPLC grade) was purchased from E. Merck (Darmstadt, F.R.G.).

Stock solutions

Standard solutions of betaxolol (1 μg/ml) and metoprolol (1 μg/ml) were prepared in methanol and kept at 4°C. Under these conditions the solutions were stable for several weeks.

Equipment

Chromatography was carried out on a Micromeritics 7000 liquid chromatograph connected to an automatic injector (Micromeritics 725) and a fluorimetric detector (Kontron SFM 23 B). The fluorimetric detector was set with an excitation wavelength of 275 nm and emission wavelength of 305 nm.

Chromatographic conditions

The mobile phase was acetonitrile—acetate buffer 0.03 *M*, pH 5.6 (40:60 v/v) pumped at a flow-rate of 1.0 ± 0.01 ml/min through a stainless-steel column (15 cm × 4.6 mm I.D.) packed in our laboratory [3] with Spherisorb CN, 5 μm (Batch 17/143) (Phase Separations, Queensferry, U.K.). Acetate buffer was prepared by adding 1.8 ml of glacial acetic acid to 1 l of distilled water and adjusting the pH to 5.6 with 10 *M* sodium hydroxide.

Extraction procedure

A 1-ml volume of blood and 1 ml of distilled water were added to a conical tube containing 50 ng of the internal standard metoprolol (50 μl of a 1 μg/ml solution in methanol). This mixture, made alkaline (pH 11) with 200 μl of 2 *M* sodium hydroxide, was shaken on a Vortex mixer and then extracted with distilled diethyl ether (7 ml) on a rock-and-roll shaker for 15 min. Following centrifugation (1000 *g*, 5 min at 4°C), 6.5 ml of the upper organic phase were transferred to a second tube and evaporated to dryness at 37°C under a gentle stream of nitrogen. The dry extract was then dissolved in 880 μl of 0.03 *M* acetate buffer by agitation on a Vortex mixer. This solution was transferred to an injection vial with a volume of 880 μl [4,5], and injected onto the column by means of an automatic injector equipped with a 500-μl loop.

For the quantification of betaxolol in urine, the same procedure was followed, but, owing to the higher concentrations of the drug in this medium, the volume analysed was scaled down to between 0.1 and 0.5 ml.

A calibration curve prepared with blood (or urine) spiked with 10, 20, 40 and 80 ng (from a methanolic solution of 1 μg/ml) and 50 ng of metoprolol as internal standard, was run with each series of samples. Each point in the calibration curve was prepared in duplicate.

Quantification

All determinations were performed by calculating the peak area ratios of betaxolol to the internal standard. The integration of the peaks and the calculations of the concentrations were performed by a Sigma 10 Perkin Elmer integrator after the definition of the response factor, between betaxolol and metoprolol.

Patients

The patients whose blood betaxolol concentrations are reported were participating in a multicentre long-term study to assess the efficacy and tolerance of betaxolol. All were outpatients, suffering at the beginning of the study from essential hypertension with a diastolic pressure > 100 mm Hg. The dose could vary from 10 to 40 mg/day, taken as a single oral administration. These patients were, in principle, on monotherapy. In these ambulatory patients blood was sampled to monitor the betaxolol concentration at the same time as the periodic clinical control. The full results of this two-year study will be reported elsewhere.

The present report also includes a second group of twelve hypertensive patients, from another long-term study, to whom betaxolol was administered as polytherapy. This makes it possible to check for possible analytical interferences.

RESULTS AND DISCUSSION

Two chromatograms obtained from a blank blood extract and from a spiked (20 ng) blood extract are presented in Fig. 1. The blank blood extract showed no endogenous interfering peaks. The retention times of metoprolol and betaxolol were 6.4 and 7.9 min, respectively. The absolute sensitivity (signal-to-noise ratio = 3) of this method, checked using standard solutions, was 0.5 ng/ml. However, from a practical point of view, the minimum quantifiable level is 1 ng/ml of blood.

The precision of the method was determined by spiking ten 1-ml aliquots of drug-free blood with four different amounts of betaxolol, i.e. 5, 50, 80 and 500 ng. After the addition of 50 ng of internal standard, the samples were processed as previously described. The coefficient of variation ranged from 11% to 3% for the concentrations 5—500 ng (Table I).

The recovery of the method was not calculated in the present study, but it is estimated to be equal to that reported in the previously described methods in which the same extraction procedure was utilized [2].

Interfering peaks from other cardiovascular drugs which can be administered concomitantly with this beta-blocker were checked. Guanethidine, furosemide, lidocaine, quinidine and diltiazem did not interfere with either betaxolol or metoprolol.

This method is suitable for an automatic sample processing and data acquisition system. Under these conditions 40 samples a day can easily be analysed, the rate-limiting step remaining the preparation of the samples for injection.

The blood concentrations observed in the patients on monotherapy are reported in Table II, and a representative chromatogram of a patient's blood sample appears in Fig. 2.

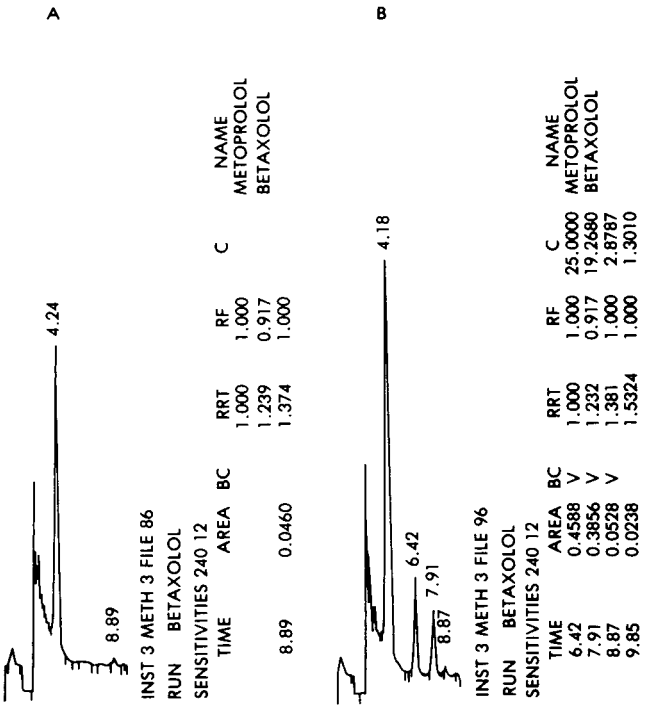

Fig. 1. Chromatograms of (A) 1 ml of blank blood extract, and (B) 1 ml of spiked blood extract containing 20 ng of betaxolol and 50 ng of internal standard (metoprolol). RRT = relative retention time, RF = response factor, C = concentration.

TABLE I

REPRODUCIBILITY OF THE HPLC METHOD FOR THE MEASUREMENT OF BETAXOLOL AT DIFFERENT CONCENTRATIONS IN BLOOD

Theoretical concentration (ng/ml)	Number of measurements	Mean concentration obtained (ng/ml)	Standard deviation	Coefficient of variation (%)
5	10	4.7	0.5	11
50	11	48.8	3.1	6
80	10	79.4	2.8	3.5
500	11	508.5	17.0	3

It is interesting to observe the consistency of the blood concentrations of betaxolol, within the same subjects, monitored over intervals of two to four months.

The very narrow intra-subject fluctuation of the betaxolol blood concentration has been already observed in many studies, which also showed minimal (two to three times) inter-individual variation of the blood betaxolol concentrations for the same given dose [6—8]. In subjects reported in this study, the inter-individual differences for betaxolol blood concentrations may appear greater. However, it must be considered that different doses were administered, that the sampling was done either before or after the drug intake, and that the subjects studied were outpatients.

TABLE II

CONCENTRATIONS OF BETAXOLOL IN BLOOD OF HYPERTENSIVE PATIENTS UNDERGOING CHRONIC TREATMENT WITH THE DRUG AT DIFFERENT DOSES

Subject	Weight (kg)	Dose (mg)	Date	Time from last dose (h)	Betaxolol plasma conc. (ng/ml)
B.M.	108	40	30.07.82	28.0	27.8
		40	29.11.82	1.5	42.4
B.E.	47	10	28.06.82	1.5	29.1
		10	04.10.82	4.5	33.9
		10	07.02.83	1.5	33.7
V.M.	79	10	20.09.82	3.0	16.1
		10	24.01.83	2.0	17.2
C.M.	80	40	21.06.82	1.5	100.7
		40	25.10.82	1.5	99.3
		40	28.02.83	2.0	136.5
M.T.	48	20	28.06.82	24.0	61.7
		20	04.10.82	24.0	73.2

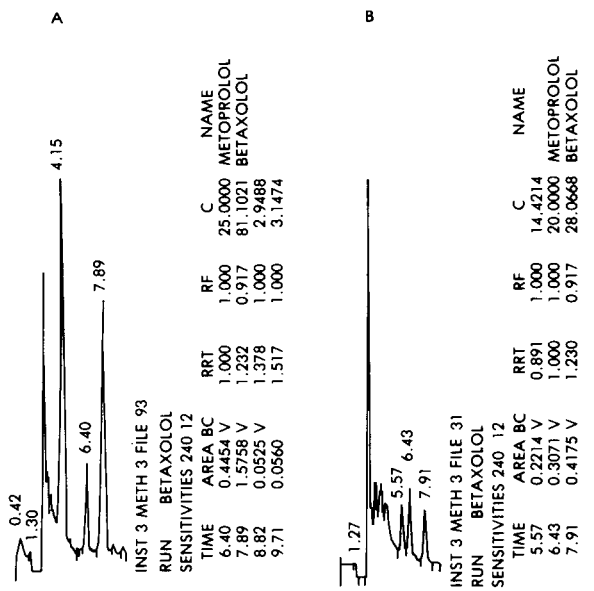

Fig. 2. Chromatograms of (A) 1 ml of blood from a patient receiving betaxolol 20 mg/day, and (B) 0.1 ml of urine from a patient receiving betaxolol 20 mg/day. Abbreviations as in Fig. 1.

For the second group of twelve patients, the individual blood concentrations are not reported because they include subjects sampled both after the first dose and after 16—30 days of treatment, which gives no comparable data. The most important observation concerns the fact that they were on polytherapy, and that clonidine, verapamil, pentobarbital, digoxin, chlortalidone, clofibrate, reserpine, caffeine, triamterene, cyclophosphamide and trimetonine were

associated with betaxolol. None of these drugs appeared to interfere with betaxolol quantification.

