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REVIEW

HIGH-PERFORMANCE LIQUID COLUMN CHROMATOGRAPHY OF NUCLEOTIDES, NUCLEOSIDES AND BASES

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CONTENTS

1. INTRODUCTION

Nucleotides, nucleosides and bases are essential constituents of nucleic acids and enzyme cofactors required for the proper functioning of cells, tissues and organs. The importance of nucleotides, nucleosides and bases is demonstrated by the severe symptoms which result from defects in purine or pyrimidine metabolism, such as mental retardation, cardiovascular diseases, renal failure, gout and toxemia [1].

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The development of high-performance liquid chromatography (HPLC) has facilitated the isolation and quantification of the nucleic acid constituents in biological fluids and tissues; separations which previously required several hours by open-column methods can be achieved rapidly using HPLC. With the on-line detection systems, the characterization as well as the accurate quantification of the solutes of interest can be accomplished.

The three major HPLC modes used in the analysis of the nucleotides, nucleosides and bases are: ion-exchange, either cation or anion; reversed-phase and ion-pairing. In cation exchange, the stationary phase contains fixed anionic sites. These interact electrostatically with cationic solutes which are thus retained. Conversely, in anion exchange, the fixed sites are positively charged and anions are retained [2]. In the reversed-phase mode which is used for the separation of non-polar and slightly polar compounds, hydrophobic interactions determine the extent of retention [3, 4]. The more polar or ionic solutes, which favor the aqueous eluent, elute faster in reversed-phase HPLC. In a technique known as ion-pairing chromatography, compounds are added to the mobile phase which contain both a lipophilic moiety that can interact with the non-polar reversed-phase stationary phase and an ionic moiety that can pair with ionic compounds of an opposite charge. Thus, greater retention of charged solutes can be achieved on reversed-phase systems [5].

Nucleosides as bases are relatively weak bases (low pK_{ab} values) and weak acids (high pK_{aa} values) (Table 1) [6]. Since these compounds are positively

TABLE 1

pK_{ab} AND pK_{aa} VALUES OF THE BASES AND NUCLEOSIDES

Data taken from ref. 6. Values are mentioned only for the first gain (pK_{ab}) or loss (pK_{aa}) of a proton.

	pK_{ab}	pK_{aa}
Bases		
Adenine (Ade)	4.15	9.8
Guanine (Gua)	3.2	9.6
Hypoxanthine (Hyp)	2.0	8.9
Xanthine (Xan)	0.8	7.5
Cytosine (Cyt)	4.45	12.2
Uracil (Ura)	−3.4*	9.5
Thymine (Thy)	− *	9.9
Nucleosides		
Adenosine (Ado)	3.5	12.5
Guanosine (Guo)	1.6	9.2
Inosine (Ino)	1.2	8.8
Xanthosine (Xao)	<2.5	5.7
Cytidine (Cyd)	4.15	12.5
Uridine (Urd)	− *	9.2
Thymidine (Thd)	− *	9.8

*Extremely low pH needed for the protonation of the species. Since both nitrogens in uracil and thymine are involved in amide tautomerism, very little basic strength remains (basic N not involved in tautomerism).

charged below their pK_{ab}, they can be separated by cation exchange at these pH values. Since they are neutral between the pK_{ab} and pK_{aa}, they can be analyzed by reversed phase. Above their pK_{aa}, they are negatively charged so they can readily be chromatographed on anion-exchange columns.

On the other hand, the nucleotides are strong acids. At a pH of 2.0, the monophosphates have one negative charge on their phosphate moiety, the diphosphates two, and the triphosphates three. At a pH of 7.0 and above, the nucleotides gain an additional negative charge due to the secondary phosphate dissociation. Thus, these compounds naturally lend themselves to separations on anion exchangers. Recently, attempts have been made to separate the nucleotides by ion-pair reversed-phase chromatography due to the adaptability of these stationary phases to rapid solvent changes.

We will briefly review the steps required for the determination of nucleotide, nucleoside and base levels in biological samples, present some of the chromatographic separations achieved with each of the different modes, and illustrate applications of these separations in the clinico-biochemical field.

2. ANALYSIS OF NUCLEOTIDES, NUCLEOSIDES AND BASES

A. Sample preparation

a. Extraction from cells. In order to study the free nucleotide, nucleoside or base content of a certain volume or number of cells, it is important to extract those compounds into a liquid medium. Desirable reagents for extraction procedures are those that can: (1) lyse the cell; (2) precipitate the protein (to stop the enzymatic degradation of the nucleotides as well as prevent the clogging of the chromatographic column); (3) give the best recovery of the compounds of interest; and (4) provide a neutral environment for the storage of those compounds.

Perchloric acid is commonly used to extract the nucleotides from biological cells [7—13]. Normally, the resulting acidic supernatant is neutralized with potassium hydroxide [8—12] or an amine—Freon^{R} solution [13, 14]. When only the deoxyribonucleotides are of interest, the neutralized extract can be treated with periodate—methylamine. This will ensure the removal of the ribonucleotides from the sample [9].

b. Extraction from biological fluids. Protein removal is probably the most important step in the analysis of nucleosides and bases in biological matrices. Conventionally, perchloric acid or trichloroacetic acid have been used. However, recently, serum [15—17], plasma [18] and urine [15, 16] samples have been ultrafiltered through membrane cones which can retain high-molecular-weight proteins. This method is preferred since it does not alter the pH of the medium, dilute the sample or interfere with the UV absorbance of sample constituents.

Gehrke et al. [19] developed a novel extraction procedure for the analysis of ribonucleosides in urine. The samples are passed through a boronate gel column. The ribonucleosides are retained on the column as *cis*-diol boronate complexes and subsequently eluted with 0.1 *M* formic acid.

B. Chromatography

Today, most chromatographic systems comprise a solvent delivery system, a gradient programmer, an injector, a column, several detector devices, one or more recorders and an integrator.

Within a chromatographic mode, the optimization of a given separation usually requires modification of the mobile phase rather than the stationary phase. The pH of the eluent plays a major role in determining the extent of dissociation of a solute [20, 21]. Charged compounds have classically been resolved by ion exchange. The larger the negative charge on a solute, the greater the retention on an anion exchanger. Elution of highly charged compounds requires eluents of high ionic strength, hence the use of ionic strength gradients in nucleotide separations.

On the other hand, non-polar compounds are retained on hydrophobic reversed-phase stationary phases. Thus, the nucleosides and bases, predominantly neutral between their pK_{ab} and pH 7 (limit of the stationary phase), lend themselves to separations by reversed-phase liquid chromatography. Gradients are often used to decrease the polarity of the system and speed up the elution of those compounds which are retained the longest.

In the ion-pair mode used for the separation of nucleotides on reversed-phase columns, the pH of the mobile phase, percentage organic modifier, and especially the nature and concentration of the pairing agent need to be considered to achieve the proper resolution of the solutes of interest [22].

C. Characterization of chromatographic eluates

This step is extremely important in the HPLC analysis of biological mixtures. Initially, compounds are identified by retention times and co-chromatography with the reference compounds. In addition, absorbance ratios at various wavelengths [23], stopped-flow UV spectra [24] or fluorimetric selectivity [25, 26] may be determined. A scintillation flow-monitor may be used for the detection of radioactive compounds [11]. Chemical tests, such as sample treatment with periodate, are used to identify *cis*-diolic compounds such as ribonucleosides and ribonucleotides [25]. Specific enzymatic tests [27, 28] can also be performed (Table 2) and the decrease in the peak area of the substrate and/or increase in the area of the product can be determined.

In the future, when the problems of interfacing liquid chromatography with mass spectrometry are resolved, the tandem operation of these two techniques will become one of the most powerful tools for the combined separation and identification of eluates.

D. Quantification

After the separation and identification of chromatographic eluates, their quantification can be achieved. One must first determine the response factor R (moles/area) of the reference compounds. This is essentially the slope of the calibration curve, in which known amounts of a compound, injected from solutions of different concentrations, are plotted against the peak area obtained. Using the external calibration method, one can write

$$\text{Amount of X in sample} = (\text{area})_X \cdot R_{ES} \cdot \text{dilution factor} \qquad (1)$$

TABLE 2

EXAMPLES OF ENZYMATIC TESTS PERFORMED IN THE IDENTIFICATION OF NUCLEOTIDES, NUCLEOSIDES AND BASES

Enzyme	Substrate	Product
Assays for specific group of substrates		
5'-Nucleotidase	Nucleoside monophosphate	Nucleoside
Alkaline and acid phosphatase	Nucleotide	Nucleoside
Purine nucleoside phosphorylase	Nucleoside + P_i	Base
Assays for specific substrate(s)		
Xanthine oxidase	(1) Hypoxanthine (2) Xanthine	Xanthine Uric acid
Adenosine deaminase	Adenosine	Inosine
Adenosine kinase	Adenosine	Adenosine monophosphate
Guanase	Guanine	Xanthine

where X and ES refer to the unknown and external standard, respectively.

The internal calibration method is often more desirable when variable recovery of compounds from biological matrices is involved. A known amount of internal standard is added to the sample prior to the extraction step. Thus

$$\text{Amount of X in sample} = \frac{(\text{area})_X \cdot R_X}{(\text{area})_{IS} \cdot R_{IS}} \cdot (\text{amount})_{IS} \cdot \text{dilution factor} \qquad (2)$$

where X and IS refer to the unknown and internal standard, respectively.

The major requirement for this method is that all compounds quantified have the same recovery as the internal standard itself — a condition often hard to achieve.

3. SELECTED CHROMATOGRAPHIC SEPARATIONS

A. Nucleotides

a. Ion exchange. The pioneering studies of Cohn [29], who separated nucleic acid components by ion-exchange chromatography, of Anderson [30], who developed an automated effluent monitoring device, and of Kirkland and Felton [31, 32] and Horváth and co-workers [33, 34], who investigated the parameters which affect column efficiency, marked the beginning of a new era in the science of chromatography. The introduction, in the late sixties, of stainless-steel columns packed with pellicular material (ion-exchange resins coated on 50-μm glass spheres) capable of withstanding pressures of up to 27 MPa (4000 p.s.i.), combined with the use of pumps to force the mobile phase through the chromatographic column, made possible the rapid separation of complex mixtures [33].

The ion-exchange mode of HPLC gained considerable importance for the

separation of nucleotides in investigations of the composition and structure of the nucleic acids. Since the nucleotides also regulate various cellular functions, the quantification of the free nucleotide concentrations in cells was essential in biomedical research. The early work of Horváth et al. [33], who achieved the separation of mono-, di- and triphosphate ribonucleotides in 90 min on pellicular anion-exchange resins illustrated the power and great potential of the new HPLC technique. Prior to that, the same separation on conventional open columns required a minimum of 20 h [33]. During this long period of time, several compounds could decompose.

The development of microparticulate packings, in 1973, set another milestone in the history of chromatography. The efficiency of the column was increased while sufficient sample loading capacity was maintained. Hartwick and Brown [35] achieved excellent resolution of the mono-, di- and triphosphate 5'-nucleotides of adenine, guanine, hypoxanthine, xanthine, cytosine, uracil and thymine, using a microparticulate, chemically bonded strong anion exchanger (Partisil 10-SAX, 10 μm). The low-strength eluent consisted of 0.007 *F* KH_2PO_4 (pH 4.0) and the high-strength eluent of 0.25 *F* KH_2PO_4, 0.25 *F* KCl (pH 4.5). The conditions of analysis were: a 15-min elution with the low-concentration buffer followed by a 45-min linear gradient to 100% of the high-strength buffer at a flow-rate of 1.5 ml/min (Fig. 1).

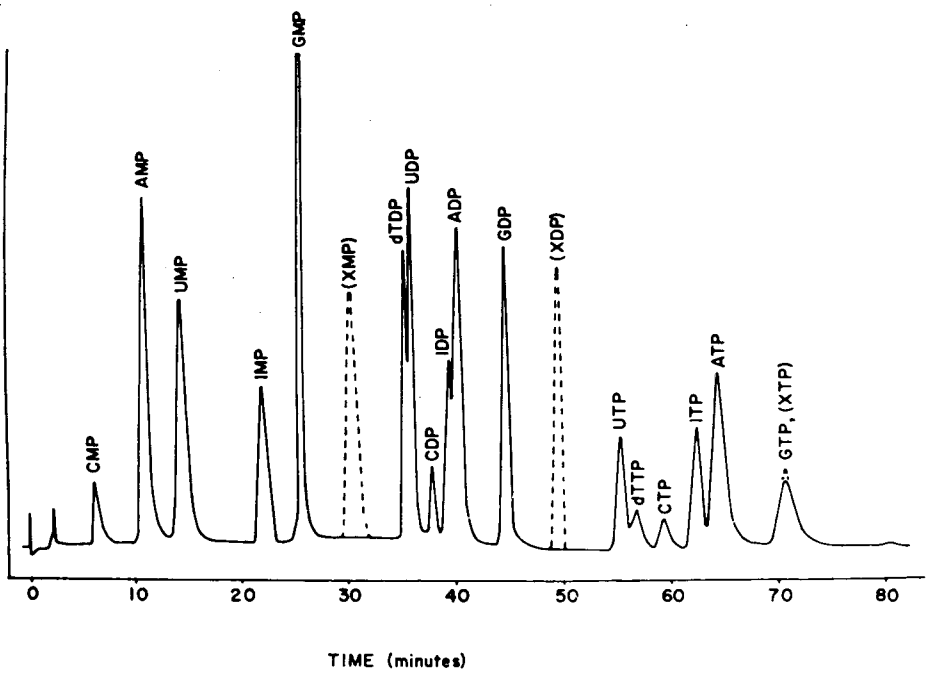

Fig. 1. Separation of mono-, di- and triphosphate nucleotides of adenine, guanine, hypoxanthine, xanthine, cytosine, uracil and thymine. Column, Partisil 10-SAX; temperature, ambient; detector sensitivity, 0.08 a.u.f.s. Eluents: (low 0.007 *F* KH_2PO_4, pH 4.0; (high) 0.25 *F* KH_2PO_4, 0.50 *F* KCl, pH 4.5. Gradient, linear, 0—100% of high-concentration eluent in 45 min; flow-rate, 1.5 ml/min. Dashed lines indicate elution positions of XMP, XDP and XTP. (Ref. 35.)

More recently, the 2′-, 3′-, 5′-ribonucleotides and cyclic ribonucleoside monophosphates of cytosine, adenine and guanine [7], the ribo- and deoxyribonucleoside triphosphates [8], as well as mono- and diphosphates [13] have been resolved using ion exchange. Floridi et al. [12] succeeded in analyzing simultaneously a mixture of bases, ribonucleosides and ribonucleotide mono-, di- and triphosphates. Similar separations were later achieved by Bakay et al. [11] and Nissinen [36]. The chromatographic conditions for these analyses are listed in Table 3.

b. Reversed phase. The use of the ion-exchange mode for the separation of the nucleotides had several disadvantages. Most analyses required one or two hours. The use of buffer gradients resulted in considerable baseline drift. An appreciable amount of time was needed to equilibrate the column at initial conditions after a chromatographic run. The separations were not always reproducible [37]. On the other hand, the development by Halász and Sebastian [38] of chemically bonded, microparticulate reversed-phase packing materials resulted in enhanced stability of stationary phases. In addition, equilibration times were very short and the chromatograms obtained extremely reproducible [28].

Since the nucleotides are negatively charged, their retention on reversed-phase systems seemed, a priori, impossible. However, several researchers attempted to separate, if not all compounds at once, a selected group of nucleotides with similar characteristics.

Using a low pH buffer, Horváth et al. [21] resolved some ribonucleoside monophosphates on a Partisil 10/25 ODS-2 column (Whatman). Krstulović et al. [39] separated the cyclic nucleoside monophosphates on a μBondapak C_{18} column (Fig. 2). Finally, Schweinsberg and Loo [40] were able to resolve ATP, ADP and AMP from other nucleotides, nucleosides and bases. Since adenine nucleotides predominate in erythrocytes, the method can be applied to the study of their levels in lysates with no interference from other red blood cell constituents. Chromatographic conditions for these analyses are given in Table 4.

c. Ion-pairing. The reversed-phase mode had obvious limitations. Although rapid separations of certain types of compounds were achieved, all ribonucleoside mono-, di- and triphosphates could not be resolved simultaneously as many of these compounds were not retained on the column. The addition to the mobile phase of molecules with a hydrophobic moiety that could adsorb on to the stationary phase and an ionic group that could pair with the negatively charged nucleotide, resulted in the greater retention of nucleotides on reversed-phase columns. This chromatographic technique was termed ion-pairing.

In 1977, Hoffman and Liao [41] succeeded in separating the ribonucleoside mono-, di- and triphosphates of cytosine, uracil, guanine and adenine in 35 min on Spherisorb ODS (10 μm, Spectra-Physics; re-coated to 13.9% by weight carbon loading). Gradient elution was used and the mobile phase contained tetra-*n*-butylammonium hydrogen sulfate as the ion-pairing reagent.

Recently, shorter separations for these compounds have been achieved by Juengling and Kammermeier [37], and by Knox and Jurand [22], who used eluents with higher pH values and greater initial amounts of the organic modifier.

TABLE 3

CHROMATOGRAPHIC CONDITIONS FOR SELECTED NUCLEOTIDE ANALYSES USING ION EXCHANGE

Compounds* resolved	Stationary phase	Mobile phase	Temp. (°C)	Flow-rate (ml/min)	Analysis time (min)	Ref. No.
2′-, 3′-, 5′-nucleotides and cyclic ribonucleotides of Cyt, Ade and Gua	Micropak AX-10 (30 cm × 4 mm, 10 μm, Varian)	0.01 *M* KH_2PO_4 (pH 3.0)	Ambient	2.0	40	7
Ribo-monophosphates and deoxyribonucleoside triphosphates of Cyt, Ura, Ade and Gua	Two Partisil 10-SAX in series (25 cm × 4.6 mm, Whatman)	A: 0.2 *M* $NH_4H_2PO_4$ (pH 3.2) B: 0.6 *M* $NH_4H_2PO_4$ (pH 4.4) Linear 2-h gradient from A to B	40	1.0	105	8
Ribo- and deoxyribonucleoside mono-, di- and triphosphates	Aminex A-29 (30 cm × 4 mm, Bio-Rad)	A: 0.025 *M* sodium citrate (pH 8.2) B: 0.5 *M* sodium citrate (pH 8.2) 30 min A followed by 2-h linear gradient to B	50	0.3	220	13
Bases, nucleosides, nucleotides — mono-, di- and triphosphates	Aminex A-14 (20 ± 3 μm, filled in 50 × 0.6 cm thick-walled glass column, Bio-Rad)	A: 0.1 *M* 2-methyl-2-amino-1-propanol (MAP), 0.1 *M* NaCl (pH 9.9) B: 0.1 *M* MAP, 0.4 *M* NaCl (pH 10) Linear gradient by placing 200 ml of A in mixing chamber and 200 ml of B in reservoir	55	100 (ml/h)	225	12
Bases, nucleosides, nucleotides	Aminex A-25 (17.5 ± 2 μm, in a 50 × 0.2 cm column, Bio-Rad)	A: 0.08 *M* $Na_2B_4O_7$, 0.05 *M* NH_4Cl (pH 9.1) B: 0.01 *M* $Na_2B_4O_7$, 0.5 *M* NH_4Cl (pH 9.0) 45 ml of A in cylindrical mixing chamber and 45 ml of B in reservoir	60	0.5	125	11

*For abbreviations, see Table 1.

TABLE 4

CHROMATOGRAPHIC CONDITIONS FOR SELECTED NUCLEOTIDE ANALYSES USING REVERSED PHASE

Compounds* resolved	Stationary phase	Mobile phase	Temp.	Flow-rate (ml/min)	Analysis time (min)	Ref. No.
Cyt, Ura, Ade and Gua ribonucleoside monophosphates	Partisil 10/25 ODS-2 (Whatman)	Phosphate buffer (pH 2.2)	25°C	4.0	3	21
Cyclic nucleoside monophosphates of Cyt, Ura, Gua, Hyp and Ade	μBondapak C_{18} (Waters)	A: 0.02 *M* KH_2PO_4 (pH 3.7) B: methanol—water (3 : 2) Gradient linear from 0% to 25% B in 30 min	Ambient	1.5	25	39
ATP, ADP, AMP and other nucleotides, nucleosides and bases	μBondapak C_{18}	A: 0.06 *M* K_2HPO_4, 0.04 *M* KH_2PO_4 (pH 6.0) B: 3 × [0.08 *M* $K_2H PO_4$, 0.05 *M* KH_2PO_4 (pH 6.0)], 1 × methanol Gradient concave from A to B in 30 min	Ambient	1	35	40

*For abbreviations, see Table 1.

TABLE 5

CHROMATOGRAPHIC CONDITIONS FOR SELECTED NUCLEOTIDE ANALYSES USING ION-PAIRING

Compounds* resolved	Stationary phase	Mobile phase**	Temp.	Flow-rate (ml/min)	Analysis time (min)	Ref. No.
Ribonucleoside mono-, di- and triphosphates of Cyt, Ura, Gua and Ade	Spherisorb ODS (Spectra-Physics) recoated	A: 0.025 *M* TBHS, 0.05 *M* KH_2PO_4, 0.08 *M* NH_4Cl (pH 3.9) B: 0.025 *M* TBHS, 0.1 *M* KH_2PO_4, 0.2 *M* NH_4Cl (pH 3.4) + 30% methanol 40-min concave gradient	–	1	35	41
Ribonucleoside mono-, di- and triphosphates of Ura, Gua and Ade	LiChrosorb RP-8 (Merck)	Acetonitrile—water (24:86), 0.65% KH_2PO_4, 0.3% TBAP (pH 5.8) Isocratic	–	2	8	37
Ribonucleoside mono-, di- and triphosphates of Gua and Ade	ODS-Hypersil (Shandon Southern Products)	Methanol—water (12:88), 75 m*M* phosphate, 1.25 m*M* C11AA (pH 5.65) Isocratic	25°C	–	15	22
Ribonucleoside mono-, di- and triphosphates of Ura, Gua and Ade	$Co(en_3)^{3+}$ bonded to silica	0.037 *M* $Na_2HPO_4 \cdot 7 H_2O$ with 1 m*M* $MgSO_4 \cdot 7 H_2O$ (pH 6.4)	–	1	15	42

*For abbreviations, see Table 1.

**TBHS = tetra-*n*-butylammonium hydrogen sulfate; TBAP = tetrabutylammonium phosphate; C11AA = aminoundecanoic acid.

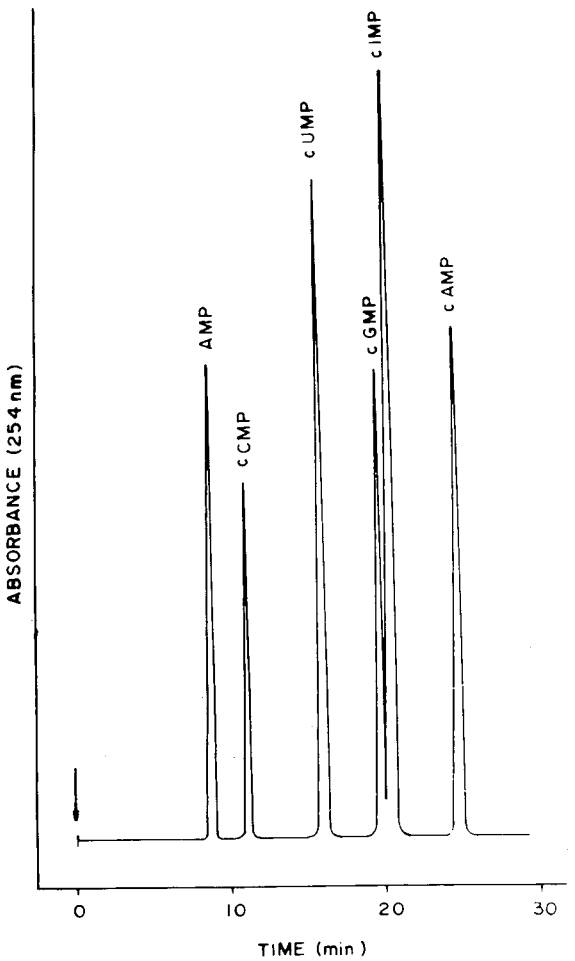

Fig. 2. Separation of reference compounds detected at 254 nm. AMP, adenosine 5′-monophosphate; cCMP, cytidine 3′, 5′-cyclic phosphate; cUMP, uridine 3′,5′-cyclic phosphate; cGMP, guanosine 3′,5′-cyclic phosphate; cIMP, inosine 3′,5′-cyclic phosphate. Concentration, approx. 10 nmol each. Chromatographic conditions: column, μBondapak C_{18}; low-concentration eluent, 20 mmol/l KH_2PO_4 (pH 3.7); high-concentration eluent, methanol—water (3:2, v/v); gradient, linear from 0% to 25% of the high-concentration eluent in 30 min; flow-rate, 1.5 ml/min; temperature, ambient; attenuation, 0.1 A full-scale. (Ref. 39.)

Whereas these separations were achieved on reversed-phase columns with C_{18} or C_8 chains bonded to the silica backbone, Chow and Grushka [42] resolved a nucleotide mixture on a column in which a $Co(en_3)^{3+}$ moiety had been bonded to silica. The $Co(en_3)^{3+}$ formed outer-sphere complexes with the anionic nucleotides. In this separation, Mg(II) ions were used in the mobile phase since they form inner-sphere complexes with the nucleotides and thus, compete with the stationary phase for these solutes. The mechanism of retention involved, therefore, a special type of ion-pairing.

The chromatographic conditions for the ion-pair separations discussed here are given in Table 5.

TABLE 6

CHROMATOGRAPHIC CONDITIONS FOR SELECTED NUCLEOSIDE AND BASE ANALYSES USING ION EXCHANGE

Compounds* resolved	Stationary phase**	Mobile phase	Temp. (°C)	Flow-rate	Analysis time (min)	Ref. No.
Ura, Cyt, Ade, Gua, $2Me_2G$, 2MeG, 7MeG, 5MeC, 1MeA	CE: Durrum DC-1A (14 ± 2 μm, Durrum)	0.1 *M* ammonium formate (pH 4.3)	55	0.41 ml/min for 50 min then 0.79 ml/min	2 h	45
Ribo- + deoxyribonucleosides (major and rare)	CE: M-71 (10—12 μm, Beckman, Munich, G.F.R.)	0.4 *M* ammonium formate (pH 4.6)	40	7.2 ml/h	180	46
Bases and ribonucleosides	AE: Aminex A-28 (8—12 μm, Bio-Rad)	$5 \cdot 10^{-3}$ *M* citrate, $5 \cdot 10^{-2}$ *M* phosphate (pH 9.25), 55% ethanol	70	—	30	47
Bases and deoxyribonucleosides	AE: OSTION LGAT 0800 (10—12 μm, Union of Chemical and Metallurgic Manufacture, Czechoslovakia)	0.005 *M* ammonium formate (pH 4.5)	50	2 ml/h	100	48

*$2Me_2G$ = N^2,N^2-dimethylguanine; 2MeG = N^2-methylguanine; 7MeG = 7-methylguanine; 5MeC = 5-methylcytosine; 1MeA = 1-methyladenine. For other abbreviations, see Table 1.

**CE = cation exchange; AE = anion exchange.

TABLE 7

CHROMATOGRAPHIC CONDITIONS FOR SELECTED NUCLEOSIDE AND BASE ANALYSES USING REVERSED PHASE

Compounds* resolved	Stationary phase	Mobile phase	Temp.	Flow-rate (ml/min)	Analysis time (min)	Ref. No.
Bases, methylated and non-methylated nucleosides	μBondapak C_{18}	A: 0.02 *M* KH_2PO_4 (pH 5.6) B: methanol—water (3 : 2) Gradient linear from A to B in 87 min	Ambient	1.5	30	49
Methylated and non-methylated nucleosides	μBondapak C_{18}/Porasil	0.01 *M* $NH_4H_2PO_4$ (pH 5.0), 1% methanol	24°C	1.0	30	19
Hyp, Thy, Thd, oxypurinol, allopurinol	μBondapak C_{18}	0.025 *M* ammonium acetate (pH 5.0)	23.5°C	2.0	25	51
Ado, dAdo	μBondapak C_{18}	0.05 *M* KH_2PO_4, 10% methanol (pH 4.5)	Ambient	2.0	10	52
Ado from other nucleotides and nucleosides	μBondapak C_{18}	0.007 *F* KH_2PO_4 (pH 5.8), 10% methanol	Ambient	2.0	7	53

*For abbreviations, see Table 1.

B. Nucleosides and bases

a. Ion exchange. Ion exchange, the classical mode for the chromatography of the nucleotides, was used initially by Uziel et al. [43] and Singhal and Cohn [44] for the separation of nucleosides and bases. In 1969, Horváth et al. [34] reported the rapid separation of those compounds on pellicular cation-exchange columns. Prior to that, attempts to utilize gas chromatography for the same separations were not entirely successful. Although thin-layer and paper chromatography were widely used, quantitative analyses at the subnanomole level [34] could not be achieved.

Recently, major and rare tRNA bases [45] as well as ribo- and deoxyribonucleosides [46], have been separated by cation exchange. On the other hand, mixtures of bases and ribonucleosides [47] or deoxyribonucleosides [48] have been resolved on anion-exchange columns. As can be seen in Table 6, these analyses require the use of low flow-rates and elevated temperatures and the time needed for the separation is rather long.

b. Reversed phase. Reversed phase is at present the most commonly used liquid chromatographic mode for the separation of nucleosides and bases. The combination of microparticulate, chemically bonded packings with proper mobile phase selectivity has resulted in extremely efficient systems, capable of rapidly resolving many compounds.

Hartwick and co-workers [28, 49], in their study of UV-absorbing serum constituents, were the first to establish the chromatographic conditions necessary for the separation of bases as well as major and modified nucleosides (Fig. 3). Gehrke et al. [19] devised a separation for the analysis of ribonucleosides in urine. These analyses were especially important in view of the potential role of the methylated nucleosides as clinical markers for cancer [19, 50].

Several other assays were developed for the quantification of plasma levels of hypoxanthine, thymine, oxypurinol, thymidine [51], adenosine and deoxyadenosine [52] or serum levels of adenosine [53]. As can be seen from Table 7, these separations are fairly rapid and can be achieved at ambient temperature.

c. Ion-pairing. Since the nucleosides and bases can readily be separated by reversed phase, very few analyses have required the ion-pair mode [16, 54]. One such example is the ion-pair separation of five bases, by Ehrlich and Ehrlich [54]. Two of these, namely cytosine and 5-methylcytosine, which eluted close to the void volume, could not be resolved by reversed phases. Using LiChrosorb RP-18 (10 μm, Rheodyne) and an eluent of 5 n*M* heptanesulfonate in 2.5 n*M* potassium phosphate at pH 5.6 (25°C, flow-rate 2.0 ml/l), both bases were retained and separated from each other as well as from uracil, guanine and thymine. The analysis required only 7 min.

4. SELECTED CLINICO-BIOCHEMICAL APPLICATIONS

A. Cancer research and chemotherapy

a. Basic cancer research. Changes in the levels of the nucleosides and bases have been observed in the physiological fluids of leukemic and other cancer patients [25, 55]. In addition, changes in the levels of the methylated purines and pyrimidines may be noticeable, due to the enhanced activity of tRNA methyltransferase in certain neoplasms [19].

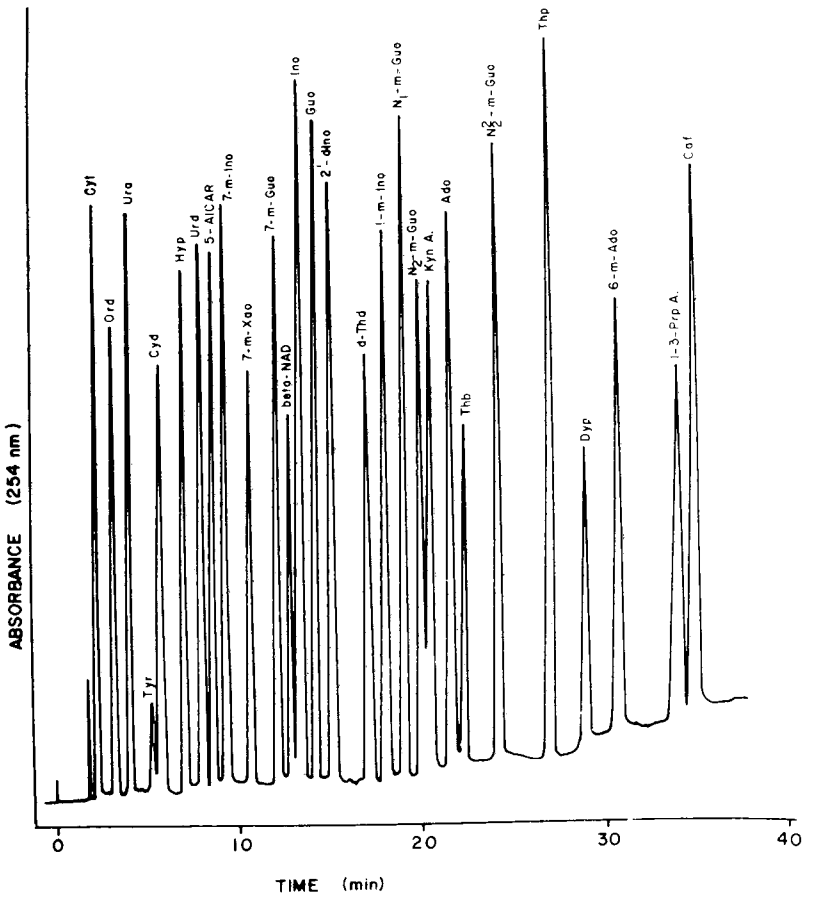

Fig. 3. Separation of 0.1—0.5 nmol of 28 nucleosides, bases, nucleotides, aromatic amino acids and metabolites. Injection volume: 40 μl of a solution $1 \cdot 10^{-5}$ mol/l in each standard. Column: chemically bonded reversed-phase (C_{18}) on 10-μm totally porous silica support. Eluents: low-strength, 0.02 mol/l KH_2PO_4 (pH 4.5); high-strength, 60% methanol. Gradient: slope 0.69%/min (0—60% methanol in 87 min), linear. Temperature, ambient; flow-rate, 1.5 ml/min. (Ref. 49.)

Gehrke et al. [19] observed elevations of 1-methylinosine, adenosine and N^2,N^2-dimethylguanosine in the urine of leukemic and breast cancer patients. In addition, the breast cancer urine chromatograms exhibited increased levels of N^2-acetylcytidine. Krstulović et al. [50] also noted the presence of 1-methylinosine and N^2-methylguanosine in the serum of some individuals with breast cancer, whereas these compounds could not be detected in the serum of normal controls (Fig. 4).

The occurrence of 7-methylguanine and O^6-methylguanine in the DNA of animals treated with carcinogens prompted Herron and Shank [56] to devise a fast chromatographic analysis for these two methylated purine bases. The assay was accomplished in 10 min on a strong cation-exchange (Partisil-10 SCX) column with an ammonium phosphate buffer eluent at pH 2.0. Both

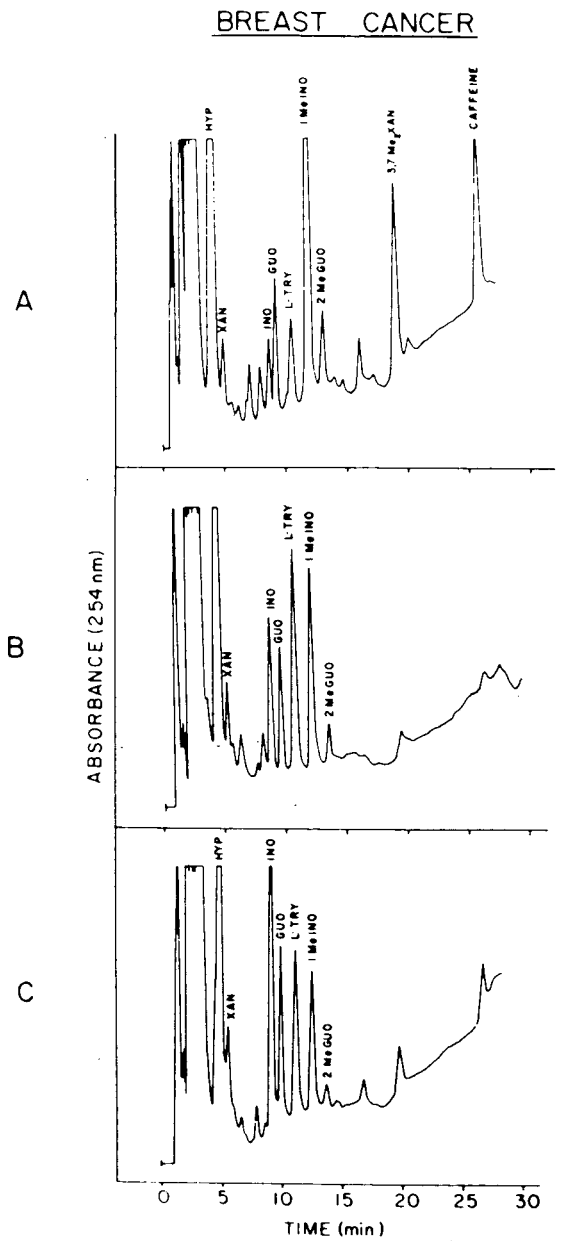

Fig. 4. (A) Chromatogram of a serum sample taken post-operatively from a non-fasting patient with breast cancer and metastasis to the bone and lymph nodes. The patient was treated by radiation and chemotherapy. (B) Chromatogram of a serum sample taken post-operatively from a non-fasting patient with breast cancer and metastasis to the bone. The patient was treated by radiation and chemotherapy. (C) Chromatogram of a serum sample taken post-operatively from a fasting patient with breast cancer with metastasis to the lung and bone. The patient was treated by radiation and chemotherapy. Chromatographic conditions for A, B and C same as in Fig. 3. (Ref. 50.)

methylated compounds can be seen in the liver DNA hydrolysate of a rat exposed to 25 μg/kg dimethylnitrosamine or the colon DNA hydrolysate of a rat treated with 163 mg/kg 1,2-dimethylhydrazine.

b. Cancer chemotherapy. In order to study the mode of action of the drugs administered during chemotherapy, assays for the drugs, their metabolites and the naturally occurring constituents in blood were needed. Brown [27], and later Scholar et al. [57], studied the effects of 6-mercaptopurine and 6 methylmercaptopurine ribonucleoside (MMPR) on Sarcoma 180 cells. A marked decrease in the adenine and guanine nucleotide pools was observed, reflecting the inhibition of their formation from IMP (Fig. 5).

In a study by Cohen et al. [13], chromatography of S-49 or murine lymphoma L5178Y cell extracts revealed that the concentrations of GTP and dGTP decreased whereas IMP increased after incubation with mycophenolic acid. These changes were attributed to the inhibition of inosinate dehydrogenase by mycophenolic acid.

Plunkett et al. [10] observed the accumulation of deazaUTP in a chromatographed extract of a brain tumor excised after intravenous infusion of deazauridine. Since other ribonucleotides were simultaneously resolved, fluctuations in the cellular concentrations of CTP could be determined and used as a sensitive indicator of the inhibitory action of deazaUTP.

Gelijkens and De Leenheer [58] optimized the separation of 5-fluorouracil (5-FU) from its deoxyribo- and ribonucleosides and nucleotides. With this new assay, the antineoplastic agent 5-FU and its metabolites in tissue extracts can readily be monitored.

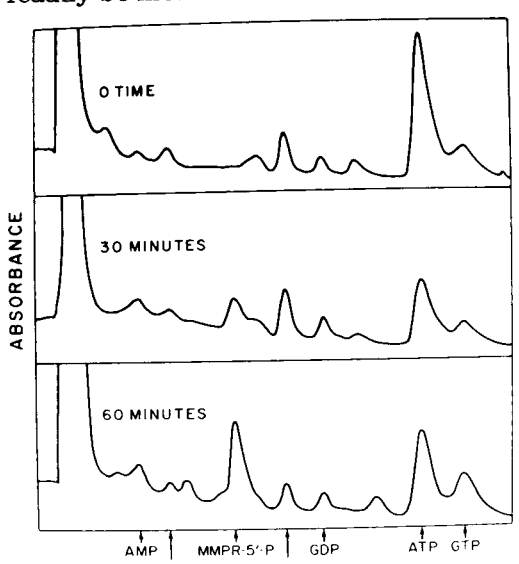

Fig. 5. Effect of 6-mercaptopurine (6-MP) and 6-methylmercaptopurine ribonucleoside (MMPR) on nucleotide patterns of Sarcoma 180 cells. Washed Sarcoma 180 cells (2 g) were incubated for 60 min with 6-MP plus MMPR. Final incubation volume was 12 ml. Extracts were prepared at 0, 30, and 60 min. Aliquots of extracts made at each time period were analyzed on a Varian Aerograph LCS-1000. Top tracing was obtained with an extract of cells prior to incubation with drug; middle and bottom tracings were from extracts of cells incubated for 30 and 60 min, respectively, with a combination of 6-MP and MMPR. (Ref. 57.)

B. Clinical studies

The levels of the nucleotides, nucleosides and bases have been quantified in the plasma, serum and urine of patients with various purine metabolic disorders. Subjects with gout and renal failure [1, 23] have been found to exhibit higher levels of hypoxanthine and xanthine than normal controls, but treatment with allopurinol reduced the oxypurine levels in the plasma of the gouty patients [1].

It was found by Bakay et al. [11] that individuals with hypoxanthine-guanine phosphoribosyltransferase deficiency had no detectable concentrations of IMP in their skin fibroblasts. Concomitantly, ATP, GTP and UTP were markedly lower than in the controls. The data reflect the lack of hypoxanthine conversion into IMP and, subsequently, other nucleotides. Whereas no free adenosine can be detected in normal human whole blood, erythrocytes or serum, this nucleoside was clearly identified in the serum of patients known to be deficient in adenosine deaminase [53] (Fig. 6). Thus, adenosine could serve as a marker for this enzyme defect, known to result in severe alterations of the immune system [59].

In order to monitor the metabolites excreted by an artificial kidney upon hemodialysis, the levels of some purines and pyrimidines were determined in the serum, dialysate and urine of a patient during a 6-h period [15]. The analytical data for the serum and dialysate were found to be essentially identical. Thus, the dialysate could be used to monitor blood composition during the time of treatment.

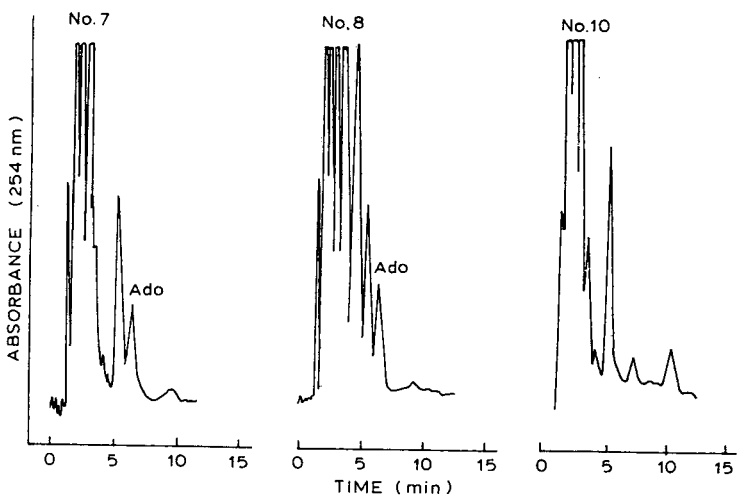

Fig. 6. Samples Nos. 7 and 8: 50 μl of serum extract from two patients suffering from adenosine deaminase deficiency; 45 and 55 picomoles are contained under the adenosine peak of sample numbers 7 and 8, respectively. Sample No. 10 shows the injection of 50 μl of serum extract from a control patient. Integrator setting, 2; column packing, μBondapak C_{18}; temperature, ambient; detector sensitivity, 0.02 a.u.f.s. Eluent, anhydrous methanol—0.007 *F* KH_2PO_4 (pH 5.8) (1 : 9). Flow-rate, 2.0 ml/min. (Ref. 53.)

C. Enzyme assays

Deficiencies in adenosine deaminase and purine nucleoside phosphorylase have often been associated with severe combined immunodeficiency. The early diagnosis of these deficiencies can help the clinician in administering proper therapy to control certain symptoms precipitated by the disease.

Hartwick and Brown [60] developed a rapid assay for adenosine deaminase in erythrocytes. Since the metabolism of adenosine includes the conversion of adenosine to inosine and, in turn, that of inosine to hypoxanthine, there was a need to separate adenosine, inosine and hypoxanthine from each other as well as from other red blood cell constituents. The decrease in the substrate adenosine, when added to an aliquot of erythrocyte lysate, corresponded to the increase in both inosine and hypoxanthine (Fig. 7). It is interesting to note that, using this technique, the activities of several enzymes could be monitored simultaneously.

More recently, Halfpenny and Brown [61] optimized an assay for purine nucleoside phosphorylase (PNPase) in erythrocytes. The substrate inosine, incubated with the cells, was converted into hypoxanthine. Xanthine oxidase was added to the incubation metium to prevent the accumulation of hypoxanthine which would inhibit the forward reaction of PNPase. Uric acid, hypoxanthine, xanthine and inosine were resolved for this assay with no interference from other compounds (Fig. 8).

Krstulović et al. [62] devised an assay for acid and alkaline phosphatase in serum using AMP as the substrate. After inhibition of 5′-nucleotidase by addition of Ni^{2+}, one could assay either for acid phosphatase by buffering the serum to pH 4.8, or for alkaline phosphatase by buffering to pH 9.8. Large increases in the activity of alkaline phosphatase could be observed in the serum of patients with cirrhosis or hepatitis, as compared to the normal serum.

The chromatographic conditions pertaining to all applications are listed in Table 8.

5. CONCLUSION

Whereas the nucleotide, nucleoside and base separations may be challenging and interesting to the chromatographer who seeks the understanding of retention mechanisms, they are truly essential to researchers in the clinical field. The compounds monitored and quantified may help to diagnose a disease or deficiency. The levels of nucleotides, nucleosides and bases may also reveal the mode of action of certain drugs administered in the course of chemotherapy. In addition, the rate of metabolism as well as the metabolic pathways of the drug itself may be determined. The requirements for clinical assays include rapid separation of the compounds of interest as well as reliable quantification. Although any HPLC mode can be used, reversed-phase is, at the present, the mode of choice for the analysis of nucleosides and bases. Ion-pairing is becoming increasingly popular for the separation of the nucleotides. However ion-exchange is still, to this date, the only mode which provides simultaneous analysis of the bases, nucleosides and nucleotides in samples. Since the existing separations are time-consuming, it is hoped that the ion-pairing technique will provide improved resolution as well as faster analyses.

TABLE 8

CONDITIONS FOR SELECTED APPLICATIONS

Mode*	Compounds resolved	Stationary phase	Mobile phase	Temp.	Flow-rate (ml/min)	Analysis time (min)	Ref. No.
CE	Methylated purines	Partisil 10-SCX (25 cm × 4.5 mm I.D.)	0.05 *M* ammonium phosphate (pH 2.0)	–	2.0	10	56
AE	Ribonucleoside mono-, di- and triphosphates	Pellicular AX (3 m × 1.0 mm I.D.)	A: 0.015 *M* KH_2PO_4 B: 0.25 *M* KH_2PO_4 in 2.2 *M* KCl 50 ml of A in mixing chamber, B pumped into mixing chamber at a flow-rate of 5 ml/h	–	12 ml/h	80	27
AE	3-Deazauridine-5′-triphosphate other ribonucleoside triphosphates	Partisil 10-SAX	A: 0.005 *M* $NH_4H_2PO_4$ (pH 2.8) B: 0.750 *M* $NH_4H_2PO_4$ (pH 3.7) Isocratic with 40% of B for 10 min, linear to 100% B in 24 min	–	2.0	30	10
RP-IP	5-FU and metabolites	RSIL-C_{18} HL (ODS) 5-μm (15 cm × 0.32 cm)	0.02 *M* KH_2PO_4 (pH 5.0) with 5% methanol	–	0.8	10	58
RP	Ado	μBondapak C_{18}, 10 μm (25 cm × 4.6 mm I.D.)	Methanol—KH_2PO_4 (pH 5.8) (1:9)	Ambient	2.0	7	53
RP	Purine and pyrimidine bases	μBondapak C_{18}, 10 μm (60 cm × 4 mm I.D.)	A: 25 m*M* sodium acetate (pH 4.50) B: 0.1 *M* acetic acid in methanol Gradient concave in 90 min	–	1.0	80	15
RP	Adenosine deaminase	μBondapak C_{18}, 10 μm (30 cm × 4 mm I.D.)	14% methanol, 86% 0.01 *M* KH_2PO_4	Ambient	2.0	6	60

RP	Purine nucleoside phosphorylase	Partisil 5-ODS, 5 μm (25 cm × 4 mm I.D.)	0.02 *F* KH_2PO_4 (pH 4.2), 3% methanol	Ambient	2.0	7	61
RP	Acid and alkaline phosphatase	RP-8, 7 μm (25 cm × 4.6 mm I.D.)	Methanol, 0.02 *M* KH_2PO_4 (pH 5.5) (1:9)	Ambient	1.5	7	62

*AE = anion exchange; CE = cation exchange; RP = reversed phase; IP = ion-pairing.

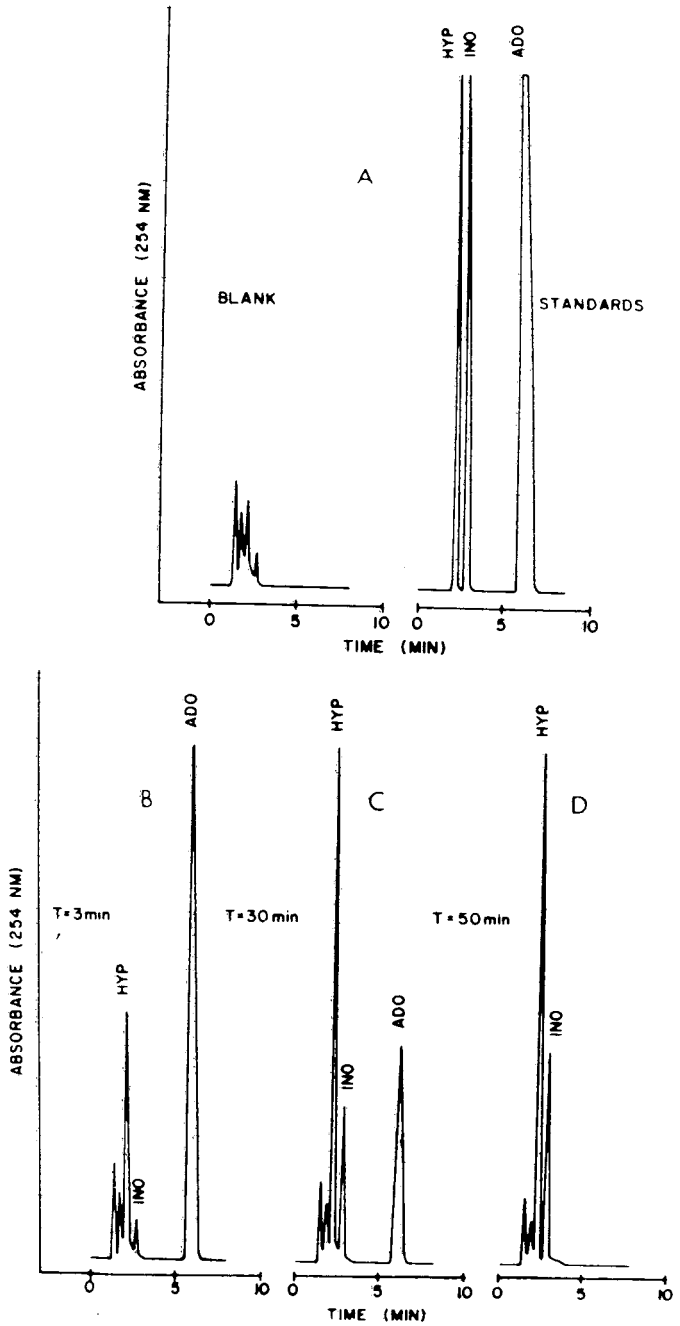

Fig. 7. The separation of the components of human erythrocytes by reversed-phase HPLC. In A a blank erythrocyte lysate is shown along with three standards: Hyp, Ino and Ado. In B, C and D, the decrease in the substrate (Ado) peak area is shown as a function of time. The chromatographic conditions are: isocratic elution, flow-rate 2.0 ml/min. Mobile phase: 86% 0.01 *F* KH_2PO_4 (pH unadjusted), 14% methanol. In each of the above chromatograms, the injection volume was 5 μl, at an attenuation of 64 on the Hewlett-Packard integrator. (Ref. 60.).

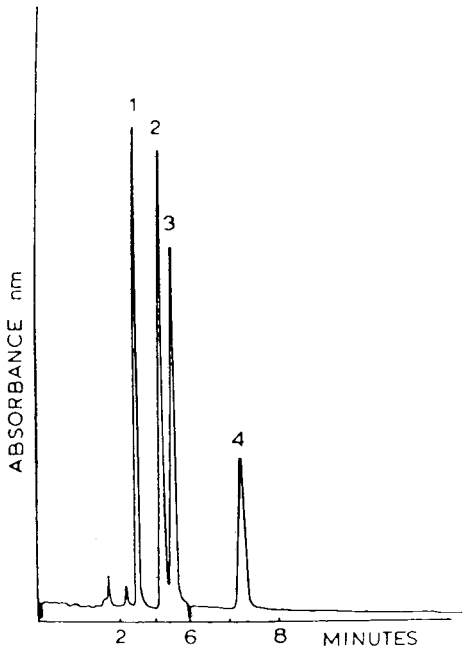

Fig. 8. Separation of the components of the reaction studied by HPLC (see text). Chromatographic conditions: isocratic elution, 2 ml/min; 0.02 *F* KH_2PO_4 (pH 4.2), 3% methanol. Peaks: 1 = uric acid; 2 = hypoxanthine; 3 = xanthine; 4 = inosine. (Ref. 61.)

6. SUMMARY

The latest advances in the HPLC analyses of nucleotides, nucleosides and their bases in biological samples are discussed. Included are sample preparation, chromatographic procedures, identification of peaks, quantification and selected chromatographic separations for each class of compounds. The merits of the various HPLC modes for each type of separation and applications in clinical and biochemistry are presented.

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A GAS CHROMATOGRAPHIC—MASS SPECTROMETRIC STUDY OF PROFILES OF VOLATILE METABOLITES IN HEPATIC ENCEPHALOPATHY

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SUMMARY

Volatile organic substances present in blood plasma and cerebrospinal fluids of certain control groups of human subjects and cirrhotic patients some of whom were suffering from hepatic encephalopathy were quantitatively analysed and identified. A rapid, reproducible, direct injection capillary column gas chromatographic method was developed for the concentration and detection of such volatiles at mg/l and lower concentrations. Of at least forty volatiles detected, twenty-one were identified. The mean concentration of one of these, 3-methylbutanal, was found to be significantly elevated ($p < 0.01$) in chronic encephalopathics (2.37 ± 0.79 mg/l, $n = 18$), when compared to the controls (0.30 ± 0.08 mg/l, $n = 20$). Furthermore, the concentration of this component increased with the clinically diagnosed severity of the encephalopathic state. The presence of 3-methylbutanal is related to leucine, a branched-chain amino acid linked with hepatic encephalopathy.

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INTRODUCTION

Hepatic encephalopathy is a complex neuropsychiatric syndrome, probably multifactorial in origin. It is generally believed that the major factor in this disease involves toxic nitrogenous substances formed in the intestine by bacterial action on proteins [1]. However, the nature of these toxic substances is unknown and is the subject of much speculation [2, 3]. Originally, it was felt that increased blood ammonia levels in encephalopathy were responsible for the resulting toxicity [4] but hyperammoniaemia is not always associated with the incidence of coma [5]. Abnormalities of amino acid metabolism [6, 7], particularly the variable elevation of the concentrations of aromatic amino acids such as phenylalanine, tryptophan and methionine and recently the consistent depression in the levels of the branched-chain amino acids (BCAA) valine, leucine and isoleucine [8—11] have been noted. This decrease in levels of the BCAA's has been attributed so far to the effect of high levels of circulating insulin in the blood resulting in an increase of muscle BCAA uptake [12].

Since hepatic encephalopathy has been linked to deranged protein metabolism and it is unlikely that the amino acids are, in themselves, toxic, it becomes necessary to consider the possibility that one or more of their metabolites may be toxic. There is some prior evidence to suggest that the metabolism of amino acids produces volatile substances such as low-molecular-weight aldehydes, ketones and alcohols [13]. Some of these components may be related to the resulting toxicity in hepatic encephalopathy. The study of the volatile components in the body fluids of subjects with hepatic encephalopathy would therefore appear to be of interest.

The study of the volatile components in body fluids, in general, is a relatively new field [14—18] with potential as a novel means of studying disease. Various methods exist for the study of low-molecular-weight substances of biological origin. These methods apply either solvent extraction, headspace analysis or direct injection techniques, and are either very time consuming or non-quantitative. Goldberg and Sandler [19] have developed a simple, rapid and quantitative method for the analysis of body fluid volatiles, which in slightly modified form, has been used in this study. With such a method it should be possible to produce metabolic profiles to distinguish between the diseased and normal state or act as indicators of certain diseases. This study shows the application of the method to hepatic encephalopathy as one such disease.

EXPERIMENTAL

Analysis for volatiles

A gas chromatographic (GC) method of analysis for body fluid volatiles [19] developed in our laboratory, was based on the direct injection of 100 μl of sample, removal of water in a condenser at 0°C and subsequent sample trapping and concentration on a small, cooled precolumn packed with Tenax GC.

The present method is a modification of the above with the Tenax GC precolumn being replaced with a 1 m × 0.5 mm I.D. stainless-steel capillary tube immersed in liquid nitrogen. Recoveries of low-molecular-weight volatiles were

improved over the previous method. After trapping for 5 min the sample is injected on to the column by means of a microvolume switching valve and heating of the trap to 100°C.

The volatiles are separated by GC using wall-coated, open-tubular columns and a flame ionization detector. The column was a 100 m × 0.5 mm I.D. nickel tube prepared according to the method of Bertsch et al. [20]. It was coated with Ucon 50 HB 5100 and had an efficiency of 100,000 theoretical plates, based on the peak for 2-pentanone (k = 5). The carrier gas (nitrogen) flow-rate (1.5 ml/min) was pressure controlled at 153 kPa. The column was held at 50°C for 10 min, then programmed at 2°C/min, to 150°C and held at 150°C to the end of the analysis. Make-up nitrogen was added at the detector base to give a total nitrogen flow-rate of 25 ml/min. Hydrogen and air flow-rates were set at 25 ml/min and 300 ml/min, respectively.

Quantitative analysis of volatiles

The concentrations of the volatiles in the sample were determined using an internal standard, *o*-xylene, added to produce a concentration of 2.6 mg/l (0.5 ml *o*-xylene standard, 132 mg/l, added to 2 ml sample). Calibration standards of 1 mg/l of various volatiles were analysed and calibration coefficients were calculated. An Autolab System 1 (Spectra Physics, Technical Marketing Assoc., Toronto, Canada) computing integrator with the calculation accessory was used to measure the peak areas and perform the calculations, giving the concentrations of the volatiles in mg/l.

Volatile identification

Components were identified by analysis on two columns of differing polarities (OV-17 and Ucon 50 HB 5100). Further confirmation of the component identification was achieved by connecting the concentrating system to a Du-Pont 21-492 mass spectrometer. The column used in this portion of the study was a glass open-tubular column 50 m × 0.5 mm I.D. coated with OV-17 (Alltech, Arlington Heights, IL, U.S.A.). The operating conditions were otherwise maintained as described above.

Sampling

Blood samples were drawn into heparinized green stopper B-D vacutainers, cooled in ice and transferred into ice-chilled stoppered bottles. Samples not analysed immediately were frozen until needed. Skin disinfection prior to blood sampling was performed with soap and water rather than 2-propanol to eliminate possible contamination of the sample from the latter source.

Cerebrospinal fluid (CSF) samples were obtained by lumbar puncture. The CSF samples were taken only when necessary for other tests and therefore very few samples were analysed.

Subjects and samples

Samples of blood were collected from human subjects clinically classified as to their state of health into one of two major groups, (1) non-encephalopathic controls (20 subjects) and (2) encephalopathic (18 subjects). The control group was divided into four subgroups as follows: six normals under no dietary

control, five subjects undergoing a colonoscopy examination who were sampled after a fast, four non-dietary controlled cirrhotics and five post portacaval shunt patients on protein-reduced maintenance diets. All the post shunt patients had experienced previous episodes of encephalopathy. The encephalopathic group was divided into two subgroups, one consisting of thirteen patients in grades 1 and 2 (mild) hepatic encephalopathy, and the other consisting of five patients in grades 3 and 4 (severe) hepatic encephalopathy. Blood samples, taken from these subjects on a number of different occasions while they were in the same clinical state, were analysed.

Three CSF samples from one patient in grade 4 hepatic coma were taken on different days and analysed by the same procedure. The results were compared with those from samples taken from two different non-encephalopathic control subjects.

Amino acid analysis

Plasma samples from ten individuals in the study were deproteinized with equal volumes of 15% sulphosalicylic acid, containing 200 μmol/l norleucine as internal standard. The free neutral amino acid concentrations were determined on 300 μl of plasma using a Beckman Spinco amino acid analyser Model 116/119.

RESULTS

The method developed for the analysis of the volatile components was found to be reproducible to within ± 0.06 mg/l at the 95% confidence level when standard solutions were used. When the same blood plasma sample was analysed on three consecutive days the reproducibility was within ± 0.07 mg/l with 95% confidence [21]. An analysis of variance showed no significant differences between standard solution or blood plasma sample replications.

A quantitative comparison of the chromatographic profiles of the volatiles showed that there were distinct differences between non-encephalopathic (Fig. 1a) and encephalopathic subjects (Fig. 1b). Subsequent quantitative analysis confirmed this. The methods used for identification of components and their plasma and CSF concentrations in the control and encephalopathic subjects are given in Table I.

Two components, 3-methylbutanal and furfural, exhibited significant concentration differences between groups. In blood samples from encephalopathics the former was present at about eight times the concentration in the control groups and the latter at a concentration of about 50% of that in the control group.

In CSF the average concentration of 3-methylbutanal in the fluid of the subject in grade 4 hepatic encephalopathy (25.13 mg/l) was found to be almost twenty times higher than that for the control subjects (1.28 mg/l). Furfural was not detected in any of the CSF samples.

When the results from patients in grades 1 and 2 hepatic encephalopathy were compared with those for grades 3 and 4, it was found that the concentration level of 3-methylbutanal increased with the severity of the encephalopathic state. The mean concentration of 3-methylbutanal in mild encephalo-

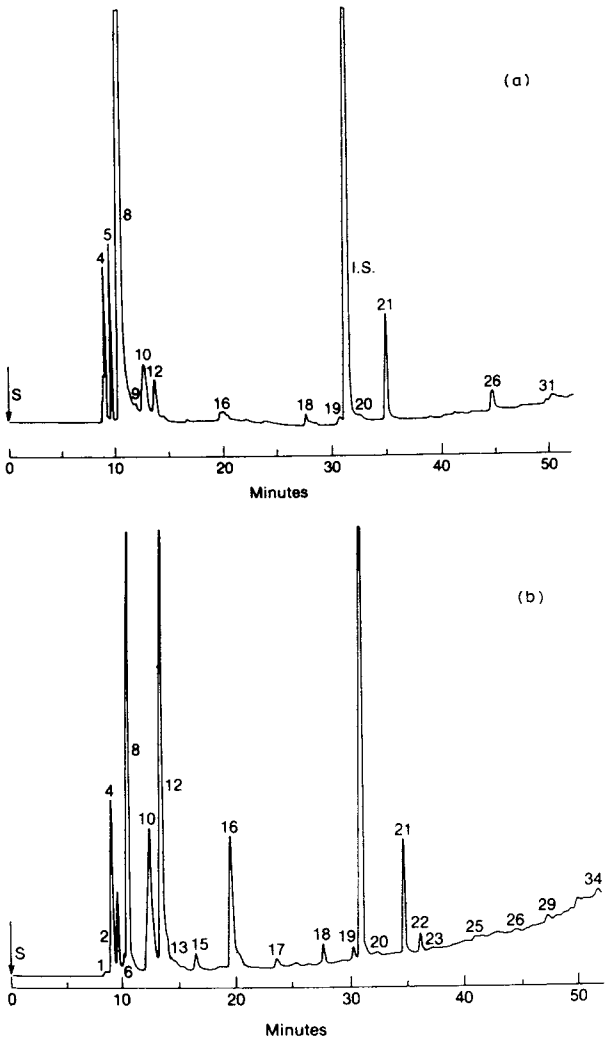


Fig. 1. Chromatograms of plasma volatile metabolites in (a) normal and (b) encephalopathic subject. Conditions: 100 m × 0.25 mm I.D. nickel column coated with Ucon 50 HB 5100; nitrogen carrier gas at a flow-rate of 1.5 ml/min; 50°C for 10 min then programmed to 150°C at 2°C/min. Peaks are identified in Table I.

pathics, grades 1 and 2 (1.30 ± 0.32 mg/l, $n = 13$) was significantly less than ($p < 0.025$) the mean for grades 3 and 4 encephalopathic subjects (5.13 ± 2.46 mg/l, $n = 5$). The results are presented graphically in Fig. 2.

Plasma amino acid analysis showed that the ratio of BCAA to aromatic amino acids was 3 for normal subjects and between 0.58 and 1.93 for cirrhotic subjects whether encephalopathic or not. Subjects in grade 4 encephalopathy had high plasma BCAA levels, yet others [22, 23] have claimed that increasing the plasma BCAA level would be beneficial in the management of hepatic encephalopathy.

TABLE I

CONCENTRATIONS (mg/l ± S.E.M.) OF IDENTIFIED VOLATILE COMPONENTS IN PLASMA AND CSF

Peak No.	Component	Method of identification*			Control group (mg/l)		Encephalopathic group (mg/l)	
					Plasma (n = 20)	CSF (n = 2)	Plasma (n = 18)	CSF (n = 3)
4	Ethanal	U	O	M	0.76 ± 0.15	2.26	0.75 ± 12	1.19
6	Propanal	U			0.04 ± 0.01	0.01	0.04 ± 0.01	0.04
8	Propanone	U	O	M	9.66 ± 2.55	1.84	17.44 ± 6.59	157.61
10	Butanone + 2-methylbutanal**	U	O	M M	2.32 ± 0.83	0.24	1.33 ± 0.25	1.60
11	Ethanal + 2-propanol**	U U	O O	M	3.11 ± 1.93	ND	0.13 ± 0.03	ND
12	3-Methylbutanal	U	O	M	0.30 ± 0.08	1.28	2.37 ± 0.79***	25.13
13	2-Butanal			M	0.05 ± 0.01	0.02	0.05 ± 0.02	1.15
14	2-Pentanone + 3-Pentanone**	U U	O		0.05 ± 0.02	ND	0.46 ± 0.37	0.04
16	3-Methyl-1-butanal	U			0.20 ± 0.06	0.05	0.22 ± 0.06	0.13
18	1-Hexanal			M	0.07 ± 0.01	0.01	0.16 ± 0.07	2.46
19	3-Heptanone	U			0.09 ± 0.02	ND	0.06 ± 0.01	0.01
20	2-Heptanone	U			0.05 ± 0.01	0.02	0.08 ± 0.02	0.08
21	Furfural			M	0.53 ± 0.06	ND	0.28 ± 0.04***	ND
23	Methylpyrrole			M	0.03 ± 0.01	ND	0.07 ± 0.02	0.06
25	4-Heptanone + 2-octanone**	U	O	M	0.07 ± 0.03	0.01	0.04 ± 0.01	0.04
28	Benzaldehyde			M	ND	ND	0.06 ± 0.03	0.24

*U = Identified by chromatography on Ucon 50 HB 5100 column; O = identified by chromatography on OV-17 column; M = identified by combined gas chromatography—mass spectrometry.

**Components not resolved on Ucon column.

***Significant difference between plasma concentrations ($p < 0.01$).

DISCUSSION

Low-molecular-weight volatile substances in body fluids are present due to the metabolism of higher-molecular-weight components such as amino acids, fatty acids and carbohydrates. Changes in the concentration of the volatiles parallel changes in the concentrations of the high-molecular-weight component. The volatiles may therefore be used as an indirect measurement of the metabolism of amino acids, fatty acids and sugars. Furthermore, the volatiles give the total of the metabolism of related substances. Treatment of hepatic encephalopathy consists of protein withdrawal and elimination or modification of the colonic bacteria.

It has been shown [24] that in the bacterial degradation of certain amino acids, volatile substances may be produced. In subjects suffering from hepatic encephalopathy, the bacterial formation of volatiles from amino acids must be

considered. Therefore, it is the metabolic pathways of bacteria which must be used in explaining the presence of those volatile components directly related to hepatic encephalopathy.

Two components, 3-methylbutanal and furfural show significant differences in plasma concentrations when encephalopathic and non-encephalopathic subjects are compared. Only the former is present in the CSF.

Thus, on the basis that CSF is the transfer agent to the brain for such substances, only 3-methylbutanal could have a neurological effect in hepatic encephalopathy. Pilotti et al. [25] have shown that 3-methylbutanal is toxic in the sense that it inhibits cell multiplication in vitro at concentration levels comparable to those found in the blood plasma of the encephalopathic subjects in this study and below the levels found in CSF samples of such a subject. Rats injected with sufficient 3-methylbutanal to give a blood concentration of this substance comparable to patients with hepatic encephalopathy showed EEG patterns similar to encephalopathics [26].

Fig. 2 shows the concentration of 3-methylbutanal for all the subject groups. Two points of interest are the relatively high levels of 3-methylbutanal in the normal group as compared to the colonoscopy subjects and the low levels of this component in the shunt group as compared to the encephalopathics. The high level in the normals would indicate that 3-methylbutanal is of dietary origin, either endogenous or exogenous, since the colon group was sampled after a fast. Furthermore, the fact that high levels of 3-methylbutanal in the normals are not associated with encephalopathy shows that other components such as ammonia, fatty acids, amino acids or mercaptans [2, 3] must be involved. A person susceptible to encephalopathy is apparently sensitive to toxic substances not removed due to the absence of the normal liver function. The low levels in the shunt patients seem to bear this out. These patients, with improper liver function, become encephalopathic when their protein intake is high. Together with the onset of encephalopathy, the plasma 3-methylbutanal increases. The formation of this aldehyde is thus related to the metabolism of ingested protein, in particular the BCAA's.

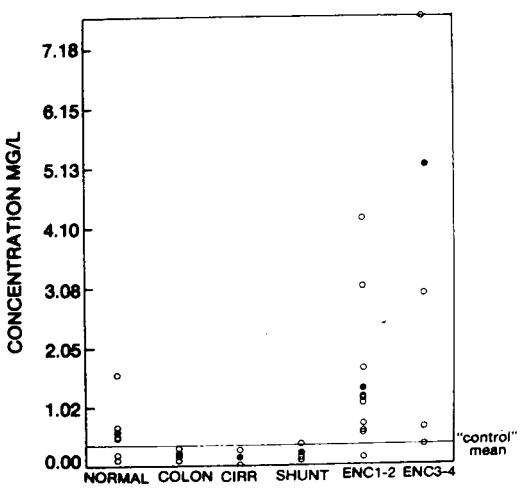
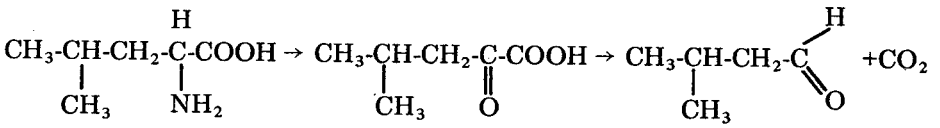


Fig. 2. 3-Methylbutanal concentrations in blood plasma in all groups in individual subjects. Group description as in text. (○) Subject mean; (●) group mean ± S.E.M.

It is proposed that 3-methylbutanal is formed by the catabolism of leucine as follows:



The literature supports the possible formation of this compound from leucine. Yabumato and Jenning [27] have proposed the formation of 3-methylbutanal from leucine, 2-methylbutanal from isoleucine and methylpropanal from valine. That is, all three BCAA's have been considered capable of producing volatile branched-chain aldehydes. Yu and Spencer [28] have demonstrated the production of [^{14}C] 3-methylbutanal from [^{14}C] leucine using an enzyme extract from tomato. Non-enzymatically, these volatiles may be formed from their corresponding amino acids by the Strecker degradation [29], which has been postulated in several biological systems. Recently [30] it has been shown that 3-methylbutanal is formed by isolated bacteria grown on agar supplemented with yeast extract.

CONCLUSION

A quantitative, reproducible method for the analysis of body fluid volatiles has been developed. This has been applied to the study of hepatic encephalopathy.

ACKNOWLEDGEMENTS

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NEW POLAR ACID METABOLITES IN HUMAN URINE

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SUMMARY

A sequence of chromatographic methods (thin-layer chromatography, high-performance liquid chromatography and glass capillary gas chromatography) was used to separate the acid fraction of human urine. The power of this method to separate and detect previously unknown compounds and the elucidation of their final structure with mass spectrometry is exemplified by the identification of N-acetyl-2-aminooctanoic acid as a metabolic compound in the urine of healthy individuals.

In addition, the conjugate of glycine with indolepropionic acid, N-formylanthranilic acid, succinoylphenylalanine, δ-hydroxyvaleric acid, δ-hydroxycapric acid, 3-hydroxyadipic acid, and higher homologues were detected in a polar fraction of human urine.

INTRODUCTION

The acid fraction of urine is of extreme complexity. A perfect separation — following appropriate derivatisation — is not possible even with glass capillary gas chromatography (GC). Therefore, we recently combined two different chromatographic systems — thin-layer chromatography (TLC) and glass capillary GC — for separation [1]. This enabled us to identify a number of previously unknown compounds [2, 3].

The separation power was then enlarged by applying a third, different technique—high-performance liquid chromatography (HPLC). The combination of these three methods in a sequence allowed the determination even of trace compounds which are usually hidden under large peaks in the GC runs of unfractionated samples. The power of this technique is described in this paper dealing with the structure elucidation of previously unknown γ- and δ-hydroxy mono- and dicarboxylic acids, as well as amino acid conjugates, obtained from a polar fraction of urinary acid compounds.

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MATERIALS AND METHODS

Isolation, derivatisation and chromatography

Urine work-up. One litre of urine, acidified to pH 1, was extracted three times with 300 ml of ethyl acetate; the combined extracts were dried over sodium sulfate and evaporated in vacuo. The residue was dissolved in methanol and methylated by adding diazomethane in diethyl ether. This solution was concentrated in a nitrogen atmosphere.

Thin-layer chromatography. About half of the solution of the methylated compounds, corresponding to 500 ml of urine, was chromatographed on twenty thick-layer plates (1 mm, 20 × 20 cm), made from silica gel 60 HF_{254} (E. Merck, Darmstadt, G.F.R.) using a mixture of diethyl ether—cyclohexane (5 : 3) as mobile phase. Eight zones were marked under UV light (254 nm) and then scraped off. The R_F values for the zones were as follows: zone 1, 0.64—1.00; zone 2, 0.54—0.64; zone 3, 0.48—0.54; zone 4, 0.42—0.48; zone 5, 0.24—0.42; zone 6, 0.20—0.24; zone 7, 0.12—0.20; zone 8, 0.00—0.12.

High-performance liquid chromatography. The four most polar fractions (zones 5—8) were subjected to HPLC. They were chromatographed on a Waters Assoc. (Milford, MA, U.S.A.) μBondapak C_{18} (10 μm) column (300 mm × 3.9 mm I.D.) using a Spectra-Physics liquid chromatograph Model 3500 B and a Model 230 UV detector. A linear gradient of methanol—water starting with a percentage ratio of 30:70 up to 99:1, with a flow-rate of 1.2 ml/min, was used. Fractions were cut according to signals on the UV detector (254 nm).

