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BIOMEDICAL APPLICATIONS

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CHROMBIO. 1682

HAWKINSINURIA — IDENTIFICATION OF QUINOLACETIC ACID AND PYROGLUTAMIC ACID DURING AN ACIDOTIC PHASE

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(First received November 22nd, 1982; revised manuscript received February 17th, 1983)

SUMMARY

A second Australian family with the genetic disease Hawkinsinuria has been identified. Affected members excrete hawkinsin and *cis*- and *trans*-4-hydroxycyclohexylacetic acid. An infant in this family presented with metabolic acidosis and excreted quinolacetic acid and pyroglutamic acid in the urine together with the tyrosine derived phenolic acids reported in the original index case. It is thought that quinolacetic acid is accumulated as a by-product of the partially defective enzyme, 4-hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27) and that pyroglutamic acid indicated lowered glutathione levels.

INTRODUCTION

Niederwieser and co-workers [1—3] have described the excretion of hawkinsin, 2-(2-L-cystein-S-yl-1,4-dihydroxy-5-cyclohexen-1-yl)acetic acid, and *cis*- and *trans*-4-hydroxycyclohexylacetic acid (4-HCHAA) in the urine of a child (L.H.), suffering from transient tyrosinaemia, and in the urine of her mother. When the child was weaned from breast feeding to artificial feeding with a concomitant higher intake of phenylalanine and tyrosine, a serious metabolic acidosis characterised by high levels of 4-hydroxyphenylpyruvic acid (4-HPPA), 4-hydroxyphenyllactic acid (4-HPLA) and 4-hydroxyphenyl-

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acetic acid (4-HPAA) was induced. It was postulated that a partial defect in 4-hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27), normally responsible for the oxidation, decarboxylation and rearrangement of 4-HPPA to homogentisic acid, prevented the final rearrangement of an intermediate. They proposed that either quinolacetic acid [2-(1-hydroxy-4-oxo-2,5-cyclohexadien-1-yl)acetic acid] or the epoxide [2-(1,2-epoxy-4-oxo-5-cyclohexen-1-yl)-acetic acid] accumulated and was disposed of by either conjugation with cysteine through the intervention of glutathione to produce hawkinsin, or by a series of reductions to produce the *cis*- and *trans*-4-HCHAA [1].

A second infant (K.G.) with Hawkinsinuria has been described [4]. In this paper we report on the urinary excretion of pyroglutamic and quinolacetic acid in addition to the 4-hydroxyphenolic acids while this child was acidotic.

MATERIALS AND METHODS

Solvent extraction of organic acids

Sodium chloride (0.5 g) was added to 1 ml of urine, followed by sufficient hydrochloric acid to bring the pH to 1. The solution was then extracted twice with ethyl acetate (2 × 2 ml) and once with diethyl ether (2 ml) and the pooled organic phases evaporated to dryness under a gentle stream of nitrogen at room temperature. The dried urinary extract was then silylated with 125 μl of bis-(trimethylsilyl)trifluoracetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) (60 °C, 30 min).

Identification of organic acids

Urinary metabolites were identified using gas chromatography—mass spectrometry (GC—MS) in which a Varian Aerograph (2700) gas chromatograph was interfaced, via a jet separator, to a DuPont 491B mass spectrometer fitted with a dual electron impact (EI)/chemical ionisation (CI) source. The gas chromatograph was fitted with a 1.8 m × 6 mm glass column packed with 3% SE-30 on 80—100 mesh dimethyldichlorosilane-treated Chromosorb W. A helium flow-rate of 25 ml/min was used, and during the analyses the temperature was programmed from 80°C to 250°C at 6°C/min.

Nuclear magnetic resonance spectra

Nuclear magnetic resonance (NMR) spectra were recorded with a Hitachi Perkin-Elmer R-24 spectrometer operating at 60 MHz using tetramethylsilane (TMS) as an internal standard. Chemical shifts are quoted on the δ scale and the signals described in terms of the number of protons, multiplicity, coupling constants and assignment.

Synthesis of 2-(1-hydroxy-4-oxo-2,5-cyclohexadien-1-yl)acetic acid

The synthesis was achieved using a modification of the original procedure described by Siegel and Keckeis [5]. *tert*-Butyl bromoacetate was prepared by the method of McCloskey et al. [6] and purified by distillation at reduced pressure in the presence of magnesium oxide using alkali washed glassware and the fraction boiling at 40°C/4 mm Hg was collected as a colourless oil, yield 74%, GC—MS (isothermal at 40°C) *m/e* 181 ($M^{+81}Br - 15$), 179 ($M^{+79}Br - 15$).

tert.-Butyl bromoacetate (3.5 g) was dissolved in anhydrous diethyl ether (100 ml) and activated zinc powder (2.3 g) added. The zinc powder had been washed with dilute hydrochloric acid, water, alcohol and anhydrous diethyl ether then dried in vacuo before activating with a single crystal of iodine by heating in vacuo. The reaction was initiated, while being vigorously stirred under reflux, by the addition of a few drops of an ethereal solution of freshly prepared methyl magnesium iodide. Refluxing continued for 90 min whereupon the solution was cooled and decanted from the excess zinc. An ethereal solution of 1,4-benzoquinone (1 g) was added slowly to the cooled solution of the organozinc compound. The precipitate was filtered and washed with anhydrous diethyl ether followed by addition to hot water (200 ml). The aqueous solution was then filtered to remove the zinc hydroxide and extracted with diethyl ether (3 × 70 ml). The ether extracts were dried over anhydrous magnesium sulphate and the ether removed by distillation to give a brown residue which was chromatographed on Merck alumina (activity II). Elution with 20% chloroform—light petroleum yielded *tert.*-butyl 2-(1-hydroxy-

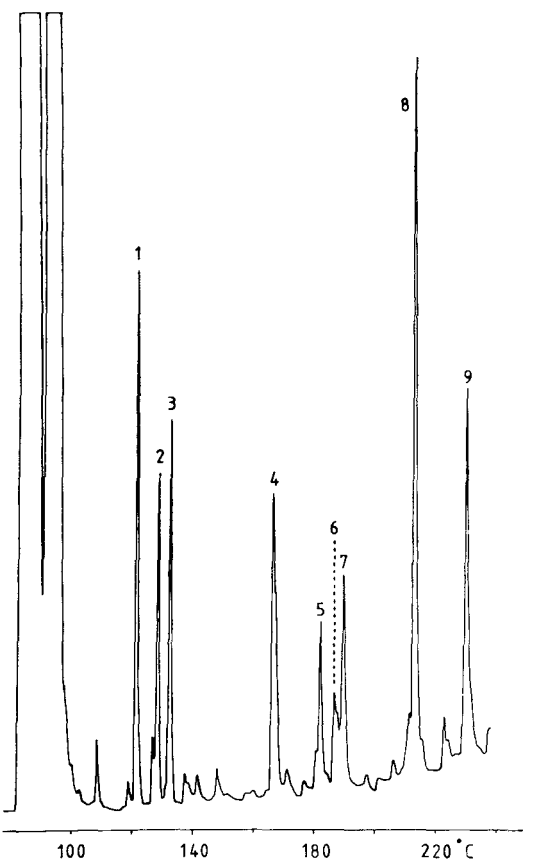

Fig. 1. GC of urinary organic acids as per-TMS derivatives excreted by K.G. while acidotic. Peaks: 1, 3-hydroxybutyric acid; 2, urea; 3, acetoacetic acid; 4, pyroglutamic acid and adipic acid; 5, 4-HPAA; 6, quinolacetic acid; 7, suberic acid; 8, 4-HPLA; 9, unknown, molecular weight 434.

4-oxo-2,5-cyclohexadien-1-yl)acetate (0.69 g, 33%) which was crystallised from chloroform—pentane; m.p. 63—65°C; MS (50°C, 70 eV) *m/e* (%) 168 (63), 150 (17), 126 (31), 124 (31), 123 (38), 122 (17), 109 (base peak); NMR (60 MHz, C^2HCl_3) δ 1.35 (9-H, s, *tert.*-butyl methyls), 2.52 (2-H, s, methylene α to acid), 4.42 (1-H, s, hydroxyl), 6.07 (2-H, d, J = 10 Hz, C-3 and C-5 protons), 6.90 (2-H, d, J = 10 Hz, C-2 and C-6 protons).

A sample of *tert.*-butyl 2-(1-hydroxy-4-oxo-2,5-cyclohexadien-1-yl)acetate was hydrolysed by dissolution in 0.1 *M* hydrochloric acid in formic acid and allowed to stand at room temperature for an hour. The solvent was removed by freeze-drying and the brown oily residue washed twice with water. NMR analysis of the crude hydrolysate indicated the production of the lactone [7]. This was then silylated with BSTFA—1% TMCS yielding di-TMS quinolacetic acid.

RESULTS AND DISCUSSION

The GC—MS analysis of the silylated urinary organic acids from the child (K.G.) showed that the urine contained three compounds (pyroglutamic acid and two unknowns) not reported in the original index case (Fig. 1). The molecular weights of the two unknowns were determined by GC—CIMS, to be 312 a.m.u. and 434 a.m.u. The mass spectrum of the first unknown was shown to be very similar to that of the di-TMS derivative of quinolacetic acid as prepared by us (Fig. 2). The di-TMS derivative gave signals at *m/e* 312 (M^+), 297 ($M—CH_3$), 269, 255, 253, 222 (M—HOTMS), 204, 194 (M—HCOOTMS), 181 ($M—CH_2COOTMS$), 147, 75, 73. The identity of the

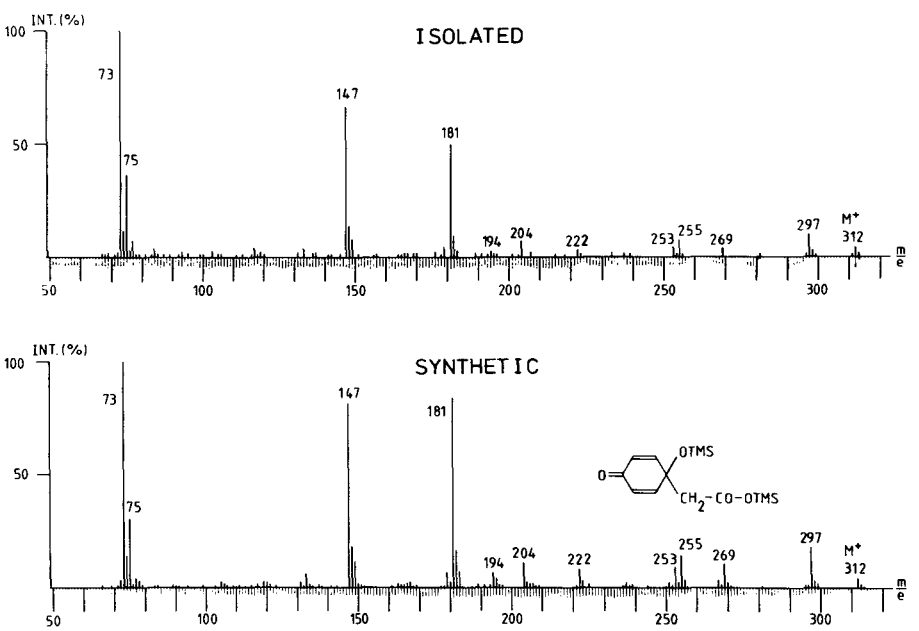

Fig. 2. EI mass spectra obtained from GC—MS of urinary quinolacetic acid and synthetic quinolacetic acid as di-TMS derivatives.

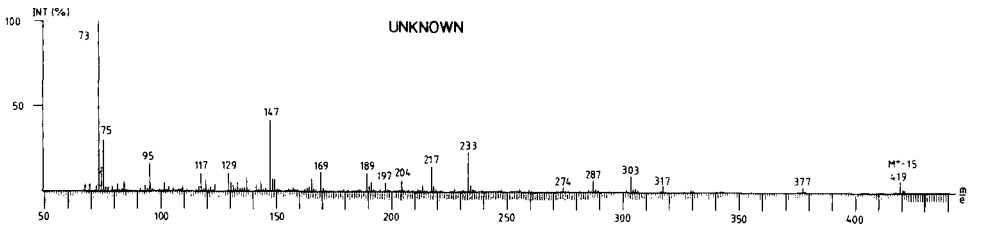

Fig. 3. EI mass spectra obtained from GC—MS of the unknown compound (molecular weight 434) excreted by K.G.

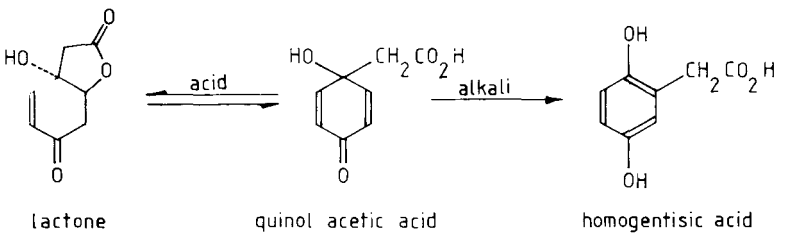

Fig. 4. Under acidic conditions quinolacetic acid may undergo an intramolecular Michael addition to form a lactone. Under alkaline conditions both the lactone and the free acid rearrange to homogentisic acid.

second unknown (Fig. 3) remains undetermined. Saito et al. [7] have noted that quinolacetic acid can undergo an internal Michael addition to form a lactone and that both the lactone and the free acid may, under alkaline conditions, be irreversibly rearranged to homogentisic acid (Fig. 4). Therefore it is likely that under the conditions of the urine extraction any free quinolacetic acid will be, at least partially, converted to the lactone form. The silylation conditions used are such that the lactone is in equilibrium with the acid form which is then, for the purposes of our work, irreversibly silylated to produce the di-TMS derivative of quinolacetic acid. This was demonstrated when the lactone obtained from the acid hydrolysis of the *tert.*-butyl ester was silylated to yield quantitatively the di-TMS derivative of quinolacetic acid. Finally, the silylated derivatives of crystalline samples of authentic quinolacetic acid and the lactone received from Professor Saito proved to have mass spectra identical with samples synthesised by us and found in the urine.

A small sample of urine excreted by the original index case (L.H.) during an acute phase of her illness was obtained from Professor Danks. The GC trace of the urinary organic acids (Fig. 5) contained a large peak which we now identify as pyroglutamic acid. The excretion of pyroglutamic acid by both children while acidotic probably indicates lowered glutathione levels. Glutathione normally exerts feedback inhibition on γ-glutamylcysteine synthetase [8] and lowered levels, as are seen for example in glutathione synthetase deficiency, may lead to a modified γ-glutamyl cycle, with increased formation of pyroglutamic acid (5-oxoproline), which exceeds the capacity of 5-oxoprolinase [8]. This supports Niederwieser's supposition that hawkinsin results from the conjugation of glutathione with the accumulated en-

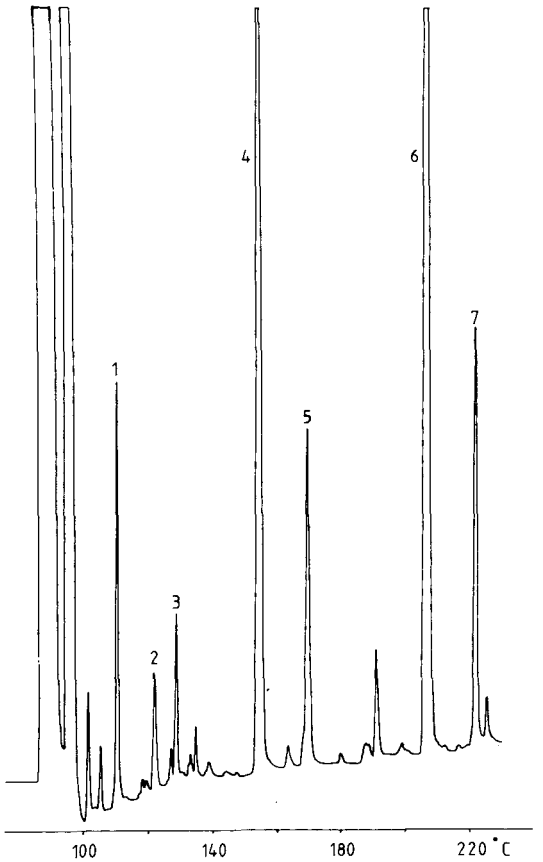

Fig. 5. GC of urinary organic acids as per-TMS derivatives excreted by L.H. while acidotic. Peaks: 1, lactic acid; 2, 3-hydroxybutyric acid; 3, urea; 4, pyroglutamic acid; 5, 4-HPAA; 6, 4-HPLA; 7, 4-HPPA.

zyme intermediate [1]. An alternative explanation might be that the accumulated intermediate metabolite inhibits glutathione synthetase, with the same result. Neither quinolacetic acid nor the unknown were detected during re-examination of urine from the original case (L.H.). This failure may perhaps be due to extensive storage and handling of the urine sample.

The finding of quinolacetic acid in the acute phase of Hawkinsinuria does not necessarily imply that this is an intermediate in the conversion of 4-HPPA to homogentisic acid. It is possible that an epoxide intermediate had been converted to quinolacetic acid in the acidic conditions of extraction and derivatisation.

ACKNOWLEDGEMENTS

We would like to express our thanks to Professor Saito, Department of Synthetic Chemistry, Kyoto University, Kyoto, Japan for his very generous gift of samples of quinolacetic acid and the lactone. Also to Professor Danks,

Genetics Research Unit, Royal Children's Hospital Research Foundation, Melbourne, Australia for allowing us to examine urine excreted by L.H.

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CHROMBIO. 1673

ANALYSIS OF 1,2-DIOLS OF LINOLEIC, α-LINOLENIC AND ARACHIDONIC ACID BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY USING CYCLIC ALKYL BORONIC ESTERS

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(First received December 1st, 1982; revised manuscript received February 3rd, 1983)

SUMMARY

Arachidonic acid is metabolized by hepatic and renal cortical microsomes in the presence of NADPH to vicinal dihydroxyeicosatrienoic acids as some of the major metabolites. Other polyunsaturated, long-chain fatty acids might be metabolized to vicinal dihydroxy acids (1,2-diols) in the same way. To facilitate identification of 1,2-diols in biological samples, a series of unsaturated 1,2-diols were synthesized from linoleic, α-linolenic and arachidonic acid and the electron-ionization mass spectra of cyclic methane- and *n*-butaneboronic ester derivatives and of trimethylsilyl (TMS) ether derivatives were compared. The TMS ether derivatives gave rise to weak molecular ions but prominent informative fragmentation ions were formed by α-cleavage as well as cleavage between the carbons with the TMS ethers. The TMS ether derivative of methyl 15,16-dihydroxy-9,12-octadecadienoate had a considerably larger carbon value than the other C_{18} diols, while the cyclic boronates were poorly separated on gas chromatography. The methane- and *n*-butaneboronic acid derivatives showed strong molecular ions and a characteristic but not very informative fragmentation, although the position of the hydroxyls could be deduced from one or two fragments formed by α-cleavage. Linoleic and α-linolenic acid are metabolized in the rabbit to many polar products by hepatic and renal cortical microsomes and NADPH. 12,13-Dihydroxy-9-octadecenoic acid and other metabolites of linoleic acid were identified by gas chromatography—mass spectrometry.

INTRODUCTION

Cyclic boronic ester derivatives were introduced by Brooks and Watson [1] for the analysis of bifunctional compounds by gas chromatography—mass spectrometry (GC—MS). Cyclic alkyl boronic esters are formed under mild conditions and have suitable GC—MS properties [2]. They have been used successfully for analysis of various 1,2- and 1,3-diols, steroids, prostaglandins, catecholamines and cannabinoids (see ref. 3 for review). Stable GC—MS deriva-

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tives are only formed with bifunctional compounds, which allow formation of the cyclic boronic esters [3]. This selectivity is advantageous and may reduce the need for extensive purification of biological samples.

Arachidonic acid is metabolized by hepatic and renal microsomes to vicinal diols as major metabolites [4—6]. These diols are formed by epoxidation catalyzed by cytochrome *P*-450 (LM_2 or $PB\text{-}B_2$) and the epoxides are converted to 1,2-diols by epoxide hydrolase [7]. As shown in the present report, linoleic and α-linolenic acid are also metabolized to polar metabolites by hepatic and renal cortical microsomes and NADPH. It seemed likely that cyclic boronic esters might be a useful complement to TMS ether derivatives for identification of some of these unknown metabolites and also for studying the metabolism of arachidonic acid to 1,2-diols. This prompted a systematic study of the mass spectra of cyclic boronic esters of unsaturated 1,2-diols, chemically prepared from linoleic, α-linolenic and arachidonic acid. The results show that these cyclic boronic esters have a rather non-specific fragmentation pattern. They can nevertheless be used for identification of metabolites in biological samples by comparison with authentic material as demonstrated for a 1,2-diol of linoleic acid, which is formed by renal cortical microsomes and NADPH.

MATERIALS AND METHODS

[1-^{14}C]Arachidonic, [1-^{14}C]linolenic and [1-^{14}C]linoleic acids (all 56—58 mCi/mmol), were from the Radiochemical Centre, Amersham, Great Britain. Arachidonic acid and NADPH were from Sigma, St. Louis, MO, U.S.A. Linoleic acid, α-linolenic acid and bis(trimethylsilyl)trifluoroacetamide (BSTFA) were from Supelco, Bellefonte, PA, U.S.A. *m*-Chloroperoxybenzoic acid (85%, technical) was from Aldrich, Beerse, Belgium. Methane- and *n*-butaneboronic acid were from Applied Science Labs., State College, PA, U.S.A. Thin-layer chromatography (TLC) was performed with glass plates, precoated with a 0.25-mm thick layer of silica gel 60 (10 × 20 cm plates), which, together with most other chemicals, were from Merck, Darmstadt, G.F.R. Equipment for high-performance liquid chromatography (HPLC) was from Laboratory Data Control, Riviera Beach, FL, U.S.A. (cf. ref. 9), and the following columns were used (Waters Assoc., Milford, MA, U.S.A.): octadecasilane bonded to 10-μm silica gel (μBondapak C_{18}, 300 × 7.8 mm) and 10-μm silica gel (μPorasil, 300 × 3.9 mm). Radioactivity was measured by liquid scintillation counting (PLD PRIAS, Packard, Downers Grove, IL, U.S.A.) using Ready Solv HP (Beckman, Fife, Scotland) as scintillator.

Synthesis of 1,2-diols

14,15-Dihydroxy-, 11,12-dihydroxy-, 8,9-dihydroxy- and 5,6-dihydroxy-$C_{20:3}$ were synthesized as described previously [7—9]. The other diols were obtained as follows: 30—70 mg of α-linolenic and linolenic acid were methylated with diazomethane. After evaporation, the fatty acid methyl esters were reacted with 1.1 equiv. of *m*-chloroperoxybenzoic acid in 1—2 ml of dichloromethane at room temperature overnight. The reaction was ended by washing with 10% Na_2SO_3 and with 5% $NaHCO_3$ in water (cf. ref. 10). After evaporation, the residue was purified by preparative TLC (silica gel 60,

15% ethyl acetate in hexane). The epoxides were converted to 1,2-diols as described [7, 9, 11] and purified by TLC (50% ethyl acetate in hexane; R_F values are given in Table I). [5,6,8,9,11,12,14,15-$^2H_8$]-14,15-Dihydroxy-5,8,11-eicosatrienoic acid was synthesized as described [9].

Experimental

Male white New Zealand rabbits (2—3 kg) were killed by a blow to the head. Microsomes were prepared from a 20% tissue homogenate as described [9] and resuspended so that 1 ml of microsomal suspension corresponded to microsomes from 0.4 g of hepatic tissue or 0.8 g of renal cortical tissue. The microsomes were used immediately after preparation. The microsomal suspensions (typically 10 ml) were incubated with 1 m*M* NADPH and the fatty acid (0.18 m*M*, added in 50 μl of ethanol) for 15 min at 37°C under a normal atmosphere. An aliquot of the microsomal suspension (0.5 ml) and NADPH were incubated with 1-^{14}C-labelled fatty acid (1—2 μCi) in the same way and the two incubations combined at the end. Proteins were precipitated with methanol (4 volumes) and spun down. The pellet was washed once with methanol. After evaporation, the residue was dissolved in water and extracted twice with dichloromethane at pH 3. The organic layers were combined, washed with water and dried over Na_2SO_4. Following evaporation, the residue was dissolved in water and extracted twice with ethyl acetate at pH 3. After evaporation, the residue was dissolved in methanol—water, centrifuged, and the clear supernatant was purified by reversed-phase HPLC (μBondapak C_{18}; methanol—water—acetic acid, 73:27:0.2, v/v; flow-rate 2 ml/min per fraction). The renal metabolites of $C_{18:2}$, which were analyzed by GC—MS, were further purified by normal-phase HPLC (μPorasil; gradients of chloroform in hexane as described below).