Importance of the type of spectrophotofluorimeter

A marked difference both in sensitivity and selectivity was observed in relation to the type of spectrofluorimeter used (Kontron versus filter fluorimetric detector). While running samples from a patient who should have been on monotherapy, we observed an unknown interference eluting with betaxolol. This interference was present only in the chromatogram obtained with the filter fluorimetric detector (Schoeffel GM 970 monochromator). The

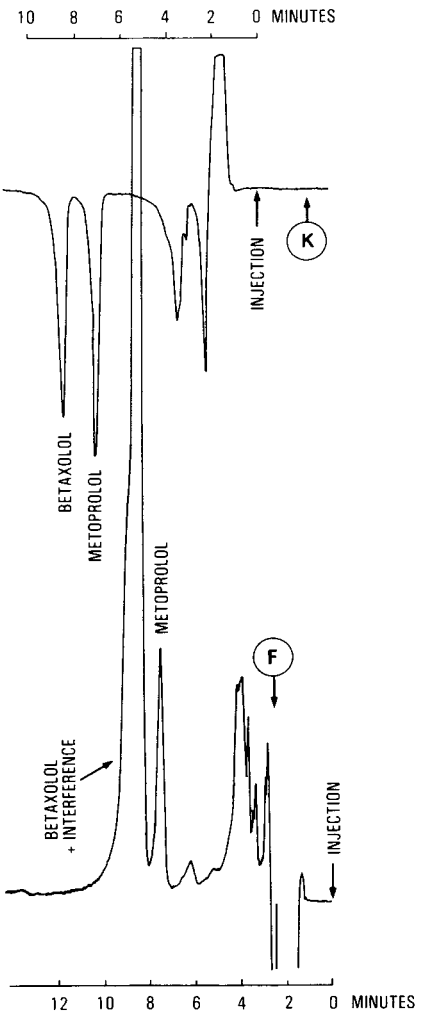

Fig. 3. Chromatograms obtained from the plasma of a patient supposed to be on monotherapy. The trace obtained with the filter fluorimetric detector (F) shows a peak interfering with betaxolol. On the other hand, the trace obtained with the Kontron (K), which is working in series with the other detector, does not show any interfering peak. The working conditions were: (A) Schoeffel filter detector, excitation 220 nm, no emission filter; (B) Kontron, excitation 275 nm, emission 305 nm.

chromatogram obtained from the Kontron detector, which was working in series with the other fluorimeter, was, however, perfectly clean (Fig. 3).

We presumed that the lack of selectivity was due to the configuration of the filter fluorimetric detector: excitation filter at 220 nm and no emission filter, this configuration being that giving the best sensitivity. By adding an emission filter at 320 nm we reduced the sensitivity of the detector by factor of two. By changing the excitation wavelength using a 275-nm filter, with or without the emission filter at 320 nm, in order to have the same configuration as the Kontron detector, the sensitivity was decreased by a factor of five. Nevertheless, the addition of the emission filter did not improve the specificity of the detector, and the interference could not be separated from betaxolol. It is likely that the intrinsic geometry of the double-monochromator detector (Kontron) gives more sensitivity and selectivity than the filter detector.

CONCLUSION

The method described is suitable for routine monitoring of betaxolol. The time required for the preparation of 40 samples for the injection is less than 2 h. Moreover, using automatic injection and an appropriate data system, manual intervention by the operator is minimal. The steady-state concentrations of betaxolol, during chronic treatment, usually do not fall below 10 ng/ml, even if the patient is treated with 10 mg of the drug. The sensitivity of the proposed method should therefore be more than adequate for the quantification of the drug. Moreover, a certain number of drugs which can be associated with a beta-blocker like betaxolol, do not seem to interfere with its analytical quantification. For interferences which cannot be separated with HPLC, it is always possible to use the gas chromatographic method with capillary column [2].

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Note

Rapid and sensitive analysis of terazosin in plasma, peritoneal dialysis solution, and urine using high-performance liquid chromatography with fluorescence detection

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Terazosin hydrochloride dihydrate {2-[4-(2-tetrahydrofuranyl)carbonyl]-1-piperazinyl-6,7-dimethoxy-4-quinazolinamine monohydrochloride, dihydrate, Fig. 1} is an antihypertensive agent [1, 2] under current development at Abbott Laboratories under the registered trademark Vasocard. The high potency of this drug necessitated the development of a very sensitive assay in order to quantify the low plasma levels which follow a therapeutic dose (1—3 mg).

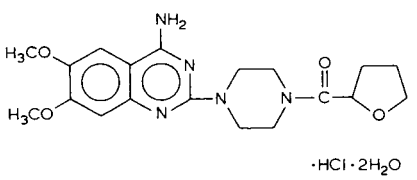

Fig. 1. Chemical structure of terazosin hydrochloride dihydrate.

The high-performance liquid chromatographic (HPLC) method reported here utilizes extraction of plasma, dialysis solution, or urine samples for lowest background interference and highest assay sensitivity. A much faster alternative sample preparation method for small volumes of plasma or serum (0.1—0.2 ml) using acetonitrile precipitation of proteins is also presented. Following extraction, the lower quantifiable concentration of terazosin was about 0.25 ng/ml using a 1-ml plasma or dialysis solution sample, and was about 1 ng/ml using a 0.2-ml urine sample. The assay was reproducible in the range of 1—50 ng/ml with a mean relative standard deviation of about 7% and 4% for plasma

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and dialysis solution, respectively. The urine assay was reproducible within the range of 10—500 ng/ml with a mean relative standard deviation of about 3%. Following acetonitrile precipitation, the serum assay was reproducible within the range of 50—1000 ng/ml with a mean relative standard deviation of about 1%.

EXPERIMENTAL

Chemicals and equipment

Terazosin hydrochloride dihydrate (THD) and prazosin hydrochloride (PH), the internal standard, were obtained from Abbott Labs. (North Chicago, IL, U.S.A.). Peritoneal dialysis solution (Dianeal PD-2 with 2.5% dextrose) was obtained from Travenol Labs. (Deerfield, IL, U.S.A.). Reagent-grade phosphoric acid, sodium hydroxide, and buffer salts were obtained from Mallinckrodt (St. Louis, MO, U.S.A.). All solvents were HPLC grade from J.T. Baker (Phillipsburg, NJ, U.S.A.). A Vortex Genie mixer or SMI Multi-tube Vortex mixer from American Scientific Products (Division of American Hospital Supply, McGaw Park, IL, U.S.A.) was used for sample preparation.

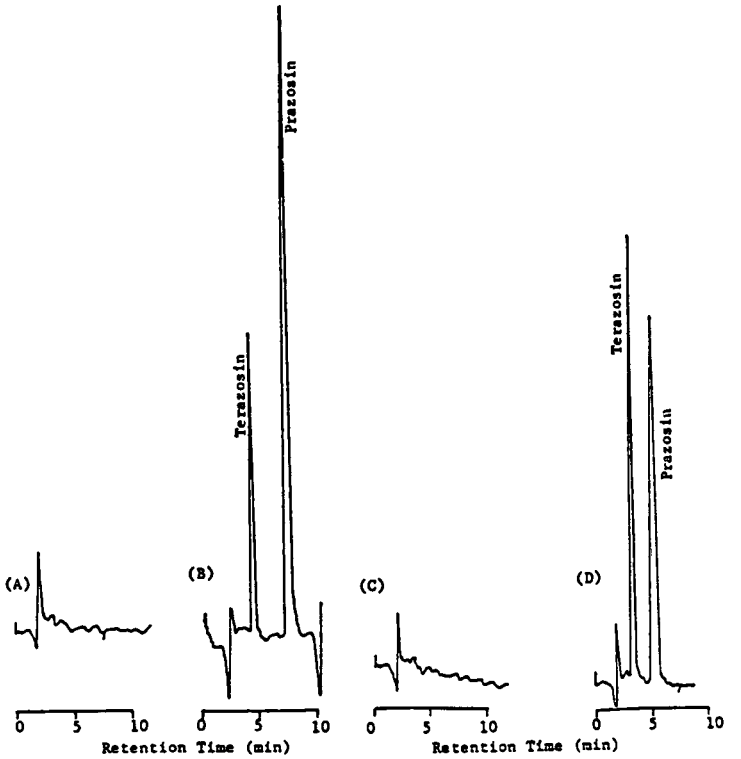
Stock solutions

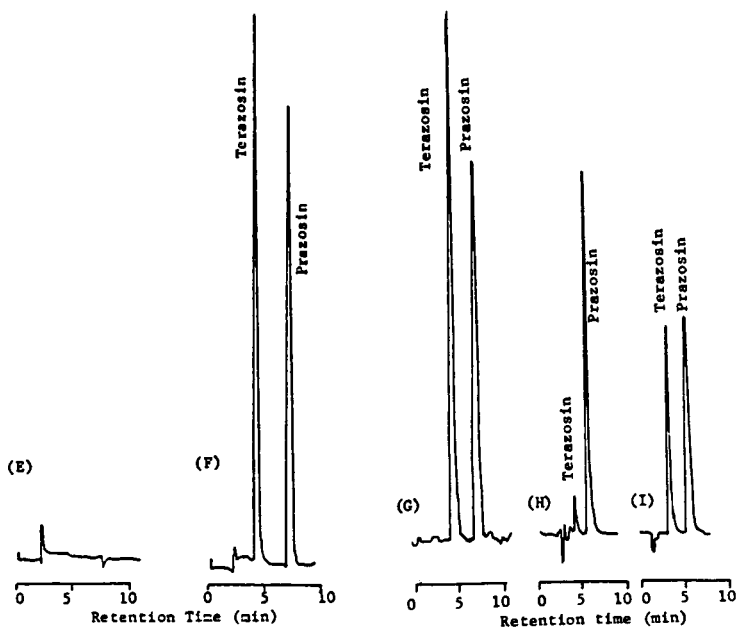
For convenience in the preparation of eluents for HPLC, stock solutions of 0.1 *M* sodium phosphate buffer, pH 7.0 and 5.0, were usually prepared. Eluents were filtered through a Nuclepore (Pleasanton, CA, U.S.A.) 400-nm polycarbonate membrane after preparation, and degassed by vacuum sonication just before use.

Stock solutions of 1 *M* sodium hydroxide and 0.1 *M* phosphoric acid were prepared in distilled water. Stock solutions of THD equivalent to 200 μg/ml terazosin (11.9 mg of THD is equivalent to 10.0 mg of terazosin) and PH (80 μg/ml), the internal standard, were prepared in methanol. Standard solutions of terazosin at 20 μg/ml in methanol, and 2, 0.2, and 0.01 μg/ml in methanol—water were prepared by serial 1:10 dilutions of the 200 μg/ml terazosin stock solution. Standard solutions of PH at 200 ng/ml in acetonitrile and 4 ng/ml in 20% (v/v) ethyl acetate in benzene were prepared.