Thirty-seven fractions were obtained. The solvent was removed under reduced pressure and the residue dissolved in a few drops of methanol.

Gas chromatography—mass spectrometry. Each of the 37 fractions was investigated by GC—MS using the following conditions: LKB 2091 mass spectrometer combined with a Pye-Unicam gas chromatograph; WCOT capillary column, 25 m long, filled with OV-101; temperature, 100—300°C, programmed at 2°C/min; data system, PDP 11, LKB 2130.

High-resolution mass spectrometry. High-resolution data were obtained by peak-matching with a Varian Model 312 mass spectrometer combined to a MAT SS 200 data system.

Synthesis of comparison compounds

N-Acetyl-2-aminooctanoic acid (1a) [4]

2-Bromocaprylic acid. Bromine (8 g) was added to a mixture of 7.2 g of caprylic acid and 450 mg of red phosphorus under stirring. Within the next 30 min, an additional amount of 8 g of bromine was added. The mixture was heated for 48 h on a water-bath; 900 mg of water were then added and the mixture heated to 150°C for 10 min.

2-Aminooctanoic acid. The crude 2-bromocaprylic acid was added to a solution of 45 g of ammonium carbonate in 7 ml of water and 20 ml of concentrated ammonia and heated for 24 h to 50°C. The mixture was poured into a porcelain vessel and concentrated over a burner until the temperature reached 110°C. After cooling to 60°C, the residue was dissolved in methanol and precipitated in the refrigerator (4.1 g).

2-Aminooctanoic acid methyl ester. A 1.1-g portion of the 2-aminooctanoic acid was suspended in 20 ml of methanol; 1 g of concentrated sulfuric acid and 20 ml of tetrachloromethane were added. This mixture was refluxed for 8 h using a water separator, then concentrated in vacuo and poured into 100 ml of a saturated solution of potassium carbonate. The solution was extracted three times with 50 ml of diethyl ether; the ethereal extract was dried over sodium sulfate and concentrated in vacuo; 0.7 g of an oily residue remained.

N-Acetyl-2-aminooctanoic acid (1a). To 0.7 g of 2-aminooctanoic acid were added 5 ml of acetic anhydride and left for 24 h at room temperature. The excess acetic anhydride was distilled off; 0.8 g of 1a remained.

3,3'-Indolepropionylglycine methyl ester (2a)

3,3'-Indolepropionic acid (95 mg), prepared from gramine and malonic acid diethyl ester [5], and 62 mg of glycine methyl ester hydrochloride were suspended in acetonitrile; 110 mg of dicyclohexylcarbodiimide and a few drops of pyridine were added. The mixture was stirred for 2 h. The solution was filtered off from dicyclohexyl urea, and the filtrate evaporated to dryness. The residue was dissolved in diethyl ether and washed with dilute hydrochloric acid and a saturated aqueous solution of sodium hydrogen carbonate. After drying (sodium sulfate) and evaporation, 100 mg of 2a in the form of a viscous oil remained.

N-Formylanthranilic acid (3a)

Anthranilic acid (1.4 g, 0.01 mol) was added to 20 ml of formic acid and refluxed for 1 h. The solution was evaporated in vacuo. The solid residue (3a) was crystallized from dilute ethanol. Yield 1.5 g; m.p. 164°C (167°C [6]).

(N-Succinoylmonomethyl ester)-phenylalanine methyl ester (5b)

Phenylalanine methyl ester hydrochloride (2.15 g) was dissolved in 20 ml of pyridine to which 1.5 g of succinic acid monomethyl ester monochloride were added under stirring. After a few minutes the warm solution was poured into a mixture of ice and hydrochloric acid. The solution was extracted three times with ethyl acetate. The combined organic extracts were washed with a 1 *N* sodium carbonate solution and dried with sodium sulfate. After evaporation a residue of 2.2 g of 5b remained which was recrystallized from diethyl ether; m.p. 76—80°C.

N-2(3-Phenylpropionic acid methyl ester)-succinimide (4a)

Phenylalanine methyl ester hydrochloride (1 g) was mixed with well-powdered succinic anhydride. This mixture was heated in a test-tube above the melting point until the development of gaseous HCl and water was finished. The cooled residue was dissolved in 1 *N* sodium carbonate which was extracted three times with diethyl ether. After evaporation, 0.6 g of residue remained; 4a was recrystallized from ether, m.p. 80°C (needles).

5-Oxotetrahydrofuryl-2-acetic acid (11a) [7]

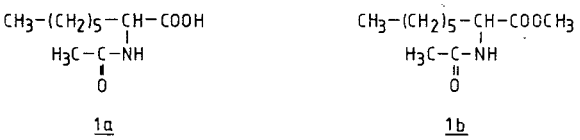
3-Hexenedioic acid (1 g) was dissolved in half-concentrated sulfuric acid and heated for 3 h to 110°C. After cooling the solution was extracted with diethyl

ether. The extract was evaporated. A residue, containing a mixture of 5-oxo-tetrahydrofuryl-2-acetic acid and 3-hexenedioic acid, remained.

RESULTS AND DISCUSSION

Characterization of N-acetyl-2-aminooctanoic acid

The power of the applied combined separation methods will be exemplified by the detection and final characterization of N-acetyl-2-aminooctanoic acid (1a).



1a 1b

Fig. 1 shows the profile of the methylated acid fraction using glass capillary GC alone. N-Acetyl-2-aminooctanoic acid methyl ester (1b) was not detectable. The region where it is buried under the other compounds is marked by an arrow.

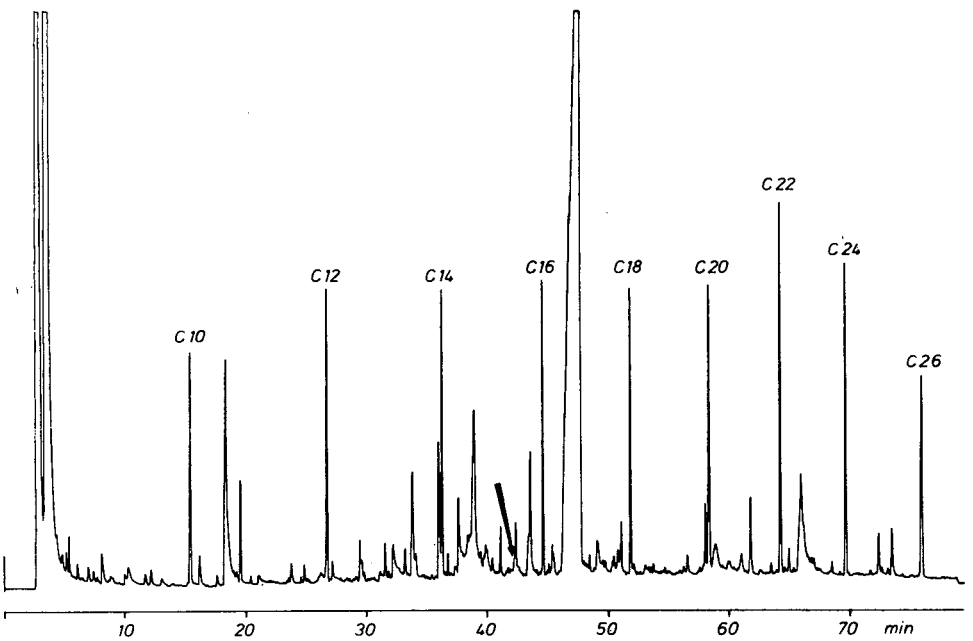


Fig. 1. Glass capillary gas chromatogram of the methylated acid fraction of human urine.

After TLC separation 1b was detected in fraction 7 (Fig. 2) by glass capillary GC—MS. Its mass spectrum is represented in Fig. 3. The mass spectrum is characterized by key ions of mass 156 and 114, produced by α-cleavage followed by loss of ketene and further degradation to an ion of mass 30.

Another α-cleavage reaction produces the ion of mass 88. The ion of mass 43

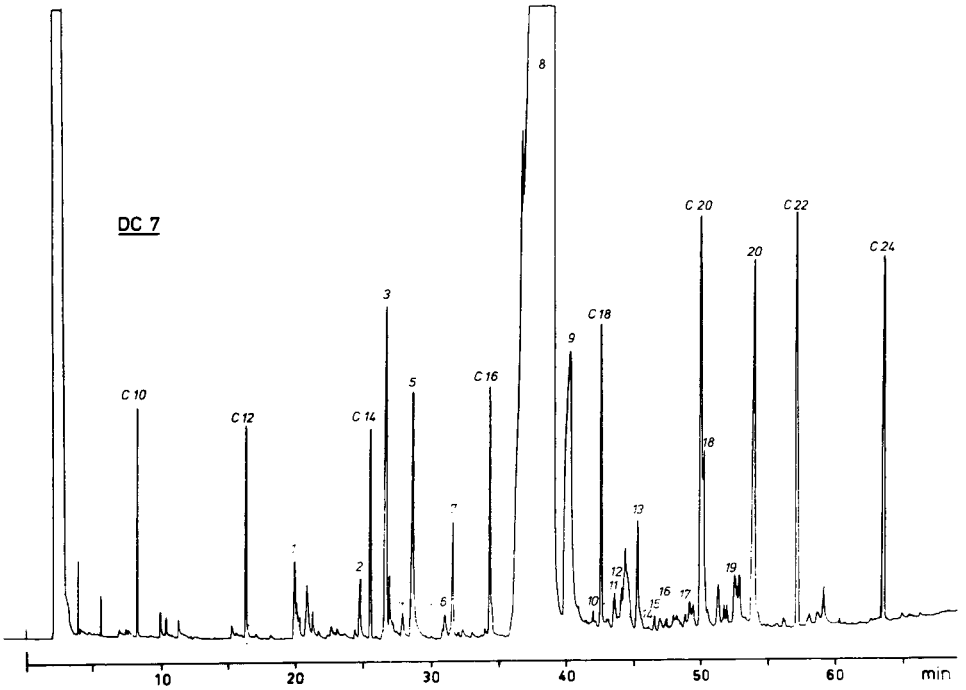


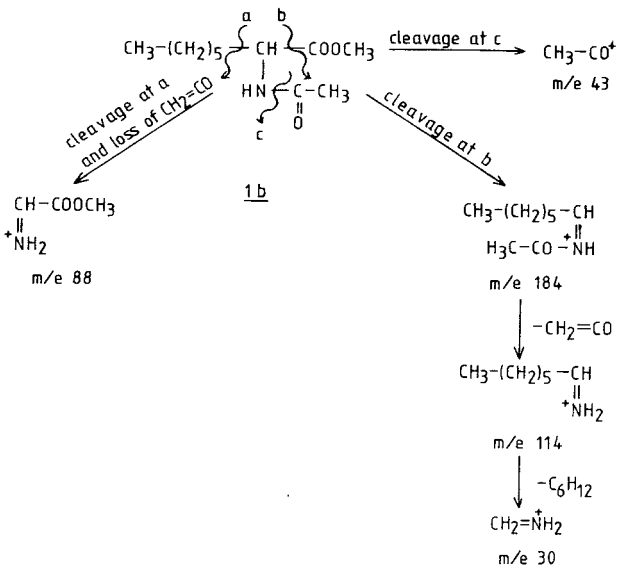
Fig. 2. Glass capillary gas chromatogram of fraction 7 of the thin-layer separation of the urinary acids. The numbers above the peaks correspond to the compounds listed in Table I.

TABLE I

SUBSTANCES CORRESPONDING TO THE NUMBERS ON THE GLASS CAPILLARY GAS CHROMATOGRAM OF FIG. 2

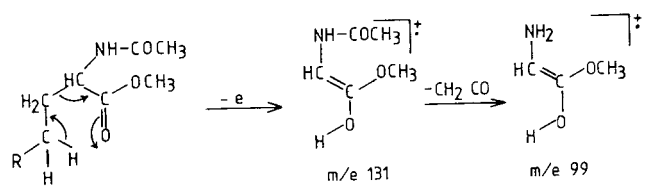
No.	RI*	Mol. wt.	Substance
1	1280	158	5-Oxotetrahydrofuryl-2-acetic acid
2	1383	171	3-Methylcrotonylglycine
3	1424	234	Citric acid
4	1452	234	Isocitric acid
5	1471	183	2-Furoylglycine
6	1523	186	4-(5-Oxotetrahydrofuryl-2)-butyric acid
7	1537	215	N-Acetylaminooctanoic acid
8	1650	193	Hippuric acid
9	1745	196	3-(3-Hydroxyphenyl)-3-hydroxypropionic acid
10	1784	207	3-Methylhippuric acid
11	1825	209	Hydroxyhippuric acid
12	1838	208	Ferulic acid
13	1873	241	5-Methoxycarbonylfuran-2-carbonyl-glycine
14	1892	261	N-2-(3-Phenylpropionic acid)-succinimide
15	1905	223	3-Methoxyhippuric acid
16	1930	223	4-Methoxyhippuric acid
17	1976	219	3-Indolyllactic acid
18	2006	219	Cinnamoylglycine
19	2065	205	5-Hydroxy-3-indolylacetic acid
20	2110	293	N-Phenylacetylglutamic acid

*RI = Kováts' index.



indicates the presence of the acetyl group, that of mass 60 a $-NH-COCH_3$ group connected to an alkyl chain with several carbon atoms [8].

References to the chain length of R were obtained by the peaks of mass 131 and 99, resulting possibly from loss of C_6H_{12} followed by loss of ketene:



To confirm these assumptions high-resolution data were needed. This, however, demanded the accumulation of enough sample to carry out peak matching. Knowledge of the mass spectrum (Fig. 3) made a control of further enrichment possible by running mass spectra of fractions obtained by HPLC separation (Fig. 4). It must be emphasized that the peaks indicated in this chromatogram do not correlate with the compounds found in the GC runs, since UV-inactive

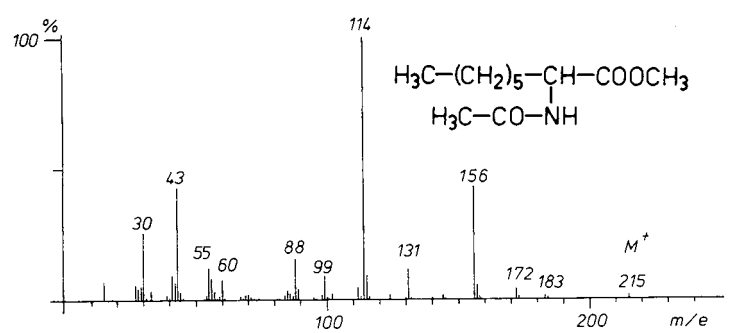


Fig. 3. Mass spectrum of N-acetyl-2-aminooctanoic acid methyl ester (1b).

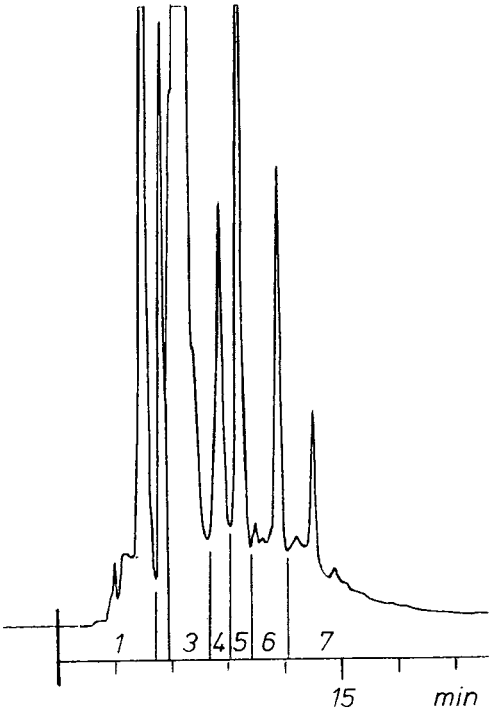


Fig. 4. HPLC separation of the thin-layer fraction 7.

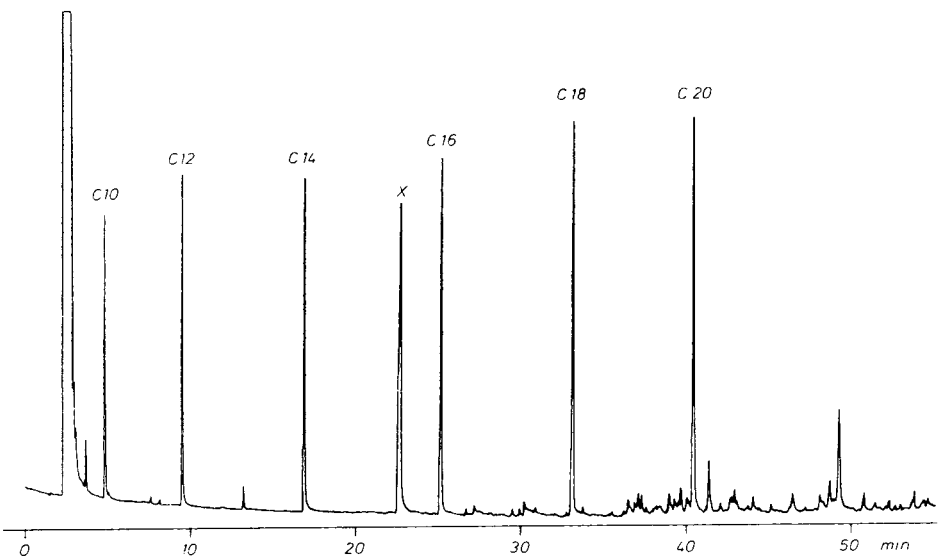


Fig. 5. Glass capillary gas chromatogram of the HPLC fraction 7, shown in Fig. 4. The peak of the enriched compound 1b is indicated by X.

compounds are not registered. Nevertheless, the peaks in the HPLC chromatogram allowed the same distinct section in subsequent runs, always to be collected.

Each fraction of the HPLC chromatogram was again investigated by GC—MS. HPLC fraction 7 contained the unknown compound (Fig. 5) in nearly pure form. By direct insertion of this HPLC fraction into the high-resolution mass spectrometer, the molecular formulae of the key ions listed in Table II were obtained by peak matching.

The high-resolution data confirmed that the chain corresponded to C_6H_{13}. The mass spectrum and the high-resolution data did not allow any conclusion about the arrangement of the six carbon atoms. To clarify this point, the most probable isomer, the compound with a straight C_6 chain, was synthesized.

To confirm the identity of the natural product with the synthetic sample the mass spectra of both components and their Kovats' indices were compared and found to be identical.

N-Acetyl-2-aminooctanoic acid was found to occur in human milk [9] and in *Aspergillus atypique* [10]. Our investigations revealed that it is obviously also a normal metabolite in healthy individuals.

In a similar way the structures of the other so far unknown compounds were determined.

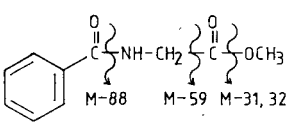
TABLE II

DATA OF THE UNKNOWN COMPOUND OF FIG. 5, OBTAINED BY HIGH-RESOLUTION MASS SPECTROMETRY

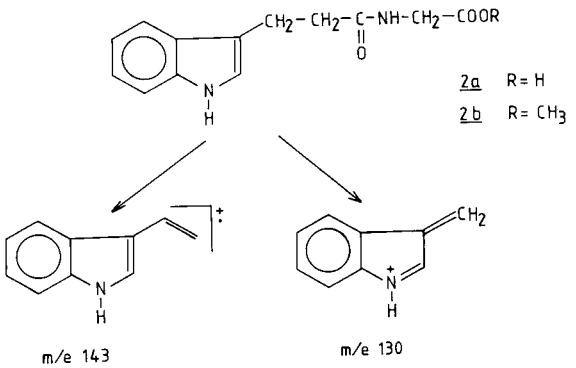
Mass	Molecular formula
215	$C_{11}H_{21}NO_3$
156	$C_9H_{18}NO$
114	$C_7H_{16}N$

Amino acid conjugates

Amino acid derivatives, especially conjugates of glycine, are common metabolic products [11]. Besides hippuric acid, the main component in the acid fraction of urine, 3- and 4-hydroxy-hippuric acid, α-picolinuric acid as well as 5-carboxy-furan-2-carbonyl-glycine [3] are known to occur in normal urine in low amounts. These methyl esters of glycine conjugates are usually easy to detect by the presence of key ions at M−31 (M−OCH_3) and/or M−32 (M−CH_3OH), M−59 (M−$COOCH_3$) and M−88 (M−$NHCH_2COOCH_3$).



This is not so in the case of the methyl ester of the conjugate of glycine with indolylpropionic acid (2b), detected in the HPLC fraction 6 of TLC zone 8, the mass spectrum of which is represented in Fig. 6. The main peaks correspond to the indolyl part of the molecule:



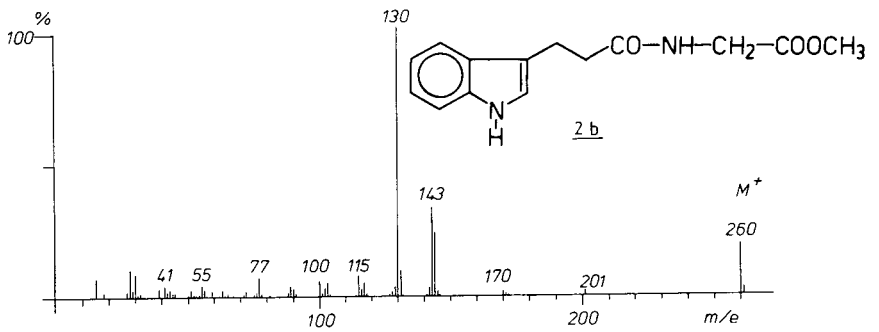


Fig. 6. Mass spectrum of the conjugate of indolylpropionic acid methyl ester with glycine (2b).

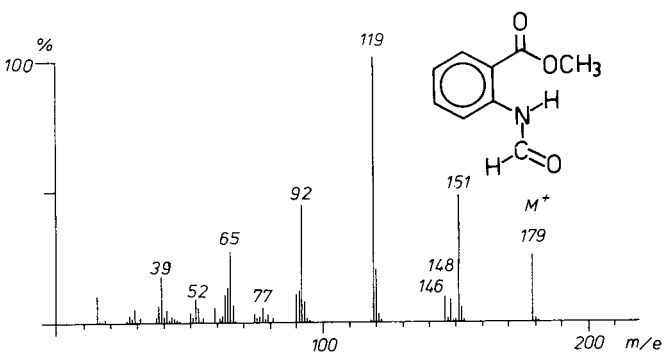
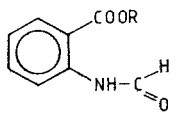


Fig. 7. Mass spectrum of N-formyl-anthranilic acid methyl ester (3b).

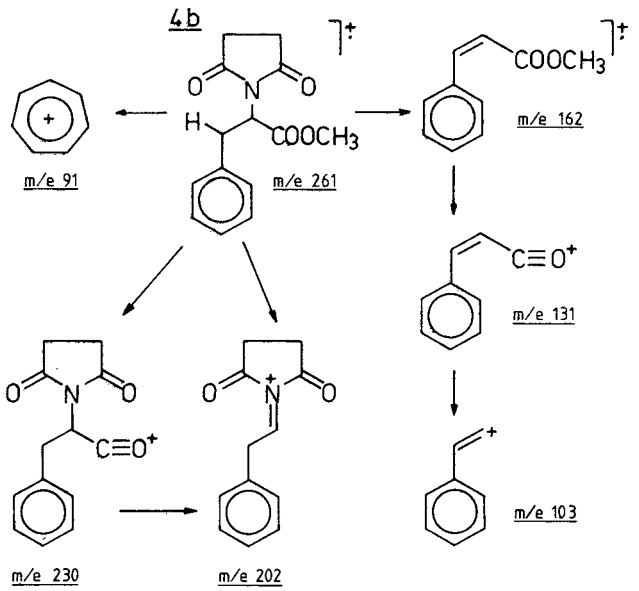
The presence of 2 was not known previously although it is well known that indolepropionic acid occurs in urine [12]. Anthranilic acid is a long-known urinary metabolite [13]. Its N-formyl derivative 3a has now been detected in human urine in the form of its methyl ester 3b in TLC zone 5. The mass spectrometric degradation of its methyl ester (3b) (Fig. 7) starts with the loss of the formyl group in the form of CO, thus producing the molecular ion of anthranilic acid which is degraded in the same way as described for this compound [14].



3a R = H

3b R = CH_3

In HPLC fraction 6 of TLC zone 7 we detected another previously unknown acid compound. The mass spectrum (Fig. 8) showed ions at *m/e* 162, 131, 103 and 77, reminiscent of the ions found in the mass spectrum of cinnamic acid methylate, indicating (together with the ion of mass 91) the presence of a substituted phenylpropionic acid derivative:



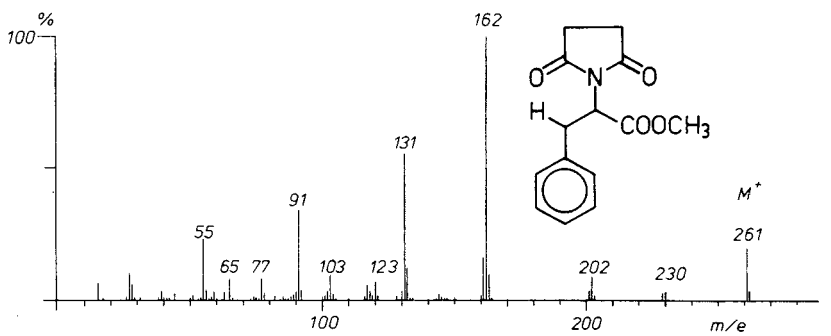


Fig. 8. Mass spectrum of N-(3-phenylpropionic acid methyl ester)-succinimide (4b).

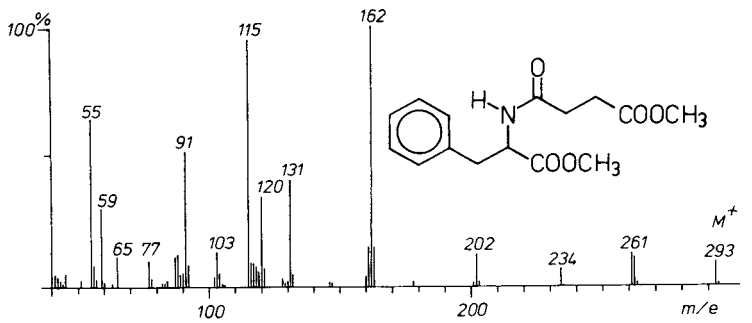
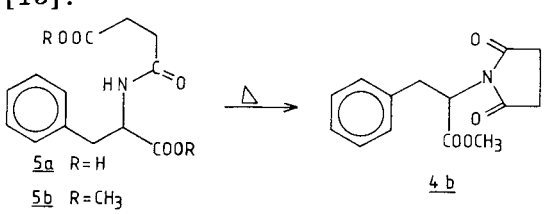


Fig. 9. Mass spectrum of the dimethyl ester of N-succinoylphenylalanine (5b).

Peak matching of the molecular ion gave values in agreement with the molecular formula $C_{14}H_{15}NO_4$. The ion at *m/e* 162 was determined to be $C_{10}H_{10}O_2$. Thus the substituent should be $C_4H_5O_2N$. Succinimide corresponds most likely with this formula. The combination of both parts allows the structure of 4b to be deduced. This assignment was proved by synthesis and comparison of the spectroscopic data and Kovats' indices. In the same fraction, but only in traces, a compound was detected, whose mass spectrum (Fig. 9) resembled strongly that of 4b but which showed a molecular ion 32 mass units higher than that of 4b. The compound was assumed to be the dimethyl ester of the succinoyl derivative of phenylalanine (5b). A synthesis proved this assumption. The imide 4b is probably an artefact of the dimethyl ester 5b, formed in the injector of the gas chromatograph: 5b is partly cyclized to 4b in the gas chromatograph [15].

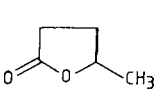


5a R=H
5b R=CH3

4b

γ- and δ-hydroxycarboxylic acids

In a previous paper we reported the detection of compounds in urine, that are characterized by intense ions of mass 85 and 99 in their mass spectra. We tentatively regarded these compounds to be γ- and δ-lactones [1]. The improved separation technique now allowed us to obtain spectra free of by-products, enabling us to recognize even small peaks free from background, important for the deduction of the structure of lactones. Thus, γ-valerolactone



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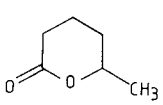

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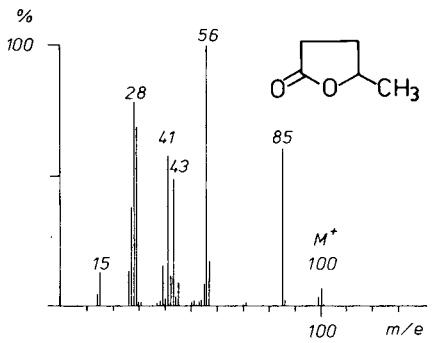


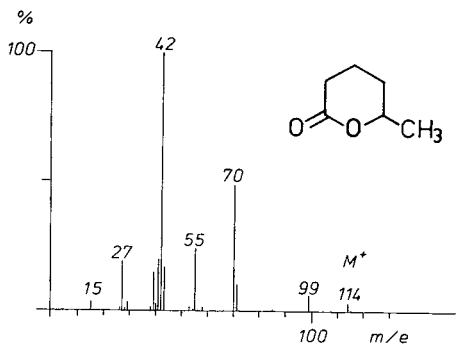
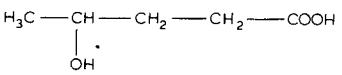


Fig. 10. Mass spectrum of γ-valerolactone.

Fig. 11. Mass spectrum of δ-caprolactone.

(6) (Fig. 10) and δ-caprolactone (7) (Fig. 11) could be identified by comparison with mass spectra already published [16, 17]. These lactones are probably not originally present in urine, but are produced from the corresponding hydroxy compounds 8 and 9 by acidifying the urine sample.



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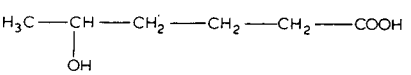
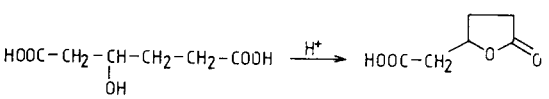


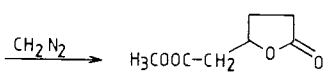
9

The occurrence of 5-hydroxyhexanoic acid has been reported in two cases of hypoglycaemia, but not in urine of healthy individuals [18].

Further compounds with the typical key ion of mass 85, indicating a γ-lactone, were found in the same fraction of the thin-layer chromatogram. The small amounts of sample prohibited recording spectra free of background. Nevertheless, we succeeded in identifying one of these compounds, 3-hydroxyadipic acid (10), which is converted obviously to 5-oxo-tetrahydrofuryl-acetic acid methyl ester (11b) first by treatment with acid to 11a and then by methylation with diazomethane:



10 11a



11b

The mass spectrum of 11b (Fig. 12) does not show a molecular ion; the ions of highest masses correspond to loss of H_2O and CO. The spectrum is further characterized by loss of $\cdot OCH_3$, CH_3OH and CO. The main ion is that of mass 85:

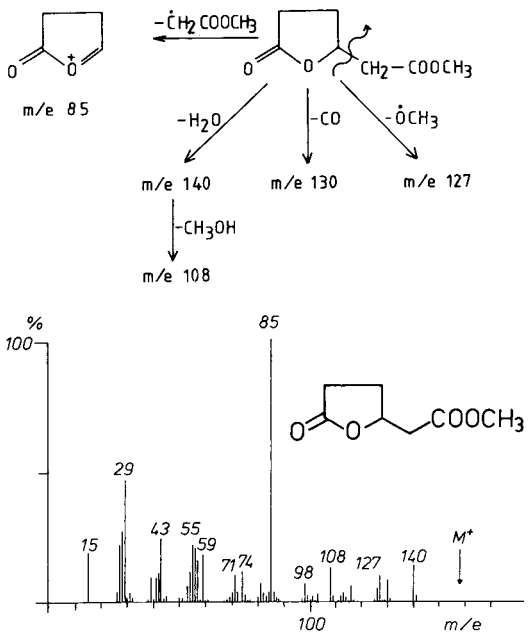


Fig. 12. Mass spectrum of 5-oxo-tetrahydrofurylacetic acid methyl ester (11b).

The validity of the structural assignment could be proved by synthesis. 4-Hydroxyoctanedioic acid, 4-hydroxyundecanedioic acid and 4-hydroxydodecanedioic acid were identified by mass spectra and Kováts' indices too (Table III).

TABLE III

KOVÁTS' INDICES OF SOME γ-LACTONES

No.	RI	Mol. wt.	Substance
1	1280	158	5-Oxotetrahydrofuryl-2-acetic acid methyl ester
2	1871	228	4-Hydroxyundecanedioic acid γ-lactone
3	1968	242	4-Hydroxydodecanedioic acid γ-lactone

GENERAL REMARKS

All the compounds described were found in the urine of healthy male and female adults, aged 20—35 years, living on a normal diet. Since we have not yet investigated profiles of acids in plants we are unable to distinguish between compounds introduced into the body by nutrition and which pass through the body unchanged and those that are produced in the body or are metabolised.

The identification of unknown compounds necessitates in most cases the use of the described combination of chromatographic and spectroscopic techniques. Then, after structure elucidation, identification should be possible by simple low-resolution GC—MS. In some cases even the measurement of the RI using glass capillary columns should allow an identification.

ACKNOWLEDGEMENTS

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CHROMBIO. 1018

VOLATILES OF EXOGENOUS ORIGIN FROM THE HUMAN ORAL CAVITY

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SUMMARY

The volatiles found in the headspace above male and female saliva were examined by combined gas chromatography—mass spectrometry. This has led to the identification of a number of constituents of exogenous origin. The most likely source of these products are atmospheric and water pollutants as well as food stuffs and cosmetic products. Volatiles from saliva represent a potential medium for the detection of reproductive states as well as

local and systemic diseases. Consequently, knowledge of compounds not arising from the body's metabolic process is important to prevent their identification as anomalous metabolites.

INTRODUCTION

Volatile compounds present in breath halitus emitted through the oral cavity provide significant information pertaining to three areas: (a) clinical detection and diagnosis of oral and systemic disorders; (b) the relationship between odor production to menstrual hormonal variations; and (c) the potential for human odor communication. Compounds in expired air have already received attention as a diagnostically important media for measuring pathological changes involving periodontal tissue destruction [1—3]. In addition, characteristic volatiles are reported to occur in the expired air of patients afflicted with various systemic disorders including cirrhosis of the liver [4, 5], uremia [6], diabetes mellitus [7] and large bowel cancer [8].

Anecdotal information as well as scientific reports have evoked considerable speculation concerning the possibility of human chemical communication [9—11]. Previous investigations pertaining to this possibility have conjectured that the vagina and/or the axillae are the odor producing areas of significance [12—16].

Recently, it has been reported from this laboratory that monthly changes in circulating sex steroids found in ovulating females influence the levels of volatile sulfur compounds of mouth air [17]. Subsequently, we have undertaken an investigation of volatiles in the headspace above incubated saliva from cycling females to determine the patterns of other compounds that may also be indicative of ovulation. The initial results of this study identified a number of volatile components, some of which appear to be artifacts ascribed to environmental contaminants, deodorant products and diet. To eliminate misinterpretation, it is important to recognize these exogenous materials in biological samples.

EXPERIMENTAL

Subjects

Volunteers were drawn from among the employees of the Monell Chemical Senses Center as well as the patient populations of the Veterans Administration Hospital and Out-Patient Clinic, the School of Dental Medicine, University of Pennsylvania and the Family Planning Service, Hospital of the University of Pennsylvania, Philadelphia, PA, U.S.A. The patient population consisted of male and female subjects (ages 20—40 years) with good general health.

All female subjects received a complete physical and pelvic examination. They were required to maintain an accurate basal body temperature chart during complete cycles prior to and during sample collection.

The intake of foods which could possibly introduce exogenous sources of compounds (i.e., members of the Alliceae family — garlic, onion, etc., or Cruciferae family — broccoli, cabbage, etc.) were recorded on a daily basis. In

addition, the usage of any medication whether prescription or over the counter, as well as tobacco consumption (frequency and type) were recorded. Male subjects were also requested to record foods, medication and tobacco consumption in the same manner as the female subjects.

Each subject also received a thorough oral exam to determine the health status of oral soft tissues. The oral health status was based on the determination of four universally accepted and highly reproducible indices [18]. Only subjects displaying low index ratings (i.e., good oral health) were allowed to participate in the study.

Salivary collection

Each subject donated a maximum of 10 ml of gum base (polyvinyl acetate, Life Savers Inc.) stimulated saliva within a 10-min interval. Subjects were instructed to abstain each morning prior to sample collection from food, liquid and desist from smoking and exercising oral hygiene. Collected samples were stored at −10° C until instrumental analysis was conducted.

Collection of volatiles

For each analysis, a 5-ml aliquot of saliva was placed into an individual 25-ml, 2-neck, round bottom flask, adapted with PTFE joints and equipped with a nitrogen line and collection tube. Diethyl phthalate was employed as the internal standard and added to each saliva sample prior to the onset of collection. Volatile organic compounds were collected and concentrated from the headspace of saliva, maintained at 37° C, using Tenax (Applied Science Labs., State College, PA, U.S.A.) polymer traps (150 mm × 1.5 mm I.D.). Collection times employed for each sample included two back-to-back collections of 1.5 h followed by one of 21 h. The remaining 5 ml of saliva were frozen at −60° C until needed for duplicate gas chromatography and gas chromatography—mass spectrometry (GC—MS) analyses [3].

Using the above procedure, the salivary volatiles were continuously collected during the indicated periods of incubation. Preliminary work with recovery of various standard mixtures of volatile organic compounds (acetic, propionic, isovaleric, butyric and heptanoic acids; CH_3SSCH_3, pyridine, picolines, benzaldehyde, 3-hydroxy-2-butanone, furfural alcohol, phenol, *p*-cresol, decanol, tetradecanol, indole, diphenylamine) from physiologic saline solutions shows that collection efficiency increases with time and decreases with compound polarity.

Reference compounds

The employed 99% pure internal standard diethyl phthalate was supplied through the courtesy of International Flavors and Fragrances (Union Beach, NJ, U.S.A.). Reagent grade allyl isothiocyanate was obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Nanograde methylene chloride was employed as the solvent in dilution studies with the other reference materials.

GC—MS of incubated saliva headspace

GC—MS was used to study the mixture of organic compounds in the headspace above incubated saliva.

While the first 1.5-h and 21-h samples were generally used for GC—MS analyses, the second 1.5-h sample was employed for calculation of chromatographic peak areas.

The GC—MS system consisted of a Perkin-Elmer 990 gas chromatograph interfaced to a Hitachi RMU-6L mass spectrometer via a Watson-Biemann separator [19]. The separator was set at 270°C, the ionization chamber at 175°C and the ionization voltage was maintained at 70 eV. Mass spectra were scanned from *m/z* 12 to 400 in 6 sec. Identification of all compounds was confirmed by comparison of their mass spectra and chromatographic retention times with those from commercially available samples. Relative GC retention times were obtained by comparison of headspace components in relation to authentic samples with a series of C_2—C_{18} fatty acid ethyl esters to obtain their "ethyl ester index" [20].

Organic materials collected on the Tenax tubes were desorbed from the polymer by rapidly heating the tube to 240°C and maintaining that temperature for 15 min. The organics were transferred and condensed onto the front 15 cm of a chromatographic column which was cooled with dry ice. After desorption was complete, the dry ice was removed, the Tenax tube was removed from the injection port, the carrier flow was resumed through the column and the oven of the chromatograph was brought to its starting temperature of 70°C. The separation was effected on a 3.3 m × 2 mm I.D. Carbowax 20M glass column. Analysis conditions were as follows: injection port 260°C, flame ionization detector 260°C, and a helium carrier gas flow-rate of 40 ml/min. The temperature program employed was initially held at 70°C for 4 min, increased from 70°C to 230°C at 4°/min and the final temperature of 230°C was held for 12 min. The same amplifier sensitivity of $2 \cdot 10^{-10}$ A full scale was employed for each analysis. Peak areas were calculated by triangulation (height × width at half height).

RESULTS

Volatiles found in the salivary headspace of saliva collected from 17 subjects (3 females and 14 males) with essentially normal oral health are shown in Table I. Those compounds which appear to be exogenous are distinguished by an asterisk.

The GC profiles shown in the figures and the compounds listed in Table I are representative of the salivary volatiles seen for males and females with good to moderate oral health. As reported previously, in both males and females, oral degenerative diseases such as periodontitis, yield qualitative and quantitative changes in the salivary volatiles [3]. In addition, preliminary data from the salivary volatiles of the three females discussed also here indicate that quantitative changes occur in these volatiles during the menstrual cycle; however, daily examination of salivary volatiles from males over comparable 30-day intervals has not yet been completed.

Perhaps the most interesting of the compounds in Table I is allyl isothiocyanate which appeared in 5 of 50 saliva samples obtained from one female across two menstrual cycles. Fig. 1 is a typical GC profile of the headspace volatiles from this subject. The average amount of allyl isothiocyanate isolated

TABLE I

COMPOUNDS FOUND IN THE SALIVARY HEADSPACE

All the compounds listed except for heptadecane and an unknown sesquiterpene were also confirmed by their relative chromatographic retention times.

Compounds	Fig. 1	Fig. 2	Identified by mass spectrum
Acetone	A	A	yes
Ethyl alcohol	B	B	yes
Benzene	C	C	yes
Tetrachloroethylene*	D	D	yes**
Toluene*	E	—	yes
C_2—C_4 alkylbenzenes*	—	—	yes**
Dimethyldisulfide	—	F	yes
Styrene*	—	G	yes**
Acetoin	—	H	yes
Limonene	I	—	yes
Allyl isothiocyanate*	J	—	yes
Dimethyltrisulfide	—	K	yes
2-Ethyl-1-hexanol*	L	L	yes
Benzaldehyde	M	M	yes
β-Bourbonene	N	—	yes
β-Caryophyllene	O	—	yes
Heptadecane	P	P	yes**
Unknown sesquiterpene	Q	—	yes**
Naphthalene*	R	—	yes**
Isopropyl dodecanoate*	—	S	yes
Benzyl alcohol	T	T	yes
Phenylethanol	U	—	yes
Butylated hydroxytoluene (BHT)*	V	—	yes**
Dodecanol	W	W	yes
Phenol	X	X	yes
Isopropyl myristate*	—	Y	yes
p-Cresol	Z	Z	yes
Tetradecanol	a	—	yes
Isopropyl palmitate*	—	b	yes
Indole	c	c	yes
Skatole	—	d	yes
Diphenylamine	e	e	yes

*Probable exogenous constituent.
**These compounds compared with literature mass spectrum only, other compounds compared with authentic sample mass spectrum, as well.

from the saliva samples was 124 ± 24 (S.E.M.) ng with a range of 70—200 ng in 10.8 l of headspace. The mass spectra of both authentic allyl isothiocyanate and the compound recovered from a saliva sample are shown in Fig. 2. The probable origin of this component is the gourmet mustard consumed by this subject. Allyl isothiocyanate is a known constituent of mustard seeds [21].

Fig. 3 is a GC pattern from a male subject in whose salivary headspace sample isopropyl esters of C_{14} and C_{16} fatty acids were identified. These esters are known components of deodorant and cosmetic preparations [22], and have

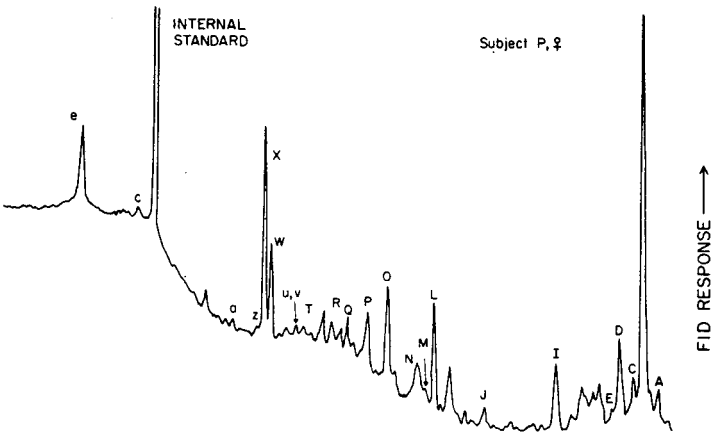

Fig. 1. Gas chromatogram of volatiles collected from the headspace above incubated whole saliva (90 min) of female subject P. This saliva sample was taken on day 15 of the subject's menstrual cycle. Volatiles were collected on Tenax and chromatographed on a 3.3-m 10% Carbowax 20M column with diethyl phthalate as the internal standard. See Table I for peak identification.

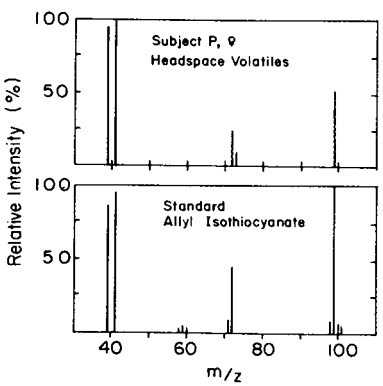

Fig. 2. Mass spectra of allyl isothiocyanate from the salivary volatiles of subject P (top) and reagent grade allyl isothiocyanate (bottom). Mass spectra of both were obtained as the compound eluted into the ion source from a 3.3-m 10% Carbowax 20M column.

been identified in other body fluids [16]. The subject revealed that the saliva sample was collected during the time he was applying a spray deodorant. Consequently, this sample was contaminated from the residual spray.

The aromatic hydrocarbons have been identified by other investigators as man-made emissions in urban air [23—26]. They may also arise, in part, from the Tenax tube traps, since toluene, styrene and alkylbenzenes have been occasionally observed in our blanks.

Butylated hydroxytoluene has been previously reported in saliva [27] and other body fluids [28]. It is an antioxidant used in a variety of foods. This compound has been sporadically observed in our samples.

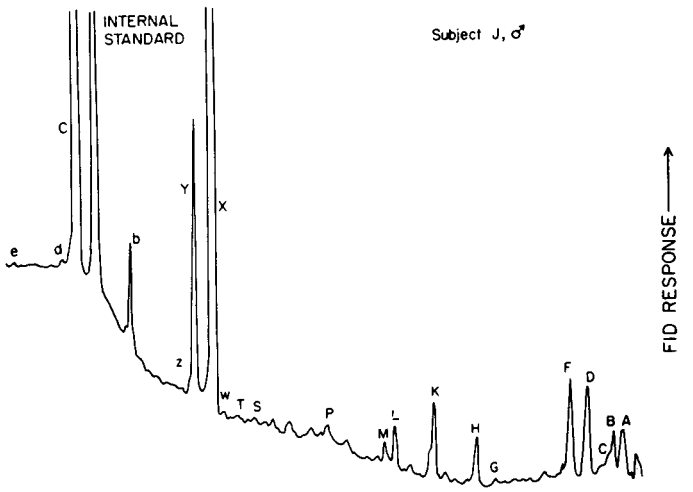

Fig. 3. Gas chromatogram of volatiles collected from the headspace above incubated whole saliva (90 min) of male subject J. Volatiles were collected on Tenax and chromatographed on a 3.3-m 10% Carbowax 20M column with diethyl phthalate as the internal standard. See Table I for peak identification.

The presence of tetrachloroethylene in Philadephia drinking water has been demonstrated by the EPA as well as our own analyses. This compound is also commonly found in urban air samples [23]. In addition, 2-ethyl-1-hexanol, a plasticizer, has been reported in body fluids [16].

DISCUSSION

The volatiles emitted by humans are gaining in importance as potential diagnostic aids [29]. However, a large number of exogenous organic compounds gain access to the body each day, some of which are stored by and only slowly expelled in secretions and excretions. Consequently, impurities make up some of the volatiles that are profiled by current headspace and GC—MS techniques.

Previous studies of volatiles produced within the oral cavity have employed small aliquots of mouth air for analysis [2, 30, 31]. These studies sought the nature of oral metabolites as well as those responsible for oral malodors. In the latter case, volatile sulfur compounds are involved in oral malodor and a gas chromatograph specifically equipped with a flame photometric detector is the method of choice for isolating and detecting the low levels of these compounds [1, 2]. In order to exploit the full potential of saliva as a diagnostic medium in the area of detection of reproductive and pathologic states, it is important to investigate a wide array of organic constituents present in healthy individuals. In our research, it has been found that part of the normal profile consists of organic constituents from diverse sources, e.g., urban air, drinking water, diet and cosmetic preparations.

Pollutants from man-made emissions in urban air, as well as chlorinated organic constituents found in municipal drinking water, have received considerable attention in the popular press because of their potential

detrimental health effects. The organic compounds identified in this study (i.e., aromatic hydrocarbons, tetrachloroethylene, etc.) have also been found in volatiles from plasma and axillary secretions [32—34, 16].

The role of diet as it affects the volatiles being profiled using GC—MS techniques has recently been reviewed [29]. Notable for the odors that they impart to the body, breath and urine are members of Alliceae (i.e., garlic, onions, etc.) and Cruciferae (cauliflower, cabbage, broccoli, etc.). Mustard is a member of the Cruciferae family and it appears that the occurrence of allyl isothiocyanate in our sample was directly related to the subject's preference for gourmet mustard*. In addition, this compound has been seen only on one other occasion in hundreds of other saliva samples analyzed in our laboratories.

Another constituent which appears clearly dietary in origin is butylated hydroxytoluene (BHT). Approximately 0.1 mg/kg body weight of phenolic antioxidants are consumed daily by man. Moreover, BHT can be metabolized by the human body and has been shown to inhibit the activation of certain carcinogens [35, 36].

The presence of the isopropyl esters was related, in our study, to the chance spraying of deodorant in the presence of the saliva being collected. However, in another study involving the analysis of secretions from the axillae, these compounds were present even after 16 days of not using any scented soaps or deodorant [16]. Since we have not seen these esters in detectable quantities in headspace of any other samples, they may not be absorbed into plasma, and hence into saliva, in any appreciable quantity. That saliva does reflect plasma levels of certain organic constituents has recently been shown [27]. In addition, a number of systemic diseases are manifested directly in the oral cavity by increased exfoliation of mucosal cells, ulceration and alterations of bacterial populations [37, 38]. Consequently, ongoing studies may show saliva to be an ideal sample which can be collected in a non-invasive manner and provide insight into body function.

ACKNOWLEDGEMENTS

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QUANTITATIVE GAS CHROMATOGRAPHY—CHEMICAL IONIZATION MASS SPECTROMETRY OF 2-KETOGLUTARATE FROM URINE AS ITS O-TRIMETHYLSILYL-QUINOXALINOL DERIVATIVE

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SUMMARY

Quantitation of 2-ketoglutarate in urine as its O-trimethylsilyl-quinoxalinol derivative by gas chromatography—chemical ionization mass spectrometry is described. This technique, with ammonia as reactant gas, produces no fragmentation and allows only the detection of the protonated molecular ion. It gives the greatest known sensitivity, and could be applied to the determination of urinary 2-ketoglutarate in normal children and in various metabolic disorders, such as dihydrolipoyl dehydrogenase defect, pyruvate carboxylase deficiency and type I glutaric acidemia.

INTRODUCTION

2-Ketoglutaric acid is a normal metabolite of amino acids such as lysine, hydroxylysine and tryptophan. Its concentration in cells and serum results from a steady-state between three pathways: (1) in the citric acid cycle, production by the reaction of isocitrate dehydrogenase and degradation to succinyl-CoA by the reaction of 2-ketoglutarate dehydrogenase; (2) formation during the oxidative deamination of amino acids by the reaction of glutamate dehydrogenase; and (3) transamination from glutamate as part of the malate—aspartate cycle.

Changes of 2-ketoglutarate concentration in serum or urine may result from disorders involving those metabolic pathways, and especially increased values were observed in the blood of patients bearing tumors of various organs [1, 2] and in urines of patients presenting type I glycogenosis [3, 4] and lactic acidosis [5, 6].

Because of the unstable nature of the free acid, most analytical methods

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have utilized derivatization, with, for instance, 2,4-dinitrophenylhydrazine [7, 8], ethoxyamine [9] or *o*-phenylenediamine [10—13]. The most sensitive method, described by Langenbeck [13], consisted of gas chromatographic (GC) quantitation of the O-trimethylsilyl-quinoxalinol derivative with a nitrogen-selective detector; but this derivative showed considerable non-linearity if less than 250 pmol were injected on to the column.

Our purpose was to establish a more sensitive and reliable method of 2-ketoglutarate quantitation by *o*-phenylenediamine derivatization (Fig. 1) and analysis by gas chromatography—chemical ionization mass spectrometry.

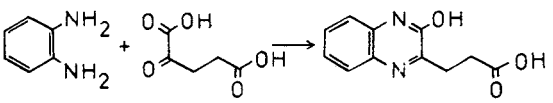


Fig. 1. Chemical structure of the quinoxalinol derivative of 2-ketoglutaric acid: 3-(2'-hydroxycarbonylethyl)-2-quinoxalinol.

EXPERIMENTAL

Reagents

All solvents and reagents were of analytical grade and used without further purification; 2-ketoglutaric acid (monosodium salt) and benzoylformic acid were purchased from Sigma (St. Louis, MO, U.S.A.). Ethyl acetate, chloroform, pyridine, diethyl ether, methylene chloride and *o*-phenylenediamine were obtained from Fluka (Buchs, Switzerland) and bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) from Pierce (Rockford, IL, U.S.A.). The OV-101 liquid phase was purchased from Varian (Palo Alto, CA, U.S.A.). Chromosorb W AW DMCS (80—100 mesh) from Merck (Darmstadt, G.F.R.) and the CPSil 5 capillary column from Chrompack (Middelburg, The Netherlands).

Derivatization

Urine samples were, whenever possible, 24-h collections, and were kept frozen at −80°C. Creatinine concentrations were determined by the automated Jaffé method [14].

For the derivatization of 2-ketoacids in standard solutions or in urine, we utilized our previously described method [15]: 0.1 ml of standard solution or urine and 0.1 ml of internal standard solution (1 μg ml^{-1} benzoylformic acid) was heated for 1 h at 90°C in a Reacti-Therm (Pierce) with 0.2 ml of a 1% solution of *o*-phenylenediamine in 4 *M* hydrochloric acid. The pH of the solution was adjusted to 1 ± 0.1 by addition of 5 *M* sodium hydroxide and extracted twice with 1.5 ml of ethyl acetate. The two extracts were collected and dried for 2 h over anhydrous sodium sulphate and evaporated to dryness at room temperature under a gentle stream of nitrogen. The residue was taken up in 0.1 ml of a (BSTFA + TMCS)—pyridine (1:1) mixture and heated at 90°C for 30 min. One microlitre of the solution was applied on the Ros injector.

Instruments

GC analyses were performed on a Varian Model 3700 gas chromatograph with 10% OV-101 as stationary phase, coupled with a CDS 111 integrator (Varian). The elution conditions for 2-ketoglutarate and benzoylformate derivatives were: 2 min isothermally at 220°C and linear temperature programme from 220°C to 270°C at 4°C/min, injector and flame ionization detector temperatures at 250°C, and carrier gas (nitrogen) at 26 ml min^{-1}.

Analyses by gas chromatography—mass spectrometry (GC—MS) were performed on a quadrupole Ribermag (Rueil-Malmaison, France) R 10-10 B mass spectrometer coupled with a Girdel (Suresnes, France) Model 31 gas chromatograph and a PDP 8a computer with a Sidar data system (Ribermag). The 2-ketoacid derivatives were resolved on a capillary glass column (25 m × 0.25 mm) coated with CPSil 5. The injector was an all-glass Ros type (Girdel). Injector and column temperatures were 250°C and 220°C, respectively, and the carrier gas was helium at a flow-rate of 1.5 ml min^{-1}. The mass spectrometer was run at 70 eV with an ion energy of 6V, a multiplier voltage of 1.8—2.5 kV and an emission current of 0.2 mA. Chemical ionization mass spectra were obtained using ammonia at 10^{-4} Torr.

Quantitation by Sidar data system

For automatic quantitation, the Sidar system programme previously described [15] was used by measuring the chromatographic peak areas at *m/z* 363.30 and 295.10 for the respective O-trimethylsilyl-quinoxalinol derivatives of 2-ketoglutarate and benzoylformate (internal standard).

RESULTS

Reinvestigation of 2-ketoglutarate derivative extraction

The literature was conflicting for the extraction of the quinoxalinol derivative of 2-ketoglutarate [11, 12] and, according to Hoffman and Haustein [11], ethyl acetate was found to be the best extraction solvent compared to chloroform, methylene chloride or diethyl ether (Table I). This extraction procedure

TABLE I

EXTRACTION OF 2-KETOGLUTARATE (KG) AND BENZOYLFORMATE (BF) DERIVATIVES BY DIFFERENT SOLVENTS

Recoveries of these two derivatives are expressed as percentage recovery in the best solvent (ethyl acetate).

	KG/C_{19}*	Recovery of KG (%)	BF/C_{19}**	Recovery of BF (%)
Diethyl ether	1.33 ± 0.05	83	1.48 ± 0.04	83
Ethyl acetate	1.60 ± 0.03	100	1.78 ± 0.03	100
Chloroform	0.43 ± 0.04	27	1.60 ± 0.03	90
Methylene chloride	0.30 ± 0.02	19	1.57 ± 0.06	88

*KG/C_{19} = ratio of peak area of 2-ketoglutarate derivative to that of nonadecane.
**BF/C_{19} = ratio of peak area of benzoylformate derivative to that of nonadecane.

was tested by analysing three duplicates for each solvent and measuring the chromatographic peak areas of 2-ketoglutarate and benzoylformate derivatives (at a concentration of 1 mg ml^{-1}) compared to the area of nonadecane added to the (BSTFA + TMCS)—pyridine mixture at a concentration of 750 μg ml^{-1}.

The extraction of 2-ketoglutarate derivative as a function of pH was also investigated, the optimal extraction pH appearing to be 1 ± 0.1 as shown in Fig. 2.

Typical chromatogram of standard solution

Fig. 3 shows a typical mass chromatogram of O-trimethylsilyl-quinoxalinol derivatives of 2-ketoglutarate and benzoylformate with single-ion monitoring (SIM) optimized, respectively, on the masses at *m/z* 363.30 and 295.10.

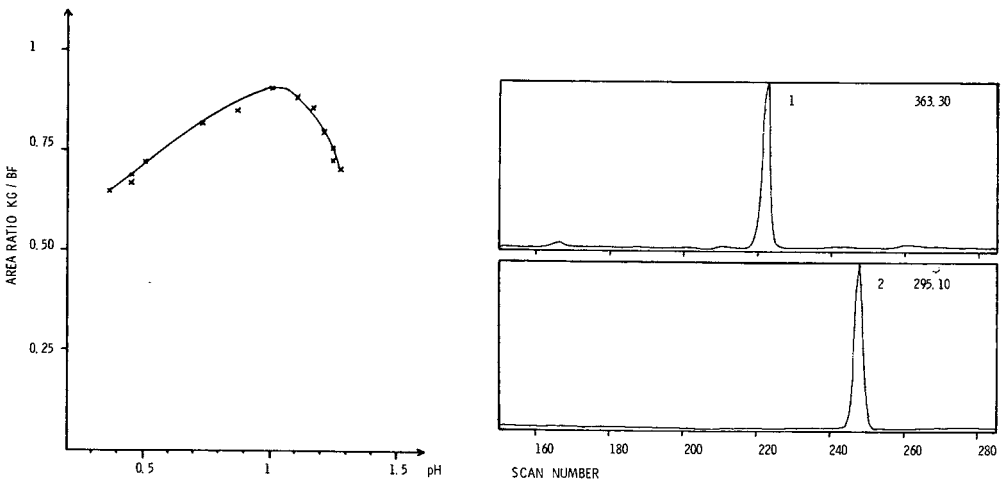


Fig. 2. Extraction of 2-ketoglutarate derivative (KG) with ethyl acetate as a function of pH. The extraction of benzoylformate derivative (BF) is independent of pH.

Fig. 3. Typical mass chromatogram of a standard solution at a concentration of 250 ng ml^{-1} for each product: 1 = derivative of 2-ketoglutarate; 2 = derivative of benzoylformate.

Mass spectra of O-trimethylsilyl-quinoxalinol derivatives

The two derivatives exhibit similar mass spectra with only the protonated molecular ion (MH^+) due to the great affinity of proton for the quinoxalinol structure (Fig. 4); the ammonium adducts were not detected. The electron impact mass spectrum of 2-ketoglutarate derivative was identical to the spectrum reported by Langenbeck et al. [16], the electron impact mass spectrum of the benzoylformate derivative being shown for comparison in Fig. 5. In the latter spectrum, the fragments at *m/z* 63, 90, 102 and 117 correspond to structures 1, 2, 3 and 4 (Fig. 6) proposed by Kovacik et al. [17], and the fragment at *m/z* 217 to structure 5 [16]. Structures 6, 7, 8 and 9 are proposed for the respective fragmentations at *m/z* 132, 203, 205 and 221 (Fig. 6).

Calibration curve

The calibration curve was automatically computed by the Sidar system after

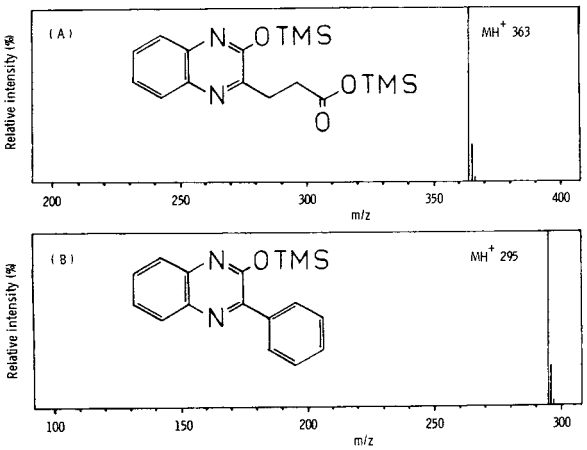


Fig. 4. Chemical ionization (ammonia) mass spectra of (A) 2-O-trimethylsilyl-3-(2'-O-trimethylsilyl-carbonylethyl)-quinoxalinol (derivative of 2-ketoglutarate), and (B) 2-O-trimethylsilyl-3-phenyl-quinoxalinol (derivative of benzoylformate).

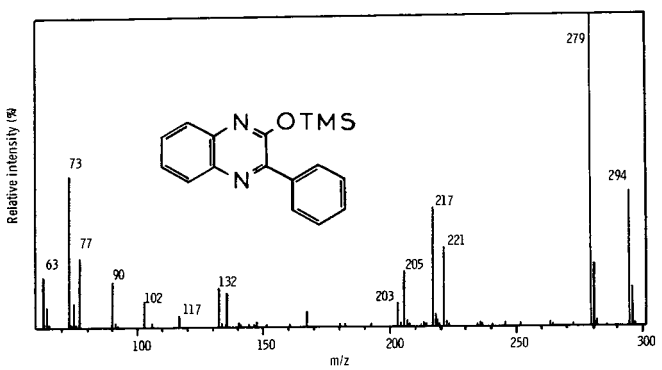
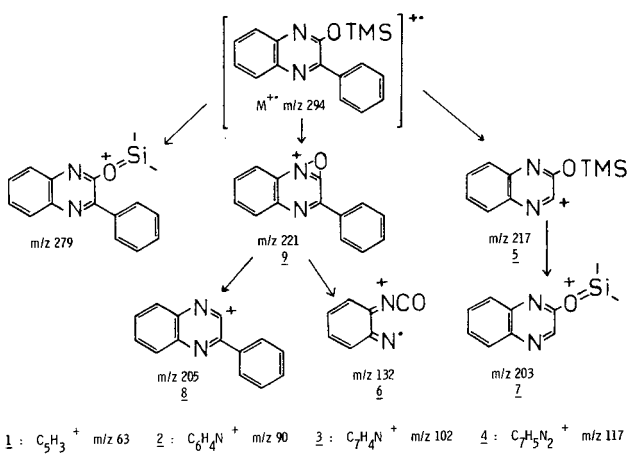


Fig. 5. Electron-impact mass spectrum of benzoylformate derivative.



1 : $C_5H_3^+$ m/z 63 2 : $C_6H_4N^+$ m/z 90 3 : $C_7H_4N^+$ m/z 102 4 : $C_7H_5N_2^+$ m/z 117

Fig. 6. Fragmentation pattern of benzoylformate derivative.

injection of six standard solutions in a concentration range from 50 ng ml^{-1} to 20 μg ml^{-1}. The obtained equation of the curve was $y = 0.26x + 0.1235$, and the correlation coefficient 0.99996. The limit of detection was in the order of 1 pg injected on to the column; in this case, the signal-to-noise ratio was 3.4.

Determination of urinary 2-ketoglutarate

The detection limit in urine as in standard solution was 7 pmol/ml (1 ng ml^{-1}). Forty urine samples of children from two months to ten years old were analysed by this method; the normal excretion was found to be 125 ± 100 μmol l^{-1} or 60 ± 55 mmol/mole of creatinine.

Twenty urines from a child (F.L.) who presented a dihydrolipoyl dehydrogenase defect were analysed, and 2-ketoglutarate excretion was found to be in the range of 820—2430 mmol/mole of creatinine.

An irregular accumulation of this product was also observed in five cases (T.K., D.K., S.P., C.E. and M.K.) of pyruvate carboxylase defect, and in this metabolic disorder the excretion was in the range 340—2200 mmol/mole of creatinine. Phenyllactic acid was also elevated in all the analysed urine samples of pyruvate carboxylase defects, but its concentration is not reported here.

Finally, 2-ketoglutarate was quantitated in different urine samples of a child (B.M.) who presented a type I glutaric acidemia [18]; the range was 220—930 mmol/mole of creatinine.

Accuracy and precision

The accuracy and precision of the method were determined by analysing standard solutions. Six samples from the same solution were analysed; the accuracy of the method was in the order of 2%. Each sample was analysed six times and the measured precision of the instrumentation was 3%.

Recovery of 2-ketoglutarate derivative from urine

The recovery of 2-ketoglutarate derivative was determined by analysing urines to which standard solutions of 2-ketoglutarate (in the range 200 pg to 20 ng) had been added. Each sample was analysed three times and the results obtained are shown in Table II.

TABLE II

RECOVERY OF 2-KETOGLUTARATE (KG) FROM URINE

Urinary concentration of KG (pg per 100 μl)	Added KG in 100 μl (pg)	Urinary theoretical concentration (pg per 100 μl)	Urinary measured concentration (pg per 100 μl)	Recovery (%)
7500	200	7700	7620 ± 110*	99 ± 1.4
7500	500	8000	7760 ± 180	97 ± 2.2
7500	1000	8500	8840 ± 150	104 ± 1.8
7500	5000	12,500	12,630 ± 230	101 ± 1.8
7500	20,000	27,500	26,670 ± 510	97 ± 1.8

*Mean ± S.D.

DISCUSSION

Various internal standards have been used for 2-ketoglutaric acid quantitation: 2,6-dimethylphenol, *p*-propylphenol [10] and trimellitic acid [8] in high-performance liquid chromatography (HPLC), undecyl-, tetradecyl- and hexadecylcyanides [11], *p*-nitrophenylphenyl ether [19] or 2-ketovaleric and 2-ketocaproic acids [12] in GC. In our opinion, only a structure analogous to 2-ketoglutarate such as 2-ketovalerate should be retained. However, this product is not suitable for 2-ketoglutarate quantitation because of its very different retention time. Thus, another 2-ketoacid, benzoylformic acid, was preferred since this compound has a suitable retention time, and it is not physiological: duplicate runs were performed in which no internal standard was added to six urine samples, and no benzoylformate was detected.

The normal excretion of 2-ketoglutarate in urine found by our technique was intermediate between the values of Liao et al. [10] and those of Langenbeck et al. [12]. However, the former authors did not utilize a structural analogue, and the latter reported values for adults.

The deficiency of dihydrolipoyl dehydrogenase (E_3 component of the pyruvate dehydrogenase complex) is also characterized by a decreased activity of 2-ketoglutarate dehydrogenase in all tissues [20], as a result of the probable identity of the E_3 components of pyruvate and 2-ketoglutarate dehydrogenase complexes [21]. However, other mechanisms may be involved in the secondary deficiency of 2-ketoglutarate dehydrogenase activity during metabolic defects. Thus, glyoxylic acid [22] and branched-chain 2-ketoacids [23, 24] inhibit 2-ketoglutarate dehydrogenase with a K_i of around 1.5 mmol l^{-1}, and their serum concentrations may exceed this value for K_i as, for instance, during maple syrup urine disease [25]. Such a mechanism might apply to pyruvate carboxylase deficiencies, which present a urine accumulation of glyoxylic and branched-chain 2-ketoacids [15].

The explanation of 2-ketoglutarate elevation in type I glutaric acidemia is more difficult; however, it is possible to relate this increase to a disturbance in the metabolism of glutamate and 4-aminobutyric acid. Leibel et al. [26] have observed a decreased activity of glutamate decarboxylase and low levels of 4-aminobutyrate in the brain of a child presenting this disease. Glutaric acid inhibits glutamate decarboxylase [26, 27], and is also a strong inhibitor of glutamate dehydrogenase [28]. In this hypothesis, the 2-ketoglutarate accumulation might be secondary to an elevation of glutamate by inhibition of glutamate decarboxylase and of glutamate dehydrogenase.

CONCLUSION

The utilization of the gas chromatography—chemical ionization (ammonia) mass spectrometry procedure applied to the O-trimethylsilyl-quinoxalinol derivative of 2-ketoglutarate allows this product to be quantitated in biological fluids such as urine with the greatest known sensitivity. This method is very specific and reliable, as compared to detection by a nitrogen-selective detector in GC analysis [13], or HPLC [10]. Thus, it is possible to direct the diagnosis of some metabolic disorders by quantitation of 2-ketoglutarate as well

as of pyruvate and other aliphatic 2-ketoacids [15], and to make these two measurements in the same urine extract by utilising a mixture of 2-ketovalerate and benzoylformate as internal standards, and proceeding at two different elution temperatures. The derivatization technique described here may be applied to determinations of 2-ketoglutarate and its homologue, 2-ketoadipate, in deproteinized plasma; this work is now in progress.

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CHROMBIO. 1013

GAS CHROMATOGRAPHIC—MASS FRAGMENTOGRAPHIC DETERMINATION OF HOMOPANTOTHENIC ACID IN PLASMA

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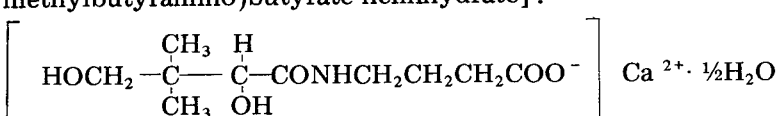
SUMMARY

A gas chromatographic—mass fragmentographic method was developed for the determination of homopantothenic acid in plasma. Acidified plasma was deproteinized by extraction with chloroform and subsequently the aqueous layer was extracted with ethyl acetate. The organic layer containing homopantothenic acid was reduced to dryness, and the resulting residue was redissolved in N,O-bis(trimethylsilyl)trifluoroacetamide—pyridine solution to allow trimethylsilylation. Aliquots of this solution were injected into the gas chromatograph—mass spectrometer and analyzed by the selected ion monitoring method using L-ascorbic acid as an internal standard. The detection limit for homopantothenic acid was 5 ng/ml of plasma.

A precise and sensitive assay for the determination of homopantothenic acid in plasma was established.

INTRODUCTION

Homopantothenic acid, which is a derivative of γ-aminobutyric acid, was first discovered in nature by Biserte et al. in 1955 [1]. It is known that homopantothenic acid improves the metabolism of glucose in brain and the higher functions of the brain [2—7]. This compound has been used clinically as a calcium salt, calcium hopantenate [calcium D-(+)-4-(2,4-dihydroxy-3,3-dimethylbutyramino)butyrate hemihydrate].



$C_{20}H_{36}CaN_2O_{10} \cdot \frac{1}{2}H_2O$

A colorimetric method for the assay of homopantothenic acid has been reported [8] in which homopantothenic acid is hydrolyzed to γ-aminobutyric acid in an alkaline solution and reacted with sodium 1,2-naphthoquinone-4-sulfate, then determined colorimetrically. This method, however, was not found to be sensitive enough for the determination of homopantothenic acid in plasma after administration of calcium hopantenate.

Therefore, we investigated the determination of homopantothenic acid in plasma by two methods, gas—liquid chromatography (GLC) with a flame-ionization detector and gas chromatography—mass fragmentography (GC—MF). The former method was not adequate because of poor sensitivity, while the second method provided high precision and sensitivity. This paper describes these results.

EXPERIMENTAL

Materials

Calcium hopantenate was synthesized and purified in our laboratory [9, 10]. N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) and pyridine for the silylating solvent were purchased from Pierce (Rockford, IL, U.S.A.). L-Ascorbic acid and the other chemicals used were obtained from Wako Pure Chemicals (Osaka, Japan). Ethyl acetate and chloroform were liquid-chromatography grade materials.

The samples of blood were collected from healthy men.

Instrumental

For GC—MF, a JEOL JMS D-300 mass spectrometer with an electron-impact ion source connected to a JEOL JGC-20kP gas chromatograph (Tokyo, Japan) was used.

The coiled glass column (1.0 m × 2.0 mm I.D.) of the gas chromatograph was packed with 3% OV-17 on Chromosorb W AW (80—100 mesh) (Gaschro Kogyo Co., Tokyo, Japan) and conditioned at 280°C for 24 h. The injector, column and ion source temperatures were 250°C, 220°C and 230°C, respectively. After homopantothenic acid and its internal standard had been detected, the temperature of the column was raised to 280°C for 3 min to burn out the remaining materials and then was returned to the operational temperature before the next analysis. The carrier gas was helium and the flow-rate was 30 ml/min.

The mass spectrometer was used under the following conditions: ionization energy, 70 eV; ionization current, 300 μA; accelerating voltage, 3.0 kV; ion multiplier voltage, 1.4 kV. The fragment ions selected for mass fragmentographic analysis were the ions at *m/z* 434 and 449, which are produced from the molecular ion by the loss of a methyl group, $[M-CH_3]^+$, for the respective trimethylsilyl derivatives of homopantothenic acid and the internal standard, L-ascorbic acid.

The mass spectra of these derivatized compounds were recorded under the same conditions as described above.

For GLC, a Shimadzu GC4-CM gas chromatograph with a flame-ionization detector (Kyoto, Japan) was used.

The conditions of the gas chromatograph were as follows: a coiled glass column (1.0 m × 3.0 mm I.D.) packed with 3% OV-17 on Chromosorb W AW (80—100 mesh); injector and detector temperatures of 280°C; an initial column temperature of 170°C and a temperature rise of 6°C/min to 290°C; flow-rate of nitrogen as carrier gas, 60 ml/min.

Analytical procedure

Blood samples were collected in heparinized containers and centrifuged to separate the plasma.

The plasma (1.0 ml) was diluted to 2.0 ml with distilled water, adjusted to pH 2.0 with 5 *N* HCl and shaken vigorously with 20 ml of chloroform at room temperature for 10 min. The aqueous layer was separated, neutralized with sodium hydroxide solution followed by the addition of 1.0 ml of distilled water, and centrifuged at 2000 *g* for 10 min to remove proteins. The supernatant was readjusted to pH 2.0 with 5 *N* HCl and was shaken vigorously with 40 ml of ethyl acetate for 20 min. The ethyl acetate layer containing homopantothenic acid was separated, evaporated at 30°C, transferred to a 1.0-ml reaction vial by washing with methanol and concentrated to dryness in a water-bath under nitrogen gas. Then the residue was mixed with methanol containing 10.0 μg of L-ascorbic acid as internal standard and concentrated to dryness again under the same conditions as described above. The residue was dried thoroughly over phosphorus pentoxide under reduced pressure and subjected to trimethylsilylation at 70°C for 20 min by the addition of a freshly prepared solution of 100 μl of pyridine containing 20% BSTFA. After cooling, 1—2 μl of this solution were injected into the GC—MF apparatus.