GC—MS derivatives

An ethereal solution of diazomethane was used for methylation. Methane- and *n*-butaneboronic acid were dissolved in dimethoxypropane (Fluka; 2.5 mg/ml) and 30 μl were used for derivatization. Initially the reaction mixture was heated to 60—70°C for a few minutes but this step was subsequently omitted since 10 min at room temperature seemed to be sufficient. A fraction of the dimethoxypropane solution was injected directly into the GC—MS system. Silylation was performed with 10 μl of BSTFA in pyridine (1:1) for about 10 min at 70°C and then evaporated to dryness under nitrogen; the residue was dissolved in hexane. Methoximes were prepared as described [7].

GC—MS analysis

Analysis were usually performed on a Finnigan 4000 quadrupole mass spectrometer equipped with a Finnigan Incos data system. The GC column was 1% SP-2100 (90 cm), operated isothermally at 210—230°C. The electron energy was 70 eV and the temperature of the ion source was set to 300°C. Occasionally, samples were run on an LKB 2091 mass spectrometer using a GC column with 1% SE-30. C values were extrapolated from the retention times of the following saturated fatty acid methyl esters: C_{18}, C_{20}, C_{22} and C_{24}.

TABLE I

SALIENT FEATURES OF THE TMS ETHER METHYL ESTER DERIVATIVES OF UNSATURATED 1,2-DIOLS FROM LINOLEIC α-LINOLENIC AND ARACHIDONIC ACID

Compound	R_F*	C values			Salient features of the TMS derivatives§ *m/z* (relative intensity)							
		MeB	BuB	TMS	M+§§	a—90	a	b	c	d	d—90	$[R_m]^+$ TMS§§§
Methyl 9,10-$(HO)_2$-$C_{18:1}$	0.57	19.9	22.1	21.1	472	225(1.3)	315(0)	259(39)	213(33)	361(4.0)	271(19)	230(0)
Methyl 12,13-$(HO)_2$-$C_{18:1}$	0.62	19.9	22.0	21.3	472	185(9.3)	275(25)	299(23)	173(32)	40(0)	311(1.2)	270(9.3)
Methyl 9,10-$(HO)_2$-$C_{18:2}$	0.55	19.9	22.1	21.4	470	223(0)	313(0)	259(43)	211(9.3)	361(4.4)	271(16)	230(0)
Methyl 12,13-$(HO)_2$-$C_{18:2}$	0.63	19.8	22.0	21.4	470	183(17)	273(15)	299(21)	171(49)	401(0.2)	311(27)	270(9.3)
Methyl 15,16-$(HO)_2$-$C_{18:2}$	0.53	19.9	22.1	22.2	470	143(30)	233(21)	339(5.3)	131(97)	441(0)	351(0.9)	310(8.2)
Methyl 5,6-$(HO)_2$-$C_{20:3}$	0.50**	21.0	23.7	22.7	496***	305(64)	395(0)	203(100)	293(5)	305(64)	215(29)	174(0)
Methyl 8,9-$(HO)_2$-$C_{20:3}$	0.66	21.0	23.7	22.6	496***	265(26)	355(26)	243(59)	253(11)	345(22)	255(100)	214(26)
Methyl 11,12-$(HO)_2$-$C_{20:3}$	0.69	21.0	23.6	22.7	496***	225(21)	315(31)	283(16)	213(41)	385(3)	295(64)	254(11)
Methyl 14,15-$(HO)_2$-$C_{20:3}$	0.70	21.0	23.6	22.8	496***	185(17)	275(59)	323(7)	173(100)	425(0)	335(0.9)	294(10)

*TLC on glass plates, precoated with silica gel 60 (system: 50% ethyl acetate in hexane).

**R_F for the δ5-lactone was 0.41.

***Complete mass spectra have been published [4—7] and mass spectra of the octadeuterated analogues [9].

§The fragmentation ions *m/z* a, b, c and d refer to: R_m—CH(OTMS)—CH(OTMS)—R_w, where R_m and R_w are the side-chains containing the methyl ester and the omega end, respectively. Relative intensities within parentheses. TMS stands for $Si(CH_3)_3$.

§§The intensity of the molecular ions is usually low.

§§§See text for explanation and refs. 12—14.

RESULTS

Synthesis of 1,2-diols

Methyl linoleate was oxidized by *m*-chloroperoxybenzoic acid to methyl 9(10)oxido-12-octadecaenoate and methyl 12(13)oxido-9-octadecaenoate, which were separated by TLC. The mass spectra of both epoxides showed signals at *m/z* 310 (M^+), 292 ($M^+ - 18$), 279 ($M^+ - 31$) and 261 [$M^+ - (18 + 31)$]. The C value was 19.3. The least polar epoxide (R_F 0.62) also showed a strong signal at *m/z* 239 ($M^+ - 71$, loss of carbons 14—18) and it was hydrolyzed to methyl 12,13-dihydroxy-9-octadecaenoate (Table I). The other product (R_F 0.57) showed a relatively strong signal at *m/z* 199 ($M^+ - 111$, loss of carbons 11—18) and it was hydrolyzed to methyl 9,10-dihydroxy-12-octadecaenoate (Table I).

Methyl linolenoate was oxidized by *m*-chloroperoxybenzoic acid to several products as judged from TLC, but only two spots contained the monooxygenated products. The least polar one was methyl 12(13)oxido-9,15-octadecadienoate (R_F 0.56). The mass spectrum of this compound showed strong signals at *m/z* 308 (M^+), 290 ($M^+ - 18$), 277 ($M^+ - 31$) and 259 [$M^+ - (18 + 31)$], 239 ($M^+ - 69$, loss of carbons 14—18) and 221 [$M^+ - (69 + 18)$]. After hydrolysis, it was converted to methyl 12,13-dihydroxy-9,15-octadecadienoate (Table I). The other product (R_F 0.50) was a mixture of methyl 9(10)oxido- and 15(16)oxido-octadecadienoate, as indicated by a mass spectrum with strong signals at *m/z* 308 (M^+), 290 ($M^+ - 18$), 279 [$M^+ - 29$, loss of CH_2CH_3 from the 15(16)oxide], 277 ($M^+ - 31$), 261 [$M^+ - (29 + 18)$] and at *m/z* 199 [$M^+ - 109$, presumably cleavage between carbons 10 and 11 of the 9(10)-oxide]. After hydrolysis, this material was converted to methyl 9,10-dihydroxy-12,15-octadecadienoate and methyl 15,16-dihydroxy-9,12-octadecadienoate, which were separated by TLC (Table I).

TMS ether methyl ester derivatives

Important features of the TMS ether methyl ester derivatives of the unsaturated vicinal dihydroxy acids, which were derived from linoleic, α-linolenic and arachidonic acid are summarized in Table I and will only be discussed briefly. Complete mass spectra of the 1,2-diols derived from arachidonic acid have been published previously [4—7] but data are included in Table I for comparison.

The TMS ether methyl ester derivatives of these unsaturated 1,2-diols showed a characteristic fragmentation. The molecular ion (M^+) was usually relatively weak and weak signals were also observed at *m/z* $M^+ - 15$, $M^+ - 31$ and $M^+ - 90$. The position of the vicinal TMS ethers could be deduced from either one or two fragments formed by α-cleavage (i.e. a and d in Table I) and by cleavage between the carbons carrying the TMS ethers (fragments b and c in Table I). Many of these fragments can also lose trimethylsilanol, and the fragments a—90 and d—90 were observed in almost all scans and were often relatively intense (Table I). Occasionally, signals at *m/z* b—90 (cf. Fig. 2D) or c—90 were also observed.

The mass spectra of several of the 1,2-diols showed prominent fragmentation ions, which were presumably formed by a common rearrangement reac-

tion. The TMS ether methyl ester derivative of 14,15-dihydroxy-$C_{20:3}$ showed a strong signal at *m/z* 294 ($M^+ - 202$) and racemic methyl [14-^{18}O]- and [15-^{18}O]-14,15-dihydroxy-$C_{20:3}$ showed a signal at *m/z* 294 but not at *m/z* 296 [5]. The mass spectrum of methyl [5,6,8,9,11,12,14,15-$^2H_8$]-14,15-dihydroxy-$C_{20:3}$ showed that *m/z* 294 had shifted to *m/z* 300 (cf. ref. 9). It is therefore likely that the signal at *m/z* 294 originates from $[R_m]^+Si(CH_3)_3$, where R_m denotes the side-chain formed by α-cleavage and containing the methyl ester. $Si(CH_3)_3$ is presumably transferred to the carbonyl group [12—14]. Other examples of this rearrangement are given in Table I (cf. *m/z* 310 in Fig. 2D).

The C values of the TMS ether methyl ester derivatives of 1,2-diols with the same number of carbon atoms were similar with one exception. 15,16-Dihydroxy-9,12-octadecadienoic acid had a C value that was 0.8 longer than that of the other C_{18} diols (Table I). This is presumably due to the TMS ethers being located rather close to the ω end (cf. ref. 15).

Cyclic alkyl boronic ester derivatives of 1,2-diols

General features. The *n*-butaneboronic acid methyl ester derivatives (BuB) showed strong molecular ions, and signals were noted at $M^+ - 31$ (loss of OCH_3), $M^+ - 57$ (loss of C_4H_9), $M^+ - 84$ (loss of C_4H_9BO), $M^+ - 102$ (loss of $C_4H_9B(OH)_2$) and $M^+ - 115$ (i.e. loss of 84 + 31). These signals were observed in all the mass spectra but with variable intensity. The methaneboronic acid derivatives (MeB) also showed strong molecular ions, and signals were noted at $M^+ - 31$ (or $M^+ - 32$ in a few cases), $M^+ - 42$ (loss of CH_3BO), $M^+ - 60$ (loss of $CH_3B(OH)_2$) and $M^+ - 73$ (i.e. loss of 42 + 31). The signal at $M^+ - 15$ of the MeB derivatives were much weaker than $M^+ - 57$ in the mass spectra of the BuB derivatives. Signals at $M^+ - 88$ (loss of 57 + 31) or $M^+ - 46$ (loss of 15 + 31) in the mass spectra of the BuB and MeB derivatives, respectively, were inconspicuous and could usually not be observed.

The mass spectra of the MeB and BuB derivatives showed fragments that presumably were formed by α-cleavage (e.g. a and b in Table II). These important fragments often gave strong signals and in some cases strong signals were also found at b—32 (presumably loss of methanol). Due to the natural abundance of ^{10}B and ^{11}B, which is 1:4.2, a boron-containing fragment (Y) gave signals both at *m/z* Y—1 and Y, which gave them a characteristic appearance [3].

The C values of the MeB and BuB derivatives are given in Table I and show little variation between diols with the same number of carbons, even if the cyclic alkyl boronic esters are situated near the ω end.

Mass spectra of individual 1,2-diols. For brevity, many of the general features discussed above will not be repeated in the description below.

1,2-Diols derived from linoleic acid

Methyl 9,10-dihydroxy-$C_{18:1}$. A mass spectrum of the MeB derivative is shown in Fig. 1A. Important signals are noted at *m/z* 241 ($M^+ - 111$) and at *m/z* 209 (241 — 32). The BuB derivative showed corresponding strong signals at *m/z* 283 ($M^+ - 111$) and 251 (283 — 32). The MeB derivative showed a

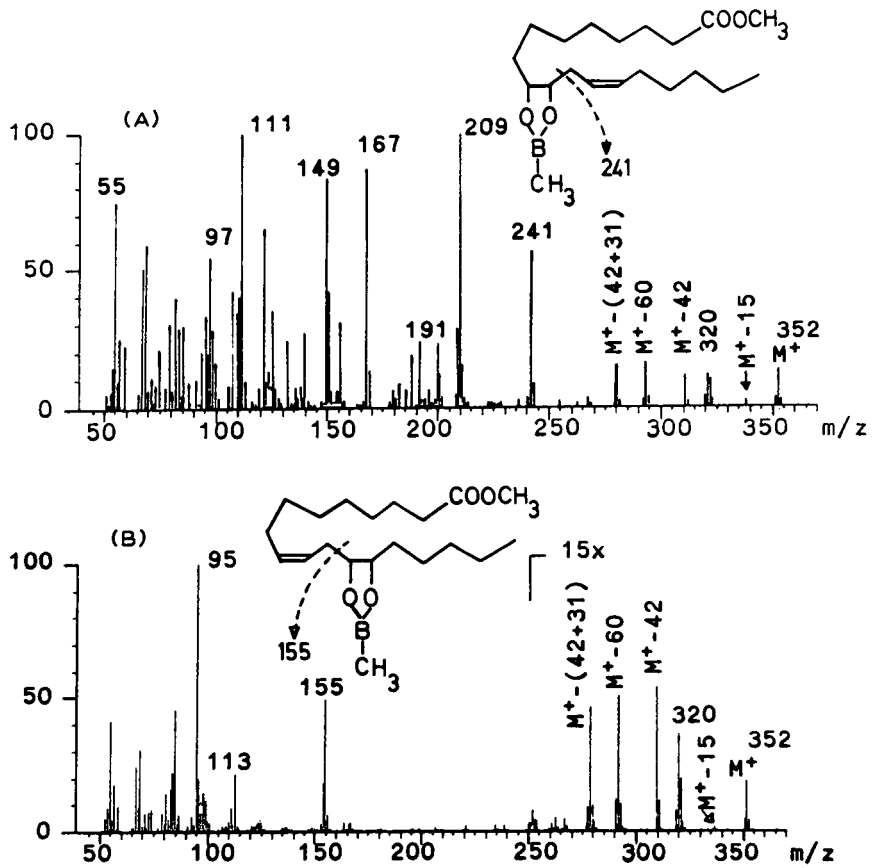

Fig. 1. Electron-ionization mass spectra of the cyclic methaneboronic ester methyl ester of 9,10-dihydroxy-12-octadecaenoic acid (A) and 12,13-dihydroxy-9-octadecaenoic acid (B).

rather strong signal at *m/z* 320 ($M^+ - 32$) but the corresponding fragment of the BuB derivative was inconspicuous.

Methyl 12,13-dihydroxy-$C_{18:1}$. A mass spectrum of the MeB derivative is shown in Fig. 1B. A fragment formed by α-cleavage could be *m/z* 155 (cleavage between carbons 11 and 12). This fragment could be expected to shift to 197 in the BuB derivative. A mass spectrum of the BuB derivative is shown in Fig. 5, although the diol in this case was isolated from a biological source (see below). This mass spectrum was, however, identical with that of the authentic material and a strong signal is noted at *m/z* 197.

1,2-Diols derived from α-linolenic acid

Methyl 9,10-dihydroxy-$C_{18:2}$. A mass spectrum of the BuB derivative is shown in Fig. 2A. The signals at *m/z* 235 and 283 might be due to α-cleavage (insert in Fig. 2A). A strong signal is found at *m/z* 251 (283 − 32). These three signals presumably correspond to the signals at *m/z* 193 (235 − 42), 241 (283 − 42) and 209 (241 − 32) of the MeB derivative.

Methyl 12,13-dihydroxy-$C_{18:2}$. A mass spectrum of the BuB derivative is shown in Fig. 2B. The signals at *m/z* 195 and 323 might be due to α-cleavage

(insert in Fig. 2B). Strong signals are also noted at *m/z* 291 (323 — 32) and 290 (M^+ — 102). The MeB derivative showed strong signals at *m/z* 281 (323 — 42), 153 (possibly 195 — 42) and 249 (281 — 32).

Methyl 15,16-dihydroxy-$C_{18:2}$. A mass spectrum of the BuB derivative is shown in Fig. 2C. The fragments formed by α-cleavage presumably give rise to the signals at *m/z* 363 (M^+ — 29, loss of C_2H_5), 332 (M^+ — (29 + 31)) and 155 (insert in Fig. 2C). The MeB derivative showed a very strong signal at *m/z* 113 (155 — 42), but a signal at *m/z* 321 (M^+ — 29) was not observed.

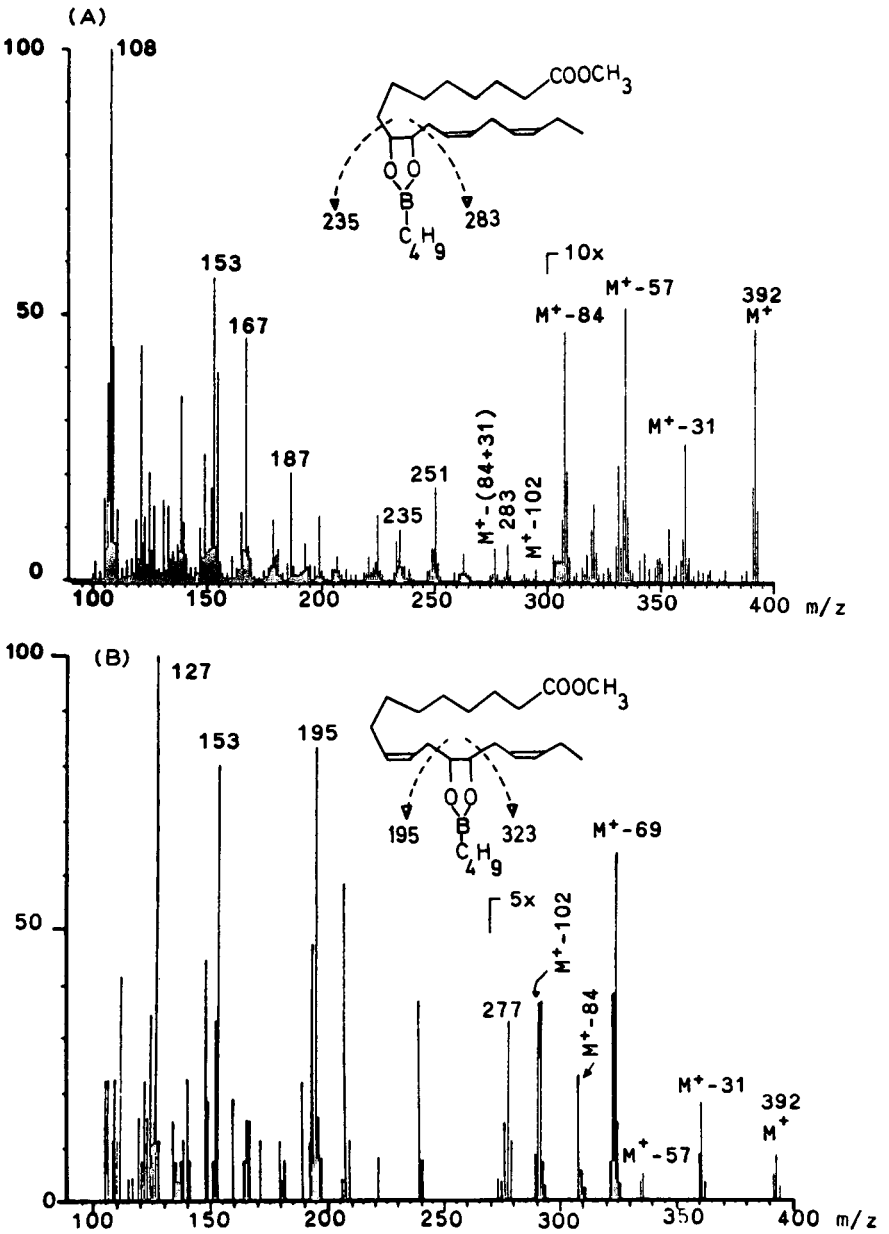

Fig. 2.

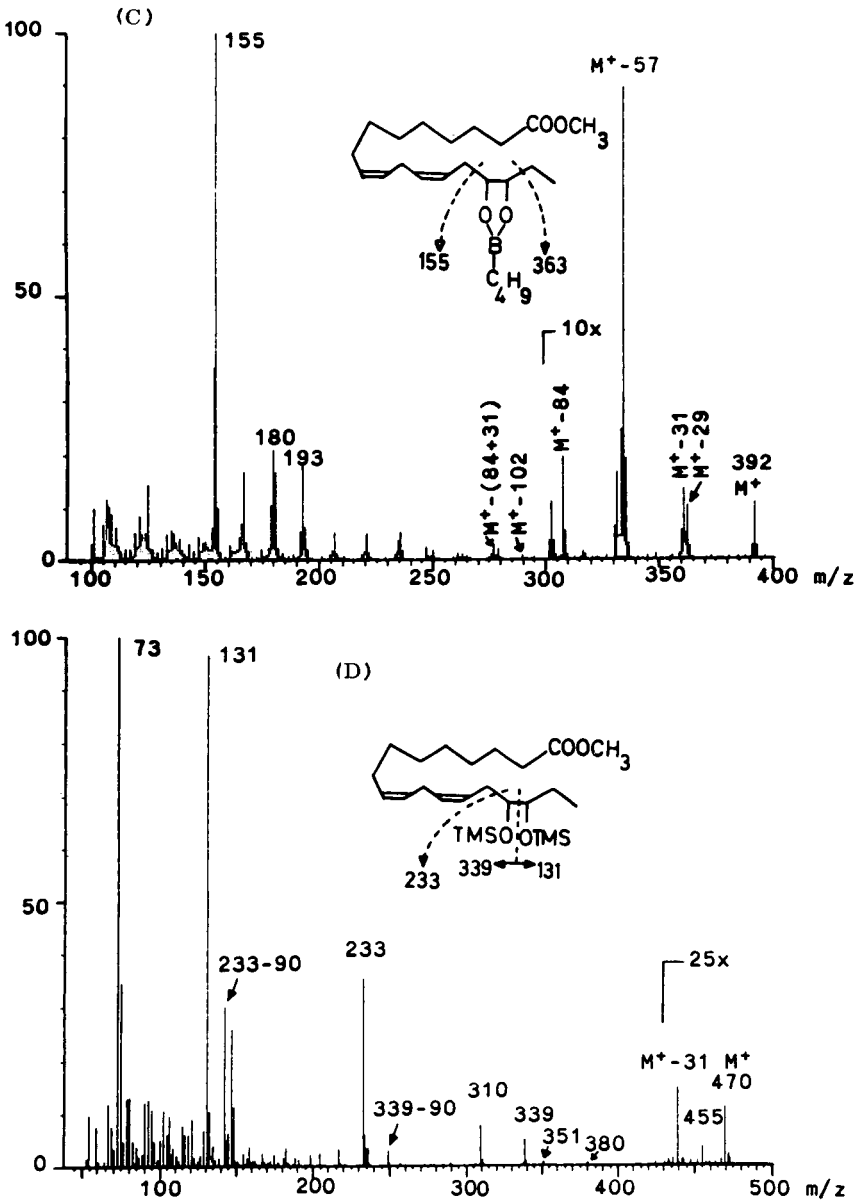

Fig. 2. Electron-ionization mass spectra of three vicinal dihydroxy acids, which were derived from α-linolenic acid. A, B and C show mass spectra of *n*-butaneboronic acid methyl ester derivatives of 9,10-dihydroxy-, 12,13-dihydroxy- and 15,16-dihydroxy-$C_{18:2}$, respectively. D shows a mass spectrum of the TMS ether methyl ester derivative of 15,16-dihydroxy-9,12-octadecadienoic acid. TMS stands for $Si(CH_3)_3$.