Chromatographic systems and conditions

HPLC separations were performed using a Waters Assoc. (Milford, MA, U.S.A.) Model M6000 or M6000A or Beckman (Fullerton, CA, U.S.A.) Model 110A reciprocating pump at a flow-rate of 1—2 ml/min for solvent delivery. The reversed-phase HPLC columns used in this study were Waters Assoc. 300 or 150 × 3.9 mm I.D. columns which were repacked with IBM C 1 (Danbury, CT, U.S.A.) 5-μm particle size or Spherisorb ODS (Phase Separation, Queensbury, U.K.) 10-μm particle size packing by Analytical Sciences (Santa Clara, CA, U.S.A.). The eluents were composed of 22—25% (v/v) acetonitrile and 6% (v/v) tetrahydrofuran in 0.004—0.02 *M* sodium phosphate, pH 5—7.0 (see legends of Figs. 2 and 3 for specific columns and conditions). A Waters Assoc. WISP 710B or Perkin-Elmer (Norwalk, CT, U.S.A.) Model ISS-100 automatic sampler was used for sample processing. The HPLC system was operated at ambient temperature, and the effluent was monitored for fluorescence with a


(A)
(B)
(C)
(D)
Terazosin
Prazosin
0 5 10
Retention Time (min)


(E)
(F)
(G)
(H)
(I)
Terazosin
Prazosin
0 5 10
Retention Time (min)
Retention time (min)

Schoeffel (Westwood, NJ, U.S.A.) Model 970 LC fluorometer using a 370-nm emission filter after excitation at 250 nm.

Sample preparation procedures

Extraction. A suitable volume of plasma, dialysis solution, or urine up to 1 ml was combined with 100 μl of 1 *M* sodium hydroxide in a culture tube and mixed well. Five ml of 20% (v/v) ethyl acetate in benzene containing 2—50 ng/ml of PH (as appropriate) was added to each tube and mixed for 5 min on a vortex-type mixer to extract terazosin into the organic (upper) phase. Following centrifugation to separate phases (if necessary), 4—4.5 ml of the organic phase were transferred to a second culture tube and evaporated to dryness in a water bath at 40—50°C under a gentle stream of air. The residue was redissolved in 300 μl of 0.1 *M* phosphoric acid or mobile phase and an aliquant was injected into the chromatograph.

Acetonitrile precipitation. A small volume (100—200 μl) of plasma was combined with 2 vols. of cold (5°C) acetonitrile containing 200 ng/ml of PH, the internal standard, and mixed immediately to precipitate proteins. Following centrifugation at 5°C for 5—10 min to sediment the precipitate, the supernatant was decanted into a second culture tube and evaporated to dryness in a water bath at 40—50°C under a gentle stream of air. The residue was redissolved in 400 μl of HPLC eluent and an aliquant was injected into the chromatograph.

Calibration curves for plasma, serum, dialysis solution, and urine

Sets of standard plasma, serum, dialysis solution, and urine samples were prepared by the addition of known amounts of THD to blank plasma, serum, dialysis solution, or urine. The chromatographic peak height ratios of terazosin/prazosin were subjected to linear regression versus the corresponding terazosin concentrations. The resulting equation was used to calculate the concentration of terazosin in the test samples. The terazosin levels, sample size, and the PH levels added as internal standard to the samples may be varied to suit the concentration ranges of the intended analyses.

Recovery

Recovery of terazosin from the extraction procedures was determined by comparing the peak height ratios of test samples to blank samples which were spiked with terazosin at the same concentration following extraction.

Fig. 2. Chromatograms from human plasma (1 ml), dialysis solution (1 ml), and human urine (0.2 ml) samples prepared by the extraction method. (A) Blank plasma; (B) blank plasma spiked with THD equivalent to 5 ng/ml terazosin and 20 ng/ml PH; (C) blank dialysis solution; (D) blank dialysis solution spiked with THD equivalent to 10 ng/ml terazosin and 20 ng/ml PH; (E) blank urine; (F) blank urine spiked with THD equivalent to 50 ng/ml terazosin and 100 ng/ml PH; (G) 0.5-h postdosing plasma spiked with 20 ng/ml PH; (H) 3—6 h postdosing peritoneal dialysate spiked with 20 ng/ml PH; (I) 8—12 h postdosing urine spiked with 50 ng/ml PH from a patient with renal insufficiency who received an oral dose of THD (1 mg free base). Chromatographic conditions were as follows: column, 150 × 3.9 mm I.D., IBM C1; mobile phase, 22% acetonitrile and 6% tetrahydrofuran, pH 7.0; flow-rate, 1 ml/min; detector sensitivity, 0.2 μA; injection volumes, 100 μl (A, B); 60 μl (C, D, G, H); 15 μl (E, F).

RESULTS AND DISCUSSION

Fig. 2 shows typical chromatograms for extracted samples of blank human plasma (A), blank human plasma spiked with THD equivalent to 5 ng/ml terazosin and 20 ng/ml of PH, the internal standard (B), blank dialysis solution (C), blank dialysis solution spiked with THD equivalent to 10 ng/ml terazosin and 20 ng/ml PH (D), blank human urine (E), and blank human urine spiked with THD equivalent to 50 ng/ml terazosin and 100 ng/ml PH (F). Fig. 2 also shows 0.5 h postdosing plasma spiked with 20 ng/ml PH (G), 3—6 h postdosing peritoneal dialysate spiked with 20 ng/ml PH (H), and 8—12 h postdosing urine spiked with 50 ng/ml PH (I) from a patient with renal insufficiency who received an oral dose of THD (1 mg free base). Fig. 3 shows typical chromatograms of dog serum prepared by the acetonitrile precipitation method: blank (A), blank spiked with THD equivalent to 50 ng/ml terazosin and 100 ng/ml PH (B), and a 2-h postdosing serum sample from a dog which was administered an oral dose of THD (0.6 mg/kg free base) and methyclothiazide (0.3 mg/kg), spiked with 100 ng/ml PH (C).

To determine the precision and accuracy of the assay methods, replicate samples (3—4) were analyzed at five concentrations of the plasma, serum, dialysis solution, and urine assays. The results of these analyses are summarized in Table I for extraction data, and Table II for acetonitrile precipitation data.

A comparison of various weighting factors showed that 1/response-squared ($1/R^2$) weighting was most similar to 1/variance weighting for the plasma

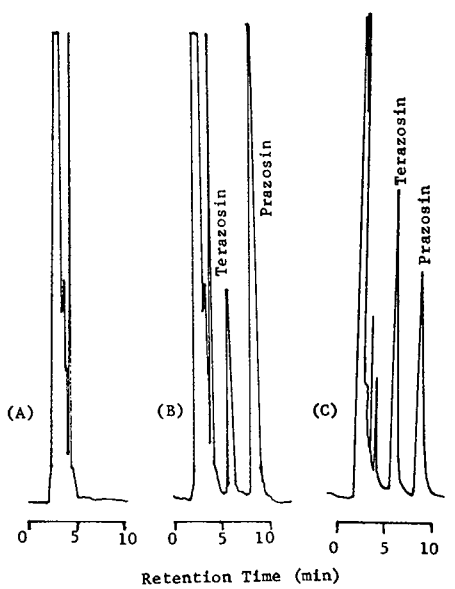


Fig. 3. Chromatograms from dog serum samples (0.2 ml) prepared by the acetonitrile precipitation method. (A) Blank; (B) blank spiked with THD equivalent to 50 ng/ml terazosin and 100 ng/ml PH; (C) 2-h postdosing sample from a dog which was administered an oral dose of THD (0.6 mg/kg free base) and methyclothiazide (0.3 mg/kg), spiked with 100 ng/ml PH. Chromatographic conditions were as follows: column, 300 × 3.9 mm I.D., Spherisorb ODS; mobile phase, 25% acetonitrile and 6% tetrahydrofuran, pH 5.0; flow-rate, 1.6 ml/min; detector sensitivity, 0.2 μA; injection volumes, 100 μl (A, B); 50 μl (C).

standard curve, while 1/concentration-squared ($1/C^2$) weighting was most similar to 1/variance weighting for the dialysis solution and urine standard curves (Table I). Using $1/R^2$ weighting (Table I), the mean predicted plasma concentrations ranged from 99% to 101% of the calculated concentrations. The relative standard deviations (R.S.D.) of the peak height ratios ranged from 2.8% to 17.8% (mean = 6.9%). This large range resulted from the 1 ng/ml concentration. If this point were excluded, the R.S.D. would range from 2.8% to 6.6% (mean = 4.2%). The mean predicted dialysis solution concentrations ranged from 94% to 105% ($1/C^2$ weighting), with an R.S.D. range of 2.9—6.6% (mean = 4.4%). The mean predicted urine concentrations ranged from 91% to 128% ($1/C^2$ weighting). This large range resulted from the 50 ng/ml point. If this point were excluded, the mean predicted concentrations would range from 91% to 96% of the calculated concentrations. The urine assay had an R.S.D. range of 2.5—5.1% (mean = 3.3%). The data from Table I were subjected to linear regression analysis, and standard curves were constructed which were linear from 1 to 50 ng/ml terazosin in plasma or dialysis solution (r = 0.999) or from 10 to 500 ng/ml terazosin in urine (r = 0.985).

A comparison of various weighting factors for the acetonitrile precipitation method showed that $1/C^2$ weighting was most similar to 1/variance weighting

TABLE I

PRECISION AND ACCURACY DATA OF THE PLASMA, DIALYSIS SOLUTION, AND URINE STANDARD CURVES FOR THE EXTRACTION METHOD

Calculated concentration of terazosin (ng/ml)	Observed mean peak height ratio	Predicted mean concentration of terazosin [Percent of theory] (ng/ml)	Relative standard deviation (%)
Plasma			
1.00	0.087	0.999 [100]	17.8
5.00	0.408	5.029 [101]	3.9
10.00	0.803	10.001 [100]	6.6
30.00	2.401	30.095 [100]	2.8
50.00	3.954	49.632 [99]	3.6
		Mean =	6.9
Dialysis solution			
1.00	0.116	0.994 [99]	6.6
5.00	0.596	5.066 [101]	2.8
10.00	1.233	10.462 [105]	2.9
30.00	3.560	30.179 [101]	5.5
50.00	5.550	47.042 [94]	4.2
		Mean =	4.4
Urine			
10.00	0.232	9.552 [96]	2.5
50.00	1.255	64.163 [128]	5.1
100.00	1.783	92.349 [92]	3.3
300.00	5.273	278.656 [93]	2.7
500.00	8.569	454.607 [91]	2.8
		Mean =	3.3

TABLE II

PRECISION AND ACCURACY DATA OF THE SERUM STANDARD CURVE FOR THE ACETONITRILE PRECIPITATION METHOD

Calculated concentration of terazosin (ng/ml)	Observed mean peak height ratio	Predicted mean concentration of terazosin [Percent of theory] (ng/ml)	Relative standard deviation (%)
50.00	0.443	50.17 [100]	0.3
100.00	0.902	99.61 [100]	0.8
250.00	2.273	247.27 [99]	2.6
500.00	4.641	502.33 [100]	1.1
1000.00	9.325	1006.84 [101]	0.8
			Mean = 1.1

for the serum standard curve. Using $1/C^2$ weighting for the serum assay (Table II), the mean predicted concentrations ranged from 99% to 101% of the calculated concentrations with an R.S.D. range of 0.3—2.6% (mean = 1.1%). Linear regression analysis of these data showed that the curve was linear (r = 0.999) from 50 to 1000 ng/ml of terazosin.