Calibration curve

A calibration curve for homopantothenic acid analyzed by the GC—MF method was prepared by adding known amounts of calcium hopantenate (0.05, 0.10, 0.50, 1.00, 2.00, 5.00 and 10.0 μg/ml) to plasma (1.0 ml) and then analyzing by the same extraction procedure.

The calibration curve was obtained by plotting the ratio of the peak height of the trimethylsilyl derivative of homopantothenic acid to that of the trimethylsilyl derivative of L-ascorbic acid as an internal standard against concentration. This calibration curve was linear.

RESULTS AND DISCUSSION

The investigation of the extraction procedure for calcium hopantenate from the aqueous solution using several kinds of organic solvents showed that this compound could be recovered quantitatively from aqueous solution acidified with hydrochloric acid with ethyl acetate, in its free form — homopantothenic acid. Therefore, the following procedure was employed. The samples of plasma were deproteinized by extraction with chloroform under acidic conditions and the supernatant was extracted with ethyl acetate. This procedure was found to be the simplest and most rapid, and to be the most reliable with the highest recovery of homopantothenic acid. Furthermore, it was found that homopantothenic acid was not tranferred to the chloroform layer under the

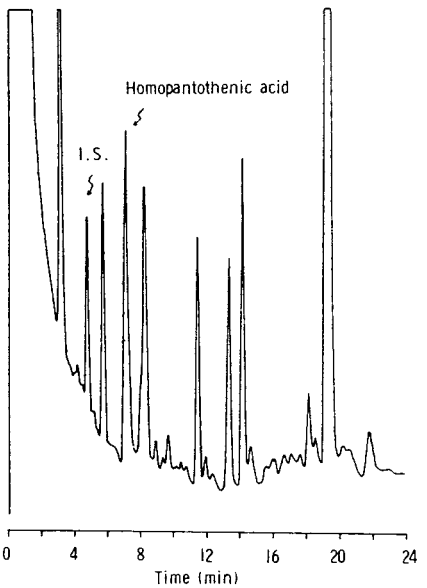


Fig. 1. Gas chromatogram showing the separation of homopantothenic acid extracted from human plasma with L-ascorbic acid as internal standard (I.S.). The result is for the trimethylsilyl derivative.

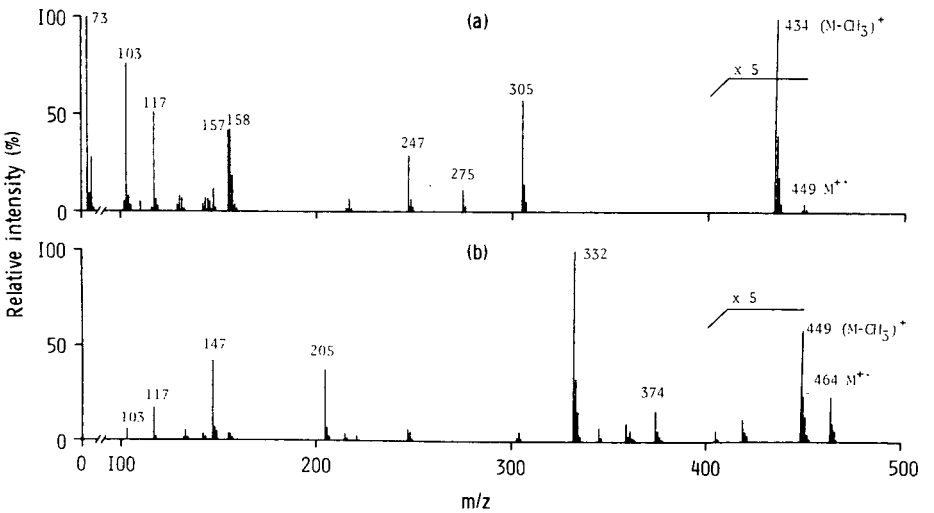


Fig. 2. Mass spectra of the trimethylsilyl derivatives of (a) homopantothenic acid and (b) L-ascorbic acid.

acidic condition described above.

On the basis of these results, a method for assay of homopantothenic acid was investigated for the subsequent experiments.

The GLC method was examined first for the determination of homopantothenic acid extracted from human plasma. To obtain a derivative of homopantothenic acid suitable for GLC, the procedures of trimethylsilylation

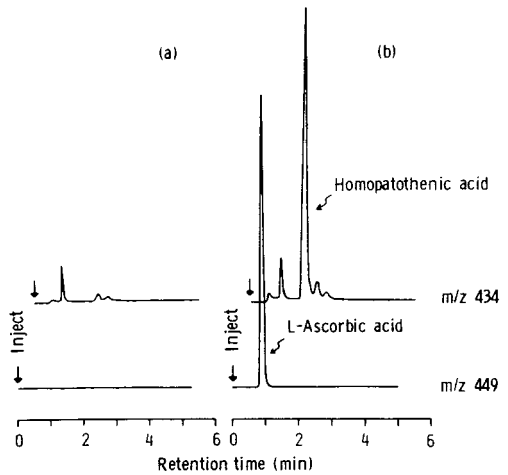


Fig. 3. Mass fragmentograms showing the separation of (a) control and (b) homopantothenic acid extracted from human plasma with L-ascorbic acid as internal standard. Results are for the trimethylsilyl derivatives.

with BSTFA or N,O-bis(trimethylsilyl)acetamide, and those of methylation with diazomethane or an on-column methylating agent (2% phenyltrimethylammonium hydroxide in methanol) were compared. The trimethylsilylation procedure with BSTFA was found to result in a quantitative reaction and a higher detection sensitivity. On the other hand, L-ascorbic acid was chosen as internal standard since it can be trimethylsilylated in the same way as homopantothenic acid. These trimethylsilyl derivatives were found to be stable at 5°C for 1—2 days.

The GLC method based on trimethylsilylation gave a good separation of homopantothenic acid from human plasma components, as shown in Fig. 1. The retention times of the trimethylsilyl derivatives of homopantothenic acid and the internal standard L-ascorbic acid were 7.2 and 4.8 min, respectively. The detection limit of homopantothenic acid in this GLC method was 1.0 μg/ml of plasma. This sensitivity is poor and not suitable for the assay of the plasma levels of homopantothenic acid found after administration of calcium hopantenate.

Next, the method for assay of trimethylsilyl homopantothenic acid by GC—MF was investigated. L-Ascorbic acid was also used as internal standard for the determination using the multiple-ion detection technique. The mass fragment ions detected for GC—MF were the $[M—CH_3]^+$ ion at *m/z* 434 and 449 in the mass spectra of the trimethylsilyl derivatives of homopantothenic acid and L-ascorbic acid, respectively (Fig. 2), since their ions at *m/z* 305 and 332, which are present in higher intensity than each $[M—CH_3]^+$ ion, were not separated clearly in some samples. In addition, the ions at *m/z* 449 are present in the spectra of L-ascorbic acid and homopantothenic acid, but since the two compounds were well separated by GC, there is no interference in the quantitation.

The GC—MF separation pattern of the trimethylsilyl derivative of homo-

TABLE I

RECOVERIES ON EXTRACTION OF HOMOPANTOTHENIC ACID FROM PLASMA

Each value is the mean of three determinations.

Added (μg/ml)	Recovery from plasma (%)
0.05	90.8
0.10	89.9
0.50	88.7
1.00	90.1
2.00	89.8
5.00	89.6
10.00	89.6
Mean ± S.D. (%)	89.7 ± 3.2

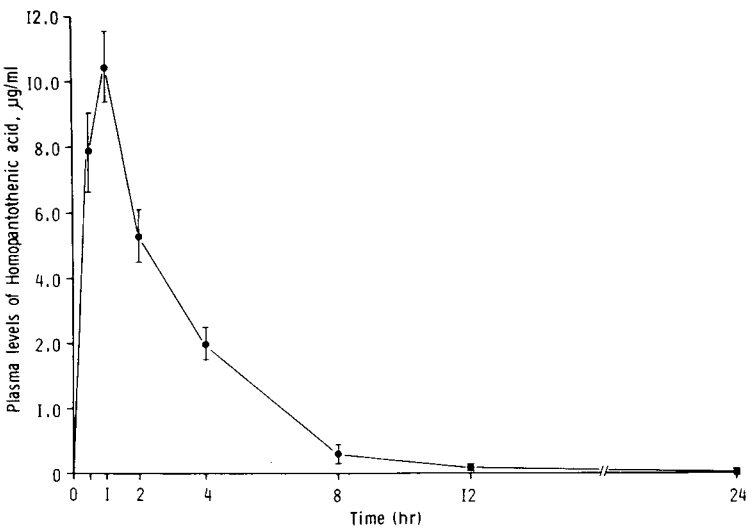


Fig. 4. Plasma levels of homopantothenic acid after oral administration of calcium hopantenate in a dose of 750 mg to ten healthy volunteers. Each point is the mean ± S.D. of ten men.

pantothenic acid extracted from human plasma following addition of 0.10 μg/ml calcium hopantenate and that of a control human plasma extract are shown in Fig. 3. The retention times of homopantothenic acid and the internal standard as their trimethylsilyl derivatives were 2.2 and 0.8 min, respectively.

The detection limit for homopantothenic acid in this GC—MF method was 5 ng/ml of plasma, which was sufficiently high. The reproducibility of the method was ± 3.1%. Thus, the present method appears to be satisfactory for the determination of homopantothenic acid in plasma.

In addition, γ-aminobutyric acid had no appreciable influence on the measurement of homopantothenic acid with the present method, because the retention time of γ-aminobutyric acid (0.6 min) was different from that of

homopantothenic acid. On the other hand, homopantothenic acid was not decomposed to γ-aminobutyric acid under the procedure described above.

Known amounts of calcium hopantenate were added to human plasma, and then the recovery of homopantothenic acid was determined. As shown in Table I, the overall recovery of homopantothenic acid was 89.7 ± 3.2%.

Furthermore, the following experiments were conducted. The stability of calcium hopantenate in the freezed-stocked plasma and that of homopantothenic acid in the cooled-stocked extract of plasma were examined. No significant decomposition was observed.

Finally, a 750-mg dose of calcium hopantenate was administered orally to healthy volunteers, and the concentration of homopantothenic acid in the plasma was determined by the present GC—MF method. The results obtained are shown in Fig. 4.

The present method also can be applied to plasma of animals. The results obtained for the chromatographic separation, recovery, precision and sensitivity were in good agreement with those obtained with human plasma.

The present GC—MF method is precise and has a higher sensitivity than the colorimetric or GLC method.

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CHROMBIO. 1019

ANALYSIS OF THE MONOSACCHARIDE COMPOSITIONS OF TOTAL NON-DIALYZABLE URINARY GLYCOCONJUGATES BY THE DITHIOACETAL METHOD

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(Received April 21st, 1981)

SUMMARY

The component aldoses, uronic acid, and hexosamines in the total non-dialyzable urinary glycoconjugates were determined by the dithioacetal method, and normal levels of these monosaccharides are presented. The molar ratios of fucose/galactose, glucuronic acid/galactose, and galactosamine/glucosamine for cancer patients were lower, and that of mannose/galactose was higher, than normal values.

INTRODUCTION

Human urine contains a variety of carbohydrates, including mono- and oligosaccharides, mucopolysaccharides, glycolipids, and glycoproteins, and there have been a number of studies on their structures, biosynthesis, and metabolism. However, analytical study from the clinical viewpoint seems insufficient, except for the major carbohydrates such as glucose in diabetes mellitus and mucopolysaccharides in some hereditary diseases. Under these circumstances our

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project aims at the establishment of correlation between microheterogeneity of carbohydrate chains in these glycoconjugates and pathological conditions. In previous papers we reported a general method for the rapid analysis of monosaccharides [1, 2] and products of periodate oxidation of carbohydrates [3, 4] as trimethylsilylated dithioacetals. This paper discusses the problems in applying this method to the analysis of monosaccharide compositions in total non-dialyzable urinary glycoconjugates and presents preliminary results obtained for urines from normal subjects and cancer patients.

MATERIALS AND METHODS

Chemicals

Ethanethiol and chlorotrimethylsilane were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Trifluoroacetic acid (TFA) and hexamethyldisilazane were from Wako Pure Chemicals (Osaka, Japan). All other chemicals, solvents, and samples of carbohydrates were of the highest grade commercially available.

Urine samples

Urine samples were collected before breakfast from sixteen normal volunteers and fifteen cancer patients. The patients include those with carcinomas of the lung (3), stomach (2), colon (3), ovary (2), breast (3), liver (1), and pancreas (1), where the numbers in parentheses are those of patients. No cases of metastasis were included. Age distribution for the normal volunteers were as follows; twenties (4), thirties (3), forties (2), fifties (2), sixties (2), seventies (2), and eighties (1). A 50-ml portion of each urine sample was dialyzed in a Visking tube for 24 h against running water. The non-dialyzable fraction was lyophilized and a part of the residue was subjected to component analysis.

Apparatus

Gas chromatography was performed on a Shimadzu 4BMPF instrument equipped with a flame ionization detector. A Scott capillary column (50 m × 0.28 mm I.D.) coated with silicone SF-96 was used at 225°C, and the flow-rate of the carrier (nitrogen) was controlled at 1 ml/min by the use of a 100:1 splitter. Scavenger gas (nitrogen) was continuously mixed with the eluates, and the mixtures were introduced into the detector. Peaks were integrated by a Shimadzu E1A integrator.

Hydrolysis of total non-dialyzable urinary glycoconjugates

For the analysis of the component aldoses and uronic acid, the lyophilized non-dialyzable fraction (ca. 1 mg) of a urine sample was weighed into a small ampoule, to which was added a 2 *M* solution of TFA (200 μl). The ampoule was flushed with nitrogen for a few minutes, sealed, and heated for 6 h at 100°C. After cooling, the ampoule was opened, and the hydrolysate was evaporated to dryness under reduced pressure in a desiccator containing pellets of sodium hydroxide.

For the analysis of the component hexosamines the lyophilized non-dialyz-

able fraction (ca. 1 mg) was hydrolyzed similarly in 4 *M* hydrochloric acid (200 μl) for 6 h at 100°C, and the hydrolysate was worked up in the same manner as described for the TFA hydrolysate.

Analysis of the component monosaccharides

The procedure for the analysis of the component aldoses and uronic acid [1] is schematically shown in Fig. 1, together with that for hydrolysis. An aqueous solution (100 μl) of 10^{-3} *M* 3-O-methylglucose (internal standard) was added to each hydrolysate, and the insoluble materials were centrifuged off. The

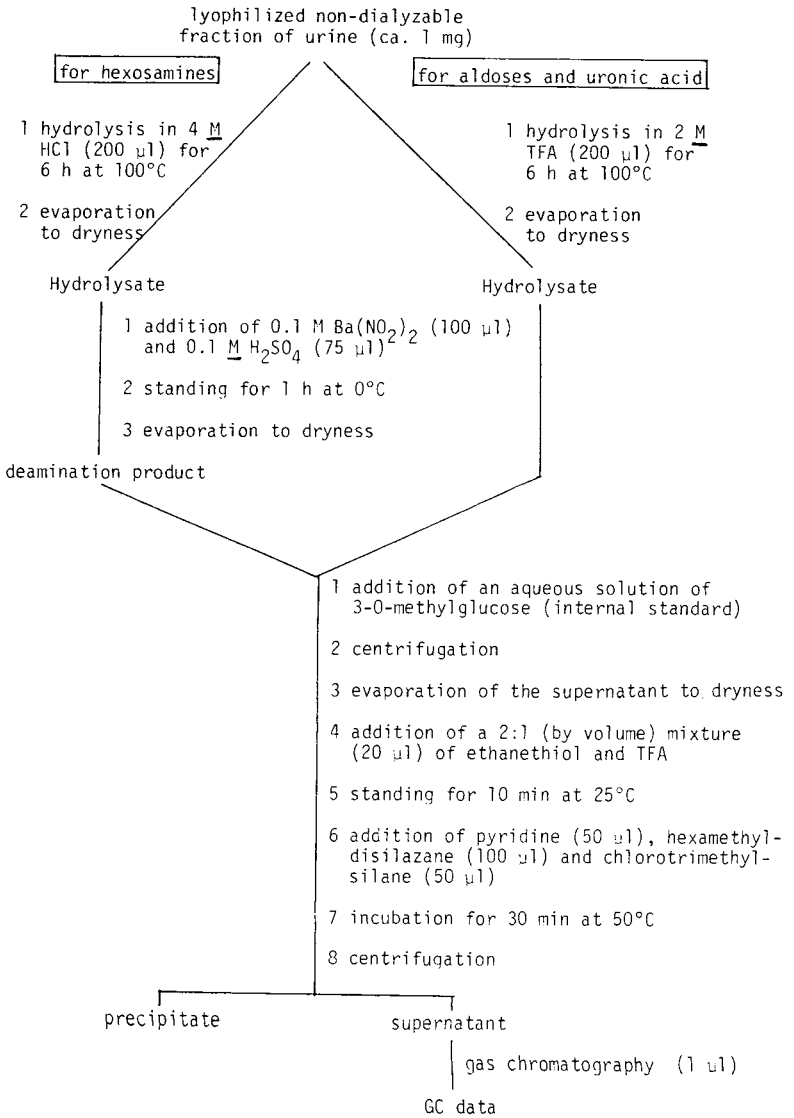

Fig. 1. Procedure for the determination of the component monosaccharides in total non-dialyzable urinary glycoconjugates.

supernatant was transferred with a small volume of washing fluid to a reaction tube (5 cm × 5 mm I.D.) carrying a polyethylene stopper, and evaporated to dryness under reduced pressure in a desiccator containing sodium hydroxide. To the residue from the TFA hydrolysate was added a 2:1 (by volume) mixture (20 μl) of ethanethiol and TFA, and the mixture was kept for 10 min at 25°C with constant swirling. Pyridine (50 μl), hexamethyldisilazane (100 μl), and chlorotrimethylsilane (50 μl) were added in this order, and the mixture was incubated for 30 min at 50°C with occasional shaking. By these sequential treatments, the component aldoses and uronic acid, as well as the internal standard, were converted quantitatively to their trimethylsilylated diethyldithioacetals. The reaction mixture was centrifuged, and a 1-μl sample of the supernatant was injected into the gas chromatography column. The amounts of the component aldoses and uronic acid were determined by comparing their peak areas with those obtained for a standard mixture of authentic monosaccharides under the same conditions.

The component hexosamines were analyzed by another reported procedure [2]. The hydrochloric acid hydrolysate was deaminated with 0.1 *M* barium nitrite (100 μl) and 0.1 *M* sulfuric acid (75 μl) for 1 h at 0°C. The component hexosamines were converted to the corresponding 2,5-anhydrohexoses by this treatment. The mixture was evaporated to dryness under reduced pressure, and the residue was subjected to sequential derivatization of mercaptalation and trimethylsilylation, in the same manner as described above. The amounts of the component hexosamines were calculated similarly by comparing the peak areas with those of a standard mixture.

RESULTS AND DISCUSSION

Gas chromatograms

The procedure used in this work allowed the simultaneous determination of aldoses and uronic acids in ca. 2 h, including the derivatization processes, giving a single peak for each standard sugar. Fig. 2a shows a typical example of a gas chromatogram obtained for the component aldoses and uronic acid of the glycoconjugates in a normal urine sample. Peaks 2, 4, 7, 8, and 9 were assignable to xylose, fucose, glucose, mannose, and galactose, respectively, as the aldose components, and peak 6 to glucuronic acid as the only uronic acid component. The non-dialyzable fraction of urine contains glycoproteins and mucopolysaccharides as the major glycoconjugates, together with small amounts of higher heterooligosaccharides. On the basis of accumulated knowledge of the chemical composition of glycoconjugates, fucose, mannose, and galactose found in the above chromatogram were undoubtedly the component monosaccharides in glycoproteins and heterooligosaccharides, whereas xylose and glucuronic acid (both in part) were contained in mucopolysaccharides. Glucuronic acid could also be freed from its conjugates with various phenolic metabolites. Although human urine contains free glucose, especially in large quantity in diabetes mellitus, peak 7 in this chromatogram was exclusively of the bound glucose, as the urine sample was dialyzed prior to component analysis. It is also apparent that the glucose detected was not an artifact arising from dialysis in a cellophane tube, because the blank test using no urine sample gave no trace of glucose. It

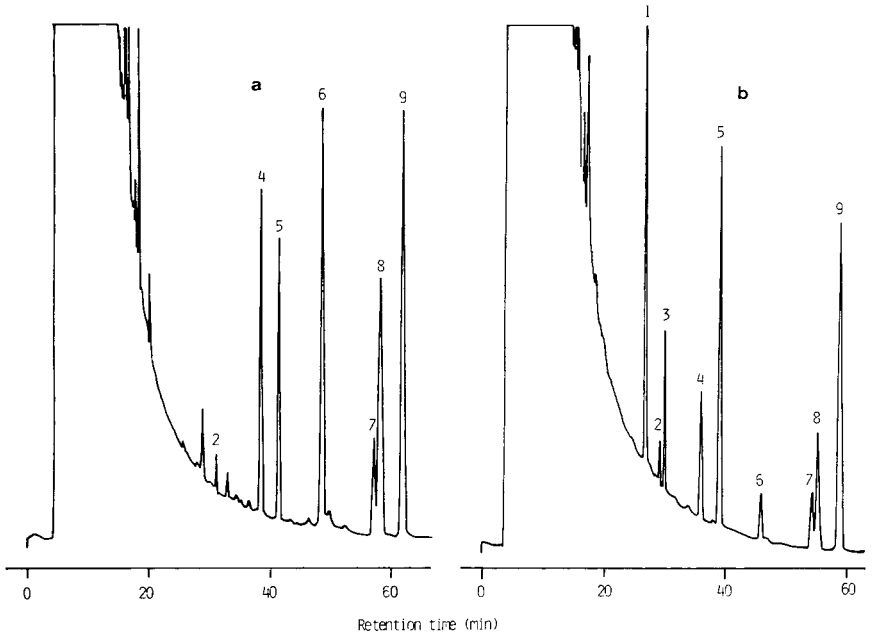

Fig. 2. Typical gas chromatogram obtained for (a) the component aldoses and uronic acid and (b) the component hexosamines in the total non-dialyzable glycoconjugates from a normal urine sample. The component aldoses and uronic acid were analysed as their trimethylsilylated diethyldithioacetals. The component hexosamines were selectively converted to their 2,5-anhydrohexoses with nitrous acid and analysed as the trimethylsilylated diethyldithioacetals of the latter, together with the derivatives of the remaining aldoses and uronic acid. Peak assignment: 1 = glucosamine, 2 = xylose, 3 = galactosamine, 4 = fucose, 5 = 3-O-methylglucose (internal standard), 6 = glucuronic acid, 7 = glucose, 8 = mannose, 9 = galactose.

is not certain from where this bound glucose was derived, but at least it was not a constituent of mucopolysaccharides.

Since hexosamines were not derivatized quantitatively under the conditions used for the aldoses and uronic acid, these hydrolysates were converted to the corresponding 2,5-anhydrohexoses and subsequently derivatized to the trimethylsilylated dithioacetals of the latter. Fig. 2b shows the chromatogram for the same urine sample. The peaks for glucosamine (peak 1) and galactosamine (peak 3) appeared in the pentose region, well separated from each other and also from pentoses. Peaks of some aldoses and glucuronic acid were smaller than those in Fig. 2a due to partial decomposition of these sugars with hydrochloric acid. Both hexosamines may be released from all kinds of non-dialyzable glycoconjugates.

The urine samples from cancer patients and other normal volunteers gave similar chromatograms, but the peak intensities varied among subjects.

Conditions for hydrolysis

For the analysis of component aldoses and uronic acid, TFA was used as the catalyst for hydrolysis because this acid minimized non-hydrolytic degradation. In addition, it is easily removable by evaporation under reduced pressure in a desiccator containing alkali. Fig. 3a shows the course of liberation of these component monosaccharides, as hydrolyzed in 2 *M* TFA at 100°C. The amounts of all these monosaccharides increased gradually to reach plateaus at

TABLE I

RECOVERIES OF THE MONOSACCHARIDES ADDED TO THE HYDROLYSATE OF THE NON-DIALYZABLE FRACTION OF A URINE SAMPLE

n = 5 in each experiment.

Monosaccharide	Average amount found in the hydrolysate (nmol/mg)	Experiment 1			Experiment 2		
		Amount of monosaccharide added (nmol/mg)	Average amount found for total monosaccharide (nmol/mg)	Recovery (%)	Amount of monosaccharide added (nmol/mg)	Average amount found for total monosaccharide (nmol/mg)	Recovery (%)
Xylose	11.2	5	16.0	99	10	20.8	94
Fucose	45.8	25	69.8	99	50	93.6	98
Galactose	112.3	50	159.3	98	100	222.6	105
Glucose	24.2	10	31.5	92	20	41.8	95
Mannose	47.8	25	70.5	97	50	100.2	102
Glucuronic acid	379.8	200	550.4	95	400	750.2	96
Galactosamine	43.5	25	64.5	94	50	90.6	97
Glucosamine	120.3	50	171.3	101	100	204.8	93

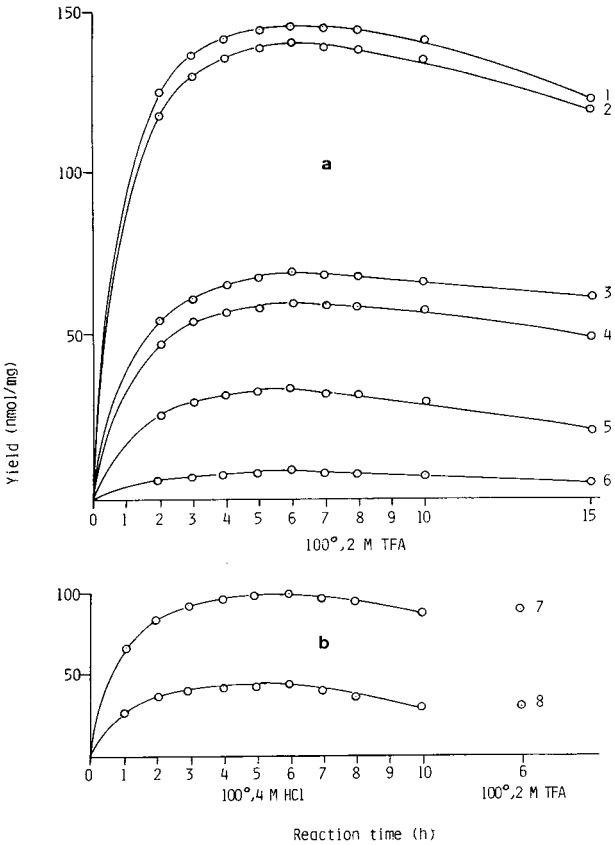

Fig. 3. Course of liberation of (a) the component aldoses and uronic acid and (b) the component hexosamines in the total non-dialyzable glycoconjugates from the same urine sample as used in Fig. 1, as estimated from the peaks of their derivatives. 1 = Glucuronic acid, 2 = galactose, 3 = mannose, 4 = fucose, 5 = glucose, 6 = xylose, 7 = glucosamine, 8 = galactosamine.

ca. 6 h, but prolonged heating resulted in gradual decomposition.

The component hexosamines were hydrolyzed with more difficulty than the aldoses and uronic acid, hence 4 *M* hydrochloric acid was used instead of 2 *M* TFA. The time course shown in Fig. 3b indicates that the appropriate heating time was also 6 h in this case.

Accuracy and precision of component analysis

Correlation of the present method to the conventional alditol acetate method in the determination of the component aldoses and uronic acid was satisfactory, giving the ratios of observed values between 0.94 and 1.05 for all sugars.

Table I shows the recoveries of aldoses added to the hydrolysate of the non-dialyzable fraction of a normal urine sample, as determined by the procedure in Fig. 1. It is indicated that this procedure was satisfactorily accurate. On the other hand Table II gives the coefficients of variation for ten determinations of the component monosaccharides. These data indicate high repeatability of this procedure.

TABLE II

PRECISION OF THE DETERMINATION OF THE COMPONENT MONOSACCHARIDES IN TOTAL NON-DIALYZABLE URINARY GLYCOCONJUGATES

$n = 10$.

Monosaccharide	Amount of monosaccharide (nmol/mg)		Coefficient of variation (%)
	Average	S.D.	
Xylose	10.3	0.37	3.6
Fucose	47.5	1.57	3.3
Galactose	109.7	3.51	3.2
Glucose	23.9	1.12	4.7
Mannose	45.6	2.23	4.9
Glucuronic acid	401.5	20.9	5.2
Galactosamine	42.5	2.17	5.1
Glucosamine	126.2	6.18	4.9

Comparison of the monosaccharide compositions for normal subjects and cancer patients

Table III compares the amounts of individual monosaccharides in total non-dialyzable urinary glycoconjugates for normal subjects and cancer patients. It also gives the molar ratios of these component monosaccharides.

The variation in the amount of xylose was relatively small for normal subjects, but its amout for cancer patients varied over a wide range.

It is noticeable that the average amount of fucose for individual cancer patients was significantly lower than the normal average, with the probability level less than 0.005. The decrease in the molar ratio of fucose to galactose was much more prominent in cancer. Carcinomas of the lung, breast, liver, and pancreas showed an especially marked decrease. The outstanding reduction of the fucose/galactose ratio in cancer is an unexpected finding, as the fucose content in serum glycoprotein was reported to be rather higher in cancer patients [5], suggesting increased activity of GDP-fucosyltransferase in tissues and sera of cancer patients. Therefore, the low level of bound fucose in total non-dialyzable urinary glycoconjugates should be attributed to either an increase in α-fucosidase activity or anomalous production of low-fucose glycoconjugates in secretory organs.

Both mannose and galactose are known to be the major component aldoses of glycoproteins and accordingly hereooligosaccharides derived thereof, but they are not contained in mucopolysaccharides. Their molar ratio is associated with the core structure of the carbohydrate chains in glycoproteins. The results in Table III indicate that the mannose/galactose ratio was varied in a relatively narrow range in both normal subjects and cancer patients, and the average values for the latter were significantly higher than the former. Carcinomas of the stomach, colon, ovary, and liver showed especially high values of the mannose/galactose ratio. The increase in this ratio might be related to the production of a carcinoembryonic antigen [6], which is a kind of glycoprotein

TABLE III

MONOSACCHARIDE COMPOSITIONS OF TOTAL NON-DIALYZABLE URINARY GLYCOCONJUGATES FOR NORMAL SUBJECTS AND CANCER PATIENTS

Monosaccharide composition	Cancer patients (n = 15)			Normal subjects (n = 16)	
	Average	S.D.	p in t-test*	Average	S.D.
Amount of monosaccharide (nmol/mg)					
Xylose (Xyl)	10.5	6.8	<0.4	12.1	2.4
Fucose (Fuc)	31.6	16.6	<0.005	54.4	18.4
Galactose (Gal)	119.1	47.5	<0.6	127.4	29.6
Glucose (Glc)	47.6	46.1	<0.1	25.1	10.5
Mannose (Man)	50.5	19.2	<0.8	48.5	12.7
Glucuronic acid (GlcUA)	184.6	127.3	<0.001	432.3	110.9
Galactosamine ($GalNH_2$)	31.3	14.8	<0.05	41.9	10.8
Glucosamine ($GlcNH_2$)	129.0	66.2	<0.975	128.3	20.9
Molar ratio					
Xyl/Gal	0.10	0.08	NS**	0.10	0.03
Fuc/Gal	0.27	0.07	<0.001	0.42	0.08
Glc/Gal	0.53	0.56	<0.05	0.19	0.06
Man/Gal	0.45	0.10	<0.025	0.38	0.04
GlcUA/Gal	1.52	0.86	<0.001	3.71	1.52
$GalNH_2$/Gal	0.27	0.08	<0.05	0.35	0.13
$GlcNH_2$/Gal	1.11	0.43	<0.9	1.08	0.39
$GalNH_2/GlcNH_2$	0.26	0.09	<0.02	0.33	0.06

*Aspin-Welch t-test was done to judge significant difference between the average of individual cancer patients and that of individual normal subjects.
**Not significant.

having a high mannose/galactose ratio (0.7—0.9) [7].

The amount of glucuronic acid, as well as the glucuronic acid/galactose ratio, was decreased greatly for most samples from cancer patients. Glucuronic acid is the constituent of various glucuronides of phenolic metabolites and mucopolysaccharides, hence the decrement may be assigned to either the former, the latter, or both. However, we could not estimate from the present data alone to what extent each glycoconjugate contributed to the formation of this uronic acid.

Of the two hexosamines found in non-dialyzable glycoconjugates, glucosamine showed no significant alteration of its content in cancer patients. But the average amount of galactosamine for cancer patients was rather smaller than that for normal subjects. The galactosamine/galactose and galactosamine/glucosamine ratios for cancer patients showed a more marked reduction compared to those for normal subjects.

Thus, the gas chromatographic procedure devised on the basis of the dithioacetal method made it possible to determine rapidly the monosaccharides in

non-dialyzable urinary glycoconjugates. The monosaccharide compositions obtained for cancer patients by this procedure are attractive for diagnostic purposes. However, a larger number of samples from both cancer patients and patients with other diseases should be analyzed, before diagnostic evaluation is established. A series of such analyses is now in progress.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF 25-HYDROXYVITAMIN D_2 AND 25-HYDROXYVITAMIN D_3 IN HUMAN PLASMA

USE OF ISOTACHYSTEROLS AND A COMPARISON WITH GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A high-performance liquid chromatographic (HPLC) method for estimating plasma 25-hydroxyvitamin D_2 (25-OHD_2) and 25-hydroxyvitamin D_3 (25-OHD_3) is described. The method involves plasma extraction, Lipidex 5000 chromatography and HPLC on straight-phase Zorbax-SIL, collecting the 25-OHD_2 + 25-OHD_3 fractions. These secosteroids are isomerised to their isotachysterol derivatives and re-run in the same HPLC system, monitoring at 290 nm. ^{3}H-Labelled 25-OHD_3 is used as an internal standard. The method was evaluated in terms of reproducibility, and recovery of added secosteroids was quantitative. Values obtained using this method were in close agreement with those values obtained on the same plasma sample using gas chromatography—mass spectrometry.

INTRODUCTION

Of the derivatives of vitamin D* circulating in human plasma, the 25-hydroxylated metabolite is in the highest concentration [1]. Since it is relatively easy to measure, estimates of plasma levels of 25-hydroxyvitamin D (25-OHD) have therefore been widely used for the assessment of vitamin D status in a variety of clinical situations [2]. Until recently, most methods for the estimation of

*Systematic and trivial names of vitamin D and its derivatives used in this paper are as follows: Vitamin D_2 (9,10-seco-5,7,10(19),22-ergostatetraen-3β-ol): D_2. Vitamin D_3 (9,10-seco-5,7,10(19)-cholestatrien-3β-ol): D_3. 25-Hydroxyvitamin D_2: 25-OHD_2. 25-Hydroxyvitamin D_3: 25-OHD_3. Isotachysterol isomer formed from 25-OHD_2: 25-$OHITS_2$. Isotachysterol isomer formed from 25-OHD_3: 25-$OHITS_3$.

25-OHD in human plasma have relied upon competitive protein-binding assays with and without chromatography prior to assay (reviewed in ref. 3). The majority of these assays, being derived from the procedure of Haddad and Chyu [1], did not distinguish between 25-OHD_2 and 25-OHD_3. Over the last five years, the introduction of high-performance liquid chromatography (HPLC) has greatly simplified and improved the specificity of methods for the estimation of plasma levels of 25-OHD_2 and 25-OHD_3 [4]. The sensitivity of ultraviolet (UV) detection, used in these HPLC methods, can be enhanced by forming isotachysterol isomers* prior to HPLC [5]. This paper describes the application of this procedure to the estimation of 25-OHD_2 and 25-OHD_3 in human plasma.

Few of the previous HPLC methods have been evaluated in terms of specificity, except that the eluent HPLC fractions have been collected and subjected to mass spectrometry (MS) [6], gas chromatography (GC) and mass spectrometry [7, 8], and UV spectra have been obtained [6—8] in order to demonstrate the purity of the final HPLC fraction. This paper compares the results obtained by the HPLC method described with the results obtained by a mass fragmentographic procedure [9].

MATERIALS AND METHODS

Materials

Pure secosteroids were obtained from the following sources: vitamin D_2 (Koch-Light Labs., Colnbrook, Great Britain), 25-OHD_2 and 25-OHD_3 (Dr. J.A. Campbell, The Upjohn Company, Kalamazoo, MI, U.S.A.). These secosteroids were purified by HPLC before use. 25-Hydroxy[23,24-^{3}H]vitamin D_3 (specific activity around 110 Ci/mmole) supplied by the Radiochemical Centre (Amersham, Great Britain) was found to be radiochemically pure on receipt. Radioactive steroids were re-purified every three months by HPLC. Lipidex 5000 was obtained from Packard Instrument Co. (Reading, Great Britain). Other reagents were as specified by Seamark et al. [9, 10] and were analytical reagent grade wherever possible. HPLC was carried out with a Model 750/03 pump, a Rheodyne 7125 injection valve, and Model SF770 variable-wavelength (190—700 nm) detector (Schoeffel Instruments) all supplied by Applied Chromatography Systems (Luton, Great Britain). A Zorbax-SIL (5 μm, 250 × 0.46 mm) column from DuPont (U.K.), Hitchin, Great Britain was eluted with a solvent system of hexane—isopropanol (9:1) as described by Seamark et al. [5]. Mass fragmentography was carried out using an LKB 2091 gas chromatograph—mass spectrometer as described by Seamark et al. [9].

All glassware was silanised by soaking overnight in 1% (v/v) dimethyldichlorosilane in toluene and washed with ethanol. Blood was taken from apparently healthy volunteers of both sexes into heparinised containers; the plasma was separated and analysed immediately. Liquid scintillation counting was carried out using an Intertechnique Model SL30 (Kontron Intertechnique, St. Albans, Great Britain) and 5 ml of NE250 liquid scintillation fluid. At least 10,000

*See p. 351.

counts were collected. Tritium counting efficiency in this system was 40% with a background count rate of around 30 cpm.

Method

The procedure is summarised in Fig. 1. Plasma (2 ml), to which approximate-

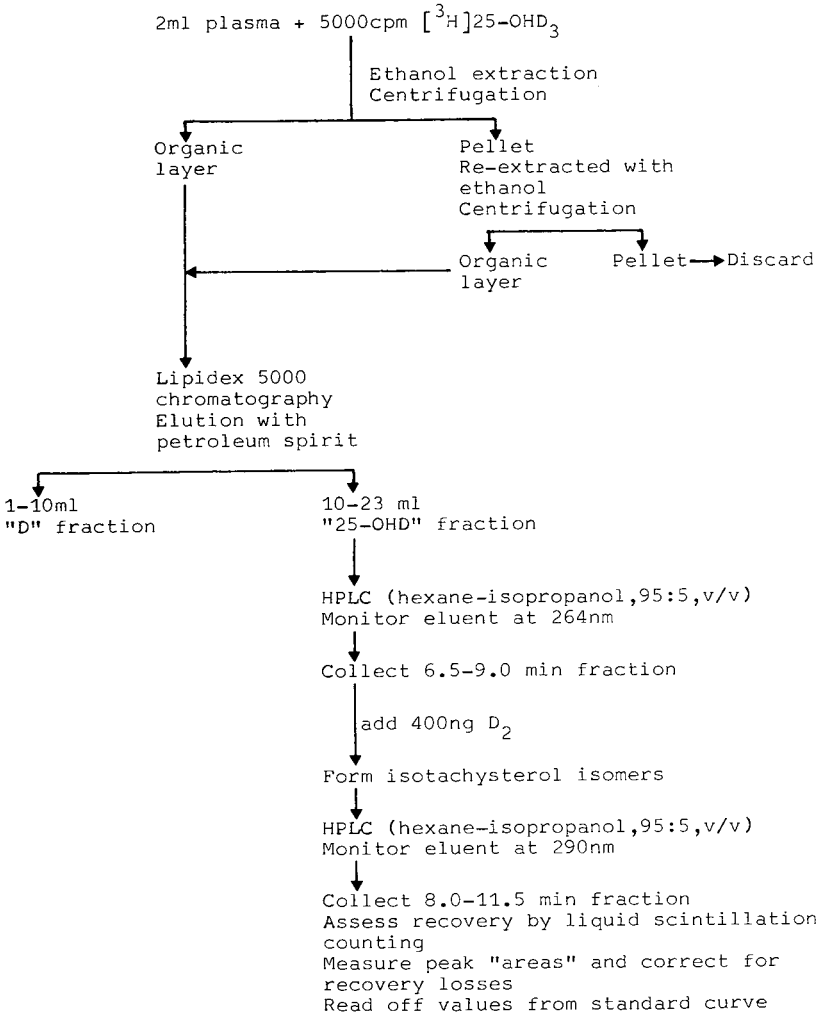

Fig. 1. Flow diagram for the two-stage HPLC assay for 25-OHD$_2$ and 25-OHD$_3$.

ly 5000 cpm of ^{3}H-labelled 25-OHD$_3$ had been added, was extracted with 18 ml of redistilled ethanol by mechanical shaking for 4 min. The precipitated protein was centrifuged (2000 *g* for 10 min), the supernatant removed and the protein pellet re-extracted with 10 ml of redistilled ethanol. The two ethanol extracts were combined and evaporated to dryness in a rotary evaporator at 40°C.

The residue was applied to a Lipidex 5000 column (7 cm × 0.5 cm I.D.) in 3 × 0.5 ml of petroleum spirit and the "25-OHD" fraction [9] was collected.

The solvent was evaporated to dryness, dissolved in 100 μl of hexane—isopropanol (95:5, v/v) and injected into the HPLC system [Zorbax-SIL, eluted with hexane—isopropanol (95:5, v/v) at a flow-rate of 1.5 ml/min], monitoring the effluent at 264 nm. The fraction containing 25-OHD_2 and 25-OHD_3 (usually between 6.5 and 9.0 min after injection) was collected and evaporated to dryness. Chloroform (75 μl) and vitamin D_2 (400 ng) were added and the isotachysterol isomers prepared using the HCl procedure described by Seamark and coworkers [5, 10]. Once formed, these isomers were dissolved in 100 μl of hexane—isopropanol (95:5, v/v) and injected into the HPLC system using the same column and eluting solvent as before, monitoring the effluent at 290 nm. The fraction containing the isotachysterol isomer of 25-OHD_3 (25-$OHITS_3$) (around 8.0—11.5 min) was collected and counted for radioactivity to assess overall recovery. Peak "areas" [5] for the isotachysterol isomers 25-$OHITS_2$ and 25-$OHITS_3$ were measured and corrected for recovery losses, assuming that the recovery of 25-OHD_3 was the same as that for 25-OHD_2.

A standard curve covering the range 0—100 ng of 25-OHD was obtained by injecting 5000 cpm of ^{3}H-labelled 25-OHD_3 plus increasing amounts of standard 25-OHD_2 and 25-OHD_3 which, together with 400 ng of vitamin D_2, had been isomerised to the isotachysterol derivatives. Peak "areas", corrected for recovery as described above, were measured and plotted against the amount of 25-OHD added. The response was linear over the range examined and equations of the lines were $y = 0.28x + 0.12$ (correlation coefficient 0.9999, for 25-OHD_2) and $y = 0.28x + 0.11$ (correlation coefficient 0.9996, for 25-OHD_3), where y represents the corrected peak area and x represents the mass in ng. 25-$OHITS_2$ and 25-$OHITS_3$ have the same UV absorbance per unit mass and thus, in the absence of standard 25-OHD_2, standard curves produced using 25-OHD_3 can be used to quantitate both 25-hydroxylated vitamins.

EVALUATION OF THE METHOD

The formation of isotachysterol derivatives of 25-OHD_2 and 25-OHD_3 increased their retention times on the straight-phase Zorbax-SIL column used.

TABLE I

RETENTION TIMES OF SOME SECOSTEROIDS AND THEIR CORRESPONDING ISOTACHYSTEROL DERIVATIVES ON A STRAIGHT-PHASE ZORBAX-SIL COLUMN

The solvent system used was hexane—isopropanol (95:5, v/v) at a flow-rate of 1.5 ml/min.

Secosteroid	Retention time (min)
D_3	2.00
D_2	2.00
25-OHD_2	6.80
25-OHD_3	8.36
ITS_3	1.78
ITS_2	1.78
25-$OHITS_2$	8.78
25-$OHITS_3$	11.20

Table I shows the retention times of vitamins D_2 and D_3, 25-OHD_2, 25-OHD_3, 25-$OHITS_2$ and 25-$OHITS_3$. Formation of these isotachysterol isomers did not significantly alter the separation of the calciferols in the HPLC system used here. Samples of the HPLC traces obtained when monitoring the effluent at 264 nm before isomerisation and when monitoring at 290 nm after isomerisation are shown in Fig. 2.

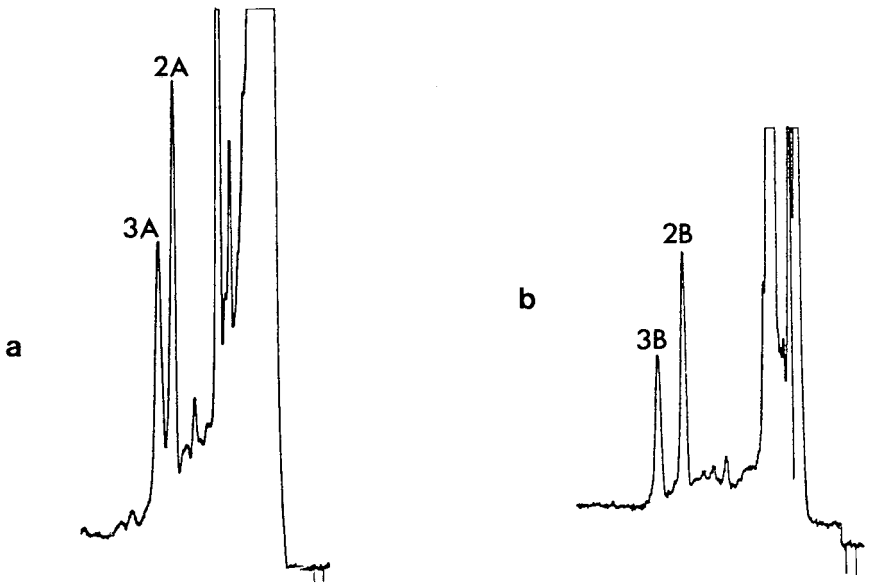

Fig. 2. HPLC traces obtained before and after isotachysterol formation. (a) First HPLC run, monitoring at 264 nm, 0.01 a.u.f.s. 2A and 3A indicate the positions of 25-OHD_2 and 25-OHD_3, respectively. (b) Second HPLC run (after isotachysterol formation), monitoring at 290 nm, 0.01 a.u.f.s. 2B and 3B indicate the positions of 25-$OHITS_2$ and 25-$OHITS_3$, respectively.

A single plasma sample was analysed in duplicate using four different volumes of plasma, ranging from 5 to 1 ml. Fig. 3 shows the amounts of 25-OHD_2 and 25-OHD_3 in each sample plotted against the volume of plasma used. Straight lines were obtained for both 25-OHD_2 and 25-OHD_3, indicating that values obtained were independent of the volume of plasma used for analysis. For a plasma sample containing around 20 ng/ml of 25-OHD_3 it should be possible to use around 0.5 ml of sample for analysis, but in practice, it was decided to use 2 ml to enable 25-OHD_2 to be measured as well.

Varying amounts of standard 25-OHD_2 and 25-OHD_3 were added in ethanol to 2-ml plasma samples in duplicate. After equilibration at 37°C for 15 min, the samples were analysed. The mean values for 25-OHD_2 and 25-OHD_3, together with the calculated recoveries, are given in Table II. In each case recoveries were quantitative and the amount of one secosteroid added did not affect the recovery of the other. The use of ^{3}H-labelled 25-OHD_3 to monitor the recovery of 25-OHD_2 appeared to give satisfactory results. Recoveries of added tritium at various stages in the method are given in Table III. Intra-assay and inter-

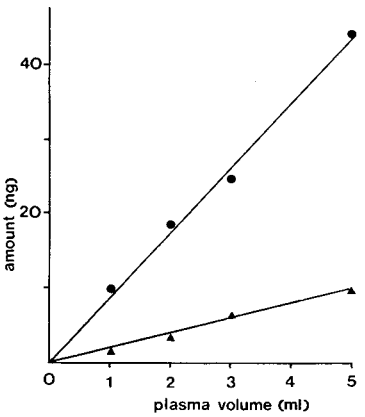

Fig. 3. Effect of analysing different volumes of plasma on the estimated amount (ng) of 25-OHD_2 (•) and 25-OHD_3 (▲). Correlation coefficients were 0.9950 (25-OHD_3) and 0.9910 (25-OHD_2); neither was significantly different from 1.000 ($p > 0.5$). Intercepts were +0.4 ng (25-OHD_3) and −0.8 ng (25-OHD_2); neither was significantly different from zero ($p > 0.5$).

TABLE II

RECOVERIES OF STANDARD SECOSTEROIDS ADDED TO PLASMA

25-OHD_3			25-OHD_2		
Added (ng/ml)	Observed (ng/ml)*	Recovery (%)	Added (ng/ml)	Observed (ng/ml)*	Recovery (%)
0	19.9	—	0	0	—
10	29.1	92.0	10	10.2	102.5
20	40.0	100.5	15	14.3	95.3
30	51.3	104.5	25	23.8	95.3
50	65.5	91.2	30	31.0	103.3
0	19.0	—	35	38.3	109.3

*Mean of duplicate assays is recorded.

TABLE III

RECOVERIES, AT VARIOUS STAGES IN THE METHOD, OF ^{3}H-LABELLED 25-OHD_3 ADDED TO PLASMA

Results are expressed as mean ± standard deviation. n = number of samples.

Stage in method	Recovery (%)
After extraction	78.4 ± 5.9 (n = 10)
After Lipidex column	69.5 ± 2.9 (n = 12)
After first HPLC	60.5 ± 3.5 (n = 8)
After second HPLC	48.0 ± 3.6 (n = 8)

assay variability studies were also carried out and the results are given in Table IV.

TABLE IV

INTRA-ASSAY AND INTER-ASSAY VARIABILITY

	Secosteroid value (ng/ml)	C.V. (%)
Intra-assay		
25-OHD_3	19.2 ± 0.95	4.9 (n = 10)
25-OHD_2	14.3 ± 0.6	4.1 (n = 10)
25-OHD_2	3.9 ± 0.3	8.3 (n = 10)
*Inter-assay**		
25-OHD_3	18.7 ± 1.6	8.8 (n = 6)
25-OHD_2	4.2 ± 0.5	11.6 (n = 6)

*Plasma sample stored at −20°C, analysed over a period of four weeks.

The specificity of the HPLC assay described here was evaluated by comparing the values for 25-OHD_2 and 25-OHD_3 in plasma obtained from normals and from volunteers taking oral vitamin D_2 supplements, with values obtained on the same plasma using a GC—MS method previously described [9]. In addition, concentrations of 25-OHD_2 and 25-OHD_3 were also estimated after the first HPLC separation using the effluent trace monitored at 264 nm. Concentrations of 25-OHD_2 and 25-OHD_3 after only one HPLC separation, without isomerisation, were higher than those obtained by GC—MS. On occasion it proved impossible to discern a single peak at the appropriate retention times. However, the second HPLC separation, after isomerisation to the isotachysterol

TABLE V

PLASMA CONCENTRATIONS OF 25-OHD_3 AND 25-OHD_2 IN TWELVE PLASMA SAMPLES ASSAYED BY THREE DIFFERENT PROCEDURES

Sample	25-OHD_3 (ng/ml)			25-OHD_2 (ng/ml)		
	$HPLC_{264}$	→ $HPLC_{290}$	GC—MS	$HPLC_{264}$	→ $HPLC_{290}$	GC—MS
1	35.0	16.8	15.8	50.5	22.5	21.3*
2	44.3	8.8	11.5	27.5	6.3	5.4*
3	28.0	12.8	13.0	3.0	1.5	1.6
4	54.8	27.1	21.3	27.2	3.4	3.0
5	24.0	10.3	8.7	22.8	5.9	4.5
6	18.5	12.8	13.0	3.0	3.1	2.8
7	25.0	12.5	14.9	19.0	6.4	6.1*
8	24.5	12.2	16.9	28.5	14.2	12.7*
9	26.0	13.8	15.8	24.1	15.7	13.7*
10	I**	40.0	46.2	45.6	4.9	2.6
11	I	18.4	18.2	30.4	3.1	3.2
12	41.5	29.4	26.9	I	ND***	ND

*Plasma samples from volunteers taking vitamin D_2 (3000 IU/day, orally).
**I = interference, making the estimation impossible.
***ND = Not detectable.

isomers, gave clearer traces and produced lower values which were in close agreement with those obtained by GC—MS. The values obtained are given in Table V. Regression analysis performed on these results gave correlation coefficients of 0.9941 (25-OHD_2) and 0.9639 (25-OHD_3) and the equation of the lines were y (GC—MS) = $0.93x$ ($HPLC_{290}$) − 0.32 (for 25-OHD_2) and y (GC—MS) = $1.06x$ ($HPLC_{290}$) + 0.34 (for 25-OHD_3). Normal values obtained from healthy volunteers of both sexes, using plasma taken in Great Britain in August are given in Table VI.

TABLE VI

NORMAL VALUES FOR 25-OHD_2 AND 25-OHD_3 IN PLASMA

Plasma was taken from six females and five males (aged between 18 and 40 years) in August. Results are expressed as mean ± S.D.

	Plasma concentration (ng/ml)
25-OHD_3	23.9 ± 11.0
25-OHD_2	4.4 ± 1.3

DISCUSSION

Although the estimates of the concentration of 25-OHD in plasma, obtained after Lipidex chromatography and a single HPLC run monitoring the effluent at 264 nm (see Table V), were in many cases of the right order of magnitude, it is clear that these values do not reflect the lower, presumably more accurate, concentrations measured by mass fragmentography. In our hands, therefore, a single straight-phase HPLC run after one column chromatographic separation is inadequate. The interpolation of a specific chemical reaction — formation of isotachysterol isomers — between the first and second HPLC separation, increases the specificity and enhances the sensitivity of detection [5]. This type of alteration in chromatographic mobility after chemical transformation has been used in classical chromatography systems for steroids [11] and for organic acids [12] and is recommended as one procedure for use in establishing the identity of steroids [13]. Improvement in specificity can also be achieved by the use of reversed-phase systems for the second HPLC separation, without chemical transformation [6], or by the use of different solvent systems on an LH-20 column prior to HPLC [14]. Dual-column methods require the use of two separate HPLC systems. The method described here has the advantage that only a single straight-phase HPLC system is required. It is thus simpler than many of the previously published HPLC assays for 25-OHD.

Plasma concentrations of 25-OHD_2 and 25-OHD_3 using this method are in reasonable agreement with those obtained by a mass fragmentographic method [9] and agree with the results reported by Shepard et al. [15], although our mean value for 25-OHD_2 is perhaps high for Great Britain. Total 25-OHD values measured here were in agreement with values obtained on samples collected during the summer months in Great Britain using competitive protein-binding assays [16, 17] and HPLC in the U.S.A. [15].

A number of HPLC assays for 25-OHD_3 [7, 8, 18—20] or total 25-OHD [7, 8, 21] have been described, none of which have measured 25-OHD_2 directly. Shepard et al. [15] and Jones [6] describe HPLC methods which measure 25-OHD_3 and 25-OHD_2. Jones [6] used a straight-phase HPLC separation followed by a reversed-phase HPLC separation, and validated the method against a competitive protein-binding assay and obtained mass spectra from trapped fractions from the second HPLC column.

The majority of HPLC assays described above have utilised UV absorbance at 254 nm for quantitation. This wavelength is not the absorption maximum of vitamin D and its metabolites. The method described by Jones [6], however, monitored the effluent at 264 nm, which is the absorption maximum. The formation of isotachysterol isomers increased the absorbance at 290 nm approximately two-fold over that at 254 nm [5] and thus increased the sensitivity of detection.

The use of HPLC with UV detection has become increasingly widespread in the assay of 25-OHD_2 and 25-OHD_3. The cost of HPLC equipment is less than a mass spectrometer and requires less expertise to operate and maintain. The HPLC assays described here give comparable specificity to that obtained by mass fragmentography and, on occasions where specificity is in doubt, effluent fractions can be collected and checked on the mass spectrometer. HPLC assays are more expensive than competitive protein-binding assays which in general are relatively non-specific and measure only total 25-OHD. The HPLC assay described here is able to distinguish between 25-OHD_2 and 25-OHD_3, and the accuracy and degree of precision are higher than most published competitive protein-binding assays for 25-OHD. This simplified HPLC assay is estimated to have a minimum detection limit of approximately 0.5 ng/ml using a 2-ml plasma sample.

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CHROMBIO. 1021

SIMPLE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE CONCURRENT DETERMINATION OF THE AMINE METABOLITES VANILLYLMANDELIC ACID, 3-METHOXY-4-HYDROXYPHENYLGLYCOL, 5-HYDROXYINDOLEACETIC ACID, DIHYDROXYPHENYLACETIC ACID AND HOMOVANILLIC ACID IN URINE USING ELECTROCHEMICAL DETECTION

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SUMMARY

A simple method for the concurrent analysis of the noradrenaline metabolites vanillylmandelic acid and 3-methoxy-4-hydroxyphenylglycol, the dopamine metabolites dihydroxyphenylacetic acid and homovanillic acid, and the serotonin metabolite 5-hydroxyindoleacetic acid in human urine is described. Following organic extraction of the metabolites from acidified urine, they are separated by single-step gradient elution high-performance liquid chromatography on a reversed-phase column. Detection and quantification are achieved with an electrochemical detector using a carbon-paste electrode; samples can be injected at 40-min intervals. Optimisation of analytical parameters is described, and examples of the application of the method in the fields of clinical chemistry and clinical neuroscience are given. This provides a convenient method for the concurrent study of the metabolism of three major biogenic amines, and is readily adaptable for studies on cerebrospinal fluid and brain tissue.

INTRODUCTION

The urinary excretion of the metabolites of catecholamines and 5-hydroxytryptamine is used in screening for a variety of disease states including phaeochromocytoma, neuroblastoma and carcinoid syndrome [1, 2]. In particular, the ability to detect elevated dopamine as well as noradrenaline metabolites is of considerable clinical significance in assessing the malignancy of phaeochromocytoma [3].

Traditional methods for these determinations, usually spectrophotometric, have lacked sensitivity and specificity. Normal variations in the excretion of

these compounds, or smaller deviations therefrom, are of interest in the context of research in neurology and psychiatry (see ref. 4, for example). This has led to the development of much more specific and reliable methods which, however, require expensive equipment (for example, gas chromatograph—mass spectrometer) and measure one or at most two metabolites of interest.

High-performance liquid chromatography (HPLC) on microparticulate reversed-phase columns offers a powerful technique for separating related compounds which are water-soluble but with some hydrophobic character. UV-absorption detection lacks the requisite sensitivity for the measurement of endogenous amine metabolite levels. Electrochemical (EC) detection offers a major increase in sensitivity, and is also somewhat selective for these metabolites.

Recently HPLC—EC methods for vanillylmandelic acid (VMA) [1], 3-methoxy-4-hydroxyphenylglycol (MHPG) [5], dihydroxyphenylacetic acid (DOPAC) [6], homovanillic acid (HVA) [7] and 5-hydroxyindoleacetic acid (5HIAA) [8] have all been described. This paper presents a simple method for the determination of all five metabolites following organic extraction from acidified urine, using single-step gradient chromatography. Samples can be injected at 40-min intervals; the simplicity of the apparatus and the procedure makes the method suitable for clinical laboratory screening as well as for clinical neuroscience research purposes.

EXPERIMENTAL

HPLC apparatus

This was made up from modular components supplied by Anachem (Luton, Great Britain) as follows: Altex 110A single-piston pump, external flow through pulse dampener; Rheodyne 7120 injection valve equipped with 20-μl sample loop; 15 cm × 4.6 mm column packed with Hypersil ODS 5 μm, at ambient temperature; Bioanalytical Systems (BAS) electrochemical detector LC-12 packed with CP-O carbon paste with LC-2A or LC-4 control box, reference electrode Ag/AgCl (RE1); Bryans 28000 series, single-pen recorder; for solvent switching an Altex slider valve adapted for automatic operation using two pneumatic activators and solenoid air valves, with timing device based on motor-driven cams operating microswitches (Radiospares).

Materials

The HPLC solvent was 0.1 *M* (final) sodium phosphate buffer (pH 3.0) containing 5 or 15% AnalaR methanol (BDH, Poole, Great Britain). Ethyl acetate (special for chromatography) and other reagents (AR where available) were obtained from BDH. Standards of the amine metabolites were obtained from Sigma (Poole, Great Britain). Working standard solutions contained VMA, 5HIAA, DOPAC and HVA, all at 25 μg/ml and MHPG at 50 μg/ml (as free glycol). β-Glucuronidase (Bacterial type II 48,000 units/g) was obtained from Sigma and arylsulphatase from Boehringer (London, Great Britain).

Sample preparation

Enzymatic hydrolysis of conjugated metabolites (for total metabolite determination) was achieved as previously described [4] by incubation of 0.45 ml of urine with 50 µl of water or mixed standard, 80 µl of 1 mol/l Tris—acetate buffer (pH 6), 25 µl of β-glucuronidase (100 mg/ml, freshly prepared) and 15 µl of arylsulphatase at 37°C for 16 h.

The incubate (or, for free metabolites, urine with added buffer, and water in place of the enzymes) was adjusted to pH 1 by the addition of 30 µl of concentrated HCl. The metabolites were then extracted successively with 1.5- and 1.0-ml portions of ethyl acetate, 1.2 and 1.0 ml, respectively, of the organic phase being recovered.

Solvent was removed from the pooled extracts at room temperature under a stream of oxygen-free nitrogen. Residues were taken up in 0.5 ml of distilled water for chromatography, and stored at −40°C where necessary.

HPLC conditions

The buffer flow-rate was 1 ml/min, the EC detector voltage 0.72 V (vs. Ag/AgCl reference), sensitivity was 100 nA f.s.d., and injection volume 20 µl. The 5% methanol buffer was automatically switched to 15% methanol buffer 5.5 min after injection (this solvent front reached the detector 8 min later); 24 min after injection the solvent was switched back to 5% methanol and the next sample could be injected 15 min later. Traces were recorded at 1 V f.s.d. and quantification was by peak height measurement, using the method of standard additions.

Analysis of an enzyme blank confirmed that none of the peaks on the chromatograms arose from the added enzymes.

RESULTS AND DISCUSSION

Method development

In preliminary experiments using relatively large amounts (20 µg) of catecholamine metabolite standards and a Cecil UV detector at 280 nm it was established that VMA, MHPG, DOPAC and HVA could be well resolved on a 15-cm column of Hypersil ODS 5 using McIlvaine buffers (0.2 *M* Na_2HPO_4 + 0.1 *M* citric acid, pH 2.7). The retention times could be controlled by varying the pH or concentration of added methanol. Using the EC detector at 0.72 V, 20-ng samples were readily quantified, an increase of 1000-fold in sensitivity over the UV detector. (Catechol and hydroxyindole compounds can be detected at ca. 0.5 V; the higher voltage is necessary to detect the methoxyhydroxy derivatives.)

Increasing the methanol concentration (Fig. 1), or increasing the flow-rate, reduced the retention times for all five metabolites. Increasing the pH of the eluting buffer at 10% methanol reduced the retention of the acidic metabolites (by shifting the equilibria towards the ionised species), but not that of the neutral metabolite MHPG (Fig. 1). The anomalous effects of pH on MHPG and DOPAC at low methanol concentrations may well be due to interactions with the ionic species present in the buffer. This was also sug-

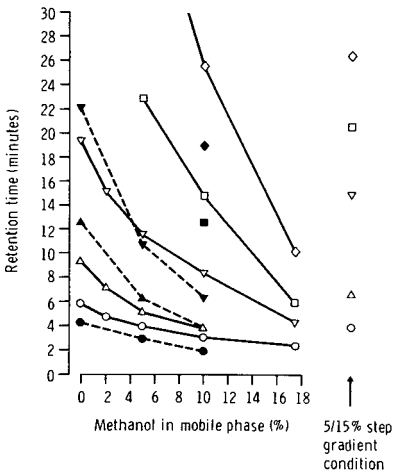

Fig. 1. Effect of added methanol on retention times. Standards of VMA (○ ●), MHPG (△ ▲), DOPAC (▽ ▼), 5HIAA (□ ■) and HVA (◇ ◆) were chromatographed using citrate—phosphate buffers (see text) at pH 2.7 (open symbols) and pH 4.0 (closed symbols) with increasing concentrations of methanol as indicated. Retention times for the final conditions with phosphate buffer (pH 3) and a step gradient of methanol are indicated at the right.

gested by the increased retention times found when 0.1 *M* phosphate buffer was substituted for citrate—phosphate (because of a report [9] that citrate ions had a detrimental effect on the life of reversed-phase columns).

Chromatography of urine extracts in 0.1 *M* phosphate buffer (pH 3) yielded peaks corresponding to the five metabolites. Addition of 5% methanol to this buffer reduced the retention time of HVA, the last peak to elute, from 96 to 60 min; further increases of methanol resulted in a loss of resolution in the area of VMA and MHPG. Variation of pH and methanol in a manner analogous to that in Fig. 1 did not yield suitable isocratic conditions, and thus a single-step gradient elution using 5 and 15% methanol was adopted; the resultant retention times are also shown in Fig. 1. The conditions described under Methods enabled a complete analysis to be made within 40 min with good resolution of the peaks of interest.

The change of solvent during the analysis resulted in a hump on the baseline; this was due to the 2-μm stainless-steel inlet filter on the line in which the second solvent stood before the switch. Changing the filters to sintered glass resulted in the abolition of the baseline disturbance (see Figs. 3 and 4). Recovery of standards added to urine was of the order of 120%, i.e. more was recovered when taken through the method in urine than in an aqueous solution. Salt saturation of aqueous standards did not improve recovery, and thus the method of standard addition (spiking) was used for each urine. The amount added was about one-third of the normal urinary concentration (see Fig. 3). Recovery between urine samples was consistent (Table I), so that a few spiked urines could be used to quantitate all those analysed in one series.

Application of the method

Using the method as described, VMA, MHPG, DOPAC, 5HIAA and HVA can be determined following a single HPLC injection using the EC detector at the least sensitive setting of 100 nA f.s.d. A linear relationship was obtained (Fig. 2) between detector response and increasing amounts of standards added to urine samples before extraction. Table I shows the recoveries through the extraction of standards added to urine (i.e. as a percentage of aqueous standards directly chromatographed). A typical trace from a urine specimen from a normal control subject is shown in Fig. 3. The average deviation from the mean of samples analysed in duplicate ranged from 6.3 to 8.8% for the various metabolites (mean of 15 pairs of determinations).

Fig. 4 shows a trace from a urine specimen from a patient with phaeochromocytoma (subsequently histologically confirmed). VMA excretion was 20.6 mg per 24 h in agreement with the clinical chemistry laboratory

TABLE I

APPARENT RECOVERIES FROM URINE

The data represent recoveries of standards added to urine relative to unextracted standards. Results are expressed as mean ± S.D. of four observations for each metabolite. The results are not corrected for incomplete recovery of organic phases; true recoveries can be obtained by dividing means by 0.9.

Metabolite	Recovery
VMA	72.5 ± 7.3%
MHPG	56.8 ± 6.6%
DOPAC	80.1 ± 8.1%
5HIAA	61.2 ± 7.0%
HVA	74.6 ± 11.5%

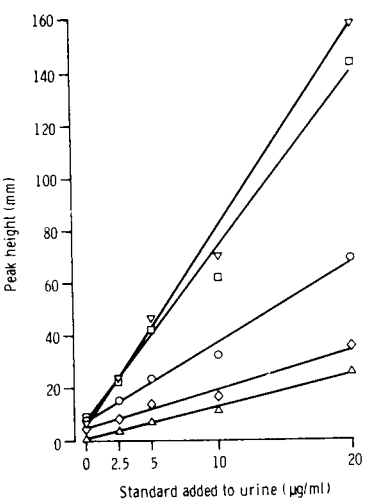

Fig. 2. Effect of adding varying concentrations of standard to a urine sample before extraction and chromatography. Symbols as in Fig. 1.

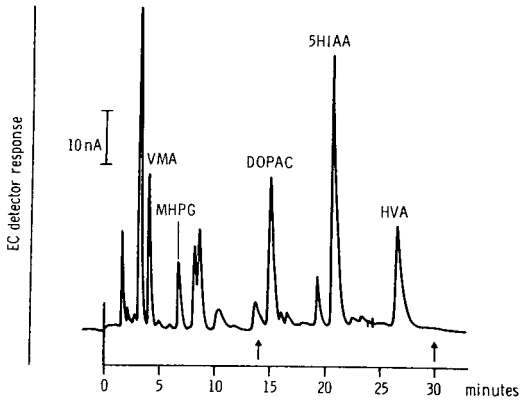

Fig. 3. Chromatogram of a urine extract from a normal subject. Unhydrolysed urine was spiked with standards of approximately one-third of the concentration of each metabolite (except MHPG, which is largely conjugated). Chromatographic conditions: 150 × 4.6 mm column of Hypersil ODS 5; solvent 1 ml/min of 0.1 *M* sodium phosphate (pH 3.0), initially 5% methanol. First arrow: same buffer but with 15% methanol arriving at detector following switch. Second arrow: return to initial conditions.

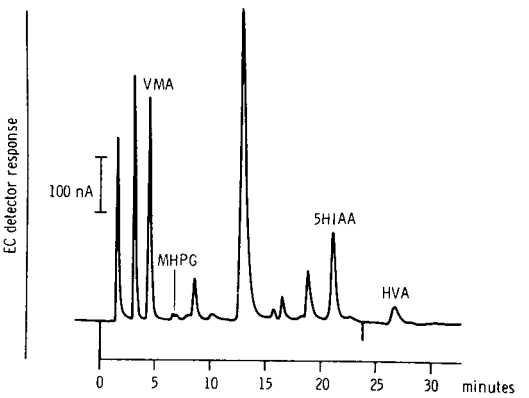

Fig. 4. Chromatogram of urine extract from a patient with phaeochromocytoma. Unhydrolysed and unspiked sample. Note change of ordinate scale. Chromatographic conditions as in legend to Fig. 3. Metabolite levels from this patient are given in the text.

result of 24.4 mg per 24 h, and conjugated VMA was present (total VMA 28.7 mg per 24 h). Total MHPG excretion was 15.1 mg per 24 h (free: 2.8 mg per 24 h, a normal proportion). This represents about a six-fold elevation of VMA and MHPG; total metanephrines were 36 mg per 24 h, again about a six-fold elevation. 5HIAA and HVA excretion were also increased to a lesser extent; conjugated HVA was also present.

Fig. 5 shows the urinary excretion (expressed per 48 h) of the five metabolites, free and total, in a subject who received 100 mg of carbidopa three times daily for one week (shown shaded), and the excretion in the week following.

During the carbidopa administration there was an increase in the output of conjugated metabolites; in particular, conjugated VMA, HVA and 5HIAA were detected in substantial amounts. These are normally a very small propor-

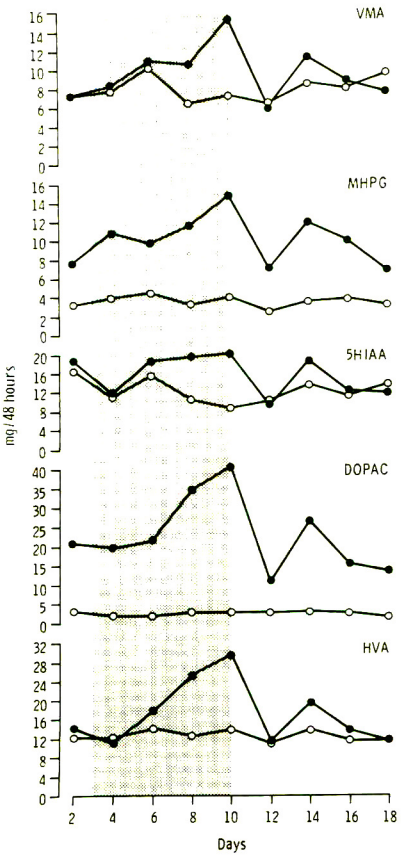

Fig. 5. Effect of carbidopa on excretion of amine metabolites in a normal subject. Carbidopa 100 mg three times daily was administered at 08.00, 14.00, 22.00 h for 7 days (shaded area). Excretion of free (○) and total (= free + conjugated, •) amine metabolites was determined. Consecutive 24-h urine samples were analysed; results are presented for 48-h periods for clarity.

tion of total output of these compounds. This argues against the conclusion of a previous study [10] which interpreted a reduction in free 5HIAA and in total MHPG excretion in ten subjects on this regimen as indicating effective blockade of extracerebral synthesis of serotonin and noradrenaline.

Simple and cheap modular apparatus can thus be used to give a rapid, accurate and specific determination of the biogenic amine metabolites VMA, MHPG, DOPAC, HVA and 5HIAA in urine. Our columns lasted for more than a year with occasional repacking of the inlet end. The carbon-paste electrodes last from 1—2 weeks to 1—2 months before requiring repacking, probably due to the relatively high methanol concentrations used in part of the running cycle. Glassy carbon electrodes are more stable, and would be preferred in this application where the ultimate in sensitivity is not required.

Similar principles are applicable to the determination of amine metabolites in cerebrospinal fluid [11] and post-mortem brain [12]. Cerebrospinal fluid can be injected directly after a filtration step [13] and for both brain and

cerebrospinal fluid the reduced number of peaks and virtual absence of VMA means that isocratic elution can be used. This methodology should find wide application in both the clinical and the research laboratory.

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CHROMBIO. 1022

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR BASE AND NUCLEOSIDE ANALYSIS IN EXTRACELLULAR FLUIDS AND IN CELLS

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SUMMARY

High-performance liquid chromatography based methods for the study of the metabolism of purine and pyrimidine bases and nucleosides have been developed. These methods, using 200—50 μl samples of extracellular fluids and employing isocratic separations, can measure a wide range of compounds. Hypoxanthine, xanthine and uridine concentrations in plasma from normal men are relatively stable. Species differences have been detected: concentrations of cytidine are higher in rat and mouse serum than in man, since the concentrations of uridine are similar; purine/pyrimidine ratios may be different. Fetal calf serum used for tissue culture contains about a 40 times higher concentration of hypoxanthine than the less-effective calf serum. Use of the methods appears to be justified in the assessment of the metabolic damage due to severe hypoxia and/or ischaemia.

INTRODUCTION

Interest in the metabolism of purines, pyrimidines, their nucleosides and nucleotides has been recently stimulated by developments in the study of inborn errors of metabolism [1] and in the biochemistry of "hypoxia". During ischaemia there is a reduction in the adenine nucleotide concentration, especially ATP, in cells (see ref. 2). Such reductions in ATP are associated with diminished erythrocyte survival [3] and renal survival [4]. However, measurement of adenine nucleotides requires tissue samples, which are difficult to obtain in serial or in clinical studies. Methods for the estimation of ATP metabolites, the purines and their nucleosides, in extracellular fluid are therefore required since there is extensive evidence [2, 4] of purine release by cells during hypoxia and by organs during ischaemia.

The isolation from biological material and subsequent identification of many

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purines, pyrimidines and their nucleosides, especially from urine [5], followed the development of ion-exchange chromatography by Cohn [6]. Despite development such methods were not suitable for physiological and pathological work, especially on small or on serial samples. There were at least two major problems; the low concentration of purines and their nucleosides in extra- and intracellular fluids, and the large number of closely interrelated and rapidly changing metabolites. The development of high-performance liquid chromatography (HLPC) with sensitive ultraviolet absorbance detectors can now provide methods of sufficient sensitivity and specificity to measure the large numbers of interrelated compounds in body fluids. The practicability of the methods allows large numbers of samples to be analysed and thus the detection of rapid changes.

Brown et al. [7] reported a method for the analysis of purine and pyrimidine bases and their nucleosides by HPLC, and Hartwick and Brown [8] further developed this type of method for the selective analysis of adenosine. This group and others showed that good separations of standard compounds could be achieved by HPLC with reversed-phase columns which have generally been prepared commercially. However, the application of these methods to biological material has been limited perhaps because of the use of gradients or the limited sensitivity even of recent methods [9].