1,2-Diols derived from arachidonic acid

Important features of the MeB and BuB derivatives are summarized in Table II, which also shows the fragments that are presumably formed by α-cleavage (a and b in Table II). Signals at b—32 were noted in several of

TABLE II

SALIENT FEATURES OF THE CYCLIC *n*-BUTANEBORONIC ACID AND METHANEBORONIC ACID DERIVATIVES OF 1,2-DIOLS DERIVED FROM ARACHIDONIC ACID

Compound	Relative intensities of *n*-butaneboronic acid derivatives									
	M^+ 418	M^+-31 387	M^+-57 361	M^+-84 334	M^+-102 316	M^+-115 303	a* (%)	b* (%)	Base peak (100%)	Fragments in the upper mass range and their relative intensities (%)
Methyl 5,6-$(HO)_2$-$C_{20:3}$	0.5	1.1	0.3	1.6	2.3	0.6	317(4.0)	227(7.2)	55	336(1.9) 306(1.6) 279(1.4) 252(5.3) 195(34)**
Methyl 8,9-$(HO)_2$-$C_{20:3}$	2.3	0.2	0.7	0.6	1.4	4.3	277(5.9)	267(2.4)	67	317(1.4) 289(2.4) 277(5.7) 263(2.3)
Methyl 11,12-$(HO)_2$-$C_{20:3}$	1.2	0.2	0.5	0.6	1.2	1.7	237(4.0)	307(3.8)	69	281(1.4) 275(1.4)** 263(1.2)
Methyl 14,15-$(HO)_2$-$C_{20:3}$	0.8	0.1	2.7	1.5	2.7	3.2	197(33)	347(2.9)	55	336(6.8) 289(3.6) 276(3) 263(3.6)
	Relative intensities of methaneboronic acid derivatives									
	M^+ 376	M^+-31 345	M^+-42 334	M^+-60 316	M^+-73 303		a* (%)	b* (%)	Base peak (100%)	Fragments in the upper mass range and their relative intensities (%)
Methyl 5,6-$(HO)_2$-$C_{20:3}$	0.5	1.9	1.8	1.4	0.6		275(3.9)	185(12)	111	302(0.6) 294(2.6) 275(3.9) 236(4.8) 153(66)**
Methyl 8,9-$(HO)_2$-$C_{20:3}$	1.5	0.1	0.5	1.1	0.8		235(7.9)	225(0)	67	291(0.8) 275(1.4) 261(3.6)
Methyl 11,12-$(HO)_2$-$C_{20:3}$	0.6	0.1	0.4	0.8	0.8		195(2.6)	265(2.8)	69	291(0.4) 275(0.6) 265(2.9) 233(1.9)**
Methyl 14,15-$(HO)_2$-$C_{20:3}$	0.7	0.1	1.2	2.0	1.5		155(30)	305(1.7)	95	294(3.1) 273(0.4)** 263(1.1) 261(1.2)

*The fragmentation ions a and b refer to R_m—CH OB(X)OCH—R_w, where R_m and R_w denote the side-chains carrying the methyl ester and the omega end, respectively, and X = CH_3 or C_4H_9.

**These signals might, at least partly, be formed from b with loss of 32 (see text).

the mass spectra but they were not very intense in most of them. Mass spectra of some of the diols showed strong ions, which presumably are formed by rearrangement reactions.

Methyl 5,6-dihydroxy-$C_{20:3}$. Mass spectra of the MeB and BuB derivatives are shown in Fig. 3 and the fragmentation is summarized in Table II. The relatively strong signals at *m/z* 336 ($M^+ - 82$) of the BuB derivative and *m/z* 294 ($M^+ - 82$) of the MeB derivative were also noted in the mass spectra of 14,15-dihydroxy-$C_{20:3}$ but not in any of the other diols.

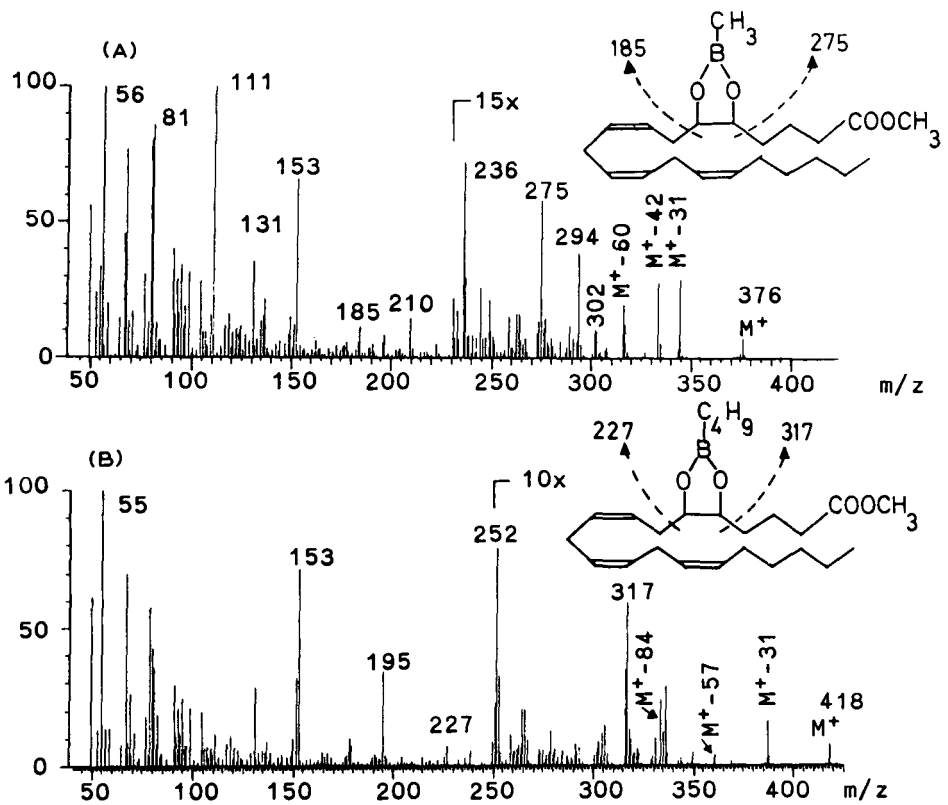

Fig. 3. Electron-ionization mass spectrum of the cyclic methaneboronic (A) and *n*-butaneboronic ester methyl ester (B) of 5,6-dihydroxy-8,11,14-eicosatrienoic acid.

Methyl 8,9-dihydroxy-$C_{20:3}$. The MeB and BuB derivatives are summarized in Table II and the mass spectra revealed few other interesting features.

Methyl 11,12-dihydroxy-$C_{20:3}$. A mass spectrum of the BuB derivative has been published previously [4]. The mass spectrum of the MeB derivative was also without conspicuous rearrangement ions.

Methyl 14,15-dihydroxy-$C_{20:3}$. A mass spectrum of the BuB derivative of the diol from a biological source has been published previously [4]. As shown by Table II, both the BuB and the MeB derivatives showed strong signals at $M^+ - 82$, i.e. at *m/z* 336 and 294, respectively. These fragments were the strongest in the upper mass range of these mass spectra (cf. Table II). The

BuB derivative of methyl [5,6,8,9,11,12,14,15-$^2H_8$]-14,15-dihydroxy-$C_{20:3}$ showed signals at *m/z* 426 (M^+), 369 ($M^+ - 57$), 355 ($M^+ - 71$), 342 ($M^+ - 84$) and 338 ($M^+ - 88$, possibly 57 + 31). The latter was the strongest signal but it can hardly correspond to *m/z* 336 of the protium form of the diol. The deuterated compound therefore seems to form partly other fragmentation ions than the protium form, when the BuB derivative is used.

Metabolism of linoleic and α-linolenic acid by hepatic and renal cortical microsomes and NADPH

[1-^{14}C]Linoleic and [1-^{14}C]linolenic acid were metabolized to many polar metabolites by rabbit renal cortical or hepatic microsomes and NADPH. Virtually no metabolism was noted in the absence of this cofactor. Fig. 4 shows the elution of radiolabelled metabolites from a reversed-phase HPLC column. Three peaks of radioactivity eluted (I—III in Fig. 4). Hepatic and renal cortical microsomes gave a very similar elution pattern. It is notable that peak II from the incubation of α-linolenic acid had the same elution volume as authentic [^{14}C]-9,10-dihydroxy- and -15,16-dihydroxy-$C_{18:2}$, but the various metabolites of α-linolenic acid were not further analyzed in this study.

Some metabolites of linoleic acid, which were formed by renal microsomes and NADPH, were purified and analyzed by GC—MS (GC column with 3% SP-2100). The material of peak II was subjected to normal-phase HPLC (a linear gradient of 30% chloroform in hexane to 90% chloroform in hexane in 60 min with 0.4% acetic acid, flow-rate 1 ml/min) and a major peak eluted after 58—60 min. The mass spectrum of the BuB derivative of the methyl ester is shown in Fig. 5. Signals were noted, inter alia, at *m/z* 394 (M^+), 363

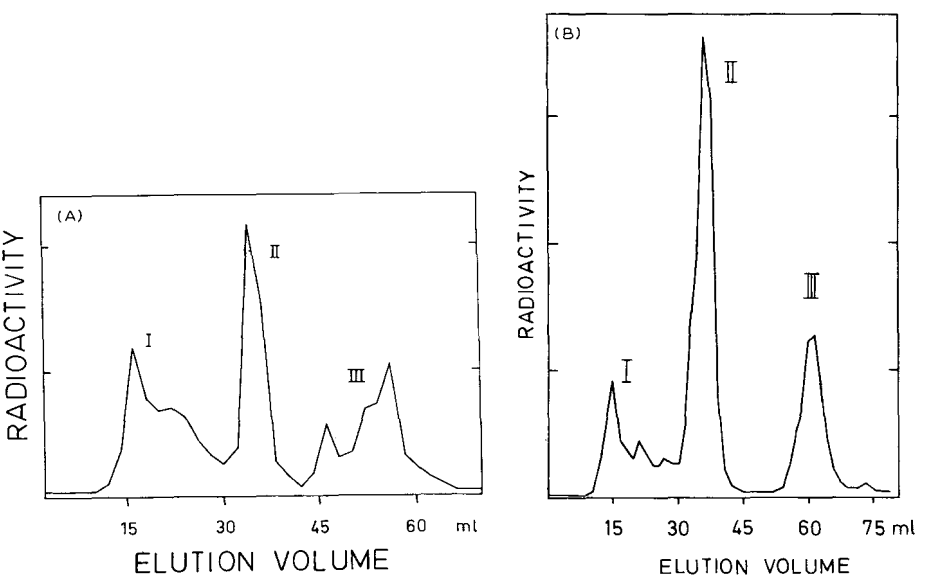

Fig. 4. Reversed-phase HPLC of metabolites formed by hepatic microsomes and NADPH from [^{14}C]linoleic (A) and α-[^{14}C]linolenic acid (B) (μBondapak C_{18}). Virtually no metabolites were formed if NADPH was omitted from the incubations.

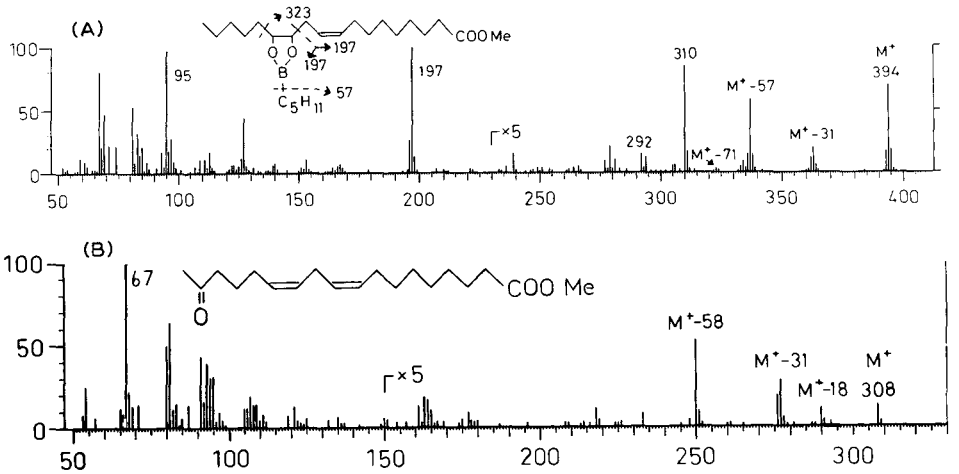

Fig. 5. Mass spectra of two metabolites of linoleic acid formed by renal cortical microsomes of the rabbit in the presence of NADPH. (A) *n*-Butaneboronic acid methyl ester derivative of 12,13-dihydroxy-9-octadecenoic acid. (B) Methyl ester derivative of 17-oxo-linoleic acid.

($M^+ - 31$), 337 ($M^+ - 57$), 323 ($M^+ - 71$), 310 ($M^+ - 84$), 279 [$M^+ - (84 + 31)$], 239, and a very strong signal at *m/z* 197. The two fragments formed by α-cleavage are indicated by the insert in Fig. 5. The C value was 22.2. This mass spectrum was identical with that of the authentic 12,13-dihydroxy-9-octadecenoic acid derivative.

The material of peak III was found to contain several metabolites. One was presumably 17-oxo-9,12-octadecadienoic acid (17-oxo-linoleic acid). It eluted after about 13 min on normal-phase HPLC (gradient as above). The mass spectrum of the methyl ester of this metabolite is shown in Fig. 5B. Signals were noted at *m/z* 308 (M^+), 290 ($M^+ - 18$), 277 and 278 ($M^+ - 31$ and $M^+ - 32$), 250 ($M^+ - 58$, presumably loss of CH_3COCH_3) and 67 (base peak). The C value was 19.3. The methoxime methyl ester derivative showed signals, inter alia, at *m/z* 337 (M^+), 306 ($M^+ - 31$) and 87 [base peak, presumably $CH_3C(NOCH_3)CH_3$]. The C value was 20.1. Assuming that the double bonds have not changed, as indicated by UV analysis, this compound was tentatively identified as 17-oxo-linoleic acid. Another metabolite peak III was 13-hydroxy-9,11-octadecadienoic acid, since the TMS ether methyl ester derivative gave a mass spectrum that was almost identical with that previously reported [16].

DISCUSSION

The present work compares the use of TMS ether derivatives and cyclic alkyl boronic ester derivatives for GC—MS analysis of 1,2-diols, which were chemically derived from three long-chain polyunsaturated fatty acids. The biological interest in these diols is based on the metabolism of arachidonic acid to vicinal diols by cytochrome *P*-450 in a reconstituted system [4, 6, 7] and by isolated renal cells and hepatocytes [9]. The present work also demon-

strates that linoleic and α-linolenic acid are metabolized to many polar products by cytochrome-*P*-450-containing microsomes and NADPH. It may be of interest to determine these metabolites, and the results of the present study might be helpful.

There are several features which make the cyclic boronates useful for analysis of 1,2-diols in biological samples. First, the cyclic boronates are only formed with a restricted number of bifunctional compounds, mainly 1,2-diols, 1,3-diols and α-hydroxy carboxylic acids [3]. The very formation of these derivatives provides important structural information. Furthermore, their electron-ionization mass spectra are easily recognized by the occurrence of abundant molecular ions with the characteristic isotope distribution and the fragmentation of the alkyl boronic esters. The mass spectra also indicate the position of the boronic esters by one or two fragments formed by α-cleavage.

The cyclic boronic ester derivatives also have some limitations. They are of little value for analysis of fatty acids with two or more vicinal diols, since diagnostically reliable fragmentation ions are not formed [17]. The mass spectra of the boronate derivatives of some of the polyunsaturated diols were difficult to interpret due to strong fragmentation ions, which presumably are formed by a rearrangement mechanism (e.g. 5,6-dihydroxy- and 14,15-dihydroxy-$C_{20:3}$ as discussed above). The present report also indicates that the *n*-butaneboronic ester derivative of octadeuterated 14,15-dihydroxy-$C_{20:3}$ can form partly other fragmentation ions than the protium form. This may limit the use of this derivative for mass fragmentography employing the deuterated internal standard.

The TMS ether methyl ester derivatives of the 1,2-diols gave rise to rather weak molecular ions, but still had many advantages. Strong fragmentation ions were formed by α-cleavage and by cleavage between the vicinal diols, giving up to four diagnostically very useful fragmentation ions. In this respect the TMS ether derivatives were clearly superior to the boronic esters. The TMS derivatives of vicinal diols also give important rearrangement ions, which were originally observed by Capella [12] and Capella and Zorzut [13]. These fragments, denoted $[R_m]^+Si(CH_3)_3$ in Table I, are of considerable practical value since they are intense, and often occur in the mass spectra of some metabolites of the 1,2-diols, e.g. metabolites formed by ω and ω—1 oxidation [7].

Long-chain fatty acids can be metabolized by hepatic and renal cortical microsomes, fortified by NADPH, to many products. ω and ω—1 oxidation have been studied in detail and both saturated and unsaturated fatty acids are oxygenated in this way by cytochrome *P*-450 [4, 18, 19]. Unsaturated fatty acids are also oxygenated by cytochrome-*P*-450-catalyzed epoxidation and by subsequent hydrolysis to vicinal diols [4—7, 20]. Finally, unsaturated fatty acids may undergo lipid peroxidation and autooxidation.

12,13-Dihydroxy-9-octadecenoic acid was identified as a metabolite of linoleic acid in rabbit renal cortical microsomes fortified with NADPH. This metabolite is presumably formed by ω6 epoxidation and by hydrolysis. Another metabolite, 17-oxo-linoleic is likely formed by ω—1 oxidation. In many respects, the metabolism of linoleic seems to be analogous to the cytochrome-

P-450-linked oxygenation of arachidonic acid. It will be of interest to determine whether α-linolenic acid and other polyunsaturated fatty acids of the $\omega 3$ series are metabolized in a similar fashion.

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CHROMBIO. 1671

DETERMINATION OF N^{τ}-METHYLHISTAMINE IN URINE BY GAS CHROMATOGRAPHY USING NITROGEN—PHOSPHORUS DETECTION

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SUMMARY

The determination of N^{τ}-methylhistamine in urine, using gas chromatography with nitrogen—phosphorus detection and the homologue N^{τ}-ethylhistamine as internal standard, is described. A comparison between the present method and a previously described stable isotope dilution mass fragmentographic method resulted in a regression line of $Y = 0.023 + 0.944X$ μmol/l with a correlation coefficient of 0.996.

The 24-h excretions of 35 normal adults on a free diet ranged from 0.4 to 1.8 μmol. Patients with mastocytosis, chronic myelocytic leukemia, anaphylactic reactions and a patient after bronchial provocation showed above normal values.

INTRODUCTION

Histamine, which is synthesized and stored by mast cells and basophils, is an important mediator of immediate hypersensitivity reactions. In man, the major metabolic pathway for the rapid degradation of histamine includes N-methylation, catalyzed by histamine methyltransferase (EC 2.1.1.8), leading to N^{τ}-methylhistamine. (For the nomenclature of substituted imidazoles see ref. 1.) About 70% of subcutaneously injected histamine is primarily metabolized by this methylation pathway, followed by subsequent deamination and dehydrogenation to N^{τ}-methylimidazoleacetic acid [2]. Both N^{τ}-methylhistamine and N^{τ}-methylimidazoleacetic acid are excreted in the urine.

In order to study the role of histamine during various pathological pro-

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cesses, several methods for the determination of histamine and histamine metabolites in body fluids have been described [3—6]. Notably those investigations in which the determination of histamine in plasma have been used, are hampered by the rapid metabolic degradation of histamine, resulting in large fluctuation in concentration within a short period of time [7]. As most studies have the aim to determine an increased production or liberation rate of histamine, it seems more practical to determine the major metabolites of histamine in the urine. In this respect the determination of N^{τ}-methylhistamine in urine has proved to be a useful parameter for the diagnosis and the follow-up of various pathological states in which histamine is involved, including mastocytosis, anaphylactic reactions, bronchial obstructive reactions, chronic myelocytic leukemia and bacterial overgrowth [3, 8].

The methods of determination of N^{τ}-methylhistamine described until now have been based on thin-layer chromatographic separation followed by fluorometric estimation of its dinitrofluorobenzene derivative [9], high-performance liquid chromatographic separation with fluorometric detection of dansylated N^{τ}-methylhistamine [10], and gas chromatographic separation in combination with mass fragmentographic quantitation of a perfluoroacylated derivative [3, 11]. These methods are fairly time-consuming, or need expensive apparatus like a mass spectrometer.

In this paper we report an assay of N^{τ}-methylhistamine in urine by gas chromatography with a nitrogen—phosphorus selective detector, which is more adequate for use in routine clinical laboratories. The results of the present method were compared to those obtained by the previously described determination of N^{τ}-methylhistamine, using stable isotope dilution mass fragmentography [3].

MATERIALS

N^{τ}-Methylhistamine was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; diethylsulphate was from Fluka A.G., Buchs, Switzerland; heptafluorobutyric anhydride was from Pierce Chemical Co., Rockford, IL, U.S.A.; all other organic solvents and reagents were of analytical grade from Merck, Darmstadt, G.F.R.

METHODS

Synthesis of N^{τ}-ethylhistamine

The internal standard N^{τ}-ethylhistamine was synthesized analogously to the preparation of N^{τ}-methylhistamine-d_3 [3], using diethylsulphate instead of dimethylsulphate-d_6.

Preparation of standards

A standard solution, containing 20 μmol/l N^{τ}-methylhistamine in 0.01 mol/l HCl was prepared and stored at 4°C. From the internal standard a stock solution was prepared containing 50 μmol/l N^{τ}-ethylhistamine in 0.01 mol/l HCl. Various amounts of the standard solution, corresponding to 0, 1, 2, 4, 6 and 8 nmol of N^{τ}-methylhistamine, were pipetted into 15-ml glass stop-

pered tubes. After the addition of 100 μmol (5 nmol) of the internal standard solution, the mixtures were evaporated to dryness at 120°C under a stream of air. All pipetting was done with Oxford adjustable push-button pipettes with polypropylene disposable tips.

Extraction of urine samples

To 4 ml of urine, 0.1 ml of 6 mol/l HCl and 100 μl of the internal standard solution were added. The solution was mixed and the volume reduced to about 0.5 ml under a stream of air at 120°C. After cooling, 0.25 ml of a 10 mol/l NaOH solution and 0.3 g of NaCl were added. The solution was shaken, and extracted twice with 4 ml of chloroform by vortexing for 5 min and subsequent centrifugation. The combined chloroform layers were dried over anhydrous sodium sulphate. After the addition of 0.1 ml of acetic acid, the solution was evaporated to dryness at 40°C under a stream of nitrogen.

Derivatization

To the dry sample (standard or extract of urine) in a 15 ml glass stoppered tube, 0.1 ml of heptafluorobutyric anhydride and 0.1 ml of acetonitrile were added. The tubes were capped and incubated overnight at room temperature.

Isolation of the derivatives

The solutions were evaporated to dryness at room temperature under a stream of nitrogen, and the residues dissolved in 1 ml of 0.75 mol/l sodium phosphate buffer pH 7.0. The derivatives were extracted into 4 ml of ethyl acetate by vortexing for 2 min. After centrifugation the aqueous layers were discarded. To the ethyl acetate 1 ml of 0.1 mol/l HCl solution was added, and the derivatives containing an imidazole moiety back-extracted into the aqueous layer. The ethyl acetate layers were discarded, and the aqueous layers adjusted to pH 7.0 by the addition of 1 ml of 0.75 mol/l sodium phosphate buffer pH 7.0. Four millilitres of ethyl acetate were added, and after vortexing and centrifugation, the aqueous layers discarded.

The ethyl acetate layers were dried over anhydrous sodium sulphate and evaporated to dryness under a stream of nitrogen at room temperature. The residues were dissolved in 100 μl of ethyl acetate, and 2-μl aliquots injected into the gas chromatograph.

Gas chromatography

Gas chromatography with nitrogen—phosphorus selective detection was performed with a Hewlett-Packard 5880 A gas chromatograph, equipped with a Model 7672 A autosampler and a 25 m × 0.22 mm I.D. SP-2100 coated, Carbowax-deactivated, fused silica column (Hewlett-Packard). The gas chromatographic conditions were as follows: injector temperature 260°C; oven temperature starting at 160°C for 6 min, then programmed to 220°C at 20°C/min, followed by 4 min at 220°C; detector temperature 300°C. The carrier gas was helium, split ratio 1:15 and the flow-rate 0.45 ml/min. The quartz insert of the injector was filled with a plug of glass wool and deactivated by rinsing with a solution of 0.05% (w/v) Carbowax 20M in chloroform and drying at 150°C.

Mass fragmentographic determination of N^{τ}-methylhistamine

The determinations of N^{τ}-methylhistamine by isotope dilution mass fragmentography were performed as described previously [3] with the following minor modifications: a Finnigan MAT 44-S mass spectrometer equipped with a Varian 3700 gas chromatograph was used under the conditions, split ratio 1:5, ionizing energy 70 eV, ion source temperature 200°C. The standard Finnigan MAT interface (open split coupling) was used.

RESULTS AND DISCUSSION

Methodology

The problems associated with the gas chromatographic analysis of imidazole compounds have been discussed previously [3, 4]. Adsorption, resulting in peak tailing and memory effects can be overcome either by introducing an acyl group into the imidazole ring, or by using a chromatographic system deactivated by Carbowax 20M. In the isotope dilution mass fragmentographic determination of N^{τ}-methylhistamine [3], the first solution was selected. The reaction of N^{τ}-methylhistamine with heptafluorobutyric anhydride (HFBA) in the presence of pyridine results in bis-HFB-N^{τ}-methylhistamine [3]. While investigating the use of this derivatization for the gas chromatographic analysis of N^{τ}-methylhistamine with the homologue N^{τ}-ethylhistamine as internal standard, it was found that this reaction often gave rise to varying yields for each compound. It was decided therefore to use the mono-HFB derivatives, which are quantitatively formed at room temperature. These derivatives, in combination with a Carbowax 20M deactivated insert and a Carbowax deactivated fused-silica capillary column, gave symmetrical gas chromatographic peaks, both of N^{τ}-methylhistamine-HFB and of N^{τ}-ethylhistamine-HFB.