Mean recoveries of terazosin from the extraction procedure were as follows: plasma, 94% and 95% at 5 and 50 ng/ml, respectively; dialysis solution, 99% and 91% at 5 and 50 ng/ml, respectively; urine, 88% and 85% at 50 and 500 ng/ml, respectively. Recovery of terazosin from the acetonitrile precipitation procedure averaged 102% at 1000 ng/ml.

Results of plasma stability experiments have shown quantitative recovery of terazosin from samples stored at room temperature (about 23° C) for at least 30 days and from refrigerated (5° C) or frozen (—20° C) plasma samples stored for at least 60 days.

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CHROMBIO. 2243

Note

High-performance liquid chromatographic analysis of naloxone in human serum

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(First received March 19th, 1984; revised manuscript received June 15th, 1984)

Naloxone is an opiate antagonist widely used for the reversal of narcotic overdose. Recently, case reports have shown naloxone to be possibly effective in reversing some of the detrimental hemodynamic effects of septic and hypovolemic shock [1—4]. Other investigational uses of naloxone include management of alcohol intoxication [5, 6], schizophrenia [7, 8], mania [9] and Alzheimer's disease [10, 11]. At the present time little is known concerning the pharmacokinetic disposition of naloxone [12, 13] and information concerning therapeutic and toxic serum concentrations and dosage adjustment in concomitant disease states is lacking. The limited clinical pharmacologic knowledge concerning this drug can be attributed, in part, to the absence of suitably sensitive, specific and rapid analytical methods capable of measuring naloxone in biological samples following therapeutic doses.

Two analytical methods for naloxone in biological media have been reported, namely, a gas—liquid chromatographic method [14] and radioimmunoassay [15]. However, while both report sensitivities consistent with concentrations observed following typical 0.4-mg bolus doses, concerns of complex derivatization and extraction in the case of the chromatographic procedure, and the lack of general availability of specific antibody in the case of the radioimmunoassay procedure, make these methods difficult to utilize in clinical situations. In the present paper we report a sensitive, rapid and reproducible high-performance liquid chromatographic (HPLC) analytical method applicable to the measurement of naloxone in patient samples following typical dosing schedules.

EXPERIMENTAL

Chemicals and reagents

Naloxone·HCl was obtained as a pure, unformulated standard from Endo

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Labs. (Lot No. 82-037, Garden City, NJ, U.S.A.) and was used directly. The internal standard, codeine sulfate USP, was obtained from Merck (Rahway, NJ, U.S.A.). All extraction and chromatography solvents were HPLC grade (Omnisolve, MCB, Cincinnati, OH, U.S.A.) and all other chemicals and reagents were analytical-reagent grade (J.T. Baker, Phillipsburg, NJ, U.S.A.) and were used as received. Distilled water was purified by passing through a reversed-osmosis four-filter system (Millipore, Bedford, MA, U.S.A.). Stock standard solutions of naloxone·HCl (0.01 mg/ml) and codeine sulfate (0.02 mg/ml) were prepared individually in methanol. These were protected from light and refrigerated at 4°C and were determined to be stable for at least one month.

Chromatographic conditions and instrumentation

A Waters Assoc. (Milford, MA, U.S.A.) Model 202 liquid chromatograph, equipped with a Model M6000A pump and a Model U6K injector, and interfaced with a Tracor Model 970A (Austin, TX, U.S.A.) variable-wavelength detector was used for the analysis. Chromatography was performed on a 30 cm × 4 mm I.D. stainless-steel μBondapak C_{18} reversed-phase column, 5 μm particle size (Analytical Systems, Santa Clara, CA, U.S.A.). The mobile phase consisted of 13% acetonitrile in 0.1 *M* sodium dihydrogen phosphate buffer, pH 4.8, at a flow-rate of 1.5 ml/min and a pressure of 1700 p.s.i. The separation was run at ambient temperature using a detector wavelength of 220 nm.

Extraction procedure

To 2.0 ml of a serum sample or standard in a 13 × 100 mm glass culture tube was added 1.0 ml of carbonate buffer, pH 8.95 (500 ml of 0.01 *M* sodium bicarbonate and 40 ml of 0.1 *M* sodium carbonate), 15 μl of internal standard solution (300 ng of codeine sulfate in methanol) and 2.0 ml of benzene. The tube was vortexed for 45 sec and centrifuged at 1500 *g* for 5 min. The upper organic layer was pipetted into a 10 × 75 mm glass culture tube and dried under a stream of filtered air at room temperature. The residue was then reconstituted with 50 μl of mobile phase and 40 μl were injected into the chromatograph.

Quantitation

Standard curves were generated over the range 10—80 ng/ml naloxone concentrations in serum and unknown concentrations were determined by calculating peak height ratios of drug:internal standard. Linear regression analysis, using a Hewlett-Packard Model 9825A computer (Palo Alto, CA, U.S.A.) was used to compute the standard curves on a daily basis.

Recovery

Recovery studies were performed by comparing peak height ratios from 10, 20 and 60 ng/ml naloxone serum standards, to which internal standard was added after extraction, to comparable unextracted standards.

Precision

Between-run variability of the method was assessed by comparing standard curves from five consecutive daily runs. Within-run variability was evaluated from five replicate analyses of 15 and 50 ng/ml control samples.

RESULTS AND DISCUSSION

Typical chromatograms from a human serum blank, a spiked human serum sample and a patient specimen are shown in Fig. 1. Internal standard was added to all three samples. Under the analytical conditions described, retention times for naloxone and codeine were 6.3 and 7.7 min, respectively. No significant interferences near the drug and internal standard peaks were observed from extracted human serum blanks. Peak shape was generally symmetrical and allowed a calculation of naloxone concentrations from peak height ratio measurements. Standard curves prepared from spiked human serum were linear over the range 10—80 ng/ml and statistics for a typical daily curve demonstrated a slope of 0.0044, an intercept of —0.001 and r^2 of 0.993. The mean r^2 observed for nine standard curves was 0.987 with a standard deviation of 0.014.

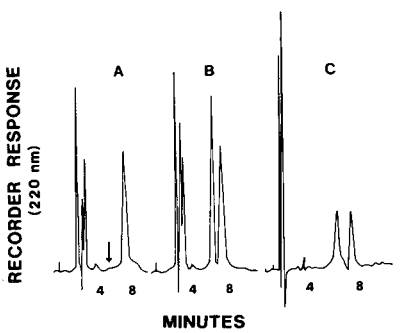

Fig. 1. High-performance liquid chromatograms of (A) extracted blank human serum, to which codeine, internal standard, had been added; (B) spiked human serum sample of 20 ng/ml naloxone; and (C) patient sample containing 12 ng/ml. All were run at 0.01 a.u.f.s. and the arrow indicates the expected elution of naloxone.

The percent recovery from 10, 20 and 60 ng/ml serum standards was 67%, 70%, and 65%, respectively, and the mean recovery was 67% with a standard deviation of 2%. The between-run coefficients of variation for control concentrations (n=5) of 20 and 40 ng/ml were 13.9% and 10.4%, respectively. The within-run coefficients of variation for 15 and 50 ng/ml controls (n=5) were 9.0% and 8.3%, respectively. The detector response demonstrated linearity up to 2000 ng/ml and the practical lower limit of sensitivity, which demonstrated a 3:1 signal-to-baseline noise ratio, was 5 ng/ml from a 2.0-ml serum sample.

Fig. 2 illustrates the application of the method to the analysis of serum samples over the typical time course of naloxone therapy in a septic shock patient. Naloxone was initiated as a 0.4-mg bolus followed by a continuous infusion of 2 mg/h. This infusion rate was increased in stepwise increments of 2 mg/h over 80 min until the rate reached 10 mg/h. Prior to each increase in the in-

fusion rate an additional 0.4-mg bolus was administered. Serial blood samples were drawn over a 6-h time period and naloxone was detected for 1 h after stopping the infusion.

The procedure presented here provides a satisfactory method for the analysis of clinical naloxone samples. It avoids the lengthy organic extraction and derivatization procedures needed in the gas chromatographic assay [14] and circumvents the difficulty in obtaining specific antibodies for the radioimmunoassay procedure [15]. There is also a question of cross-reactivity by metabolites in the immunoassay method. Since the primary metabolite of naloxone in humans is the 3-glucuronide [16], it is not extracted in the present system and, as such, does not interfere in the HPLC analysis. Additionally, other narcotic agents with similar chemical structures were evaluated and all, including morphine, meperidine, and hydromorphone, eluted with the solvent front in this system.

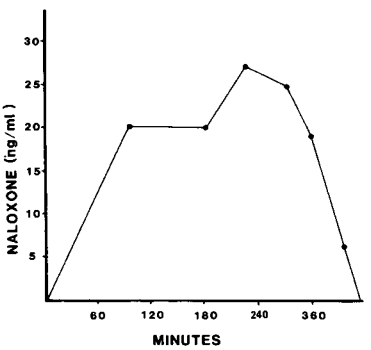

Fig. 2. Time course of serum naloxone concentrations in a patient in septic shock receiving typical intravenous administration (see text for details of dosing protocol).

In summary, the present procedure appears to be selective, sensitive, reproducible and rapid enough to support typical clinical pharmacology studies with naloxone and continuing clinical studies are presently being conducted in our laboratory.

ACKNOWLEDGEMENT

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Note

Determination of bupivacaine in human plasma by high-performance liquid chromatography

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The efficacy and safety of regional anaesthesia with local anaesthetics have improved considerably during recent years. However, the undesirable effects of these drugs, especially those of central nervous system and cardiac toxicity, can still cause severe problems [1]. Since these effects are directly related to the concentrations of local anaesthetics in the systemic circulation, their determination in plasma is of paramount importance. Due to its favourable properties, bupivacaine is one of the most frequently used local anaesthetics in many regional anaesthetic techniques, especially in obstetric anaesthesia [2]. Until now, it has been assayed by gas chromatography [3—6]. In the present communication, an assay for bupivacaine using high-performance liquid chromatography (HPLC) with a simple one-step extraction procedure is described. This assay permits the determination of bupivacaine concentrations in plasma following the administration of this drug by various regional anaesthetic techniques.

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EXPERIMENTAL

Reagents and materials

All chemicals and solvents were of analytical or HPLC grade (E. Merck, Darmstadt, F.R.G.) and were used without further purification. Bupivacaine·HCl and the internal standard 1-pentyl-2-(2′,6′-xylylcarbamoyl)piperidine (PXP) (Fig. 1) were supplied by Astra Läkemedel (Södertälje, Sweden). All glassware used for sample preparation was acid-washed and rinsed extensively with double-distilled water. Silanization of the glassware is not necessary, although it is recommended so as to decrease the variation of the results. The PTFE linings of the screw caps of culture tubes were ultrasonicated in methanol and then in double-distilled water.