The present paper describes the development and application of a flexible isocratic HPLC method of high sensitivity and resolution using readily available 5- and 3-μm packings and standard equipment. Purine and pyrimidine bases and their nucleosides were quantitated in a wide variety of extracellular fluids and cell samples. These methods have been proved to be suitable for serial physiological and pathological studies over a period of three years.

MATERIALS

Purine and pyrimidine bases, nucleosides and other standards were obtained from Sigma, London, Great Britain, unless otherwise stated. Solvents and other chemicals were obtained from BDH, Poole, Great Britain; AnalaR grade was used unless otherwise stated. The methanol used in HPLC mobile phases was from Rathburn Chemicals, Walkerburn, Great Britain. Allopurinol used as an internal standard was supplied by Burroughs Wellcome, London, Great Britain. For HPLC, only all-glass double-distilled water, which was stored in glass containers and checked for the absence of plasticiser peaks on HPLC, was used.

Enzymes used in microchemical identification were xanthine oxidase (E.C. 1.2.3.2) from Koch-Light Labs., Colnbrook, Great Britain, and guanase (E.C. 3.4.5.3) and purine-nucleoside phosphorylase (E.C. 2.4.2.1.) from International Enzymes, Windsor, Great Britain.

A liquid chromatograph Model ALC 200 (Waters Assoc., Hartford, Great Britain) incorporating a U6K injector, M600A pump and Model 440 absorbance detector operating at 254 and 280 nm was used for all chromatography.

METHODS

Column packing

We have used ODS-Hypersil (Shandon Southern Ltd., London, Great Britain) packing. These are silica microspheres 5—7 μm, and more recently 3 μm, in diameter with a bonded phase of octadecyl groups. Both prepacked columns (250 × 5 mm) and laboratory packed columns were employed, the latter proving to be more robust. Columns were packed by upwards displacement at $4.3 \cdot 10^3$ kPa (6000 p.s.i.) [10] using a CPIII column slurry packer (Jones Chromatography, Llanbradach, Great Britain). The packing suspension and packing solvent was isopropanol. Columns were subsequently equilibrated with 200—300 ml of 60% (v/v) methanol—water and 200—300 ml of 30% (v/v) methanol—water before use.

After flushing to remove buffer salts, columns were stored overnight in 5—20% (v/v) methanol—water containing 0.2% (w/v) sodium azide. Columns were cleaned after every 100—200 samples, or if a noticeable loss of efficiency occurred, with 60% (v/v) methanol—water, isopropanol, and aqueous 0.2% (w/v) EDTA. This, and periodic repacking of 1—3 mm of the column top, restored a column to a usable efficiency, with a theoretical plate count of about 8000—16,000 per 250 mm, as measured with hypoxanthine.

Mobile phases

All buffer solutions were filtered through a Millipore 0.5-μm filter and degassed under vacuum, using all-glass containers at each stage. Mobile phases containing organic solvent, 0.9—1.0% (v/v) methanol, gave good peak shapes and consequent quantitation as well as prolonging column life.

The pH of the mobile phase had to be chosen carefully if peaks of interest were to be separated from contaminants, which sometimes varied in extracts of the same type from different people. Changes in pH and ionic strength altered the behaviour of some contaminants more than that of purines and pyrimidines. The mobile phase for the best resolution of the components of an extract had sometimes, therefore, to be determined. Generally 0.01 mol/l potassium dihydrogen phosphate (pH 6.5) containing 1% (v/v) methanol, which gave good resolution of standard purines, pyrimidines and their nucleosides, was a starting point. Concentrations of potassium dihydrogen phosphate ranged from 0.001 to 0.05 mol/l with the pH adjusted to 3.5—6.75 with 10 mol/l sodium hdyroxide or orthophosphoric acid. For analysis of the nucleosides thymidine and adenosine somewhat higher concentrations of methanol, 5—10% (v/v), gave optimal sensitivity and resolution.

The retention of compounds on the columns could be decreased by increasing the temperature of the column and mobile phase from the usual ambient temperature of 21°C, to 55°C. Although increased retention was found at 0°C, peak shapes were poor and high viscosity of the mobile phase caused marked pressure increases. A simple water jacket for the column (Wright Scientific) coupled to a standard TU14 Tempunit (Techne) is now used and gives more reproducible retention of compounds.

Quantitation of compounds

Complex chromatograms are obtained from biological samples; thus an internal standard with a retention time close enough to compounds of interest to allow easy quantitation may be obscured by contaminating peaks. Since complex samples can take about 30 min to analyse by HPLC, it is not practicable to inject a solution of standard compounds before each sample. Moreover, retention of a compound was variable throughout a day, although such variations are reduced by controlling the column temperature, so that simple measurements of peak height were inaccurate and measurement of peak area by graphical methods impossible, due to the small width of most peaks. However, column efficiency as measured by the number of theoretical plates (N) remains constant during a day, although varying by ± 10% on column storage. For these reasons N was measured with hypoxanthine and a mobile phase of 0.01 mol/l potassium dihydrogen phosphate (pH 6.5) to which 1% (v/v) methanol was added, at 1.0 ml/min. For each compound of interest, retention and peak height were recorded. It can be shown that for any peak, concentration, C, is related to peak height, H, and retention time, t_R, as follows

$$C \propto H\, t_R / N^{1/2}$$
$$C = F\, H\, t_R / N^{1/2}$$

where F is a constant for each compound dependent on detector response and instrument settings. For each compound of interest F was measured and found to be constant (± 2%) and independent of N, retention time and, for most compounds, mobile phase. However, when peak shape changed with pH, with for example guanine, F had to be measured again. This somewhat unusual method of measurement was satisfactory.

The calculation of concentrations in cells was initially based on cell number determined by counting, or on cell protein as measured with the Folin phenol reagent. In order to obtain concentrations that were comparable with concentrations in extracellular fluids and which bore some relation to available kinetic constants of metabolising enzymes and membrane "carriers", results were expressed relative to cell volume determined from existing median values [11].

Identification of purine bases and nucleosides in extracts

The identity and purity of peaks was established by a variety of methods:

(1) Retention relative to an internal standard which remained relatively constant throughout the life of the column.

(2) Ratios of absorbance measured as peak heights at 254 and 280 nm which was a useful characteristic for many compounds [12].

(3) Peak shape, there was characteristic tailing of adenine.

(4) Response to changes in pH of the mobile phase. Cytidine, xanthine, and guanine change their retention and in the case of guanine, peak shape, with pH changes whereas hypoxanthine and uridine do not change.

(5) Treatment with the relatively specific enzyme preparations described above caused the disappearance of the appropriate substrate and appearance of its product on the chromatogram. Incubation was at 37°C, after adjusting the pH of the extract with solid Tris or 2 mol/l hydrochloric acid to an optimum for the relevant enzyme. The efficiency of enzyme treatment and duration of

incubation needed was checked with standard compounds at the appropriate concentration. When resolution from contaminants was impracticable, peaks could be measured before and after enzyme treatment and the concentration of the substrate thereby determined.

(6) Absolute concentrations of urate were high in plasma and amniotic fluid but not in cerebrospinal fluid. Urate has a high absorbance at 280 nm compared to that at 254 nm, thus providing an identifiable endogenous internal standard with a short retention time eluting before most of the purine bases of interest and uridine. The added internal standard, allopurinol, eluted after the purine bases and uridine but before inosine (Fig. 1).

(7) The addition of standard compounds to extracts sometimes showed that the retention time of an unknown was not exactly identical with that of the added standard.

The procedures commonly used were relative retention time, 254/280 nm absorbance ratio and enzyme treatment. The only measured components not identified by all the above procedures were uridine and cytidine. Their chromatographic behaviour, however, was distinctive.

Estimation of the efficiency of extraction and overall precision of analysis of hypoxanthine, xanthine and inosine from plasma

The extraction procedure for plasma, amniotic fluid, cerebrospinal fluid or blood cells was an adaptation of that of Khym [13]. Two volumes of ice-cold fresh trichloracetic acid (TCA) solution, 6—14% (w/v) depending on the type of sample analysed, were added to the fluid or cell suspension and the resulting precipitate removed by centrifugation. The supernatant was then extracted three times with 1.5 volumes of water-saturated diethyl ether to remove the TCA. The aqueous layer, now pH 4—5, was passed through a 0.5-μm Millipore filter prior to HPLC. About 0.2 ml of biological sample was adequate for this procedure although volumes of 20 μl have been extracted satisfactorily. All extracts were stored at —20° C.

The use of Tris and phosphate buffers to neutralise the TCA in the extracts was associated with the appearance of ultraviolet-absorbing compounds with similar retention times to allopurinol on ODS-Hypersil columns; these impurities could not be eliminated. Since high sensitivities were needed for purine base and nucleoside analyses, especially in cerebrospinal fluid in which concentrations were often less than 1 μmol/l, neutralisation of extracts with buffers could not be used; the longer extraction of TCA with diethyl ether was necessary.

In order to estimate the efficiency of the extraction method, a 5-ml sample of human blood plasma was incubated for 30 min with xanthine oxidase (0.5 ml, Koch-Light) to remove endogenous hypoxanthine and xanthine. The reactions were stopped by adjustment of the pH to 2.5 with concentrated hydrochloric acid. Known amounts of solutions of hypoxanthine, xanthine, both 1.3—30.1 μmol/l, and inosine 0.6—16.3 μmol/l were added to aliquots of this plasma and mixed thoroughly. Two volumes of 6% (w/v) TCA were added to the plasma and the extracts analysed at least twice [14].

Precautions in sample handling

The stability of TCA extracts was similar to that of solutions of standards. Purine base concentrations showed no definite change over periods of as long as six months. Brown and Miech [15] noted little change even in nucleotide concentrations of comparable extracts stored at −20°C for over one month.

Purine release from blood cells into plasma is detectable almost immediately after sampling [16], as suggested by earlier experimental work [17], and affects intracellular nucleotide concentrations significantly by about 40 min after sampling [18]. If consistent results were to be obtained from plasma extracts the rapid removal of blood cells by centrifugation within 15 min of sampling, with care taken to avoid cell lysis, was important; thereafter plasma was stable at −20°C. In general most samples except amnotic fluid were extracted within 48 h. In order to separate debris and contaminating cells all samples were better centrifuged. Only slight changes in the HPLC pattern were noted with samples of amniotic fluid when stored for over 24 months; these changes were not consistent. Cell preparations were routinely extracted upon receipt. The suspending medium was removed by centrifugation and aspiration, and the cellular pellet was resuspended in the appropriate volume of TCA solution.

RESULTS

The isocratic separation of standards is shown in Fig. 1, with the difficult separation of hypoxanthine from guanine in Fig. 2. Good resolution of the components of plasma and cerebrospinal fluid extracts was always possible (Figs. 3 and 4) with a mobile phase of appropriate pH and ionic strength. Sensitivity for hypoxanthine was about 1 pmol but depended on the volume injected and the concentrations of interfering compounds; this was especially important in samples from patients with renal failure.

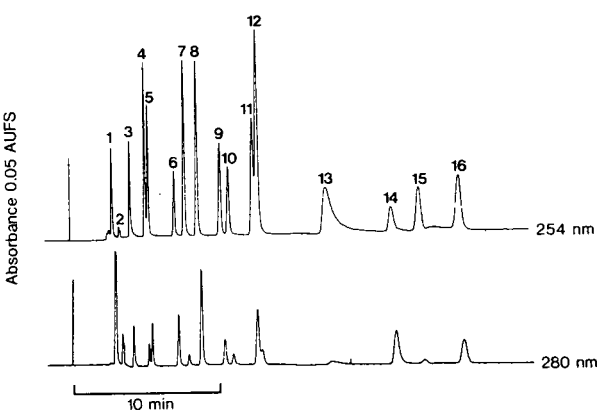

Fig. 1. Chromatogram of standard compounds (30—100 pmol). Peaks: 1 = orotic acid, 2 = uric acid, 3 = cytosine, 4 = uracil, 5 = pseudouridine, 6 = cytidine, 7 = hypoxanthine, 8 = xanthine, 9 = uridine, 10 = oxypurinol, 11 = thymine, 12 = allopurinol, 13 = adenine, 14 = 7-methylguanine, 15 = inosine, 16 = guanosine. Conditions: 25 × 0.5 cm column of Shandon C_{18} 3-μm ODS; mobile phase, 1 ml/min, 0.004 mol/l KH_2PO_4 (pH 5.8) with 1% (v/v) methanol at 30°C column temperature. N = 14,000.

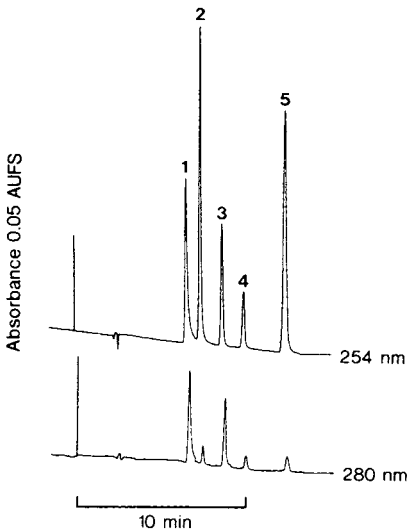

Fig. 2. Chromatogram of standard compounds (30—100 pmol) showing separation of guanine (1), from hypoxanthine (2), xanthine (3), uridine (4) and allopurinol (5). Mobile phase adjusted to pH 3.2; otherwise conditions and column performance as in Fig. 1.

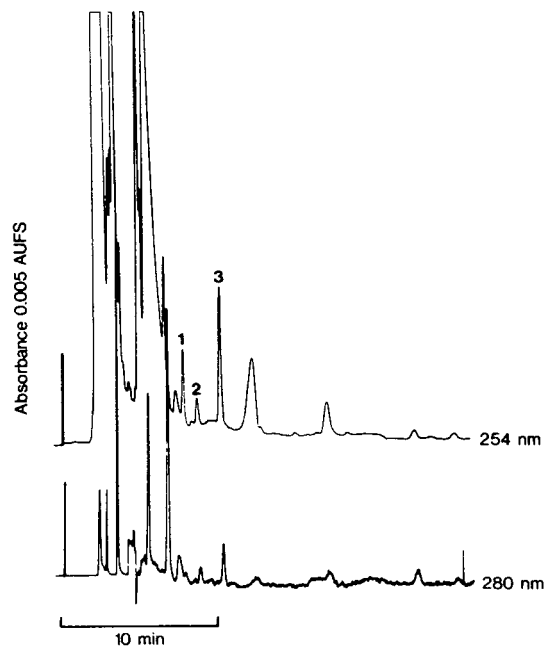

Fig. 3. Chromatogram of an extract of plasma (20 μl) using EDTA as an anticoagulant showing hypoxanthine (1), xanthine (2) and uridine (3). Conditions and column performance as in Fig. 1.

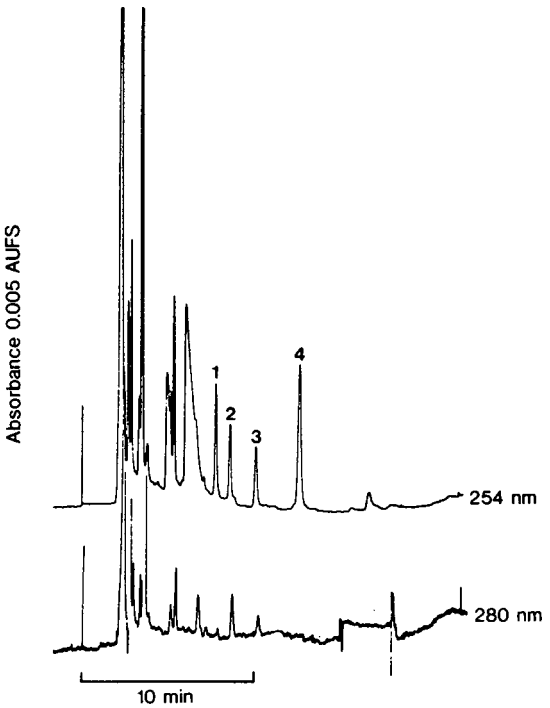

Fig. 4. Chromatogram of an extract of cerebrospinal fluid (20 μl) showing hypoxanthine (1), xanthine (2), uridine (3) and the internal standard allopurinol (4). Conditions and column performance as in Fig. 1.

Estimation of the efficiency of extraction and overall precision of analysis of hypoxanthine, xanthine and inosine from plasma

The mean recovery ± S.D. of hypoxanthine was 99.4 ± 3% (n = 12) in the concentration range 1.3—10 μmol/l and 100.3 ± 2% (n = 10) in the concentration range 10—30 μmol/l. For xanthine the mean recovery ± S.D. was 115 ± 15% (n = 18) in the concentration range 1—30 μmol/l. The presence of interfering peaks in this extract accounted for the higher and more variable values. The mean recovery ± S.D. for inosine was 82.3 ± 8% (n = 18) in the concentration range 1—16 μmol/l.

Estimates of precision obtained by repetitive analyses of single pools may be lower than those obtained during practical applications of a method. A series of samples were analysed in duplicate; using the differences between duplicate estimates [14] on a series of 29 different plasma samples the S.D. was ± 1.8 for a mean of 37.4 μmol/l, a coefficient of variation of 4.9%. For xanthine, the mean, S.D. and coefficient of variation were 3.8 ± 0.3 μmol/l, and 8.7% on 22 sets of duplicate analyses.

Using the difference between duplicate HPLC estimations on the same extract from a series of different plasma samples, the coefficients of variation for hypoxanthine were 2.4% (n = 18), 2.9% (n = 14), 1.3% (n = 14) and 1.9% (n = 12) for the concentration ranges 3—12, 20—25, 38—50 and 70—95 μmol/l, respectively. Similarly, for xanthine the coefficient of variation was 7.6% (n = 32) in the concentration range 0.1—50 μmol/l, for uridine 13.4% (n = 18) in

the concentration range 0.1—30 μmol/l and for inosine 6.0% (n = 8) in the concentration range 0.1—30 μmol/l. Using the above results for hypoxanthine, it appears that about half the error in the results may be due to the extraction and about half due to the HPLC.

Purine base, urate and uridine concentrations in extracellular fluids

The concentrations of hypoxanthine, xanthine, uridine and urate in plasma from four normal human males were obtained from serial samples of blood taken through an indwelling needle at 0, 30, 60, 90, 140 and 210 min in the middle of a working day. The concentrations showed no systematic or large differences in this "normal" working day. The results are therefore shown as mean ± S.D. (Table I); adenine, cytidine and inosine were not detected in these samples.

TABLE I

CONCENTRATIONS OF HYPOXANTHINE, XANTHINE, URATE AND URIDINE IN PLASMA FROM SERIAL SAMPLES FROM FOUR MEN

Samples from an indwelling needle were obtained at 0, 30, 60, 90, 150 and 210 min during a working day. Separation was by HPLC and detection by absorbance at 254 nm.

Subject	Concentration (μmol/l, mean ± S.D.; n = 6)			
	Hypoxanthine	Xanthine	Urate	Uridine
R	2.04 ± 0.6	0.61 ± 0.21	230 ± 30	4.37 ± 0.34
V	1.53 ± 0.14	0.68 ± 0.12	230 ± 30	4.21 ± 0.67
M	1.19 ± 0.27	0.92 ± 0.45	250 ± 30	3.04 ± 0.52
D	1.92 ± 0.75	0.85 ± 0.34	240 ± 50	3.35 ± 0.55

TABLE II

PURINE, CYTIDINE AND URIDINE CONCENTRATIONS IN FETAL CALF, CALF, RAT AND MOUSE SERA

Samples were from separate batches of material for tissue culture. The numbers in parentheses are separate batches in which the compound was detected. Analysis was by HPLC with detection at 254 nm.

	Concentration (μmol/l; mean ± S.D.)				
	Hypoxanthine	Xanthine	Uridine	Urate	Cytidine
Fetal calf	74.7 ± 31.9 (9)	92.0 ± 28.9 (9)	5.1 ± 2.1 (6)	130 ± 80 (7)	ND*
Calf	1.8 ± 1.9 (3)	0.3	3.8 ± 2.0 (3)	13 ± 9 (3)	1.3
Rat	0.7 ± 0.3 (9)	0.4 ± 0.5 (4)	3.9 ± 2.3 (9)	75 ± 40 (8)	9.7 ± 3.3 (9)
Mouse	1.1 ± 0.8 (3)	0.8 ± 0.5 (3)	5.0 ± 3.1 (3)	6.1 ± 1.4 (3)	3.6 ± 2.6 (3)

*ND = not detectable, i.e. a concentration of less than about 0.1 μmol/l.

Cytidine was found in rat and mouse sera (Table II) as well as in more limited samples from pig, rabbit, guinea pig, chicken, rhesus monkey, dog and sheep. Uridine as well as hypoxanthine and xanthine concentrations have been comparable in all species so far studied although the concentrations of hypoxanthine were low in rat plasma. The very low concentration (< 0.1 μmol/l) of cytidine in human plasma was confirmed using ultrafiltrates prepared with a filter with an exclusion limit of 25,000 (Amicon, Woking, Great Britain).

TABLE III

PLASMA AND CELLULAR CONCENTRATIONS OF PURINES, THEIR NUCLEOSIDES AND URIDINE IN BLOOD FROM FOUR NORMAL MEN

Plasma and erythrocytes were prepared by centrifugation, lymphocytes and polymorphonuclear neutrophil leucocytes (PMN) by a dextran—Ficoll based method; compounds were separated by HPLC and estimated from their absorbance at 254 nm.

	Concentration (μmol per l of plasma or cell volume; mean ± S.E.M.)					
	Hypoxanthine	Guanine	Xanthine	Uridine	Inosine	Guanosine
Plasma	1.5 ± 0.2	0*	0.46 ± 0.09	3.2 ± 0.6	0	0
Erythrocytes	11.0 ± 5.8	0	0	1.5 ± 0.3	0	0
Lymphocytes	139 ± 22	84 ± 21	0.2 ± 0.1	35 ± 7	64 ± 10	0
PMN	375 ± 98	71 ± 1.8	9.7 ± 5.5	63 ± 9.9	80 ± 13	20 ± 8

*0 indicates a concentration of less than about 0.1 μmol/l.

The most striking finding on analysing sera used for tissue culture purposes is the variable but approximately 40-fold higher concentrations of hypoxanthine and xanthine in fetal calf sera (Table II).

Cellular concentrations of hypoxanthine, guanine, xanthine, uridine, inosine and guanosine

The erythrocytes, polymorphonuclear neutrophil leucocytes and lymphocytes from human blood are frequently studied after standard separation methods (see ref. 19). The major purine base and nucleoside contents of erythrocytes and leucocytes with related plasma samples from four normal men are shown in Table III. Uridine concentrations in plasma are higher than in erythrocytes whereas hypoxanthine concentrations are higher in erythrocytes.

The pattern of purine bases has shown differences between different types of cells separated by standard methods; in lymphocytes the guanine concentration can be relatively high [20] whereas in polymorphonuclear neutrophil leucocytes xanthine may be relatively high. The relatively high rates of protein synthesis in lymphocytes and of oxidative and peroxidative metabolism in polymorphonuclear neutrophil leucocytes are probably related to these patterns. Overall concentrations would probably be lower in vivo because some nucleotide breakdown is to be expected during the standard separation methods used to produce lymphocyte and especially polymorphonuclear neutrophil leucocyte preparations.

Chromatography of the regulatory nucleotide A2'p5'A2'p5'A on ODS-Hypersil

A variety of nucleotides can be chromatographed on reversed-phase columns using our system. The most novel of these is the nucleotide A2'p5'A2'p5'A, the core of a variety of phosphorylated derivatives from interferon-treated cells [21]. The core material, which was supplied by Dr. I.M. Kerr (NIMR), had a retention time relative to adenosine of 0.81; similar relative times were 0.56 for A2'p5'A and 0.51 for adenine. Good separations and adequate retention was achieved with a mobile phase of 0.004 mol/l potassium dihydrogen phosphate with 10% (v/v) methanol (pH 6.5) at 1 ml/min. Extracts from a variety of tissues contained no major components with a similar retention time to the core nucleotide. Estimation of these compounds by the selective removal of

phosphate groups and estimation of the core nucleotides in tissue extracts may therefore be possible if sensitivity is adequate.

DISCUSSION

Clinical studies of blood plasma using xanthine oxidase and an oxygen electrode by Saugstad [22] have shown raised concentrations of oxypurines after hypoxia. However, this method failed to detect any oxypurines in cerebrospinal fluid from 15 of 39 patients [23]. The present HPLC method has been sensitive enough to estimate hypoxanthine in more than 100 cerebrospinal fluid samples.

The use of thin-layer chromatography (TLC) and densitometry [24] allowed the detection of raised concentrations of hypoxanthine in newborn urine after hypoxia but could not detect any increase in xanthine. A spectrophotometric method detected an increase in xanthine but not in hypoxanthine [25]. However, using the HPLC method we have shown an increase in both hypoxanthine and xanthine concentration of amniotic fluid, derived from fetal urine [2] and in urine from hypoxic newborn (unpublished). The identification of adenine in human plasma using TLC [26], despite the failure of other workers to detect this compound, suggests that results of purine analysis using TLC should only be accepted with caution.

The recent availability of better packing materials for HPLC has allowed much greater sensitivity and resolution than those previously demonstrated with isocratic systems [27]. This was needed for inosine concentrations in plasma. Even a recent HPLC method [9] for hypoxanthine required two isocratic runs and 500 μl of plasma (cf. Fig. 3).

For repetitive analysis of extracellular fluids for those compounds, hypoxanthine and uridine, that are exchanged between cells [28], isocratic conditions are more practicable than gradients [29, 30]. We found that a gradient was required only for separation of adenosine. However, due to co-chromatographing impurities plasma extracts required further purification before estimation of adenosine by HPLC. A single gradient separation of one TCA extract was impracticable.

It was necessary to select a suitable batch of reversed-phase material for the separations which were needed. The most difficult relevant separation was hypoxanthine from guanine (Fig. 2); this was achieved with resolution to the baseline with the initial batch of ODS-Hypersil. However, some later batches, despite giving a more consistent performance, better peak shapes and efficiencies, did not resolve these two compounds. Less trimethylsilylation or "capping" of the C_{18}-coated packing material allowed this separation to be made but not others (for example, xanthine and uridine) that are also biochemically important. There was associated with less trimethylsilylation a marked tailing of the peaks. It was generally possible with a selection of columns and mobile phases to achieve the necessary separation in a biochemical sample because not all the bases were always present. For example, guanine was not present in extracellular fluids in amounts comparable to hypoxanthine.

The use of external purines by cells is well established [28]. High concentrations of hypoxanthine in fetal calf serum would reduce the energy cost of cell

growth in culture [31] and avoid the need for the long pathway of de novo purine synthesis. Hypoxanthine may therefore be one of the factors responsible for the capacity of fetal calf serum to establish fibroblast cultures from human cell samples [32].

The results in Table II suggest that there may be differences between human, rat and mouse cells in the concentrations of extracellular purines relative to pyrimidines to which these cells are adapted. Experimentally large imbalances of purines relative to pyrimidines are capable of causing metabolic upset [33].

The applications of the present series of methods to physiological and pathological problems in man [2, 16, 34] and pig [35] has provided evidence for their reliability. The relative constancy of the results in plasma (Table I) suggest that physiological and pathological changes could be detected relatively easily.

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SIMULTANEOUS DETERMINATION OF D- AND L-THYROXINE IN HUMAN SERUM BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A method for the determination of D- and L-thyroxine in human serum is described. The method involves extraction of thyroxine from serum and the separation of thyroxine enantiomers on a reversed-phase, high-performance liquid chromatographic column by use of a chiral eluent containing L-proline and cupric sulfate. Satisfactory resolution of the enantiomers of thyroxine, triiodothyronine, and reverse triiodothyronine can be achieved in 12 min and, employing amperometric detection to monitor the separation, the detection limit for serum thyroxine is in the range of 1—3 ng per injected sample.

INTRODUCTION

The principal naturally occurring thyroid hormone is the levo-enantiomer of thyroxine (3,3′,5,5′-L-tetraiodothyronine, LT_4) [1]. The dextro-isomer, DT_4, considered to have only a fraction of the biological activity of LT_4, has been used extensively to reduce serum cholesterol levels in euthyroid hyperlipidemic subjects [2]. Recently it was shown that either 4 mg of DT_4 or 0.15 mg of LT_4 produced a similar degree of pituitary thyrotrophin suppression and an equal stimulation of basal metabolic rate [3]. Since no adequately sensitive method was available to measure specifically the D and L forms of iodothyronines, it was impossible to define whether (1) LT_4 contaminated the administered DT_4, (2) DT_4 was converted in vivo to LT_4, or (3) DT_4 and/or a dextro-metabolite had true biological activity. In order to investigate these questions we developed a sensitive high-performance liquid chromatographic (HPLC) technique which is capable of rapidly separating the optical isomers of both thyroxine

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and the two triiodothyronines, 3,3',5'-triiodothyronine (T_3) and 3,3',5-triiodothyronine (reverse T_3, rT_3), and can, when combined with an efficient serum extraction method, allow the specific measurement in human serum of both endogenous LT_4 and exogenous DT_4.

In the past, the specific determination of iodothyronine enantiomers was dependent on techniques such as direct measurement of optical rotation [4] or stereospecific oxidation with L-amino acid oxidase [5]. More recently Lankmayr et al. [6] coupled iodothyronines with L-leucine and resolved the resultant diastereomers by reversed-phase HPLC. We have employed a more direct approach by the use of a chiral eluent, adapting the principle first described by Hare and Gil-Av [7], who successfully employed it to separate underivatized amino acid enantiomers on an ion-exchange column.

To permit the application of this technique to our goal of measuring in human serum the thyroxine enantiomers, we required both a detection method capable of measuring iodothyronines in subnanogram quantities and a serum extraction procedure adequately efficient to allow satisfactory HPLC. Recently reported HPLC techniques for detecting iodothyronines by UV light have a sensitivity threshold of 8—10 ng [8, 9]. The post-column catalytic detection system of Nachtmann et al. [10], although adequately sensitive, requires the construction of special equipment and can not be used with a mobile phase containing metallic ions. Electrochemical detection, as reported by Hepler et al. [11], has the potential for measuring T_4 and T_3 down to the 0.12—0.18 ng range. We have successfully employed this detection principle using commercially available amperometric equipment [12]. The serum extraction techniques described by Hepler and Purdy [13] and by Bongiovanni et al. [14] were found to be unsatisfactory for our purposes. Consequently we had to develop a novel extraction method to allow for the first time the specific determination in human serum of the enantiomers of thyroxine.

MATERIALS AND METHODS

Reagents and materials

All solvents were analytical reagent grade. Methanol, ethyl acetate and acetonitrile were supplied by J.T. Baker (Phillipsburg, NJ, U.S.A.). Water was de-ionized and glass-distilled. Sodium acetate trihydrate, cupric sulfate pentahydrate, silver nitrate, L- and D-proline were supplied by Sigma (St. Louis, MO, U.S.A.), who also provided the D- and L-enantiomers of T_3 and T_4. The D- and L-enantiomers of rT_3 were a generous gift from Dr. H. Rokos, Henning Berlin GmbH, Berlin, G.F.R. Sep-Pak silica cartridges were supplied by Waters Assoc. (Milford, MA, U.S.A.).

The mobile phase consisted of an acetonitrile—0.1 *M* sodium acetate solution (30—35:70—65, v/v) containing 0.004 *M* cupric sulfate, 0.008 *M* L- or D-proline and 0.002 *M* silver nitrate. The 2:1 molar ratio of proline to copper was as employed by Hare and Gil-Av [7]. The addition of silver nitrate allowed chromatography at a lower amperometric offset reading. Litre solutions of chiral eluent (without acetonitrile) were routinely prepared by dissolving in water 13.6 g of sodium acetate, 1.0 g of cupric sulfate, 0.92 g of L-proline and 0.34 g of silver nitrate. Prior to chromatography appropriate volumes of ace-

tonitrile were added to the copper—proline solution and the mixture degassed with a vacuum pump for 10 min. Standards for HPLC consisted of DT_3, LT_4 and DT_4 dissolved in methanol at final concentrations of 0.25—1.25 μg/ml for DT_3 and 0.5—2.5 μg/ml for L and DT_4.

Chromatographic instruments

An Altex Model 110A solvent metering pump (Altex Scientific, Berkeley, CA, U.S.A.) combined with a Rheodyne 7120 sample injection valve (100-μl loop) was used for the chromatographic separation. The reversed-phase column (25 cm × 4.6 mm I.D.) employed was an Ultrasphere I.P. (5 μm average particle diameter), a product of Altex. Amperometric detection was performed with electrochemical equipment (TL-5 Kel-F glassy carbon thin-layer cell, LC-4 electronic controller), from Bioanalytical Systems Inc. (West Lafayette, IN, U.S.A.), coupled to a Fisher Series 5000 Recordall pen recorder. Gradient elution and thermostating were not necessary and separations were performed isocratically at room temperature (20—22°C).

Serum extraction

To each 1-ml serum sample are added sequentially 100 μl of ^{125}I-labeled T_4 (20,000 cpm), 3 ml of 5% trichloroacetic acid, and 4 ml of ethyl acetate. Each tube is vortexed vigorously to allow complete mixing of ethyl acetate and precipitated serum proteins. After spinning for 5 min at 1500 *g*, the upper organic layer is transferred with a glass pipette to a 15-ml borosilicate collection tube. Ethyl acetate extraction is then repeated twice using two further 3-ml volumes. The extract is reduced under nitrogen in a water-bath evaporator to a final volume of about 1.5 ml. This 1.5-ml extract is then applied with a glass transfer pipette to a Sep-Pak silica cartridge equilibrated with 5 ml of ethyl acetate. After an 8-ml ethyl acetate wash, the extract is eluted with 4 ml of methanol—ammonium hydroxide (90:10), reduced to dryness under nitrogen and reconstituted in 100 μl of methanol prior to injection on to the column.

Chromatographic procedure

A flow-rate of 1.4—2.0 ml/min was generally used at a pressure of 262—310 bars for the Ultrasphere I.P. column. The potential applied to the detector cell was +0.78 V and detection was routinely performed at a setting on the electronic controller of 2—5 nA/V. Standard solutions of 20—25 μl volume containing 2.5—50 ng of DT_4 and LT_4 and 1.25—25 ng of DT_3 or LT_3 were injected on to the column using Pressure-Lok liquid syringes (0—25 μl, 0—50 μl) Series B110 from Precision Sampling Corporation. Standard curves for DT_4 and LT_4 were constructed from duplicate determinations of standard samples and the peak height (nA) was plotted against the known injected amount of thyroxine (ng).

RESULTS

Optimization of chromatographic conditions

Chromatography was routinely performed with L-proline as the chiral

constituent of the mobile phase. Best results were found to occur when the column and thin-layer cell (with voltage applied) were allowed to equilibrate with the mobile phase for 1—2 h prior to injection of samples. Fig. 1 shows the separation of the enantiomers of T_3 and T_4 obtained within 25 min on isocratic elution with an acetonitrile—copper-proline-acetate (35:65) mobile phase. Since L-proline was used in the chiral eluent, the first peak of each pair represents the L-enantiomer. The use of D-proline would result in reversal of the order of appearance of compounds from the analytical column.

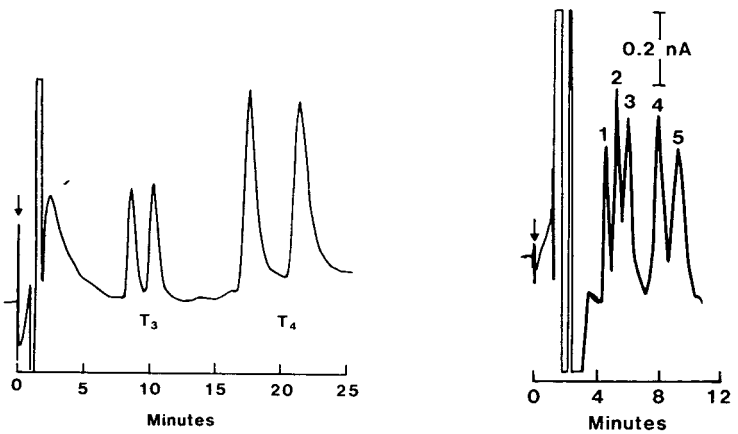

Fig. 1. Chromatogram of the enantiomers of T_3 and T_4. Column was injected with 10 ng each of DT_3 and LT_3 and 20 ng each of DT_4 and LT_4. Mobile phase, 30% acetonitrile; flow-rate, 2 ml/min; detector sensitivity, 5 nA/V.

Fig. 2. Separation of LT_3 (1), LrT_3 (2), DrT_3 (3), LT_4 (4) and DT_4 (5). Column injected with 7.5 ng of each of LT_3, LrT_3 and DrT_3 and 15 ng of each of LT_4 and DT_4. Mobile phase, 35% acetonitrile; flow-rate, 1.5 ml/min; detector sensitivity, 2 nA/V.

To allow a more rapid analysis, the conditions were altered to enable a separation within 12 min of the T_3, rT_3 and T_4 enantiomers. Fig. 2 shows the HPLC pattern obtained when 7.5 ng of LT_3, LrT_3 and DrT_3 and 15 ng of LT_4 and DT_4 were injected onto the column. In this example peak 2 is represented by LrT_3 but it could equally have been DT_3 since these compounds coelute under these conditions. If D-proline was exchanged for L-proline in the mobile phase, then an unknown peak at position 2 with L-proline could be resolved into peaks at either position 1 or 3, representing DT_3 and LrT_3. Judicious choice of the chiral constituent of the mobile phase could therefore permit the chromatographic identification of any of the four triiodothyronine enantiomers.

For quantitative purposes a mobile phase containing 34—36% acetonitrile was employed at a flow-rate of 1.4—1.6 ml/min. Optimal tracings were obtained when conditions (as in Fig. 2) were manipulated to allow the LT_4 peak to appear at about 8 min after injection. Fig. 3 shows a typical calibration curve for the quantitative detection of LT_4 and DT_4. With peak height plotted against concentration, the linearity of the standard curves in the range 2.5—100 ng was determined by calculating the correlation coefficient, which was found to be 0.998 for both the T_4 enantiomers. At a sensitivity setting of 2 nA/V on

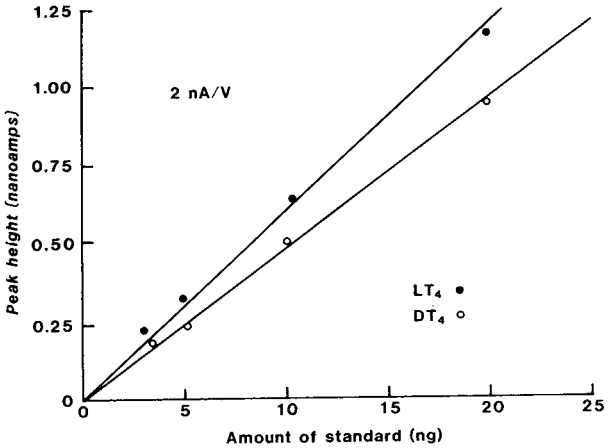

Fig. 3. Calibration curves for assay of LT_4 and DT_4. Conditions as in Fig. 2. Each point represents the average of duplicate determinations which agreed to within 3% of each other.

the electronic controller, the detection limit for either LT_4 or DT_4 was 1—3 ng, while for either of the T_3 or rT_3 enantiomers as little as 0.5—1.5 ng could be detected. Because at 2 nA/V there tended with repeated injections to be increasing fluctuation in the electronic baseline, routine quantitation was performed at the higher setting of 5 nA/V with its consequently higher detection limit of 3—5 ng for T_4.

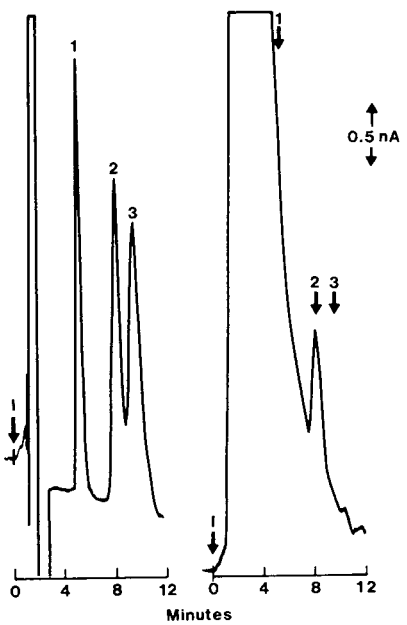

Fig. 4. Chromatogram of a serum extract from a euthyroid patient (right panel) compared with (on left) that derived from a standard solution containing 30 ng of DT_3 (1), 60 ng of LT_4 (2), and 60 ng of DT_4 (3). The single peak seen in the serum extract elutes in the position of LT_4. Mobile phase, 35% acetonitrile; flow-rate, 1.5 ml/min; detector sensitivity, 5 nA/V; injection volumes, 25 μl.

Quantitation of T_4 in serum samples

LT_4, labeled with radioactive iodine (^{125}I), was used to reflect T_4 recovery from serum samples. On average the ethyl acetate extraction recovered 85—95% of the counts while the silica column typically retained 65—70% of the extracted counts which were completely removed by the methanol—ammonium hydroxide (90:10). Using serum samples spiked with known amounts of LT_4, the recoveries were found to be linear over the range 15—200 ng of T_4 (1.5—20.0 μg/dl serum). The overall recovery from 75 consecutive serum extractions was 65 ± 6%. The value of T_4 obtained by electrochemical detection was found to correlate well ($R = 0.84, n = 48, p < 0.001$) with values obtained by conventional nonstereospecific radioimmunoassay of serum total thyroxine.

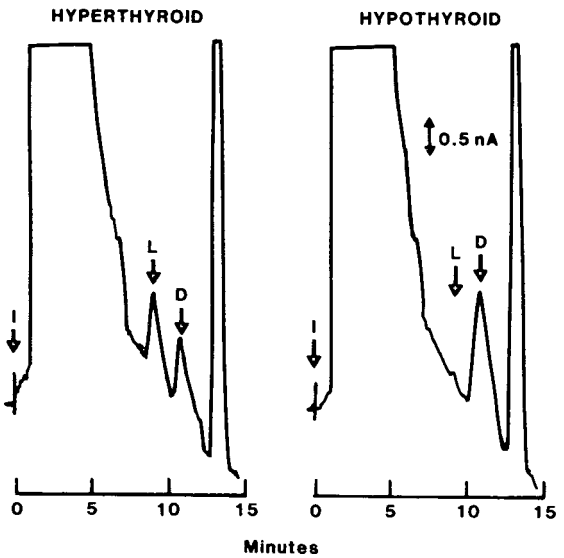

Fig. 5. Chromatograms of serum extracts from hyperthyroid and hypothyroid patients after the addition to the serum of DT_4 (100 ng/ml). Conditions as in Fig. 4, but injection volume for hyperthyroid was 15 μl and for hypothyroid 30 μl. The large peak at 13 min is due to an unknown serum constituent.

The technique was first applied to the determination of the enantiomeric state of circulating T_4 in serum from patients known to be either euthyroid or hyperthyroid as determined by clinical and biochemical assessment. In both these circumstances, a single T_4 peak was seen with the retention time of standard LT_4. Fig. 4 demonstrates the HPLC pattern seen in a euthyroid serum sample and compares the tracing with that using standards of DT_3, LT_4 and DT_4.

When known amounts of DT_4 were added in vitro to either hypothyroid or hyperthyroid human serum, this was reflected in the HPLC tracing by the appearance of a second peak with a retention time identical to that of authentic DT_4 (Fig. 5). Similarly, when a euthyroid patient was orally administered 10 mg of DT_4 and blood drawn 4 h after ingestion, the HPLC pattern obtained from the serum (Fig. 6) showed an identical configuration to that obtained when DT_4 was added in vitro to serum.

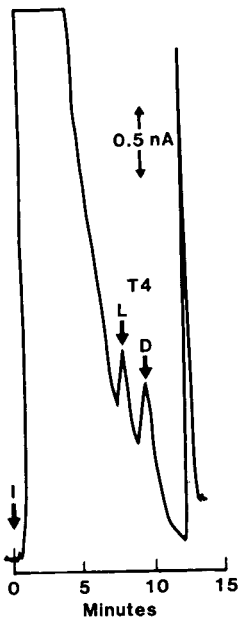

Fig. 6. Chromatogram of serum extract from euthyroid patient given 10 mg of D-thyroxine 4 h prior to venipuncture. Conditions as in Fig. 4. Injection volume, 25 μl.

DISCUSSION

Although multiple HPLC techniques have now been described [6, 8—11, 14] which are capable of separating T_3 from T_4, only the method described by Lankmayr et al. [6] has the stereoselective capability of separating the T_3 and T_4 optical isomers. The technique described in this paper does not require the precolumn synthesis of diastereomers and, by using underivatized samples, it permits the direct quantitation of the iodothyronine enantiomers. The serum extraction we have described is both simple and efficient and has allowed for the first time a stereospecific determination in human serum of circulating thyroxine.

Like the technique of Lankmayr et al. [6] the present method can be used to determine the LT_4 contamination of pharmaceutical preparations of DT_4. Analysis by this technique of currently available U.S. preparations of DT_4 has revealed no evidence of contamination with LT_3 but has demonstrated an LT_4 content of 0.4—0.5%. This minor degree of LT_4 contamination is comparable to that estimated using the classical L-amino acid oxidase method [15] and would not of itself account for the biological effects seen in our recent studies of DT_4 treatment in hypothyroid subjects [3].

The more exciting possibility that ingested DT_4 may in vivo be converted to more bioactive LT_4 has never been investigated, largely because currently available anti-DT_4 antibodies cannot differentiate between DT_4 and LT_4. The presently described technique provides for the first time a methodology capable of verifying or refuting this possibility. Current sensitivity limits allow the detection of serum T_4 levels down into the hypothyroid range. However,

further refinement of the thin-layer electrochemical detection system [11] will be necessary before the technique can be applied to the direct quantitation in human serum of either T_3 or reverse T_3.

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SEPARATION OF BILIRUBIN SPECIES IN SERUM AND BILE BY HIGH-PERFORMANCE REVERSED-PHASE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance, reversed-phase liquid chromatographic (HPLC) procedure has been developed for the separation of at least three major bilirubin fractions in bile and four fractions in human serum. This procedure was unlike most others, in that serum was not totally deproteinized prior to injection onto the HPLC column; instead, serum was treated with an excess of sodium sulfate solution to precipitate primarily proteins larger than albumin. Injection of the filtered and diluted supernatant onto a reversed-phase column then resulted in the separation of the bilirubin species in a 24-min gradient elution run. Both the initial aqueous acidic mobile phase and the final isopropyl alcohol-based mobile phase contained 5% methoxyethanol (v/v) to facilitate elution of albumin still present in the treated sample. Bilirubin species eluting from the column were detected by absorbance at 450 nm.

Results of a number of chromatographic separations of pathological sera indicated a wide variation in the relative proportions of the four bilirubin fractions observed. A correlation of the sum of the areas of the bilirubin peaks observed by HPLC was found with the total bilirubin value obtained by a standard reference procedure.

INTRODUCTION

The formation of diazo derivatives of bilirubin is the basis for most clinical methods of analyzing bilirubin in serum and bile [1—3]. In general, the diazo methods yield a single value for the total concentration of all the bilirubin species present in a sample and, in certain modifications, an additional lesser value which is considered to be proportional only to the concentration of the more water-soluble bilirubin esters or conjugates. Yet, it has been well established for some time that bilirubin exists in bile as at least three major chemical forms [4—6]: free, monoester, and diester with glucuronic acid as the predominant ester group. Besides these three, in human serum there is a fourth form of bilirubin which is more tightly associated with protein [7—12]. In

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addition, minor amounts of other esters have been found in biological fluids [4, 6, 13, 14]. Thus, the use of a single colorimetric measurement to characterize the sum of several chemical species may be clinically useful but yields little accurate information about the relative proportions of each of the individual species, which may also be of clinical significance.

Chromatographic methods would appear to be ideal for studying the distribution, as well as the clinical significance, of the individual bilirubin species in serum. Paper chromatography has been applied extensively to this problem and has been reviewed by With [15]. Thin-layer methods have also been used [5, 16—19]. In general, these methods do separate several bilirubin species; however, quantification and identification of the separated fractions found in biological fluids are problematical because of the instability of the pigments.

The open-column, reversed-phase chromatographic procedure of Cole and co-workers [20, 21] and Billing [22, 23] is the basis for most early work with column chromatography. Unfortunately, this procedure is not entirely satisfactory because with pathological serum samples, a substantial amount of the yellow color due to bilirubin is lost with the precipitated proteins [24—28]. Kuenzle and co-workers [7—12] modified this procedure by chromatographing serum without first deproteinizing. As a result, they observed four bilirubin fractions: unconjugated bilirubin (α), bilirubin monoconjugate (β), bilirubin diconjugate (γ), and a fourth fraction (δ) strongly associated with protein. This last fraction is distinct from the complex of unconjugated bilirubin (α) and albumin known to exist in serum and aqueous solution [29—32]. Perhaps because of the large volume of serum required and difficulties in quantifying the separated pigments, this work seems to have been neglected in the more recent literature.

In the last few years, several high-performance liquid chromatographic (HPLC) methods have been reported for the separation of bilirubin species in various biological fluids [33—36]. These methods either have not been applied to serum or have required extensive deproteinization prior to chromatography. Difficulties with protein elution from modern, silica-based chromatographic materials are well known [37—40]. In general, most large proteins appear to associate irreversibly with the column, either because of adsorption on free silanol sites or because of a strong partitioning of the hydrophobic side chains of the proteins into the hydrophobic bonded phase. Since proteins are present in serum at 6—8% by weight and since high-performance columns can be destroyed in a short time by this association, common practice is to remove the proteins prior to the chromatographic separation of serum components. This procedure makes it impossible to observe bilirubin species which are attached, either in a very strong complex or by a covalent bond, to protein.

To overcome this limitation of HPLC for the analysis of bilirubin species in serum, we used a solvent system first described by Mönch and Dehnen [37] for the elution of proteins with molecular weights as high as $4 \cdot 10^5$ from a reversed-phase column. This system allowed albumin and that fraction of bilirubin (δ) most tightly associated with it to be separated from other bilirubin species present in serum. Unfortunately, serum contains an appreciable fraction of protein with molecular weight $> 10^6$ daltons [41], and some would not pass through the column, again causing irreversible loss of efficiency in a

short time. To circumvent this effect, prior to the chromatography, we have resorted to a classical precipitation of the higher-molecular-weight proteins in serum by dilution with a sodium sulfate solution [42]. With this separation for serum, some yellow color was still lost with the precipitated higher-molecular-weight proteins. However, the resulting chromatographic separation was reproducible, and the column was extremely stable, a single column having been used for more than 250 injections of serum without serious degradation of resolution or efficiency.

EXPERIMENTAL

Materials

Ascorbic acid, 2-methoxyethanol, caffeine, sodium benzoate, and phosphoric acid were all reagent grade or better, obtained from Kodak Laboratory Chemicals (Rochester, NY, U.S.A.). Caffeine—benzoate solutions were formulated according to Tietz [43]. Sodium phosphate, mono- and dibasic, and anhydrous sodium sulfate were obtained from MCB Reagents (East Rutherford, NJ, U.S.A.). Sodium sulfate solutions were prepared by dissolving 27.7 g of the anhydrous salt in ca. 80 ml of hot distilled water. The pH was then adjusted to 7.0 ± 0.2 with dilute sulfuric acid or dilute sodium hydroxide. The solution was diluted to volume in a 100-ml volumetric flask and kept in a 37°C water bath. Each day a 10% (w/v) aqueous ascorbic acid solution was prepared in a 50-ml volumetric flask with 1 ml of 2 *M* phosphate buffer (pH 6.7) added. This solution was adjusted to pH 5.8 ± 0.2 with sodium hydroxide.

Human serum albumin (fraction V) and unconjugated bilirubin were obtained from Sigma (St. Louis, MO, U.S.A.). A master standard solution of unconjugated bilirubin in 5% albumin solution was made in a manner similar to that of Tietz [44]. Serial dilutions of this standard (5—150 mg/l) were made using 5% human serum albumin in distilled water (w/v).

From these albumin—bilirubin solutions, standards for injection onto the HPLC column were prepared by diluting 0.25 ml of the appropriate solution in a 10-ml volumetric flask with 7 ml of sodium sulfate solution, 0.50 ml of the 10% ascorbic acid solution, and distilled water. Because of instability in room light, all sera, bile and standard solutions of bilirubin were handled under yellow light. Under these conditions, the standards in sodium sulfate solution were stable at room temperature for ca. 15 h (< 3% loss).

The initial mobile phase for the HPLC separation was prepared by adding 2 *M* phosphate solution (sodium salt, pH 6.7, heated to prevent precipitation) and 2-methoxyethanol to a large Erlenmeyer flask to give, upon dilution with distilled water, a solution 0.05 *M* in phosphate and 5% in 2-methoxyethanol (v/v). The pH of the solution was then adjusted by means of a combination glass electrode and pH meter to 2.0 ± 0.1 by the addition of phosphoric acid. The second mobile phase was 5% in 2-methoxyethanol and 95% in HPLC grade isopropyl alcohol (v/v) obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). Phosphoric acid was then added to this solution in the ratio of 25 ml to 1 l of solution. Before use, both mobile phases were thoroughly degassed by stirring under a stream of helium.

Glass-fiber filters were obtained from Gelman (Ann Arbor, MI, U.S.A.)

(Type A-E, 25 mm, No. 61630) and 0.45-μm filters were obtained from Millipore (Bedford, MA, U.S.A.) (Type HA, 25 mm). The HPLC column was stainless steel, 25 cm × 4.6 mm I.D., prepacked with 10-μm silica to which octylsilane had been bonded (LiChrosorb RP-8). This was purchased from Brownlee Labs. (RP-10A) via Rheodyne (Berkeley, CA, U.S.A.). For some separations, an RP-8 guard column, also from Brownlee Labs., was used ahead of the analytical column.

Apparatus

Either of two filtration units obtained from Amicon Corporation (Model MMC or Model 12) was used with the Gelman filter placed over a Millipore filter in the apparatus to remove precipitated proteins from sodium sulfate-treated serum samples. Both units were pressurized with helium.

The chromatography system consisted of a Waters Intelligent Sample Processor (WISP 710B, Waters Assoc., Milford, MA, U.S.A.) to inject samples, a Waters Model 660 programmer to control the gradient and two Waters Model 6000 pumps that had been upgraded to 6000A standards by the substitution of a 6000A reference valve and multisolvent inlet ports for those originally fitted. The chromatographic column was maintained at 41°C in an oven obtained from a DuPont 830 liquid chromatograph. After elution from the column, the eluent passed through a variable-wavelength spectrophotometer (Perkin-Elmer LC-55) set at 450 nm and then through a second spectrophotometer set at 280 nm (HM Holochrome UV Monitor, Gilson Medical Electronics). The outputs of both detectors were monitored with strip-chart recorders while peak areas were obtained simultaneously by means of an on-line computer system.

Procedure

Bilirubin—albumin—sodium sulfate standard solutions were prepared as described above and injected directly into the chromatography system. Bile samples were first diluted 1:10 with distilled water. Then a 0.25-ml aliquot of this diluted bile was added to a 25-ml volumetric flask containing 0.5 ml of ascorbic acid solution and 0.5 ml of 5% albumin solution. Sodium sulfate solution (14 ml) was then added, and the solution was diluted to the mark with distilled water.

Serum was diluted with sodium sulfate solution at 37°C in the ratio of 3.5 ml to 0.25 ml of serum to precipitate high-molecular-weight proteins. The diluted serum was then heated at 37°C in sealed vials, with occasional shaking, for several minutes before filtration through the Amicon filtration unit. The effluent from the Amicon filtration unit was fed directly into a 10-ml volumetric flask containing 0.5 ml of ascorbic acid solution. A second 3.5-ml aliquot of sodium sulfate solution was then passed through the precipitate and combined with that in the flask. The contents were diluted to volume with distilled water for injection into the chromatographic system.

For the chromatographic separation, 500-μl aliquots of the diluted and sulfate-treated sample were injected onto the chromatography column, which was maintained at 41°C. The serum components were eluted over 16 min at a flow-rate of 1.4 ml/min by a linear gradient from 100% initial mobile phase to 80%

final mobile phase—20% initial mobile phase. The mobile phase composition was then held constant for 8 min, by which time all components had eluted from the column. Before injection of the next sample, 100% initial mobile phase was allowed to flow for a 7-min equilibration period.

RESULTS AND DISCUSSION

Chromatography system

The phosphoric acid—methoxyethanol-based chromatography eluent system was adopted from the work of Mönch and Dehnen [37] after a series of preliminary experiments indicated that albumin would not elute reproducibly from a reversed-phase column when a more common acetate-buffered system (pH 4.5) was used. More important, with the acetate system a substantial amount of the yellow material present in pathological sera did not elute from the reversed-phase column. This suggested that some bilirubin species might be strongly associated with albumin. In fact, Kuenzle and co-workers [7—12] demonstrated almost 20 years ago that in pathological serum there was a bilirubin fraction (δ) which was very tightly associated with protein.

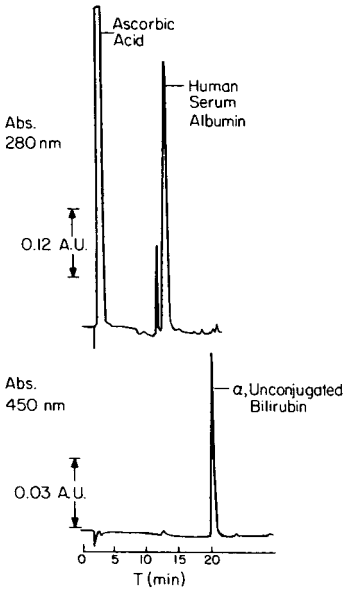
The present chromatography system has enabled the elution and separation of both albumin and unconjugated bilirubin, as shown in the chromatogram for a standard sample in Fig. 1. With fresh columns it was sometimes necessary to condition the column with several injections of a bilirubin standard solution before running samples and standards for a calibration curve. During this initial equilibration period, the peak height of the albumin peak (detected at 280 nm) would often decrease by as much as 10% of its initial value and then stabilize at a constant height. This effect may have been caused by residual adsorptive sites which, once coated with protein, did not interfere further in the analysis.

The small absorbance on the 450-nm trace in Fig. 1 at the retention for albumin was mostly due to the slight inherent yellow color of human serum albumin used to prepare the standard solution and not to unconjugated bilirubin. This peak was minimally enhanced in fresh standards, if at all, when compared with an injection of the same concentration of albumin with no unconjugated bilirubin present. Thus, in this chromatography system, the interaction of the column with bilirubin and albumin was strong enough to break up the complex known to exist between unconjugated bilirubin and albumin in aqueous solution.

In contrast, a typical pathological serum sample separated into four well-defined yellow fractions (Fig. 2). In this case, there was a substantial amount of yellow material which eluted at the same retention as that for albumin. Since interaction with the column did not break this association, it had to be stronger than those existing between albumin and the other bilirubin species in serum. In accord with notation adopted by Kuenzle and co-workers [7—12], we have labeled the peaks (α) unconjugated bilirubin, (β) monoconjugated bilirubin, (γ) diconjugated bilirubin, and (δ) protein-bound bilirubin.

Prechromatography treatment with sodium sulfate

The use of sodium sulfate as a protein precipitant was necessary for a reproducible chromatographic separation and long life of the column. When sodium



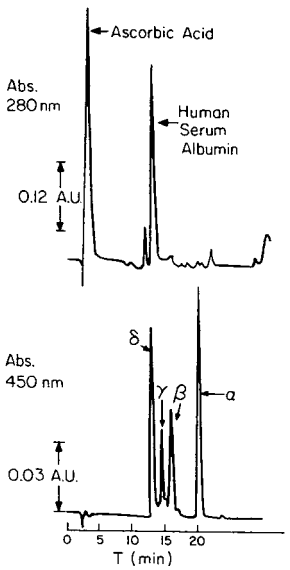


Fig. 1. HPLC separation of unconjugated bilirubin (α) in human serum albumin solution.

Fig. 2. HPLC separation of pathological serum.

sulfate was not added to samples, the initial separation of serum on fresh HPLC columns was similar to that given in Fig. 2, i.e., four well-defined bilirubin fractions. However, as few as eight 50-μl injections of undiluted serum could cause a substantial loss of resolution. In particular, peak broadening and tailing became progressively more severe, and the absolute area of the peaks decreased. These problems were traced to the incomplete elution of proteins with molecular weight higher than that of albumin. Mönch and Dehnen [37] had demonstrated that certain proteins with molecular weights as high as $4 \cdot 10^5$ should elute from the present system, but there are some proteins present in serum with molecular weights greater than 10^6.

For the system to reproducibly separate serum samples, it was necessary to remove these interfering proteins by pretreating sera with a 14-fold volume excess of sodium sulfate at 277 g/l, similar to the method of Yeoman [42]. The precipitate which was formed could not be easily removed by centrifugation and so was separated by filtration. In this procedure, a Gelman fiber filter served as a prefilter to increase the capacity of the filtering system so that blockage of the 0.45-μm filter would not occur. If a sample was cloudy after this treatment, it was filtered through a second 0.45-μm filter. If it was still cloudy after the second filtration, it was discarded, because cloudiness indicated incomplete removal of the interfering proteins, which would lead to rapid column degradation.

This treatment did cause some loss of yellow material which was adsorbed to the precipitated and filtered proteins. These losses may have been partly related to sample storage conditions prior to analysis. Many times, samples

stored frozen (−4°C) for several weeks were used to develop the procedure. Recent experiments have indicated that under these conditions, changes in the chromatographic pattern could occur. Freshly drawn samples are recommended.

Column performance

With this precolumn separation, column lifetime has been dramatically improved. Over a period of a month, more than 400 injections of standards and serum samples diluted with sodium sulfate and separated from higher-molecular-weight proteins were made on the same reversed-phase column. Column resolution was not appreciably degraded during this time and retentions were remarkably stable. In addition, we have found that different columns from the same manufacturer yield nearly identical separations. Unfortunately, we did not find that columns from other manufacturers would afford the same reproducible and long-term stable separation. Since nominally similar columns from different manufacturers have been known to exhibit different selectivities, this result was not surprising. Even with the Brownlee columns, we have found that only fresh columns, i.e., unused for other analyses with different solvent systems, would reliably yield a stable chromatographic system. Extraneous material irreversibly adsorbed to the column bed and/or protein denatured on the column surface from other analyses has, in our experience, led to unsatisfactory separation and/or poor column lifetime.

Standard calibration curve for α-bilirubin

The addition of ascorbic acid at an acidic pH along with albumin minimized oxidation and increased the stability of standard solutions of unconjugated bilirubin when diluted with sodium sulfate for injection onto the chromatography column. However, acidic pH values for the master albumin—bilirubin standard solutions should be avoided because precipitation and/or aggregation of the concentrated bilirubin standards could occur, leading to poor calibration curves. In our experiments, the sodium sulfate diluted standards (pH $\approx$ 6.0), automatically injected interspersed with serum samples in overnight runs, yielded linear calibration curves similar to that shown in Fig. 3. All standards were prepared and diluted for injection at the same time and then remained in the automatic injector in either subdued incandescent light or yellow filtered light for up to 17 h before injection. As indicated in Fig. 3, this procedure produced a linear calibration of area versus concentration with a minimal negative intercept corresponding to about 1—2 mg/l of bilirubin. Peak heights were not generally usable for quantification because of band broadening at the higher concentrations.

Identification of bilirubin components

Fig. 4 shows chromatograms of pathological human serum, adult human bile, dog bile, and rabbit bile. The bile samples, as noted in the Experimental section, were first diluted with human serum albumin and sodium sulfate to closely imitate the matrix from which serum samples were injected, as well as to stabilize the conjugates against degradation. For the bile samples, no appreciable peak was found at the retention for the δ-component, only a minimal

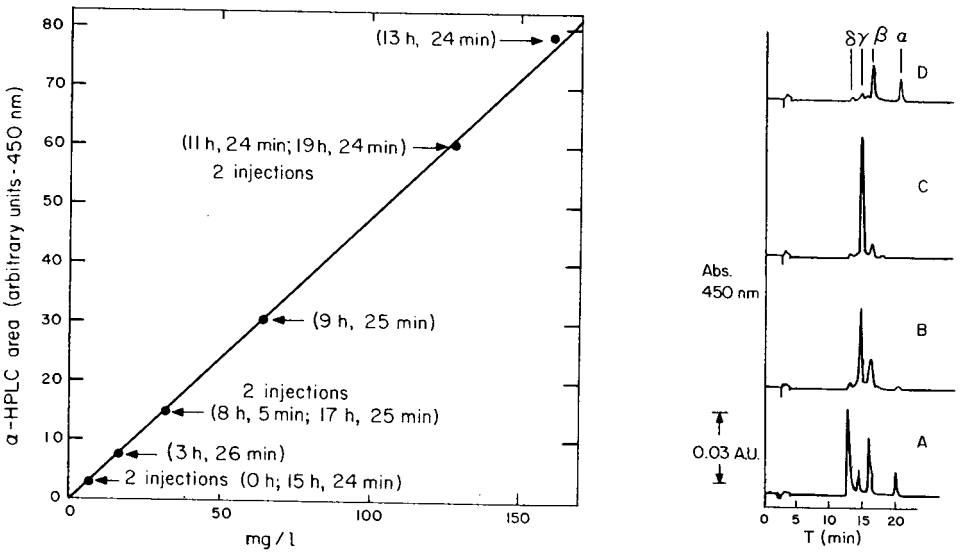


Fig. 3. Calibration curve for unconjugated bilirubin (α). Concentrations are for standard solutions in 5% albumin before dilution with sodium sulfate, as noted in the text. In each case 500 μl of the diluted standard were injected. In parentheses are the relative times in hours for the injection of each standard. The line was fitted by a linear least-squares program (Texas Instruments 58C) with correlation coefficient = 0.9995 and intercept equivalent to −0.9 mg/l unconjugated bilirubin.

Fig. 4. HPLC separation of bile and serum: (A) human serum, (B) human bile, (C) dog bile, (D) rabbit bile.

absorbance due mostly to the yellow color of the human serum albumin added to the samples. If the δ-component is formed only with albumin or protein, this result should be expected, because bile does not contain much protein and thus is less likely to contain protein-bound bilirubin species.

The δ-component, which is both yellow and diazo positive, has been discussed only inferentially in the literature [28, 36, 45], except for the work of Kuenzle and co-workers [7—12]. In both our work and the earlier work, the major distinguishing feature of this material was its tight binding to protein. The material has been isolated in impure form from pathological sera by treatment with caffeine—benzoate reagent followed by diafiltration. The details of this isolation as well as the results of various physical and chemical tests on the properties of the δ-fraction can be found elsewhere [46]. In brief, the material is composed of one or more bilirubin derivatives, very tightly, if not covalently, bound to a protein, most likely albumin.

The γ-fraction was identified as a diconjugate of bilirubin by comparison of its retention with that of the major component in isolates of bilirubin glucuronide from human bile, by the procedure of Wu et al. [47]. In addition, we found (Fig. 4) that the γ-fraction predominated in samples of adult human bile and in the sample of dog bile. Both of these results agree with the work of others [5].

The β-fraction of bilirubin has not been isolated, but much circumstantial evidence indicated that it was a monoconjugate of bilirubin. First (Fig. 4), it predominated in the bile of rabbits which had been treated by ligation of their bile ducts. That a monoconjugate of bilirubin predominates in lower species was observed previously [18]. In addition, we found that this fraction, β, as well as γ, diconjugated bilirubin, decreased in concentration during the keeping of pathological serum samples at room temperature in the dark for periods of up to 72 h. In the same time period, the concentration of unconjugated bilirubin, α, increased. The large loss with time of γ, the slight decrease in β, and the large increase in α were consistent with hydrolysis of the esters. In that case, β was logically a monoconjugate.

Precision and accuracy

The large injection volume (500 μl) was chosen as a compromise to obtain adequate sensitivity for detecting bilirubin at low, nominally normal values, while not overloading the column with albumin, which can shorten column lifetime. Because of the amount of sodium sulfate needed to completely precipitate the higher-molecular-weight proteins, the injection volume chosen allowed detection of bilirubin at 1—2 mg/l, depending upon which species were present. The precision of the measurements was about the same order of magnitude as illustrated in Table I. In this table, the precision of the average area obtained for each of the bilirubin peaks for triplicate injections of the same aliquot of serum is given along with the average area. For the Table, ca. 2200 area units were equivalent to 10 mg/l of unconjugated bilirubin. Thus, it is evident that the precision and accuracy are limited to about ±1.0 mg/l. Since levels less than 10 mg/l are considered benign or normal, this sensitivity and precision should be adequate for most purposes.

TABLE I

HPLC PRECISION STUDY

Three replicate injections.

Serum No.	δ	γ	β	α	Total area*
18	20066 ± 362	4873 ± 59	12467 ± 250	6100 ± 116	43507 ± 435
9	7087 ± 32	1985 ± 33	6347 ± 121	11200 ± 529	26618 ± 612
8	9013 ± 76	1004 ± 122	7013 ± 161	6260 ± 238	23291 ± 675
1	25663 ± 51	4652 ± 47	10549 ± 158	5226 ± 99	46091 ± 323
7	4967 ± 40	1393 ± 110	6233 ± 50	8233 ± 354	20827 ± 416
4	1977 ± 166	3948 ± 146	262 ± 227	17067 ± 563	23253 ± 233
45	36367 ± 315	2760 ± 58	3467 ± 42	12933 ± 65	55527 ± 55
17	1876 ± 186	1553 ± 5	6833 ± 14	16767 ± 50	27029 ± 243

*Peak area at 450 nm given in arbitrary units (ca. 2200 area units = 10 mg/l bilirubin).

Sample stability

Although the sample preparation conditions given in the procedure did stabilize unconjugated bilirubin for 15—20 h, such was not the case for the con-

jugated forms, β and γ. Although the conditions chosen minimized changes in these components, some hydrolysis of γ to β and β to α was observed over 15 h and appeared to be sample or serum dependent. In most cases, this amounted to a change in peak area for individual peaks of less than 10%, with a lesser overall change in the total peak area because the areas are compensating, i.e., as the conjugates hydrolyze, unconjugated bilirubin (α) increases. The effect, then, appeared to be the result of gradual hydrolysis of esters in the sample, rather than oxidation, as the sample awaited injection.

Separation of sera

Fig. 5 shows chromatograms of three pathological sera. These demonstrate the wide variations in concentration of the four bilirubin fractions that have been observed in more than 200 different pathological sera chromatographed by this procedure. In general, without a prior knowledge of the disease state of the patient from whom the sera were drawn, no consistent pattern of the four species even at the same level of total bilirubin has been observed. Any of the four peaks has been found to be the greatest in area in a particular sample, with the exception of that due to the diconjugate (γ). At nominally normal values of total bilirubin ($\leqslant$ 10 mg/l), bilirubin has been present almost entirely as the unconjugated (α) form with perhaps a marginal amount of the δ-component present. These observations are in general agreement with the more complete investigations of Kuenzle and co-workers [7—12].

The present procedure is only qualitative, or semi-quantitative at best, because some yellow material is still retained with the proteins precipitated by the sodium sulfate treatment. Also, during processing of samples with total bilirubin levels >130 mg/l, a rather large and variable fraction of the total bilirubin, particularly the β- and γ-fractions, was lost with the precipitate. In some cases, as much as 60% was lost. However, with sera elevated to <130 mg/l, less yellow material was lost with the precipitated proteins and the recovery was

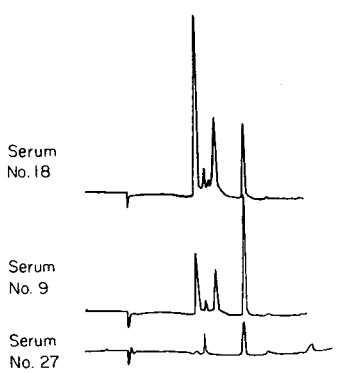


Fig. 5. Comparison of pathological sera.

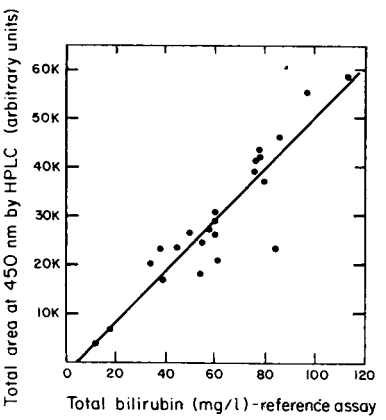


Fig. 6. Correlation plot of HPLC area (450 nm) versus total bilirubin: r = 0.915 for all points by linear least squares; r = 0.960 if low point at 84 mg/l neglected.

less variable. This is indicated in Fig. 6, in which the total area of all bilirubin peaks separated by HPLC in a number of serum samples is plotted versus the total level of bilirubin as determined by the Jendrassik—Grof reference diazo procedure as modified by Doumas et al. [3]. There is a good correlation but not a predictive one because of the considerable amount of scatter. The scatter still seemed to be caused primarily by irregular losses in the precipitation step. Scatter is also expected because of the assumption, implicit in Fig. 6, that the extinction coefficients of all bilirubin species at 450 nm in the HPLC eluent are equal. This is most likely not true.

CONCLUSIONS

Four bilirubin fractions in serum and three in bile have been separated rapidly on a reproducible and stable reversed-phase chromatography system. The major advantages over the earlier, open-column procedure are the smaller sample size required (here 250 μl but potentially as little as 50 μl), the stability of the HPLC reversed-phase column and the relative speed of the analysis. The procedure yields semi-quantitative information on the concentration of the four species in serum and has demonstrated that they vary widely in different pathological sera.

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CHROMBIO. 1039

SIMULTANEOUS DETERMINATION OF BLOOD CONCENTRATIONS OF METHOHEXITAL AND ITS HYDROXY METABOLITE BY GAS CHROMATOGRAPHY AND IDENTIFICATION OF 4'-HYDROXY-METHOHEXITAL BY COMBINED GAS—LIQUID CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

A simple, sensitive and selective method is described for the simultaneous determination of low concentrations (less than 50 ng/ml) of underivatized methohexital and its hydroxy metabolite in small (0.1 ml) samples of human and rat plasma or whole blood by gas chromatography with nitrogen-selective detection.

Moreover, the main metabolite in rat and man was identified as 4'-hydroxymethohexital by comparison of chromatograms from gas—liquid chromatography (GLC) with data obtained from GLC—mass spectrometry and ^{1}H-nuclear magnetic resonance spectrometry of this metabolite, produced both by incubating methohexital with isolated rat liver microsomes and by isolating this metabolite from rat urine.

INTRODUCTION

Methohexital [α-*dl*-1-methyl-5-allyl-5(1'-methylpentyn-2'-yl) barbituric

0378-4347/81/0000—0000/$02.50

acid], Brevimytal®*, is an ultra-short-acting barbiturate that has found widespread use in clinical situations in which rapid and complete recovery from a short anaesthesia is desired [1].

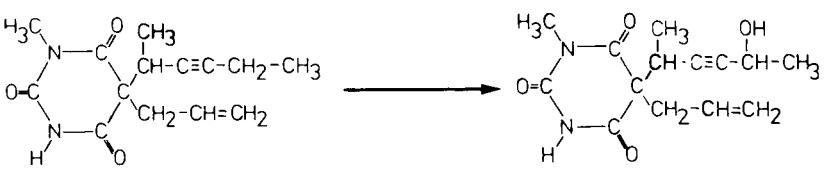
Several authors have described methods for the determination of methohexital and its metabolites in biological fluids. Brand et al. [2] studied plasma and adipose tissue concentrations of methohexital in man. The lack of sensitivity of their ultraviolet assay method required the administration of very high doses of at least 1200 mg to measure concentrations for up to 5 h after intravenous administration. Bush et al. [3] measured whole blood concentrations of methohexital following oral administration of 10 mg/kg by an optical density differences method. This assay method needs 10-ml samples of blood.