Using a simple chloroform extraction from NaCl-saturated, alkalinized urine, many amines are extracted and subsequently derivatized to their HFB derivatives. When such a crude derivatized extract is further purified by adding 1 ml of phosphate buffer pH 7.0 and extraction with 4 ml of ethyl acetate, a chromatogram as shown in Fig. 1a is obtained. Indeed, many peaks are observed, making the determination of N^{τ}-methylhistamine with N^{τ}-ethylhistamine as internal standard troublesome.

In the same way as described for the determination of N^{τ}-methylimidazoleacetic acid, it was thought it would be possible to exploit the typical pK value of the imidazole ring for a direct clean-up of the crude derivatized extract [4]. In this method apolar interfering impurities were separated from the protonated imidazole-ring-containing compounds, by their selective extractability in an organic solvent at pH 1.0. However, such a clean-up procedure of the HFB derivatives at pH 1.0, using ethyl acetate as organic solvent, resulted in an almost quantitative extraction of N^{τ}-methylhistamine-HFB and the internal standard into the organic phase. In studying this phenomenon we found that ion-pair extraction of protonated substituted histamines and the heptafluorobutyrate anion occurred. To prevent the formation of this ion pair we investigated two post-derivatization clean-up methods. In the first method a methylation step for heptafluorobutyric acid was introduced.

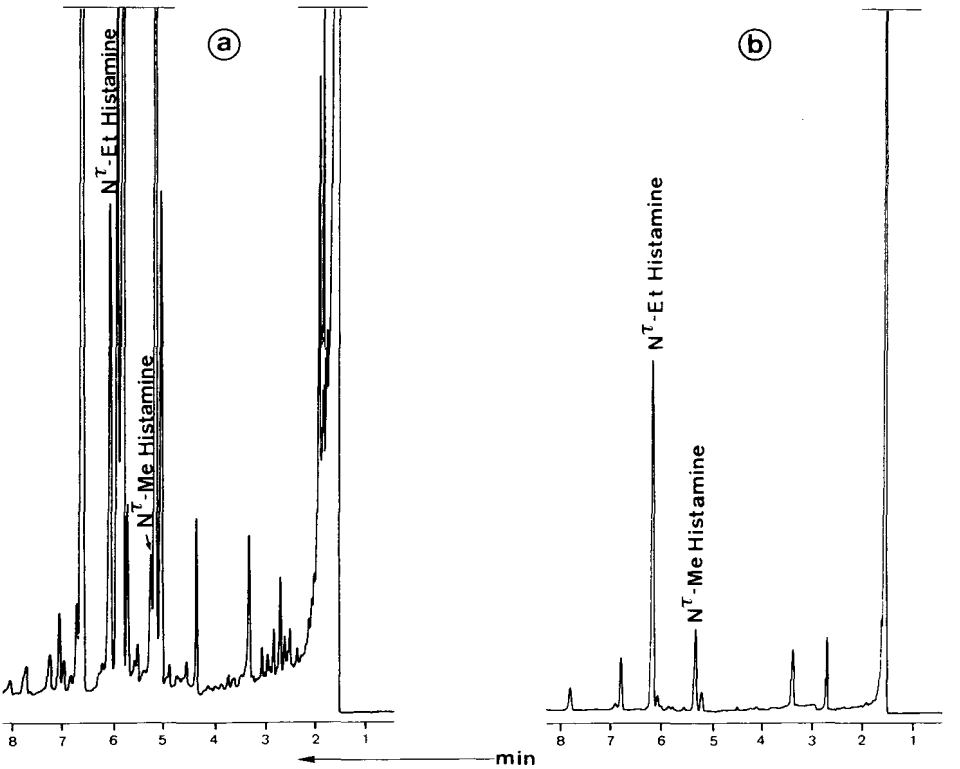

Fig. 1. Gas chromatograms of a urine of a normal person without a clean-up procedure after derivatization (a), and with an additional clean-up procedure after derivatization (b). The peak of N^{τ}-methylhistamine corresponds to an amount of about 1.3 pmol on column.

To the derivatized and evaporated extract, 1 ml of 1 mol/l HCl in methanol was added and the mixture heated at 60°C for 1 h. The solution was evaporated to dryness at room temperature under a stream of nitrogen. In the second method an extraction of the derivatives with ethyl acetate and buffer pH 7.0 was included, prior to the extraction at pH 1.0. In this preextraction the heptafluorobutyrate anion will remain in the aqueous layer. Both methods resulted in a considerably clean-up, with a high recovery of N^{τ}-methylhistamine-HFB and the internal standard. For routine analysis we prefer the second method in which three extraction steps at pH 7.0, pH 1.0 and pH 7.0 are used. A chromatogram of the same urine as shown in Fig. 1a, processed by the procedure described above, is shown in Fig. 1b. The overall recovery of N^{τ}-methylhistamine and N^{τ}-ethylhistamine amounted to 60%. Using this procedure, in combination with capillary gas chromatography and nitrogen—phosphorus detection, a specific and sensitive assay was obtained, without interference from extraneous compounds.

The calibration graph for the determination of N^{τ}-methylhistamine in urine was linear in the range investigated (0—8 nmol). An example of the typical data for a regression line is $Y = 0.197X - 0.001$ (Y = amount of N^{τ}-methylhistamine, X = peak height ratio), $r = 0.999$. When it was expected or found

that the concentration of N^{τ}-methylhistamine in urine was more than 2 μmol/l, 1 ml or less of this urine was taken instead of 4 ml in the extraction procedure.

The within-day reproducibility of this method was investigated by analyzing 20 aliquots of a pool urine. A concentration of 0.809 ± 0.028 μmol/l (mean ± 1 S.D.) was found, corresponding to a coefficient of variation of 3.5%. For the day-to-day variation these data were 0.801 ± 0.037 μmol/l (n = 10), coefficient of variation 4.7%. As a quality control in each series, two samples of a pool urine were incorporated, to one of which 4 nmol of N^{τ}-methylhistamine were added. When a difference greater than 2 S.D. between the value of this pool and the mean value, or a relative recovery of the spiked pool beyond 90—110% was found, the series was re-analyzed.

Correlation of the gas chromatographic and mass fragmentographic methods

To investigate the correlation between the gas chromatographic determination of N^{τ}-methylhistamine and the stable isotope dilution mass fragmentographic method previously described [3], 25 urine samples in the normal range (0.1—1.5 μmol/l) were analyzed by both methods. Fig. 2 shows the results of this correlation study. The regression line was calculated by the Deming method, as discussed by Wakkers et al. [12]. The correlation was found to be satisfactory, indicating that the present method is a good alternative to the mass fragmentographic determination.

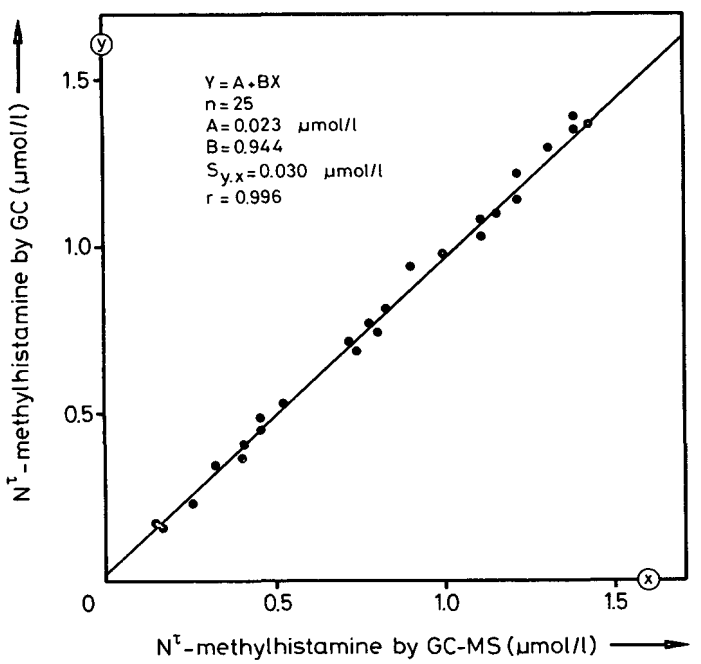

Fig. 2. Correlation between urinary N^{τ}-methylhistamine values obtained by a mass fragmentographic method (GC—MS) and the present gas chromatographic method (GC).

Urinary excretions of N^{τ}-methylhistamine

Urines were kept refrigerated during the collection period, and frozen at −20°C until assayed. Urines sent from elsewhere were collected in 6 mol/l HCl (10 ml/l urine). The N^{τ}-methylhistamine in urine stored at −20°C remains stable for at least six months. When non-preserved urines were kept at room temperature for several days, we observed in some cases a considerable increase in the N^{τ}-methylhistamine concentrations. This phenomenon was attributed to bacterial growth.

Since histamine may be present in the diet or may be synthesized from histidine by microorganisms in the lumen of the alimentary tract, Granerus [13] suggested that the investigations of histamine metabolism in man should be performed under standardized dietary conditions. To study the dietary influence in the N^{τ}-methylhistamine excretion, the 24-h excretions were determined in a group of eight normal persons, on a free diet and on a standardized diet. The standard diet, which was used the day before and on the day of the urine collection, contained 55 g of protein, and no bacterially processed foodstuffs like cheese or sauerkraut. The mean 24-h excretion of N^{τ}-methylhistamine on the free diet amounted to 1.27 μmol, range 0.85—1.57 μmol. Under the dietary conditions described above the values were 1.03 μmol and range 0.74—1.33 μmol. The difference between the excretion with and without a prescribed diet shows that, depending on the nature of the histamine investigation, it may be preferable to use dietary conditions.

In order to permit the measurement of N^{τ}-methylhistamine in urines collected over short time periods, N^{τ}-methylhistamine excretion may be expressed in relation to urinary creatinine. Table I shows the N^{τ}-methylhistamine excretions of some patients with an increased production or liberation rate of histamine. All patients were on a free diet. The normal range is based on

TABLE I

EXCRETION VALUES OF N^{τ}-METHYLHISTAMINE DURING VARIOUS PATHOLOGICAL STATES

Diagnosis	Specimen (h)	Urinary N^{τ}-methylhistamine	
		μmol per 24 h	μmol/mol creatinine
Systemic mastocytosis	24	29.40	5160
Systemic mastocytosis	24	16.10	1456
Systemic mastocytosis	24	8.92	610
Chronic myelocytic leukemia	24	103.0	10360
Chronic myelocytic leukemia	24	9.10	855
Chronic myelocytic leukemia	24	7.32	814
Anaphylactic reaction to:			
Acetylsalicylic acid	2		520
Glafenine	3		382
Iodamide	1		423
Iodamide	1		368
Iodamide	1		274
Normal range		0.4—1.8	40—160

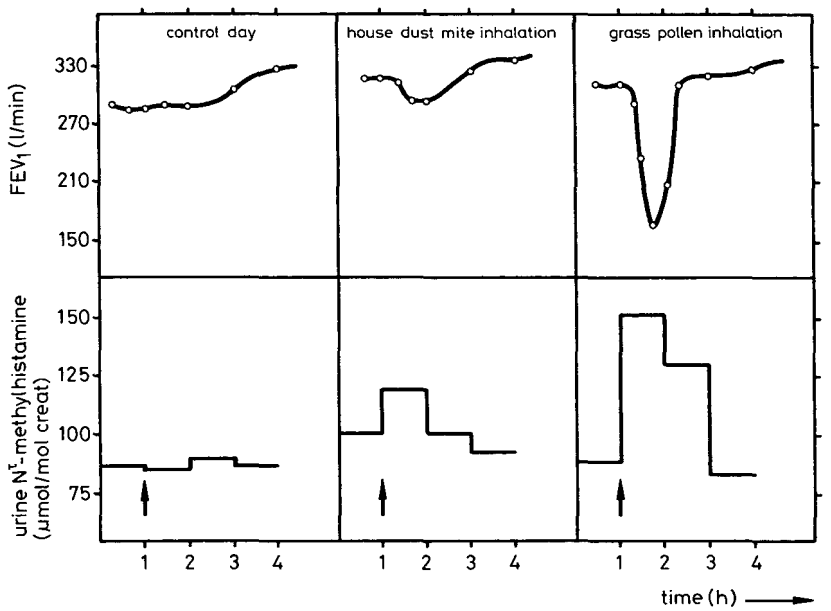

Fig. 3. The relationship between lung function (FEV_1) and urinary N^{τ}-methylhistamine excretion under different allergen challenges in the same patient. The arrows indicate the start of the 10-min allergen inhalation.

the determination of the excretions of 35 healthy adults on a free diet. The urines of the patients with an anaphylactic reaction were collected over a period directly after the onset of the reaction. The excretion of N^{τ}-methylhistamine of an asthmatic patient, on a control day and after bronchial provocation reactions, in relation to a parameter for the lung function, the forced expiratory volume during 1 sec (FEV_1), is shown in Fig. 3. The urines were collected in 1-h fractions. The association between the N^{τ}-methylhistamine excretion and the FEV_1 value was in agreement with the experiences of Löwhagen et al. [8].

In summary, it can be concluded that the described method permits the accurate determination of N^{τ}-methylhistamine in urine for clinical-chemical purposes, without the need of expensive gas chromatographic—mass spectrometric equipment. Although the extraction and clean-up procedures are somewhat more laborious than those of the formerly described mass fragmentographic procedure [3], in our hands it was possible to analyse up to 40 urine samples within one day by one technician, making use of an autosampler.

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CHROMBIO. 1672

DETERMINATION OF CATECHOLAMINES IN HUMAN SERUM BY MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH MICRO PRECOLUMN AND DUAL ELECTROCHEMICAL DETECTION

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SUMMARY

A dual electrochemical detector having two working electrodes (anode and cathode) in parallel—opposed configuration suitable for micro high-performance liquid chromatography was developed for the selective and sensitive detection of catecholamines on the basis of their electrochemical reversibility and catalytic amplification by recycling oxidation and re-reduction. The micro high-performance liquid chromatographic system with a micro alumina precolumn for enriching catecholamines and the dual electrochemical detector in parallel—opposed configuration was successfully utilized for the determination of catecholamines in healthy human serum injected directly after ultrafiltration.

INTRODUCTION

An innovative approach to electrochemical detection in high-performance liquid chromatography (HPLC) involves the use of two working electrodes operated simultaneously at different potentials. Several studies have reported the use of such dual electrochemical detection for HPLC [1—6].

In preceding papers [7, 8], a dual electrochemical detector (DECD) having two working electrodes (anode and cathode) in series configuration was designed for use in micro high-performance liquid chromatography (MHPLC), and the MHPLC system with a micro alumina precolumn for enriching and the DECD in series configuration was successfully utilized for the selective determination of catecholamines in healthy human urine directly injected. In the present work, a DECD based on a thin-layer electrolytic cell with two working electrodes (anode and cathode) in parallel—opposed configuration was designed and constructed to gain the catalytic amplification of electrochemical response in MHPLC. The direct injection analysis of catecholamines

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in healthy human serum was tried by using the MHPLC system with an alumina precolumn and such a DECD.

EXPERIMENTAL

Apparatus

The direct injection analytical system used is similar to that in the former study for urine analysis [7] and is shown schematically in Fig. 1. The dif-

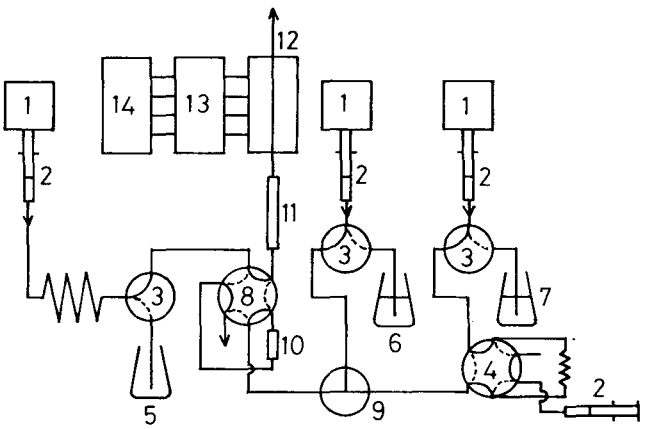

Fig. 1. Block diagram of the MHPLC system with micro precolumn and dual electrochemical detector in parallel—opposed configuration. 1 = Micro feeder, 2 = micro syringe, 3 = three-way valve, 4 = sample injector, 5 = mobile phase, 6 = buffer solution, 7 = water, 8 = six-way valve, 9 = mixing joint, 10 = micro precolumn, 11 = micro separation column, 12 = parallel—opposed twin-electrode thin-layer electrolytic cell, 13 = dual potentiostat, 14 = dual pen recorder.

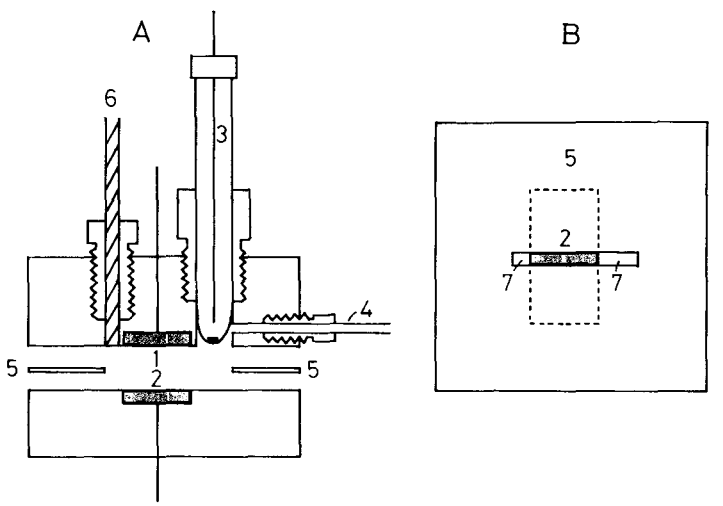

Fig. 2. Construction of parallel—opposed twin-electrode thin-layer electrolytic cell and connection with micro separation column. (A) Side view of cell, (B) top view of spacer. 1, 2 = Working electrode (glassy carbon), 3 = reference electrode (Ag/AgCl), 4 = counter electrode (platinum tube), 5 = spacer (PTFE sheet), 6 = micro separation column, 7 = hole.

ferences are as follows: the solution filter was removed, the pre-concentration way was reversed, the 200-μl sample loop was set and the DECD in parallel—opposed configuration was applied. The design of the twin-electrode thin-layer electrolytic cell in parallel—opposed configuration for DECD and connection with the micro separation column are shown in Fig. 2. The thin-layer cavity was constructed of two fluorocarbon resin blocks separated by a PTFE sheet 50 μm thick and 2 mm wide. Two working electrodes were made with glassy carbon plates 1 cm long and 2 cm wide contained in each block. The reference electrode, silver/silver chloride electrode, was held in a cylindrical hole in one of the blocks. A platinum tube served both as the counter electrode and the exit line.

The micro separation column for analysis was filled with octadecylsilica (Yanapak ODS-T, 10 μm) in a PTFE tube 15 cm $\times$ 0.5 mm I.D. The micro precolumn for enrichment was made by packing alumina (E. Merck, LiChrosorb Alox T, 30 μm) in a PTFE tube 2 cm $\times$ 0.5 mm I.D. The other instruments used are the same as in the previous paper [7].

Reagents

Analytical-reagent grade chemicals were used without further purification. All solutions were prepared from distilled, deionized water. For standard samples, noradrenaline (NA), adrenaline (AD) and 3,4-dihydroxybenzylamine hydrobromide (DHBA) were dissolved in Britton-Robinson (B-R) buffer pH 1.8. The buffer solution for pretreatment of the micro precolumn and pH adjustment of the sample was 1 *M* Tris buffer pH 8.7 containing 0.25% EDTA (disodium salt) and 0.05% sodium hydrogen sulfite for stabilizing catecholamines. The mobile phase for analysis was the B-R buffer pH 1.8 containing 2 m*M* sodium 1-heptanesulphonate (HSA) for ion-pairing, 0.1 m*M* EDTA (disodium salt) for masking iron ion and 50 m*M* sodium perchlorate for smoothing base currents.

Procedures

The human blood sample was drawn in a serum separation tube by venipuncture. After incubation at room temperature (ca. 20°C) for about 10 min to induce coagulation, the tube was centrifuged, separating the serum from the blood cells. Then, 500 μl of the raw human serum were taken in a test tube, to which had been added 0.5 mg of solid EDTA (disodium salt), 0.5 mg of solid sodium hydrogen sulfite, and 10 μl of 25 pg/μl DHBA standard solution. The former solid was added to complex any possible metal ions present, the latter solid to serve as an antioxidant and the DHBA solution to serve as an internal standard. The test tube was stoppered, shaken for 1 min by hand and a large portion of the mixed sample solution was transferred to an ultrafiltration cell (Millipore, type XX42 013 10). The ultrafiltration was carried out by stirring the solution with a magnetic stirrer under a nitrogen pressure of 2.5 kg/cm^2 in an ice bath. The filtrated serum was taken with a micro syringe and injected into the sample loop (200 μl) of the sample injector. The sample was delivered by water at a flow-rate of 33 μl/min and mixed with Tris buffer (pH 8.7) at the same flow-rate in the mixing joint to adjust the sample to pH 8.6. The sample was injected into the micro precolumn

for 10 min for enrichment with a mixed flow of water and buffer solution and then the micro precolumn was washed for a further 10 min with a flow of water only by stopping the flow of the buffer solution. By switching the six-way valve, the mobile phase was introduced into the micro separation column through the micro precolumn at a flow-rate of 8.3 μl/min. In this procedure, the adsorbed catecholamines are eluted from the micro precolumn and simultaneously separated by the micro separation column.

Selective and sensitive detection of catecholamines

Consider a reversible or quasi-reversible redox couple. The anode and cathode of the twin electrode thin-layer cavity are set at potentials where the reductant is oxidized and its oxidant is reduced, respectively. The reaction product of the anode is detected at the cathode positioned across from the anode. The reversible and/or quasi-reversible species are selectively detected at the cathode from many irreversible species. The selective detection of reversible species from quasi-reversible species may be achieved if the potentials of anode and cathode are properly chosen. The catalytic amplification in electrochemical detection may be obtained by recycling the oxidation and re-reduction between the anode and cathode at low flow-rates. In this manner the number of electrons exchanged in the detection process may be increased, and the sensitivity enhanced.

The separated catecholamines were introduced into the twin electrode thin-layer electrolytic cell in parallel–opposed configuration, in which the lower and upper working electrodes were set at the potentials (V vs. Ag/AgCl) of 0.60 and 0.20, respectively. The catecholamines were selectively detected with high sensitivity by monitoring the re-reduction current at the cathode and determined by comparing with the response of internal standard.

RESULTS AND DISCUSSION

Electrochemical behaviors of catecholamines

The electrochemical behaviors of NA, AD and DHBA on the glassy carbon electrode in the mobile phase used in MHPLC were studied by cyclic semi-integral voltammetry (CSIV) [9, 10] and semi-differential voltammetry (CSDV) [11–14]. CSIV and CSDV measure, respectively, the semi-integral (m) and semi-derivative (e) of current with respect to time vs. applied potential (E) under the same experimental conditions as in conventional cyclic voltammetry. Fig. 3 shows typical cyclic semi-integral and semi-derivative voltammograms of NA as an example. Voltammograms similar to those for NA were observed for AD and DHBA. The anodic and cathodic peak potentials (V vs. Ag/AgCl) in semi-derivative voltammograms were 0.48 and 0.46 for NA, 0.48 and 0.46 for AD, and 0.51 and 0.47 for DHBA, respectively. Therefore, the anode potential of 0.60 V and the cathode potential of 0.20 V were chosen for selective detection of three catecholamines in dual electrochemical detection.