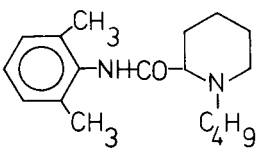

Fig. 1. Chemical structure of bupivacaine. The internal standard (PXP) carries a pentyl group on the piperidine nitrogen.

Sample preparation

The internal standard solution (4 μl), containing 3.2 μg of PXP (1.8 μg for lower concentrations of bupivacaine) was added to 1-ml plasma samples in screw-capped (lined with PTFE) culture tubes using a Hamilton Microlab M automatic pipette. The samples were alkalinized with 100 μl of sodium hydroxide solution (2 mol/l) and extracted into 7 ml of hexane by rotating slowly for 20 min. The hexane phase was separated from the aqueous phase by centrifuging at 1000 *g* for 10 min; it was transferred to a conical glass tube and evaporated to dryness under a gentle nitrogen stream. The residues were redissolved in 50 μl of methanol and then further diluted with 50 μl of water. An 85-μl aliquot of this final solution was injected onto the chromatograph.

Chromatography

A model 6000A high-pressure solvent delivery system (Waters Assoc., Milford, MA, U.S.A.) coupled with an automatic injector (WISP 710 B, Waters Assoc.), a μBondapak C_{18} reversed-phase column (30 cm × 3.9 mm I.D., particle size 10 μm, Waters Assoc.) and a filterphotometer (Model 160, Beckman Instruments, Berkeley, CA, U.S.A.) was used. The mobile phase, consisting of 60 parts of methanol and 40 parts of 50 m*M* sodium phosphate buffer (adjusted to pH 5.0 with phosphoric acid), was passed through a 0.45-μm filter (RC 55, Schleicher and Schüll, Dassel, F.R.G.) before use. The flow-rate was 1.0 ml/min. The effluent was monitored either at 0.003 or 0.005 a.u.f.s. at 254 nm and recorded at 10 mV with a chart speed of 1 mm/min. All chromatography was performed at ambient temperature.

Calibration

Calibration curves were constructed by adding known amounts of bupivacaine·HCl (50, 100, 200, 400, 600, 1000, 1400, 2000, 2400, 2800, 3200 ng)

to 1-ml aliquots of pooled human plasma. The peak height ratios of bupivacaine·HCl to the internal standard were plotted against the concentrations of bupivacaine·HCl. The least-squares linear regression line was fitted through the data points. The bupivacaine concentrations of the unknown samples were determined by using the regression equation of the calibration curve which was assayed concurrently with the unknown samples.

Application of the method

In order to test the method under clinical conditions, plasma concentrations of two patients, who received either 187.5 or 250 mg of bupivacaine·HCl for brachial plexus block, were determined. Blood samples were taken frequently following the block from an antebrachial vein using silanized plastic syringes with heparin (approximately 10 I.U./ml of blood) as anticoagulant. The plasma was separated by centrifugation and stored at −20°C until assayed.

RESULTS AND DISCUSSION

The resolution of the chromatographic system was checked daily by the injection of 20 μl of a mixture containing bupivacaine and the internal standard PXP. Retention times were 8.5 and 12.5 min for bupivacaine and internal standard, respectively (Fig. 2). Using the extraction method described, endogenous plasma components did not interfere with either bupivacaine or the internal standard (Fig. 2). The detection limit for bupivacaine from plasma samples, using a signal-to-noise ratio of 4, was approximately 50 ng/ml.

The linearity of the detector response was assessed by injecting 100 μl of aqueous bupivacaine solutions with concentrations ranging from 50 to 5000

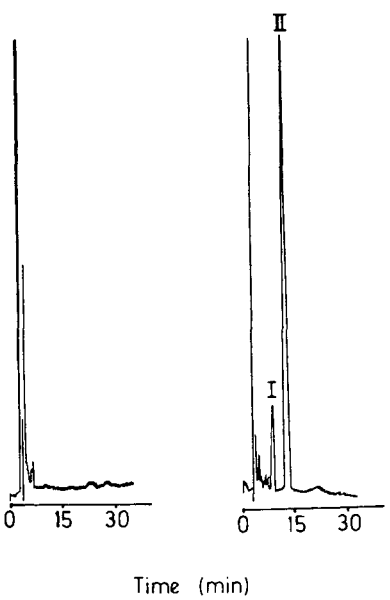

Fig. 2. Chromatograms of blank plasma (left) and plasma spiked with 300 ng/ml bupivacaine·HCl (I) and 3200 ng/ml internal standard (II) (right).

ng/ml. The absolute peak heights of bupivacaine were plotted against the corresponding concentrations. The relationship was linear (r = 0.999) with a y-intercept close to zero (0.00214). The regression line of the plasma calibration curve also showed excellent linearity (r = 0.998) with a small intercept on the y-axis (0.00843).

The precision of the assay was evaluated in a blind study in the concentration range of approximately 70—3000 ng/ml. The experimentally determined concentrations agreed well with the theoretical concentrations (Table I). The day-to-day variation of the assay stayed in an acceptable range. The slope of the standard curve showed a coefficient of variation of 5.9% (n = 12) within a time period of two months.

TABLE I

PRECISION OF THE ASSAY

Concentration of bupivacaine·HCl (ng/ml)		Accuracy* (%)
Theoretical	Experimental	
72.3	77.4 ± 5.0**	6.9
239.3	237.6 ± 8.45	0.7
939.0	944.3 ± 11.1	0.6
1671.6	1652.1 ± 42.8	1.2
2498.6	2466.4 ± 54.6	1.3
3156.0	3157.8 ± 41.4	0.06

*Calculated according to ref. 7.
**Mean ± S.D. (n = 5).

The recovery of the extraction procedure was estimated by comparing the bupivacaine peak height ratio in a plasma extract with that in an aqueous solution of the same concentration. The recovery of bupivacaine in the concentration range 70—4000 ng/ml was 94.5 ± 4.44% (n = 14).

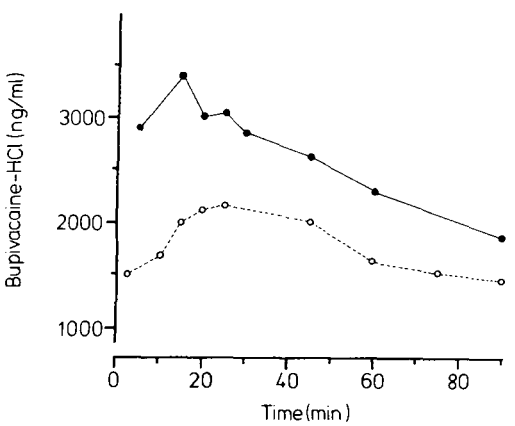

Fig. 3. Plasma concentrations of bupivacaine in two patients who received the drug for brachial plexus block: 187.5 mg (○), or 250 mg (●).

The application of the assay is demonstrated for two patients who received a brachial plexus block with a dose of either 187.5 or 250 mg of bupivacaine (Fig. 3). The plasma concentration—time profile for bupivacaine showed that this method is sensitive and specific enough to determine concentrations of bupivacaine in the systemic circulation following such an anaesthetic technique.

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CHROMBIO. 2251

Note

Detection of nalbuphine in plasma: an improved high-performance liquid chromatographic assay

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Nalbuphine is a partial agonist opiate derived from oxymorphone. Although approximately 0.8 times the potency of morphine at a dose of 8 mg [1], a "ceiling" effect on respiratory depression is observed at a dose of approximately 0.5 mg/kg, and very much larger doses have been employed in clinical practice [2]. Thus in a 70-kg man doses ranging from 5 to 200 mg may be used, resulting in widely variable plasma concentrations even at peak effect.

A method for the high-performance liquid chromatographic (HPLC) analysis of nalbuphine concentrations in plasma has been described using an electrochemical detector [3]. However, the method did not prove to be sufficiently sensitive or consistent for our clinical use; emulsification is likely to occur during extraction and interference peaks are common. In addition, the use of sodium hydroxide for control of pH makes uniform recovery of nalbuphine difficult. The method was therefore modified as described.

METHODS AND MATERIALS

Reagents

Ethyl acetate, toluene, isopropanol (glass-distilled) and methanol (HPLC grade) were all obtained from Rathburn (Walkerburn, U.K.). Potassium dihydrogen orthophosphate, boric acid, borax and hydrochloric acid were all AnalaR grade from BDH (Poole, U.K.). Nalbuphine hydrochloride powder was obtained from the Pharmaceutical Development Section of Endo Labs. (Garden City, NY, U.S.A.) and the internal standard, naloxone hydrochloride powder, from Sigma.

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Extraction procedure

To 2 ml of plasma in a 10-ml screw-topped test-tube were added 50 μl of an aqueous solution of naloxone, the internal standard (4 μg/ml) and 1 ml of 0.5 *M* borate buffer (pH 8.0). The plasma was then extracted twice with 5 ml of ethyl acetate—toluene—isopropanol (79:20:1, v/v/v) by mixing on a rotary tumbler at 40 rpm for 15 min and centrifuging for 5 min at 750 *g*. The organic layers were transferred to a clean tube by Pasteur pipette; 1 ml of 0.1 *M* hydrochloric acid was added, mixed on a rotary tumbler and the tube centrifuged. The organic layer was discarded and the acid layer brought to pH 8.0 with 2 ml of 0.5 *M* borate buffer. The aqueous layer was extracted twice with 5 ml of the ethyl acetate—toluene—isopropanol mixture. After centrifugation the organic top layer was collected and evaporated to dryness in tapered reaction vials under a gentle stream of nitrogen. The sample residue was then redissolved in 500 μl of methanol and injected onto the column using a 100-μl sample loop.

Nalbuphine calibration curves were prepared by spiking blank human plasma with nalbuphine at concentrations ranging from 1 to 100 ng/ml and with naloxone at a fixed concentration of 100 ng/ml. These samples were extracted and chromatographed as described below and the peak height ratios of nalbuphine relative to internal standard were plotted against the nalbuphine concentration.

High-performance liquid chromatography

A ConstaMetric III pump (Laboratory Data Control) was used in conjunction with an automatic injection system (Magnus Scientific M7110 automatic injector) and a precolumn (5 × 0.5 cm) and column (15 × 0.5 cm) both packed with Spherisorb 5-μm C_8 (HPLC Technology). The eluent was 55% potassium dihydrogen orthophosphate (0.01 *M*) and 45% methanol (HPLC grade) with a flow-rate of 0.8 ml/min. A BioAnalytical Systems LC4A electrochemical detector with a glassy carbon electrode (TL5) operating in the oxidative mode at 0.75 V was used. The current sensitivity was set at 5 nA and chromatograms were recorded on a Kipp & Zonen BD9 flat bed recorder.

RESULTS

The recovery of nalbuphine from extracted plasma at various concentrations was determined by comparing the peak height ratios of extracted nalbuphine samples with naloxone as an external standard, to those obtained with unextracted primary standard. The actual recovery determined was 76 ± 3% (mean ± S.E.M., $n = 7$).