Sunshine et al. [4] developed a gas chromatographic method to measure blood concentrations after clinical doses (1.5—2.0 mg/kg). However, the sensitivity of their procedure only allowed the measurement of unchanged drug concentrations for 10 min following intravenous injection (5 μg/ml).

Using gas chromatography with a nitrogen-selective detector Breimer [5] succeeded in measuring human plasma concentrations down to 50 ng/ml, which made it possible to study the pharmacokinetics after therapeutic doses in man for longer time periods.

Distribution and metabolism studies with methohexital have been performed in rat [6], dog [6] and man [2, 4]. Radioactively labelled methohexital was used in such studies. In the rat and dog 4′-hydroxymethohexital was identified as the main metabolite and assayed by thin-layer chromatography (TLC) and paper chromatography.

In this paper we described a simple and sensitive gas chromatographic method for the simultaneous measurement of methohexital and its hydroxy metabolite in small plasma or whole blood samples of man and rat. The main metabolite, 1-methyl-5-allyl-5(1′-methyl-4′-hydroxypentyn-2′-yl)barbituric acid (Fig. 1), formed by allylic oxidation in rat and man was identified by combined gas—liquid chromatography—mass spectrometry (GLC—MS) and ^{1}H-nuclear magnetic resonance spectrometry (^{1}H-NMR).



methohexital
(M.W. 262)

OH-methohexital
(M.W. 278)

Fig. 1. Structural formulae of methohexital and 4′-hydroxymethohexital.

*Eli Lilly GmbH, Lahn Giessen, Reg. No. B 156-1, as methohexital (sodium salt).

MATERIALS AND METHODS

Chemicals and drugs

Methohexital sodium (Brevimytal®-Natrium) was purchased from Eli Lilly (Lahn Griessen, G.F.R.) and hexobarbital-sodium (Evipan® -Na) was purchased from Bayer (Leverkusen, G.F.R.).

The organic solvents light petroleum (b.p. 50—70°C), diethyl ether, propanol-2, benzene, chloroform, acetone and the chemicals for the in vitro reaction, NADPH tetrasodium salt, tris(hydroxymethyl)aminomethane for the preparation of Tris—HCl buffer and concentrated hydrochloric acid were obtained from E. Merck (Darmstadt, G.F.R.).

Absolute ethanol was purchased from C. Roth (Karlsruhe, G.F.R.).

OV-17 (phenylmethyl silicone, 50 : 50) and PPE-21 (poly-*m*-phenyl ether high polymer) were from Chrompack (Middelburg, The Netherlands).

Apparatus

A Hewlett-Packard Model 5710 gas chromatograph, equipped with a nitrogen-selective detector (HP 18789A) was used. The column (1.8 m × 2 mm I.D., borosilicate glass, silanized with 10% dimethyldichlorosilane) was supported with 3% OV-17 on resilanized Gas-Chrom Q (100—120 mesh). Temperatures were: injection port, 250°C; column, 220°C; detector, 350°C. Gas flow-rates were: air, 60 ml/min; hydrogen, 3 ml/min; carrier gas, helium, 30 ml/min.

In routine measurements plasma or whole blood concentrations of the drug and its metabolite were calculated with a Hewlett-Packard automation system (Model 3385A), which was calibrated with samples containing known amounts of methohexital and internal standard.

An LKB 2091—2130 gas chromatograph—mass spectrometer—computer system was used for the unambiguous identification of the compounds eluted from the gas chromatograph. It was equipped with a capillary SCOT column (8 m × 0.5 mm I.D. Duran 50 glass, Cab-O-Sil, coated with the mixed stationary phase 1.6% PPE-21—2.6% OV-17) and a modified pyrolysis sluice system (Becker, Model 767) [7]. Details of the solid injector and the preparation of the column have been previously reported [8]. Temperatures were: injection port, 240°C; column, 170—220°C; ion source, 200°C; separator, 210°C. The carrier gas (helium) flow-rate through the column was 5 ml/min; electron energy, 70 eV; accelerating voltage, 3.5 kV; and trap current, 50 μA.

The proton magnetic resonance (^{1}H-NMR) spectra were recorded on a 100-MHz Jeal INM-PS-100 instrument in deuterated chloroform with tetramethylsilane as internal standard.

Assay of methohexital and 4'-hydroxymethohexital

Extraction procedure for plasma. To 1.0 ml plasma were added 1.0 ml of distilled water and 0.05 ml of ethanol containing 0.5 μg of the internal standard hexobarbital. Subsequently the mixture was extracted twice for 15 sec with 5 ml of a mixture of light petroleum (b.p. 50—70°C)—diethyl ether—propanol-2 (50 : 50 : 2) on a Cenco whirlmixer.

The organic solvent layers were transferred to a conical tube and evap-

orated to dryness at 40°C under a light stream of nitrogen. The residue was dissolved in 0.05 ml of absolute ethanol and 2—3 µl of this solution were injected into the gas chromatograph.

Extraction procedure for whole blood. To 0.1 ml of whole blood 0.3 ml of distilled water and 0.1 ml of standard solution containing 0.5 µg of hexobarbital were added. After homogenization and extraction for three times with 3-ml portions of light petroleum—diethyl ether—propanol-2 (50 : 50 : 2) on the whirlmixer, the organic solvent layers were treated as described above.

Preparation of calibration graphs. The concentrations of methohexital and 4'-hydroxymethohexital were calculated with the aid of calibration graphs, which were prepared by adding known amounts of methohexital and 4'-hydroxymethohexital to 1.0 ml of blank plasma or 0.1 ml of blank whole blood. The samples were analysed by the same procedure described above and the ratios of the peak areas of methohexital and its metabolite to the internal standard were plotted against the known concentrations.

Determination of recovery. For the determination of the extraction yields of methohexital and 4'-hydroxymethohexital from plasma at different concentrations, various amounts of methohexital and the metabolite were added to 1-ml portions of blank human plasma and carried through the extraction procedure described above, except that hexobarbital was used as an external standard. The relative peak area ratios were calculated and compared with the ratios obtained by GLC of standard amounts of methohexital and 4'-hydroxymethohexital.

Incubation of methohexital. The standard incubation mixture contained 6 µmol of methohexital, 4 µmol of NADPH and a suitable amount of enzyme solution, mostly 0.2 ml of the purified microsomal fraction of rat liver homogenate [9]. To this mixture Tris—HCl buffer (pH 7.4) was added to give a total volume of 2 ml. The reaction was carried out in a Warburg shaking water-bath at 37°C for 15 min. The incubation was stopped by deep-freezing the reaction mixture. After adjusting the pH to 4—5 by adding 0.1 *N* HCl, extraction was carried out four times as described before. Further details for this reaction will be reported in a following paper [10].

Isolation of 4'-hydroxymethohexital from rat urine. To accumulate sufficient metabolite for characterization three male Wistar rats each received 1 g of methohexital sodium in 1 l of drinking water during three days. During this time urine was collected. Portions of 200 ml of the pooled urine were adjusted to pH 3—4 with 4 *N* HCl and extracted three times with 200 ml of diethyl ether. The combined organic layers were dried over sodium sulfate and evaporated in vacuo. The brown—yellow viscous residue was redissolved in 2 ml of diethyl ether and applied to the column or the TLC plate.

Column chromatography. The ether extract was passed through a column of silica gel 60 (Merck; 44 × 3.7 cm) conditioned with the mobile phase diethyl ether—light petroleum (50 : 50, v/v). The effluent fractions were checked by GLC. The fractions containing the largest amounts of metabolite were combined and evaporated. Recrystallisation of the yellow residue with diethyl ether—light petroleum yielded 4'-hydroxymethohexital as colourless needles (m.p. 100—102°C).

Thin-layer chromatography. Portions (500 µl) of the ether extract were placed as a band on a PSC-plate of silica gel 60 (Merck; 20 × 20 cm) precoated for preparative-layer chromatography (layer thickness 2 mm); the plate was developed three times with benzene—chloroform—acetone—ethanol (80 : 10 : 5 : 5, v/v). 4′-Hydroxymethohexital (R_F = 0.34) was extracted from the silica gel with diethyl ether.

RESULTS AND DISCUSSION

Gas chromatographic sensitivity and selectivity

The sensitivity and the selectivity of the alkali-flame ionization detector (nitrogen detector) for nitrogen-containing compounds allows the relatively simple and rapid determination of low concentrations of methohexital and 4′-hydroxymethohexital in small plasma and whole blood samples. The detection limit is about 1 ng per single injection. Typical gas chromatograms obtained after extraction of 1 ml of human blank plasma and plasma containing 1.06 µg of methohexital and 1.12 µg of 4′-hydroxymethohexital are shown in Fig. 2. In Fig. 3 the corresponding gas chromatograms obtained after extraction of 0.1 ml of rat blank whole blood containing 2.85 µg/ml methohexital and 21.56 µg/ml 4′-hydroxymethohexital are shown. There is no interference with endogenous constituents. A derivatization procedure is not required. In patients with liver cirrhosis in some cases an interfering peak with a retention time of about 4.27 min can be observed, which may prohibit an accurate determination of the metabolite. In these cases a decrease of the column temperature to 210°C yields better results.

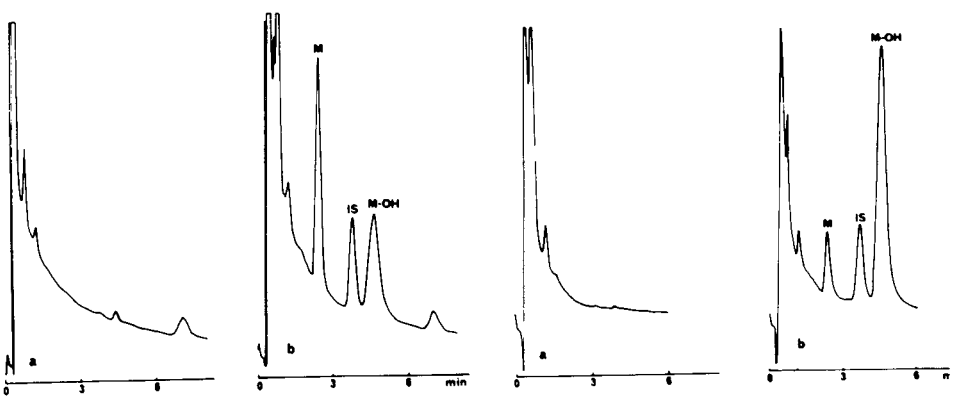


Fig. 2. Gas chromatograms of (a) a 1.0-ml extract of human blank plasma and of (b) human plasma containing 1.06 µg/ml methohexital (M) and 1.12 µg/ml 4′-hydroxymethohexital (M-OH). IS = internal standard, hexobarbital, 0.5 µg/ml.

Fig. 3. Gas chromatograms of (a) a 0.1-ml extract of rat blank whole blood and (b) of rat whole blood containing 2.85 µg/ml methohexital (M) and 21.56 µg/ml 4′-hydroxymethohexital (M-OH). IS = internal standard, hexobarbital, 5.0 µg/ml.

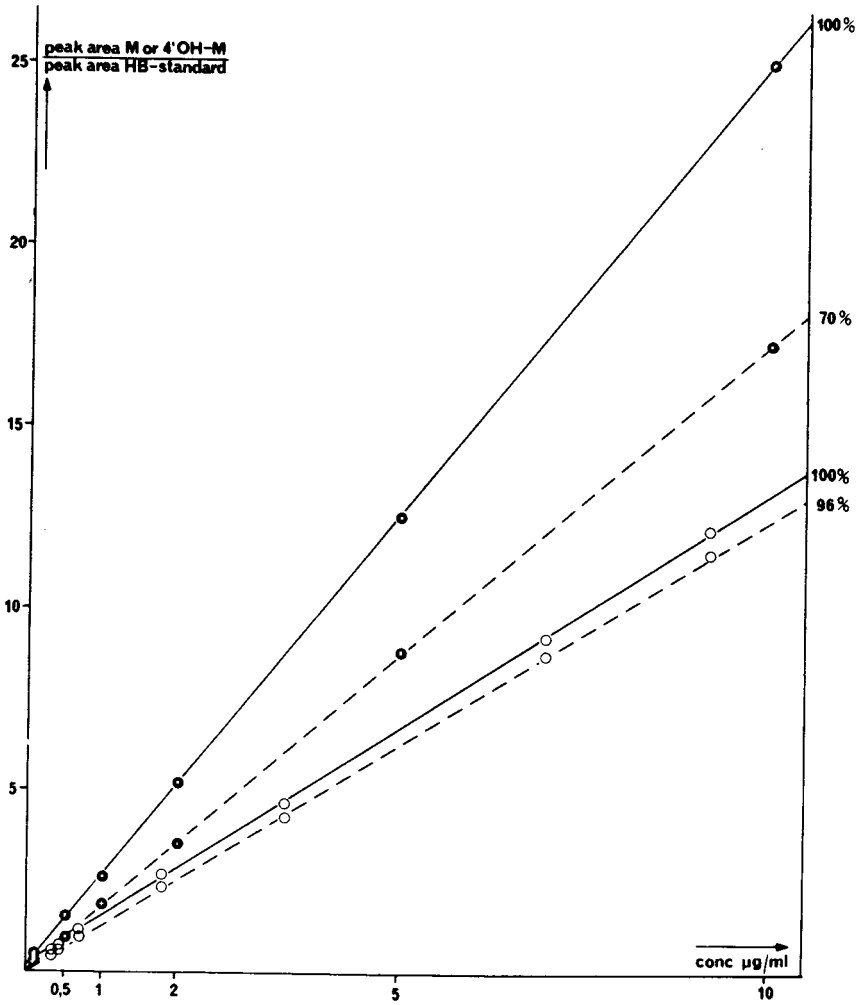


Fig. 4. Peak area ratios of methohexital (M, •) and 4'-hydroxymethohexital (4'-OH-M, ○) as a function of known concentrations for the determination of the recovery from human plasma. The human plasma curves (— — —) were obtained in the same way as described for the preparation of the calibration graphs, except that 0.5 µg of hexobarbital (HB) was added after extraction. The standard curves (———) were obtained by comparing known standard amounts of M and 4'-OH-M with 0.5 µg of HB.

Extraction procedure and precision

The extraction of methohexital and 4'-hydroxymethohexital with a mixture of light petroleum (b.p. 50—70° C)—diethyl ether—propanol-2 (50 : 50 : 2, v/v) proved to be quite suitable. The extraction yields are satisfactory and constant over a large concentration range. For plasma samples, good linearity is obtained for concentrations of methohexital and the hydroxy metabolite between 50 ng/ml and 5 µg/ml. In whole blood samples the calibration curve is linear between 100 ng/ml and 20 µg/ml. The corresponding correlation coefficients were in all cases better than 0.998.

In addition, the mean recoveries were determined for the same concentration range using hexobarbital as an external standard. For plasma, extraction yields of methohexital and its hydroxy metabolite are 70% and 96%, respectively, with standard deviations in the range 5% or less (n = 5) (Fig. 4).

Identification of the hydroxy metabolite

Although there are several reports on enzymic oxidation at allylic and benzylic positions [11], examples of enzymic oxidation at a position alpha to an acetylenic bond are rather scarce. Lindeke et al. [12] have reported enzymic oxidation alpha to a triple bond in the metabolism of N-(5-pyrrolidinopent-3-ynyl)succinimide in vitro. Propynylic oxidation in the metabolism of 7-alkynyl-substituted theophyllins [13] and the occurrence of N-methylbenzylamine as a metabolite in vivo of pargyline (N-benzyl-N-methyl-propynyl-2-amine) [14] must also implicate α-acetylenic oxidation.

Welles et al. [6] subjected pooled urine extracts from dogs, treated intravenously with [^{14}C]methohexital, to paper chromatography. They succeeded in isolating the main metabolite and characterising it as 4′-hydroxymethohexital by melting point, ultraviolet spectrometry, catalytic hydrogenation, iodoform formation of the reduced metabolite, and elemental analysis. We can, furthermore, report the mass and ^{1}H-NMR spectrum of this metabolite obtained by incubation of methohexital with rat liver microsomes and by isolating this metabolite from rat urine.

The analysis of these samples by means of GLC (Figs. 2 and 3) and a GLC—MS—computer system (Fig. 5) showed that methohexital and 4′-hydroxymethohexital are eluted from the gas chromatograph without chemical altera-

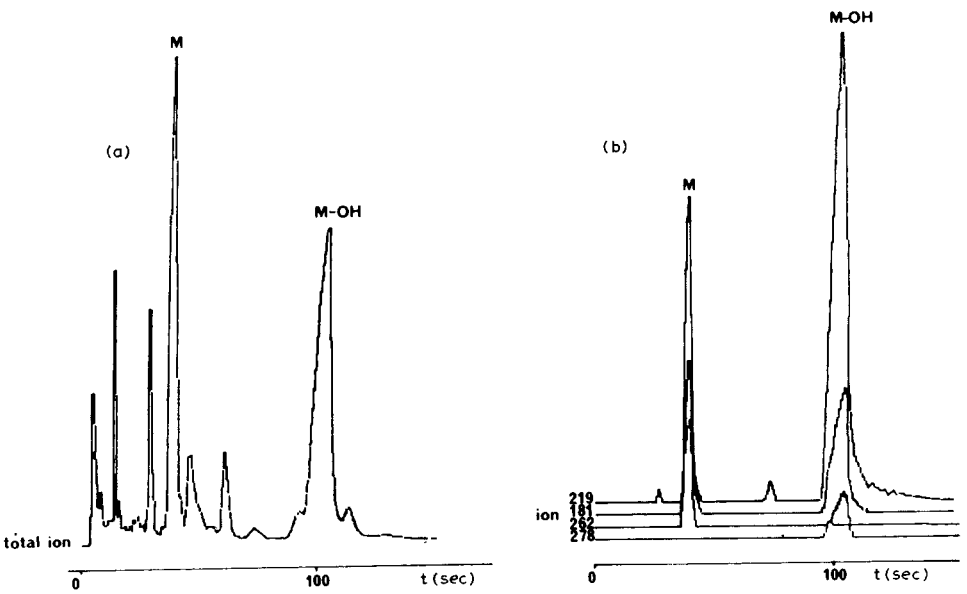


Fig. 5. (a) Total ion current and (b) mass chromatogram for four selected m/e values characteristic of methohexital (M) and its 4′-hydroxy metabolite (M-OH): m/e = 181, m/e = 219 and m/e = 262 for M and M-OH, and m/e = 278 for M-OH only.

tion under the conditions described. The mass spectra of the eluted compounds (peaks M and 4'-OH-M in the obtained chromatograms) and those after direct sample introduction, contain the molecular ions *m/e* = 262 for methohexital (M) and *m/e* = 278 for 4'-hydroxymethohexital (4'-OH-M), as shown in Fig. 6.

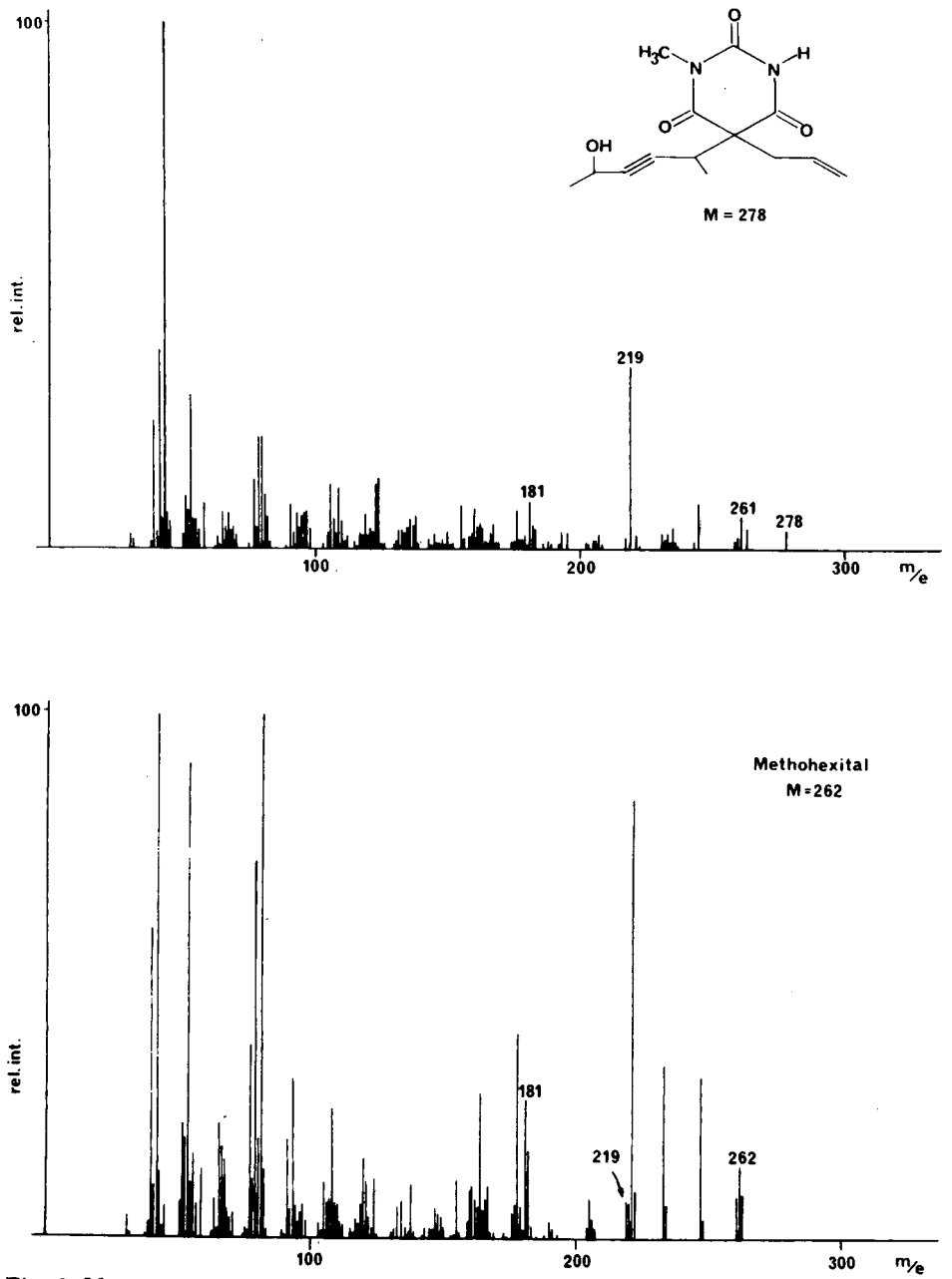


Fig. 6. Mass spectra at 70 eV of methohexital (below) and its metabolite 4'-hydroxymethohexital (above).

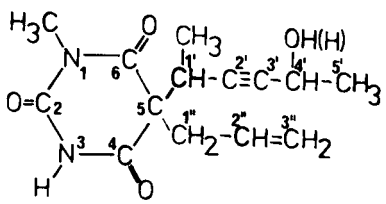
Fig. 5b shows a mass chromatogram of four characteristic *m/e* values: the molecular ions, *m/e* = 262 for methohexital and *m/e* = 278 for the metabolite; *m/e* = 181 for both formed by the loss of the 2-pentynyl chain as a radical from methohexital and the hydroxylated pentynyl chain in hydroxymethohexital; and *m/e* = 219 due to a fragment produced by the loss of an allyl radical and a molecule of water in the metabolite. In methohexital the loss of isocyanic acid, HCNO, followed by ring contraction of the barbiturate ring leads to this *m/e* value.

Further evidence for the proposed structure of 4′-hydroxymethohexital was obtained from the ^{1}H-NMR spectrum of the isolated metabolite. Comparison of the ^{1}H-NMR spectrum of the metabolite 4′-OH-M with that of the parent drug M (Table I) showed that hydroxylation has occurred in the 4′-position. In 4′-OH-M an OH signal becomes evident at δ = 4.40 ppm. The C(4′)H_2 signal in M (δ = 2.05 ppm) is lost upon hydroxylation. A C(4′)H signal appears in 4′-OH-M at δ = 2.72 ppm. The triplet from C(5′)H_3 from M disappears and shifts as a doublet under the doublet from C(1′)-CH_3 in 4′-OH-M.

TABLE I

CHEMICAL SHIFTS FOR THE ^{1}H-NMR SPECTRA OF METHOHEXITAL AND 4′-HYDROXYMETHOHEXITAL IN DEUTERATED CHLOROFORM

Tetramethylchlorosilane was used as internal standard.



	δ (ppm) methohexital	δ (ppm) 4′-hydroxymethohexital
C(5′)H_3	1.00 (t, 3H)*	
C(1′)-CH_3	1.24 (d, 3H)	1.30 (t, 6H)*
C(4′)H_2	2.05 (q, 2H)	
C(4′)H		2.72 (m, 3H)
C(1″)H_2	2.70 (m, 2H)	
C(1′)H	3.00 (q, 1H)	3.10 (q, 1H)
N(1)-CH_3	3.22 (s, 3H)	3.22 (s, 3H)
C(4′)-OH		4.40 (broad m, 1H)
C(3″)H_2	5.08 (m, 2H)	5.08 (m, 2H)
C(2″)H	5.42 (m, 1H)	5.42 (m, 1H)
N(3)H	8.78 (broad m, 1H)	9.24 (broad m, 1H)

*s = singlet; d = doublet; t = triplet; q = quartet; m = multiplet.

CONCLUSIONS

The assay described is simple and rapid and allows the simultaneous quantitative determination of methohexital and 4′-hydroxymethohexital in plasma or whole blood. Endogenous substances do not interfere with the method and, with the exception of a short extraction procedure, time-consuming clean-up or derivatisation procedures are not necessary. This method also proves quite suitable to determine the drug and its metabolite in other biological fluids such as bile and urine, and it has been successfully applied to a study of the pharmacokinetics of methohexital and 4′-hydroxymethohexital in humans and rats [15, 16].

ACKNOWLEDGEMENT

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ANALYSIS OF THE METABOLITES OF ETHYL LOFLAZEPATE BY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

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SUMMARY

A gas chromatographic assay with electron-capture detection (GC—EC) is described for the metabolites of ethyl loflazepate (Victan), a new benzodiazepine with a potent anti-anxiety activity, in biological fluids. Since the parent drug undergoes a first-pass effect, pharmacokinetic data may only be obtained by measuring the total levels of two of the major metabolites. Accurate data can not be obtained for the metabolites separately since one of them (M1) is chemically transformed to the other (M2) during plasma sampling, storage and extraction.

A sensitive, specific and accurate GC—EC assay is developed using a synthetic analogue of M2 as an internal standard. The limit of detection in plasma is approximately 2 ng/ml and the precision about 3% (within-run and between-run).

The method is applied to plasma samples collected after oral administration of 2 mg and 4 mg of the drug in tablet form to human volunteers. The results obtained are correlated with those from an existing gas chromatographic—mass spectrometric assay. A very good correlation between the results (inter-laboratory comparison) is obtained, validating both techniques.

INTRODUCTION

Ethyl loflazepate (CM 6912, Victan) is a novel benzodiazepine characterized by its potent anti-anxiety activity, clearly demonstrated in animal models as well as in patients [1]. Acute toxicity studies established the very low toxicity of the drug, the LD_0 in rat and mouse being higher than 4 g/kg, whereas human therapeutic active doses are scheduled at lower than 0.1 mg/kg. No significant toxic effects were observed during the subacute and chronic toxicity studies performed in rat and baboon [1].

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The chemical structures of the parent drug and its identified metabolites are shown in Fig. 1. Metabolites M1 and M2 are common metabolites for the rat, the baboon and the dog. A marked species difference was observed between the rat and the other species in the formation of hydroxylated metabolites. The monkey and the dog form predominantly 2′-fluorooxazepam whereas the rat forms the 4′-hydroxylated compound. A comparable difference has been described in the literature on benzodiazepines [2].

Preliminary in vitro studies revealed the high esterase activity of intestinal wall and liver homogenates and plasma when ethyl loflazepate was introduced as a substrate.

Considering the presence of an extensive first-pass effect, it seemed unlikely that the unchanged drug would be detected after oral administration. Never-

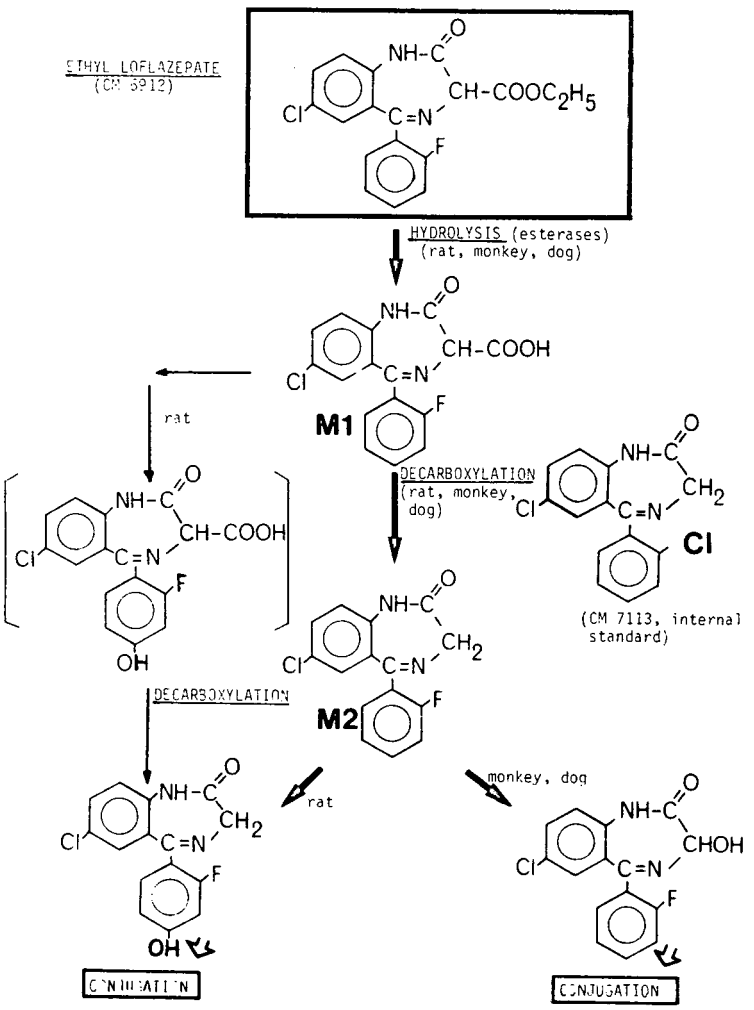

Fig. 1. Tentative metabolic pathway of ethyl loflazepare (rat, monkey and dog). M1 and M2 are monitored for pharmacokinetics. CM 7113, the chlorinated homologue of M2, is used as internal standard.

theless, this surmise had to be confirmed. Thus, the assay of three compounds — parent drug, M1 and M2 — was investigated. The carboxylic acid metabolite M1 is chemically speaking very unstable and is decarboxylated rapidly to form M2 during plasma sampling, storage and extraction.

It is well known [3—7] that dipotassium clorazepate can not be directly analyzed by gas chromatography and is rapidly transformed in an acid or neutral aqueous medium to N-desmethyldiazepam. For that reason, M1 was converted quantitatively to M2 before the analysis and total (M1 + M2) levels were measured.

A previous paper [8] reported the development of gas chromatographic—mass spectrometric (GC—MS) method for the drug and its metabolites. The absence of the parent drug in human plasma after oral administration was clearly demonstrated. Preliminary pharmacokinetic data obtained on human volunteers were described in the same paper.

This present paper deals with the development of a GC method with electron-capture (EC) detection for the monitoring of total (M1 + M2) levels in biological samples. This technique will be developed and used for field pharmacokinetic studies. The GC—EC and GC—MS techniques will be evaluated for precision and reproducibility and their correlation determined.

EXPERIMENTAL

Apparatus and chromatographic conditions

A Pye-Unicam gas chromatograph equipped with a ^{63}Ni electron-capture detector was used. The all-glass column (1.80 m × 0.4 cm I.D.) was packed with 3% OV-1—OV-17 (1 : 3) on Gas-Chrom Q (80—100 mesh). The temperature settings were: oven, 270°C; injection port, 300°C; detector, 300°C. Nitrogen was used as carrier gas with a flow-rate of 60 ml/min. The column was conditioned as described elsewhere [9]. A Hewlett-Packard 3352 B laboratory data system, a 18652 A/D convertor and a Teletype teleprinter were connected to the chromatograph.

All GC—MS analyses were run on a Ribermag 1010B gas chromatograph—mass spectrometer system coupled on-line to the Ribermag SIDAR data system (Rueil Malmaison, France). GC separations were performed on a packed column (1.5 m × 0.18 cm I.D.) filled with 3% OV-1 on Chromosorb W AW (80—100 mesh). Helium was used as carrier gas at a flow-rate of 25 ml/min. The oven temperature was 245°C. The mass spectrometer was operated in the chemical ionization (CI) mode using ammonia as the reagent gas. Quantitative analyses were performed in the "switched" single-ion detection mode focusing the respective MH^+ ions as described previously [8]. Quantification was performed automatically after data acquisition using the SIDAR software.

Reagents

All reagents were of analytical grade. The inorganic reagents were all prepared in double-distilled water.

Concentrated sulfuric acid "Ultrex" was obtained from J.T. Baker (Phillipsburg, NJ, U.S.A.). Sodium hydroxide, potassium bicarbonate, potassium hydroxide, dipotassium hydrogen phosphate and a buffer solution at pH 10

were obtained from Merck (Darmstadt, G.F.R.). The aqueous solution of 1 *N* potassium hydroxide—1 *M* dipotassium hydrogen phosphate was washed with diethyl ether before its use in the clean-up procedure.

Organic solvents used were acetone, diethyl ether, *n*-hexane, methanol, *n*-heptane and toluene. All solvents had the label "pestipur" and were obtained from Solvent Documentation Synthèse (Peypin, France).

A chlorinated homologue of M2 was used as internal standard. The structure of this compound, code number CM 7113, is shown in Fig. 1.

Derivatization procedure

The analytes were derivatized before GC—MS analysis. GC—EC analysis did not involve a derivatization procedure. All details of the derivatization procedure used were as described in detail previously [8, 10]. It involves butylation of the NH moiety in the 1-position of the 1,4-diazepine cycle, using 1-iodobutane in the presence of methanol, N,N-dimethylacetamide and tetrabutylammonium hydroxide.

Standard solutions

Standard solutions of M2 and CM 7113 (internal standard) were prepared as follows. An exactly weighed 10-mg amount of the compound was dissolved in 2.5 ml of acetone and 2.5 ml of methanol in a 10-ml volumetric flask and diluted with acetone—hexane (20 : 80, v/v). The dilutions required were prepared with the acetone—hexane mixture.

Standard solutions of M1 were made up in double-distilled water buffered to pH 10 (0.1 *M* potassium bicarbonate adjusted with potassium hydroxide). They were freshly prepared every day and stored at 4°C.

Biological sampling

Blood samples were collected in 10-ml vacutainer tubes containing 10 mg of ammonium oxalate and 8 mg of potassium oxalate. The samples collected were shaken and immediately centrifuged at 5000 *g* for 10 min; the plasma was stored at −20°C pending analysis.

Determination of M2

Into a 30-ml glass centrifuge tube containing 2.5—10 ng of internal standard, 0.5—2 ml of plasma and 2 ml of pH 10 buffer solution were added. The mixture was shaken for 5 min with 8 ml of diethyl ether and centrifuged for 5 min at 3000 *g*. The organic layer was transferred to a fresh centrifuge tube. The extraction was repeated with 8 ml of ether. The ether phases were combined and evaporated to dryness under vacuum with a nitrogen leak. To the residue were added 2.5 ml of 1 *N* sulfuric acid and 10 ml of hexane. The tube was shaken for 10 min and centrifuged for 5 min. The hexane layer was carefully removed by aspiration without removing any of the acid phase. This aqueous phase was adjusted to pH 10 by means of 1 *N* sodium hydroxide—1 *M* potassium hydrogen phosphate solution and extracted twice with 8 ml of ether as described before but with 10 min of centrifugation. The ether phases were pooled and evaporated to dryness. To simplify the extraction, both first and final steps of extraction can be performed once using 10—

14 ml of ether for each. The residue was reconstituted with 50 μl of toluene and 2—3-μl aliquots were injected for analysis.

Determination of (M1 + M2) levels

Into a centrifuge tube containing 2.5—10 ng of internal standard, 0.5—2 ml of plasma were added. The mixture was adjusted to pH 2 by means of 0.25 ml of 1 *N* sulfuric acid and then incubated in a 37°C water-bath for 30 min with continuous agitation (in this step M1 is transformed to M2). The pH was adjusted to 10 with 1 ml of 1 *M* potassium hydrogen phosphate and a sufficient amount of 1 *N* sodium hydroxide—1 *M* potassium hydrogen phosphate. The M2 formed was then extracted together with the M2 initially present as described above.

The concentrations were calculated from the calibration curves after simultaneous determination of specimens of control, blank, plasma to which known amounts of M2 or/and M1 (ranging from 2 to 40 ng/ml of plasma) were added. The curves were constructed by calculating the peak area ratio of M2 to that of internal standard, and plotting the ratio against the amount of (M1 + M2) spiked.

RESULTS AND DISCUSSION

A previous paper [8] confirmed the absence of the parent drug in human plasma after oral administration of the drug. A GC—MS technique was used for that study. The same paper reported preliminary pharmacokinetic data measuring total (M1 + M2) levels. The sensitivity and accuracy of the technique were discussed in detail before. The GC—EC technique developed in this present paper will be used for the complete pharmacokinetic study of the drug after single oral and intravenous administrations and after chronic oral treatment. This technique will therefore be discussed in detail and correlated for sensitivity, specificity and accuracy with the existing GC—MS technique.

Specificity

M1 is not soluble in organic solvents and would thus remain in the aqueous phase during extraction of M2. Hence, it would not interfere in the determination of M2. As explained further in this paper, it is impossible to avoid degradation of M1. Anyway, neither during the analysis of M2 nor of (M1 + M2), were interfering peaks observed in the GC—EC trace (Fig. 2). These chromatograms reveal a complete separation between M2 and its internal standard. Moreover, none of the endogenous substances interfere during the assay. Thus, the method described can be considered to be specific enough for pharmacokinetic studies.

Recoveries and limits of detection

Using diethyl ether for extraction and by performing double extractions for each step, M2 and its internal standard were quantitatively recovered. Normal sulfuric acid, used in the clean-up procedure, back-extracted quantitatively the components from the hexane layer.

Fig. 2. GC—EC trace obtained from a subject treated with an oral dose of 2 mg of the drug (A) before administration, (B) 1 h after dosing. Peak 1 represents (M1 + M2); peak 2, 15 ng/ml of the internal standard.

The limits of detection were about 1 ng/ml of plasma for M2 and about 2 ng/ml of plasma for total (M1 + M2) levels. With single extractions the recoveries were slightly less quantitative but did not considerably influence the detection limits. Moreover, single extractions were less time-consuming.

Precision

The precision of the assay was determined within run (WR) and between runs (BR) and was found to be excellent. The coefficients of variation (C.V.) with $p = 0.05$ are summarized in Table I.

TABLE I

PRECISION OF THE GC—EC METHOD

Compounds analyzed	Concentration (ng/ml)	*N*	C.V. (%) $p = 0.05$	Note*
M2	2	5	2.8	WR
M2	10	5	1.7	WR
M2	17	8	1.7	WR
M1	50	10	1.4	BR
(M1 + M2)	8.7 (as M2)	6	2.7	WR

*WR = within run; BR = between run.

In vitro stability kinetics of M1 and M2 in plasma

An amount of M1 or M2 was added to about 10 ml of plasma buffered at pH 10 (final concentrations were about 15 ng/ml for M2 and 75 ng/ml for M1). Aliquots of spiked plasma were then distributed to ten fresh tubes, after which they were stored in a 4°C refrigerator or a −20°C freezer. The concentrations of free M2 were determined at set time intervals (one tube for each determination). In the case of M1, concentrations of total (M1 + M2) were also analyzed after decarboxylation of M1 as described above.

The results of this study are presented in Table II. M2 was found to be stable at −20°C as well as at 4°C. As expected, M1 was not stable in plasma. When it was stored at 4°C, after 4—6 days it was quantitatively transformed to M2. At −20°C it seemed to be stable during conservation but there was an instantaneous degradation. This transformation may have occurred during the sample preparation during which the mixture was shaken and the working conditions were at room temperature. We assume that such a transformation may also have occurred during the extraction procedure and during biological sampling. As a consequence of these observations, M1 levels can not be measured precisely and separately from M2. Therefore, pharmacokinetic parameters must be based upon total (M1 + M2) levels in plasma and urine. For preparation of standard curves best results were obtained by spiking control plasma with M1 as reference compound. An example of such a calibration curve is reproduced in Fig. 3.

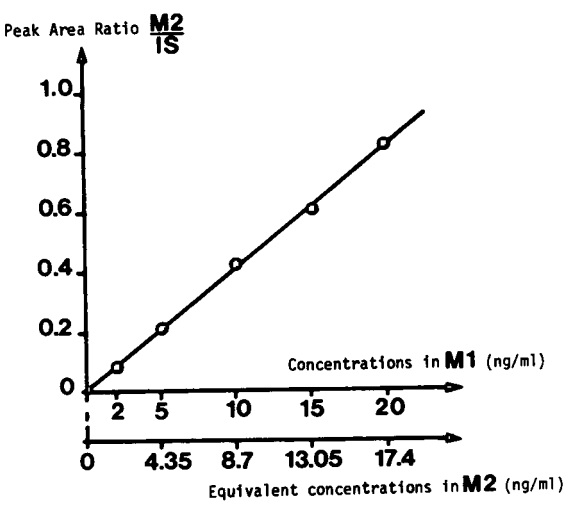

Fig. 3. Calibration curve obtained after spiking control plasma with M1 and decarboxylation—extraction.

Application of the GC—EC method to a human pharmacokinetic study of ethyl loflazepate

The GC—EC method was applied to human plasma samples collected after an oral administration of 2 mg or 4 mg of the drug. Plasma levels of total (M1 + M2) were determined at the following times: 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, 12, 24, 48, 72, 96 and 144 h after drug intake. Fig. 4 reproduces the

TABLE II

STABILITY KINETICS OF M1 AND M2 IN PLASMA

Results are expressed as percentage of compound recovered relative to the initial concentration.

Compound added to control plasma	Temperature of conservation (°C)	Time of conservation (days)															
		0	1	2	3	4	5	6	8	10	13	15	16	20	22	25	30
M1	4	68.2	43.2	31.1	N.A.*	8.1	N.A.	3.5	N.A.	5.4	N.A.	0.7					
	−20	N.A.	65.7	68.3	67.3	N.A.	N.A.	65.1	67.5	65.1	67.5	N.A.	65.1	65.1	65.1		
M2	4	103	102.4	102	N.A.	97.6	N.A.	95.8	N.A.	97	N.A.	101	N.A.	101	N.A.	102	
	−20	102	101	N.A.	N.A.	N.A.	N.A.	96.5	N.A.	101	N.A.	99.4	N.A.	100	N.A.	102	97

*N.A. = not analyzed.

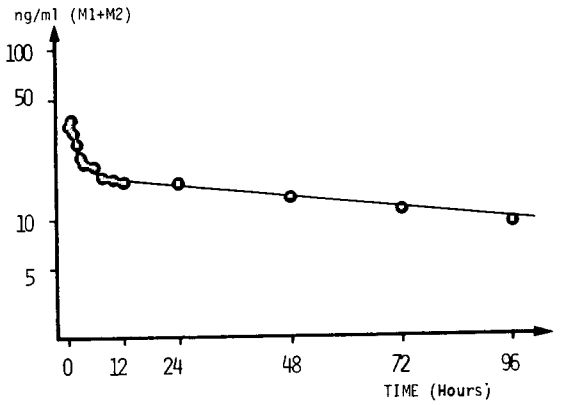

Fig. 4. Plasma concentration vs. time curve for a subject treated orally with 2 mg of the drug in tablet form.

plasma level vs. time curve obtained after a 2-mg administration. A maximum plasma level of about 40 ng/ml was attained 1 h after administration, showing a rapid absorption of the drug from the gastrointestinal tract. The active metabolites monitored were slowly eliminated from the body. The half-life of the terminal phase was found to be 122 h for this subject. These values correlate well with the values of the same parameters, reported previously, after GC—MS analysis of (M1 + M2) levels in four volunteers [8].

Comparison of the results obtained by GC—EC and GC—MS

Since a GC—MS assay technique existed, the plasma samples collected were run simultaneously at the two different facilities using the techniques available (GC—EC and GC—MS). A very good correlation was obtained between the two techniques over the entire concentration range studied. Fig. 5 summarizes the results obtained for the samples collected after the 2-mg administration. The data from Fig. 5 were fitted to a linear regression. The

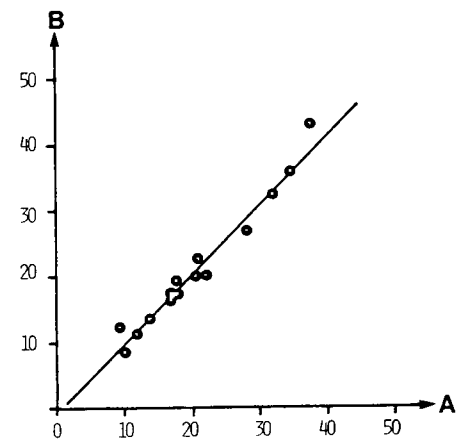

Fig. 5. Correlation of total (M1 + M2) levels assayed by GC—EC and GC—MS (concentrations in ng/ml). A = results obtained by GC—MS; B = results obtained by GC—EC.

best-fit corresponds with the equation $y = 1.06\ x - 1.21$, where y = concentration obtained after the GC—MS analysis and x = concentration obtained after GC—EC analysis. The correlation coefficient r was 0.989 ($n = 16$). Another linear regression was made on a total of 43 data points obtained after analysis of one subject treated with 2 mg and two subjects treated with 4 mg. The equation obtained was $y = 1.06\ x + 1.24$ with $r = 0.947$.

CONCLUSIONS

The inter-laboratory comparison of total (M1 + M2) plasma levels was very satisfactory considering the fact that two different techniques were used on a relatively large number of samples. The sensitivity, precision and reproducibility of the GC—EC assay were at least as good as those described previously for the GC—MS technique.

The GC—EC technique will be used to study the pharmacokinetic profile of ethyl loflazepate in man and animals.

The contribution of M1 to the total (M1 + M2) levels will be investigated. This study will probably need the administration of radiolabeled drug, chemical stabilization of the carboxylic acid moiety of M1, and analysis by high-performance liquid chromatography with a radioactivity detector.

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CHROMBIO. 1015

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF PAPAVERINE IN WHOLE BLOOD

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SUMMARY

The development and application of an assay method for papaverine in whole blood is reported. A single, simple extraction procedure at pH 10.0 using chloroform—*n*-hexane (2:3) as the solvent, results in pure extracts which can be chromatographed without further purification. Chromatography is performed on a nitrile-bonded phase, using *n*-hexane—dichloromethane—acetonitrile—propylamine (50:25:25:0.1) as mobile phase. This method is characterized by a between-day precision of 4% at the 200 ng/ml level and a detection limit of 5 ng/ml, and was successfully applied in a pharmacokinetic study.

INTRODUCTION

Papaverine is a smooth-muscle relaxant, mainly used as a peripheral vasodilator. In order to study the pharmacokinetics of a new sustained-release capsule, various published gas chromatographic (GC) and gas chromatographic—mass spectrometric (GC—MS) methods [1—4] were tested in our laboratory. At concentrations below 50 ng/ml several difficulties, mainly adsorption problems (on glassware, on the column and the GC—MS interface), were encountered. Additional attempts on various packed columns all resulted in non-zero intercepts of the calibration curves. It was therefore decided to use high-performance liquid chromatography (HPLC). When this research was started, no HPLC method for papaverine had been published, so we had to develop one. The chromatographic system used in this study was developed as one of a set of preferred HPLC systems for basic drugs, published in a previous paper from this laboratory [5]. In order to obtain statistically relevant pharmacokinetic curves, a great number of samples need to be estimated, hence necessitating a rapid and simple procedure. Since the conventional extraction

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procedures for papaverine are tedious and time-consuming, special attention has been paid to the extraction step.

EXPERIMENTAL

Reagents

All drugs were of pharmacopeial purity. Acetonitrile, hexane and dichloromethane were all liquid-chromatographic grade and purchased from Fluka (Buchs, Switzerland). Propylamine was synthesis grade and obtained from Merck-Schuchardt (Hohenbrunn, G.F.R.). All other reagents were analytical grade and purchased from E. Merck (Darmstadt, G.F.R.).

Apparatus

A Varian 5020 liquid chromatograph was used, equipped with a loop injector (sample loop 100 μl), a standard UV detector (254 nm), a Varian 9176 recorder and a Varian CDS 111 chromatographic data system.

Chromatography

Chromatography was performed on a Varian Micropak CN-10 column (particle size = 10 μm) (30 mm × 4 mm I.D.) at 30°C. The mobile phase consisted of *n*-hexane—dichloromethane—acetonitrile—propylamine (50:25:25:0.1). The flow-rate was 2 ml/min and the detector attenuation 0.01 or 0.02 a.u.f.s. The efficiency of the column was tested before use with a mixture of toluene and 2,6-dinitrobenzene as test samples and a mixture of hexane—ethyl acetate (99:1) as mobile phase. At a flow-rate of 1 ml/min, 2,6-dinitrobenzene eluted with a retention time of 16.1 min, giving about 10,000 theoretical plates and an asymmetry factor of 1.1. Almost daily injection of 25—40 extracts of blood samples during 4 months, caused no deterioration of the column.

Extraction procedure

To 4 ml of blood standard or sample, 100 μl of internal standard solution were added in silanized centrifuge tubes equipped with PTFE-covered screw-caps. After vortexing, 10 ml of phosphate buffer solution (μ = 0.4) were added. The mixture was homogenized by vortexing, and 5 ml of chloroform—hexane (2:3) were added. Partitioning was performed by gently shaking the tubes longitudinally in a shaking bath for 30 min. After centrifugation, 3 ml of the organic phase were transferred to a clean silanized vial with conical bottom, and evaporated to dryness under a gentle nitrogen stream at 45°C. Each extract was reconstituted just prior to chromatography with 250 μl of dichloromethane; 100 μl were injected.

Assay standards

Blood standards were prepared by spiking drug-free blood with papaverine · HCl solutions in double-distilled water, to give final concentrations of 10—600 ng/ml.

Internal standard solution

Each day a fresh solution containing 800 μg of mepyramine maleate per 100

ml of double-distilled water was prepared from a fresh stock solution.

Quantitation

The peak area ratio of papaverine to the internal standard was used for quantitation. The peak area ratios for the blood standards, which were taken through the entire assay procedure, were plotted against concentration to obtain standard calibration curves.

A new calibration curve was made with each sample set. Detector response was linear in the concentration range studied. The sequence of the samples was chosen at random and the analyses were performed blindfold. The concentration was determined from the standard curve.

RESULTS AND DISCUSSION

Extraction procedure

Most extraction procedures for papaverine are based on the method of Axelrod et al. [6]. As reported by Guttmann et al. [2], and also in our hands, the extracts yield various interfering peaks. Guttmann et al. [2] proposed an alternative procedure based upon ion-pair extraction of papaverine with di-(2-ethylhexyl)phosphoric acid as ion-pairing reagent. This ion-pairing and adduct-forming reagent has been studied systematically by us and has been found to be very useful for the extraction from aqueous solution of various basic drugs with very different structures [7]. However, when applied to biofluids a lot of endogenous compounds are coextracted, which necessitates a clean-up of the extract [2, 8]. Furthermore, since papaverine is moderately hydrophobic, the use of such a powerful extractant was considered to be unnecessary. The use of a less apolar ion-pairing reagent, sodium *n*-octylsulphate, which we also found to be applicable to the extraction of basic drugs in general, resulted in very pure extracts, but due to interactions with plasma proteins low recoveries were obtained [8]. During these preliminary experiments it was also observed that, provided an appropriate organic solvent, a pH < 11 and phosphate buffer instead of NaOH were used to alkalinize the sample, pure extracts could be obtained. Contrary to the extraction procedure of Axelrod et al. [6] and those emanating from it, interfering peaks could thus be avoided. Table I represents the overall recoveries at the 200 ng/ml of blood level obtained for papaverine and mepyramine, which was selected as internal standard (see further), using phosphate buffers ($\mu = 0.4$) of pH 5.0, pH 6.0 and pH 10.0. It can be seen that, with the chloroform—hexane (2:3) mixture as the

TABLE I

EFFICIENCY (PERCENTAGE OVERALL RECOVERY) OF THE EXTRACTION OF PAPAVERINE AND MEPYRAMINE FROM WHOLE BLOOD AT DIFFERENT pH VALUES

Solvent	pH 5.0		pH 6.0		pH 10.0	
	Papaverine	Mepyramine	Papaverine	Mepyramine	Papaverine	Mepyramine
Chloroform	44.9	84.3	27.9	85.3	21.4	80.1
Chloroform—hexane (2:3)	87.7	33.0	90.4	52.1	91.5	91.2

solvent and at pH 10.0, acceptable, reproducible (mean coefficient of variation = 2.1%) and comparable extraction efficiencies for both the analyte and the internal standard are obtained. The chloroform—hexane (2:3) mixture has furthermore the advantage over plain chloroform, that it has a lower density than blood, which makes it easy to recover after partitioning and phase separation. A simple, single extraction at pH 10.0, using the chloroform—hexane (2:3) mixture as the solvent, was hence considered as being an efficient work-up procedure.

Chromatography

A previous paper from this laboratory [5] reported on the selection of preferred HPLC systems for basic drugs in general. It was concluded that a combination of a nitrile-bonded phase with either acetonitrile—water—propylamine (90:10:0.01) or heptane (or hexane)—dichloromethane—acetonitrile—propylamine (50:50:25:0.1) as mobile phase, are very efficient for the chromatography of basic drugs in general. The latter mobile phase, being the most apolar element, was used in this study as initial investigation eluent and optimized for the determination of papaverine in whole blood. Care was taken to ensure complete resolution of papaverine and caffeine which might be present in the samples, and also to ensure minimal analysis time. The selected mobile phase was hexane—dichloromethane—acetonitrile—propylamine (50:25:25:0.1). At a flow-rate of 2 ml/min, caffeine and papaverine gave retention times of 2.9 and 3.4 min, respectively. Several drugs were tested for use as internal standard. Their retention times relative to papaverine are given in Table II. Mepyramine was chosen as internal standard since it eluted near to, but completely separated from, papaverine, and since it is reproducibly extracted from blood by the method described, with the same efficiency as papaverine. All drugs with a retention time differing by $< 10\%$ can interfere.

TABLE II

RETENTION TIMES, RELATIVE TO PAPAVERINE, OF VARIOUS DRUGS

	Relative retention time*
Papaveraldine	0.62
Ethaverine	0.64
Dioxyline	0.65
Cocaine	0.77
Imipramine	0.80
Promethazine	0.87
Carbetapentane	0.89
Yohimbine	0.94
Methapyrilene	0.97
Procaine	0.98
Heroin	1.12
Mepyramine	1.17
Thonzylamine	1.18
Fluphenazine	1.36
Strychnine	2.95

*Papaverine = 1.0.

This presents no problem, however, since in the pharmacokinetic study only volunteers not undergoing drug therapy were accepted. If the method should be used for routine papaverine monitoring, however, it is essential to know the complete medication history of the patient.

Precision and detection limit

The within-day precision of the method was evaluated at the 25 ng/ml and 250 ng/ml levels by analysing replicate spiked samples (n = 6). Coefficients of variation of, respectively, 5.6% and 5.5% were found. The between-day precision was determined to be 6.4% and 4.0% at the 50 ng/ml and 200 ng/ml levels, respectively. The detection limit at a signal-to-noise ratio of 3 was estimated to be 5 ng/ml, which was considered to be sufficient for our purpose. However, it should be possible to measure much lower concentrations if the entire organic phase can be recovered, if the entire reconstituted extract is injected, and if a variable-wavelength detector, set at the maximum absorbance wavelength of papaverine, is used.

Pharmacokinetic study

The method was used to measure 400 blood samples from ten healthy volunteers, each taking four different papaverine formulations with a minimum one-week "wash-out". Fig. 1a illustrates a chromatogram of a volunteer specimen with added internal standard. The blood sample was withdrawn 30

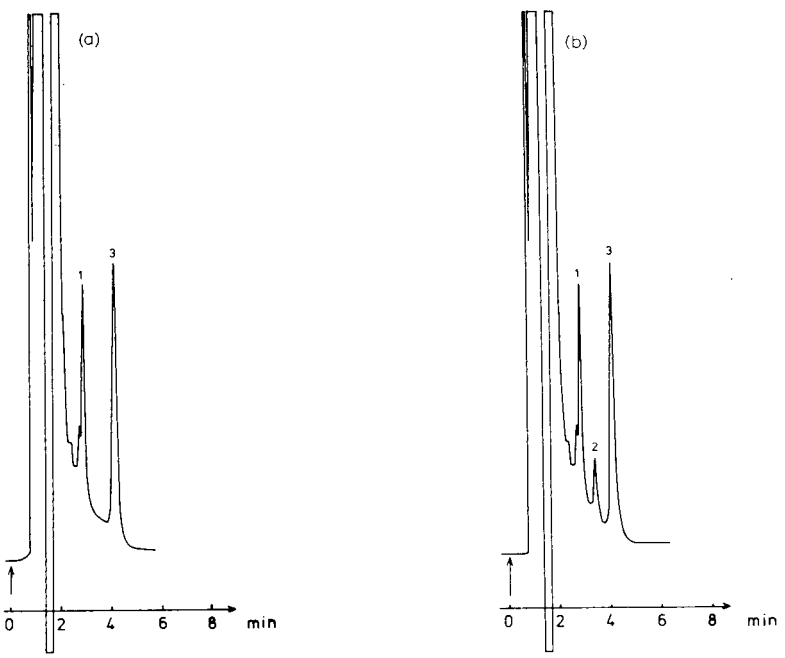

Fig. 1. (a) Chromatogram of blood from a volunteer before papaverine administration, spiked with internal standard. Peaks: 1 = caffeine, 3 = internal standard. (b) Chromatogram of blood from the same volunteer withdrawn 30 min after ingestion of a 300-mg dose of papaverine · HCl. Peaks: 1 = caffeine, 2 = papaverine (concentration found: 12 ng/ml), 3 = internal standard.

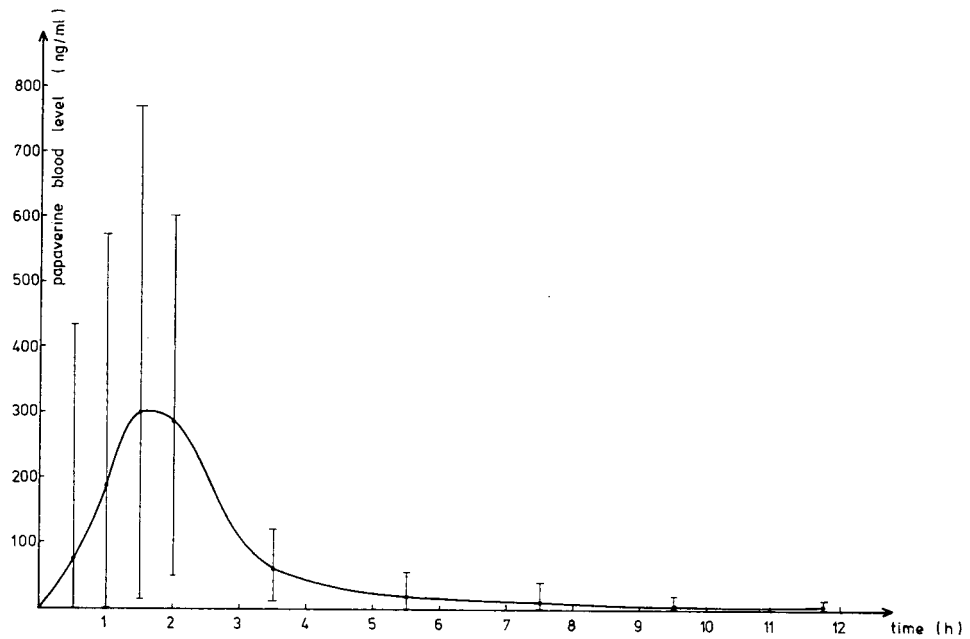

Fig. 2. Mean time course and extreme values of papaverine levels in the blood of ten volunteers each receiving a single 150-mg dose of papaverine · HCl.

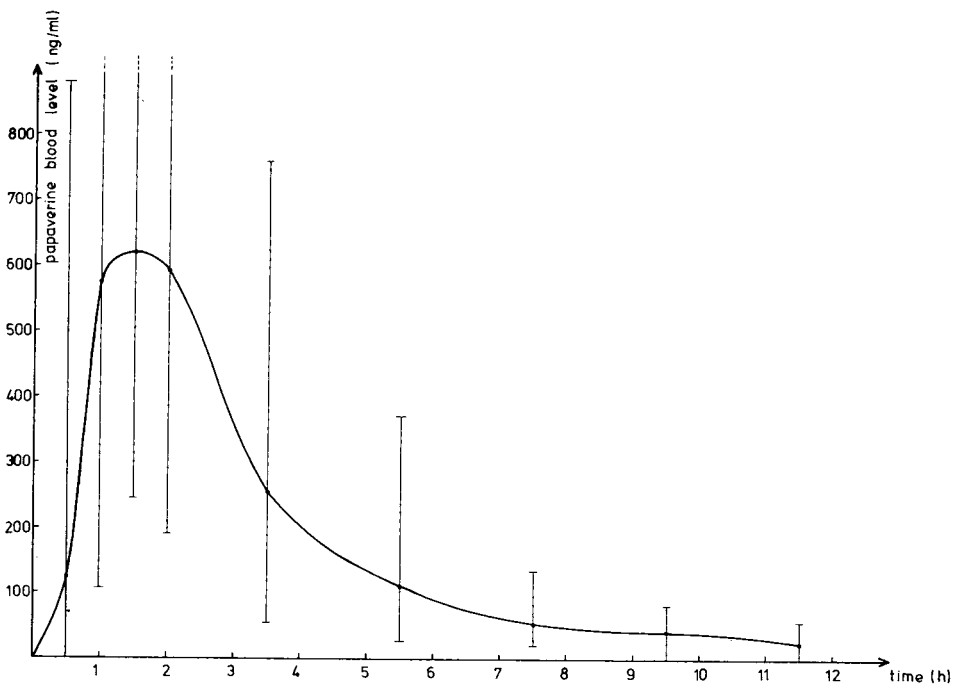

Fig. 3. Mean time course and extreme values of papaverine levels in the blood of ten volunteers each receiving a single 300-mg dose of papaverine · HCl.

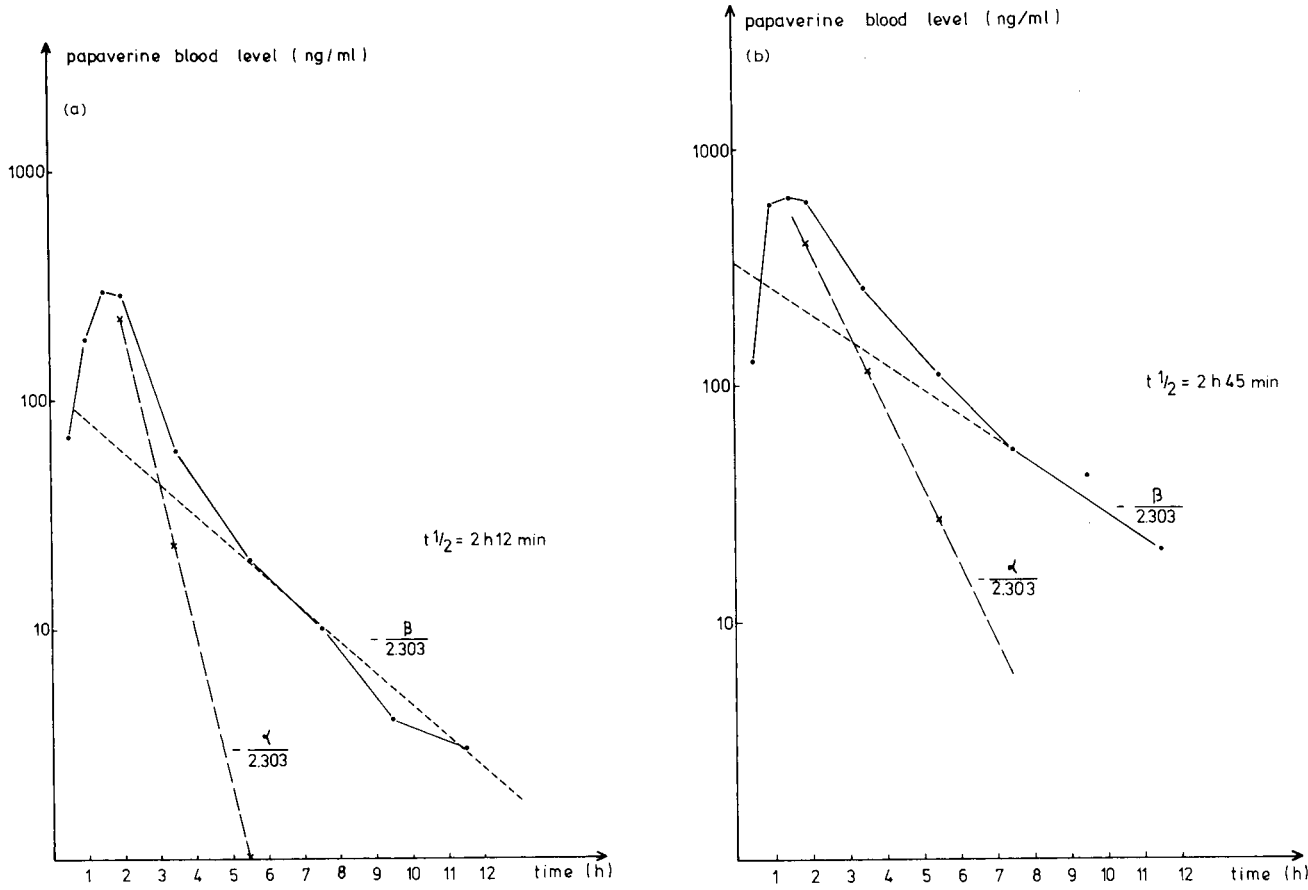

Fig. 4. (a) Mean time course (logarithmic coordinates) of papaverine levels in the blood of ten volunteers each receiving a single 150-mg dose of papaverine · HCl. (b) Mean time course (logarithmic coordinates) of papaverine levels in the blood of ten volunteers each receiving a single 300-mg dose of papaverine · HCl.

min after ingestion of a single classical capsule containing 300 mg of papaverine HCl and lactose as the only ingredients. The concentration found was 12 ng/ml. Fig. 1b shows the chromatogram of blank blood from the same volunteer, withdrawn before papaverine intake and spiked with internal standard. The results of the pharmacokinetic study are reported in Figs. 2 and 3 as mean blood—time profiles. Only the results obtained with classical capsules containing 150 or 300 mg of papaverine · HCl and lactose as the only ingredients are shown. As could be expected, a similar curve was obtained for both doses. The extreme values obtained show the inter-subject variability of the papaverine blood levels to be very important. From Figs. 2 and 3 it can also be seen that the peak level is reached between 1.5 h and 2 h after ingestion of the drug. The results of the pharmacokinetic study are plotted on semi-logarithmic coordinates (Fig. 4). This figure suggests that the change in the concentration of the drug in the body as a function of time can be described by a two-compartmental model. The elimination half-life, determined graphically, is about 2.5 h.

CONCLUSION

A simple, fast and reproducible method for the determination of papaverine in whole blood has been developed. During the editorial review of this paper, we became aware of an article by Gautam et al. [9], dealing with the HPLC determination of papaverine in plasma and urine. These authors, claiming their method to be the first HPLC method for that purpose, used a single extraction with chloroform—isopropanol (95:5) followed by reversed-phase chromatography on a C_8 column. While their method is more or less similar in simplicity and reproducibility, our method is more sensitive. Furthermore, we obtained larger extraction recoveries and, although Gautam et al. [9] used a faster flow-rate (which also means that larger eluent volumes are needed), the time needed for the chromatographic step is longer than in our method. While Gautam et al. [9] presented no pharmacokinetic data, the procedure developed by us has been shown to be successfully applicable to a pharmacokinetic study. The results of the pharmacokinetic study show that there is an important inter-subject variability in papaverine blood levels (and elimination of the drug, proceeding in two phases). The half-life of the drug was estimated to be about 2.5 h.

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CHROMBIO. 1025

DETERMINATION OF CEFOTAXIME AND DESACETYLCEFOTAXIME IN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method is described for the analysis of the anti-bacterial agent cefotaxime and desacetylcefotaxime in physiological fluids. Plasma or serum samples were mixed with chloroform—acetone to remove proteins and most lipid material. The aqueous phase was then freeze-dried, reconstituted in mobile phase and chromatographed on a reversed-phase column using UV detection at 262 nm. Urine was analysed directly after centrifugation to remove particulate matter. The detection limit was 0.5—1.0 μg/ml for serum and 5 μg/ml for urine. The method has been applied to the analyses of cefotaxime and desacetylcefotaxime in plasma, serum, urine, cerebrospinal fluid, saliva, and pus from infected wound secretions. Two additional metabolites, which are lactones in which the β-lactam ring has been opened, could be separated by this method.

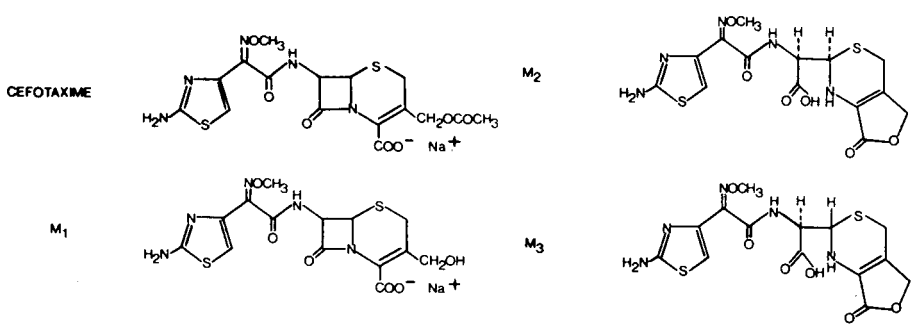
INTRODUCTION

Cefotaxime (sodium 7-[{[2(2-amino-4-thiazolyl)-2-methoxyimino]acetyl}-amino]-3-acetoxymethyl-8-oxo-5-thia-1-azatricyclo-[*4,2,0*]-octene 2-carboxylate, developed by Hoechst, Frankfurt, G.F.R., and Roussel-Uclaf, Paris, France) is a semi-synthetic cephalosporin, active against a variety of gram-positive and gram-negative bacteria, including some strains resistant to other antibiotics such as ampicillin, tetracycline and chloramphenicol [1]. Microbiological methods are widely used for the analysis of penicillins and cephalosporins. For cefotaxime, the organism *Escherichia coli* V6311 provides a sensitive (0.1 μg/ml) assay method [2] but it is not completely specific since the organism is sensitive to desacetylcefotaxime. *Proteus morganii* NCTC 11354 is metabolite resistant and will measure cefotaxime even in the presence of high metabolite concentrations [3].

An alternative method of assay which would allow the determination of metabolites in the presence of cefotaxime was required for biological fluids. A reversed-phase high-performance liquid chromatographic (HPLC) method was developed. HPLC has been used for the determination in biological fluids of most of the commonly prescribed cephalosporins including cephaloridine [4], cephalothin [5], cefazolin [5], cefuroxime [6, 7], cefoxitin [8], cefatrizine [9], cephaloglycin [10], and cephalexin [11, 12]. Although the aqueous solubility of cephalosporins is generally too high to allow their extraction by organic solvents from biological fluids, an ion-pair method [13] in which cephalothin and desacetylcephalothin are extracted from biological specimens with tetraheptylammonium chloride has been used to determine these compounds in serum. In our laboratories a reversed-phase method was developed for the analysis of a semi-synthetic cephalosporin, HR 580 (Hoechst), in serum and in urine [14]. Preliminary details of methods for the analysis of cefotaxime and metabolites have been reported [3, 15—17].

In the methods described by Reeves et al. [15], White et al. [3] and Borner and Lode [16], accuracy, precision and recovery data are not reported. Bergan and Solberg [17] report accuracy data for cefotaxime at 10 μg/ml. The present paper reports accuracy and precision for both cefotaxime and desacetylcefotaxime in plasma and urine over a wide concentration range. Plasma sample preparation procedures are discussed in relation to the suitability of the various methods for use with autoinjectors. The reversed-phase method described allows separation of cefotaxime from the metabolites M_2 and M_3 as well as from desacetylcefotaxime (M_1). Evidence for the structure of the epimeric lactones M_2 and M_3 has been presented in a separate publication [18].

The method reported below has been applied to the analysis of cefotaxime, M_1, M_2, and M_3 in plasma, serum, urine, cerebrospinal fluid, saliva and pus from infected wound secretions, from patients receiving intramuscular and intravenous doses of cefotaxime.



EXPERIMENTAL

Apparatus and materials

The chromatographic system consisted of a reciprocating pump, a variable-wavelength detector (262 nm), an automatic injector (WISP, Waters Assoc., Northwich, Great Britain), and a computing integrator (SP 4100, Spectra

Physics, St. Albans, Great Britain). The separation column was a 100 mm × 3 mm I.D. stainless-steel tube packed with ODS Spherisorb (5 μm diameter, Phase Separations, Queensferry, Great Britain). The mobile phase consisted of a mixture of methanol—water—glacial acetic acid (12:87:1, v/v). All chemicals and solvents were of analytical reagent grade (Fison's Scientific Apparatus, Loughborough, Great Britain) and were used without further treatment. Water was distilled in all glass apparatus. Stock aqueous solutions of cefotaxime and desacetylcefotaxime (M_1) were prepared weekly and stored at 0—4°C.

Preparation of columns

Packing material (1.1 g) was added to isopropanol (10 ml) and the mixture was poured into a Micromeritics column packing chamber (Micromeritics, Norcross, GA, U.S.A.). The column was packed by forcing the slurry into the column tube by means of a constant-pressure pump (MCP 71, Olin Energy, Sunderland, Great Britain) set at 20,700 kPa.

Analysis of plasma samples

Plasma or serum (1 ml) was mixed with chloroform—acetone (1:3, v/v) (8 ml) on a vortex mixer for 30 sec. After centrifuging (2000 *g*, 5 min) a measured volume from the upper aqueous layer was freeze-dried. The dry residue was reconstituted in mobile phase (100 μl) and, after centrifuging (2000 *g*, 5 min), 20 μl of the supernatant were injected. To obtain the concentrations of cefotaxime, M_1, M_2, and M_3, the external standard, the non-linear calibration programme of the SP 4100 was utilized. Standard samples for use with this programme were prepared by adding cefotaxime and M_1 to control human plasma to give six samples within the concentration range 0—200 μg/ml; these samples were processed and chromatographed as described above. The SP 4100 computed equations relating peak area to concentration which best fitted the data and applied these to calculate the concentrations of cefotaxime and M_1 in test samples. Pure samples of M_2 and M_3 were not available, so the concentrations of these metabolites were computed from the cefotaxime standard curve. A typical chromatogram is illustrated in Fig. 1.

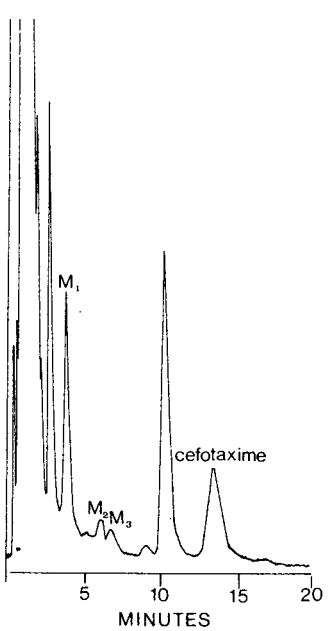
Analysis of urine samples

Urine samples were centrifuged (200 0 *g*, 5 min) and a portion (20 μl) was analysed as described. Calibration samples were prepared by adding known amounts of cefotaxime and M_1 to control urine. A typical chromatogram is illustrated in Fig. 2. All biological samples were stored at −20°C until required for analysis.