According to the theory [9, 10], the wave height (m_d) in semi-integral voltammogram is represented by $m_d = nAFC\sqrt{D}$, where n is the electron transfer number, A the electrode area, F Faraday's constant, C the concen-

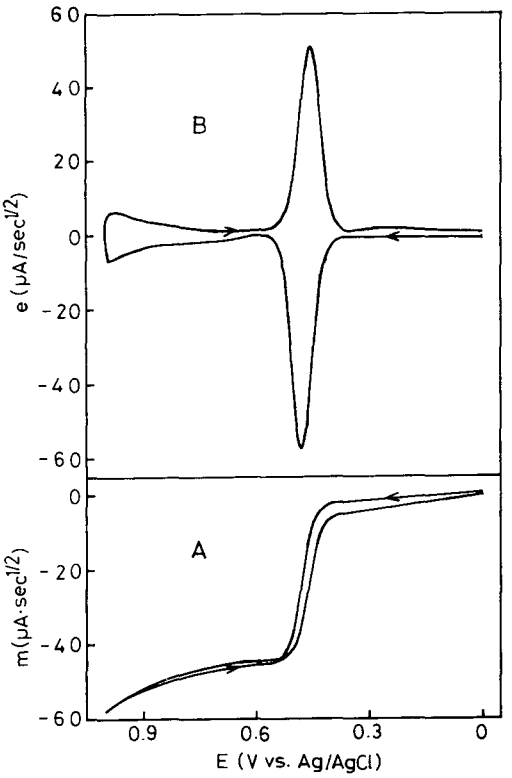

Fig. 3. Cyclic semi-integral and semi-derivative voltammograms of 1.0 m*M* noradrenaline in the B-R buffer pH 1.8 containing 2 m*M* HSA, 0.1 m*M* EDTA and 50 m*M* sodium perchlorate at a scan rate of 100 mV/sec. (A) Cyclic semi-integral voltammogram, (B) cyclic semi-derivative voltammogram.

tration and D the diffusion coefficient. The wave heights ($\mu A \cdot sec^{1/2}/mM$) in semi-integral voltammograms for oxidation of NA, AD and DHBA were 41.5, 41.0 and 44.5, respectively, while that for one electron reduction of $Fe(CN)_6^{3-}$ measured in the same medium was 20.9 $\mu A \cdot sec^{1/2}/mM$. It is clear that the three catecholamines investigated show nearly reversible electrode reactions in the medium used and the number of electron transfers involved is two.

Catalytic amplification in parallel—opposed dual electrochemical detection

Micro high-performance liquid chromatograms after precolumn enrichment were measured by using one or two working electrodes of the twin-electrode thin-layer electrolytic cell in parallel—opposed configuration. The flow-rate of mobile phase was 8.3 μl/min, and the applied potentials of the anode and cathode were 0.60 V and 0.20 V, respectively. The anodic peak currents of NA, AD and DHBA in measurements using one working electrode (anode) were amplified to 2.3, 2.8 and 2.7 times, respectively, on measurements using two working electrodes (anode and cathode). It should be noted that these values of amplification are much larger than those (1.1—1.2 times) obtained by using the twin-electrode thin-layer electrolytic cell in series configuration

[8]. The better amplifications are expected at slower flow-rates of mobile phase. The details will be described elsewhere [16].

Quantitation of catecholamines by the internal standard method

The MHPLC system with precolumn and DECD in parallel—opposed configuration was used for the quantitative analysis of the mixtures of NA, AD and DHBA. Typical chromatograms of standard catecholamines obtained from a 200-μl injection of 500 pg/ml of each of NA, AD and DHBA using the micro ODS column are shown in Fig. 4, in which parts A and B are, respectively, the anodic and cathodic chromatograms. All three catecholamines gave both anodic and cathodic chromatographic peaks as expected. The peak current ratios of re-reduction to oxidation of catecholamines were 0.69, 0.67 and 0.74 for NA, AD and DHBA, respectively, under the conditions shown in Fig. 4. These ratios in parallel—opposed configuration coincided with 0.68 and 0.68 for NA and AD, respectively, obtained by using the DECD in series configuration [8]. It is interesting that these ratios, collection efficiencies, in our DECD were much higher than those (< 0.37) observed in the similar series dual electrode detector in conventional HPLC [6]. This seems to

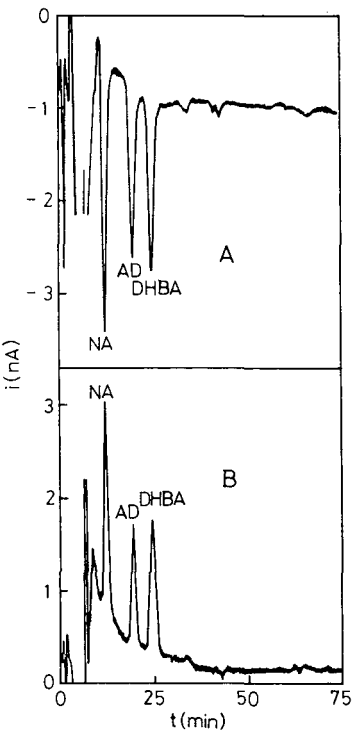

Fig. 4. Typical chromatograms of standard catecholamines by the MHPLC system with micro precolumn and dual electrochemical detector in parallel—opposed configuration. (A) Anodic response, (B) cathodic response. Sample: 200 μl of a standard solution of 500 pg/ml each of NA, AD and DHBA. Mobile phase: B-R buffer pH 1.8 containing 2 m*M* HSA, 0.1 m*M* EDTA and 50 m*M* sodium perchlorate. Flow-rate of mobile phase: 8.3 μl/min. Potentials (V vs. Ag/AgCl): anode (+) 0.60, cathode (+) 0.20.

depend on the differences in flow-rates used of 8.3 μl/min and 1 ml/min, and in the thickness of the thin-layer channel of 50 μm and 130 μm, respectively, in the former and latter. The electrolytic efficiency in the DECD in parallel–opposed configuration was about 83% at a mobile phase flow-rate of 8.3 μl/min. The better electrolytic efficiencies are expected at slower flow-rates of mobile phase. The details will be described elsewhere [16].

Both the anodic and cathodic peak height ratios of NA and AD to DHBA were linear with each amount of catecholamines injected, with good correlation, as shown in Table I. The linear dynamic range was about 10^3 and the

TABLE I

RELATIONSHIPS BETWEEN ANODIC AND CATHODIC PEAK HEIGHT RATIOS TO DHBA AND AMOUNT OF CATECHOLAMINES USING THE MHPLC SYSTEM WITH MICRO PRECOLUMN AND DUAL ELECTROCHEMICAL DETECTOR IN PARALLEL–OPPOSED CONFIGURATION

Mobile phase: B-R buffer pH 1.8 containing 2 m*M* HSA, 0.1 m*M* EDTA and 50 m*M* sodium perchlorate. Flow-rate of mobile phase: 8.3 μl/min. Potentials (V vs. Ag/AgCl): anode (+) 0.60, cathode (+) 0.20. Amount of DHBA injected as an internal standard: 200 pg.

Species		Relationship*	Correlation coefficient
NA	Anodic	$Y = -8.10X + 0.01$	0.999
	Cathodic	$Y = 7.81X - 0.07$	0.998
AD	Anodic	$Y = -4.64X - 0.01$	0.999
	Cathodic	$Y = 4.34X$	0.998

* Y = peak height ratio to DHBA; X = amount of catecholamine measured in ng.

TABLE II

PRECISION FOR DETERMINATION OF CATECHOLAMINES BY MHPLC WITH MICRO PRECOLUMN AND DUAL ELECTROCHEMICAL DETECTOR IN PARALLEL–OPPOSED CONFIGURATION

Sample: 200 μl of 500 pg/ml each of NA, AD and DHBA. Potentials (V vs. Ag/AgCl): anode (+) 0.60, cathode (+) 0.20.

Number	Peak height ratio to DHBA			
	NA		AD	
	Anodic	Cathodic	Anodic	Cathodic
1	1.75	1.71	1.03	0.98
2	1.87	1.67	1.05	1.00
3	1.77	1.62	1.05	0.97
4	1.80	1.58	1.04	0.93
5	1.78	1.64	1.00	0.98
6	1.85	1.62	1.06	0.96
7	1.81	1.60	1.03	0.94
8	1.81	1.65	1.02	0.99
Mean	1.81	1.64	1.04	0.97
Relative S.D.	2.2	2.5	1.9	2.5

minimum detectable amount in the proposed system was about 3 pg for NA and AD. This detection limit using the parallel—opposed DECD was about three times better than that [7, 8] using the series DECD. The precision for determination of catecholamines by the proposed system is shown in Table II. The relative standard deviations (%) for repetitive determination at the 100 pg level using the anodic and cathodic responses were 2.2 and 2.5 for NA, and 1.9 and 2.5 for AD, respectively.

Catecholamines in human serum

In order to examine the applicability of the proposed system for completely direct injection analysis of catecholamines in human serum, experiments were carried out to see whether raw human serum can be directly injected into a micro alumina precolumn. We were unable to establish the conditions to preconcentrate catecholamines without stopping up the precolumn. However, satisfactory results were obtained when raw human serum was ultrafiltrated before injection into the precolumn, as described under Procedures. Fig. 5 shows chromatograms of 200-μl injections of ultrafiltrated

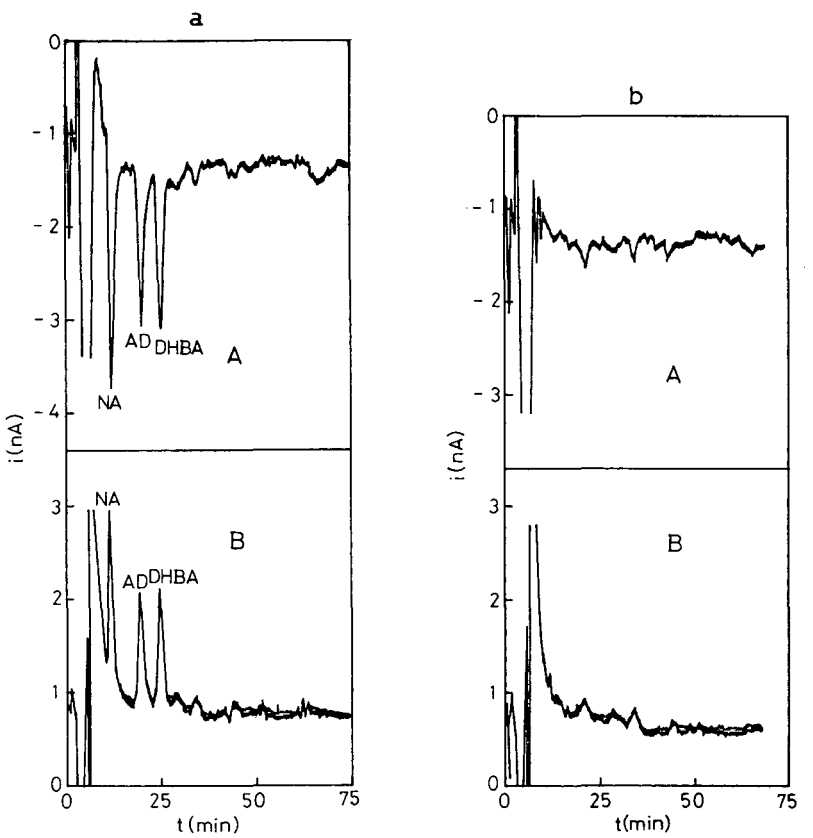

Fig. 5. Chromatograms of ultrafiltrated blank human serum and that spiked with standard catecholamines. (A) Anodic response, (B) cathodic response. Sample: (a) 200 μl of ultrafiltrated blank human serum spiked with 100 pg each of NA, AD and DHBA, (b) 200 μl of ultrafiltrated blank human serum. Other conditions are the same as in Fig. 4.

blank human serum and that spiked with 100 pg each of NA, AD and DHBA. The blank serum was prepared by storing the raw human serum for a long time (about three months) in the freezer at −8°C, which may introduce slow spontaneous oxidation or enzymatic conversion of catecholamines. Parts A and B in Fig. 5 represent, respectively, the anodic and cathodic chromatograms. The chromatograms of blank human serum spiked with catecholamines compare well with those of standard catecholamines (see Fig. 4). This indicates that the component of human serum does not seriously interfere the determination of catecholamines by the proposed system.

Typical chromatograms of catecholamines in human serum from healthy individuals obtained using the proposed procedures of internal standard addition and ultrafiltration, and the MHPLC system with precolumn and parallel–opposed DECD are shown in Fig. 6. Of particular interest in parts A are the peaks appearing as the shoulder of NA in Fig. 6b and the background of AD in Fig. 6a, respectively. By recording the cathodic current, the interferences from the compounds responsible for these peaks could be removed, as shown in parts B, on the basis of their electrochemical irreversibility. The precision for determination of catecholamines in the same healthy human serum by the proposed method is shown in Table III. The relative standard deviations for the determination of catecholamines in healthy human serum

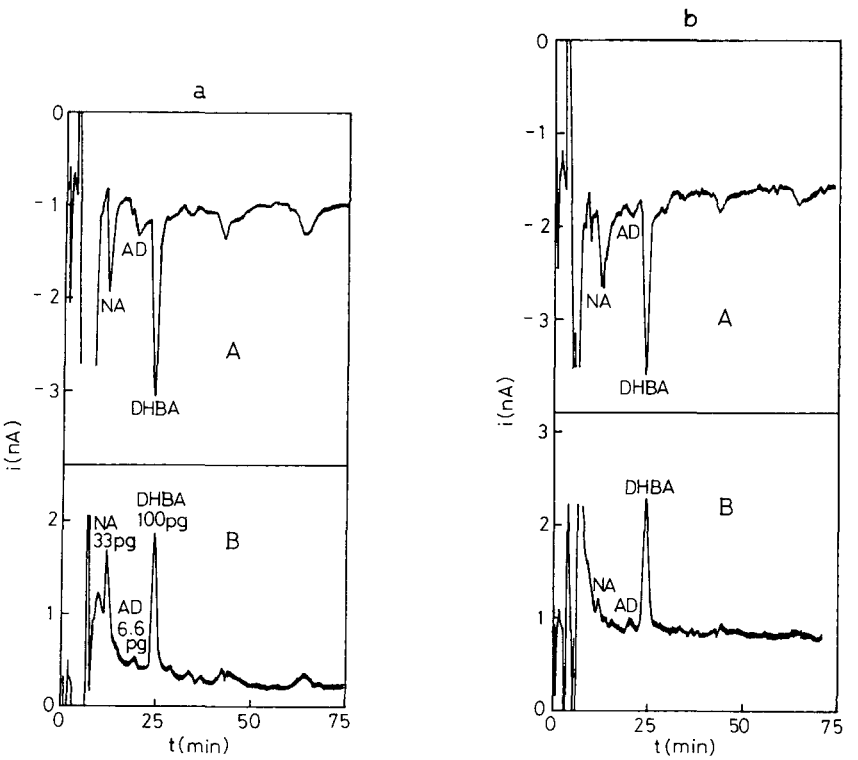

Fig. 6. Typical chromatograms of catecholamines in human serum from healthy individuals by internal standard addition. (A) Anodic response, (B) cathodic response. Sample: 200 μl of human serum spiked with 100 pg of DHBA. Other conditions are the same as in Fig. 4. a and b represent two different individuals.

TABLE III

PRECISION FOR DETERMINATION OF CATECHOLAMINES IN THE SAME HEALTHY HUMAN SERUM BY INTERNAL STANDARD ADDITION USING THE MHPLC SYSTEM WITH MICRO PRECOLUMN AND DUAL ELECTROCHEMICAL DETECTOR IN PARALLEL—OPPOSED CONFIGURATION

Number	Concentration (pg/ml)			
	NA		AD	
	Anodic	Cathodic	Anodic	Cathodic
1	162	184	59	42
2	159	178	62	37
3	187	165	67	33
4	160	177	94	38
5	160	169	113	34
6	146	170	89	30
Mean	162	174	81	36
Relative S.D.	8.3	4.0	26.5	11.8

using the cathodic responses were 4.0% for NA and 11.8% for AD. The precision for determination using the cathodic responses was about two times better than that using anodic responses. This depends on the difference in the possibility of interference from other electrochemical species present in human serum. The serum concentrations for NA and AD of the normal sample in Table III were 174 pg/ml and 36 pg/ml, respectively. These values compare well with the normal plasma concentrations (mean ± standard deviation) for NA and AD of 210 ± 100 pg/ml (n = number of samples = 1883) and 38 ± 20 pg/ml (n = 800), respectively, estimated by fluorometric detection [15].

CONCLUSIONS

The DECD in parallel—opposed configuration with anode and cathode is a powerful tool for selective detection of reversible and/or quasi-reversible species from many irreversible species and can provide an enhancement in sensitivity by recycling oxidation and re-reduction between the anode and cathode at slow flow-rates of mobile phase.

The determination of catecholamines in healthy human serum could be performed on direct injection of only 200 μl of an ultrafiltrated sample by using the proposed MHPLC system.

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PROTEINASE INHIBITORY ASSAYS OF SERUM USING HIGH-PERFORMANCE SIZE EXCLUSION CHROMATOGRAPHY

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SUMMARY

A size exclusion column (Spherogel TSK-2000 SW) was utilized in a high-performance size exclusion chromatographic assay to determine the proteinase inhibitory capacity of human sera. Values from assays using this technique agreed well with the standard spectrophotometric inhibitory assays. Nanogram to milligram amounts of protein, namely, α_1-proteinase inhibitor, elastase, trypsin, chymotrypsin and their corresponding complexes with the inhibitor, were fractionated in less than 15 min. The nitrated or oxidized α_1-proteinase inhibitor was shown to retain its ability to form stable complexes with trypsin or chymotrypsin; however, they lost the inhibitory activity against elastase and instead they behaved as common protein substrates for this enzyme. The present chromatographic procedure was unable to detect any peptide released when the native inhibitor and any of the proteinases reacted to form a complex. Moreover, dissociation of the α_1-proteinase inhibitor—elastase complex in an alkaline pH did not result in the formation or release of any peptide.

INTRODUCTION

α_1-Proteinase inhibitor (α_1-PI) is the major serine proteinase inhibitor in mammalian plasma. Among the proteinases inhibited by α_1-PI are trypsin, chymotrypsin and elastase. Although the mechanism of inhibition has not been definitely elucidated, inhibition entails the formation of a 1:1 molar complex between α_1-PI and proteinase. The standard assays used to determine the proteinase inhibitory capacity of α_1-PI or the inhibitor-containing sample such as the serum itself, are spectrophotometric procedures which measure residual proteinase activity after incubation of the enzyme with the inhibitor or the serum. This is accomplished by measuring the change of absorbance at a particular wavelength which occurs upon enzymatic hydrolysis of an appropriate substrate. Presented in this paper is a versatile and rapid method of high-performance size exclusion chromatography

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(HPSEC) which can be employed without the use of substrates, not only to assay for proteinase inhibitory capacity of pure α_1-PI, but also that of whole or unfractionated serum. Since the assay was able to differentiate the proteinase inhibitory activities of sera from (a) pregnant women, (b) women on oral contraceptives and (c) diluted sera simulating severe α_1-PI deficiency from that of (d) normal male sera, it is anticipated that this method will have a wide range of clinical and diagnostic applications. Moreover, investigations involving inhibitor-proteinase interaction, such as complex formation and dissociation, and digestion of oxidized or nitrated α_1-PI by elastase are readily demonstrable with this technique.

MATERIALS AND METHODS

Apparatus

This size exclusion chromatographic system employed consisted of a Beckman Spherogel TSK-2000 SW column (300 mm × 7.5 mm, fractionation range 1500—70,000), a Beckman Model 110 A pump and an Altex Model 210 injection valve. The proteinases were detected by their absorbance at 230 nm (unless otherwise indicated) with a Hitachi Model 100-10 variable-wavelength spectrophotometer and their areas were determined with a Hewlett-Packard recording integrator. The chart speed was 0.5 cm/min and the attenuation setting was at 3.

Buffers

The mobile phase was 0.1 *M* sodium phosphate buffer, pH 6.5, which had been made from deionized-redistilled water. The buffer solution was Millipore filtered (0.20 μm and degassed under house vacuum). The proteins were eluted at a flow-rate of 1.0 ml/min. All other buffer solutions used were Millipore filtered and degassed under vacuum. Protein stock solutions were filtered with disposable Amicon Sterilet (0.20 μm).

Proteins

α_1-PI was purified from out-dated human plasma as described previously [1]. Porcine pancreatic elastase and bovine pancreatic trypsin and α-chymotrypsin were obtained from Sigma (St. Louis, MO, U.S.A.). Protein concentrations were determined by using their extinction coefficients at 280 nm ($A_{280}^{1\%}$) which for α_1-PI [2], trypsin [3], chymotrypsin [4] and elastase [5] were 5.3, 15.4, 20.7 and 20.2, respectively.

High-performance size exclusion chromatographic inhibitory assays

Known amounts of plasma, serum, α_1-PI, elastase, trypsin, chymotrypsin and their respective incubation mixtures were applied to the column by syringe suction. Using 0.05 *M* Tris—HCl buffer, pH 8.0, 400 μl of the protein solutions were prepared and passed through a 20-μl sample loop to ensure complete loop loading, and 20 μl containing 1/20 of the protein were injected on to the column. The proteins were monitored by their absorbance at 230 nm. To assess complex formation, a 1:1 molar mixture of α_1-PI and elastase or trypsin or chymotrypsin were incubated in a total volume of 400 μl of 0.1

M Tris—HCl buffer, pH 8.0, at room temperature (25°C) for 15 min. As in the spectrophotometric assay [6], the incubation time was varied in some instances from 1 to 30 min in order to ensure maximum complex formation.

Serum was obtained from a male, a pregnant woman, and a woman receiving oral contraceptives. Each sample was filtered with an Amicon Sterilet (0.2 μm) to remove any particulate matter. Elastase (20 μg) was reacted with various volumes of serum (10—50 μl) in a total of 400 μl of 0.05 *M* Tris—HCl buffer, pH 8.0, for 15 min at 25°C. A 20-μl aliquot was subjected to HPSEC. The proteins were monitored by their absorbance at 280 nm rather than 230 nm. The detector range setting was 0.2 absorbance units and the recorder attenuation setting was 1.0. Corresponding incubation mixtures were assayed by the spectrophotometric procedure described previously [6].

Additional procedures

The spectrophotometric assays for trypsin, chymotrypsin or elastase, the proteinase inhibitory assay for α_1-PI, the nitration with tetranitromethane (TNM) or oxidation with the N-chlorosuccinimide (NCS) of α_1-PI, were described in previous publications [7]. Chymotrypsin in 0.05 *M* Tris—HCl buffer, pH 8.0, was treated with 100-fold molar excess of the inhibitor phenylmethanesulfonyl fluoride for 10 min at 25°C [8], and excess reagent was removed by dialysis against the same buffer in the cold room.

RESULTS

Comparison of the HPSEC and spectrophotometric inhibitory assays of human sera

The HPSEC proteinase inhibitory assay of serum (Fig. 1b) was possible because of the fact that no absorbing (280 nm) material was observed in the area where elastase (Fig. 1c) was eluted. On the other hand, when plasma (Fig. 1a) was used, a major protein peak appeared at the position where the enzyme also elutes. For this reason only sera was utilized for the assay. The inhibitory assay is based simply on the fact that the elastase peak disappears quantitatively when incubated with varying amounts of serum prior to chromatography. As can be seen in Fig. 1d and e, 59 and 100% of elastase (2 μg) disappeared and presumably formed a complex with α_1-PI when incubated for 15 min with 0.5 μl and 1.5 μl of normal male serum, respectively. From Fig. 2, the calculated amount of different serum samples required to inhibit completely 1 mg of elastase were for (a) a normal male, 2.5 ml; (b) a pregnant woman, 0.50 ml; (c) a woman receiving oral contraceptives, 0.55 ml; and (d) normal male serum diluted to 10% with 0.05 *M* Tris—HCl buffer, pH 8.0, 25 ml.

It is evident that the HPSEC assay is superior to the spectrophotometric one since it can achieve 100% inhibition (Fig. 2) while the latter only 70—85%. Moreover, as more serum was incubated with the enzyme, the spectrophotometric assay becomes inefficient as indicated by an apparent decreasing inhibitory activity. This is not the case in the HPSEC assay.

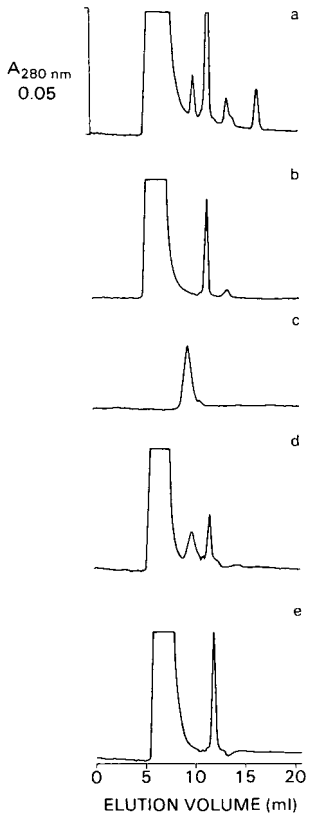

Fig. 1. HPSEC of human plasma, serum, porcine elastase and mixtures of serum and elastase. (a) 2.5 μl of plasma; (b) 1.0 μl serum; (c) 2 μg elastase, retention time 9.43 min; (d) 0.5 μl serum incubated with 2 μg of elastase for 15 min at 25°C; (e) 1.5 μl serum incubated with 2 μg of elastase. The samples were prepared for injection into the column as described in the Methods section.