As can be seen from the chromatograms of extracts from spiked plasma samples (Fig. 1), the retention times of the naloxone and nalbuphine were 7.5 and 10 min, respectively. A small tail peak was present on the nalbuphine peak of the extracted and unextracted standards, but this did not appear to affect the linearity of the calibration curve, the regression equation of which was $Y = 0.0177X - 0.0092$ from five separate determinations, with a correlation coefficient of 0.9999.

The reproducibility was checked by analysing plasma samples spiked with several concentrations of nalbuphine. The results are shown in Table I.

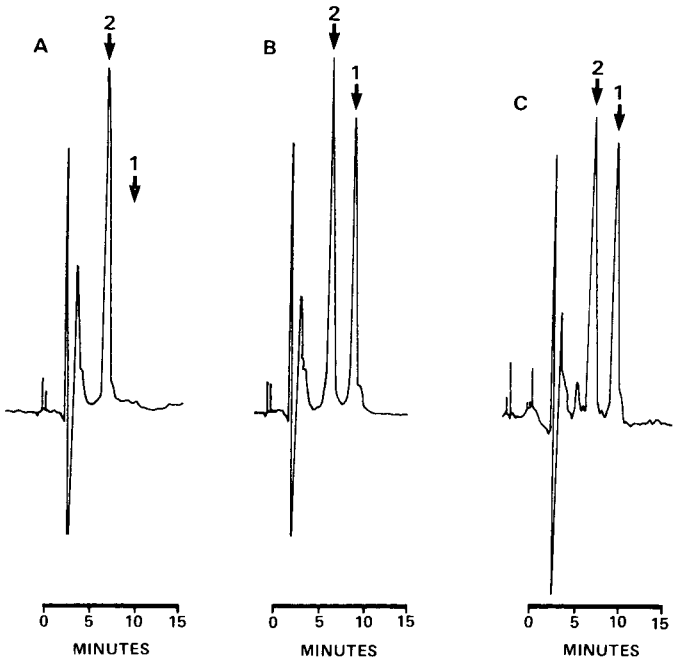

Fig. 1. Typical chromatograms of (A) control plasma to which internal standard (100 ng/ml) has been added, (B) control plasma to which have been added nalbuphine (50 ng/ml) and internal standard (100 ng/ml), and (C) patient plasma taken 1 h after administration of 0.24 mg/kg nalbuphine to which naloxone (100 ng/ml) has been added. Arrows 1 and 2 indicate the retention times of nalbuphine and naloxone, respectively.

TABLE I

REPRODUCIBILITY OF ASSAY

Added (ng/ml)	Found (ng/ml, mean ± S.E.M., n = 4)	S.E.M. (%)
10	10.26 ± 0.05	0.5
25	23.72 ± 0.40	1.7
50	47.91 ± 0.30	0.6
100	110.75 ± 0.49	0.4

DISCUSSION

This method was based on a modification of that presented by Lake et al. [3]. It was found that the extraction mixture ethyl acetate—isopropanol (9:1) with plasma was prone to the formation of emulsions which obstructed adequate removal of the organic phase. Addition of toluene to the extraction mixture resulted in an almost emulsion-free separation. In addition, there was great difficulty measuring chromatograms of plasma extracts due to interference peaks. The inclusion of a back-extraction in the extraction procedure produced a chromatogram free of interference from endogenous plasma sub-

stances. It was also found that the extraction pH was an important factor in nalbuphine recovery. Lake et al. [3] employed 0.1 *M* sodium hydroxide to adjust the plasma to pH 8.0; however, we obtained better pH control with 0.5 *M* borate buffer, with a correspondingly more consistent recovery.

The recovery was only 76% compared with a reported 94% or greater by Lake et al. [3]. However, the actual improvement in clarity of the chromatograms obtained by a more thorough extraction procedure would seem to compensate for this.

Using the method described in this paper, levels of nalbuphine down to 0.1 ng/ml of plasma were measured. The level of detection could have been increased by injection a more concentrated sample into the chromatogram, i.e. redissolving the final dried-down sample in 200 μl instead of 500 μl of methanol. This was not possible with our automated injection system as about 300 μl of final sample are required to inject 100 μl onto the column.

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Note

Determination of carboxybupranolol, the major metabolite of bupranolol, in human plasma by high-performance liquid chromatography

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Bupranolol [1-*tert.*-butylamino-3-(2-chloro-5-methylphenoxy)propan-2-ol, Fig. 1, R = CH_3] is used clinically as a β-adrenoceptor antagonist [1]. In human subjects, oral doses of bupranolol are almost completely excreted as the carboxy metabolite, carboxybupranolol [1-*tert.*-butylamino-3-(2-chloro-5-carboxyphenoxy)propan-2-ol, Fig. 1, R = COOH], which is also the major drug-related component in plasma [2].

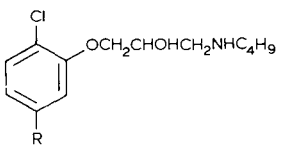

Fig. 1. Chemical structure of bupranolol (R = CH_3) and carboxybupranolol (R = COOH).

After oral doses of 40 mg bupranolol to human subjects, unchanged bupranolol was not detected above a limit of detection of 1 ng/ml using an electron-capture—gas chromatographic procedure [3]. Bupranolol has been detected in lower ng/g concentrations by a high-performance liquid chromatographic—ultraviolet (HPLC—UV) procedure [4] in rat plasma and tissues after administration of a relatively large intravenous dose (1.85 mg/kg). Studies of [^{14}C] bupranolol in both humans and animals indicated extensive first-pass metabolism of the drug and the rapid formation of carboxybupranolol; these results suggested either that bupranolol is pharmacologically active, although present at very low concentrations in plasma, or that (a) metabolite(s) of bupranolol also contributes to the pharmacological activity [2, 3, 5]. Since carboxybupranolol has been identified as the major metabolite of bupranolol in

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both humans and animals [2, 6], and since this compound has some pharmacological activity [7] it may be measured as an indication of the absorption of bupranolol from dose formulations of the drug.

In order to obtain reliable pharmacokinetic and bioavailability data, the method of analysis of carboxybupranolol in plasma must be sufficiently sensitive for the accurate determination of circulating concentrations of the compound. This paper describes an HPLC method for the measurement of carboxybupranolol in plasma over the concentration range 45—623 ng/ml. The assay procedure involves the extraction from plasma of the drug as an ion-pair, followed by chromatography in a reversed-phase mode, coupled with fluorimetric detection. The latter is ideal for carboxybupranolol analysis in that it is sensitive and highly specific thereby minimising the possibility of interference from other bupranolol metabolites, or other drugs administered concurrently with bupranolol.

EXPERIMENTAL

Reagents

Methanol was HPLC grade; sodium lauryl sulphate was Prima reagent grade; chloroform and propan-2-ol were Distol grade. All other reagents were of analytical grade. All solutions of inorganic reagents were prepared in freshly glass-distilled water. Standard solutions of the hydrochloride salt of carboxybupranolol (obtained from Dr. R. Bonn, Pharma-Schwarz GmbH, Monheim, F.R.G.) were prepared at concentrations of 1 mg/ml and 0.01 mg/ml in methanol. Solutions of deschlorocarboxybupranolol [1-*tert.*-butylamino-3-(5-carboxyphenoxy)propan-2-ol] hydrochloride, used as the internal standard for the assay, were made at similar concentrations. All standard solutions were stored in the dark at 4°C.

Sample preparation procedure

Plasma samples (0.1—0.5 ml) were transferred into conical centrifuge tubes and the volume adjusted to 0.5 ml with control plasma; the samples were spiked with internal standard (30 μl, containing 300 ng deschlorocarboxybupranolol hydrochloride). The pH of the samples was adjusted by the addition of sodium dihydrogen orthophosphate buffer (1 *M*, pH 3, 0.5 ml). Sodium dioctyl sulphosuccinate (DSS) (1%, w/v, 1.0 ml) was added as an ion-pairing agent. Samples were extracted by manual shaking for 15 sec with chloroform—propan-2-ol (9:1, v/v, 5 ml). After centrifugation, the organic layer was carefully transferred into another centrifuge tube and was evaporated to dryness at 37°C under a stream of nitrogen. The residue was washed to the bottom of the tube with a small amount of chloroform—propan-2-ol, which was again evaporated to dryness. The residue was mixed on a vortex mixer with mobile phase (50 μl) for 15 sec, after which time the sample was transferred to an autosampler vial. The total sample was injected into the chromatograph.

High-performance liquid chromatography

The liquid chromatograph consisted of a Waters M6000A pump (Waters Assoc., Northwich, U.K.) coupled to a Perkin-Elmer 3000 fluorescence detector

(Perkin-Elmer, Beaconsfield, U.K.) operated at an excitation wavelength of 245 nm and an emission wavelength of 330 nm. Injection was via an automatic injector, Waters' Intelligent Sample Processor (WISP™, Waters Assoc.). The column was constructed of stainless steel (25 cm × 0.46 cm I.D.) prepacked with Zorbax® C_8 (mean particle diameter 6 μm, DuPont, Hitchin, U.K.). A pre-column (7 cm × 0.2 cm I.D.) constructed of stainless steel and dry-packed with pellicular Co:Pell® ODS (particle diameter 25—37 μm) (Whatman, Maidstone, U.K.) was installed in front of the analytical column to protect it from contamination.

Chromatography was performed in a reversed-phase mode using an ion-pairing mechanism. The mobile phase consisted of methanol (70%, v/v) in aqueous potassium dihydrogen orthophosphate buffer (0.1%, w/v), containing sodium lauryl sulphate (1%, w/v). The final solution was adjusted to pH 3 with phosphoric acid. The mobile phase was passed through the column at a flow-rate of 2 ml/min.

Chromatograms were recorded using a 3380A computing integrator (Hewlett-Packard, Slough, U.K.).

Under the conditions described, carboxybupranolol had a retention time of 7 min and the internal standard a retention time of 5 min (Fig. 2).

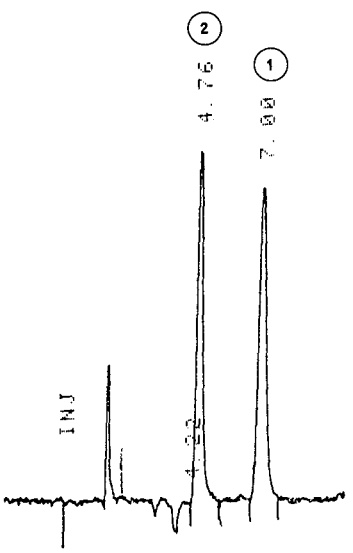

Fig. 2. Chromatogram of reference standards. Chromatographic conditions: column, 25 cm × 0.46 cm I.D., containing Zorbax C_8; mobile phase, 70% (v/v) methanol—aqueous potassium dihydrogen orthophosphate (0.1% w/v) containing sodium lauryl sulphate (1%, w/v), final pH adjusted to 3 with phosphoric acid; flow-rate, 2 ml/min; detector, fluorescence, excitation wavelength 245 nm, emission wavelength 330 nm. Peaks: 1 = carboxybupranolol; 2 = internal standard.