RESULTS AND DISCUSSION

Chromatography

Typically, for plasma samples using the conditions described, the retention times for cefotaxime, M_1, M_2, and M_3 were 13.5, 3.9, 6.2 and 6.9 min, respectively, at a flow-rate of 1.1 ml/min (Fig. 1). For urine samples, in order



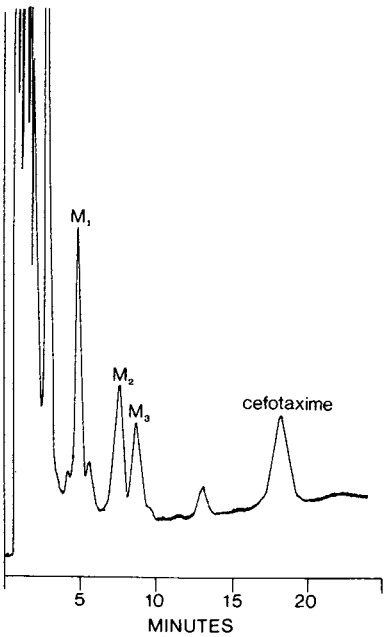


Fig. 1. Chromatogram of a plasma sample from a uraemic patient 1 h after receiving a 1-g intravenous dose of cefotaxime. 12% methanol in mobile phase. Concentrations (μg/ml): cefotaxime, 29; M_1, 70; M_2, 2; M_3, 1.

Fig. 2. Chromatogram of a urine sample from a uraemic patient, 8—12 h after receiving a 1-g intramuscular dose of cefotaxime. 10% methanol in mobile phase. Concentrations (μg/ml): cefotaxime, 127; M_1, 86; M_2, 72; M_3, 50.

to separate M_1 from early eluting endogenous components, it was necessary to lower the methanol content to 10% (Fig. 2). The concomitant increase in overall retention time, however, resulted in a mobile phase composition which was a compromise between adequate separation and a rapid analysis time. It was necessary from time to time to make small changes in the proportions of the mobile phase components in order to accommodate variations in the endogenous background in the large number of biological samples analysed.

Nucleosil ODS (Macherey-Nagel, Camlab, Cambridge, Great Britain) was a suitable alternative to Spherisorb ODS, although the retention times were longer using the former material. On Hypersil ODS (Shandon Southern, Runcorn, Great Britain) M_2 could not be separated from M_3; this material, however, gave the most efficient columns and would be the column of choice in circumstances where separation of M_2 and M_3 was not required. These selectivity differences are illustrated in Fig. 3. Heptanesulphonic acid (0.005 *M*) is a suitable alternative to acetic acid in the mobile phase and adequate separations have been obtained using sulphonic acids with columns of either ODS Spherisorb, ODS Nucleosil or μBondapak C_{18} (Waters Assoc.). In addition, acetonitrile has been used instead of methanol, the advantage of the former solvent being the lower back pressures that result from its use com-

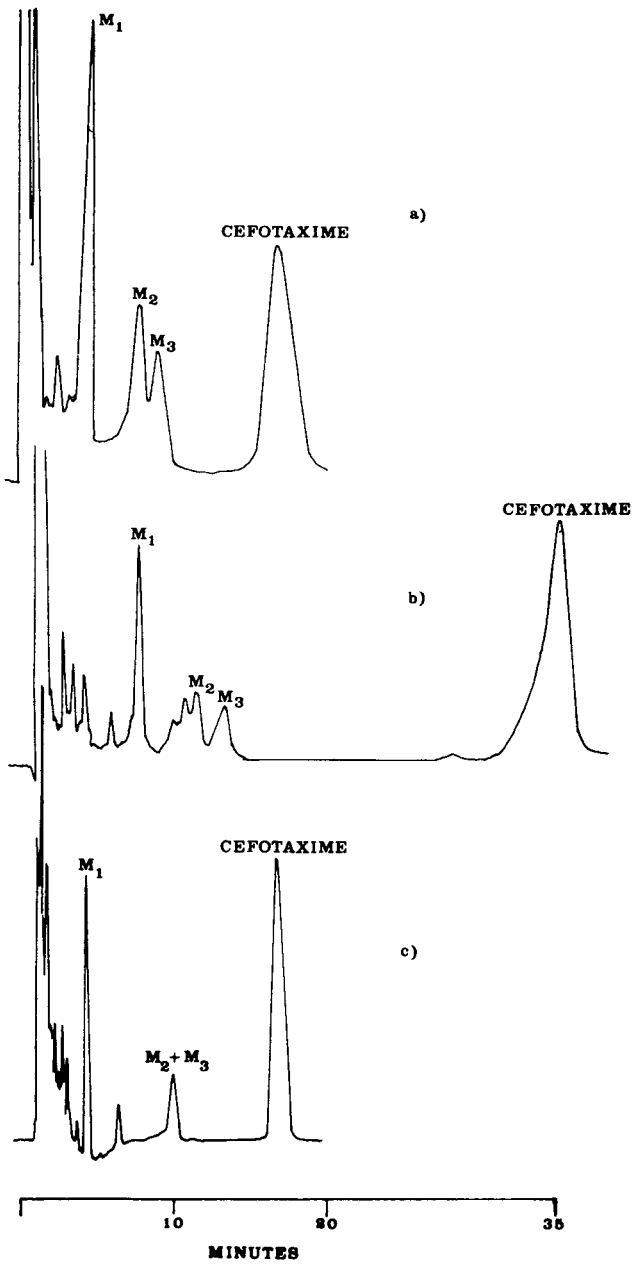


Fig. 3. Chromatograms of a urine sample from a human subject receiving cefotaxime, demonstrating selectivity differences between various reversed-phase columns. (a) Spherisorb ODS, 10 μm, 10 cm × 3 mm. Mobile phase: methanol—water—acetic acid (12:87:1). (b) Nucleosil ODS, 10 μm, 10 cm × 3 mm. Mobile phase: acetonitrile—water—acetic acid (6:93:1). (c) Hypersil ODS, 5 μm, 20 cm × 4 mm. Mobile phase: acetonitrile—water—acetic acid (10:89:1).

pared with methanol. It is likely, therefore, that an adequate separation of all four components can be obtained on most commercially available C_{18} columns, using a mobile phase consisting of a mixture of water, acetonitrile or methanol and a suitable acidic modifier giving a pH of 3—4. In any new system, the retentions of M_1, M_2 and M_3 must be determined to ensure that these metabolites do not co-elute with cefotaxime.

A strong anion-exchange column (Nucleosil 5SB, Macherey-Nagel) has also been used for plasma analysis (Fig. 4). The mobile phase was acetonitrile—

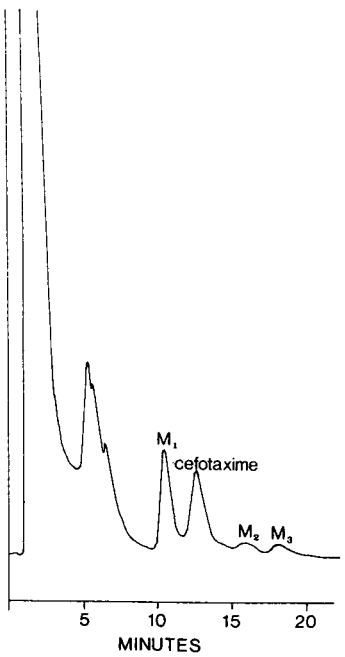


Fig. 4. Chromatogram of a plasma sample from a dog 4 h after receiving a 1500 mg/kg intravenous dose of cefotaxime. Concentrations (μg/ml): cefotaxime, 74; M_1, 84; M_2, 5; M_3, 10. Chromatography on an anion-exchange column (see text).

0.4% ammonium dihydrogen phosphate (1:4, v/v) adjusted to pH 4.7. Under these conditions the elution order is M_1, cefotaxime, M_2, M_3. Since the selectivity is very different from reversed-phase columns, anion exchange may provide a useful alternative when separation of the analytes from endogenous components cannot be achieved on a reversed-phase column.

Sample preparation

An important advantage of HPLC over gas—liquid chromatography is that aqueous biological samples can be injected directly on to a column, obviating the need for an extraction stage. In order to realise this advantage, it is necessary to remove proteins and desirable to remove lipids from plasma samples prior to analysis on a reversed-phase column. The removal of both proteins and neutral lipids was achieved by extraction of the plasma samples with a chloroform—acetone mixture. Using the proportions indicated,

the plasma, free of protein and lipid, separated as a clear upper aqueous layer after mixing and centrifuging to remove the precipitated protein. The highly water-soluble cephalosporin remained in the aqueous phase. In order to achieve acceptable precision below 5 μg/ml it was necessary to concentrate the sample by freeze-drying. A fivefold increase in final concentration was obtained by freeze-drying 0.5 ml of the aqueous phase and re-constituting in 0.1 ml.

The recovery of cefotaxime in the aqueous layer following this procedure was assessed by comparing the peak areas of cefotaxime obtained after processing plasma samples containing known concentrations of the compound with peak areas following analysis of standard aqueous solutions of cefotaxime. The recovery was quantitative (Table I).

TABLE I

THE RECOVERY OF CEFOTAXIME FOLLOWING THE PLASMA SAMPLE PREPARATION PROCEDURE

Cefotaxime added (μg/ml)	Cefotaxime found (μg/ml)	Mean recovery (%)
100.0	100.7, 96.9	99
19.9	20.4, 20.2	103
9.9	10.9, 11.8	115
5.0	4.8, 4.1	94
3.0	2.4, 2.8	87
2.0	1.6, 1.7	83
1.0	0.9, 1.0	95
Overall mean recovery		96 ± 11%

If automatic injection systems are to be used, consideration must be given to the fact that a sample may be standing in solution at room temperature for up to 16 h before analysis, in the case of an overnight run. Cefotaxime was found to be stable under these conditions, following the preparation procedure described in this report and the acetonitrile procedure. The use of acidic protein-precipitation reagents, however, causes significant degradation of cefotaxime. When 0.1 ml of 70% (w/v) trichloroacetic acid was added to 1 ml of plasma containing cefotaxime, the half-life of the drug was 7 h at room temperature.

Detection limit

In the mobile phase used, λ_{max} was 262 nm (ϵ 1.7 × 10^4). At any time, the sensitivity of the method was dependent upon the condition of the column and detector noise level. Over a period of three years, during which time several thousand assays have been carried out, plasma samples containing 0.5 μg/ml cefotaxime gave an average signal-to-noise ratio of 4:1 at 0.01 absorption units full scale deflection. The precision at this concentration was 10—15%.

Specificity

The sample preparation procedure for plasma imparted specificity as far as interference from most lipophilic compounds was concerned, since these would have partitioned into the chloroform—acetone phase. Separation of cefotaxime from the metabolites and endogenous components was achieved on reversed-phase columns as well as on a strong anion-exchange column. When lack of specificity was encountered as a result of interference from endogenous components, it was usually possible to select a different reversed-phase or anion-exchange column on which separation could be achieved.

Precision and accuracy

Cefotaxime and M_1 were added to control plasma to give six samples in the concentration range 0.5—120 μg/ml. Sufficient plasma was prepared to allow six replicate samples to be analysed at each concentration. The procedure was repeated for urine over the concentration range 5—500 μg/ml. The results are presented in Tables II and III. Accuracy, here, is defined as (amount found/amount added) × 100 (%).

TABLE II

PRECISION AND ACCURACY FOR CEFOTAXIME

Cefotaxime added (μg/ml)	Cefotaxime found (μg/ml)	Mean	S.D.	C.V. (%)	$\frac{\text{Found}}{\text{Added}} \times 100$
Plasma					
0.50	0.60, 0.54, 0.63, 0.69, 0.53, 0.54	0.59	0.06	10.8	118
1.0	1.1, 1.3, 1.2, 1.1, 1.0, 1.2	1.2	0.10	9.1	120
4.6	4.9, 4.8, 5.1, 4.8, 4.9, 4.8	4.7	0.14	3.0	107
11.8	12.1, 13.1, 15.1, 12.6, 12.5, 12.3	13.0	1.1	8.5	108
52.2	58.3, 55.9, 59.8 58.6, 57.1, 56.8	57.8	1.4	2.4	98
118.4	113.8, 125.1, 125.3, 120.6, 126.6, 116.8	121.4	5.2	4.3	103
Urine					
580	516, 538, 523 539, 531, 534	530	9.0	1.7	91
230	227, 235, 247, 241, 213, 213	229	14.3	6.2	100
115	100, 102, 116, 114, 116, 114	110	7.3	6.6	96
58	64, 58, 58, 55, 66, 64	60.8	4.4	7.2	105
8.3	7.5, 7.2, 8.0, 6.9, 7.2, 8.3	7.5	0.5	7.1	90
4.6	3.9, 4.7, 3.6, 2.5, 4.2, 3.0	3.7	0.8	22.0	80

TABLE III

PRECISION AND ACCURACY FOR DESACETYLCEFOTAXIME

Desacetylcefotaxime added (μg/ml)	Desacetylcefotaxime found (μg/ml)	Mean	S.D.	C.V. (%)	$\frac{\text{Found}}{\text{Added}} \times 100$
Plasma					
0.49	0.47, 0.50, 0.51, 0.44, 0.49, 0.49	0.48	0.025	5.2	98
0.96	1.0, 0.8, 1.1, 1.2, 1.1, 1.1	1.05	0.14	13.1	110
5.4	4.6, 4.9, 5.2, 5.4, 5.5, 4.5	5.0	0.4	8.0	93
10.8	9.1, 9.4, 9.3, 7.9, 9.4, 9.8	9.2	0.7	7.6	85
16.1	16.5, 15.6, 16.9, 16.3, 16.6	16.4	0.5	3.0	102
21.5	20.7, 23.0, 23.7, 23.1, 23.2, 22.2	22.7	1.1	4.8	105
Urine					
519	503, 502, 501, 513, 513, 511	507	5.7	1.1	98
206	200, 210, 222, 221, 197, 195	208	12.0	5.8	101
103	92, 93, 104, 105, 107, 105	101	6.7	6.6	98
51	58, 52, 50, 47, 59, 58	54	5.0	9.3	106
8.2	9.3, 8.9, 10.1, 8.9, 9.5, 10.1	9.5	0.5	5.7	116
4.5	4.1, 4.9, 3.4, 2.7, 4.6, 3.0	3.8	0.9	23.5	84

Application

The method has been applied to the analysis of cefotaxime and the three metabolites in various physiological fluids including plasma, serum, urine, cerebrospinal fluid, saliva, and pus from infected wound secretions. A typical concentration—time course curve for the elimination of cefotaxime and its metabolites from the plasma of a patient receiving cefotaxime is shown in Fig. 5.

The chloroform—acetone treatment for plasma improved column longevity, compared with our previous plasma preparation procedure, which involved freeze drying and re-constitution in methanol [13]. Using the procedure described in this paper, deterioration in column performance, as judged by peak broadening accompanied by loss of sensitivity, occurred after approximately five hundred injections of biological fluid. A column could normally be regenerated by replacing the top 3 mm of packing material.

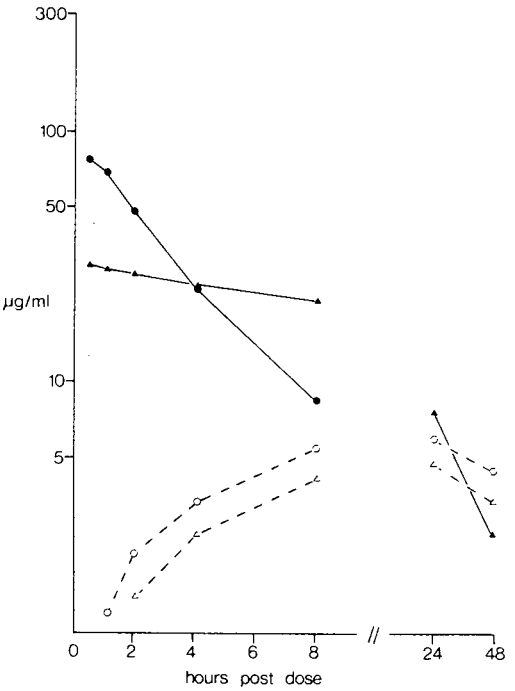


Fig. 5. The concentration—time course of cefotaxime and metabolites in the plasma of a uraemic patient after receiving a 2-g intravenous dose of cefotaxime.

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DETERMINATION OF BUMETANIDE IN THE PLASMA OF NON-HUMAN PRIMATES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A simple, sensitive and specific method for the determination of bumetanide in the plasma of non-human primates has been developed using high-performance liquid chromatography in a reversed-phase mode. The limit of accurate measurement for bumetanide in plasma was 1 ng/ml although lower concentrations could be detected. The method has been applied to plasma samples obtained from cynomolgus monkeys after intravenous doses of bumetanide of 0.03 mg/kg. In this species, mean plasma concentrations declined from 300 ng/ml at 2 min after dosing to 1 ng/ml at 180 min; the half-life of the terminal linear phase was 43 min.

INTRODUCTION

Bumetanide (3-*n*-butylamino-4-phenoxy-5-sulphamoyl benzoic acid) (Fig. 1) is a potent "high ceiling" (loop) diuretic which produces the rapid onset and short duration of action characteristic of this class [1—4]. Since doses of bumetanide in clinical usage are low, a sensitive and highly specific method of analysis for the measurement of this drug in plasma is necessary.

A wide variety of methods have been reported for the quantitative estimation of bumetanide, many of which employed the radiolabelled drug and thin-layer chromatography but did not distinguish between bumetanide and its

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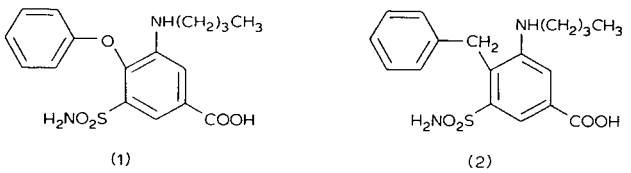

Fig. 1. Chemical structure of bumetanide (1) and internal standard (2).

metabolites; other methods are not sufficiently sensitive for the accurate determination of circulating levels of the drug. Published methods include those based on gas–liquid chromatography [5], thin-layer chromatography with fluorimetry [4] and radiometric assay [6].

In order to investigate the pharmacokinetics of bumetanide in non-human primates, an analytical method was developed using high-performance liquid chromatography (HPLC) coupled with fluorimetric detection. Fluorimetry is an ideal technique for bumetanide analysis, in that it is particularly sensitive and can be highly specific when coupled with chromatographic separation. A similar method has recently been published [7]. The major advantage of the method described in this paper is the greater sensitivity, giving a limit of accurate measurement of 1 ng/ml of plasma. The improved sensitivity is achieved by the use of a scanning fluorimeter which facilitates the measurement of the drug by using both the excitation and emission maxima of the compound. Sensitivity is further enhanced by buffering the mobile phase to pH 4.0 at which value the fluorescence of bumetanide is at a maximum.

EXPERIMENTAL

Materials

All reagents were of analytical grade and all inorganic reagents were prepared in fresh glass-distilled water. Diethyl ether was freshly redistilled prior to use. Standard solutions of bumetanide were prepared at concentrations of 0.1 μg/ml, 1.0 μg/ml and 10.0 μg/ml in methanol. A stock solution of 4-benzyl-3-*n*-butylamino-5-sulphamoyl benzoic acid (Fig. 1) for use as internal standard was prepared at a concentration of 1 μg/ml in methanol. All standard solutions were stored at 4°C.

Extraction procedure

Plasma samples (1 ml) were transferred to 10-ml pointed centrifuge tubes and spiked with internal standard (30 μl, containing 30 ng). Sulphuric acid (200 μl, 1 *N*) was added and the mixture was extracted by vortex mixing with diethyl ether (5 ml). After centrifugation of the extract at 2000 *g* for 10 min, the organic layer was transferred to another pointed centrifuge tube and evaporated to dryness under a stream of dry nitrogen at 37°C. The residue was washed to the bottom of the tube with a small volume of diethyl ether, which was again evaporated to dryness. The dry residue was redissolved in methanol (30 μl) and the total sample was injected into the liquid chromatograph.

High-performance liquid chromatography

The chromatograph consisted of an M6000A pump (Waters Assoc., Northwich, Great Britain) fitted to a Perkin-Elmer 3000 fluorimeter (Perkin-Elmer, Beaconsfield, Great Britain) operated at an excitation wavelength of 338 nm and an emission wavelength of 440 nm (the fluorescence maximum for bumetanide dissolved in mobile phase). Injection was made by syringe via a U6K universal injector (Waters Assoc.). The column was constructed of stainless steel (30 cm × 4 mm I.D.) prepacked with μBondapak C_{18}, particle size 10 μm (Waters Assoc.). A pre-column (7 cm × 2 mm I.D.) constructed of stainless steel and dry-packed with pellicular Co:Pell® ODS (particle diameter 25–37 μm, Whatman, Maidstone, Great Britain) was installed in front of the analytical column.

Chromatography was performed in a reversed-phase mode using a mobile phase of 60% (v/v) methanol in aqueous potassium dihydrogen orthophosphate (0.1%, w/v); the pH of the final mixture was adjusted to 4.0 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, Great Britain). Peak height measurements were used in preference to peak area measurements due to the inability of the integrator to assign correct areas to the smaller peaks.

Under the conditions described, bumetanide had a retention time of 5.7 min and the internal standard a retention time of 8.0 min (Fig. 2).

Calibration procedure

The calibration line was constructed from peak height ratio measurements of bumetanide to internal standard against concentration, over the concentration range 1–100 ng/ml. Samples of blank plasma (1 ml) were spiked with bumetanide at concentrations of 1, 5, 15, 30, 60, and 100 ng/ml, and with internal standard at a fixed concentration of 30 ng/ml. Samples were taken through the extraction procedures described previously.

Studies in monkeys

Five cynomolgus monkeys (*Macaca fascicularis*) were dosed with the commercial preparation of bumetanide, Burinex® (Leo Labs., Hayes, Great Britain). Each animal was dosed intravenously with 0.03 mg bumetanide per kg of bodyweight (corresponding on a mg/kg basis to the human therapeutic dose).

The animals were fasted for at least 12 h preceding drug administration and for 6 h following drug administration. Blood samples were taken from the femoral veins of the animals at 0 (pre-dose), 2, 5, 10, 20, 30, 45, 60, 80, 100, 120, 150, 180, 240, 300, 450, and 1440 min after dosing. The samples were taken into heparinised tubes and the blood cells separated by centrifugation and discarded. The separated plasma was stored at −20°C until analysis by the method described. Where less than 1 ml of plasma from the dosed animals was required for analysis, the volume was made up to 1 ml with control plasma.

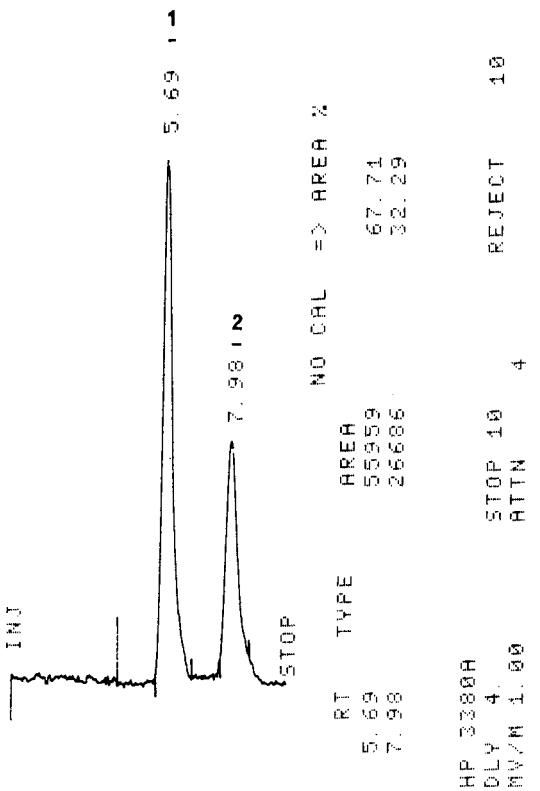

Fig. 2. Chromatogram of a standard mixture containing bumetanide (1) and internal standard (2). Column, μBondapak C_{18} (Waters Assoc.); mobile phase, 60% (v/v) methanol in aqueous potassium dihydrogen orthophosphate (0.1%, w/v), pH 4.0; flow-rate, 2 ml/min; detector, fluorescence, excitation 338 nm, emission 440 nm.

Gas chromatography—mass spectrometry

Gas chromatography—mass spectrometry (GC—MS) was carried out using a Pye 104 gas chromatograph (Pye-Unicam, Cambridge, Great Britain) linked via a single-stage, glass jet separator to a Micromass 16F mass spectrometer (VG Analytical, Altrincham, Great Britain). The mass spectrometer was operated in the electron impact mode of ionisation, with an electron beam energy of 70 eV, a trap current of 100 μA and a source temperature of 200°C. Mass spectra were obtained at 10-sec intervals and the data stored using a Display Digispec data system (VG Analytical) using floppy diskettes.

For the analysis of bumetanide samples, the GC oven was fitted with a glass column (1 m × 3 mm I.D.) packed with 1% OV-1 on Diatomite CLQ (100—120 mesh) and was operated at 230°C. Helium was used as the carrier gas at a flow-rate of 20 ml/min. The temperature of the GC—MS interface was 250°C.

Bumetanide samples for GC—MS were evaporated to dryness and the residues (ca. 2 μg) were methylated by dissolving them in Methelute (0.2 *M* trimethylanilinium hydroxide in methanol; 10 μl) (Pierce and Warriner (U.K.), Chester, Great Britain); 2.5-μl aliquots were injected for on-column reaction.

Specificity of the analytical method

Initial studies on bumetanide reported in the literature suggested that there was no metabolism of the drug in man [2, 4, 8]; more recent work shows that there is N-debutylation and oxidation in the butyl side-chain of the molecule [6,9—11]. In the absence of analytical standards of the proposed metabolites, it was not possible to test for the co-elution of these compounds with the parent drug in the chromatographic system described. However, the literature available suggests that no metabolites of bumetanide have been reported after acid—ether extraction of the drug from biological fluids, while Pentikainen et al. [6] reported than an ether extraction from an acid medium quantitatively separated bumetanide from its more polar metabolites.

Profiles of the methylated compound produced by the mass spectrometer (Fig. 3) were similar for the pure drug and for extracts of post-dose plasma samples measured as bumetanide by the HPLC method described. Three major ions corresponding to m/z 420 (molecular ion), m/z 377 ($M - C_3H_7$) and m/z 58 [$(CH_3)_2\overset{\oplus}{N} = CH_2$, formed by rearrangement] were observed in all samples, with no other major ions occurring in the plasma extracts (as would be expected if metabolites were present). Similarly, the gas chromatograms for the samples each yielded only one major peak, with the same retention time as authentic bumetanide.

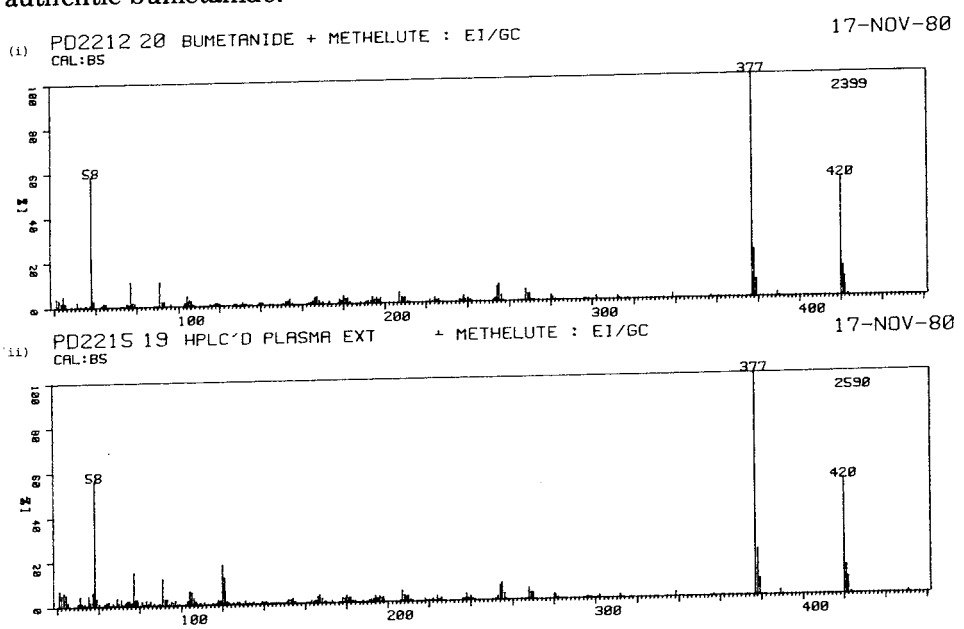

Fig. 3. Mass spectra of (i) authentic bumetanide (methylated) and (ii) post-dose plasma extract of bumetanide, separated by HPLC then methylated.

RESULTS AND DISCUSSION

Precision

Extraction and measurement at each concentration was repeated on six

TABLE I

PRECISION OF MEASUREMENT AND RECOVERY OF BUMETANIDE FROM PLASMA

Concentration added to plasma (ng/ml)	Mean peak height ratio (bumetanide/ internal standard)	Coefficient of variation (%)	Recovery (%)
1	0.06	17	77
5	0.26	4	74
15	0.80	3	68
30	1.64	1	69
60	3.08	2	69
100	5.26	2	69

occasions over the calibration range. The precision of the method for the measurement of bumetanide in plasma was indicated by the coefficients of variation of peak height ratios (Table I) which were ± 17% at 1 ng/ml, ± 1% at 30 ng/ml and ± 2% at 100 ng/ml.

Accuracy

The calibration line for the measurement of bumetanide in plasma was constructed over the range 1—100 ng/ml; six replicate extractions were made at each concentration over the range. The plot of peak height ratios against concentration was linear ($Y = a + bX$, where $a = 0.0087$ and $b = 0.0523$, correlation coefficient $r = 0.9995$) where Y is the peak height ratio and X is the concentration (ng/ml) of bumetanide. The accuracy of the method as defined by the 95% confidence limits of the least-squares regression line, i.e. taking the calibration line as an estimate of the concentration of bumetanide in plasma, was ± 44%, ± 8% and ± 2% at 5, 30 and 100 ng/ml, respectively.

Recovery

The recovery of internal standard (30 ng/ml) from plasma (1 ml) was determined by comparison of peak height ratio measurements of internal standard to bumetanide of standards taken through the extraction procedure, to those injected into the chromatograph without extraction. The mean recovery of internal standard was 66 ± 5% S.D. ($n = 5$).

The mean recovery of bumetanide from plasma was determined by comparison of peak height ratio measurements of non-extracted standards to those of extracted standards corrected for 100% recovery of internal standard, and was 71 ± 4% S.D. (Table I).

Stability of bumetanide in plasma

The stability of bumetanide in plasma under the storage conditions used (−20°C) was tested by storing 50 ng/ml plasma standards for 16 weeks. No decomposition of bumetanide was detected over this period.

Limits of detection

No interfering peaks with retention times similar to bumetanide were present

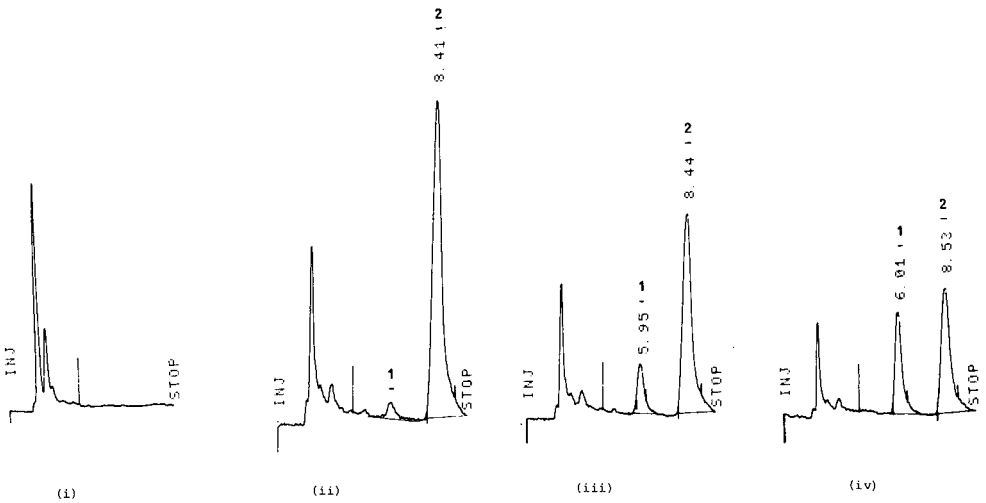

Fig. 4. Chromatograms of (i) pre-dose control plasma, and (ii—iv) plasma samples containing bumetanide at 1, 5, and 15 ng/ml, respectively, and 30 ng of internal standard (2).

TABLE II

CONCENTRATION OF BUMETANIDE IN THE PLASMA OF FIVE CYNOMOLGUS MONKEYS AFTER A SINGLE INTRAVENOUS DOSE OF 0.03 mg/kg

Concentrations are given in ng/ml.

Time (min)	Animal number					Mean ± S.D.
	1	2	3	4	5	
2	230	360	280	360	270	300.0 ± 57.9
5	120	180	140	130	100	134.0 ± 29.7
10	90	80	60	80	50	72.0 ± 16.4
20	50	30	20	50	20	34.0 ± 15.1
30	40	12	14	20	13	19.8 ± 11.7
45	24	6	8	12	6	11.2 ± 7.6
60	14	4	6	8	4	7.2 ± 4.1
80	8	3	5	4	4	4.8 ± 1.9
100	6	3	4	2	2	3.4 ± 1.7
120	4	2	3	2	2	2.6 ± 0.9
150	3	<1	3	1	<1	1.4 ± 1.5
180	3	<1	2	<1	<1	1.0 ± 1.4
240	2	<1	2	<1	<1	<1
300	1	<1	1	<1	<1	<1
450	<1	<1	<1	<1	<1	<1

in pre-dose (blank) plasma (Fig. 4). The limit of detection of bumetanide in plasma was less than 1 ng/ml and was set by instrumental noise.

Concentrations of bumetanide in the plasma of cynomolgus monkeys

The mean concentrations of bumetanide in the plasmas of five cynomolgus monkeys after an intravenous dose of 0.03 mg/kg are shown in Table II and

Fig. 5. A peak mean plasma concentration of 300 ng/ml bumetanide was measured at the first time of sampling (2 min); thereafter, mean plasma concentrations declined to the limit of accurate measurement (1 ng/ml) at 180 min after dosing. The half-life of the terminal linear section of the mean plasma bumetanide concentration—time relationship was 43 min compared with reported half-lives of 10 min in the rat [12], 9—15 min in the dog [4,12], and 60—90 min in humans [6—8,10,12]. The bumetanide half-life of 43 min is greater than that of 29 min reported for frusemide (another high-ceiling diuretic in clinical usage) in the same species [13].

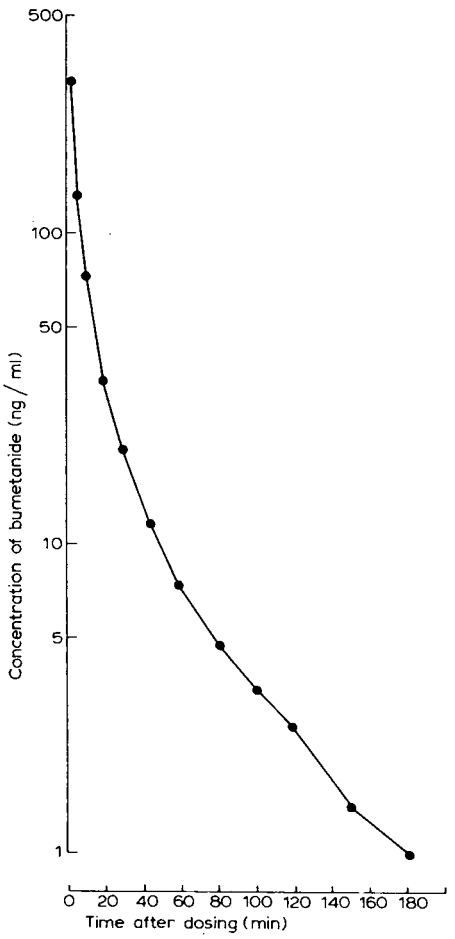

Fig. 5. Mean plasma concentrations of bumetanide after single intravenous doses of bumetanide (0.03 mg/kg) to five cynomolgus monkeys.

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CHROMBIO. 1036

Note

Improved sample preparation for the quantitative mass spectrometric determination of prostaglandins in biological samples*

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The simultaneous analysis of various prostaglandins in biological samples is difficult because the prostaglandins have to be extracted from a complex matrix, and because of the presence of many other prostaglandins and their metabolites.

We have developed a sample preparation procedure which uses both reversed-phase octadecylsilyl (C_{18}) silica and normal-phase silica cartridges (Sep-Pak^{TM}, Waters Assoc., Milford, MA, U.S.A.) to selectively extract and purify prostaglandins from biological samples. The procedure is simple and rapid. Small volumes of clear eluates are obtained which do not contain biological contaminants which would otherwise overburden the subsequent high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Deuterium- and tritium-labelled 7α-hydroxy-5,11-diketo-tetranorprostane-1,16-dioic acid (PGE-M) were a generous gift of Dr. W.J.A. VandenHeuvel, Merck Sharp & Dohme Research Labs., Rahway, NJ, U.S.A. The other deuterium-labelled prostaglandins were a generous gift of Dr. U. Axen, The Upjohn Company, Kalamazoo, MI, U.S.A.; the other tritium-labelled prostaglandins were purchased from Amersham Buchler, Braunschweig, G.F.R.

The derivatization procedures, HPLC and SE-30 glass capillary gas chromatography—mass spectrometry (GC—MS) were all carried out as previously described [1, 2].

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Sample preparation for the simultaneous determination of prostaglandins in urine or serum

The sample preparation procedure is shown schematically in Fig. 1.

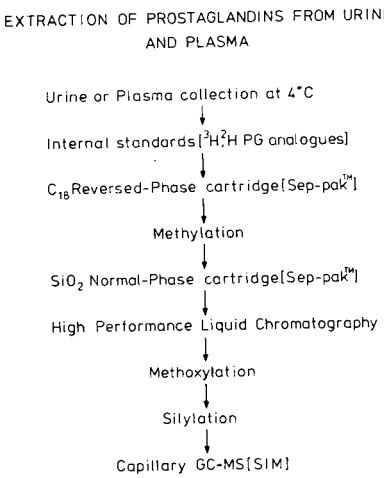

Fig. 1. Scheme of the sample preparation procedure.

Tritium- and deuterium-labelled prostaglandin analogues were added to samples of urine (10—40 ml) or plasma (5—50 ml) and the samples were then acidified to pH 3.2 with formic acid. A reversed-phase silica cartridge (Sep-Pak C_{18}) was prepared by rinsing it with 10 ml of methanol followed by 10 ml of water. The acidified sample was passed through the cartridge. The cartridge was washed with water (10 ml) to remove polar components, and the prostaglandins were then quantitatively eluted with chloroform (20 ml). Non-polar components remained on the cartridge. After methylation with diazomethane, the sample was passed through a normal-phase silica cartridge (Sep-Pak) which was then washed with chloroform (10 ml) to remove non-polar components; the prostaglandin methyl esters were eluted with chloroform—methanol (98 : 2, 20 ml). Polar components remained on the cartridge. This extract was subjected to preparative HPLC using a normal-phase silica (10 μm) column (μPorasil, Waters Assoc.). Individual prostaglandin fractions were treated to give the methoxime and silyl ether derivatives and the isotope ratios were determined with a GC—MS system equipped with a glass capillary column [1].

RESULTS

The HPLC separation of various tritiated prostaglandins added to human urine or plasma is shown in Fig. 2. Sharp separations of the prostaglandin methyl esters were obtained; the various prostaglandins occurred in fractions of usually 2 ml or, at most, 3 ml. The methyl esters of these prostaglandins span a smaller range of polarity than their parent acids, so that a HPLC gradient can be used with only a moderate change in the polarity of the eluents. These chromatographic characteristics, together with the clarity of the extract,

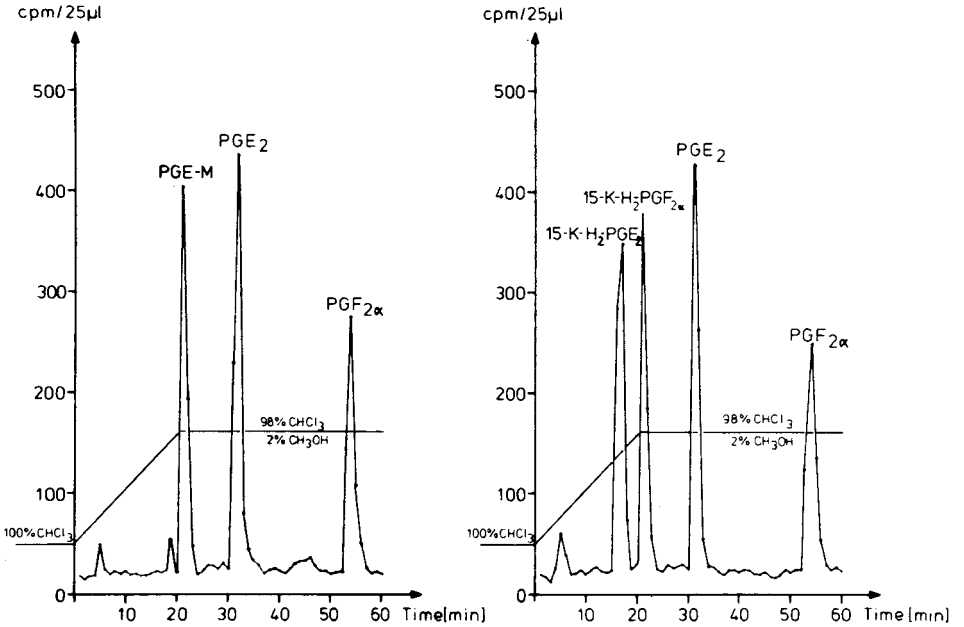

Fig. 2. HPLC separation of the tritiated methyl esters of PGE-M, PGE_2 and $PGF_{2\alpha}$ added to human urine (left), and 15-keto-13,14-dihydro-PGE_2 (15-K-H_2-PGE_2), 15-keto-13,14-dihydro-$PGF_{2\alpha}$ (15-K-H_2-$PGF_{2\alpha}$), PGE_2 and $PGF_{2\alpha}$ added to human plasma (right). A 10-µm µPorasil column (250 mm × 4.0 mm I.D.) was used. Two M6000A pumps (Waters Assoc.) were controlled by an M660 gradient controller (Waters Assoc.). The initial eluent was chloroform and the final eluent, after 20 min, was chloroform—methanol (98 : 2) (program No. 6, linear). The flow-rate was 1 ml/min. Fractions of 1 ml were collected; the radioactivity of 25-µl samples was determined by liquid scintillation spectrometry.

help make this HPLC separation a reliable and reproducible part of the analysis.

The recovery after HPLC of tritium-labelled prostaglandins (34,500 to 40,000 cpm) added to human urine was (mean ± S.D.): PGE-M, 65.7 ± 9.5% (n = 10); PGE_2, 63.8 ± 7.0% (n = 7); and $PGF_{2\alpha}$, 49.8 ± 4.9% (n = 8). The reproducibility of the method was demonstrated by adding 50 ng of each of PGE-M, PGE_2 and $PGF_{2\alpha}$ to four 40-ml samples of urine. The amounts recovered were (mean ± S.D.): PGE-M, 48.0 ± 4.8 ng; PGE_2, 53.8 ± 1.3 ng; and $PGF_{2\alpha}$, 49.0 ± 0.9 ng.

A sample preparation procedure designed to measure a number of prostaglandins in one sample will also extract other prostaglandins and other substances of similar polarity. Some of these interfering substances will also be present in individual fractions of the HPLC separation. The selected-ion chromatograms of derivatized PGE_2, $PGF_{2\alpha}$, PGE-M and 6-keto-$PGF_{1\alpha}$ from biological samples (Fig. 3) show that subsequent capillary GC—MS is needed. This separation gives accurate quantitation which is not always achieved with packed GC columns [1, 3].

DISCUSSION

The introduction of Sep-Pak C_{18} cartridges has greatly simplified the

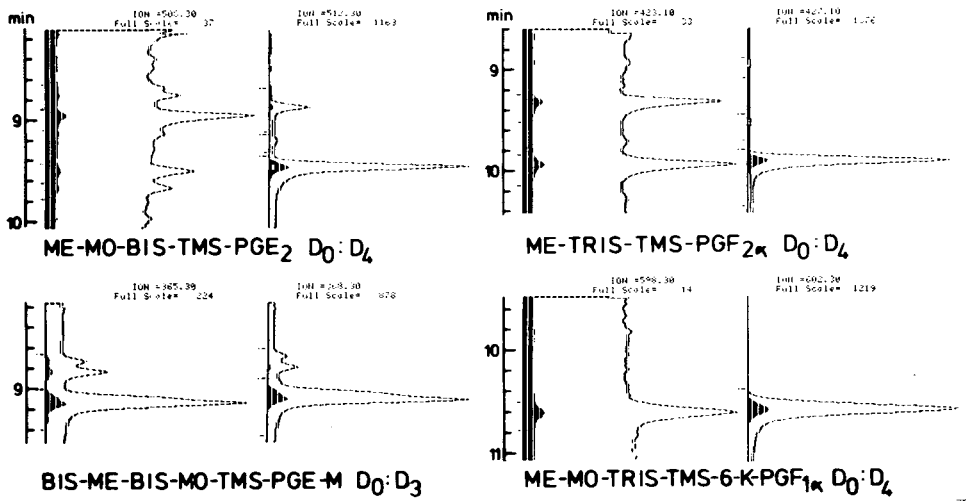

Fig. 3. Portions of typical selected-ion chromatograms of derivatized endogenous and deuterated PGE_2, $PGF_{2\alpha}$, PGE-M, and 6-keto-$PGF_{1\alpha}$ from biological samples. 6-Keto-$PGF_{1\alpha}$ was extracted from plasma, the other prostaglandins were extracted from urine. The gas chromatographic separation was obtained on an SE-30 glass capillary column 20 m long. Dwell times were 150 msec and 50 msec for the ions of endogenous and deuterated prostaglandins, respectively.

analytical extraction of drugs from plasma (see, for example, ref. 4), of natural and synthetic steroids from urine [5] and, indeed, of prostaglandins from urine [6]. All of these procedures use the C_{18} cartridge to obtain an extract from the aqueous biological medium in a polar organic solvent such as methanol or methyl formate.

In our procedure, the biological liquid is passed through the C_{18} cartridge and washed free of strongly polar components with water. Elution with chloroform, which is a very weak eluent of C_{18} silica, selectively extracts prostaglandins and, presumably, other substances of similar lipophilicity and polarity.

Our improved sample preparation procedure using Sep-Pak cartridges is simpler, more rapid and more reliable than our previous procedure which involved extraction and chromatography on open silica columns. We used the previous procedure to quantitate various prostaglandins in the urine of infants and children [2], in the urine of tumour-bearing rats [7] and in the perfusates of isolated rat kidneys [8], and to identify PGE_2 and 15-keto-13,14-dihydro-PGE_2 in human gastric juice [9].

The sample preparation procedure should also be suitable as a preliminary to radioimmunoassay or bioassay, although the methylation step has then to be excluded and the free acids separated on a suitable HPLC system (see ref. 10).

In conclusion, the extraction of biological samples for the analysis of prostaglandins is simplified and improved by the use of reversed-phase silica (C_{18}) and silica Sep-Pak cartridges. Considerable time is saved and the small volumes of eluates are free of contaminants which could overburden the subsequent HPLC.

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Note

Evaluation of the relative efficacy of various techniques for deproteinizing plasma samples prior to high-performance liquid chromatographic analysis

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The methods used for treating biological samples prior to their introduction into a high-performance liquid chromatographic (HPLC) system generally fall into one of two categories — extraction or direct injection. In the extraction method the compound of interest is removed from the biological matrix (plasma, serum, urine, etc.) using suitable solvent and pH conditions, which selectively extract the desired components and leave behind unwanted materials. The solvent is then removed by gentle evaporation and the dried residue reconstituted in a small volume of the elution solvent (or one quite similar to it) for injection on to the HPLC column.

The direct-injection technique is by far the simplest and most rapid of the two methods. In this procedure the biological sample may be injected directly on to the top of the HPLC column [1]. However, a number of reports have indicated that this results in a rapid increase in back-pressure and a deterioration of column performance [2—5], presumably due to the precipitation of plasma proteins as a result of their contact with the organic solvents and buffer salts commonly utilized in mobile phases [4]. To alleviate this problem, a number of sample preparation techniques have been described for removing proteins prior to injection of the sample. These include the use of precolumns [6], ultrafiltration devices [7, 8], and various protein precipitants such as organic solvents [9—11] and ionic salts [12—14].

Only one report dealing specifically with sample preparation procedures for the direct-injection HPLC technique has appeared to date [15]. In that study, six different methods of deproteinizing plasma were evaluated using the biuret assay to assess their efficiency. In the present report we describe a number of other potentially useful methods of protein removal, using the much more sensitive Lowry [16] method of protein determination to evaluate

their efficacy. This latter point is important since even small amounts of residual protein will build up rapidly at the head of a HPLC column under conditions of high sample throughput, thereby necessitating more frequent column regeneration or replacement.

EXPERIMENTAL

Precipitating agents

The following precipitating agents were used: acetone, B.P.C. (Evans Medical Co., Liverpool, Great Britain); acetonitrile, AnalaR (BDH, Poole, Great Britain); ethanol, AnalaR (James Burroughs, London, Great Britain); methanol, AnalaR (James Burroughs); ammonium sulfate [$(NH_4)_2SO_4$] (saturated solution), AnalaR (Hopkin and Williams, Chadwell Heath, Great Britain); trichloroacetic acid (TCA), 10% (w/v) AnalaR (BDH); perchloric acid ($HClO_4$), 6% (w/v), AnalaR (BDH); metaphosphoric acid (HPO_3), glacial, sticks, 5% (w/v) (Fisons, Loughborough, Great Britain); sodium tungstate dihydrate ($Na_2WO_4 \cdot 2H_2O$), 10% (w/v), plus 0.67 *N* sulfuric acid, both AnalaR (BDH); zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), 10% (w/v), plus 0.5 *N* sodium hydroxide, both AnalaR (BDH); zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$), 5% (w/v), plus 0.3 *N* barium hydroxide, both AnalaR (BDH); copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), 5% (w/v), plus sodium tungstate dihydrate, 6% (w/v), both analaR (BDH).

Plasma samples

All protein precipitation studies were performed using a single lot of pooled human plasma collected over lithium heparin from two healthy human volunteers. The total protein content of this pooled plasma sample was 89.3 g per 100 ml. All sample preparation techniques for a given precipitant were performed on the same day and the supernatants (or ultrafiltrates) assayed later that day.

Protein removal procedures

Precipitation methods. A series of 5-ml glass test-tubes, each containing 0.5 ml of plasma and the following quantities of precipitant — 0.1, 0.2, 0.3, 0.4, 0.5, 0.75, 1.0, 1.5 and 2.0 ml — were prepared in triplicate. In the case of those precipitants consisting of two ingredients, equal volumes of each component were added to provide the volumes of precipitant desired. The tubes were then rotated on a Vortex mixer for 30 sec, allowed to stand at room temperature for 15 min, and centrifuged at 1650 *g* for 15 min.

Each supernatant was collected, its pH measured (pH Meter Type PHM 51, Radiometer, Copenhagen, Denmark), and 0.1-ml aliquots were taken and assayed for protein content by the Lowry method [16] using suitable controls.

Ultrafiltration. To assess the efficacy of ultrafiltration in removing protein from plasma, triplicate 0.5-ml aliquots of pooled plasma were placed in Centriflo membrane cones (Type CF-25 and CF-50, Amicon Corp., Lexington, MA, U.S.A.) and centrifuged at 720 *g* for 30 min. This procedure yielded approximately 0.2 ml of ultrafiltrate from each cone. The individual ultrafiltrates were then assayed using the Lowry method [16].

RESULTS AND DISCUSSION

The efficacy of the various precipitants in removing the protein from plasma samples is shown in Table I. The data indicate that only very small quantities of 10% (w/v) trichloroacetic acid and 6% (w/v) perchloric acid are needed to remove > 98% of the protein present in plasma. At a 1 : 1 (v/v) ratio of precipitant to plasma only methanol and saturated ammonium sulfate solution failed to remove > 90% of the plasma protein. In spite of their relatively low efficacy in removing plasma proteins, the four organic solvents (methanol, ethanol, acetone, and acetonitrile) have been very popular as precipitants in the direct-injection HPLC technique because of their widespread use as mobile phase components. Their relative order of effectiveness in precipitating protein is acetonitrile > acetone > ethanol > methanol, which is approximately inversely related to their polarity.

Ammonium sulfate is a classical protein precipitant which functions as a result of its ability to compete sucessfully with protein molecules for the available water in the system. While the efficacy of this precipitant could probably be improved by controlling the pH so that the plasma proteins are at or near their isoelectric points, this procedure does not appear to be very popular because other, more efficacious precipitants are available.

The remainder of the precipitation methods examined here are more commonly used and depend upon the formation of insoluble salts. The best of these precipitants appear to be the four anionic types — trichloroacetic, perchloric, tungstic, and metaphosphoric acids. They are believed to function by forming insoluble salts with the positively charged amino groups of the protein molecules at a pH below their isoelectric point. The control of pH is especially important in the case of tungstic and metaphosphoric acids, as pointed out by Berkman et al. [17] and Briggs [18]. This is verified by the data in Table I.

The remaining three precipitants tested consisted of the heavy metal cations zinc and copper. It was once believed that these cations formed insoluble salts with protein molecules due to their interaction with the negatively charged carboxyl groups on the protein at pH values above the isoelectric point. However, the exact mechanism of this insoluble-salt formation is still unclear. These agents were proposed originally by Somogyi [19—21] and have not been widely used in conjunction with HPLC techniques due to the greater efficacy and ease of use of the anionic precipitants and the organic solvents mentioned earlier.

The CF-25 and CF-50 ultrafiltration cones were found to remove 99.8 ± 0.06% and 99.5 ± 0.31% (mean ± S.D.), respectively, of the plasma protein. These results indicate that either type of membrane (molecular weight cut-off 25,000 or 50,000) provides nearly complete removal of plasma proteins.

The method is relatively simple and offers a number of advantages over the protein-precipitation procedures that were outlined by Farese and Mager [22]. However, it should be realized that analysis of an ultrafiltrate will provide a measure of non-protein-bound drug, as opposed to total drug. In addition, separate experiments must be carried out to determine, and correct for, any binding of the drug (and the internal standard if added before ultra-

TABLE I

THE RELATIVE EFFICACY OF VARIOUS PROTEIN PRECIPITANTS

The results are expressed as the percentage plasma proteins precipitated and represent the mean of triplicate determinations.

Precipitant	pH of supernatant*	Volume of precipitant added per volume of plasma								
		0.2	0.4	0.6	0.8	1.0	1.5	2.0	3.0	4.0
10% (w/v) TCA	1.4—2.0 (3.1)	99.7	99.3	99.6	99.5	99.5	99.7	99.8	99.8	99.8
6% (w/v) $HClO_4$	<1.5 (2.0)	35.4	98.3	98.9	99.1	99.1	99.2	99.1	99.1	99.0
Tungstate—H_2SO_4	2.2—3.9 (7.0, 6.5)	3.3	35.4	98.6	99.7	99.7	99.9	99.8	99.9	100.0
5% (w/v) HPO_3	1.6—2.7 (4.8)	39.8	95.7	98.1	98.3	98.3	98.5	98.4	98.2	98.1
$CuSO_4$—Na_2WO_4	5.7—7.3 (8.0)	36.5	56.1	78.1	87.1	97.5	99.8	99.9	100.0	100.0
$ZnSO_4$—NaOH	6.5—7.5 (8.0)	41.1	91.5	93.0	92.7	94.2	97.1	99.3	98.8	99.6
$ZnSO_4$—$Ba(OH)_2$	6.6—8.3 (8.7)	45.6	80.7	93.5	89.2	93.3	97.0	99.3	99.6	99.8
Acetonitrile	8.5—9.5	13.4	14.8	45.8	88.1	97.2	99.4	99.7	99.8	99.8
Acetone	9 —10	1.5	7.4	33.6	71.0	96.2	99.1	99.4	99.2	99.1
Ethanol	9 —10	10.1	11.2	41.7	74.8	91.4	96.3	98.3	99.1	99.3
Methanol	8.5—9.5	17.6	17.4	32.2	49.3	73.4	97.9	98.7	98.9	99.2
Saturated $(NH_4)_2SO_4$	7.0—7.7	21.3	24.0	41.0	47.4	53.4	73.2	98.3	—**	—**

*Values in parentheses represent the pH of supernatants where the volume of precipitant per volume of plasma was 0.2, and 0.4 in the case of Tungstate —H_2SO_4.

**Cloudiness present in these samples precluded their accurate assay.

filtration) to the membrane. Finally, it may be necessary to consider the effect of the volume of ultrafiltrate collected on the concentration of drug in the ultrafiltrate to ensure that the binding equilibrium is not disturbed by the filtration process [23, 24], although recent reports indicate that binding equilibria are not perturbed by ultrafiltration [25, 26].

The decision as to which method to choose for a given analytical application must ultimately be determined by an assessment of such factors as the stability, recovery, membrane binding, etc., of the compound of interest and the precision of the assay. In fact, combinations of some of these methods, such as the use of mixed organic solvents [27], or organic solvents plus inorganic salts to help salt out the proteins [28, 29], may prove most suitable. Other seemingly unimportant factors such as the relative centrifugal force used to pack the precipitated proteins [30], and the temperature of the precipitants [31, 32], may prove to be the critical factors in determining the acceptability of a given preparative technique.

It is hoped that the data presented here will assist the analyst in optimizing the sensitivity of an assay and will help to resolve much of the dialog (usually undocumented) that has appeared in the literature recently [5, 11, 33–36] regarding the need for specific ratios of precipitant to plasma to achieve "complete" removal of protein.

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Note

Assay of catechol O-methyltransferase activity by high-performance liquid chromatography with electrochemical detection

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The enzymatic methylation of catecholamines and their metabolites by catechol O-methyltransferase (COMT) is one of the important degradation routes of these compounds in the human body [1]. Recently, it has been stated that COMT activity might be closely related to affective disorders such as some cases of depression, and that the *meta/para* ratio of O-methylated products might be of clinical significance [2].

Of the methods for the assay of COMT activity, fluorimetric, radiochemical or colorimetric techniques only measure the total amount of products formed. On the other hand, high-performance liquid chromatographic (HPLC) [2—5] and gas chromatographic [6] techniques have been developed as a reliable procedure for the assay of *m*-methoxy and *p*-methoxy products. HPLC with electrochemical detection has proven to be sensitive and specific for the determination of phenolic compounds, including those arising from O-methylation by COMT.

The present paper describes a procedure for the assay of COMT activity, which involves the use of norepinephrine as a substrate, periodate oxidation, deproteinization by solvent extraction, and the separation of O-methylated isomers by HPLC with electrochemical detection.

EXPERIMENTAL

Chemicals

L-Norepinephrine bitartrate and DL-normetanephrine hydrogen chloride were obtained from Wako Junyaku (Osaka, Japan). Vanillin, isovanillin, *p*-hydroxyacetanilide and S-adenosyl-L-methionine hydrogen sulfate (SAM) were purchased from Tokyo Kasei (Tokyo, Japan).

Substrate (norepinephrine) solution (340 μg/ml) and SAM solution (1.6 mg/ml) were each prepared in 50 m*M* phosphate buffer (pH 7.5). Magnesium chloride ($MgCl_2$) solution was 9.4 mg/ml in water; and the internal standard solution was *p*-hydroxyacetanilide solution (5 μg/ml) in ethyl acetate. All other chemicals were of analytical grade.

Preparation of COMT

Adult Wistar rats (150—200 g) of either sex were decapitated and their livers were homogenated in 4 volumes of 50 m*M* phosphate buffer (pH 7.5). The homogenate was centrifuged at 100,000 *g* for 30 min at 4°C. The supernatant solution was used as a crude source of COMT. Protein determinations were carried out by the method of Lowry et al. [7] with bovine serum albumin as standard.

High-performance liquid chromatography

Apparatus for the HPLC in this work was constructed from a Kyowa Seimitsu mini micro pump Type KSU, a Kyowa Seimitsu damper Type KD-300, a Yanaco voltammetric detector Type VMD 101, and a column system. The HPLC column (15 cm × 4 mm I.D., stainless steel), was prepared with LiChrosorb 5 RP-18 (E. Merck, Darmstadt, G.F.R.) using a balanced density method. The mobile phase consisted of methanol—50 m*M* phosphate buffer (pH 7.2) (3:7, v/v) and the flow-rate was adjusted to 0.3 ml/min. All separations were performed at 40°C and the cell potential was maintained at +0.9 V vs. a Ag/AgCl reference electrode.

Standard curves

A standard curve for normetanephrine was prepared by the following procedure. To 0.8 ml of 50 m*M* phosphate buffer (pH 7.5) containing normetanephrine in the range 10—50 μg were added successively 0.2 ml of COMT preparation and 0.1 ml of 3.6 *N* perchloric acid. The mixture was treated according to the procedure described in assay of COMT activity. A standard curve for norparanephrine was prepared on the basis of that obtained above for normetanephrine, and calibration graphs were constructed for authentic vanillin and isovanillin using *p*-hydroxyacetanilide as internal standard.

Assay of COMT activity

A 0.5-ml volume of substrate solution, 0.25 ml of SAM solution and 0.05 ml of $MgCl_2$ solution were added successively to a 10-ml centrifuge tube. The tube was preincubated for 5 min at 37°C. The enzyme reaction was started by adding 0.2 ml of COMT preparation (10 mg of protein per ml). The standard incubation mixture (1 ml) consisted of 50 m*M* phosphate buffer (pH 7.5), 0.5

m*M* norepinephrine, 1 m*M* SAM, 5 m*M* $MgCl_2$ and 2 mg of COMT. The mixture was incubated for 1 h except for the study of the time course. Incubation was stopped by adding 0.1 ml of 3.6 *N* perchloric acid. After centrifugation, all the supernatant fluid was decanted into another 10-ml tube containing 3 ml of 1 *M* phosphate buffer (pH 9.5). A 0.3-ml volume of 0.01 *N* sodium metaperiodate ($NaIO_4$) was added in order to oxidize O-methylated products and the mixture was allowed to stand for 10 min at room temperature. Excess $NaIO_4$ was destroyed by adding 1 ml of 0.1 *N* sodium metabisulfate ($Na_2S_2O_5$). The mixture was saturated with sodium chloride and the resulting O-methylated products (vanillin and isovanillin) were extracted with 3 ml of ethyl acetate containing *p*-hydroxyacetanilide as internal standard. The extract was evaporated to dryness under reduced pressure and the residue was redissolved in 1 ml of 1% acetic acid. A 10-μl aliquot of the solution was determined with the HPLC system as described above. Each sample was injected in duplicate. Peak height ratios of products to internal standard were measured and the amount of methylated products (normetanephrine and norparanephrine) in an unknown sample was calculated from the standard curves.

RESULTS AND DISCUSSION

Norepinephrine, one of the important catecholamines in biological fluids, was chosen as a substrate for an assay of COMT activity, because the structure of the substrate used causes marked differences in COMT activity and in its evaluation. In vitro normetanephrine and norparanephrine have been known to be enzymatically formed from norepinephrine by the COMT reaction [3]. Both O-methylated isomers can be separated by HPLC, but in this case we encountered two problems. One was the problem of deproteinization. Incomplete deproteinization often caused rapid degeneration in the resolution of the column, so a sample clean-up procedure was considered using DEAE-Sephadex A-25 chromatography [4] or Bio-Rex 70 ion-exchange resin [2]. The other problem is that the commercial availability of norparanephrine, one of the metabolites, is limited. On the other hand, it is known that normetanephrine is easily converted to vanillin by periodate oxidation [8]. This reaction attracted our attention because vanillin and isovanillin are commercially available, so normetanephrine and norparanephrine can be measured by conversion to vanillin and isovanillin, respectively. Complete deproteinization can also be expected by extracting vanillin and isovanillin with a suitable organic solvent from the reaction mixture. The technique has been successfully applied to an assay of COMT activity. The optimum conditions for the periodate oxidation of normetanephrine as described under Experimental were fixed from preliminary experiments consulting the method of Pisano [8]. Under these conditions it was expected that the reaction of norparanephrine with periodate proceeds in a similar manner to normetanephrine. Vanillin, isovanillin and *p*-hydroxyacetanilide (internal standard) were quantitatively extracted with 3 ml of ethyl acetate below pH 7.5 when saturated with sodium chloride. Vanillin and isovanillin were not very stable above pH 7.0, so they were treated in 1% acetic acid solution.

The separation of vanillin, isovanillin and *p*-hydroxyacetanilide on a LiChro-

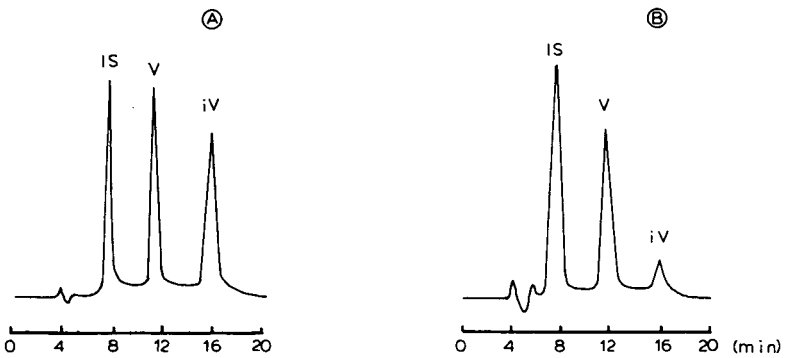

Fig. 1. Liquid chromatographic separation of vanillin (V) and isovanillin (iV) with *p*-hydroxyacetanilide (IS) as internal standard. Chromatographic conditions: column, 15 cm × 4 mm I.D. filled with LiChrosorb 5 RP-18; column temperature, 40°C; mobile phase, 30% methanol in 0.05 *M* phosphate buffer (pH 7.2); pressure, 1 MPa; flow-rate, 0.3 ml/min; detector, voltammetric +0.9 V; injection volume, 10 µl. (A) Separation of standard mixture; (B) separation of O-methylated isomers formed by COMT reaction after periodate oxidation.

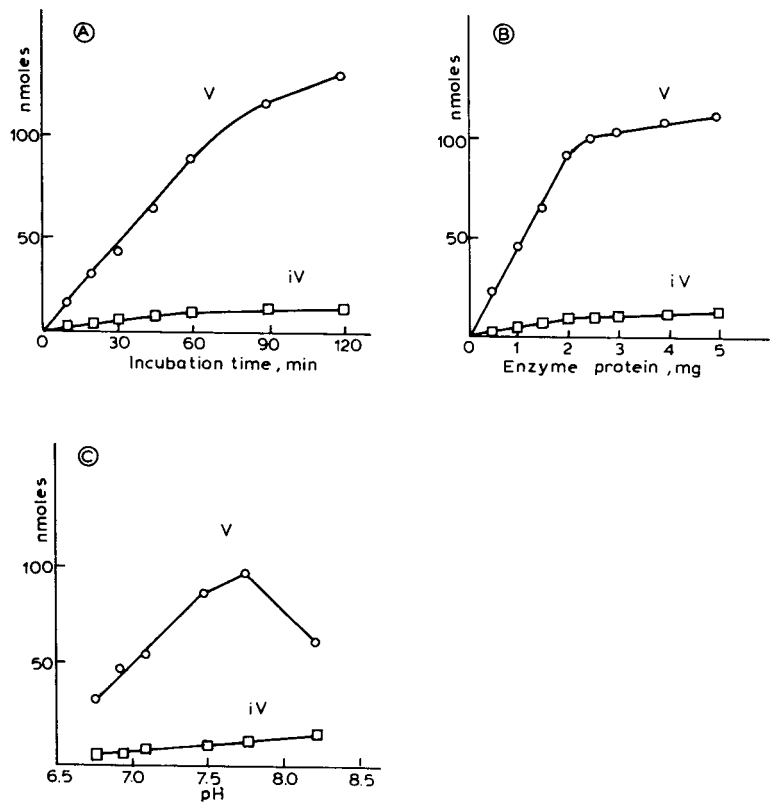

Fig. 2. The kinetic parameters of COMT from rat liver using norepinephrine as substrate. ○, Vanillin (V); □, isovanillin (iV).

sorb 5 RP-18 column is shown in Fig. 1A. To demonstrate the ability of this method, we measured the COMT activity present in the soluble fractions of rat liver (Fig. 1B). There were neither interfering compounds nor the peak derived from the substrate. In the blank tests performed by omitting SAM or norepinephrine, no O-methylated products were formed.

Some kinetic parameters of rat liver COMT were also measured by this method. The results are shown in Fig. 2. Fig. 2A shows the time course for formation of O-methylated products in enzyme incubation mixtures and Fig. 2B shows the influence of the enzyme concentration on the COMT activity. The enzyme activity was linear up to 60 min incubation time and up to about 2.0 mg of enzyme protein.

The *meta*- and *para*-methylated products were simultaneously measured in the same incubation mixture and the effect of pH on the enzyme activity and the *meta*/*para* ratios was also studied. As is shown in Fig. 2C, the COMT activity changed depending on the pH of the incubation mixture. As the pH was increased from 7.50 to 8.23, the *meta*/*para* ratio rapidly decreased from 10.31 to 5.02. These results are similar to those reported by Creveling et al. [9].

The COMT activities were measured from a series of five separately prepared samples using the same rat liver preparation. The relative standard deviations for the *meta*- and *para*-O-methylated isomers were 3.97% and 4.29%, respectively. The correlation coefficient for the standard curve obtained using normetanephrine according to the procedure described under standard curves was 0.997. Quantitative assay was possible on the chromatogram even for the injection of 1 ng (6.6 pmoles) of vanillin or isovanillin. The specific activity of the rat liver COMT was 43.3 nmoles of vanillin and 4.2 nmoles of isovanillin per mg of protein per h at pH 7.5.

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Note

Rapid separation of platelet nucleotides by reversed-phase, isocratic, high-performance liquid chromatography with a radially compressed column

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In the past, platelet adenine nucleotides have been assayed in several different ways [1—5], including a firefly luciferase [6], an enzymatic method [7], a fluorimetric procedure [8], column chromatography and high-performance liquid chromatography (HPLC) [9]. A simple, rapid and efficient method is still unavailable for routine analysis of clinical and research samples. Several years ago we described a method using ion-exchange HPLC for separating and quantitating adenine nucleotides of human blood platelets [10]. Other laboratories subsequently improved the technique for separation by employing linear gradients [11, 12]. However, no information was provided regarding reversed-phase separation of platelet nucleotides and the linear gradient methods described took considerable lengths of time for complete separation of all the nucleotides. In the present paper we describe a method for complete separation of platelet nucleotides which is carried out in an isocratic mode with minimum of elution time and maximum efficiency.

MATERIALS AND METHODS

Blood for these studies was obtained from normal voluntary donors. The procedures used to obtain blood, mix the samples with trisodium citrate—citric acid—dextrose (CCD) buffer (citrate 0.1 *M*, citric acid 7 m*M*, dextrose 0.14 *M*, pH 6.5), in a ratio of 9 parts blood to 1 part anticoagulant, and isolate platelet-rich plasma (PRP) by centrifugation at room temperature, have been described in several recent publications [13—15]. Nucleotides were determined using platelets obtained from 1-ml samples of fresh PRP. Cell count was determined by phase optics or a coulter system. Each 1-ml PRP sample was mixed with 0.5 ml of CCD and centrifuged for 1:5 min in a Beckman microfuge to obtain

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platelet pellets. Supernatant plasma was discarded and the platelet pellet precipitated with 100 μl of cold, 2 *N* perchloric acid. After sonication of the precipitate in perchloric acid at low temperature, the samples were again sedimented in a microfuge for 1.5 min. The clear supernatant thus obtained containing platelet nucleotides was separated and neutralized with 5 *N* potassium hydroxide to a pH of 5.5—8.0. The neutralized samples were subjected to a freeze—thaw cycle to achieve complete precipitation of the salt. To sediment the salt generated during neutralization all the samples were centrifuged one more time in a microfuge for 1.5 min. The clear neutral extracts were separated and subjected to HPLC for the separation of nucleotides.

Separation of nucleotides

A Waters Assoc. Model 204 high-performance liquid chromatograph was used for the separation of nucleotides. The chromatographic system consisted of a Model 440 fixed-wavelength (245 nm) UV detector, a 6000A solvent-delivery system and a UK6 universal injector. A 30-cm stainless-steel column packed with μBondapak C_{18} (10-μm particles), or a Radial-Pak C_{18} (10-μm particles) with radial compression module (RCM-100), was used for the separation of nucleotides. The solvent system consisted of HPLC grade acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) 20% (v/v), deionized distilled water of pH 7.5, 80% (v/v), and a vial of tetrabutylammonium phosphate, (Pic A) (Waters Assoc., Milford MA, U.S.A.). Final concentration of the Pic A was 0.005 *M* per liter of stock solvent used in the separation of nucleotides. Solvent flow-rate was 1 ml/min when the μBondapak C_{18} column was used and 6 ml/min when the Radial-Pak column was used. Sample size was 10 μl per injection. A Hewlett-Packard 3385A automation system was used to obtain electronic integration of the peaks. Data thus generated were further processed through a programmable Cannon calculator (Canola 1614P) to obtain values in μmol for 10^{11} platelets. Results presented are mean values for platelets of six different normal donors.

RESULTS AND DISCUSSION

Human platelets contain substantial quantities of adenine nucleotides and small quantities of guanine nucleotides. A relative absence of nucleotide precursors in platelets has also been demonstrated. The uptake of mono-, di- or triphosphate nucleotides is negligible. Metabolites such as inosine monophosphate and hypoxanthine are released to the supernatant and, as such, do not form a major component of the platelet extracts.

In the present study a mixture of various nucleotides was analyzed using the conventional μBondapak C_{18} column. Solvent elution was isocratic and the flow-rate was 1 ml/min. Complete separation of all the nucleotides at ambient temperature was achieved in approximately 24 min (Fig. 1). Retention times for hypoxanthine and adenine were close to one another, but were well separated. To achieve a better separation of nucleotides, samples were analyzed using radially compressed columns. The separation obtained at ambient temperature of various nucleotide standards on a Radial-Pak column is presented in Fig. 2. Elution time for the separation of the eight standards from the mixture

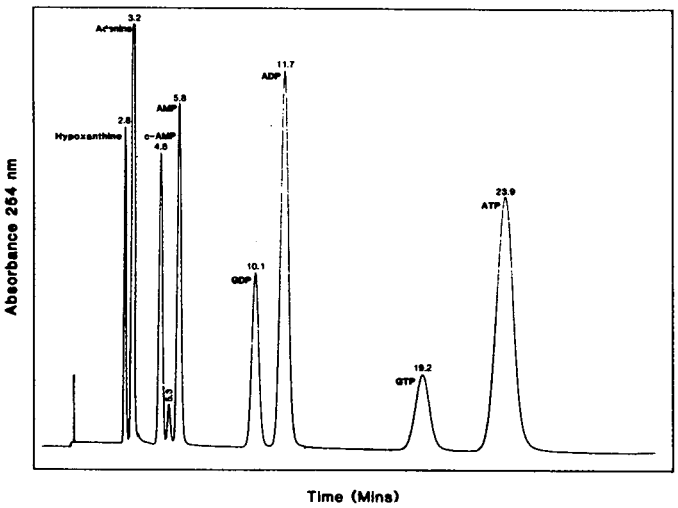

Fig. 1. Separation of various nucleotide standards from a mixture by isocratic elution using a μBondapak C_{18} column. Complete separation of all the nucleotides (including c-AMP from AMP) at ambient temperatures was achieved in approximately 24 min. Chromatographic conditions: column, 30 cm × 4 mm; packing, μBondapak C_{18}; solvent, acetonitrile—water—Pic A; detector, UV 254 nm; sample, 10 μl.