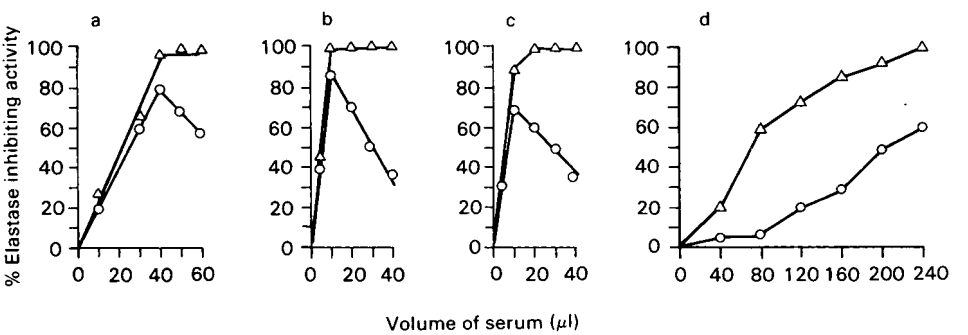

Fig. 2. Comparison of the spectrophotometric and HPSEC elastase inhibitory assays of human sera. Elastase (20 μg) was titrated with increasing volumes of (a) male serum; (b) serum from a pregnant woman, and (c) serum from a woman receiving oral contraceptives, and (d) male serum diluted to 10% with 0.1 *M* Tris—HCl buffer, pH 8.0. The elastase spectrophotometric (○——○) and HPSEC (△——△) inhibitory assays were performed as described in the Methods section. In (a) 2.5, (b) 0.50, (c) 0.55 and (d) 25 ml of serum inhibited 1 mg of elastase, respectively as calculated from the HPSEC assays.

The inability of inactivated chymotrypsin to form a complex with α_1-PI

In order to study further the application of HPSEC in the interaction of α_1-PI with proteinases, the subsequent experiments described below were performed. The chymotrypsin-inhibitory capacity of α_1-PI (Fig. 3a) against native chymotrypsin (Fig. 3b) was 98.9% (Fig. 3d). When complex formation occurred, the retention time of 6.53 min for α_1-PI (Fig. 3a) was shortened to 6.32 min (Fig. 3d). However, inactive chymotrypsin (Fig. 3c) failed to form a complex with α_1-PI as evidenced by the fact that the area of chymotrypsin did not decrease or disappear (Fig. 3e). Furthermore, both the area and retention time of α_1-PI were altered (Fig. 3e). It should be noted that when chymotrypsin was inactivated by phenylmethanesulfonyl fluoride, a 39% decrease in its area occurred (Fig. 3c).

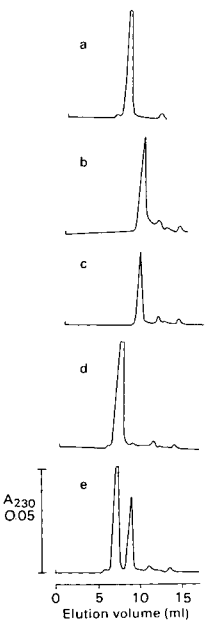

Fig. 3. The inability of inactivated chymotrypsin to form a complex with α_1-PI. Active site inactivated and native chymotrypsin were reacted with α_1-PI as described in the Methods section and 20 μl were applied to HPSEC. (a) 2.8 μg of α_1-PI control, retention time 6.53 min; (b) 1.0 μg of chymotrypsin control, retention time 8.31 min; (c) 1.0 μg of modified chymotrypsin, retention time 8.24 min; (d) 1.15:1 molar mixture of α_1-PI and native chymotrypsin, retention time of complex 6.32 min; (e) 1.15:1 molar mixture of α_1-PI and modified chymotrypsin; 2.8 μg of α_1-PI and 1.0 μg of modified chymotrypsin; retention time of α_1-PI 6.53 min; retention time of modified chymotrypsin 8.24 min. The percent elastase-inhibitory activity of (d) was 98.9%, while that of (e) was 0%.

Dissociation of the α_1-PI—elastase complex by exposure at pH 12

A 1:1 molar ratio of α_1-PI (Fig. 4a) and elastase (Fig. 4b) was incubated and HPSEC analysis showed that 95% of the elastase was inhibited by α_1-PI (Fig. 4c). The retention time of the complex was 6.38 min. After 15 min at pH 12, 65.6% of the elastase was recovered (Fig. 4d) and the retention time of the complex (6.38 min) changed to 6.49 min, indicating the formation

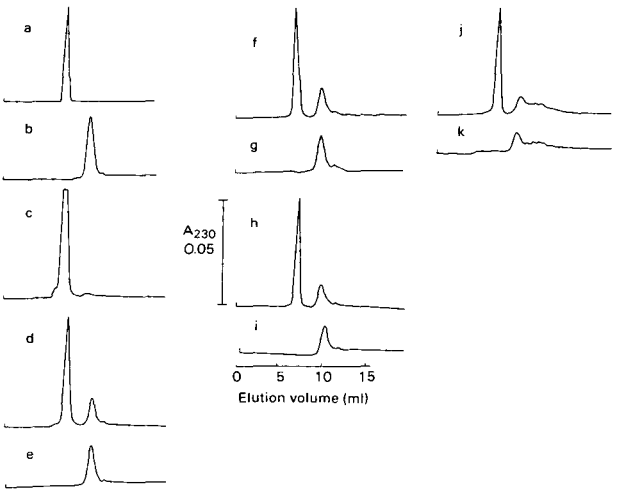

Fig. 4. Dissociation of the α_1-PI—elastase complex by exposure at pH 12. A 1:1 molar mixture of α_1-PI and elastase was incubated under standard conditions (25°C, pH 8.0, 15 min), at which point the pH was adjusted to 12, as described in the Methods section. (a) 2.5 μg of α_1-PI control; retention time 6.55 min; (b) 1.0 μg of elastase control, retention time 9.51 min; (c) incubation mixture after 15 min, and before adjustment to pH 12: 3.5 μg of complex, retention time 6.38 min; (d—k) incubation mixtures (3.5 μg total protein) and elastase controls (1.0 μg) after treatment at pH 12 for various time periods; (d) pH 12 for 15 min, retention time of complex and dissociated α_1-PI 6.49 min; retention time of elastase 9.44 min; (e) elastase control, pH 12 for 15 min, retention time 9.44 min; (f) pH 12 for 45 min, retention time of complex and dissociated α_1-PI 6.50 min; retention time of elastase 9.37 min; (g) elastase control, pH 12 for 45 min, retention time 9.38 min; (h) pH 12 for 133 min, retention time of complex and dissociated α_1-PI 6.51 min; retention time of elastase 9.29 min; (i) elastase control, pH 12 for 133 min, retention time 9.29 min; (j) pH 12 for 22 h, retention time of complex and dissociated α_1-PI 6.54 min; retention time of elastase 9.23 min; (k) elastase control, pH 12 for 22 h, retention time 9.12 min. The percent elastase liberated from the complex upon treatment at pH 12 was 65.6%, 78.1%, 81.5%, and 98% for (d), (f), (h), and (j), respectively.

of free α_1-PI (Fig. 4d). After 45 min, 133 min, and 22 h a recovery of 78.1%, 81.5% and 98% (Fig. 4f, h and j, respectively) of the elastase originally bound to α_1-PI (Fig. 4c) was observed. After 22 h, the retention time of the complex (6.38 min) had returned to that of α_1-PI (6.54 min, Fig. 4a and j) showing complete dissociation. Furthermore, only after 22 h were any low molecular weight peptides observed for either the dissociated mixture (Fig. 4j) or the elastase control (Fig. 4k), indicating that elastase was stable at pH 12 for at least 133 min. It is very possible that the low molecular weight peptides formed at 22 h incubation are autocatalytic products of elastase and not from α_1-PI. α_1-PI alone remained intact for 22 h under the same incubation condition (Fig. 4j).

HPSEC preparative elution profiles of oxidized and nitrated α_1-PI

Fig. 5 represents a typical preparative elution profile of (a) 10 mg of NCS-oxidized α_1-PI and (b) 10 mg of TNM-nitrated α_1-PI. In the case of the oxidized α_1-PI, the peak (Fig. 5a) as measured at 280 nm, was symmetrical, indicating

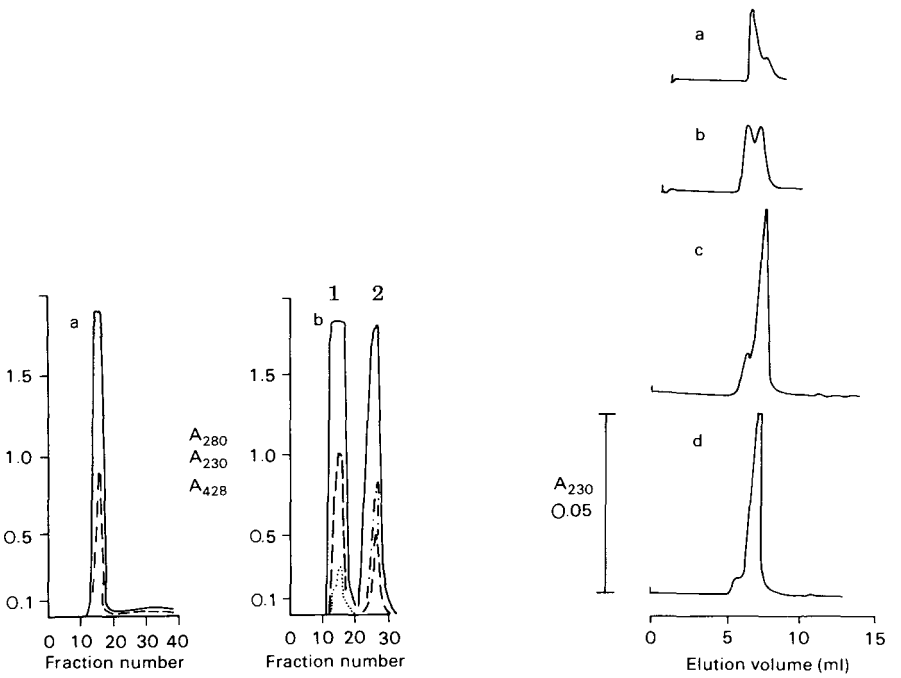

Fig. 5. HPSEC elution profiles of oxidized or nitrated α_1-PI. (a) α_1-PI (10 mg, 18.5 nmol) was oxidized with NCS as described in the Methods section. At the end of the reaction, the mixture was lyophilized, redissolved in 500 μl of distilled water, and subsequently subjected to HPSEC. The protein was eluted and fractions were collected. After the addition of 2.0 ml of 0.1 *M* sodium phosphate buffer, pH 6.5, the protein was detected by its absorbance at 230 nm (——) and 280 nm (— — —). Fractions 15—18 were pooled and the pH was adjusted to 8.0 with 4 *N* sodium hydroxide. (b) α_1-PI (10 mg, 18.5 nmol) was nitrated as described in the Methods section. The mixture was prepared, subjected to HPSEC, and processed as described in (a). In addition to 230 nm (——) and 280 nm (— — —), the nitrated inhibitor (b), peak 1 was detected by the absorbance of its nitrotyrosine residues at 428 nm (. . . .). Fractions 15—18 were pooled and the pH was adjusted to 8.0 with 4 *N* sodium hydroxide. Peak 2 represented the other product of the nitration reaction, namely, nitroformate.

Fig. 6. Polymerization of α_1-PI upon nitration. Samples of 60 μl were taken from fractions 13—16 isolated from the HPSEC fractionation of nitro-α_1-PI (Fig. 5b). The samples in μg amounts were subjected to HPSEC. (a) Fraction No. 13, retention time 5.31 min; (b) fraction No. 14, retention time 5.43 and 6.31 min; (c) fraction No. 15, retention time 5.58 and 6.44 min; (d) fraction No. 16, retention time 6.48 min.

that no polymerization occurred. Although polymerization of the nitrated α_1-PI was not clearly evident (Fig. 4b, first peak), the absorbance at either 280 nm or 428 nm showed a shoulder at fraction No. 14. The second peak (fractions 21—30), absorbing at 428 nm, represented the other product of reaction, namely nitroformate. The inability of the TSK-2000 column to separate the polymer from the monomer was due to overloading. When aliquots were removed from fractions 13—16 and microgram amounts were chromatographed, polymerization was evident (Fig. 6). Fraction No. 13 contained mostly polymer (Fig. 6a), while fractions 14, 15 and 16 (Figs. 6b, c, d) con-

tained increasing amounts of the nitrated monomer. The area of the polymer in Fig. 6a was approximately 15% of the area of the nitrated monomer (Fig. 6d), which agrees well with the values obtained after column chromatography on Sephadex G-100. To minimize the amount of polymer, fractions 16—18 were pooled for subsequent studies (Fig. 7).

The same HPSEC studies were performed with the NCS-oxidized α_1-PI. The results (not shown) revealed that only the monomeric form of the material existed.

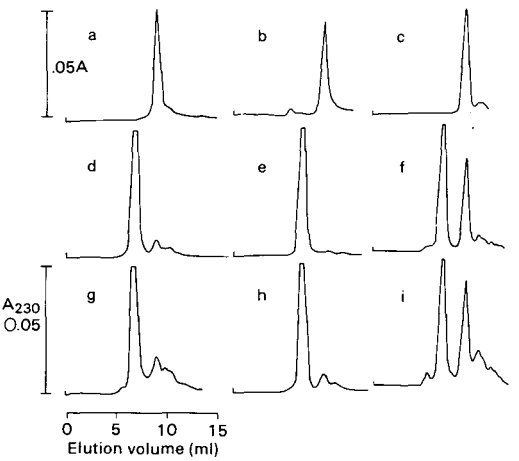

Fig. 7. Loss of complex formation between elastase and nitrated or oxidized α_1-PI. Samples of nitrated and oxidized α_1-PI (Fig. 5) were tested for their ability to form a complex with trypsin, chymotrypsin, and elastase. The HPSEC inhibitory assays were performed as described in the Methods section. (a—c) Amounts of 1 μg of trypsin, chymotrypsin and elastase, respectively, retention times (a) 8.59 min, (b) 8.37 min, (c) 8.80 min; (d, e) complex of oxidized α_1-PI with trypsin and chymotrypsin, respectively; 3.8 μg total protein, retention times (d) 6.39 min, (e) 6.35 min; (f) incubation mixture of oxidized α_1-PI with elastase, 2.8 μg of oxidized α_1-PI and 1.0 μg of elastase; retention time of oxidized α_1-PI, 6.55 min; retention time of elastase, 8.82 min; (g, h) complexes of nitrated α_1-PI monomer with trypsin and chymotrypsin, respectively, 3.8 μg total protein, retention time (g) 6.39 min, (h) 6.36 min; (i) incubation mixture of nitrated α_1-PI monomer with elastase; 2.8 μg of nitrated α_1-PI and 1.0 μg of elastase; retention time of nitrated α_1-PI 6.53 min, retention time of elastase 8.80 min. The complex formation of oxidized α_1-PI with trypsin (d), chymotrypsin (e), and elastase (f), was 85%, 98%, and 11%, respectively. The complex formation of nitrated α_1-PI with trypsin (g), chymotrypsin (h), and elastase (i) was 80%, 85%, and 0%, respectively.

The loss of complex formation between elastase and nitrated or oxidized α_1-PI

When oxidized (Fig. 7d, e, f) and nitrated α_1-PI (Fig. 7g, h, i) were reacted separately with trypsin (Fig. 7a), chymotrypsin (Fig. 7b) and elastase (Fig. 7c), the percent complex formation of the modified derivatives with the enzymes agreed well with the values previously obtained with the corresponding spectrophotometric inhibitory assays. The complex formation of oxidized α_1-PI with trypsin (Fig. 7d), chymotrypsin (Fig. 7e), and elastase (Fig. 7f) was 85%, 98%, and 11%, respectively. The complex formation of nitrated α_1-PI with trypsin (Fig. 7g), chymotrypsin (Fig. 7h), and elastase (Fig. 7i)

was 80%, 85% and 0%, respectively. Not only did both derivatives fail to inhibit elastase but they also underwent proteolysis with elastase as demonstrated by the appearance of new peptide peaks (Fig. 7f and i).

Degradation (proteolysis) of oxidized and nitrated α_1-PI by elastase

Fig. 8 represents the elution profile of mg amounts of oxidized or nitrated α_1-PI (6.8 mg) after 1.0 min of incubation with elastase (2.7 mg). When compared to unreacted controls (Fig. 8a and b, nitrated and oxidized α_1-PI, respectively), it is immediately apparent that the modified derivatives underwent proteolysis by elastase. Furthermore, in the case of the nitrated α_1-PI (Fig. 8c), the appearance of peptides containing 3-nitrotyrosine residues was observed. These peptides are now being purified for sequence determination.

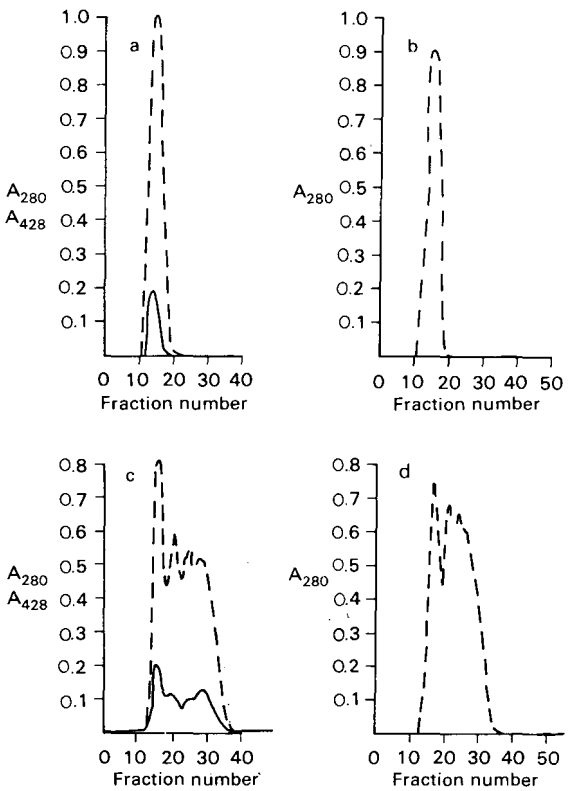

Fig. 8. Digestion of nitrated or oxidized α_1-PI by eleastase. Aliquots of 125 nmol (6.8 mg) of (c) nitrated monomer and (d) oxidized α_1-PI were reacted separately with a 1:1 molar ratio of elastase (2.7 mg) for 1.0 min at 25°C in a total of 6.2 ml of 0.05 *M* Tris—HCl buffer, pH 8.0. After 1.0 min, a 100-fold molar excess of phenylmethanesulfonyl fluoride was added to each and the mixtures were frozen and lyophilized. The samples were redissolved in 500 µl of distilled water and subjected to HPSEC. Fractions of 500 µl were collected and 100 µl of 0.1 *M* sodium phosphate buffer were added to each fraction. (a) and (b) represent undigested (no elastase added) nitrated and oxidized α_1-PI, respectively. The absorbance of each fraction at 280 nm (— — —) was measured, as well as the absorbance at 428 nm (——) for the nitrated α_1-PI—elastase mixture.

DISCUSSION

The results presented in this manuscript clearly demonstrate the usefulness of HPSEC on Spherogel as a means of assaying proteinase inhibitory activity of whole serum, as well as of purified α_1-PI as reported previously [6]. The assay is based on the quantitative disappearance of the elastase, trypsin or chymotrypsin peak when each of the enzymes was incubated with increasing amounts of serum or pure α_1-PI. Of serum or plasma, only the former can be used because the latter contains a highly absorbing peak that elutes at the same position as the enzymes. Adding increasing amounts of serum eventually interferes with the spectrophotometric inhibitory assay presumably due to the increased absorbance of serum at the wavelength employed to measure the hydrolytic products of the substrates; this is not the case for the HPSEC assay since no substrate is necessary. The HPSEC assay can be employed not only for normal male serum, but also in other conditions, whereby α_1-PI concentration is increased, such as in pregnancy and women receiving oral contraceptives [9], as well as, perhaps, in a genetic deficient state [10] as simulated by the normal serum diluted to 10%.

In contrast to the standard spectrophotometric inhibitory assay which requires about 10 μg of enzyme and 25 μg of pure α_1-PI or 25 μl of undiluted serum the present method may require only 1/10 of the above materials and, therefore, is more economical. Very often and especially in the case of the elastase spectrophotometric assay, the substrate N-*tert.*-butyloxycarbonyl-L-alanine-*p*-nitrophenylester (NBA) is relatively unstable under the assay conditions and, therefore, difficult to obtain stable baseline readings. This problem is clearly obviated when HPSEC is used, since, as already mentioned, no enzyme substrate is needed.

The versatile and rapid technique of HPSEC on the Spherogel column can be employed also, as shown in the present communication, in studies dealing with the capacity of the native α_1-PI to form complexes with native proteinases, the inability of the nitrated or oxidized α_1-PI to inhibit or form a complex with elastase, although retaining the capacity to inactivate trypsin or chymotrypsin as evidenced by complex formation with these enzymes, the loss of the capacity of the inactivated proteinases to form a complex with α_1-PI, and to detect the presence or absence of aggregation when α_1-PI is chemically modified. Thus, aggregation was shown during nitration with TNM but not oxidation with NCS.

Morii et al. [8] had observed the release of a peptide from human α_1-PI with an approximate molecular weight of 3600, when the native inhibitor formed a complex (0.05 *M* Tris—HCl buffer, pH 8.0, 25°C, 5 min) with native bovine chymotrypsin. The peptide was isolated by heating the reaction solution at 100°C for 2 min in the presence of 1% sodium dodecyl sulfate and followed by polyacrylamide gel electrophoresis. Peptides of the same molecular weight were also formed during the interaction of α_1-PI and bovine trypsin or porcine elastase [8]. Carrell et al. [11] incubated a 1:1 molar ratio of human α_1-PI and bovine trypsin in 0.025 *M* Tris—HCl, pH 7.3, at 25°C for 30 sec, and then formic acid and 95% ethanol were added to precipitate the protein. The ethanol supernatant was dried in vacuo and subjected

to two-dimensional high-voltage and conventional paper electrophoresis, whereby small peptides were observed in the chromatogram. After amino acid sequence determination, the peptides identified were to have arisen from the 37 residues in the C-terminal region of the native α_1-PI.

The above experiments were carefully repeated with the exception that HPSEC was utilized for the detection of the peptides released instead of polyacrylamide gel electrophoresis or high-voltage paper electrophoresis. Unfortunately, we were unable to detect by this method any peptide(s) released even after repeated attempts. In these experiments, the chromatographic time was extended for as long as 60 min. Standard amino acids such as lysine, glutamic acid or leucine were each eluted in 12 min, while bovine insulin (molecular weight 6000) in 10 min. In some instances, in order to decrease the possibility that the sensitivity of the technique was inadequate, the amount of the reactants, i.e., α_1-PI and enzymes, injected to the column was increased five times the amount normally detectable by this method. However, as with the other experiments, no peptide peak was observed. Moreover, dissociation of the α_1-PI—elastase complex at alkaline pH did not show any release of small peptide either. We have no explanation at this time for our negative results, although we believe that if the peptide(s) were in fact released during the interaction of α_1-PI and a proteinase, HPSEC as presently used should have been able to detect them.

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CHROMATOGRAPHIC ANALYSIS OF BLOOD LIPIDS

COMPARISON BETWEEN GAS CHROMATOGRAPHY AND THIN-LAYER CHROMATOGRAPHY WITH FLAME IONIZATION DETECTION

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SUMMARY

Intact human blood plasma lipids of different composition were analyzed by gas chromatography and thin-layer chromatography with flame ionization detection. The reproducibility of the results obtained by gas and thin-layer chromatography was compared. The main advantages and disadvantages of both methods for lipid analysis are discussed. Generally, the variability of the results measured by thin-layer chromatography in series and from day to day was greater than that obtained by gas chromatography.

INTRODUCTION

In the study of lipid metabolism and its disorders, a sufficiently rapid, selective and precise method is lacking for the determination of the level or composition of the individual lipid classes. At present, primarily various chro-

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matographic methods and combinations thereof are used for this purpose. The most frequently used methods are gas and thin-layer chromatography.