Calibration procedure

The calibration line was constructed from peak area ratio measurements of carboxybupranolol to internal standard against concentration over the concentration range 45—623 ng/ml.

Samples of blank plasma (0.5 ml) were spiked with amounts of carboxy-

bupranolol hydrochloride of 25, 50, 100, 150, 200, 250 and 350 ng per 0.5-ml sample; this was equivalent to concentrations of 45, 89, 178, 267, 356, 445 and 635 ng carboxybupranolol free base per ml. Internal standard (as the hydrochloride) was added into samples at a fixed concentration of 600 ng/ml (300 ng per 0.5 ml). The samples were taken through the extraction procedure described previously.

Studies in humans

Four human subjects were dosed with capsules containing bupranolol (300 mg as the hydrochloride salt), together with 100 ml water, at 1 h following a standardised breakfast. The study was conducted under conditions similar to those previously described [8].

Blood samples were withdrawn by venepuncture into heparinised tubes before dosing and at 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6, 8 and 10 h after dosing. The blood cells were separated by centrifugation and discarded; plasma was stored at −20° C until analysis by the method described.

RESULTS AND DISCUSSION

Precision

Extraction and measurement at each concentration were repeated on six occasions at each point over the calibration range. The precision of the method for the measurement of carboxybupranolol in plasma was indicated by the coefficients of variation of peak area ratios (Table I) which were 21% at 45 ng/ml, 6% at 267 ng/ml and 5% at 623 ng/ml.

TABLE I

BETWEEN-ASSAY PRECISION MEASUREMENTS OF CARBOXYBUPRANOLOL IN PLASMA

Concentration of carboxybupranolol (ng/ml)	Peak area ratio $\left(\frac{\text{carboxybupranolol}}{\text{internal standard}}\right)$						Mean (± S.D.)	Coefficient of variation (%)
45	0.12	0.12	0.18	0.16	0.14	0.12	0.14 (0.03)	21
89	0.30	0.32	0.23	0.36	0.26	0.21	0.28 (0.06)	21
178	0.47	0.54	0.47	0.52	0.55	0.44	0.50 (0.04)	8
267	0.83	0.71	0.81	0.79	0.85	0.82	0.80 (0.05)	6
356	1.02	1.03	1.10	0.96	1.13	1.05	1.05 (0.06)	6
445	1.16	1.19	1.26	1.15	1.15	1.17	1.18 (0.04)	3
623	1.69	1.81	1.87	1.69	1.70	1.71	1.75 (0.08)	5

Accuracy

The calibration line for the measurement of carboxybupranolol in plasma was constructed over the range 45—623 ng/ml; six replicate extractions were made at each concentration over the range. The plot of peak area ratio against concentration was linear ($Y = a + bX$, where $a = 0.031565$ and $b = 0.002732$) where Y is the peak area ratio and X is the concentration of carboxybupranolol free base (ng/ml). The accuracy of the method as indicated by the standard

error of the fitted least-squares regression line, i.e. using the calibration line to estimate the concentrations of carboxybupranolol in plasma, was ± 23 ng/ml.

Recovery

The recovery (extraction efficiency) of internal standard (600 ng/ml) from plasma (0.5 ml) was determined by comparison of peak area ratio measurements of internal standard to carboxybupranolol of standards taken through the extraction procedure, to those injected into the chromatograph without extraction. The mean recovery of internal standard was 79 ± 6% S.D. (n = 6).

The mean recovery of carboxybupranolol from plasma was determined by

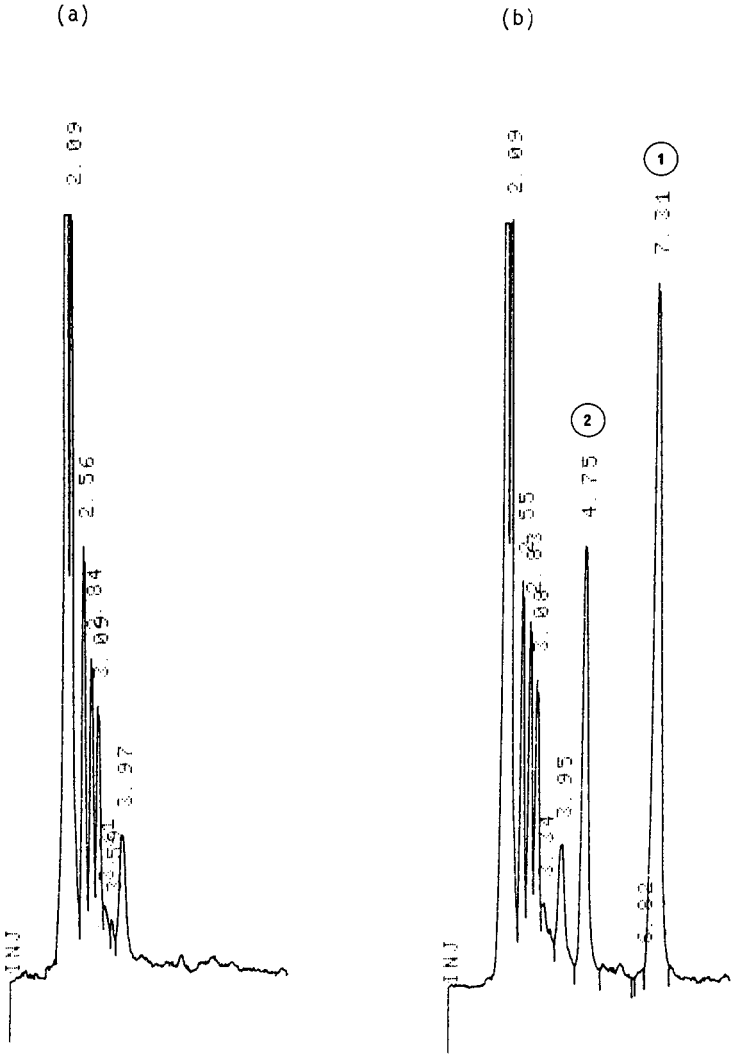

Fig. 3. Chromatograms of (a) pre-dose control plasma and (b) 1-h post-dose plasma containing carboxybupranolol at a concentration of 768 ng/ml. Experimental conditions as for Fig. 2. Peaks: 1 = carboxybupranolol, 2 = internal standard.

comparison of peak area ratios of extracted standards, corrected for 100% recovery of internal standard, to those of non-extracted standards. The mean recovery of carboxybupranolol from plasma over the concentration range 45—356 ng/ml did not differ significantly from 100%.

Stability of carboxybupranolol in plasma

The stability of carboxybupranolol in plasma under the storage conditions used (—20°C) was tested by storing plasma standards at a concentration of 267 ng/ml for nineteen days. Recovery of carboxybupranolol from the stored samples was 95 ± 5% S.D. (n = 6).

Limits of detection

No interfering peaks with retention times similar to either carboxybupranolol or internal standard were present in predose (blank) plasma (Fig. 3). The limit of detection of carboxybupranolol based on the extraction of 0.5 ml plasma, was set by instrumental noise at 20 ng/ml. The reliable limit of accurate measurement based on integrator sensitivity was 45 ng/ml, the lowest datum point on the calibration line.

Selectivity of the analytical method

No peaks interfering with the analysis were present from any control plasma investigated. Samples chromatographed without internal standard showed no interference from metabolites with the same retention time as the internal standard. Bupranolol, desmethylbupranolol and hydroxymethylbupranolol (another less important metabolite) were only weakly fluorescent at the wavelengths employed and did not interfere with either carboxybupranolol or internal standard.

Concentrations of carboxybupranolol

The mean concentrations of carboxybupranolol in the plasma of four volunteers after single oral doses of 300 mg bupranolol (as the hydrochloride

TABLE II

MEAN (± S.D.) CONCENTRATIONS OF CARBOXYBUPRANOLOL (ng/ml) IN THE PLASMA OF FOUR HUMAN SUBJECTS AFTER SINGLE ORAL DOSES OF 300 mg OF BUPRANOLOL HYDROCHLORIDE

Time after dosing (h)	Concentration (± S.D.) (ng/ml)
0.5	236 (143)
0.75	1275 (612)
1	2273 (880)
1.5	4190 (1272)
2	3304 (852)
3	2174 (741)
4	747 (213)
5	308 (88)
6	165 (45)
8	54 (39)
10	<45 (—)

salt) reached a peak of 4190 ng/ml (Table II) at 1.5 h after dosing and thereafter declined to below the limit of accurate measurement at 10 h with a mean half-life of 1.3 h (± 0.33). These concentrations were associated with drug action since concurrent pharmacodynamic measurements indicated that there was a pharmacological effect (inhibition of response to exercise stress) in the healthy volunteers after administration of 300 mg bupranolol.

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Note

Determination of sodium 2-mercaptoethanesulphonate by high-performance liquid chromatography using post-column reaction colorimetry or electrochemical detection

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Sodium 2-mercaptoethanesulphonate (mesna) is a new drug which is used to ameliorate the urotoxicity associated with oxazaphosphorine (e.g. cyclophosphamide) chemotherapy [1]. The urotoxic oxazaphosphorine metabolites are detoxified by their reaction with the free sulphydryl group of mesna [2]. In order to ensure constant protection of the bladder and urinary tract, sufficient levels of free thiol must be maintained during the period when the toxic metabolites are excreted. Monitoring the urinary levels of free thiol is therefore important clinically.

Mesna in biological samples can be measured by the non-specific Ellman's free thiol assay [3, 4] which detects other thiols (endogenous thiols and thiol drugs) as well. A high-performance liquid chromatographic (HPLC) method with ultraviolet (UV) detection has also been reported [5]; however, its sensitivity is not sufficiently high to make it suitable for application to clinical samples.

The method described here allows the determination of mesna per se in pharmaceutical and biological samples. It is based on the separation of mesna from other thiols by ion-pair HPLC followed either by post-column derivatization and colorimetric detection (system A) or by electrochemical detection (system B).

MATERIALS AND METHODS

Chemicals

Mesna was provided by WB Pharmaceuticals (Bracknell, U.K.); other thiol

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compounds (L-cysteine, DL-homocysteine, glutathione, D-penicillamine) were purchased from Sigma (Poole, U.K.) and the ion-pairing reagents heptanesulphonic acid (HSA) and tetrabutylammonium phosphate (TBAP) from Magnus Scientific (Aylesbury, U.K.). All other chemicals were of analytical grade.

HPLC equipment

Two HPLC systems were used which consisted of the same basic set-up, but utilized different ion-pairing reagents and detection systems (Table I). They comprised a Constametric III solvent pump (Laboratory Data Control, Stone, U.K.) and a Rheodyne injection valve (Rheodyne, Berkeley, CA, U.S.A.) with a 50-μl injection loop. Separation was performed on a Hypersil ODS analytical column (25 cm × 4 mm I.D., particle size 5 μm).