TABLE I

RELATIVE VALUES FOR PLATELET NUCLEOTIDES OBTAINED BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

The values obtained by other investigators using linear gradient elution are presented for comparison. Values are given in μmol per 10^{11} cells.

Method	ATP	ADP	AMP	GTP	GDP	ATP : ADP ratio
Scholar et al. 1973 [9]	5.7	3.5	—	0.9	0.9	1.6
Rao et al. 1974 [10]	7.4	4.0	3.0	—	—	1.8
Parks et al. 1975 [11]	5.0	3.5	0.8	—	—	1.4
D'Souza and Glueck 1977 [12]	3.8	3.5	0.3	0.45	0.4	1.1
Rao et al.* 1981 - present paper	4.3 ± 0.2	3.0 ± 0.2	0.6 ± 0.08	0.7 ± 0.05	0.4 ± 0.05	1.4

*Mean ± standard error (n = 6).

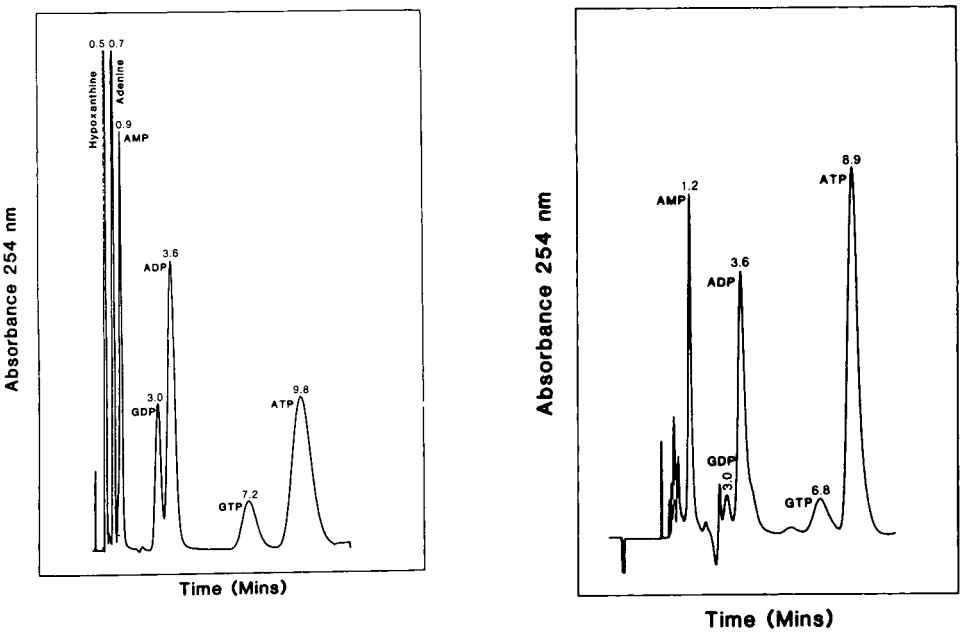

Fig. 2. Separation of various nucleotide standards from a mixture by isocratic elution with a radially compressed column. Elution time for separating all the nucleotides was less than 10 min. The range of recovery for all the nucleotides was excellent. Chromatographic conditions as in Fig. 1 except that column used was Radial-Pak.

Fig. 3. Separation of platelet nucleotides by isocratic elution using a radially compressed column. Identification of individual peaks was done by comparison of retention times of standards added to the platelet extract and assayed under identical conditions. Also, standard additions of known nucleotides to platelet extracts and their analysis served as a supplementary confirmation for peak identification. Retention times for AMP, ADP and ATP were 1.2, 3.6 and 8.9 min. The values obtained using this method for AMP, ADP, ATP and ATP: ADP ratios, agree with results obtained by others using linear gradient elution to achieve complete separation of nucleotides. Chromatographic conditions as in Fig. 2.

was less than 10 min. To test the efficacy of this analytical method, recoveries of standards added to the platelet rich plasma were measured. The range of recovery was 94—100% for all the nucleotides examined.

Nucleotide profile obtained by using a platelet extract on the Radial-Pak column is presented in Fig. 3. Complete separation was achieved in less than 9 min. In addition to adenine nucleotides, guanine nucleotides (GDP, GTP) were also separated by this method. Mean values obtained for each nucleotide are presented in Table I. Values obtained by other investigators using HPLC are presented for comparison [9—12]. Retention times for AMP, ADP and ATP were 1.2, 3.6 and 8.9 min. According to published reports elution time for complete separation of nucleotides by linear gradient techniques is over 70 min [9, 11, 12]. Values obtained for ATP in the present paper are lower than those reported by Parks et al. [11], but closer to the results of D'Souza and Glueck [12]. ATP:ADP ratios obtained by this method matched with the

values obtained by Parks et al. [11]. The value of 0.6 μM for AMP is in between the values published by Parks et al. [11] and by D'Souza and Glueck [12].

Results of these studies demonstrate that nucleotides can be separated rapidly and quantitated accurately by reversed-phase chromatography using isocratic elution. The time taken for each analysis could be further reduced by using radially compressed columns. The method is fast, efficient and provides excellent separation of platelet nucleotides. The values obtained using this method agree with results published by others using linear gradient elution to achieve complete separation of nucleotides [9, 11, 12].

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Note

Liquid chromatographic determination and time—concentration studies of riboflavin in hemodialysate from uremic patients

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During recent years, the identification and assay of chemical compounds in physiological fluids has become an important diagnostic indicator for the characterization of metabolic disorders which are often associated with disease. High-performance liquid chromatography (HPLC) has been used extensively for this purpose. Recently, reversed-phase HPLC has been applied successfully to numerous biological and clinical assays, including the analysis of uremic hemodialysate fluid samples from the artificial kidney [1, 2], the determination of various classes of biochemically active compounds in urine [3—5] and blood plasma or serum (6—9], and the separation of derivatized amino acids [10].

Determinations of riboflavin in multivitamin preparations using ion-exchange [11], normal-phase [12] and reversed-phase columns [13, 14] have been described and reviewed [15]. Recently, an HPLC method for the determination of riboflavin in urine using fluorescence detection has been described [16, 17]. These methods are selective for riboflavin, but they have not been applied to uremic hemodialysate, nor have they involved the use of on-line pre-column enrichment. Short pre-columns have been employed in liquid chromatography as effective pre-column enrichment devices and to prevent strongly retained sample components from reaching the analytical column [18]. Cantwell [19] and Mohammed and Cantwell [20] have also used a

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pre-column containing Amberlite XAD-2 to facilitate on-line clean-up prior to the determination of preservatives and drugs in pharmaceutical syrups.

Vitamin supplements are routinely prescribed to kidney patients who undergo regular dialysis treatment. Loss of water-soluble vitamins via dialysis is well accepted, while the concentration of the excreted vitamins in dialysate is extremely small. This paper describes a rapid, simple and sensitive method for the determination of nanogram amounts of riboflavin in hemodialysate using a pre-column containing Corasil-C_{18}, an analytical reversed-phase column, and a solvent switching valve. Time—concentration profiles for riboflavin loss during hemodialysis for two patients are also included.

EXPERIMENTAL

Samples and sample preparation

Samples of uremic hemodialysate were collected from a male and a female patient suffering from chronic renal failure. These samples (approximately 500 ml each) were collected twice hourly throughout the 6-h dialysis treatment from the dialysate drain of a parallel-plate artificial kidney. Five hemodialysate samples were also obtained from five other patients. These samples were frozen and stored at −25°C in polyethylene containers until assayed.

Before chromatographic separation, the dialysate samples were thawed at room temperature and filtered through 0.2-μm Nalgene Filters (Sybron Corp., Rochester, NY, U.S.A.) to remove particulate matter.

Riboflavin was obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. HPLC-grade methanol was obtained from Burdick and Jackson Labs., Muskegon, MI, U.S.A. HPLC-grade water was produced by a Milli-Q Reagent Grade Water System (Millipore, Bedford, MA, U.S.A.). Standard solutions were prepared by dissolving weighed samples in deionized distilled water.

Apparatus

The schematic diagram of the liquid chromatograph used for the determination of riboflavin is illustrated in Fig. 1. Pump P_1 was a 5000 p.s.i. Mini-Pump supplied by Laboratory Data Control, Riviera Beach, FL, U.S.A. With the Teflon rotary valve V_1 (Type 50, Rheodyne, Cotati, CA, U.S.A.) in the position shown, pump P_1 pumped solvent 1 through valves V_1 and V_2 (Valco Instruments, Houston, TX, U.S.A.), as well as the pre-column C_1, and then to waste. Simultaneously, pump P_2, an SP8700 solvent delivery system (Spectra-Physics, Santa Clara, CA, U.S.A.) pumped solvent 2 through valve V_2, the analytical column C_2, and the fluorimetric detector (Fluoromonitor™ Filter Fluorometer, American Instrument Co., Silver Spring, MD, U.S.A.). A Corning 7-51 glass primary filter (370 nm) was used. The secondary filter was a Wratten No. 8 ($\geqslant$ 460 nm). The analytical column C_2 was constructed of 25 × 0.40 cm I.D. stainless steel, packed with 7-μm (average diameter) Zorbax BP-TMS spherical particles (DuPont, Wilmington, DE, U.S.A.). Pre-column C_1 consisted of 11.5 cm × 0.15 cm I.D. stainless-steel tubing dry-packed with 37—50 μm Corasil-C_{18} (Waters Assoc., Milford, MA, U.S.A.). An SP4100 (Spectra-Physics) computing integrator/printer-plotter was used to record all chromatograms and to calculate the analytical results.

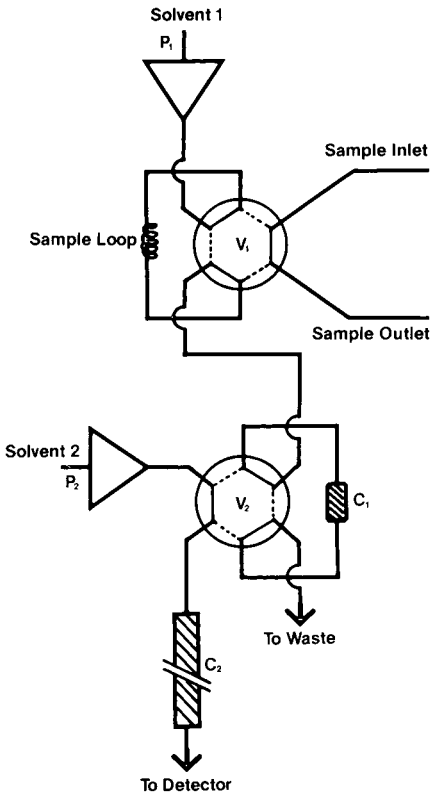

Fig. 1. Scheme of the liquid chromatograph.

Procedure

For the determination of riboflavin, solvent 1 consisted of an aqueous acetate buffer at pH 4.50 (10 mmol/l acetic acid taken to pH 4.50 with sodium hydroxide). Solvent 2 consisted of a mixture containing 65% solvent 1 and 35% methanol. With solvent 1 flowing at a rate of 2.0 ml/min, and solvent 2 passing through the analytical column at a flow-rate of 1.50 ml/min, a 1.0-ml sample of hemodialysate was injected. After 3.0 min, V_2 was switched to allow solvent 2 to pass through pre-column C_1, thus eluting riboflavin through both C_1 and C_2. Certain compounds which are strongly retained on C_1 and C_2 and which normally would elute well beyond riboflavin do not interfere with the analysis and are subsequently removed from both C_1 and C_2 by elution with 100% methanol. After 13 min, V_2 is switched back to its original position and the next injection can be made after a 5.0-min equilibration period.

RESULTS AND DISCUSSION

Fig. 2 shows a typical chromatogram for riboflavin in uremic hemodialysate. Quantitation was based on a comparison with the standard curves obtained by injecting aqueous solutions of riboflavin. Both peak height and peak area measurements yielded linear calibration curves with correlation coefficients

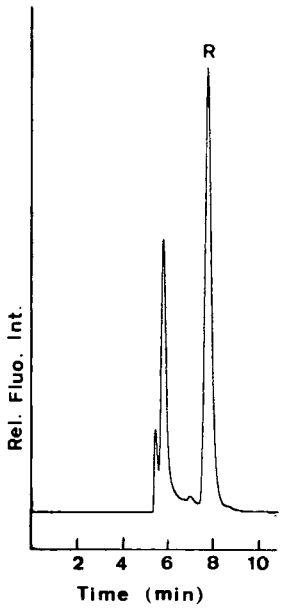

Fig. 2. Liquid chromatogram for 7.9 ng/ml riboflavin (R) in uremic hemodialysate.

of 0.999 for both peak height and peak area. Quantitative recovery was demonstrated with spiked hemodialysate, prepared by adding a known amount of riboflavin to a portion of blank hemodialysate. Recovery values reported in Table I are the averages of both height and area values.

In order to evaluate the loss of riboflavin during hemodialysis, samples of uremic hemodialysate fluid were collected at regular time intervals from two patients throughout the entire dialysis session. Concentrations of riboflavin in hemodialysate were determined by the HPLC method described in this paper and were plotted as a function of dialysis time as shown in Fig. 3. Our data for riboflavin correspond to our earlier time—concentration dialysis profiles [2], though our explanations now reflect dietary intake during the study, changes in transmembrane pressure and blood pressure during the study as well as our own level of understanding.

TABLE I

RECOVERY DATA FOR RIBOFLAVIN ADDED TO BLANK HEMODIALYSATE

Amount added (ng/ml)	Amount found (average) (ng/ml)	Recovery (%)
20.30	20.14 ± 0.01	99.2
16.20	16.12 ± 0.01	99.5
12.20	12.30 ± 0.02	100.8
8.10	8.02 ± 0.01	99.0
4.10	4.07 ± 0.02	99.3
2.00	2.03 ± 0.03	101.5

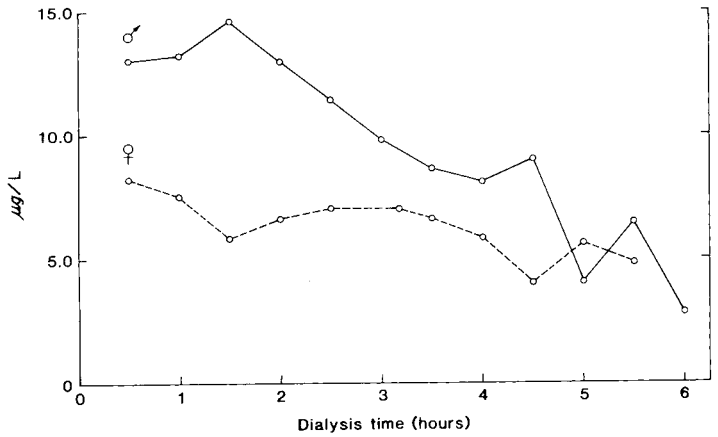

Fig. 3. Time—concentration dialysis profiles for riboflavin in hemodialysate of a male and a female patient.

With the ingestion of food (male: hours 1 and 4), the amount of riboflavin removed in the dialysate is increased and the time—concentration curve shows an upswing (hours 1.5 and 4.5) if blood pressure remains stable (Fig. 3, male), and a marked temporary downswing (hour 1.5) if blood pressure falls (Fig. 3, female). The oscillations at the end of the treatment and collection period are likely to reflect a combination of patient factors including the post-prandial state, the changing transmembrane pressure associated with membrane clotting, and the rate of change of body weight (due to the drop in total body water during treatment).

Riboflavin, the heat-resistant factor of vitamin B, is essential for normal growth and tissue maintenance, assisting in the metabolism of carbohydrates, fatty acids, and amino acids. With the recommended daily amount (RDA) for riboflavin being 0.8—2.6 mg for adults, and dialysis removing a large portion of this, vitamin supplements which include riboflavin would be justified. Riboflavin is primarily provided by dairy products and eggs, major sources of high biological proteins in the diets fed to the time—study subjects and patients with renal disease participating in nutritional therapy.

CONCLUSION

The method described in this paper permits nanogram quantities of riboflavin to be determined in uremic hemodialysate by direct injection using pre-column enrichment without sample pretreatment. Uremic hemodialysate was found to contain 2—20 ng/ml riboflavin. The use of a pre-column and a solvent switching valve eliminates the need for preliminary concentration and sample clean-up steps prior to injection. This method has been shown to be more sensitive by three orders of magnitude than the method previously applied to urine [16].

The time—concentration study presented as part of this investigation confirms our earlier data and emphasizes the need to pursue techniques for rapid determinations of nanogram quantities of vitamins in nutritional clinical

studies. Further delineation of the physiological role of vitamins in uremia is needed.

ACKNOWLEDGEMENT

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Note

Separation and identification of dansylated human serum and urinary amino acids by two-dimensional thin-layer chromatography

Application to aminoacidopathies

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Various authors have utilized the sensitivity of fluorescent labelling in the qualitative and quantitative analysis of the dansylated derivatives of amino acids and peptides [1, 2]. The isolation of these derivatives polyamide thin-layer chromatography was first described in 1967 by Woods and Wang [3], and has been adapted for micro determination [4]. Later, the optimal conditions for dansylation and its application to the separation of known amino acid mixtures or biological samples were reported. This technique, however, has never been used for analysis of human serum and urinary amino acids or for screening for aminoacidopathy.

The method described differs from classical chromatographic techniques on cellulose or silica gel, both mono- and two-dimensional, by its speed (migration length 3.5 cm), its excellent resolution, and its sensitivity (average amount deposited 50 picomoles). In addition, it can be applied to non-deionised urine samples and to non-deproteinised ultrafiltrated serum.

MATERIALS AND METHODS

Reagents

Dansyl chloride and free amino acids were obtained from Sigma (St. Louis,

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MO, U.S.A.); the other chemicals were from Merck (Darmstadt, G.F.R.). The micropolyamide plates (F 1700, 15 × 15 cm) were from Schleicher and Schüll (Dassel, G.F.R.). Micro tanks (4.5 × 3 × 9 cm) were used for migration.

The amino acid reference solutions (10^{-1} *M* in 0.1 *N* HCl) were stored at −20°C and diluted for dansylation to 10^{-3} *M* with 0.05 *M* $NaHCO_3$ solution. The mobile phases used are (I) formic acid—water (1.5:98.5), (II) benzene—acetic acid (4.5:1).

The 0.05 *M* $NaHCO_3$ solutions and the solvents can be stored up to one week, at 4°C, in airtight containers.

Sample preparation

Daily urines are collected with a preservative (chloroform—water, 4:1000, v/v). A choice had to be made between chloroform and other preservatives such as organo-mercuric derivatives, e.g. 1% merseptyl®. This latter product gives a dansylated derivative which does not interfere with other spots of the chromatogram. On the other hand, sodium azide should be ruled out because it quenches fluorescence. The serum is dialyzed by ultrafiltration (1 h, 4 bars) through a collodion membrane filter (Sartorius). Urine and serum ultrafiltrates can be stored at −20°C; they are adjusted to pH 8.5 with $NaHCO_3$ for the dansylation reaction. Biological samples and standards are dansylated by mixing (v/v) with fresh dansyl chloride solution (3.7 m*M* in acetone) and incubating for 1 h at 37°C. The dansyl amino acids can be stored for up to 1 month at −20°C in the dark.

Chromatography

The volume of dansylated standards applied is 0.1 µl, which corresponds to approximately 50 picomoles per amino acid (detection limit of dansylated glycine is 1.25 picomoles).

For urine the volume deposited, X (µl), is calculated on the basis of the daily urine volume, V (liters), corrected for the body surface area, S (m^2), of the child (adult surface = 1.73 m^2). The formula applied is $0.1 \times V \times (1.73/S)$. S is found from Dubois tables [5] based on the height (cm) and weight (kg) of the child. This formula takes into consideration diuresis, body surface and methodological sensitivity. However, it is limited, and does not take into account many parameters such as age, sex, pregnancy, inaccuracy of child samples, that are liable to modify, qualitatively and quantitatively, the aminoaciduria.

Some authors propose basing the volume on the creatinine content. This method, while valid for adults, is less applicable to infants and young children because of large individual and daily variations.

We advise, for the pathological interpretation, that a second chromatogram be prepared using a sample taken from a normal patient belonging to a similar age and population group. Thus, in the case of an adult male with a daily urine volume of 1 liter and a standard 1.73 m^2 body surface, according to the values obtained by Lewis et al. [6], the maximum difference between applied quantities will not exceed 100 picomoles for glycine and 2.5 picomoles for α-aminoadipic acid.

For plasma, 0.1 µl of dansylated ultrafiltrate is sufficient to take into account the extreme physiological values of an adult male. As in aminoaciduria,

the evaluation is based on the comparison of a chromatogram from a normal patient of similar age.

The aliquots are applied with a 1-μl microsyringe with an unbevelled needle in 0.05-μl fractions (spot 0.5 mm in diameter) at 5 mm from each contiguous side of a previously cut micropolyamide plate (4.2 × 4.2 cm). Plates carefully handled with gloves are introduced into perfectly flat presaturated tanks. The solvents are removed following each analysis.

After allowing free migration for 5 min in solvent system I, the plates are dried with hot air and then cooled to room temperature. They are then placed in solvent system II and allowed to migrate for 5 min in a direction perpendicular to the first.

Detection and interpretation

The yellow fluorescent spots are identified under light at 254 nm (Camag TL 900U). Dansyl sulfonic acid exhibits a blue fluorescence. The migration distances of the dansyl amino acids in systems I and II are measured in comparison with a dansyl glycine internal standard.

Interpretation is carried out by comparison with control chromatograms. The stability of the fluorescence in the absence of light allows easy storage of chromatograms and comparison with further sequential biological samples. The entire analysis requires 3 h when performed by an experienced operator.

RESULTS AND DISCUSSION

Thirty-nine amino acids representative of normal or pathological samples have been studied individually, pooled in structural and functional groups A, B, C, D, E, and F (Fig. 1), and in a global pool (groups A + B + C + D + E + F, Fig. 2).

Dual identification of the different spots of the dansyl amino acids was possible. Firstly the plates were developed individually for each known dansylated amino acid in order to localize its position. Secondly, the spots were identified after dansylation of the amino acid mixtures (A, B, C, D, E, and F from Fig. 1, or the global pool A + B + C + D + E + F from Fig. 2), and by checking the location of each spot.

Certain amino acids (tyrosine, lysine, cysteine, etc.) contain two reactive functions and can produce two or three spots (two monodansylated derivatives and one bisdansylated derivative). Practically, the migration distances are measured from the most fluorescent spot identified (generally the bidansylated derivative) in a separate experiment with pure commercial standards (Sigma, Cat. No. DAN L 23).

The relative migration distances, determined as the value R_x, are measured after the migration of the pooled amino acids and in comparison with a glycine internal standard (Fig. 1). Table I shows the R_x values obtained in solvent systems I and II, arranged in increasing order. The procedure, which consists of supplementing known individual amino acids, is suitable for identifying any amino acid present at abnormal levels. Fig. 2 indicates the relative positions of each amino acid studied and demonstrates the sharpness of the separation. Nevertheless, some amino acid associations remain unresolved, especially

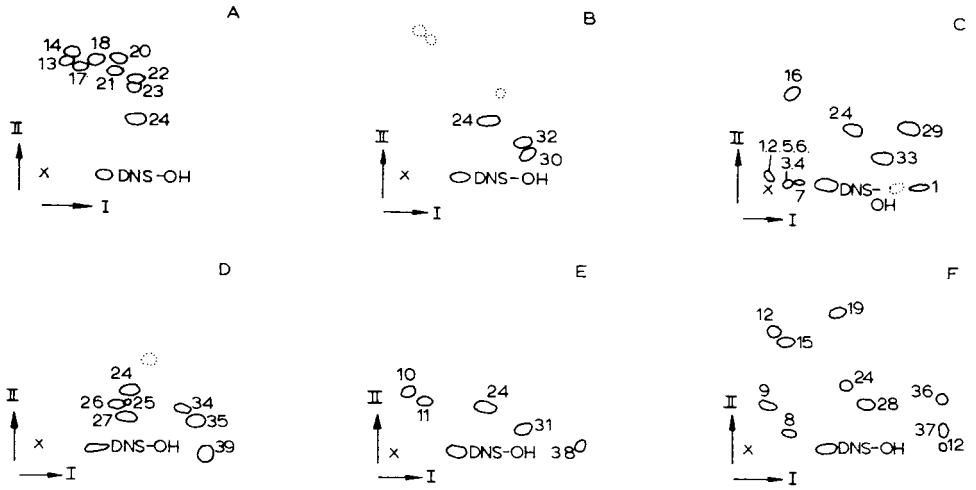

Fig. 1. Two-dimensional chromatography in solvent systems I and II of known dansylated amino acids pooled in structural and functional groups A, B, C, D, E and F. Identification was with glycine as internal standard. DNS-OH = dansyl sulfonic acid. (A) 13 = leucine, 14 = isoleucine, 17 = norvaline, 18 = valine, 20 = β-aminoisobutyric acid, 21 = GABA (γ-aminoisobutyric acid), 22 = β-alanine, 23 = alanine, 24 = glycine. (B) 30 = serine, 32 = threonine, 24 = glycine. (C) 1 = cystine, 2 = cysteine, 3 = taurine, 4 = taurocholic acid, 5 = homocysteine, 6 = homocystine, 7 = cysteic acid, 16 = methionine, 29 = L-methionine sulfoxide, 33 = DL-methionine sulfone, 24 = glycine. (D) 26 = glutamic acid, 27 = aspartic acid, 34 = glutamine, 35 = asparagine, 25 = α-aminoadipic acid, 39 = arginosuccinic acid, 24 = glycine. (E) 10 = lysine, 11 = ornithine, 31 = citrulline, 38 = arginine, 24 = glycine. (F) 8 = tyrosine, 9 = tryptophan, 12 = histidine, 15 = phenylalanine, 19 = proline, 28 = hydroxyproline, 36 = 3-methylhistidine, 37 = 1-methylhistidine, 24 = glycine.

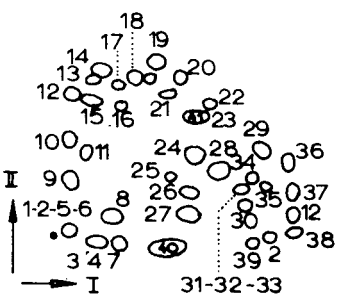

Fig. 2. Two-dimensional chromatography of a global pool of 39 known dansylated amino acids (groups A + B + C + D + E + F from Fig. 1). Identification was with glycine as internal standard. 1 = cystine (bis-dansyl), 2 = cysteine, 3 = taurine, 4 = taurocholic acid, 5 = homocysteine, 6 = homocystine, 7 = cysteic acid, 8 = tyrosine (bis-dansyl), 9 = tryptophan, 10 = lysine (bis-dansyl), 11 = ornithine (bis-dansyl), 12 = histidine (bis-dansyl), 13 = leucine, 14 = isoleucine, 15 = phenylalanine, 16 = methionine, 17 = norvaline, 18 = valine, 19 = proline, 20 = β-aminoisobutyric acid, 21 = GABA (γ-aminoisobutyric acid), 22 = β-alanine, 23 = alanine, 24 = glycine, 25 = α-aminoadipic acid, 26 = glutamic acid, 27 = aspartic acid, 28 = hydroxyproline, 29 = L-methionine sulfoxide, 30 = serine, 31 = citrulline, 32 = threonine, 33 = DL-methionine sulfone, 34 = glutamine, 35 = asparagine, 36 = 3-methyihistidine, 37 = 1-methylhistidine, 38 = arginine, 39 = arginosuccinic acid, 40 = dansyl sulfonic acid, 41 = dansylamine.

TABLE I

R_x VALUES OF DANSYLATED AMINO ACIDS SEPARATED BY TWO-DIMENSIONAL CHROMATOGRAPHY ON MICROPOLYAMIDE PLATES

	R_{x_1}*		R_{x_2}**
Tyrosine (bis-dansyl)	0	Arginosuccinic acid	0
Cysteine (bis-dansyl)	0.11	α- or β-dansylcysteine	0
Cystine (bis-dansyl)	0.11	Taurine	0
Homocysteine	0.11	Taurocholic acid	0
Homocystine	0.11	Cysteic acid	0
Tryptophan	0.13	α-Amino dansylhistidine	0.05
Lysine (bis-dansyl)	0.13	Cysteine (bis-dansyl)	0.17
Histidine (bis-dansyl)	0.19	Cystine	0.17
Leucine	0.25	Homocysteine	0.17
Isoleucine	0.29	Homocystine	0.17
Taurine	0.30	Arginine	0.24
Taurocholic acid	0.30	Tyrosine (O-dansyltyrosine)	0.27
Ornithine	0.32	1-methylhistidine	0.30
Cysteic acid	0.38	Aspartic acid	0.32
Phenylalanine	0.38	Asparagine	0.42
Tyrosine (O-dansyltyrosine)	0.39	Serine	0.46
Norvaline	0.42	Glutamic acid	0.53
Methionine	0.49	Glutamine	0.58
Valine	0.56	Citrulline	0.59
γ-Aminoisobutyric acid	0.75	DL-Methionine sulfone	0.66
DL-β-Aminoisobutyric acid	0.80	Threonine	0.67
Proline	0.88	Tryptophan	0.68
Alanine	0.92	Hydroxyproline	0.73
Glutamic acid	0.92	DL-α-Aminoadipic acid	0.80
Aspartic acid	0.93	3-Methylhistidine	0.80
β-Alanine	1.00	Glycine	1.00
Glycine	1.00	L-Methionine sulfoxide	1.08
DL-α-Aminoadipic acid	1.00	Ornithine	1.12
Hydroxyproline	1.19	Lysine (bis-dansyl)	1.32
DL-Methionine sulfone	1.33	Methionine	1.54
Threonine	1.35	Alanine	1.55
Serine	1.38	Phenylalanine	1.64
Citrulline	1.45	β-Alanine	1.70
L-Methionine sulfoxide	1.50	Tyrosine (bis-dansyl)	1.75
Glutamine	1.57	γ-Aminoisobutyric acid	1.82
Asparagine	1.72	Histidine (bis-dansyl)	1.86
Arginosuccinic acid	1.80	Norvaline	1.91
α- or β-dansyl cysteine	2.00	Leucine	1.95
1-Methylhistidine	2.02	DL-β-Aminoisobutyric acid	2.00
α-Amino dansylhistidine	2.02	Valine	2.00
3-Methylhistidine	2.02	Isoleucine	2.09
Arginine	2.08	Proline	2.09

*$R_{x_1} = \frac{\text{migration distance of dansyl amino acid}}{\text{migration distance of dansyl glycine}}$ in solvent system I (formic acid—water, 1.5 : 98.5.

**$R_{x_2} = \frac{\text{migration distance of dansyl amino acid}}{\text{migration distance of dansyl glycine}}$ in solvent system II (benzene—acetic acid, 4.5 : 1).

cysteine/cystine/homocysteine/homocystine (perhaps due to the breaking of the covalent disulfide bond when dansylated), taurine/taurocholic acid and citrulline/threonine. Interference by excess dansylsulfonic acid, with the fluorescence of cysteic acid, previously described by Lee and Safille [1], was not observed under our conditions.

The dansylamine by-product of the dansylation reaction [7] shows a yellow fluorescence at the same place as dansyl alanine. The two derivatives can be separated by a third migration, in the second dimension, with Na_3PO_4 (0.05 *M*)—ethanol (3:1) [4]. In addition, this procedure increases the resolution of the dansyl arginine/bisdansyl lysine couple.

CLINICAL APPLICATIONS

Fig. 3a shows chromatograms for serum and urine of a child with leucinosis. The serum chromatogram points out an obvious increase in leucine, isoleucine and valine. In the same way, the quality of serum chromatography of other aminoacidopathies, notably for a case of citrulinemia (Fig. 3b) and a case of phenylketonuria (Fig. 3c), emphasises the interest of analysis of a serum milieu whose composition is more constant than that of urine.

In conclusion, the major advantage of the proposed method is its qualitative evaluation of general or a particular aminoacidopathy.

In comparison with classical thin-layer chromatographic (TLC) separation on cellulose or silica gel, the proposed identification of human aminoacidopathies

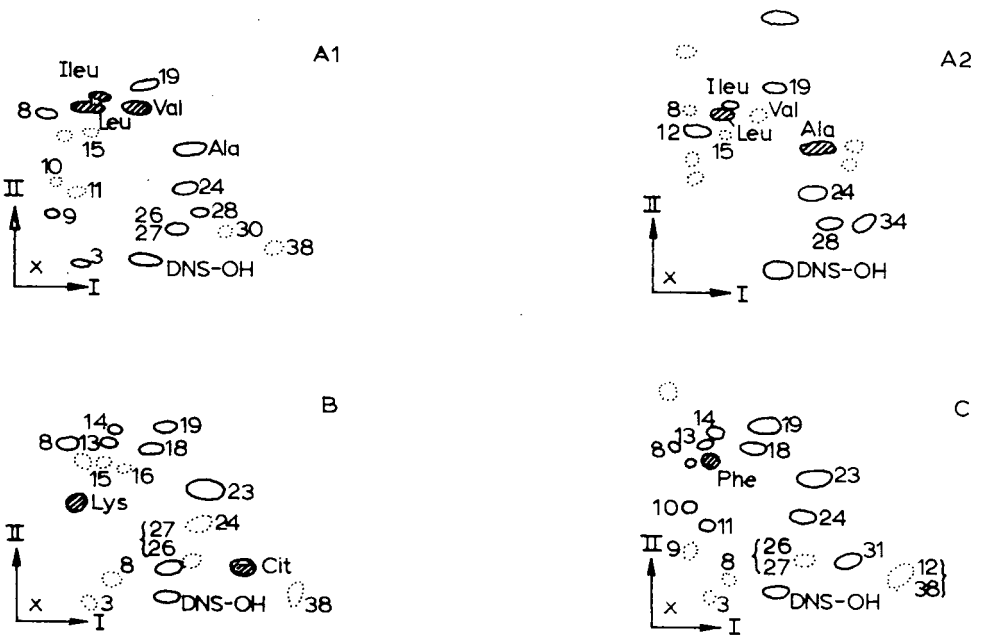

Fig. 3. Chromatograms of serum and urine from different children with leucinosis, citrullinemia and phenylketonuria: (A) leucinosis (A_1 = serum, A_2 = urine); (B) citrullinemia (serum); (C) phenylketonuria (serum).

by TLC on polyamide is faster, with greatly improved definition (no tails) and does not require deionised samples. The results justify analysis using serum, which is of a more constant composition than urine, and which shows more clearly any pathology, giving a more accurate diagnosis, especially in the case of aminoacidopathies associated with secondary nephropathy.

Quantitatively, the separated dansylated amino acids can be eluted and assayed following the technique described by Airhart et al. [8]. The sensitivity (about 1 picomole) and the rapidity of this method might provide a valuable alternative to standard autoanalyzer determination.

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Note

Improved thin-layer chromatographic assay for monitoring lecithin/sphingomyelin ratios in amniotic fluid

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Numerous methods have been proposed for assessing fetal lung maturity by analysis of the amniotic fluid. The lecithin/sphingomyelin (L/S) ratio is the test most widely used as being specific for that purpose, and one-dimensional thin-layer chromatography (TLC) seems to be the method most often used.

A number of reagents, chromogenic as well as fluorogenic, are available for the visualization. Sulfuric acid [1], phosphomolybdic acid [2], and ammonium molybdate perchloric acid—HCl reagent [3] seem to be the most frequently used reagents in the chromogenic mode, whereas 2,7-dichlorofluorescein [4], and rhodamine B [5] are used in the fluorogenic mode.

This paper presents the first report using high-performance thin-layer chromatoplates (HPTLC plates) for the separation of the phospholipids and 8-anilino-1-naphthalene sulfonic acid (ANSA) as the visualization reagent [6], a combination which seems to yield a chromatographic separation and a sensitivity superior to most of the modifications of this assay that have recently been published [7, 8].

EXPERIMENTAL

The phospholipid standards, LS-10 with L/S = 1 and LS-20 with L/S = 2, and the detection reagent 8-anilino-1-naphthalene sulfonic acid, ammonium salt, practical grade (Cat. No. A 3125) were from Sigma (St. Louis, MO, U.S.A.). For all experiments we used HPTLC plates from E. Merck, Darmstadt, G.F.R. (Cat. No. 5644). The plates were spotted by a 50-μl luer-tipped Hamilton syringe mounted in a Hamilton repeating dispenser and equipped with a 10-mm tuberculin needle. The plates were scanned with a spectrodensitometer equipped with a recorder and integrator (Schoeffel Instruments, Model SD 3000) in the fluorescence mode.

Amniotic fluid samples obtained by transabdominal amniocentesis are centrifuged within half an hour for 10 min at 2000 *g* to remove cells and sediment. A 1-ml aliquot of the supernatant is pipetted into a 10-ml disposable test-tube containing 3 ml of chloroform—methanol (2 : 1, v/v). The mixture is shaken vigorously for 3 min, and the aqueous and organic phases are separated by centrifugation for 5 min at 2000 *g*; the aqueous (upper) layer is discarded and the organic layer transferred to a 5-ml Pyrex beaker and the chloroform—methanol mixture is removed by placing the beaker in a 60°C water-bath under a stream of air from a hair-dryer. After drying the residue is re-dissolved in 100 μl of chloroform—methanol (1:1, v/v) and 5 μl are spotted on a 5 × 10 cm HPTLC plate. One microliter of standard LS-10 or LS-20 is also spotted on the plate, which is developed in chloroform—methanol—water (65 : 25 : 4, v/v) to a solvent height of 6 cm from the origin, in a saturated tank. The plate is then removed from the tank and dried under a stream of hot air until solvent removal is complete. To visualize the spots of phospholipids the plate is dipped in a 0.2% aqueous solution of the ANSA reagent. To remove excess reagent the plate is placed face downwards on lint-free tissue paper. When the plate is inspected in UV light at 365 nm the spots will appear yellow—green on a dark, uncolored background which is only faintly fluorescent (Fig. 1). It should be emphasized that this high contrast is only achieved if all of the developing solvent has been removed from the plate and the plate is examined and scanned while still wet with the ANSA reagent. If the plate is allowed to dry out subsequently during the scanning procedure, some contrast is lost and the accuracy may decrease. Therefore it must be strongly pointed out that only three spots, for example two standards and one sample, should be spotted on the same plate. The plate is scanned in the direction opposite to development. The spectrodensitometer is set with the excitation wavelength at 365 nm. The speed of the densitometer and the recorder is set at 10 mm/min. The recorder tracing of the plate (Fig. 1) is shown in Fig. 2.

RESULTS AND DISCUSSION

To determine the precision of the method two samples of amniotic fluid with L/S ratios of 1.6 and 14.9 were each analyzed ten times within one day. The results are shown in Table I. As can be seen, the coefficient of variation rises proportionally to the increase in the L/S ratio. This is due to the sharp decrease in the sphingomyelin fraction during the last few weeks of gestation. When using ANSA reagent the detection limits for lecithin and sphingomyelin are about 4 μg/ml and 1 μg/ml of amniotic fluid [6], and the superiority of this reagent over 2,7-dichlorofluorescein and rhodamine B can be explained by the improved background of the plate after the dipping procedure. The HPTLC plates give excellent separation of the substances and usually nice round spots. The length of the chromatographic run is only 6 cm (15—20 min) and the staining procedure can be performed within a few seconds; thus the main advantage of this TLC method is its speed and simplicity.

The whole analytical procedure, including the extraction, can be performed within 1 h. It should also be emphasized that the content of other lipids

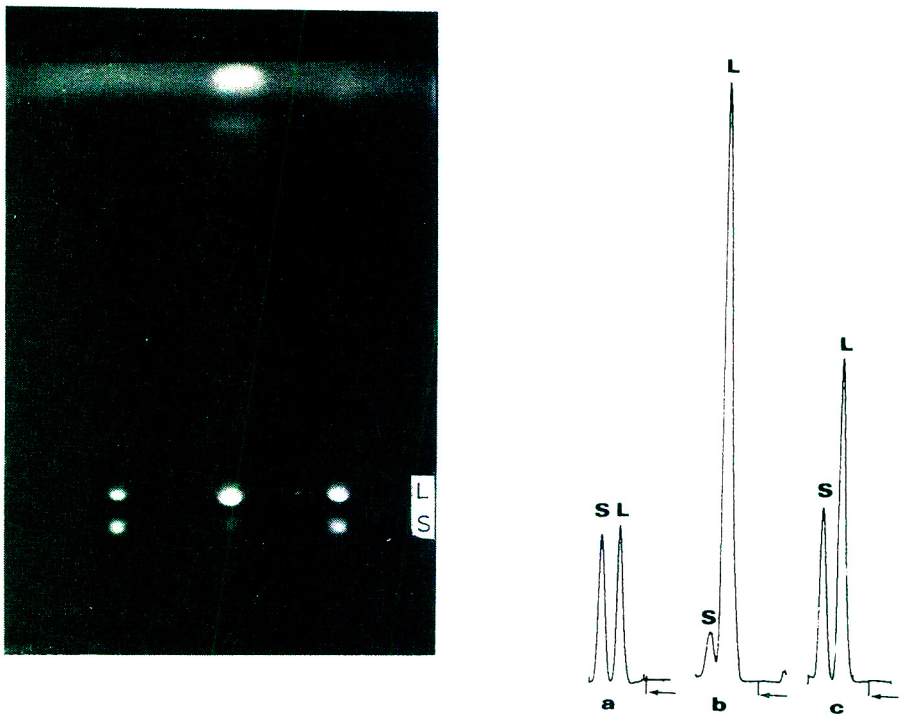

Fig. 1. HPTLC plate photographed in UV light at 365 nm. L = lecithin, S = sphingomyelin. (a) Sigma standard LS-10. (b) Extract of a "mature" (39th week of gestation) amniotic fluid; two spots of unidentified lipids are seen near the front. (c) Sigma standard LS-20.

Fig. 2. Recorder tracing of the chromatogram shown in Fig. 1. The arrows indicate the start of the scanning. L = lecithin, S = sphingomyelin.

TABLE I

THE ANALYTICAL VARIATION OF TWO STANDARDS AND TWO PATIENTS' SAMPLES ANALYZED TEN TIMES WITHIN ONE DAY

	Standard LS-10 L/S = 1.0	Amniotic fluid ("immature") L/S = 1.6	Standard LS-20 L/S = 2.0	Amniotic fluid ("mature") L/S = 14.9
Mean	0.98	1.58	1.94	14.85
S.D.	0.02	0.04	0.06	0.74
C.V. (%)	1.93	2.78	2.89	5.00

normally present in the amniotic fluid have not interfered with the results. Finally, it should be pointed out that this method, producing regular round spots, could also be used visually as a screening assay in laboratories where no scanning instrument is available.

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CHROMBIO. 1020

Note

Determination of biperiden in human serum by glass capillary gas chromatography with isothermal splitless injection and nitrogen-sensitive detection

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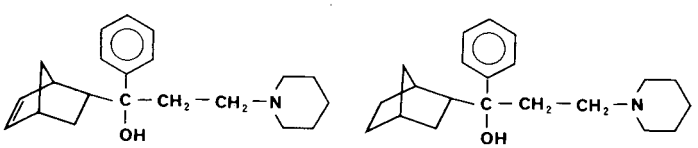
Biperiden, 1-bicyclo[2.2.1]hept-5-en-2-yl-1-phenyl-3-piperidinopropan-1-ol hydrochloride (Fig. 1A), is an anticholinergic drug which is used in the treatment of Parkinson's disease [1]. So far only one method based on gas chromatography—mass spectrometry has been reported for the determination of biperiden in serum [2]. Although the method involves a complex extraction and derivatization procedure, the sub-nanogram sensitivity required for bioavailability and pharmacokinetic studies is not obtained. In this report we describe a simpler and more sensitive method, which involves extraction of biperiden from serum with hexane and quantitation by glass capillary gas chromatography with nitrogen-sensitive detection.

EXPERIMENTAL

Materials

Biperiden hydrochloride was supplied by Orion Pharmaceutical Co. (Helsinki, Finland). Internal standard, 1-bicyclo[2.2.1]hept-2-yl-1-phenyl-3-piperidinopropan-1-ol (Fig. 1B), was obtained by catalytic hydrogenation (Pd-C) of



A B

Fig. 1. The structures of biperiden (A) and internal standard (B).

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biperiden. Hexane (p.a., E. Merck, Darmstadt, G.F.R.) and tridecane (puriss., Koch-Light, Colnbrook, Great Britain) were used without further purification.

Procedure

After adding internal standard (2.5 ng/ml), 2 ml of serum were alkalinized (pH 9.5) with 1 *M* NH_4OH and extracted twice with 5 ml of hexane. The combined hexane layers were evaporated to almost dryness with a stream of nitrogen at 40°C and the residue was dissolved in 25 μl of tridecane. The extracts were protected from light and stored in the refrigerator.

Analyses were performed using a Hewlett-Packard 5730A gas chromatograph with an HP 18704B capillary system inlet and an HP 18789A N—P flame ionization detector. The OV-101 glass capillary column, 25 m × 0.32 mm I.D., was prepared by Dr. S. Räisänen (Helsinki University, Helsinki, Finland). The column was operated isothermally at 215°C. Aliquots of 1.5 μl of extracts in tridecane were injected using splitless injection technique with a 15-sec splitless period. The injector temperature was 250°C and the detector temperature 300°C. Gas flow-rates were: carrier (helium) 2 ml/min, hydrogen 3 ml/min, air 40 ml/min and auxiliary (helium) 30 ml/min. Biperiden was quantitated by comparing peak height ratios of biperiden and internal standard to a calibration curve obtained by analyzing spiked serum samples over the range 0.25—10 ng/ml.

RESULTS AND DISCUSSION

Biperiden chromatographed well on the OV-101 glass capillary column with a detection limit of about 20 pg injected onto the column. Derivatization was not necessary to prevent adsorption as was reported to be the case with packed columns [2]. For the sensitivity required splitless injection was a prerequisite. In this case the solvent effect [3] can be utilized to prevent band broadening. This can be achieved in a case of lower boiling solvents by injecting at low temperature and by subsequent temperature programming of the column. The use of long-chain alkanes as solvents to obtain the solvent effect at higher injection temperatures, in order to make faster and simpler isothermal operation feasible, is well known although not commonly adopted for quantitative drug analysis [4—7]. At higher temperatures the injection conditions, especially the choice of solvent, are critical to obtain optimum effect and maximum peak height. In this case best results were obtained using isothermal conditions at 215°C with tridecane as the solvent. Biperiden and internal standard eluted fully separated within 5 min and samples could be analyzed with an interval of 6 min.

After injecting about 200 samples the contamination of the beginning of the column started to cause some broadening of peaks. The performance of the column could be restored by washing the first 20—30 cm of the column with hexane and then treating with 0.5% OV-101 in methylene chloride.

The specificity achieved with glass capillary gas chromatography and nitrogen-sensitive detection allowed the complex extraction procedure suggested by previous workers [2] to be replaced by a simple extraction with hexane. Gas chromatograms obtained by analyzing serum extracts are shown in Fig. 2. The

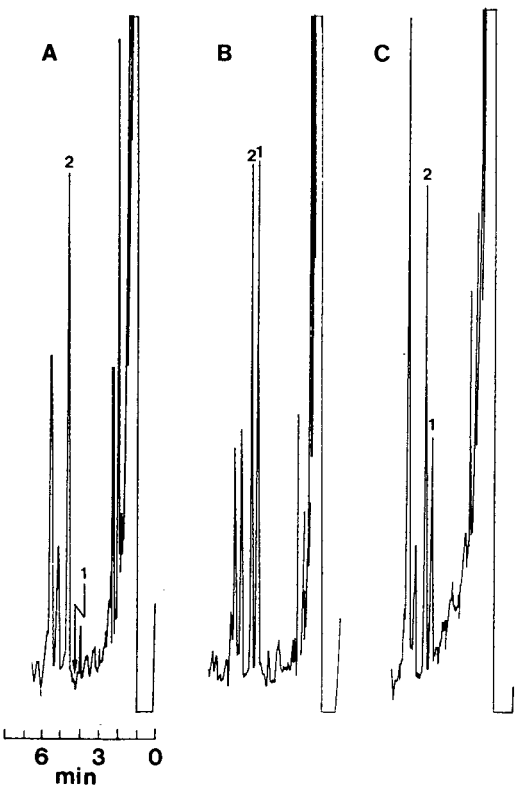

Fig. 2. Gas chromatograms obtained by analyzing serum extracts with a 25-m OV-101 glass capillary column isothermally at 215°C. (A) Blank serum with internal standard. (B) 2.5 ng/ml biperiden and internal standard added to blank serum. (C) Serum sample of a volunteer 2 h after an oral dose of 4 mg. Peaks: 1 = biperiden, 2 = internal standard.

recovery test was carried out using standard serum samples spiked with 2.5 ng/ml biperiden. Internal standard was added in tridecane at the end of the evaporation step. In reference samples both biperiden and internal standard were added to blank serum extracts. Recovery of biperiden was found to be 60 ± 6% (S.D., n = 6). Despite the relatively low recovery the precision was reasonable, even near the lower limit of quantitation. This is due to the suitable internal standard, which differed from biperiden only by the absence of the double bond in the bicycloheptane ring. Precision was studied by analyzing replicate spiked samples at the concentrations of 2.5 ng/ml and 0.5 ng/ml, and was 3.3% and 6.8% (C.V., n = 6), respectively.

Biperiden was quantitated by comparing peak height ratios with a calibration curve, which was constructed daily by linear regression after analyzing spiked serum samples in the concentration range 0.25—10 ng/ml. In this range linearity was good (typically r = 0.999). The lower limit of quantitation, 250 pg/ml, was adequate for analyzing samples up to 8 h after a single dose of 4 mg, as is demonstrated in Fig. 3. The assay method has been used successfully for moni-

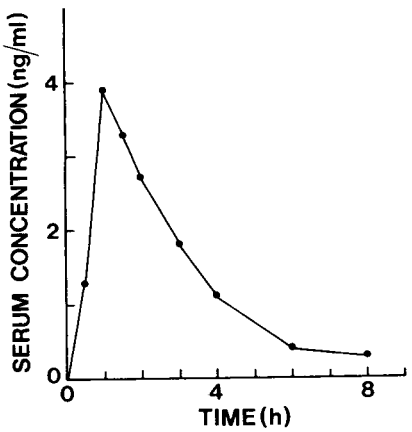

Fig. 3. Serum concentration curve from a healthy volunteer after a 4-mg dose of biperiden.

toring serum concentrations in a steady-state situation. After a dose of 4 mg twice a day of long-acting formulations, serum concentrations of 0.5—4 ng/ml were measured.

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Note

High-performance liquid chromatographic estimation of cyproterone acetate in human plasma

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Cyproterone acetate (17α-hydroxy-6-chloro-1,2α-methylenepregn-4,6-diene-3,20-dione acetate, CPA) possesses antiandrogen and progestational properties [1]. CPA is used in the treatment of hirsutism [2], severe acne [3] and precocious puberty [4]. Although the pharmacokinetic [5] and pharmacological [6] aspects have been studied, there is a dearth of information concerning peripheral blood levels and clinical effects in patients treated with CPA. For this reason a selective, rapid assay was developed utilising high-performance liquid chromatography (HPLC). HPLC allows potentially clinically important metabolites to be measured, offering an advantage over a previously reported radioimmunoassay method [7].

MATERIALS AND METHODS

Chemicals and reagents

Cyproterone acetate (Schering, Sydney, Australia) was added to pooled human plasma to prepare plasma samples and standards. Ethyl acetate and hexane were of analytical grade and distilled before use. Methanol was HPLC grade (Waters Assoc., Milford, MA, U.S.A.) and chromatographic grade alumina was obtained from Merck (Darmstadt, G.F.R.). The internal standard starting material, 17α-hydroxyprogesterone, was obtained from Sigma (St. Louis, MO, U.S.A.).

Apparatus

The chromatographic equipment consisted of a high-performance liquid chromatograph (Tracor, Austin, TX, U.S.A., Model 985 LC master, Model 951

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pump) with a 100-μl valve injection loop and a variable-wavelength detector (Model 970A).

The column was a 300 × 3.9 mm octadecylsilane reversed-phase (μBondapak, 10 μm Waters Assoc.). Retention times and peak heights were measured with a recording integrator (Model SP4100, Spectra Physics, Santa Clara, CA, U.S.A.).

Chromatographic operating conditions

The detector was set at 282 nm, determined as λ_{max} by a stopped flow scan of cyproterone acetate standard. The molar extinction coefficients of cyproterone acetate and the internal standard in the eluting solvent measured at 282 nm are 17,700 and 16,600 respectively. The sensitivity was 0.030 a.u.f.s. The flow-rate was held constant at 2 ml/min and all measurements were at ambient temperatures. The eluting solvents, water and methanol, were filtered through a glass filter (pore size, 0.5 μm; Millipore, Bedford, MA, U.S.A.) and degassed under vacuum. The LC master was set to deliver a 70:30 ratio (by volume) of methanol to water.

Internal standard

The internal standard, 17α-hydroxypregn-4,6-diene-3,20-dione 17-butanoate, was synthesized from 17α-hydroxyprogesterone using conventional methods [8, 9].

17α-Hydroxyprogesterone was esterified with butanoic anhydride and pyridine and the reaction monitored by high-performance liquid chromatography (HPLC) for completion. The product, after purification on silica gel with hexane—diethyl ether mixtures as eluent, was treated with chloranil in *tert.*-butanol to give the internal standard. The overall yield following purification on neutral alumina (hexane—diethyl ether mixtures as eluent) was 37%.

A stock internal standard solution of 250 mg/l was prepared in methanol and diluted to 12.5 mg/l.

Extraction procedure

Spiked plasma or patient sample (0.5 ml) was mixed with 0.5 ml sodium hydroxide (0.25 *M*), 100 μl of internal standard and 10 ml of ethyl acetate in an assay tube (Pyrex tube, 150 × 20 mm with PTFE-lined screw cap). After shaking (5 min) and centrifuging (5 min at 900 *g*), the organic layer was transferred to a 100 × 24 mm pyrex tube and the solvent removed in vacuo at 40°C. The residue was chromatographed on 0.5 g silica gel with 4 ml of 5% (v/v) ethyl acetate—hexane followed by 5 ml of ethyl acetate. The ethyl acetate fraction was evaporated in vacuo at 40°C, the residue dissolved in 100 μl of methanol and injected onto the column.

Stability

The effect of anticoagulants on the assay is shown in Table I. Each assay contained the same amount of CPA and each tube was assayed at the same time. Lithium heparin results agree closely with the non-extracted value and this anticoagulant was used for all patient samples.

The long-term stability of CPA with lithium heparin as an anticoagulant is

shown in Table II. Aliquots of a blood sample containing lithium heparin and spiked with CPA were centrifuged and plasma stored at −20°C at the times shown. The samples were assayed concurrently and showed no decomposition of CPA over a 24-h period.

TABLE I

EFFECT OF VARYING ANTICOAGULANT

Anticoagulant	Peak height ratio*
Theoretical	1.01
Plain tube	1.04
Lithium heparin	1.01
Oxalate	1.08
Oxalate/fluoride	1.06
EDTA	1.06

*Peak height of drug:peak height of internal standard.

TABLE II

LONG TERM EFFECT OF LITHIUM HEPARIN

Time (h)	Peak height ratio*
0	1.38
0.5	1.44
1	1.47
2	1.42
4	1.50
6	1.38
8	1.49
24	1.41

*Peak height of drug:peak height of internal standard.

RESULTS

Linearity of response and sensitivity

Pooled plasma was spiked with CPA from 0.1 μg/ml and assayed. Peak height ratio (peak height of CPA divided by peak height of internal standard) was used as the response. A least squares linear regression analysis was used to determine the slope, y-intercept, and correlation coefficient. The regression line obtained was $y = 1.735x - 0.012$ ($r = 0.999$). Concentrations as low as 0.02 μg/ml could be measured accurately. The lower limit of detection was 4 ng.

Precision and accuracy

The reproducibility of the assay was determined using spiked plasma pools. These results are given in Table III. Recoveries were calculated using plasma

pools containing 2 μg/ml and 0.5 μg/ml CPA (n = 6 for each). Recovery from the 2 μg/ml sample averaged 95.6% and 88.3% from the 0.5 μg/ml sample.

Selectivity

Interference in the assay by other drugs was studied by using either solutions of the drug or plasma samples from patients ingesting the drug (Table IV). Medroxyprogesterone acetate and 17α-hydroxypregnenolone have similar retention volumes to CPA but absorption of these compounds at 282 nm is negligible.

A typical chromatogram of extracted human plasma spiked with CPA and internal standard is shown in Fig. 1. The retention times for the assay conditions are 5.3 min and 7.5 min with baseline separation. A pooled plasma sample without CPA or internal standard shows no interfering compounds.

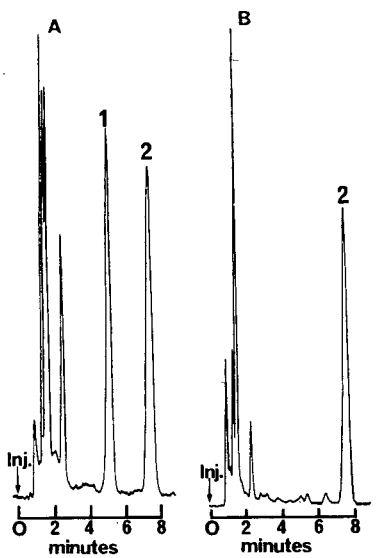

Fig. 1. (A) Chromatogram of extracted pooled plasma spiked with CPA 2.1 μg/ml (1), and internal standard (2). (B) Chromatogram of extracted blank plasma containing only internal standard (2); 0.03 a.u.f.s.

DISCUSSION

An internal standard with a larger k' value than CPA was synthesized to achieve baseline separation and an absorption maximum with close correspondence to that of CPA. The butanoate ester of 17α-hydroxypregn-4,6-diene-3,20-dione used as internal standard was separated from all endogenous plasma components and drugs tested for specificity of the assay.

CPA levels have been measured by radioimmunoassay in plasma of patients following administration of a contraceptive containing 2 mg of CPA [10]. In the latter study the maximum CPA concentration of 11.0 ± 3.4 ng/ml plasma was observed 1.6 ± 0.6 h after a single administration. We have used the HPLC

TABLE III

PRECISION OF ASSAY FOR CPA

	Intra-assay* (n = 10)			Inter-assay** (n = 10)		
Theoretical	0.55	1.11	2.21	0.55	1.11	2.21
Mean ± S.D. (μg/ml)	0.53 ± 0.015	1.06 ± 0.028	2.19 ± 0.05	0.54 ± 0.021	1.11 ± 0.039	2.14 ± 0.074
C.V. (%)	2.74	2.65	2.30	3.80	3.50	3.50

*Within-assay precision.
**Between-assay precision.

TABLE IV

INTERFERENCE IN THE ASSAY BY OTHER DRUGS

Tablet preparations and pure drugs tested		Plasma from patients ingesting these drugs tested	
Androstenedione	17α-Hydroxyprogesterone	Acetylsalicylic acid	Oxazepam
Cortisol	Medroxyprogesterone acetate	Chlorothiazide	Paracetamol
Cortisone	Methylprednisolone	Chloral hydrate	Phenytoin
Dehydroisoandrosterone	Prednisolone	Digoxin	Propranolol
Dexamethasone	Prednisone	Erythromycin	Pseudoephedrine
17β-Estradiol	Progesterone	Hydralazine hydrochloride	Salbutamol
Estriol	Spironolactone	Isoniazid	Theophylline
Ethinyl estradiol	Testosterone	Nitrazepam	Thiamine
17α-Hydroxypregnenolone			

assay to measure CPA in patients with precocious puberty treated with 75—200 mg daily (Table V).

The HPLC assay is rapid and selective. Each assay requires about 30 min from receipt of the plasma sample. It is linear to 4 μg/ml and recoveries average between 88—96%. The assay is being adapted to measure metabolites of CPA in plasma.

TABLE V

ASSAYS OF PATIENT SAMPLES

Random samples were used.

Patient	CPA (μg/ml)	Daily dose (mg)
A	0.59	75
B	0.52	125
B	0.74	125
B	0.63	125
B	0.66	200
B	0.62	200
B	0.97	200

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Note

Determination of penicillamine and other thiols by combined high-performance liquid chromatography and post-column reaction with Ellman's reagent: application to human urine

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Penicillamine (β,β-dimethylcysteine) is widely used in the treatment of a number of diseases including rheumatoid arthritis, Wilson's disease, cystinuria, and heavy-metal poisoning [1]. Many toxic side-effects have been noted and there is a wide variability in therapeutic effect. A simple, rapid and specific analytical method for the determination of penicillamine in biological fluids is needed to investigate these effects. Several approaches to the analysis of penicillamine have been described, including colorimetry [2], gas—liquid chromatography [3] and amino acid analyser methods [4], but few of these methods meet the desired criteria. More recently a high-performance liquid chromatographic (HPLC) method using electrochemical detection specific for thiols has been reported [5]. However, the expense of the detector, coupled with the expertise required for its operation, encouraged us to investigate alternative methods.

We now describe a method whereby, after chromatographic separation, thiols react with Ellman's reagent in a solid-bed post-column reactor to produce a coloured anion. Concurrently with our own work a sulphur-specific post-column

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reaction has been developed [6], which is also capable of detecting penicillamine.

EXPERIMENTAL

Apparatus

The analytical system employed in this study is illustrated in Fig. 1. Mobile phase was delivered using a Laboratory Data Control (Stone, Great Britain) Constametric III pump at 1 ml min^{-1}. Ellman's reagent was pumped into the post-column reactor by a Waters Assoc. (Northwick, Great Britain) Model 6000A pump at 0.5 ml min^{-1}. Samples were injected on to the column via a Waters Assoc. Model U6K universal injector. Changes in the absorbance of the eluent from the post-column reactor were monitored with a Varian (Walton-on-Thames, Great Britain) Vari-Chrom, variable-wavelength detector at 412 nm.

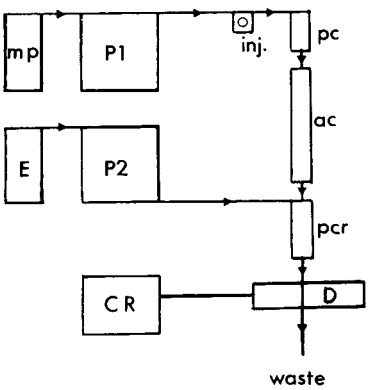


Fig. 1. Schematic diagram showing the system used in these experiments. mp = mobile phase, E = Ellman's reagent, P1 and P2 = pumps, inj. = injector, pc = precolumn, ac = analytical column, pcr = post-column reactor, D = detector, CR = chart recorder.

Chromatography was performed on a 4.5-cm precolumn connected to a 10-cm analytical column (4.6 mm I.D., stainless steel). Columns were slurry packed in methanol with 5-μm ODS Hypersil (Magnus Scientific, Sandbach, Great Britain) using a Magnus P6000 slurry packing unit.

The post-column reactor was constructed as described in ref. 7, and consisted of a stainless-steel tube, 15 cm × 2 mm I.D., filled with 40-μm glass beads (Magnus Scientific).

Post-column reduction of disulphides was performed on a column of dihydrolipoamide beads (Pierce and Warriner, Chester, Great Britain) packed into a stainless-steel column, 15 cm × 4.6 mm I.D., which is inserted between the analytical column and the post-column reactor. For best results beads were reduced with mercaptoethanol according to the manufacturer's instructions, either before use, or on oxidation.

Chemicals

All chemicals including thiols were of the purest grades available and were used without further pretreatment. Solutions of penicillamine (Sigma, Poole,

Great Britain) and other thiols used for preparing standard curves were prepared daily in mobile phase.

The mobile phase consisted of 1 g of heptanesulphonic acid (HSA; Magnus Scientific) and 150 mg of EDTA (sodium salt); (BDH, Poole, Great Britain) dissolved in 1 litre of distilled water at pH 4.

Ellman's reagent consisted of 200 mg of 5,5'-dithiobis(2-nitrobenzoic acid) (Sigma) and 10 g of tripotassium citrate made up in 100 ml of 0.25 m*M* phosphate buffer (pH 7.4). This stock solution was diluted ten-fold with distilled water immediately before use. Both solutions were de-gassed and filtered before use.

Penicillamine determination

Sample preparation was limited to the addition of 1—2 mg ml^{-1} EDTA to urine samples immediately after collection. Homocysteine (5 μg ml^{-1}) was added to urine samples as an internal standard. Penicillamine concentrations in unknowns were determined by comparison with a standard curve constructed using blank urine spiked with known quantities of penicillamine over the range 0—50 μg ml^{-1} and 5 μg ml^{-1} internal standard and treated as above. A 20-μl sample volume was injected onto the column.

RESULTS AND DISCUSSION

The system is illustrated diagrammatically in Fig. 1. Penicillamine is a polar, ionisable compound and to obtain retention during reversed-phase chromatography an ion-pair reagent (HSA) was added. The addition of a small quantity of EDTA to the mobile phase was necessary to prevent on-column oxidation of thiols. Following separation, the thiols were mixed with a stream of Ellman's reagent at the top of the post-column reactor. Reaction and further mixing occurred as the thiol and reagent flowed through the reactor. The narrow bore of the reactor and the small bead size ensure that band spreading is reduced to a minimum [7].

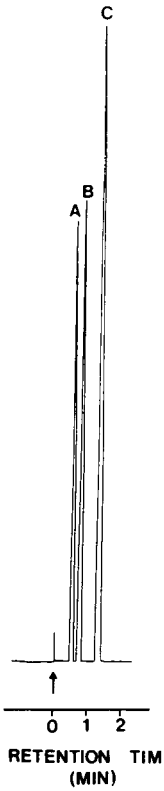
Under the conditions described the time available for reaction, before the thiol passes through the detector flow cell, is 17 sec, in which time the reaction goes to about 90% completion.

The reaction of thiols (PS^-) with Ellman's reagent (E—S—S—E) [8], shown below, produces a yellow anion (E^-) absorbing strongly at 412 nm.

$$PS^- + E—S—S—E \rightleftharpoons P—S—S—E + E^-$$

In Fig. 2 the separation and detection of a mixture of cysteine, homocysteine, and penicillamine is shown. The linear range of the system is large, allowing quantities of thiols over the range 10 ng to 150 μg (on column) to be determined.

Calibration curves for penicillamine, cysteine and glutathione over the range 0—1 μg (on column) are shown in Fig. 3. There is no response to other amino acids, or to non-SH-containing compounds, such as disulphide. The system is also insensitive to the gross contaminants present in biological fluids. Calibration curves from distilled water and urine, using homocysteine as internal standard, were identical.



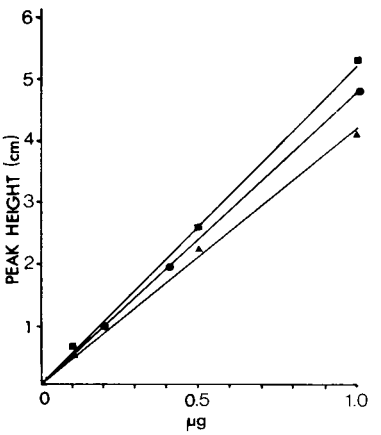


Fig. 2. Separation of a mixture of 6 μg each of cysteine (A), homocysteine (B) and penicillamine (C). Absorbance of cysteine = 1.08, homocysteine = 1.13, penicillamine = 1.56.

Fig. 3. Calibration curves for gluthathione (▪), penicillamine (●) and cysteine (▲) over the range 0—1 μg. Each point represents the average value of at least two determinations.

We have applied this method to the analysis of a number of urine samples obtained from patients treated with penicillamine (Fig. 4). For these preliminary experiments we used homocysteine as an internal standard. In future work we hope to use a penicillamine analogue. A typical HPLC trace from the urine of a patient treated with penicillamine (250 mg/day) is given in Fig. 4. Penicillamine is readily detected and we have observed levels of drug up to 60 μg ml^{-1} in patients taking between 250 and 750 mg/day.

Penicillamine solutions in HSA—EDTA are relatively stable; however, penicillamine in urine is rapidly oxidised. In common with others [5] we have found that the addition of a small quantity of EDTA at the time of sample collection prevents the oxidation. A time course experiment showing the disappearance of penicillamine in urine, with and without added EDTA, is shown in Fig. 5.

However, even at the point of collection, urine (and plasma) samples containing penicillamine will also contain penicillamine in the oxidised (disulphide) form, as well as mixed disulphides of penicillamine with cysteine and perhaps other —SH compounds. Any technique to determine total levels of penicillamine therefore requires some method of measuring the penicillamine present in disulphide form. Precolumn reduction of the disulphides electrochemically has

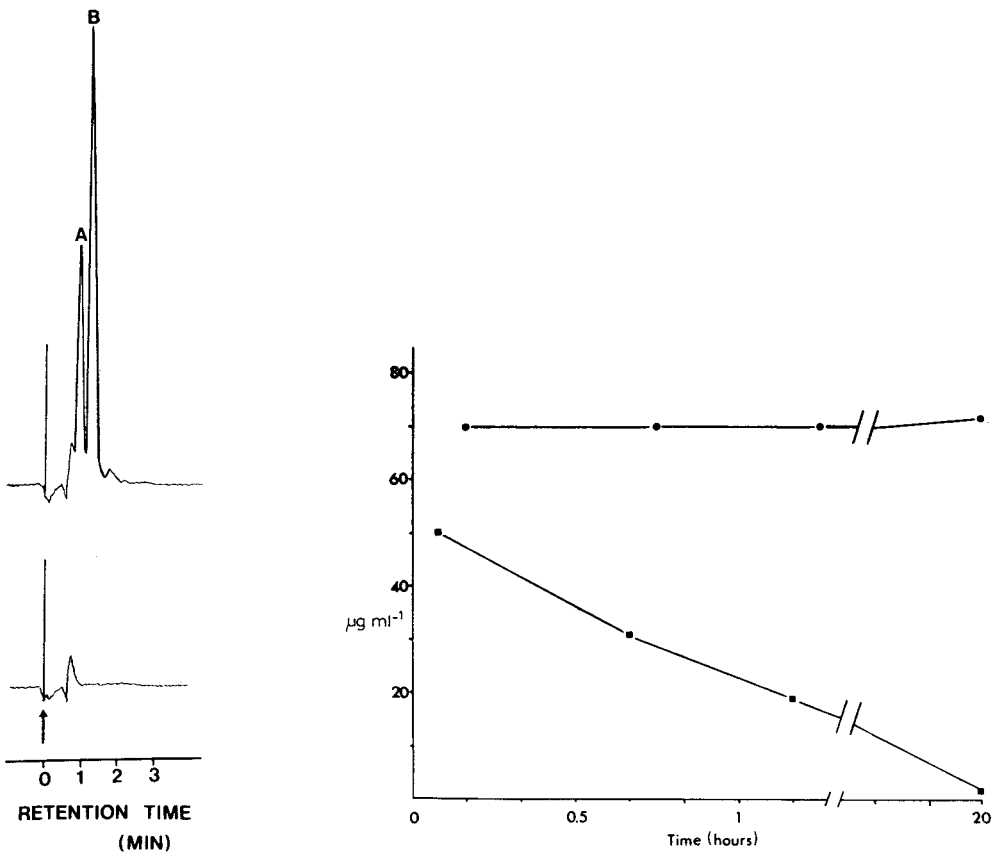


Fig. 4. (Top) Chromatogram of urine from a patient receiving 250 mg of penicillamine per day, 3.5 h after dosing. A = homocysteine, B = penicillamine. Internal standard is homocysteine 5 μg ml^{-1}; absorbance of peak = 0.054. Penicillamine absorbance = 0.103; this corresponds to 11.6 μg ml^{-1} penicillamine. (Bottom) Chromatogram of blank urine.

Fig. 5. Quantity of penicillamine detected in urine in the presence (•) and absence (▪) of EDTA over a period of 20 h.

been reported [5], and the chemical reduction of disulphides using Cleland's reagent (dithioerythritol, DTE) is well established [9]. Our attempts to use DTE to reduce disulphides before chromatographic separation were unsatisfactory because the injection of DTE rapidly destroyed the separating capacity of the analytical columns, and no satisfactory method of removing excess DTE was found. Alternatively, the disulphides might be reduced after chromatography (but before mixing with Ellman's reagent). Indeed post-column reaction and detection of disulphides has been described [10]. The availability of dihydrolipoamide covalently bound to glass beads suggested the possibility of placing a short column of these between the analytical column and the post-column reactor. Preliminary experiments showed that when the disulphides of cysteine and penicillamine were chromatographed on the system free thiols were detected in the eluent. Differences in the reactivity of the two disulphides were found, cystine being much more readily reduced than penicillamine di-

sulphide. Such differences in the reactivity of these two compounds have been noted previously [11], and were attributed to steric factors. Further work to find the best conditions for this disulphide-reducing column is in progress.

CONCLUSION

For the analyst penicillamine poses a number of problems of specific detection and stability. Our experiments with the post-column reaction of thiols with Ellman's reagent provide the basis for a rapid, sensitive and specific method for their analysis in biological fluids. The insensitivity of the system to contaminants means that sample preparation can be kept to a minimum.

Our preliminary experiments with post-column reduction of disulphides are promising and may allow the analysis of both reduced and oxidised forms of the drug in the same sample, without the need for prechromatographic chemistry.

New reagents that show marked changes in UV absorption [12] or fluorescence [13] in the presence of thiols have recently become available. Such compounds substituted for Ellman's reagent may provide even greater sensitivity and specificity. In this context we are currently examing monobromotrimethylammoniobimane and related compounds [13].

In conclusion, the procedures which we have described provide a basis for the analysis of penicillamine (and other thiols) in urine.

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Note

Assay of 5-aminosalicylate and its acetylated metabolite in biological fluids by high-performance liquid chromatography on dynamically modified silica

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(First received April 22nd, 1981; revised manuscript received July 10th, 1981)

Salicylazosulphapyridine (SASP) has for years been a cornerstone in the treatment of ulcerative colitis and Crohn's disease. Recently it has been suggested [1, 2] that the effect of this drug may be ascribed to the 5-aminosalicylate (5-ASA) moiety. This compound is formed by bacterial cleavage of SASP in the gut [3]. 5-ASA is mainly eliminated by acetylation, forming the metabolite acetyl-5-aminosalicylic acid (Ac-5-ASA) [4], which in turn is excreted by the kidneys. In order to measure these two substances in biological fluids, both spectrofluorimetry [5], colorimetry [6], and recently also an assay based on high-performance liquid chromatography (HPLC) [7] have been applied. The HPLC method, which is claimed to be both sensitive and specific, involves extraction and evaporation of the organic phase followed by redissolving in the mobile phase and injection on to the HPLC column. However, in our experience with this method, the recoveries from the extraction procedure only reached about 40% for 5-ASA and about 70% for Ac-5-ASA. Furthermore, 5-ASA, and particularly the internal standard *p*-aminosalicylate (PAS), were found to form interfering degradation products during the extraction and evaporation procedures. Finally, the assay as a whole is quite time-consuming, the chromatographic procedure alone lasting 30—40 min.

The aim of the present investigation was to develop an HPLC method involving dynamically modified silica [8, 9] in order to obtain a simple, rapid and sensitive method for the determination of 5-ASA and Ac-5-ASA in biological fluids. Furthermore, the assay should be reliable and specific enough for use in clinical experimental work.

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EXPERIMENTAL

Apparatus

A Waters liquid chromatograph consisting of a 6000A pump, a 710A WISP autoinjector, a 440 ultraviolet (UV) absorbance detector (254 nm), a 730 data module and a 720 system controller was used. The columns were thermostated in an LC 250/3 Kratos oven. Three different fluorescence detectors were used: a Perkin-Elmer LC 1000, a Perkin-Elmer 3000, or a Schoeffel FS 970 instrument.

For centrifugation an Ole Dich micro centrifuge was used.

Chemicals

5-Aminosalicylic acid, acetyl-5-aminosalicylic acid and the pharmaceutical preparations were kindly supplied by Ferring (Copenhagen, Denmark).

Acetonitrile HPLC S grade was obtained from Rathburn Chemicals (Walkerburn, Great Britain). All other chemicals were of analytical-reagent grade and obtained from E. Merck (Darmstadt, G.F.R.).