Gas chromatography (GC) of intact blood lipids was first described by Kuksis et al. in 1967 [1]. Since then, several other works devoted to this problem have been published and the method has been automated [2–6]. The use of flame ionization detection (FID) for detection and quantitation of substances separated by thin-layer chromatography (TLC) was first described roughly at the same time as GC of intact lipids [7]. Subsequently, similar works appeared, but broader application of FID in the detection and quantitation of substances separated on a thin layer was first described in the work of Okumura et al. [8, 9].

Both methods, GC and TLC—FID, are limited to a certain extent in practical applications. In GC, this is primarily a result of the small capacity of the examination and the impossibility of direct analysis of some polar lipids [4]. In TLC, reproducibility of measurements at low concentrations of the separated substances has so far been dubious. In addition, GC yielded separation of fractions of neutral lipids in dependence on their carbon number, while TLC in the given experimental arrangement permits only separation of lipid classes. This property, which is a drawback from the point of view of biochemical research, may be compensated in clinical biochemistry by the much greater capacity of this method compared with GC. TLC—FID has been proposed and successfully applied to the screening of lipid levels [10].

Our work was concerned with studying the reproducibility of the results of analysis of blood lipids by the two methods considering the suitability of application of TLC—FID in the study of disorders in lipid metabolism.

EXPERIMENTAL

Chemicals and instruments

All the solvents used were rectified and tested for purity before use. Pure substances were used for calibration of the gas chromatograph and to obtain suitable correction factors for TLC—FID. Purity was controlled by GC. Triglycerides with carbon numbers of 48 (tripalmitin), 50 (*rac*-glyceryl-1,3-palmitate-2-stearate), 52 (*rac*-glyceryl-1,3-stearate-2-palmitate) and 54 (tristearin) were products of Supelco, Bellefonte, PA, U.S.A. Cholesteryl esters with carbon numbers of 41 (cholesteryl myristate), 43 (cholesteryl palmitate), 45 (cholesteryl stearate) and 47 (cholesteryl arachate) and internal standards for GC (cholesteryl butyrate and tridecanoin) and free cholesterol were supplied by Applied Science Labs., State College, PA, U.S.A. Phospholipids *rac*-1,2-palmitoylglycerol-3-phosphorylcholine, *rac*-1-palmitoylglycerol-3-phosphorylcholine and *rac*-1-palmitoyl-2-oleyl-3-phosphorylethanolamine were supplied by Fluka, Buchs, Switzerland; sphingomyelin and phosphatidylserine were supplied by Koch-Light, Colnbrook, Great Britain. Alfol RD 18 (1-octadecanol) was a product of Condea, Hamburg, G.F.R.

Preparation of sample

Biological samples were obtained by extraction of 2 ml of blood plasma from persons with normal or increased blood lipid levels according to the

TABLE I

COMPOSITION OF MODEL LIPID MIXTURE FOR TLC—FID

1 μl of the solution was applied on the rod.

Substance	Concentration (μg/μl)	Weight (%)
Cholesterol	0.4	7.7
Cholesteryl palmitate	1.7	32.7
Tripalmitin	1.2	23.1
Palmitic acid	0.2	3.8
rac-1,2-Palmitoylglycerol-3-phosphorylcholine	1.7	32.7

TABLE II

COMPOSITION OF MODEL LIPID MIXTURE FOR GC

1 μl of the solution was injected into the gas chromatograph.

Substance	Concentration (μg/μl)	Weight (%)
Cholesterol	0.300	9.8
Cholesteryl myristate	0.025	0.8
Cholesteryl palmitate	0.250	8.2
Cholesteryl stearate	1.200	39.4
Cholesteryl arachate	0.150	4.9
Cholesteryl esters	1.625	53.3
Tripalmitin	0.025	0.8
rac-Glyceryl-1,3-palmitate-2-stearate	0.200	6.6
rac-Glyceryl-1,3-stearate-2-palmitate	0.600	19.7
Tristearine	0.300	9.8
Triglycerides	1.125	36.9

method of Folch et al. [11]. The composition of samples prepared from pure substances is given in Tables I and II.

Thin-layer chromatography

TLC—FID was carried out on an Iactroscan TH-10 (Iatron Labs., Tokyo, Japan) using silica gel S-I (10 μm particle size) and S-II (5 μm) Chromarods. Scanning speed was 3.2 cm/sec, air flow through the detector 2100 ml/min, hydrogen flow 180 ml/min for all samples.

Analysis of neutral lipids and free fatty acids. The internal standard for TLC—FID was 1-octadecanol. Each sample was dissolved in 0.5 ml of a 1% solution of the internal standard in chloroform—methanol (2:1, v/v) and 1 μl was applied on the rod. The developing chamber was saturated for 20 min;

the rods were activated by being passed through the FID prior to use. The samples of blood lipids were separated using hexane—diethyl ether—formic acid (90:10:1, v/v) at 22—25°C. The system was developed to a height of 10 cm. After drying, the rods were scanned in FID. The biological samples were analyzed on S-I rods, while the model synthetic ones were analyzed on both S-I and S-II rods to check for effect of particle size.

Analysis of phospholipids. The blood phospholipids as well as model synthetic samples were analyzed on S-II rods. The sample volume was in all cases 1 μl.

A model mixture of phospholipids was separated using chloroform—methanol—water (45:26:2.5, v/v) at 22—25°C. The chromatogram was developed to a height of 8 cm. The biological sample of human blood phospholipids was first developed in acetone to move the neutral lipids and free fatty acids (FFA) to the front of the rods, and after scanning in FID (from R_F = 0.1), the phospholipids remaining at the start were then developed in the same system as the model mixture. The second scanning was carried out under the conditions described above.

Simultaneous analysis of neutral lipids and phospholipids. Both polar and neutral lipids can be separated and assayed simultaneously according to the method of Iatron Laboratories [12]. The solution of plasma lipids prepared by the method mentioned above was spotted on Chromarod S-II and developed in chloroform—methanol—water (40:20:2.5, v/v) to a height of 5 cm (two times) and then in *n*-hexane—light petroleum—diethyl ether—formic acid (30:24:6:0.08, v/v). The scanning conditions were the same as mentioned above.

Integration and quantitative evaluation was carried out using an Autolab System I Computing Integrator (Spectra Physics, Mountain View, CA, U.S.A.).

Gas chromatography

GC analyses were carried out using the Perkin-Elmer F-30, F-17 and Sigma 1 instruments under conditions described earlier [5, 6]. The sample composition for GC is given in Table II. Prior to analysis, the samples were dissolved in a solution of cholesteryl butyrate (200 ng/μl) in chloroform—isooctane solution (20:80, v/v); 1 μl of this sample was injected into the gas chromatograph.

TABLE III

C.V. VALUES FOR THE DETERMINATION OF THE CONTENT OF THE LIPID CLASSES IN THE MODEL SYNTHETIC SAMPLE BY TLC—FID

Concentrations of individual compounds are given in Table I.

	C.V.	Cholesteryl esters	Triglycerides	Fatty acids	Free cholesterol	Phospho-lipids
S-I rods	In series*	5.5	6.0	12.1	4.6	3.8
	From day to day**	6.3	8.1	—	4.2	6.2
S-II rods	In series*	1.1	1.4	15.4	4.4	3.9
	From day to day**	3.2	3.1	30.7	4.9	5.9

*Calculated from ten runs analyzed during a single day.
**Calculated from six series of four runs analyzed over three weeks.

Quantitative data were obtained by a computer after integration by means of a Perkin-Elmer Sigma 10 Data System, as described earlier [5].

Evaluation of results

Reproducibility of the determination of all lipid classes by both methods was expressed by means of coefficients of variation (C.V.). For the neutral lipids the serial C.V. were calculated from ten measurements of each biological

TABLE IV

REPRODUCIBILITY OF THE DETERMINATION (C.V.) OF THE LIPID CLASSES IN MODEL SYNTHETIC SAMPLE BY GC

Concentrations of individual lipid classes are given in Table II.

C.V.	Free cholesterol	Cholesteryl esters	Triglycerides
In series*	0.51	1.31	0.89
From day to day**	1.33	2.75	2.73

*Calculated from ten runs analyzed during one day.
**Calculated from seven series of four samples analyzed over seven weeks.

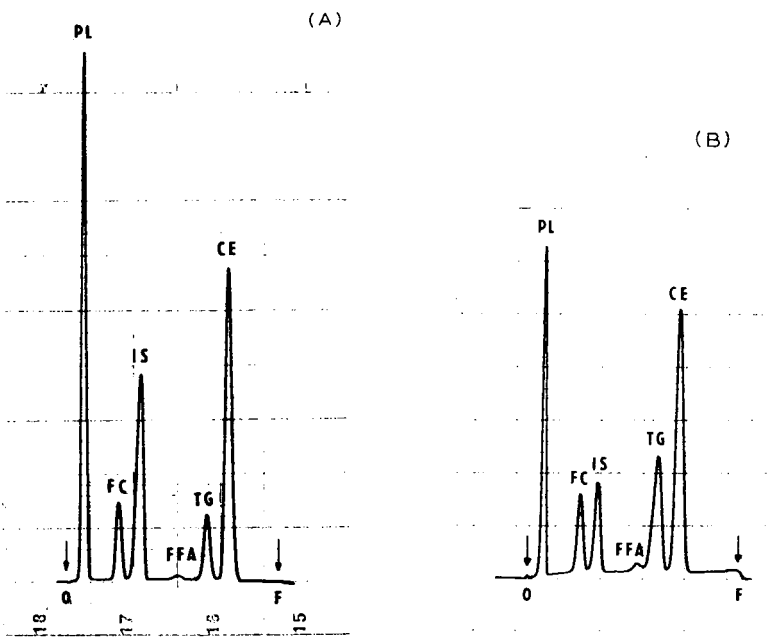

Fig. 1. Analysis of neutral lipids and free fatty acids by TLC—FID on Chromarods S-I. (A) Model sample prepared from pure saturated substances. Peak designation: PL = *rac*-1,2-palmitoylglycerol-3-phosphorylcholine, FC = cholesterol, IS = internal standard (1-octadecanol), FFA = palmitic acid, TG = tripalmitin, CE = cholesteryl palmitate. (B) Biological sample isolated from human blood plasma. Peak designation: PL = phospholipids, FC = cholesterol, IS = internal standard (1-octadecanol), FFA = free fatty acids, TG = triglycerides, CE = cholesteryl esters. 0 = start, F = front. Analytical conditions are given in the text.

TABLE V

C.V. VALUES FOR THE DETERMINATION OF THE CONTENT OF THE LIPID CLASSES IN BIOLOGICAL SAMPLES WITH VARIOUS COMPOSITION BY TLC—FID

Lipid class	Sample I			Sample II			Sample III
	Content*	Series** (C.V.)	Day*** (C.V.)	Content	Series (C.V.)	Day (C.V.)	Content
Cholesteryl esters	6.3	8.7	21.6	41.1	5.2	10.0	27.2
Triglycerides	68.0	4.3	6.4	9.4	8.7	12.4	34.5
Fatty acids	1.0	37.1	51.5	2.0	29.2	37.7	0.7
Free cholesterol	3.2	3.5	4.6	7.3	6.1	8.8	5.1
Phospholipids	21.5	4.8	6.5	40.2	4.9	7.5	32.4

*Weight per cent of the individual lipid classes in the sample.
**C.V. calculated from ten runs of the same sample measured during a single day.
***C.V. calculated from six series of four runs of the same sample measured over three weeks.

TABLE VI

REPRODUCIBILITY OF THE DETERMINATION (C.V.) OF THE LIPID CLASSES IN BIOLOGICAL SAMPLES BY GC

C.V.	Free cholesterol	Cholesteryl esters	Triglycerides
In series*	0.62	1.52	1.13
From day to day**	1.21	2.13	2.99

*Calculated from ten runs of the same sample analyzed during one day.
**Calculated from seven series of four samples analyzed over seven weeks.

sample and model synthetic sample as well. For the phospholipids only serial C.V. were calculated from five measurements of the model and biological samples. Day-to-day variation was calculated similarly from the means of four serial determinations of each sample analyzed six or seven times during three weeks (TLC) and seven weeks (GC).

RESULTS

The reproducibility of measurements of synthetic samples by TLC on S-I and S-II rods is given in Table III. The results of the analyses of comparable model samples by GC are listed in Table IV.

Biological samples were analyzed on S-I rods. The reproducibility of the results of analysis of biological samples is given in Table V. Corresponding results obtained by GC are given in Table VI.

Typical separation for model and biological samples using TLC–FID is depicted in Fig. 1A and B; separation of synthetic and biological samples by GC is given in Fig. 2A and B.

In addition to analysis of cholesterol, cholesteryl esters and triglycerides, TLC can be used to separate and determine phospholipids quantitatively and also to separate phospholipid classes. The reproducibility of the analysis of

		Sample IV			Sample V			Average	
Series (C.V.)	Day (C.V.)	Content	Series (C.V.)	Day (C.V.)	Content	Series (C.V.)	Day (C.V.)	Series (C.V.)	Day (C.V.)
4.9	8.1	37.9	4.3	7.9	36.8	2.9	3.4	5.2	10.2
3.7	6.8	14.0	5.1	8.4	3.8	3.1	4.8	5.0	7.4
32.6	41.1	1.2	36.5	57.9	2.1	10.7	16.9	29.2	41.0
5.9	10.5	5.6	6.2	13.0	6.2	4.3	7.5	5.2	8.9
6.1	9.5	41.3	5.1	7.8	51.0	3.2	5.8	4.8	7.4

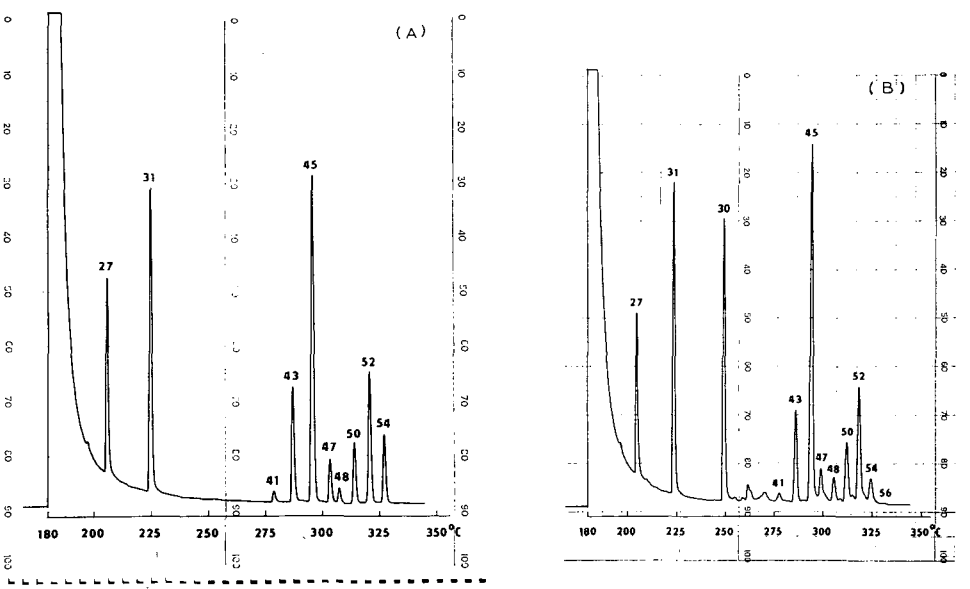

Fig. 2. Analysis of neutral lipids by GC. (A) Model sample prepared from pure saturated substances. Peak designation: 27 = cholesterol; 31 = cholesteryl butyrate (internal standard); 41 = cholesteryl myristate; 43 = cholesteryl palmitate; 45 = cholesteryl stearate; 47 = cholesteryl arachidate; 48 = tripalmitin; 50 = *rac*-glyceryl-1,3-palmitate-2-stearate; 52 = *rac*-glyceryl-1,3-stearate-2-palmitate; 54 = tristearin. (B) Biological sample isolated from human blood plasma. Peak designation: 27 = cholesterol; 31 = cholesteryl butyrate; 30 = tridecanoin (both internal standards); 41, 43, 45, 47 = cholesteryl ester fractions with carbon numbers of 41, 43, 45 and 47; 48, 50, 52, 54, 56 = triglycerides with carbon numbers of 48, 50, 52, 54 and 56. Analytical conditions: gas chromatograph Perkin-Elmer F-17, column glass 0.5 m × 1.75 mm I.D., packing 1% OV-1 on Gas-Chrom Q 100—120 mesh. Temperatures: injector and detector 340°C, oven programme from 180 to 350°C (rate 5°C/min), detector FID, attenuation 1 × 64. Carrier gas helium, flow-rate 80 ml/min. Carbon number for cholesteryl esters is defined as the sum of carbon atoms in the molecule, but for triglycerides it represents only the sum of the carbon atoms in the fatty acid moieties.

TABLE VII

REPRODUCIBILITY OF THE DETERMINATION (C.V.) OF THE INDIVIDUAL CLASSES OF PHOSPHOLIPIDS BY TLC–FID

Sample	Phosphatidyl-ethanolamine		Phosphatidyl-inositol		Phosphatidyl-serine		Phosphatidyl-choline		Sphingomyelin		Lysophosphatidyl-choline	
	Content*	C.V.**	Content	C.V.	Content	C.V.	Content	C.V.	Content	C.V.	Content	C.V.
Model mixture***	18.4	5.4	—	—	—	—	43.7	3.3	25.6	4.8	7.8	10.0
Blood Phospholipids	14.9	5.4	3.4	27.6	3.1	23.8	57.7	1.8	15.1	4.4	5.7	15.1

*Mass per cent.
**Calculated for a series of five determinations of the same sample carried out in a single day.
***The model sample also contained 4.5% lysophosphatidylethanolamine.

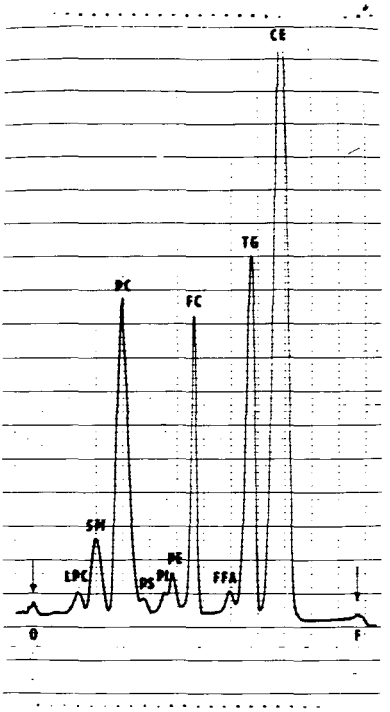

Fig. 3. Analysis of human blood plasma lipids by TLC—FID on Chromarods S-II. Peak designation: LPC = lysophosphatidylcholine, SM = sphingomyelin, PC = phosphatidylcholine, PS = phosphatidylserine, PI = phosphatidylinositol, PE = phosphatidylethanolamine, FC = cholesterol, FFA = free fatty acids, TG = triglycerides, CE = cholesteryl esters. 0 = start, F = front. The detailed analytical conditions are given in the text.

phospholipids is basically not different from the reproducibility of the determination of other components, and the results obtained here are in agreement with the literature data [13]. A survey of the results obtained is given in Table VII. The separation ability of Chromarods is clearly demonstrated in Fig. 3.

DISCUSSION

The detection limit for TLC—FID is about 50 ng per component, for GC of neutral lipids about 5 ng (12.5 μg/ml plasma for TLC—FID and 1.25 μg/ml plasma for GC under the given experimental conditions). The linear range for both methods is similar, about 0.5—20 μg. The main difference between the methods is the possibility to use GC for quantitative analysis in the non-linear range of calibration (up to 10 ng), whereas for TLC—FID such a possibility is not yet described.

It is apparent from the results given that the variability of the determination of cholesterol, cholesteryl esters, triglycerides and phospholipids by TLC—FID depends on a number of factors: quality of the separation rods, relative content and chemical composition of individual lipid classes in the sample analyzed, and also some other factors which affect separation, detection and quantitation of analyzed compounds. The dependence on the quality of the separation rods

is demonstrated in Table III. The effect of the relative content and chemical composition of the sample is apparent from comparison of the results in Tables III and V. For the biological samples higher values of the day-to-day coefficients of variation were observed in comparison with the corresponding values measured for the model synthetic sample. A possible explanation of this difference is the lower chemical stability of the unsaturated substances contained in the biological samples compared with the model samples. A similar effect was not observed in GC. However, it is not possible to make a simple conclusion from the given data, because of the simultaneous effect of several factors including the actual amount of all components analyzed. Study of an isolated effect of the individual factors was not the aim of this paper.

In agreement with other authors [14], we found that the optimal reproducibility in TLC—FID can be obtained in the range 1—10 μg of separated substance. When the sample contains smaller amounts of components, the reproducibility of the determination of these substances decreases very rapidly. The dependence of the reproducibility of the measurement on the sample size is depicted in Fig. 4. There is a similar dependence in GC in the region of non-linear calibration dependences [4, 5]. Ionization of the separated substances occurs during the GC detection process, so that loss of the separated substances results primarily from irreversible sorption or thermal decomposition during the analysis [5, 15]. In TLC—FID, the principle of detection is based on the pyrolysis of the substances that are adsorbed on the carrier, resulting in the effect of the material of thin layer and its structure. A further factor that has a negative effect on the reproducibility of analysis by TLC—FID is the reproducibility of the integration. As the whole detection in the flame ionization detector takes of the order of tens of seconds per rod, the peaks of all the

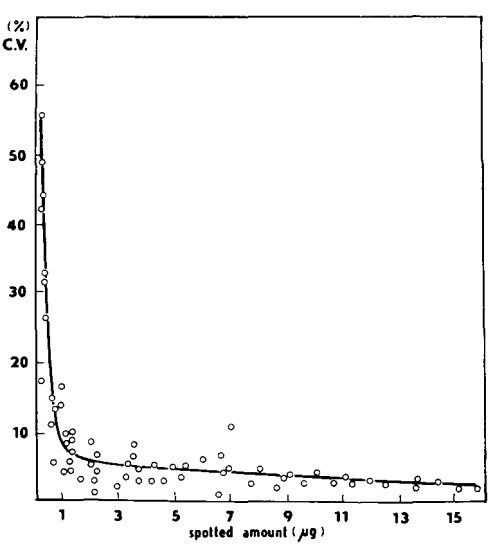

Fig. 4. Dependence of the reproducibility of the results of blood lipid analyses by TLC—FID on Chromarods S-I on the amount of substance analyzed. The dependence was measured for cholesteryl esters, triglycerides, cholesterol and phospholipids. No substantial differences between individual blood lipid classes were observed at comparable concentrations.

components are quite sharp, leading to a negative effect on the integration using common integrators, which are not constructed for such rapid processes. In spite of this lower reproducibility attained so far, TLC—FID is finding ever wider application in lipid analysis. Lower reproducibility can frequently be compensated by a greater number of determinations on a single sample, which is not a drawback, considering the high capacity of this method. A further advantage of TLC for routine clinical biochemistry of lipids is the fact that this method in the usual arrangement does not separate lipid classes into further fractions according to the molecular weight or other criteria. Together with the high capacity, this property makes TLC—FID useful for study of large sets of samples where further separation of the lipid classes is not required. The ability of TLC to analyze phospholipids and even to separate them according to class without separation of neutral lipids from the sample prior to the analysis is a further advantage (see Fig. 3).

On the other hand, GC has been found useful where high reproducibility and precision are required, or more detailed separation of lipid classes where the duration of the analysis is not an important factor. However, the development of TLC—FID suggests that further improvements in the integration could rapidly lead to a great increase in the reproducibility of measurements with this method. Some results obtained on selected rods confirm this suggestion [16, 17]. If it is possible to obtain a reproducibility in the determination of lipids comparable to that of GC, then TLC—FID will become an irreplacable tool in clinical biochemistry and in the study of lipid metabolism.

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ASSAY FOR CODEINE, MORPHINE AND TEN POTENTIAL URINARY METABOLITES BY GAS CHROMATOGRAPHY—MASS FRAGMENTOGRAPHY

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SUMMARY

A mass fragmentography (MF) assay is described for ten potential, minor urinary metabolites of codeine (C) and morphine (M). Samples were hydrolyzed, extracted, derivatized with Tri-Sil Z and analyzed by methane chemical ionization (CI)-MF. The method is sensitive to ca. 0.01 μg/ml for all compounds with the exception of normorphine (NM) which was difficult to extract with chloroform. The sensitivity of the MF assay for NM was only ca. 0.10 μg/ml. Various solvent systems were investigated for optimization of extraction efficiency of all metabolites. A separate method for the extraction of NM is reported which utilizes a solid buffer—solvent combination, i.e., potassium carbonate—isopropanol. This latter method provided the best overall recovery of NM (39.0 ± 3.4%). Gas chromatographic (GC) retention times of C, M and metabolites are reported for three liquid phases (3%) on Gas-Chrom Q (100—120 mesh). Resolution of metabolites (as trisilyl derivatives) was best on Silar-5CP and this phase was used in metabolic studies of C and M. GC resolution was not complete for all compounds; however, selection of specific ions for monitoring by MF provided the required specificity for all compounds except the 6α- and 6β-hydroxy isomers. CI spectra for all metabolites are reported. The MF assay was used for urinary analysis of samples from guinea pigs that received single doses of C (15 mg/kg) or M (8 mg/kg). Following C administration 6α- and 6β-hydrocodol, 6α,β-hydromorphol (undifferentiated), HM and M were measured. Following M administration only 6α,β-hydromorphol was found. The amount of total metabolite as percent dose for each component was calculated as < 1%.