TABLE I

DETAILS OF THE HPLC SYSTEMS USED FOR THE DETERMINATION OF MESNA

Both systems were used with conventional HPLC equipment (see Materials and methods).

	System A	System B
Mobile phase	Phosphate buffer (0.25 *M*, pH 7.4)—methanol (95:5)	Phosphate buffer (0.25 *M*, pH 7.4)
Ion-pairing reagent	TBAP (0.005 *M*)	HSA (0.005 *M*)
Detection system	Post-column detivatization with Ellman's reagent followed by colorimetric detection at 412 nm	Electrochemical detection with glassy carbon electrode, potential + 800 mV

System A. The mobile phase consisted of a mixture of aqueous phosphate buffer (0.25 *M*, pH 7.4) and methanol (95:5) containing 0.005 *M* TBAP as the ion-pairing reagent. The flow-rate was 1 ml/min. For the post-column reaction a stainless-steel column (20 cm × 4 mm I.D.) packed with glass beads (100—120 mesh, dichloromethylsilane-treated) was used [6]. The stock Ellman's reagent [7] for the post-column derivatization consisted of 5,5'-dithiobis(2-nitrobenzoic acid) (0.2%, w/v) and tripotassium citrate (10%, w/v) in phosphate buffer (0.25 *M*, pH 7.4); it was diluted 1:10 with water before use. The flowrate was 0.5 ml/min.

Absorbance was measured using a Varichrom variable-wavelength detector at 412 nm (Varian, Walton-on-Thames, U.K.).

System B. The mobile phase consisted of HSA (0.005 *M*) in aqueous phosphate buffer (0.25 *M*, pH 7.4) and was used at a flow-rate of 1 ml/min. The electrochemical detector (LCA 15, EDT Research, London, U.K.) with a glassy carbon electrode was used at a potential of + 800 mV [8].

Preparation of standard solutions and calibration graph

Standard solutions containing mesna, D-penicillamine, L-cysteine, DL-homocysteine and glutathione were made up in water or urine. EDTA (final concentration 0.1%, w/v) was added to prevent disulphide formation [9]. Calibration curves were prepared up to 3 μg of mesna (on column).

Preparation and analysis of urine samples

Urine samples were collected from patients receiving mesna (30 mg/kg body weight/dose intravenously; four doses were given each day at times 0, 3, 6 and 9 h after the cyclophosphamide dose) during cyclophosphamide therapy [10]. Control urine samples were obtained before the start of chemotherapy. Urine samples were preserved with EDTA (final concentration 0.1%, w/v) and analysed immediately.

A 50-μl volume of diluted urine (dilution range 1:3 to 1:39) was injected onto the column. The mesna concentrations were calculated using the calibration graphs.

RESULTS AND DISCUSSION

Mesna was readily detectable by both of the HPLC and detection systems described and gave a linear calibration graph up to 3.5 μg (on column) in system A and 7.4 μg (on column) in system B. Quantitative variation in system A was 15 ng at the 1-μg level and 0.8 ng at the 25-ng level in system B (n=4).

In system B with HSA as the ion-pairing reagent the retention time for mesna is relatively short since it probably does not form an ion-pair with HSA at this pH; however, a sufficient separation from other thiols (Table II) and endogenous compounds (see Fig. 2A) is achieved because a number of these compounds do form ion-pairs with HSA at pH 7.4. HSA possibly modifies the reversed-phase HPLC column giving it ion-exchange characteristics and this could be an important mechanism of separation of mesna from endogenous compounds. Since a number of chemical groups give an electrochemical response at + 800 mV (e.g. phenolic hydroxyl groups), this is important when clinical samples are analysed, where interference from drugs and their metabolites during multiple drug treatment could be a problem.

TABLE II

CAPACITY FACTORS (k') FOR SELECTED THIOL COMPOUNDS USING REVERSED-PHASE ION-PAIR HPLC AS DESCRIBED IN TABLE I

Thiol compound	System A	System B
L-Cysteine	0.1	0
DL-Homocysteine	0.1	0.3
D-Penicillamine	0.72	1.5
Glutathione	1.2	0.1
Mesna	5.0	0.2

Considerable interest in the application of electrochemical detection has resulted in numerous techniques for detection of phenolic hydroxyl groups (e.g. catecholamines); this study underlines the importance of this technique in the detection of thiol groups.

In system A the resolution is much greater (Table II) because mesna forms an ion-pair with TBAP and this results in discrete peaks for all thiol compounds tested apart from cysteine and homocysteine which are not separated under these conditions. The specific Ellman's assay only detects compounds containing free sulphydryl groups; therefore the number of possible inter-

fering substances is limited and no interfering peaks are present in the control urine samples analysed (Fig. 1A).

The elctrochemical detection has three-fold greater sensitivity when applied to urine samples (limit of detection 25 ng compared to 75 ng in system A); however, with minimum mesna levels in the urine in the order of 50 μg/ml during the first 24 h after mesna administration the sensitivity of the Ellman's assay is sufficient.

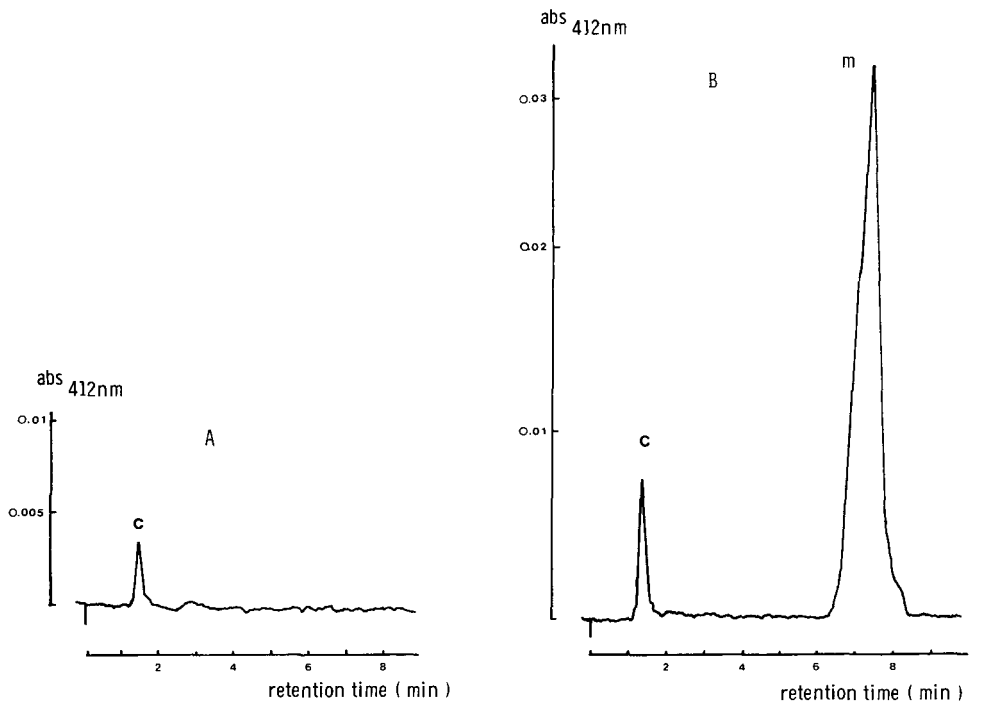

Fig. 1. Reversed-phase HPLC traces (system A) (A) of undiluted control urine showing a small peak for excreted cysteine (c), and (B) of diluted urine (1:5) from a patient receiving mesna during cyclophosphamide cancer chemotherapy. The trace shows enhanced excretion of cysteine (c) and excretion of mesna (m).

HPLC analysis of urine from patients receiving mesna (Figs. 1B and 2B) demonstrates enhanced cysteine excretion and underlines the necessity to use chromatographic separation prior to the colorimetric determination rather than the Ellman's free thiol assay alone (i.e. where total SH levels are measured) [4]. This results in artificially high "mesna" levels in urine from patients receiving mesna. The enhanced cysteine excretion during mesna administration is analogous to the treatment of cystinuria with the thiol drug penicillamine [11] and is presently being studied in our laboratory.

CONCLUSION

Both HPLC systems followed by either the post-column reaction or electrochemical detection techniques described are suitable for application to rapid

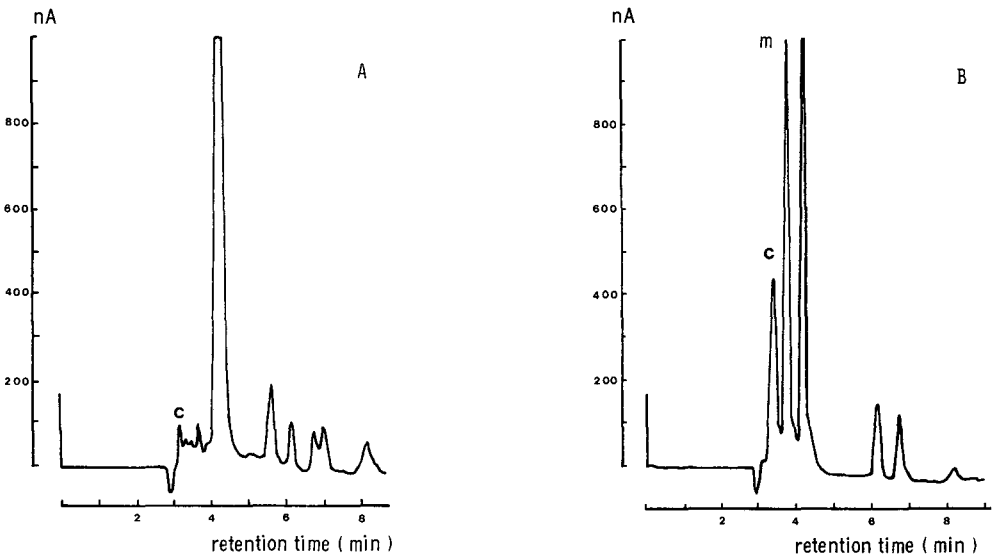

Fig. 2. Reversed-phase HPLC traces (system B) (A) of control urine showing a small peak for excreted cysteine (c), and (B) of urine from a patient receiving mesna during cyclophosphamide cancer chemotherapy. The trace shows enhanced excretion of cysteine (c) and excretion of mesna (m).

clinical assays, and may be used to monitor urinary mesna levels during oxazaphosphorine cancer chemotherapy using mesna uroprotection.

There is no reason why the ion-pairing reagents (TBAP and HSA) and the detection systems cannot be exchanged to produce the combination most suitable for a specific analytical problem.

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Journal of Chromatography	282	283 284/1	284/2 285/1	285/2 285/3 286 287/1	287/2 288/1 288/2 289	290 291 292/1	292/2 293 294	295/1 295/2 296	297 298/1 298/2 298/3	299/1 299/2 301/1	301/2 302 303/1	303/2
Chromatographic Reviews		300/1					300/2					300/3
Bibliography Section		304/1	304/2			304/3					304/4	304/5
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