Sample preparation

A 1-ml volume of plasma or urine was mixed with 4 ml of methanol. After standing for at least 30 min the mixture was centrifuged for 1 min at 15,000 *g* and 20 μl of the supernatant were injected onto the column. Faeces were collected in and extracted by 500 or 1000 ml of methanol per day depending on the amount of faeces delivered; 10 μl of the centrifuged methanol extract were injected on to the column. Ileostomy effluents were at each collection suspended in 50 ml of 0.9% sodium chloride. This suspension was treated in the same way as the plasma samples and 20 μl of the resulting supernatant were injected on to the column.

Chromatography

The column set-up has been described previously [8]. The analytical column was a Knauer column 120 × 4.6 mm I.D., packed with LiChrosorb Si 60 (5-μm particles) using the dilute slurry technique [10]. The guard column (100 × 4.6 mm I.D.) situated between the pump and the autoinjector was dry-packed with LiChroprep Si 60. Both columns were operated at 40°C. The mobile phase was acetonitrile + 0.2 *M* potassium phosphate (pH 7.5) + water (30:5:65) containing 1.25 m*M* N,N,N-trimethylhexadecylammonium bromide, and the flow-rate was 1.5 ml min^{-1}.

RESULTS AND DISCUSSION

Detection limits, reproducibility and recovery

The detection limits, i.e. the sensitivity of the method defined as three times the noise, were determined by the use of a standard solution containing 5-ASA and Ac-5-ASA. The minimal detectable quantities are given in Table I for the four detectors examined. The results show a detection limit of 20 ng of Ac-5-ASA per ml plasma when using a Perkin-Elmer 3000 detector.

The reproducibility and precision of the method were determined by adding

standard solutions to plasma samples. The results (Table II) show a recovery between 96 and 103%. The precision of the assay was excellent as the coefficient of variation never exceeded 5%. No degradation of the compounds during analysis has been observed. The use of the internal standard (PAS) was not found to improve the precision or reproducibility of the assay, and has, therefore, been omitted. The between-run and within-run coefficients of variation (n = 6) were, in the concentration range 25—5000 ng/ml, in all cases less than 5%.

TABLE I

MINIMUM DETECTABLE QUANTITIES OF PAS, 5-ASA AND Ac-5-ASA MEASURED BY THREE DIFFERENT FLUORESCENCE DETECTORS AND BY A UV DETECTOR

Apparatus	Wavelength (nm)		Minimum detectable quantity (ng) injected on to column		
	Excitation	Emission	PAS	5-ASA	Ac-5-ASA
Perkin-Elmer LC 1000	315*	430	5	20	0.8
Perkin-Elmer 3000	315	430	0.5	2	0.08
Schoeffel FS 970	315	418**	0.4	0.1	0.06
Waters 440	254		1	5	2

*14-nm bandpass filter.
**Cut-off filter.

TABLE II

ANALYSIS OF PLASMA SAMPLES WITH STANDARD ADDITION OF PAS, 5-ASA AND Ac-5-ASA

The linear regression equation for the Ac-5-ASA calibration is $x = 1.03y - 8.49$, with $r = 1.000$.

Drug	Concentration (ng/ml)	Mean amount found (n = 6) (ng/ml)	Precision (C.V., %)	Recovery (%)
PAS	100	96	4.1	96
PAS	1000	984	2.0	98
5-AS	500	478	3.5	96
5-AS	5000	5150	2.2	103
Ac-5-ASA	25	23	5.1	92
Ac-5-ASA	100	101	2.5	101
Ac-5-ASA	500	512	2.0	102
Ac-5-ASA	1000	977	0.9	98
Ac-5-ASA	5000	4865	1.4	97

Specificity

The use of fluorescence detection provides a high degree of selectivity, and no interfering peaks from endogenous metabolites have been observed in any of

the sample materials analysed. A number of drugs have been tested but only other salicylates have been found to interfere. Salicylate has a longer retention time resulting in an extended analysis time to avoid interference with the next sample. The low detection limit for 5-ASA using the Schoeffel detector (Table I) is due to the use of a cut-off filter on the emission side. However, this results in less selectivity, which, on the other hand, in this particular case is an advantage. Some typical chromatograms are shown in Fig. 1.

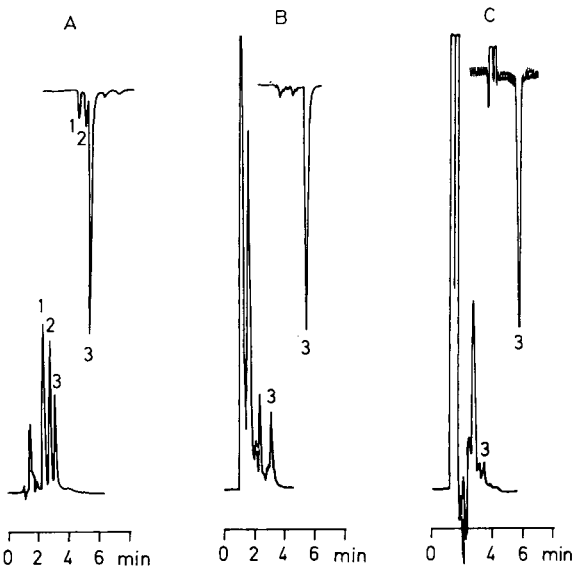

Fig. 1. Typical chromatograms of Ac-5-ASA in urine and plasma. Bottom traces: ultraviolet (254 nm) absorbance. Top traces: fluorescence detection (Perkin-Elmer 3000) at excitation 315 nm/emission 430 nm. (A) Standard mixture. 1 = PAS; 2 = 5-AS; 3 = Ac-5-ASA. (B) Urine sample. (C) Plasma sample (0.3 μg of Ac-5-ASA per ml of plasma).

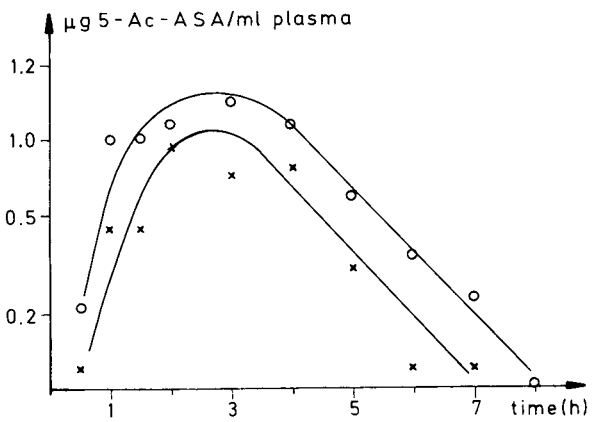

Fig. 2. Plasma concentrations of Ac-5-ASA in two patients after oral administration of 0.5 g of 5-ASA (slow release). (×), patient A; (○), patient B. See also Table III.

Application to clinical material

The assay has been applied to various biological materials (plasma, urine, faeces and ileostomy effluents) obtained from healthy volunteers and ileostomy patients receiving 500 mg of 5-ASA. Details of these clinical experiments are published elsewhere [11], but examples from this study are presented in Table III and Fig. 2. They concern the results of measurements of 5-ASA and Ac-5-ASA in the ileostomy effluents and urine and concentration—time curves for plasma Ac-5-ASA in two patients. The assay proved suitable for pharmacokinetic studies. The apparent half-life of elimination ($t_{½\beta}$) for Ac-5-ASA in plasma is about 70 min.

Once the method has been set up in the automated mode ten samples can be analysed per hour.

TABLE III

TOTAL AMOUNT OF 5-ASA AND Ac-5-ASA EXCRETED IN ILEOSTOMY EFFLUENT AND URINE FROM TWO PATIENTS AFTER ADMINISTRATION OF 0.5 g 5-ASA (SLOW RELEASE)

Patient	Time (h)	Amount of sample*			Amount eliminated (mg)		Total amount expressed as 5-ASA (mg)	
					5-ASA	Ac-5-ASA		
A	0—3	87.5	g	IE	0.0	0.0	0.0	
	3—4	3.5	g	IE	22.4	2.5	24.3	
	4—5	127.3	g	IE	240.2	91.9	312.3	
	5—6	33.1	g	IE	1.5	14.0	12.5	
	6—7	66.4	g	IE	1.8	19.3	16.9	
	7—8	84.7	g	IE	1.0	9.0	8.1	
	8—24	919	g	IE	0.0	0.0	0.0	
	0—1	45	ml	U	0.0	4.7	3.7	
	1—5	225	ml	U	0.0	81.1	63.6	
	5—8	180	ml	U	0.0	9.1	7.1	
	8—24	696	ml	U	0.0	0.0	0.0	
							448.5	(89.7%)**
B	0—3	13.7	g	IE	0.0	0.0	0.0	
	3—4	9.9	g	IE	79.7	22.7	97.5	
	4—5	14.2	g	IE	69.5	32.0	94.6	
	5—6	18.2	g	IE	75.7	38.5	105.9	
	6—7	1.9	g	IE	1.0	0.8	1.6	
	7—8	72.7	g	IE	23.7	40.9	55.8	
	8—24	426	g	IE	0.0	0.0	0.0	
	0—1	25	ml	U	0.0	6.6	5.8	
	1—5	675	ml	U	0.0	84.3	66.1	
	5—8	165	ml	U	1.4	13.5	10.6	
	8—24	313	ml	U	0.0	9.2	7.2	
	24—48	643	ml	U	0.0	0.0	0.0	
							429.8	(86.0%)**

*IE = ileostomy effluent; U = Urine.

**Percentage recovery.

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The author is grateful to Perkin-Elmer, Birkerød, Denmark, for access to the Perkin-Elmer 3000 fluorescence detector.

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Note

Determination of methyclothiazide in human plasma by high-performance liquid chromatography

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The oral diuretic—antihypertensive agent, methyclothiazide (MCT), 6-chloro-3-chloromethyl-3,4-dihydro-2-methyl-7-sulfamoyl-1,2,4-benzothiadiazine 1,1-dioxide, Aquatensen® (Wallace Laboratories, Cranbury, NJ, U.S.A.), is a member of the thiazide family of drugs. No assay method for methyclothiazide in human plasma has been published to date, but this report describes a high-performance liquid chromatography (HPLC) procedure developed in our laboratories, for the determination of the drug in human plasma.

EXPERIMENTAL

Materials

All reagents were of analytical grade. Aqueous solutions were prepared using deionized water (Milli-Q-Water System, Millipore Corp., Bedford, MA, U.S.A.). Glass-distilled methanol (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) was used for HPLC. Methyclothiazide (MCT) Lot No. JC1755 was from Wallace Laboratories (Division of Carter-Wallace, Inc., Cranbury, NJ, U.S.A.). Glass-distilled ethyl acetate (Burdick and Jackson Labs.) was used for plasma extraction. Acetophenetidine (phenacetin) and anhydrous magnesium sulfate were obtained from Sigma (St. Louis, MO, U.S.A.). Sodium hydroxide pellets were obtained from Mallinckrodt (Paris, KT, U.S.A.).

High-performance liquid chromatography

The chromatograph was a modular instrument, equipped with two Model 6000A pumps, a Model 720 system controller, a Model 710B "WISP" autosampler (all from Waters Assoc., Milford, MA, U.S.A.), a Model 770 variable-wave-

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length spectrophotometric detector and a Model LC 250/3 column oven, both from Kratos (Division of Schoeffel Instruments, Westwood, NJ, U.S.A.). A 10-μm μBondapak C_{18} column (Waters Assoc.) was used; the mobile phase was methanol—water (35:65). The column oven temperature was 35°C. A flow-rate of 1.5 ml/min was used yielding an operating pressure of approximately 1500 p.s.i. The spectrophotometric detector had an 8-μl cell volume and was operated at a wavelength of 225 nm.

Standards

A standard solution of MCT was prepared in methanol (1.0 mg/ml) and stored at 4°C. This solution was then diluted as necessary to prepare the appropriate plasma standards for each drug assay run. The internal standard of acetophenetidine (phenacetin) was also prepared in methanol (8 μg/ml) and stored at 4°C. Peak area ratios of MCT to phenacetin were determined for plasma standards.

Sample preparation procedure

To 2 ml of plasma (or standard) were added 2 ml of 0.1 *M* sodium hydroxide and 8 ml of ethyl acetate. The mixture was vigorously stirred for 30 sec on a Vortex Genie Mixer (Scientific Products, Evanston, IL, U.S.A.) and centrifuged for 10 min at 10°C and 2000 *g*.

The organic layer was drawn off and passed through a 35 mm × 6 mm column of anhydrous magnesium sulfate. The column was rinsed with an additional 2 ml of ethyl acetate. The extraction mixture was evaporated to dryness under nitrogen in a 50°C water-bath. The residue was redissolved in 100 μl of methanol, containing the internal standard phenacetin (8 μg/ml). The sampler injected a 20-μl volume onto the column of the high-performance liquid chromatograph.

Reproducibility and recovery

Reproducibility was determined for the concentration range of 5.0, 10.0, 20.0, 50.0, and 100.0 ng/ml MCT in plasma by quadruplicate analyses of samples at each concentration. Drug recovery from plasma after sample preparation was determined by comparison of the peak height ratios with those obtained from methanol solutions containing known concentrations of MCT.

RESULTS AND DISCUSSION

A linear relationship between the peak height ratio and plasma concentration of MCT exists in the range 5—100 ng/ml. The correlation coefficient r^2 is 0.9987.

The precision (reproducibility) of this method was determined by quadruplicate analyses of standard samples at each concentration. The results (Table I) show that the precision, expressed as the coefficient of variation (C.V.), was 8.3% or better for the concentration range 5—100 ng/ml.

The accuracy, calculated as the relative mean error*, was 4.7% or better for

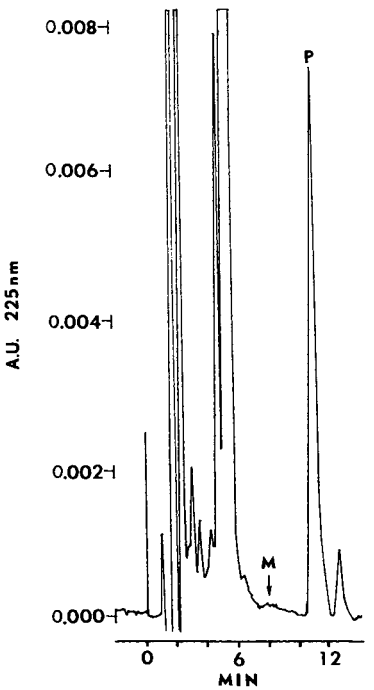
*Relative mean error = $\frac{\text{absolute value of theoretical} - \text{determined value}}{\text{theoretical value}} \times 100.$

TABLE I

PRECISION (C.V.) AND ACCURACY (M.E.) OF THE DETERMINATION OF METHYCLOTHIAZIDE IN HUMAN PLASMA IN THE RANGE 5—100 ng/ml

Theoretical concentration of MCT (ng/ml)	Calculated (mean ± S.D.)	C.V. (%)	M.E.* (%)
100	98.8 ± 4.5	4.5	1.2
50	52.5 ± 3.6	6.8	4.7
20	19.8 ± 1.2	6.1	1.0
10	10.0 ± 0.6	5.9	0.5
5	4.0 ± 0.3	8.3	25.8

*M.E. = relative mean error; see text for definition.



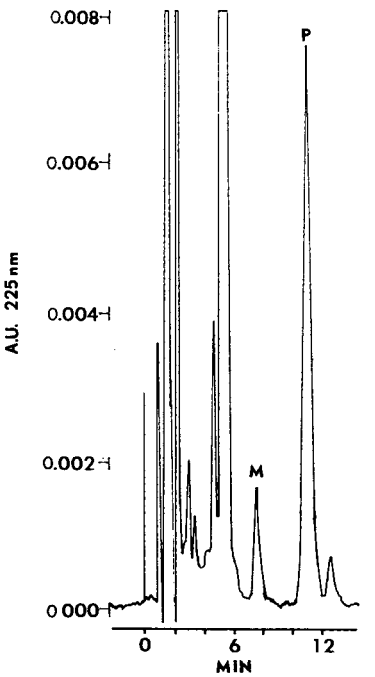


Fig. 1. Chromatogram of plasma from a patient before dosing with MCT (M). Phenacetin (P) as internal standard. Column, μBondapak C_{18}; mobile phase, methanol—water (35 : 65).

Fig. 2. Chromatogram of plasma from a patient after a single oral dose of 10 mg of MCT (M). See legend to Fig. 1 for further details.

the concentration range 10—100 ng/ml and 25.8% for the 5 ng/ml concentration. The accuracy is more commonly expressed as recovery, which for our method was 99.0—104.9% for the concentration range 10—100 ng/ml and 79.5% for the 5 ng/ml concentration.

In order to obtain a realistic estimate of the sensitivity of the assay, the limit of detection [1] was calculated on the basis of the peak height ratio value for

zero concentration as estimated from linear regression and the standard deviation for the lowest plasma concentration used. The limit of detection was found to be 1.5 ng/ml.

Complete (baseline) resolution of MCT and the internal standard phenacetin from endogenous plasma substances was considered a prerequisite for a good assay and was achieved using the mobile phase composition described (see Fig. 1). An example of an analysis of plasma obtained from a patient 3 h after an oral dose of 10 mg of MCT (two Aquatensen® tablets) is shown in Fig. 2. The determined concentration was 24 ng/ml.

Thiazides are used as step 1 therapy in the treatment of hypertension. More severe hypertension involves concomitant administration of other antihypertensive agents. We have determined that some of the common agents used, namely propranolol, prazosin, clonidine and methyldopa, do not interfere with the determination of MCT when these drugs were added at a concentration of 1 mg/ml to the sample and each sample carried through the procedure described in this paper.

The time needed for analysis was 75 min for sample preparation and 15 min for chromatographic analysis. In the automated mode many samples can be prepared within a few hours and with the automatic sampler capacity of 48 samples all samples can be analyzed in a 12-h overnight run.

CONCLUSION

An automated HPLC assay has been developed that is sufficiently sensitive, accurate and precise for the routine clinical monitoring of plasma levels of methyclothiazide. The presence of propranolol, prazosin, clonidine or methyldopa in plasma does not interfere with this assay.

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Note

Reversed-phase high-performance liquid chromatographic assay for the antineoplastic agent 9,10-anthracenedicarboxaldehyde bis(4,5-dihydro-1H-imidazol-2-yl hydrazone) dihydrochloride

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9,10-Anthracenedicarboxaldehyde bis(4,5-dihydro-1H-imidazol-2-yl hydrazone) dihydrochloride (ADAH) (Fig. 1) is an anthracene derivative with antitumor activity against several animal tumor model systems including L-1210 and P-388 murine leukemias, Lieberman plasma cell tumor, B-16 melanoma, Ridgeway osteogenic sarcoma and murine colon tumor 26 [1]. Studies in dogs show that ADAH exhibits less cardiotoxicity than adriamycin [2]. ADAH is currently undergoing clinical evaluation as an anticancer agent in humans. There is no information on the pharmacologic behavior of ADAH in humans. As a preliminary step to studies in humans a sensitive reversed-phase high-performance liquid chromatographic (HPLC) assay for ADAH in plasma and other biological fluids has been developed. The assay has been used to study the disposition of ADAH in rabbit and a patient receiving ADAH in phase II clinical trial.

EXPERIMENTAL

Drugs

9,10-Anthracenedicarboxaldehyde bis(4,5-dihydro-1H-imidazol-2-yl hydrazone) dihydrochloride (CL 216,942) was supplied by Dr. K.C. Murdock, American Cyanamid Company, Lederle Laboratories, Pearl River, NY, U.S.A.; 1-[2-(2-hydroxyethyl-amino)ethylamino]-4-hydroxy-9,10-anthracenedione (NSC 299,187) was supplied by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD, U.S.A.; β-glucuronidase was obtained from Boehringer Mannheim, Indianapolis, IN, U.S.A.

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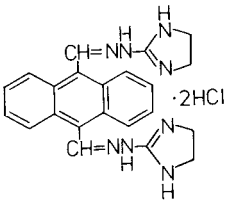


Fig. 1. Structure of ADAH.

Animal and patient studies

Male New Zealand white rabbits weighing between 2 and 3 kg were injected intravenously with ADAH in 3 ml 0.9% NaCl containing 10% (w/v) ethanol at doses between 50 and 150 mg/m^2, over a 1-min period into a peripheral ear vein using a vein infusion set with a winged adapter (Miniset, Travenol, Deerfield, IL, U.S.A.). Blood was collected into heparinized tubes at different times from a peripheral vein of the other ear vein. Urine was collected for 24 h following administration of the drug by placing the animal in a stainless-steel metabolism cage. The bile duct of one rabbit was cannulated and the bladder catheterized through the urethra under pentobarbital anesthesia. The animal was allowed to recover for 3 h before being given ADAH, and bile and urine were collected from the exteriorized bile cannula and bladder catheter for 24 h. ADAH was administered to a patient with cancer at a dose of 260 mg/m^2 as a 90-min intravenous infusion as part of a phase II clinical trial.

Preparation of samples

A 2-ml volume of plasma, or a lesser volume of urine or bile diluted to 1 ml with water, was mixed with an equal volume of 1 *M* sodium phosphate buffer (pH 10) and extracted with 8 ml of ethyl acetate. The efficiency of extraction of ADAH was 95%. In most cases a standard of 1 μg of 1-[2-(2-hydroxyethyl-1-amino)ethylamino] 4-hydroxy-9,10-anthracenedione (alkyl AAD) was added to the sample prior to extraction to provide an internal measure of extraction efficiency. The ethyl acetate extract was taken to dryness under nitrogen and the residue dissolved with gentle warming in 300 μl of methanol. A 100-μl sample was taken for HPLC. Bile and in some cases urine samples, 0.5 ml, were mixed with 50 μl of 1 *M* sodium acetate buffer (pH 4.0) and incubated overnight with β-glucuronidase, 10 U/ml, prior to extraction.

High-performance liquid chromatography

ADAH was separated by reversed-phase HPLC on a 25-cm C_2-bonded LiChrosorb RP-2 column, 5 μm particle size (E. Merck, Darmstadt, G.F.R.) with a 10-min linear gradient of 5% to 100% methanol in 0.5 *M* sodium perchlorate (pH 5.3) at a flow-rate of 2 ml/min. The sodium perchlorate solution was cleaned by passing it through a bed of silica gel and activated charcoal before use. Eluting compounds were detected at 430 nm. Alkyl AAD was measured by a second chromatographic run with detection at 500 nm. A Hewlett-Packard 1084B liquid chromatograph and variable-wavelength detector

798575A were employed. Reproducibility of injection was < 1%. Wavelengths are uncorrected values. The output from the detector was fed into a Hewlett-Packard 79850B liquid chromatograph terminal and peak areas integrated.

Pharmacokinetic analysis

Nonlinear least-square regression analysis of the data to obtain pharmacokinetic parameters [3] employed the SAS NLIN pharmacokinetic program with a weighting factor of $1/y^2$ [4]. Allowance was made for a 1-min infusion of drug in rabbit.

RESULTS AND DISCUSSION

ADAH was bound tightly to the C_2-bonded reversed-phase support and could not be eluted with acetonitrile as the mobile phase although it was eluted as a broad peak with methanol. When a gradient of 5% to 100% methanol in 10 m*M* sodium acetate (pH 4.0) buffer was used ADAH still eluted as a broad peak. A gradient of 5% to 100% methanol in 0.1 *M* to 0.5 *M* sodium perchlorate at acid pH produced a sharp ADAH peak. Optimum resolution was obtained by a linear gradient of 5% to 100% methanol in 0.5 *M* sodium perchlorate (pH 5.3). Sodium perchlorate contained contaminants which bound to the column and eluted with methanol, interfering with the assay for ADAH. These contaminants were removed by passing the sodium perchlorate solution through a bed of silica gel and activated charcoal before use.

Freshly prepared ADAH dissolved in methanol or plasma eluted as a single peak on HPLC (Fig. 2). The lower level for detection of ADAH in rabbit plasma was 25 ng/ml and the assay was linear up to least 10 μg/ml. Urine and bile samples were diluted appropriately to bring ADAH levels into this range. The coefficient of variation of the assay at 0.1 μg of ADAH per ml of plasma was ± 7.3%. Alkyl AAD was used as an internal standard for the extraction procedure. We were unable to find a suitable internal standard which

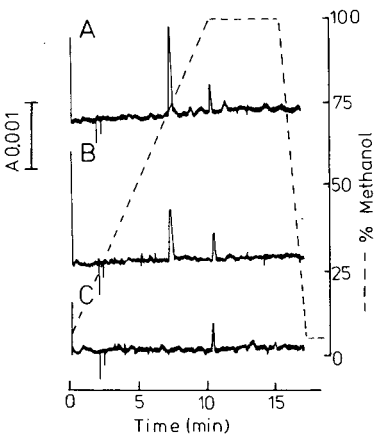


Fig. 2. Chromatograms of ADAH added to rabbit plasma: (A) 50 ng/ml; (B) 25 ng/ml; and (C) control plasma. Chromatographic conditions are described in the text. Detection at 430 nm.

could be measured at the same detector wavelength as ADAH. Alkyl AAD had therefore to be determined separately in a second chromatographic run with a detector wavelength of 500 nm.

If a sample of 10 μg of ADAH in methanol was exposed to fluorescent room light for 12 h, additional peaks were formed (Fig. 3). There was a major peak eluting before the peak due to ADAH and a smaller peak eluting after the peak due to ADAH. The formation of additional peaks also occurred with nitrogen-saturated ADAH solution exposed to light. Equilibrium between the forms represented by the different peaks appeared to have been reached by 12 h and exposure to light for periods of up to 40 h produced no further change in the pattern of the peaks. The absorption spectra of the new major peak, of parent ADAH and of the third smaller peak were similar with maxima at, respectively, 270 nm, 375 nm and 432 nm (uncorrected wavelengths, Hewlett-Packard variable-wavelength detector).

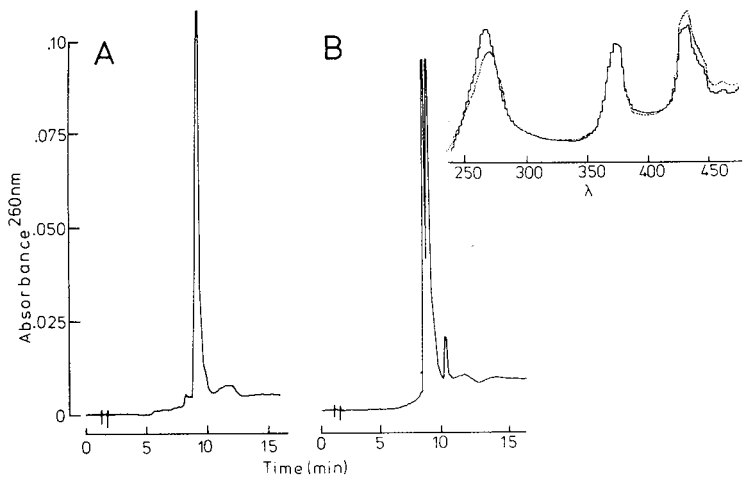
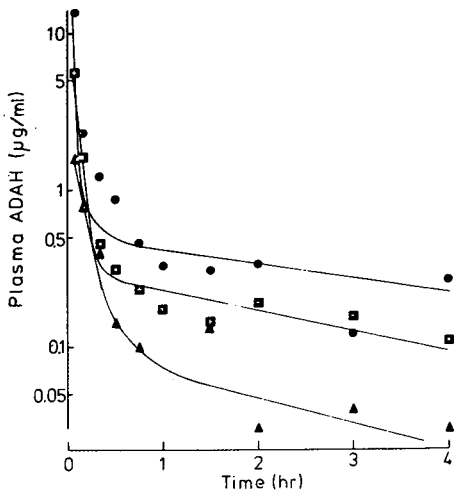


Fig. 3. Chromatograms of (A) 1 μg of ADAH prepared as a 10 μg/ml solution in methanol and stored overnight at room temperature in the dark, and (B) 1 μg of ADAH, prepared as a 10 μg/ml solution in methanol and placed on a bench surface under fluorescent room lights overnight. Inset are wavelength scans of the two major peaks, full line the first peak which eluted, dotted line the second peak which eluted (which had the same retention time as parent ADAH). Chromatographic conditions are described in the text.

ADAH is administered to humans by infusion dissolved in 5% (w/v) dextrose at a concentration of approximately 1 mg/ml. There was no detectable conversion of ADAH to other components when 1 mg of ADAH per ml of 5% dextrose was exposed to light for 4 h. However, a more dilute solution, 10 μg of ADAH per ml of 5% dextrose, showed a 13.6% conversion to a more rapidly eluting peak when exposed to light for 4 h. The nature of the additional peaks is not known. One possibility is that the peaks represent the three possible isomers of ADAH resulting from the *syn* and *anti* configurations of the hydrazone groups. When assaying ADAH in plasma, urine and bile, a second peak eluting before the parent ADAH peak was always apparent despite precautions to keep the samples dark. Consequently, in the assay for ADAH

both peaks were added together. By limiting exposure of the samples to light the peak eluting before parent ADAH was usually less than 10% of the total area.

Plasma concentrations of ADAH in the rabbit fell rapidly following intravenous injection of ADAH (Fig. 4). The data were best described by a two-compartment open model. Pharmacokinetic parameters are shown in Table I. Relatively little unchanged ADAH was excreted in the urine. The 24-h urinary excretion of ADAH at doses of 50, 100 and 150 mg/m^2 was 11.7, 5.8 and 7.4% of the dose administered, respectively. In 24 h 7.8% of ADAH administered was excreted unchanged in the bile of an animal with a cannulated bile duct receiving 100 mg of ADAH per m^2 (Fig. 5). A small amount of ADAH was excreted in the bile as a glucuronide conjugate detected by an increase in free ADAH after β-glucuronidase digestion. In the rabbit in which bile samples were collected, 2.6% of the administered dose of ADAH was excreted in the urine in 24 h as unchanged ADAH and 3.8% as a glucuronide conjugate of ADAH.



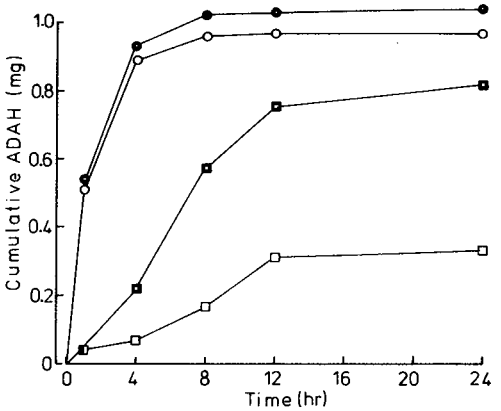


Fig. 4. Plasma ADAH concentration in rabbit plasma following intravenous injection of ADAH: (▲) 50 mg/m^2, (■) 100 mg/m^2, and (●) 150 mg/m^2. Points are experimental observations, continuous lines are computer fits to the data.

Fig. 5. Biliary (●,○) and urinary (■,□) excretion of ADAH. The rabbit received 12.5 mg of ADAH (100 mg/m^2). Bile and urine were collected at various times for 24 h through an exteriorized bile cannula and bladder catheter. The filled symbols represent total ADAH after treatment of the sample with β-glucuronidase, the open symbols free ADAH without β-glucuronidase treatment.

Control human plasma contained peaks detectable at 430 nm not seen in rabbit plasma. The lower limit for detection of ADAH in human plasma was 50 ng/ml. Chromatograms of patient plasma before and after receiving ADAH are shown in Fig. 6. Plasma concentrations of ADAH in the patient fell relatively rapidly following cessation of the intravenous infusion of ADAH but ADAH could still be detected 24 h later (Fig. 7). The concentration of ADAH in

TABLE I

PHARMACOKINETIC PARAMETERS OF ADAH IN RABBIT

Rabbits were administered ADAH at the doses shown. $t_{1/2\alpha}$ and $t_{1/2\beta}$ are the distributive and post-distributive half-lives. V_1 and V_2 the volumes of the central and peripheral compartments, and Cl the total body clearance of ADAH.

Dose (mg/m²)	$t_{1/2\alpha}$ (min)	$t_{1/2\beta}$ (min)	V_1 (l/m²)	V_2 (l/m²)	Cl (l/min/m²)
50	5.7	113.0	17.6	495.6	1.30
100	1.8	138.3	2.3	313.8	0.57
150	1.9	201.2	1.5	288.2	0.36

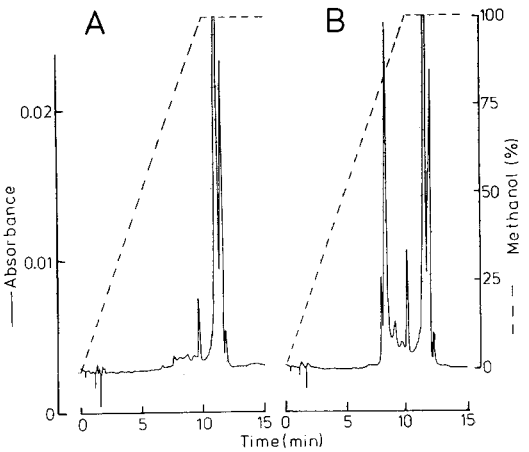


Fig. 6. Chromatograms of patient plasma: (A) before treatment; (B) 60 min after receiving a 90-min infusion of ADAH, 260 mg/m². This sample contained 1.0 μg of ADAH per ml.

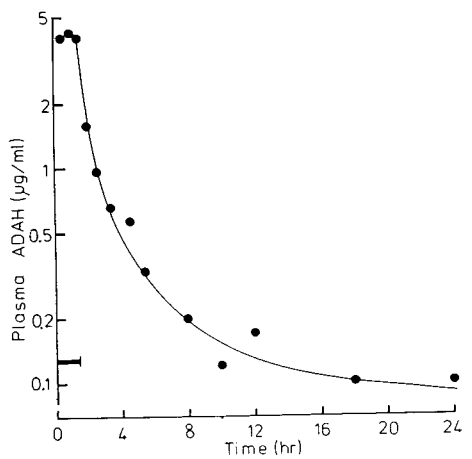


Fig. 7. Plasma ADAH concentration in a 62-kg female cancer patient receiving intravenous infusion of ADAH, 260 mg/m² over 90 min (shown by the bar).

plasma 24 h after administration was above the level, 50 ng/ml for 1 h, that completely inhibits growth of some human tumor cell lines in the in vitro soft agar colony forming assay [5].

In summary, a sensitive reversed-phase HPLC assay for ADAH has been developed which is suitable for measuring ADAH in the plasma and urine of humans receiving the drug in clinical trial. ADAH is sensitive to light and in solution exposed to light forms a second and a third component which can be separated from the parent ADAH by HPLC. The three peaks have similar absorption spectra. Studies in the rabbit have shown that plasma ADAH concentrations fall rapidly in a biphasic manner following intravenous administration of the drug, $t_{½\alpha}$ = 3.1 min and $t_{½\beta}$ = 151 min and a mean total body clearance of 743 ml/min/m^2. Between 6 and 12% of the dose of ADAH is excreted unchanged in the urine in 24 h. A further small proportion of ADAH is excreted in the urine as glucuronide conjugates. In a rabbit with a cannulated bile duct 8% of a dose of ADAH was excreted unchanged in the bile in 24 h, mostly in the first 4 h after ADAH administration. Preliminary studies in a patient receiving ADAH have shown that plasma ADAH concentrations also fall rapidly but that ADAH can still be detected in the plasma 24 h later. Plasma concentrations of ADAH have been measured in a patient receiving ADAH in Phase II clinical trial.

ACKNOWLEDGEMENTS

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Note

Rapid high-performance liquid chromatographic assay for the anthracyclines daunorubicin and 7-con-O-methylnogarol in plasma

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(First received May 4th, 1981; revised manuscript received July 15th, 1981)

The anthracyclines doxorubicin and daunorubicin** (Fig. 1) are important antitumour drugs but show a dose-limiting cardiotoxicity [1, 2]; hence, less toxic anthracyclines are being sought [3]. Rapid reliable assays are needed for in vivo studies on such compounds: here we consider daunorubicin and 7-con-O-methylnogarol. The former is a "type" compound for the group, and an assay for it in the blood of infected animals was needed for investigation of its trypanocidal effects [4]; the latter compound is the most promising semi-synthetic derivative of the anthracycline nogalamycin [5].

Fluorescence assay will not distinguish between anthracyclines and their fluorescent metabolites [6], and thin-layer chromatography is also inappropriate since hydrolysis of drug may occur [1]. High-performance liquid chromatography (HPLC) is the method of choice. Normal-phase HPLC has been reported [7—11], but the drug must be extracted into organic phase so there may be problems of recovery. Reversed-phase HPLC has also been used [7, 12—17]; in all cases an extraction (solvent extraction or adsorption on to a pre-column) was performed, yielding an organic phase which was either evaporated or back-extracted, so again there are problems of recovery. The procedure described here maximises recovery of drug and metabolites (irrespective of partition properties), and minimises transferences, by precipitation of protein from the plasma followed by direct injection of supernatant on to the HPLC column. Whilst this work was in progress, a similar method was reported for doxorubicin by Quattrone and Ranney [18].

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**Daunorubicin is the International Non-Proprietary Name for the compound given the trivial name daunomycin.

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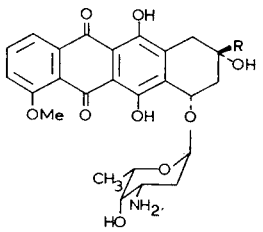
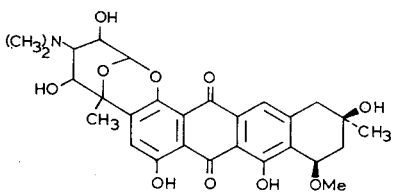


Doxorubicin	$R=COCH_2OH$
Daunorubicin	$R=COCH_3$
Daunorubicinol	$R=CH(OH)CH_3$



7-con-o-methylnogarol

Fig. 1. Structural formulae of doxorubicin, daunorubicin, daunorubicinol and 7-con-O-methylnogarol (the stereochemistry of the latter compound is not yet fully verified but is most probably that shown here [5]).

MATERIALS AND METHODS

Materials

Daunorubicin hydrochloride and daunomycinone were gifts from Dr. F. Arcamone (Farmitalia, Milan, Italy); 7-con-O-methylnogarol was a gift from Dr. P.F. Wiley (The Upjohn Company, Kalamazoo, MI, U.S.A.). Acetonitrile was HPLC grade (Fisons, Loughborough, Great Britain) and phosphoric acid was analytical grade (BDH, Poole, Great Britain). Water was double-distilled before use. All glassware was silanised to prevent adsorption of drug. All drug solutions were protected from light and were stored refrigerated.

Method

General extraction method. Acetonitrile—0.1 *M* H_3PO_4 (4 : 1) (1 ml) was added to human plasma (1 ml) in a stoppered centrifuge tube; the mixture was vortex-mixed for 10 sec then centrifuged at 2200 *g* for 10 min to pellet the precipitated protein. The supernatant (20 μl or 100 μl) was chromatographed and the peak height and peak area were compared with those from standards prepared by the addition of drug to a centrifuged plasma—acetonitrile—0.1 *M* H_3PO_4 (5 : 4 : 1) mixture.

High-performance liquid chromatography. A Pye LC-XPD pump was used with a six-port rotary injection valve (Rheodyne 7125) and either a 20-μl or 100-μl loop, and a 250 × 4.6 mm I.D. LiChrosorb RP-2 (5 μm) column. The mobile phase was 35% acetonitrile in 0.01 *M* H_3PO_4 at a flow-rate of 1 ml min^{-1} (ambient temperature) and detection was with a 25-μl mirrored flow cell in a Perkin-Elmer 3000 fluorescence spectrometer (Figs. 2 and 3).

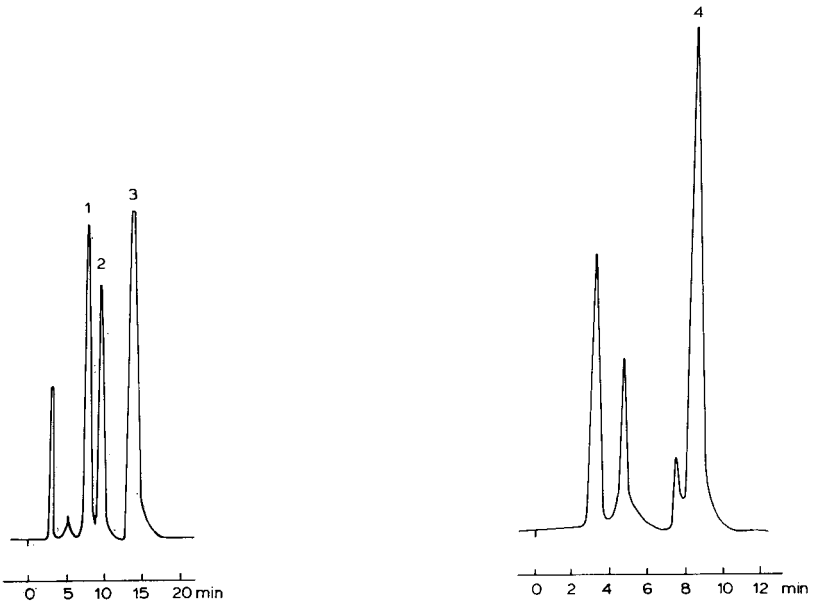


Fig. 2. Separation of daunomycinol (1, retention time 7.5 min) daunorubicin (2, 9.5 min) and daunomycinone (3, 14.0 min) in supernatant from precipitated plasma. Stationary phase, LiChrosorb RP-2; mobile phase, 35% acetonitrile in 0.01 *M* phosphoric acid; pressure, 120 bar; flow-rate, 1.0 ml min^{-1}; fluorescence detection, excitation 475 nm (15 nm slit), emission 557 nm (20-nm slit).

Fig. 3. Separation of 7-con-O-methylnogarol (4, retention time 8.3 min) in supernatant from precipitated plasma. Conditions as for Fig. 2, except excitation 471 nm (15-nm slit), emission 550 nm (20-nm slit).

Evaluation of fluorescence enhancement. Solutions containing daunorubicin (500 ng ml^{-1}) were prepared in mixtures of plasma and acetonitrile—0.01 *M* H_3PO_4 (4 : 1) in ratios from 25 : 75 to 75 : 25. This was repeated using water in place of plasma. All solutions were vortex-mixed for 10 sec then centrifuged at 100,000 *g* for 2 h. The fluorescence of each solution was determined at 30°C, with the excitation monochromator at 475 nm (5-nm slit) and the emission monochromator at 557 nm (10-nm slit).

RESULTS

The method developed here for the assay of daunorubicin in plasma uses precipitation of proteins from the plasma by addition of an equal volume of acetonitrile—0.1 *M* H_3PO_4 (4 : 1), so avoiding losses during solvent extraction and during transferences. An aliquot of the supernatant is then chromatographed. Since fluorescence detection was used, it was necessary to verify that there is no change in the fluorescence properties of the drug when it is applied to the column in this form compared to a solution in water. Preliminary experiments showed that the fluorescence of drug detected on elution from the column is enhanced in the treated plasma sample compared to the water standards. This change is presumably due to co-eluting material.

This enhancement of daunorubicin fluorescence was further investigated by determination of the fluorescence of daunorubicin (500 ng ml^{-1}) in centrifuged mixtures of plasma and acetonitrile—0.1 *M* H_3PO_4 (4 : 1) and in centrifuged mixtures of distilled water and acetonitrile—0.1 *M* H_3PO_4 (4 : 1). The fluorescence of daunorubicin in those samples containing water was independent of the ratio of water to acetonitrile—0.1 *M* H_3PO_4. In samples containing plasma, the fluorescence of daunorubicin increased as the ratio of plasma to acetonitrile—0.1 *M* H_3PO_4 (4 : 1) increased, and the fluorescence was enhanced compared to the samples containing water (for example, at a 35 : 65 ratio of plasma to acetonitrile—0.1 *M* H_3PO_4 the fluorescence was 104% of that in samples containing water, whereas at a 65 : 35 ratio it was 122% of that in the water-containing samples). The relevance of this fluorescence enhancement to the HPLC assay was next evaluated by comparison of the peaks from standards of daunorubicin in distilled water to which an equal volume of acetonitrile—0.1 *M* H_3PO_4 (4 : 1) was added, with those from solutions prepared by addition of daunorubicin to treated plasma (that is, plasma to which an equal volume of acetonitrile—0.1 *M* H_3PO_4 (4 : 1) had been added, followed by centrifugation). Hence, in the first case, the concentration of daunorubicin in water—acetonitrile—0.1 *M* H_3PO_4 (5 : 4 : 1) was accurately known, and in the second case the concentration of daunorubicin in centrifuged plasma—acetonitrile—0.1 *M* H_3PO_4 (5 : 4 : 1) was accurately known. The values of peak height for the daunorubicin in the "plasma" samples were consistently 10% higher than the corresponding values for the "water" samples. Consequently in all further work, drug standards were always prepared in plasma—acetonitrile—0.1 *M* H_3PO_4 (5 : 4 : 1). Standards typically gave calibration curves with a correlation coefficient of 0.998.

The efficiency of recovery of the assay method was next assessed by triplicate assay of spiked plasma samples containing between 10 ng ml^{-1} and 5 μg ml^{-1} daunorubicin. The plasma was treated by addition of an equal volume of acetonitrile—0.1 *M* H_3PO_4 (4 : 1) and centrifugation. The recovery of daunorubicin in the 100 ng ml^{-1} to 5 μg ml^{-1} range was 99.1% (s = 1.24, n = 5) and for the whole range, 10 ng ml^{-1} to 5 μg ml^{-1}, was 92.3% (s = 12.3, n = 8). The procedure was repeated for the aglycone (daunomycinone); for the 100 ng ml^{-1} to 5 μg ml^{-1} range the recovery was 96.6% (s = 5.82, n = 5) and for the whole 10 ng ml^{-1} to 5 μg ml^{-1} range was 95.0% (s = 5.44, n = 8). For 7-con-O-methylnogarol there was excellent recovery over the whole range from 10 ng ml^{-1} to 5 μg ml^{-1} (98.5%, s = 1.12, n = 5).

DISCUSSION

Reversed-phase HPLC was chosen as the method here since it gives the opportunity to develop an assay where manipulation of a biological sample is minimised. LiChrosorb RP-2 was used since it has been shown previously [12] that selectivity of the assay is not affected as the chain length of the bonded group is altered; RP-2 gives shorter retention times than octylsilyl- and octadecylsilyl-bonded phases. Isocratic elution with a solvent similar to that of Eksborg et al. [13] gave good separation of daunorubicin, daunorubicinol and daunomycinone, so allowing assay of parent drug and these metabolites.

An evaluation of the effect of the assay procedure on the fluorescence properties of the drug was necessary since the quantum yield of a drug (and hence fluorescence intensity) is sensitive to changes in factors such as solution composition. It was found that fluorescence was enhanced, so standards should always be prepared in a solution of the same composition as the sample.

The assay method developed here is very rapid and is carried out in a single tube, thus minimising losses. It will extract metabolites as well as parent drug. The recovery for all compounds evaluated (daunorubicin, daunomycinone and 7-con-O-methylnogarol) was 96% or greater for the 100 ng ml^{-1} to 5 μg ml^{-1} range. As concentration of drug was reduced, recovery of daunorubicin and daunomycinone decreased but recovery of 7-con-O-methylnogarol remained at 98.5%. The method is thus a very rapid, reproducible assay for anthracyclines and their metabolites in plasma, and the sensitivity compares well with that of other reported methods. We have also extended the assay to determination of anthracyclines in homogenised cell samples.

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Note

Determination of the diuretic agent metolazone in plasma by high-performance liquid chromatography

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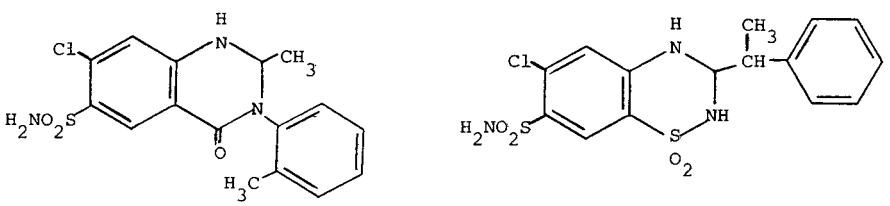
Metolazone (Fig. 1) is a recently introduced diuretic drug structurally and pharmacologically related to quinethazone [1]. It causes increased excretion of sodium and chloride, and, to a lesser extent, of potassium. On a weight basis the drug is about ten times more potent in the rat for promoting the excretion of sodium than is quinethazone or hydrochlorothiazide, but its effect on the excretion of potassium in humans is less than that produced by other diuretics [2].



Metolazone Bemetizide

Fig. 1. Chemical structures of metolazone and internal standard (bemetizide).

Metolazone has been measured in urine by high-performance liquid chromatography (HPLC) over the concentration range of 1—10 μg/ml [3, 4] and more recently by fluorimetry over the concentration range 0.1—10 μg/ml [5]. In order to obtain reliable pharmacokinetic data, accurate, precise, sensitive (< 10 ng/ml) and specific methods of measurement of drug concentrations in plasma are usually required. This paper describes an HPLC method for the measurement of metolazone in plasma over the concentration range

2—200 ng/ml. It involves a simple liquid—liquid partition extraction stage followed by chromatography in a reversed-phase mode and detection by ultraviolet (UV) absorption at 236 nm. The thiazide diuretic bemetizide is used as the internal standard and the method has been applied to the measurement of unchanged drug in the plasma of rhesus monkeys receiving oral doses of 2.5 mg of metolazone administered in solution.

Since the work described in this paper was performed, another HPLC method for the measurement of metolazone in plasma and urine has been published [6]. This method requires the use of a fluorimetric detector and although this appears to be a precise and perhaps more sensitive (1 ng/ml) method than the one described here (2 ng/ml), it requires the extraction of a larger plasma volume (2 ml instead of 0.5 ml), which is not always practical, particularly in the case of animal studies as are described here.

EXPERIMENTAL

Materials

All reagents were of analytical grade and all inorganic reagents were prepared in freshly glass-distilled water. Acetonitrile was HPLC grade (Fisons Scientific Apparatus, Loughborough, Great Britain) and diethyl ether was freshly redistilled prior to use. Metolazone (7-chloro-1,2,3,4-tetrahydro-2-methyl-4-oxo-3-*o*-tolyl-6-quinazolinesulfonamide) was provided by Sandoz, Basle, Switzerland, and bemetizide [3-(α-methylbenzyl)-6-chloro-7-sulphamoyl-3,4-dihydro-1,2,4-benzothiadiazine-1,1-dioxide], used as internal standard, was from Sanol Schwarz-Monheim, Monheim, G.F.R. Standard solutions of metolazone were prepared in methanol at concentrations of 1 μg/ml and 10 μg/ml and bemetizide at 11 μg/ml, and stored at −20°C throughout the study.

Extraction procedure

Plasma samples (0.5 ml) were transferred into 10-ml pointed centrifuge tubes and spiked with internal standard (5 μl, containing 55 ng of bemetizide). Distilled water (0.5 ml) was added together with sodium hydrogen carbonate (about 0.2 g) and the samples were extracted by mixing them with diethyl ether (5 ml) for 30 sec using a vortex mixer. After centrifugation of the extract at 2000 *g* for 10 min, the organic layer was transferred to another pointed centrifuge tube and the solvent 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 ether which was again removed by evaporation. The residue was taken up in mobile phase (40 μl, see later), centrifuged at 2000 *g* for 10 min, and the resulting clear solution transferred to autosampling vials. Aliquots (15 μl) of this solution were injected into the chromatograph.

Liquid chromatography

The liquid chromatograph consisted of a Waters M6000A pump (Waters Assoc., Northwich, Great Britain) coupled to a Pye LC3 variable-wavelength UV-absorption detector (Pye-Unicam, Cambridge, Great Britain) operated at 236 nm. Injection was via an automatic injector (Wisp™, Waters Assoc.). Chromatograms were recorded on a Hewlett-Packard 3380A recording inte-

grator (Hewlett-Packard, Slough, Great Britain) using a 1-V input and an attenuation setting of 16; peak height measurements were preferred to peak area measurements. The column was constructed of stainless steel (30 cm × 0.4 cm I.D.), pre-packed with μBondapak C_{18} (mean particle diameter 10 μm) (Waters Assoc.). 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, Great Britain) was installed in front of the analytical column to protect it from contamination and was changed routinely if the back-pressure in the system increased.

Chromatography was performed in a reversed-phase mode using a mobile phase of 40% (v/v) acetonitrile in aqueous potassium dihydrogen orthophosphate (0.1%, w/v) with a flow-rate of 2 ml/min. The retention times of metolazone and bemetizide (internal standard) were 3.6 min and 5.5 min, respectively (Fig. 2).

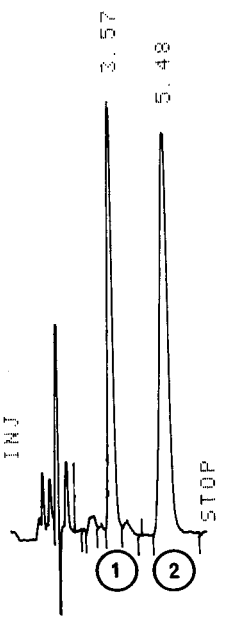


Fig. 2. Chromatogram of reference standards; peak 1 = metolazone, peak 2 = internal standard. Column, 30 × 0.4 cm I.D., pre-packed with μBondapak C_{18}; flow-rate, 2 ml/min; solvent system, 40% (v/v) acetonitrile—aqueous potassium dihydrogen orthophosphate (0.1%, w/v); detector, UV at 236 nm.

Calibration procedure

Calibration lines of peak height ratio measurements of metolazone to internal standard were constructed over the concentration range up to 200 ng/ml. Samples of blank (drug-free) plasma (0.5 ml) were spiked with metolazone to give concentrations of 2, 10, 40, 100, 140 and 200 ng/ml and with internal standard at a fixed concentration of 110 ng/ml. The samples were taken through the extraction procedure described previously.

Studies in monkeys

Plasma samples were obtained from eight adult female rhesus monkeys (*Macaca mulatta*) (bodyweights about 5—7 kg) after administration of 2.5 mg of metolazone, dissolved in a mixture of propylene glycol—water (5 ml, 2 : 3, v/v), given by oral intubation and washed in with distilled water (5 ml). During 16 h before dosing and for 6 h thereafter, each monkey was fasted, although water was allowed during this period.

Blood samples (3 ml) were withdrawn from the femoral veins of the animals into heparinised tubes before dosing and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 24, 32, 48, and 72 h after dosing. Blood cells were separated by centrifugation and discarded. The separated plasma was stored at −20°C until analysed. Metolazone was found to be stable in plasma stored at −20°C for several weeks.

RESULTS AND DISCUSSION

Precision

Extraction and measurement at each concentration was repeated on five occasions. The precision of the method for the measurement of metolazone in plasma as indicated by the coefficient of variation of peak height ratio measurements of drug to internal standard (Table I) was ± 12% at 10 ng/ml, ± 4% at 100 ng/ml and ± 6% at 200 ng/ml; the precision at the limit of detection of the method, 2 ng/ml, was ± 50%.

TABLE I

RECOVERY AND PRECISION MEASUREMENTS OF METOLAZONE FROM PLASMA

Concentration of metolazone added to plasma (ng/ml)	Recovery* (%)	Coefficient of variation (%)
10	72	12
40	73	8
100	72	4
140	72	4
200	74	6
Mean	73 ± 1 S.D.	

*Mean of five determinations at each concentration.

Accuracy

The calibration line for the measurement of metolazone in plasma was constructed from five replicate measurements at six concentrations over the range up to 200 ng/ml; plots of peak height ratio against concentration of metolazone (ng/ml) were linear ($y = 0.0110x - 0.0315$) and the value of the intercept was not significantly different from zero ($p > 0.05$).

The variance about the calibration was found to be concentration depen-

dent (inhomogeneous) and Bartlett's statistic [7] was significant at the 1% level.

The variance was homogenised by logarithmic transformation of the data, and a plot of $\log_e$ of peak height ratio against $\log_e$ concentration was linear ($\log_e y = a + b \log_e x$) where $a = -5.0429$ and $b = 1.1037$. The accuracy of the method as indicated by the coefficient of variation about the fitted line was ± 16% over the concentration range 2—200 ng/ml.

Recovery

The recovery of internal standard (110 ng/ml) from plasma (0.5 ml) was 80% ± 3 S.D. ($n = 5$). The mean absolute recovery of metolazone from plasma over the concentration range 10—200 ng/ml was determined by comparison of peak height ratio measurements of non-extracted standards to those of extracted standards corrected for losses of internal standard, and was 73% ± 1 S.D. (Table I).

Limit of detection

No interfering peaks were present in pre-dose (control) plasma with the same retention times as metolazone or internal standard (Fig. 3). The limit of detection of metolazone under the experimental conditions used (signal-to-noise ratio = 2 : 1) was 2 ng/ml when a 0.5-ml plasma sample was analysed.

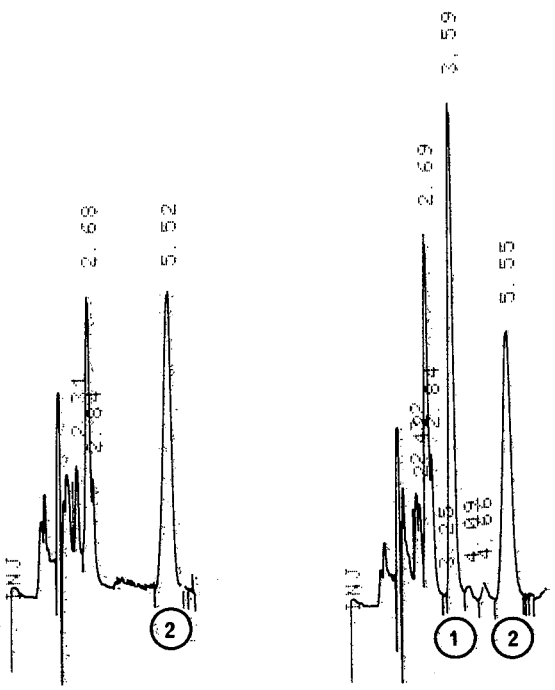


Fig. 3. Chromatograms of (left) pre-dose (control) plasma containing internal standard, and (right) 3-h post-dose plasma sample containing metolazone at a concentration of 166 ng/ml. Peak 1 = metolazone, peak 2 = internal standard. Chromatographic conditions as for Fig. 2.

Concentrations of metolazone in plasma

After single oral doses of 2.5 mg of metolazone to eight rhesus monkeys, a peak of mean concentrations of 202 ng/ml was reached at 1 h after dosing (Table II). Mean concentrations declined to 23 ng/ml at 8 h and were below the limit of detection at 24 h. The mean half-life of metolazone in plasma was 2.20 h ± 1.04 S.D. (Table III).

TABLE II

CONCENTRATION OF METOLAZONE IN THE PLASMA OF EIGHT RHESUS MONKEYS AFTER A SINGLE ORAL DOSE OF 2.5 mg

Results are expressed in ng/ml. For concentrations > 200 ng/ml, smaller aliquots of plasma (0.1—0.25 ml) were analysed.

Time (h)	Animal No.								Mean ± S.D.
	1	2	3	4	5	6	7	8	
0.5	9	58	82	274	24	395	425	270	192 ± 169
1	13	202	192	296	52	315	355	192	202 ± 122
1.5	26	206	214	222	62	305	275	182	187 ± 97
2	29	208	214	184	68	181	167	143	149 ± 67
3	166	156	184	114	127	102	105	103	132 ± 32
4	191	125	121	84	127	70	64	61	105 ± 44
6	100	58	52	43	94	37	25	36	56 ± 27
8	36	25	26	21	28	18	10	18	23 ± 8
24	< 2	< 2	< 2	9	4	< 2	< 2	< 2	< 2
32	< 2	< 2	< 2	3	< 2	< 2	< 2	< 2	< 2
48	< 2	< 2	< 2	3	< 2	< 2	< 2	< 2	< 2
72	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2

TABLE III

HALF-LIVES OF THE TERMINAL LINEAR SECTIONS OF THE PLASMA METOLAZONE CONCENTRATION—TIME RELATIONSHIPS

Animal No.	Half-life (h)	r^2
1	1.66	0.98
2	1.72	1.00
3	1.80	1.00
4	—	<0.90
5	4.48	0.92
6	2.02	1.00
7	1.47	1.00
8	2.27	0.99
Mean ± S.D.	2.20 ± 1.04	

ACKNOWLEDGEMENT

We are grateful to Sandoz Limited, Basle, Switzerland, for the opportunity to carry out these studies.

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Related articles published in *Journal of Chromatography*, Vols. 214 and 215

Since the majority of the articles published in Vol. 215 may be of interest to the readers of the *Biomedical Applications* section, the contents list of this volume has been reproduced in its entirety.

Volume 214

Volume 215

HONOUR VOLUME ON THE OCCASION OF THE 60TH BIRTHDAY OF JERKER O. PORATH

Author Index

Subject Index

Vol. 226, No. 2

JOURNAL OF CHROMATOGRAPHY
BIOMEDICAL APPLICATIONS

NEWS SECTION

MEETING

4th INTERNATIONAL SYMPOSIUM ON QUANTITATIVE MASS SPECTROMETRY IN LIFE SCIENCES

An international symposium on quantitative mass spectrometry in life sciences, sponsored by the Faculty of Pharmaceutical Sciences of the State University of Ghent, the National Foundation of Scientific Research (N.F.W.O–F.N.R.S.), and the Ministry of National Education and Dutch Culture, will be held in Ghent, Belgium, from May 11–14, 1982, at the Academieraadzaal.

Contributed papers (20 minutes) will cover the following topics: drug metabolism, clinical chemistry, biochemistry, pharmacokinetics, toxicology, ecology and isotope labeling. All papers have to be presented in English and no simultaneous translation will be provided.

Five plenary lectures will be presented by outstanding specialists in the field of quantitative mass spectrometry: W.A. Garland (Hoffmann-La Roche, Nutley, NJ, U.S.A.); E. Gelpi (Institute of Bio-Organic Chemistry, Barcelona, Spain); F.A. Muskiet (University Hospitals, Groningen, The Netherlands); R. Schaffer (National Bureau of Standards, Washington, DC, U.S.A.); and, C.C. Sweeley (Department of Biochemistry, Michigan State University, East Lansing, MI, U.S.A.).

Before and after the Symposium all correspondence should be sent to: Prof. Dr. A. De Leenheer, Laboratoria voor Medische Biochemie en voor Klinische Analyse, Rijksuniversiteit Gent, De Pintelaan 135, B-9000 Gent, Belgium; telephone: (091) 22 57 41, ext. 2662.

COURSE

COURSE IN BIOLOGICAL SEPARATION METHODS

A course in biological separation methods will be held from March 23rd to June 4th, 1982, at the Uppsala Separation School, Institute of Biochemistry, University of Uppsala in Sweden (organizers: Professors Stellan Hjertén and Paul Roos).

The course is centered around modern analytical and preparative methods for the separation of cells, virus, proteins and nucleic acids, and their characterization. The course will consist of lectures and laboratory work dealing with the following methods: moving boundary electrophoresis; free zone electrophoresis; zone electrophoresis, in both sieving and non-sieving anticonvection media; isoelectric focusing; displacement electrophoresis (isotachophoresis); molecular sieve chromatography; hydroxyapatite chromatography; hydrophobic interaction chromatography; covalent chromatography; bioaffinity chromatography; gas chromatography; HPLC; counter-current distribution (liquid phase partition); analytical and preparative centrifugation methods (centrifugation in different kinds of density

(*Continued on next page*)

gradients, determination of sedimentation coefficients and of molecular weights); immunodiffusion; immunoelectrophoresis; determination of diffusion coefficients; light scattering; spectrofluorometry; bioluminescence; and radioimmunoassay.

Knowledge of biochemistry and mathematics, corresponding to a basic university degree, is required. Good knowledge of English is necessary. The number of participants is limited to 12, 6 from Sweden and 6 from abroad.

The course fee is US $450. Living expenses to cover food and accommodation in student rooms will be a minimum of US $1600. No fellowships are available through the organizers.

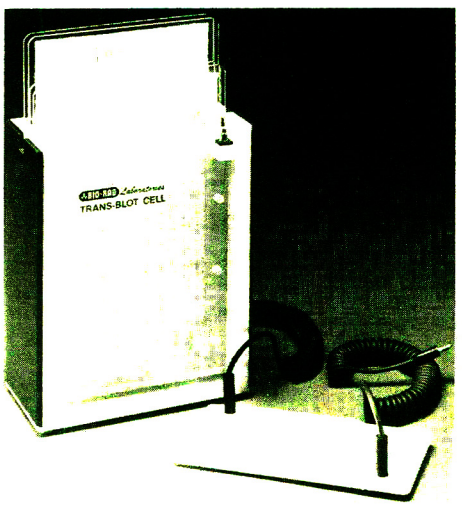
The closing date for applications is January 15, 1982. Application forms can be obtained from Eva Linder, Secretary, Institute of Biochemistry, University of Uppsala, Box 576, S-75123 Uppsala, Sweden.

MANUFACTURERS' LITERATURE

N-1605

MOBILE PHASE SELECTION BROCHURE

Supelco has issued a brochure on the selection of mobile phases for various octadecyl reversed-phase HPLC columns. This brochure, Bulletin 788, compares the mobile phase composition required for each of eight manufacturers' ODS columns, discusses the reasons for the differences, and explains how the chromatographer can adapt analyses from one brand of column to another. Because of variations in the manufacturing of most comparable ODS columns, mobile phases must often be adjusted when an ODS column is replaced, even when the replacement is supposedly an identical column from the same manufacturer.



INSTRUMENTATION

N-1607

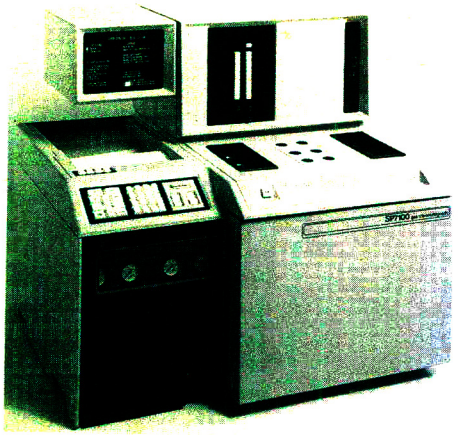
TRANS-BLOT CELL

Bio-Rad Labs. has introduced a new trans-blot cell for the rapid, efficient electrophoretic transfer blotting of DNA, RNA and protein. The cell has platinum electrodes, triple gel capacity, one-piece gel holders, and internal cooling if required. The cell allows the user to transfer electrophoretically separated DNA, RNA or protein from the gel to an immobilizing matrix in 30 min, according to the manufacturer. Once transferred, the bands are readily accessible to autoradiography, ELISA or fluorescent detection, and to preparative elution.

N-1636

NEW GAS CHROMATOGRAPH

In September, 1981, the new SP 7100 gas chromatograph designed by Delta Electronics and produced by the mother company Spectra-Physics was introduced throughout Europe. A CRT/keypad interface makes the 7100 easy to use, and the complete status of the instrument is shown on the CRT. The large oven is said to have very low temperature gradients. The oven cools down very quickly. In the 7100 system all sorts of columns can be used. TCD, FID and TID (N- and P-compounds) detectors are available with the SP 7100. A feature of the 7100 is the possibility of connecting multiple instru-



ments in a system network where each GC can communicate and interact with all the others, and at the same time can be used as a stand-alone instrument. With the 7100 system a dual-channel data system is available, based on the SP 4100 integrator.

N-1618

FOUR-SOLVENT CAPABILITY

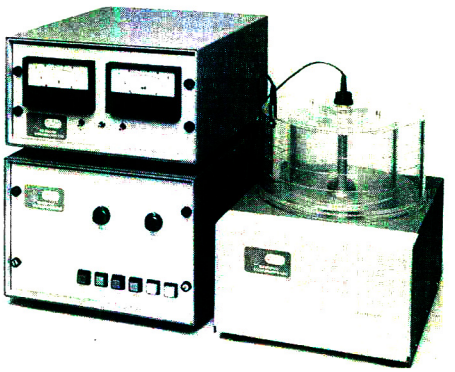
Du Pont has recently introduced an optional four-solvent capability for their 8800 series of HPLC systems. It expands the chromatographer's ability to separate complex compounds and can simplify the selection of the optimum liquid phase for the particular separation required. This is made possible by a new predictive model developed by Du Pont which optimizes the strength and selectivity of a solvent system in reversed-phase LC. Only minor modifications to the 8800 HPLC systems are required to accommodate the four-solvent capability. They can be undertaken in the field, and include modification of the three-piston pump module, the provision of extended programming capability for the system's gradient controller, and the addition of a graphics display module.

For further information concerning any of the news items, apply to the publisher, using the reply cards provided, quoting the reference number printed at the beginning of the item.

N-1603

PREPARATIVE ELECTROPHORESIS SYSTEM

The Macro-Page preparative electrophoresis system, manufactured by Birchover Instruments, is now available with accessories for the electrophoretic desorption of material from affinity matrices. Eluent composition is one of the critical parameters of affinity systems and its choice can influence the purification obtained.



Electrophoretic desorption is a mild method for the removal of material from affinity matrices and immunoadsorbents, being particularly suited for the desorption of antibodies from the high association complexes with immobilized antigen and to other high affinity complexes.

N-1640

POST-COLUMN REACTION SYSTEM

Kratos' new URS 050 post-column reaction system is a self-contained instrument system for post-column derivatization in HPLC. The URS 050 is designed for derivatization procedures requiring the addition of a single reagent at room temperature. Post-column derivatization with a reagent such as *o*-phthalaldehyde is a popular procedure used in conjunction with fluorescence detection for the HPLC separation of, *e.g.*, peptides and nucleic acids. The URS 050 incorporates a flow-induced vortex mixing chamber and a reaction chamber that is said to provide reduced levels of band broadening and improved overall resolution when compared with other systems. The standard volume of the reaction chamber is 1 ml, but the URS 050 can be equipped with reaction chambers of other volumes.

N-1601

POST-COLUMN REACTION SYSTEM

The Schoeffel Instruments Division of Kratos has introduced a universal post-column reaction system. It has been designed to satisfy a broad range of reaction conditions as might be required for a variety of post-column derivatization methods. This instrument, the Model URS 100, provides the user with control over derivatizing reagent flow-rate, total reaction time between column and detector, and reaction temperature. In cases where a two-step derivatizing reaction is needed, a second pump can be added to the system for a second reagent.

N-1606

VARIABLE-WAVELENGTH UV DETECTOR

A variable-wavelength UV detector was recently introduced by Kratos. This new SF 769 provides variable-wavelength control from 190 to 400 nm. The detector has easily interchangeable flowcells for use in standard HPLC, low pressure LC, and also microbore column LC methods. The detector noise is specified as $1.5 \cdot 10^{-4}$ a.u. typical, and $0.5 \cdot 10^{-4}$ under ideal conditions. The SF 769 provides seven selectable absorbance ranges from 0.01 to 2.0 a.u.f.s. Use of the detector at wavelengths below 200 nm is possible by purging the monochromator and optics chamber with dry nitrogen.

N-1634

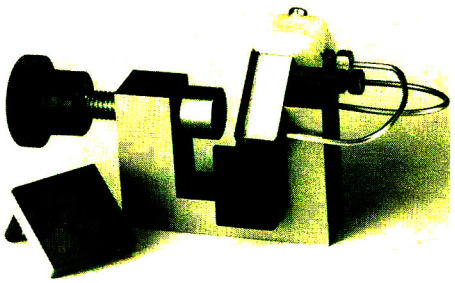
TLC SCANNER

The thin-layer chromatogram scanners of the Hydra series from Technical Associates are capable of scanning paper chromatograms, TLC plates and electrophoretic plates. The instruments scan up to six traces simultaneously. In the Hydra series three different detectors are available for any nuclide, such as the beta emitters ^{3}H, ^{14}C, ^{32}P, etc., and the gamma emitters ^{55}F, ^{125}I, and ^{99}Tc. The scanners offer a high precision at all speeds due to digital drive, according to the manufacturer. The instruments can be used for trace methodology in biochemistry, biomedical and clinical laboratory practice, radiopharmacy and nuclear medicine.

N-1612

COULOMETRIC DETECTOR

A new electrochemical detector for HPLC has been introduced by Kipp Analytica. In this detector, Model 9205, use is made of glassy carbon electrodes with a relatively large area. In this way a high degree of conversion is obtained, resulting in high detection signal levels. Thus



the sensitivity to all sorts of external factors, *e.g.*, composition of the mobile phase, the temperature and static electricity, is said to be much lower. The long term stability of the detector is said to very good; only after a long period of use is some poisoning noticeable. The manufacturer claims the cell to be extremely suited for the determination of catecholamines in biological samples.

SORBENTS AND ION EXCHANGERS

N-1600

REVERSED-PHASE TLC PLATES

A linear-K (pre-adsorbent), reversed-phase TLC plate is now available from Whatman. The analytical form of the new plate has the type indication LKC_{18} with a fluorescent indicator $LKC_{18}F$; the preparative version is called $PLKC_{18}F$. This preparative plate has a 1000-μm layer thickness and a fluorescent indicator for UV detection at 254 nm. The plates are the first application of the Whatman 'linear-K' concept. The new LKC_{18} series adds a pre-adsorbent strip along the lower edge of the plate, contiguous with the KC_{18} analytical layer, which is in every way identical to the analytical layer of the Whatman KC_{18} series of plates. The new LKC_{18} series plates are available in all analytical sizes; the preparative plates are available in a 20 × 20 cm format.

N-1620

CELL SORTING SYSTEM

Bio-Rad Labs. have introduced a cell sorting system, called Affi-Gel®. This system is said to be a new affinity chromatography technique that efficiently separates B and T cells, as well as other cell subpopulations, according to surface characteristics. The manufacturer claims that the system yields highly pure and viable cell populations. The separation is completed in 2.5 h. Each system includes a siliconized Econo-Column®, an outlet tube to control flow, polyethylene pipets, and lyophilized Affi-Gel cell sorting beads. Three different systems are available for binding cells labeled with mouse Ig, rabbit Ig, or any fluorescent ligand.

N-1641

LC COLUMNS FOR PROTEINS, PEPTIDES AND MACROMOLECULES

Rheodyne has recently introduced HPLC columns for rapid separations of proteins, peptides and synthetic macromolecules by reversed-phase and anion exchange techniques. These columns will, according to the manufacturer, perform difficult analyses such as protein digests and will make high-resolution profiles of serum and urine. The columns are packed with a silica gel that has relatively large 30-nm pores instead of ordinary 6- to 10-nm pores. These larger pores facilitate rapid separation of macromolecules. The columns are available in lengths of 3, 10 and 25 cm.

N-1639

HPLC COLUMNS FOR CARBOHYDRATE ANALYSIS

Bio-Rad Labs. has introduced two HPLC columns for carbohydrate analysis. The Animex HPX-42A column is intended for fast, high resolution analysis of oligosaccharides. The manufacturer claims an analysis time of within 20 min for oligosaccharides as large as DP-11. The Bio-Sil Amino 5S column is especially made for disaccharide analysis. Bio-Rad claims a resolution of sucrose, maltose, and lactose in 10 min. Bio-Rad now has available six columns for carbohydrate analysis.

N-1617

GUARANTEED HPLC PERFORMANCE

Kratos has introduced a new line of HPLC columns. These columns, called Unisep, are packed with 5-μm spherical silica materials. The manufacturer offers silica, C_{18} (ODS), C_8 (octyl), NH_2 (amino), and CN (cyano) columns. The materials are packed in 25 cm × 5 mm stainless-steel columns. The Unisep line is guaranteed to provide a minimum of 50,000 plates per meter, with a maximum asymmetry range of 0.8–1.3.

CHEMICALS

N-1635

DRUG STANDARDS

The Theta Corp. recently introduced Thetakits for diuretics, adrenergics, and anticholinergics. Each kit contains 20 analytical standards of drugs in the above classes, in individual syringe-accessible vials. These standards are added to the extensive line of abused and therapeutic drugs available from the manufacturer. The standards can be used in gas, liquid and thin-layer chromatography and in most kinds of spectrometric analysis.

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