INTRODUCTION

Codeine (C) and morphine (M) have been used extensively throughout the world for many years for the relief of moderate to severe pain. Additionally, C is the opiate of choice for relief of cough since it has less dependence liability than M. Despite their long and continued widespread use, metabolic and dispositional studies on these compounds continue to yield new information. Several new metabolites of M [1, 2] and C [3] have recently been identified.

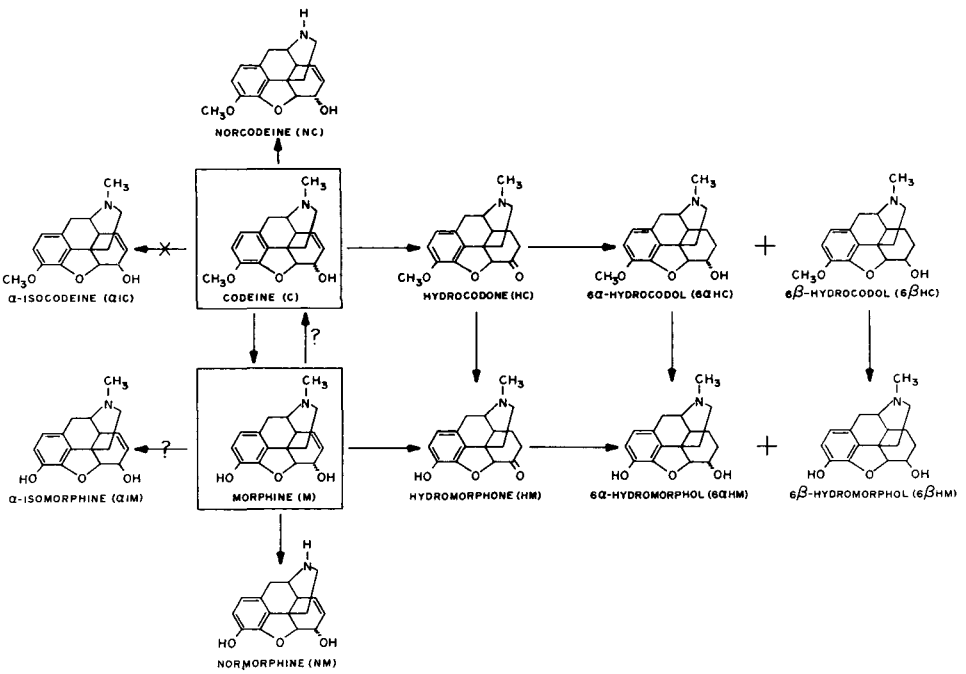

Fig. 1. Biotransformation of codeine and morphine.

The metabolic profiles for C and M (Fig. 1) which have evolved include numerous metabolites with equal or greater analgesic potency than that of the parent compound. The relationship of these metabolites to the pharmacological activity of M and C remains in question; however, measurement of these compounds in urine and blood should contribute to the understanding of their actions. Numerous assays have been described for the detection and measurement of M and C in biological fluids. These methods include assay by mass fragmentography (MF) [4–6], gas chromatography (GC) with electron-capture detection [7–10], GC with flame ionization detection [11, 12], radioisotopic techniques [13, 14], radioimmunoassay [15, 16] and high-performance liquid chromatography with electrochemical detection [17]. However, there have been no reports describing the simultaneous assay of M, C and as many as ten possible metabolites. This report describes an MF assay for the opiates of Fig. 1 in urine following the administration of M or C. Additional data are presented for the solvent extraction and GC separation of these compounds. These methods were used to demonstrate the presence of several minor metabolites in the urine of guinea pigs following a single dose of M or C.

MATERIALS AND METHODS

Chemicals

Sources for the narcotic standards were as follows: C and M (Mallinckrodt, St. Louis, MO, U.S.A.); norcodeine (NC) and normorphine (NM) (Merck, Rahway, NJ, U.S.A.); hydrocodone (HC) (Merrell National, Cincinnati, OH,

U.S.A.); hydromorphone (HM) (Knoll, Whippany, NJ, U.S.A.); cyclazocine (CY) (Sterling Winthrop, Rensselaer, NY, U.S.A.); α-isocodeine (αIC), 6β-hydrocodol (6βHC), 6α-hydromorphol (6αHM), and 6β-hydromorphol (6βHM) (Drug Addiction Laboratory, University of Virginia, Richmond, VA, U.S.A.). 6α-Hydrocodol (6αHC) and norhydrocodone (NHC) were generous gifts from Dr. Everette May, Medical College of Virginia, Richmond, VA, U.S.A. and Dr. J.W. Barnhart, Dow Chemical Co., Midland, MI, U.S.A., respectively. All compounds were analyzed by gas chromatography—mass spectrometry (GC—MS) before use.

Solvents and chemicals were of reagent grade quality. Chloroform (1% v/v ethanol) was purchased from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.) and was treated with calcium hydroxide for 12 h prior to use in order to eliminate phosgene and related impurities.

Gas chromatography

The analyses were performed on a Varian gas chromatograph Model 2700 equipped with a flame ionization detector. Glass columns (1.83 m × 2 mm) were used. Injector and detector temperatures were maintained at 275°C. Column temperature was maintained isothermally at the indicated temperatures. The air, hydrogen and nitrogen (carrier) gas flow-rates were 300, 30 and 50 ml/min, respectively. Column packings tested were 3% Silar-5CP, 3% Silar-10C, 3% OV-225 and 3% OV-17 on 100—120 mesh Gas-Chrom Q. The reported retention times represent an average of triplicate determinations.

Gas chromatography—mass spectrometry

Mass spectral data were obtained on a Finnigan Model 3300 quadrupole gas chromatograph—mass spectrometer operating in the methane chemical ionization (CI) mode. The gas chromatograph—mass spectrometer was equipped with a Finnigan Model 6000 Interactive Data System. The gas chromatograph consisted of a glass column (1.52 m × 2 mm) packed with 3% Silar-5CP on 100—120 mesh Gas-Chrom Q and was coupled to the mass spectrometer by a glass-lined stainless-steel tube and a venting valve. The electron energy was 80 eV. Methane (flow-rate 20 ml/min) was used as the carrier and reagent gas.

The temperatures of the injector, column and ion source were 250°C, 250°C and 100°C, respectively. After sample injection, the venting valve was opened for 20 sec, allowing solvent and highly volatile substances to escape without entering the ion source. MF recordings were performed. The ions selected for monitoring for each compound at its respective retention (min) were as follows: C, *m/e* 372, 2.78; M, *m/e* 430, 1.76; NC, *m/e* 268, 4.29; NM, *m/e* 326, 2.78; HC, *m/e* 372, 2.46; 6αHC, *m/e* 374, 1.72; 6βHC, *m/e* 374, 1.83; NHC, *m/e* 358, 3.79; αIC, *m/e* 372, 2.04; HM, *m/e* 430, 1.48; 6αHM, *m/e* 432, 1.02, 6βHM, *m/e* 432, 1.02; αIM, *m/e* 430, 1.26.

Although the number of ions that could be monitored simultaneously was limited to 4, by appropriate selection, all of the compounds in Fig. 1 could be determined in two separate runs. Standard curves were prepared by adding known amounts of drug and metabolites (0—4 μg/ml) to predrug control urine containing internal standard, αIC (5 μg/ml). The standard samples were

TABLE I

SOLVENT EXTRACTION EFFICIENCIES OF CODEINE (C), MORPHINE (M) AND DERIVATIVES

Samples were extracted in triplicate by Method A (Materials and Methods) and the mean ± S.E. is reported. Corrections were made for sample aliquots.

Compound	*n*-butyl chloride	Chloroform	Chloroform—isopropyl alcohol					Isopropyl alcohol—solid buffer
			95:5	90:10	85:15	80:20	70:30	
C	52.3 ± 6.4	75.9 ± 1.4	74.8 ± 3.0	71.2 ± 2.1	61.3 ± 1.2	59.5 ± 1.7	46.4 ± 1.9	77.4 ± 2.0
M	1.1 ± 0.3	36.4 ± 2.1	71.4 ± 4.3	78.7 ± 4.0	83.0 ± 4.1	81.2 ± 4.9	68.0 ± 1.2	80.6 ± 2.1
NC	0	52.0 ± 5.1	45.6 ± 0.5	57.2 ± 4.6	59.6 ± 4.3	45.0 ± 2.2	31.4 ± 0.8	ND*
NM	0	7.2 ± 0.4	7.2 ± 0.6	7.2 ± 1.0	10.5 ± 1.6	11.9 ± 0.8	13.2 ± 3.8	39.0 ± 3.4
HC	77.8 ± 7.5	59.2 ± 0.7	60.0 ± 1.6	53.0 ± 0.6	49.8 ± 1.3	46.8 ± 1.1	23.6 ± 2.1	ND
6αHC	59.3 ± 3.1	71.5 ± 0.6	66.9 ± 1.1	65.1 ± 1.6	58.5 ± 1.4	53.9 ± 0.8	43.1**	ND
6βHC	54.4 ± 6.2	86.7 ± 0.9	88.9 ± 0.8	83.3 ± 0.5	78.3 ± 0.3	72.6 ± 0.7	61.5**	ND
NHC	32.3 ± 0.7	76.2 ± 4.9	60.8 ± 3.4	53.1 ± 5.5	32.0 ± 3.8	16.6 ± 3.6	19.9 ± 0.3	ND
HM	1.9 ± 0.3	85.3 ± 6.7	88.8 ± 6.2	92.5 ± 4.7	87.4 ± 3.0	78.5 ± 4.9	62.6 ± 6.2	74.5 ± 2.2
6αHM	0	39.1 ± 2.0	77.0 ± 4.1	88.4 ± 4.9	93.7 ± 5.4	94.5 ± 6.7	73.8 ± 1.0	94.3 ± 2.8
6βHM	0	20.1 ± 3.4	64.7 ± 2.4	83.6 ± 4.2	95.7 ± 6.3	98.1 ± 6.5	76.7 ± 0.9	93.7 ± 0.5
αIM	0	17.8 ± 1.4	55.8 ± 2.5	69.6 ± 2.2	77.8 ± 0.6	79.5 ± 0.8	75.6 ± 0.6	88.4 ± 1.3

*ND = not determined.
**Only single determinations were made.

processed in similar fashion to the drug samples. A daily standard curve was constructed. Linear relationships of peak height ratios of drug or metabolite to internal standard were observed throughout the concentration range.

Extraction methods

Two general procedures (Methods A and B) were employed for the extraction of C, M and metabolites. Method A is essentially the same as that described for the analysis of metabolites of naltrexone [18]. Using this method, urine samples (10 ml) were acid-hydrolyzed (10% concentrated hydrochloric acid, v/v), pH adjusted to 10.0 ± 0.1 and buffered with 2 ml of phosphate buffer (40% K_2HPO_4, w/v). Sodium chloride was added (1 g per 10 ml), and the solution was extracted with 15 ml of an organic solvent. The extract was transferred to a tube containing 3 ml of 2 *N* hydrochloric acid and the contents were shaken for 10 min. The organic layer was removed and the pH of the solution adjusted to 10.0 ± 1 with 2 *N* sodium hydroxide solution. The solution was buffered, 1 g of sodium chloride added, and extracted with 15 ml of organic solvent. The extract was removed and evaporated to dryness at 60°C under a nitrogen atmosphere. The residue was dissolved in methanol (1 ml), transferred to acylation tubes (Regis, Morton Grove, IL, U.S.A.) and the solvent evaporated to dryness. Tri-Sil Z (Pierce Chemical, Rockford, IL, U.S.A.) (100 μl) was added. The tube was sealed and heated at 95–100°C for 2 h; after cooling, 2–5 μl were analyzed by GC–MS.

Method B utilized a solid buffer system in which the urine samples (10 ml) were acid-hydrolyzed as in Method A, followed by the careful addition of 7.5 g of anhydrous K_2CO_3. The solution was extracted with 6 ml of isopropanol and the extract transferred to a new tube and evaporated to dryness under nitrogen. Chloroform (7 ml) and 3 ml of 2 *N* hydrochloric acid were added and the contents were shaken for 10 min. The organic phase was aspirated and 1.8 g of anhydrous K_2CO_3 were added, followed by 15 ml of isopropanol. The contents were shaken for 10 min and the extract was transferred to an acylation tube and evaporated to dryness. The residue was derivatized and analyzed as in Method A.

RESULTS AND DISCUSSION

Extraction of opiates from urine

Prior to extraction, all urine samples were acid-hydrolyzed by treatment with 10% (v/v) concentrated hydrochloric acid and heating in an autoclave (115°C and 15 p.s.i.) for 30 min. This procedure is effective for the cleavage of glucuronide conjugates of morphine and related compounds. The pH values of the resulting solutions were adjusted to 10.0 ± 0.1, buffered with phosphate buffer and extracted with an organic solvent. The organic phase was back extracted into 2 *N* hydrochloric acid and the aqueous phase removed, pH adjusted to 10.0 ± 0.1, buffered and extracted with organic solvent (Method A). Analysis of the first organic extract without further clean-up was attempted but proved difficult since sensitivity for the minor metabolites was reduced because of the high endogenous background from urine. Back-extraction into acid followed by re-extraction effectively reduced background interferences to acceptable levels.

Percent recoveries of M, C and various congeners from urine were determined with several organic solvents and are listed in Table I. *n*-Butyl chloride provided lower recoveries than did chloroform for all compounds except HC. Increasing the polarity of chloroform by the addition of isopropyl alcohol generally improved recoveries for compounds with a free phenolic group, eg., M, NM, 6αHM, 6βHM, αIM and lowered recoveries for compounds with a 3-O-methyl group, e.g., C, NC, HC, 6αHC, 6βHC, NHC.

An alternative extraction method (Method B) was developed for optimization of recovery of NM, a highly polar water-soluble metabolite which was poorly extracted by Method A. Method B utilized a solid buffer, potassium carbonate, to saturate the urine sample. The buffered sample was extracted with pure isopropanol which was removed and extracted with 2 *N* hydrochloric acid. The aqueous phase was removed, carefully saturated with solid potassium carbonate and extracted with isopropanol. This extraction procedure provided the best overall recovery of NM (39.0 ± 3.4%) together with high recoveries of other opiates. Occasional problems did arise with this procedure due to carry-over of salt into the final extract. Although derivatization in the presence of salt was successful, the presence of excess bulk material in sample tubes made sampling for analysis difficult. Despite these problems, Method B was the method of choice for the detection of NM because of the improved recovery.

GC separation of M, C and metabolites

Resolution of M, C and metabolites and their trimethylsilyl (TMS) derivatives by GC was attempted on four different liquid phases (3% on Gas-Chrom Q, 100—120 mesh, 1.8 m glass column). Retention times are listed in Table II for three of the phases. The fourth phase tested, Silar-10C, was

TABLE II

RELATIVE RETENTION TIMES (RRT) OF CODEINE (C), MORPHINE (M) AND DERIVATIVES

RRT values reported are the mean of triplicate determinations. Values in brackets represent uncorrected retention times.

Compound	RRT on Silar-5CP (min)		RRT on OV-225 (min)		RRT on OV-17 (min)	
	Underivatized compound (250°C)	Silyl derivative (250°C)	Underivatized compound (240°C)	Silyl derivative (210°C)	Underivatized compound (240°C)	Silyl derivative (240°C)
C	2.30 (6.45)	5.07 (3.80)	1.87 (3.31)	4.85 (9.95)	2.14 (5.16)	2.96 (4.00)
M	—*	3.13 (2.35)	4.16 (7.36)	3.78 (7.75)	2.69 (6.46)	2.74 (3.70)
NC	3.64 (10.20)	8.07 (6.05)	2.54 (4.49)	6.95 (14.25)	2.52 (6.06)	3.44 (4.65)
NM	—	4.93 (3.70)	6.20 (10.98)	5.46 (11.20)	3.26 (7.83)	3.26 (4.40)
HC	4.11 (11.50)	4.60 (3.45)	3.09 (5.47)	4.39 (9.00)	2.94 (7.05)	3.07 (4.15)
6αHC	2.04 (5.70)	3.27 (2.45)	1.71 (3.03)	3.00 (6.15)	2.07 (4.96)	2.26 (3.05)
6βHC	2.64 (7.40)	3.07 (2.30)	2.11 (3.74)	2.98 (6.10)	2.28 (5.47)	2.37 (3.20)
NHC	6.11 (17.10)	7.00 (5.25)	4.41 (7.80)	6.17 (12.65)	3.48 (8.35)	3.56 (4.80)
HM	—	2.73 (2.05)	5.07 (8.98)	3.15 (6.45)	3.28 (7.87)	2.70 (3.65)
6αHM	—	1.87 (1.40)	—	2.05 (4.20)	2.49 (5.85)	1.89 (2.55)
6βHM	—	1.87 (1.40)	—	2.17 (4.45)	2.60 (6.10)	2.07 (2.80)
Cy	1.00 (2.80)	1.00 (0.75)	1.00 (1.77)	1.00 (2.05)	1.00 (2.40)	1.00 (1.35)

*Indicates unsatisfactory results for GC analysis.

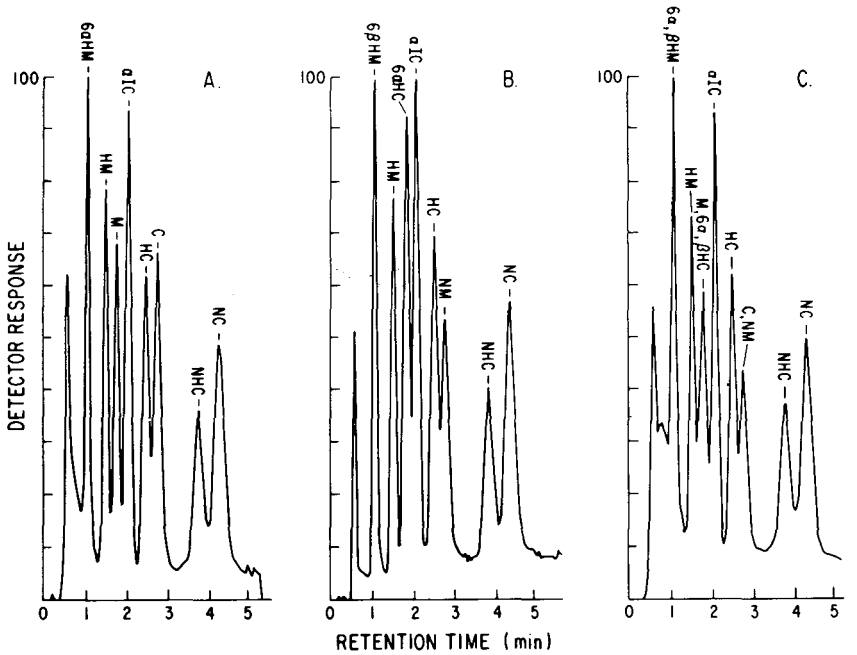

Fig. 2. GC separation of trimethylsilyl derivatives of codeine, morphine and metabolites on 3% Silar-5CP. (A) Codeine (C), morphine (M) and six derivatives; (B) eight derivatives; (C) all compounds in Fig. 1.

unsuitable for analysis of either free or TMS derivatives because of the poor chromatographic response of these compounds on this liquid phase. Silar-5CP also was not suitable for analysis of M and related compounds with free phenolic groups (underivatized); however, the analogous compounds in the C series (3-O-methyl) demonstrated good chromatographic characteristics on this phase.

Derivatization consistently improved peak shape and shortened retention times for the three phases in Table II. Resolution of all compounds as TMS derivatives was somewhat better on the more polar phases (Silar-5CP and OV-225) than on OV-17; however, all compounds in Fig. 1 were not resolvable on either of the three systems.

Silar-5CP was selected for use in continued metabolic studies on opiates since it provided equal or better separation of the compounds in Fig. 1 than did the other phases. Urine extracts were analyzed on Silar-5CP as the TMS derivatives in most cases. Under these conditions up to eight of the compounds could be resolved to near baseline resolution in a single run (Fig. 2A and B); however, when all components were present, clusters of two to three compounds eluted simultaneously. The 6α- and 6β-hydroxy derivatives of HC and HM proved the most difficult to separate by GC methods. 6αHC and 6βHC could be separated on Silar-5CP in the underivatized form, whereas 6αHM and 6βHM did not elute on this system and were not separable on OV-225 or OV-17. Prior separation of these isomers by thin-layer chromatography [18] will provide the specificity for separate determination.

TABLE III

METHANE CHEMICAL IONIZATION SPECTRA OF CODEINE (C), MORPHINE (M), AND DERIVATIVES

Compound	t_R (min)*	Mol. wt.	Methane CI spectra**			
			$(M+29)^+$	$(M+1)^+$	M^+	Prominent fragment ions
C-TMS	2.78	371	400 (14)	372 (44)	371 (29)	370 (15), 356 (20), 283 (26), 282 (100)
M-TMS_2	1.76	429	458 (19)	430 (78)	429 (55)	431 (32), 415 (37), 414 (92), 371 (16), 341 (33), 340 (100)
NC-TMS	4.36	357	386 (12)	358 (45)	357 (24)	342 (25), 269 (27), 268 (100)
NM-TMS_2	2.78	415	444 (17)	416 (74)	415 (47)	417 (38), 401 (36), 400 (93), 371 (21), 327 (36), 326 (100)
HC-TMS	2.49	371	400 (17)	372 (100)	371 (38)	373 (37), 370 (21), 356 (32)
6αHC-TMS	1.83	373	402 (13)	374 (100)	373 (56)	375 (32), 372 (28), 358 (26), 315 (19), 284 (20)
6βHC-TMS	1.72	373	402 (18)	374 (100)	373 (55)	375 (43), 372 (30), 358 (43), 315 (19), 284 (47), 285 (18)
NHC-TMS	3.83	357	386 (16)	358 (100)	357 (40)	359 (38), 356 (15), 342 (31)
HM-TMS_2	1.48	429	458 (17)	430 (100)	429 (46)	432 (21), 431 (44), 415 (33), 414 (76)
6αHM-TMS_2	1.02	431	460 (13)	432 (100)	431 (72)	433 (54), 430 (27), 417 (38), 416 (74), 373 (20), 342 (23)
6βHM-TMS_2	1.02	431	460 (19)	432 (96)	431 (76)	433 (61), 430 (33), 417 (46), 416 (100), 373 (22), 343 (33), 342 (82)
αIM-TMS_2	1.22	429	458 (25)	430 (100)	429 (49)	431 (39), 428 (22), 415 (28), 414 (82), 340 (37)
αIC-TMS	2.04	371	400 (17)	372 (100)	371 (43)	373 (48), 370 (22), 356 (29), 282 (38)

*t_R (min) were determined by GC—MS with a 1.5-m glass column packed with 3% Silar-5CP on Gas-Chrom Q (100—120 mesh). Methane was the carrier gas at a flow-rate of 30 ml/min. The column was operated isothermally at 245°C.

**m/e (relative abundance). Only ions ≥ 15% relative abundance are reported. The ions selected for monitoring by MF are underlined.

Methane CI-MS spectra of M, C and metabolites

The compounds in Fig. 1 were analyzed as TMS derivatives by GC—MS under CI conditions with methane as carrier and reagent gas. The spectra and retention times (t_R) are listed in Table III. All of the compounds displayed strong $(M+1)^+$ and $(M+29)^+$ ions. The $(M+1)^+$ ion was the most abundant ion for eight of the compounds and the $(M-89)^+$ ion (loss of TMSOH) was the most abundant ion for four compounds. The four compounds which readily eliminated TMSOH contain a C7—C8 carbon—carbon double bond and α-hydroxy configuration which apparently serves to facilitate this decomposition pathway. Only one compound, 6βHM, had an $(M-15)^+$ ion as the most abundant ion.

CI-MF analysis of M, C and metabolites

Although MF analysis was limited to the scanning of four ions per run, selection of appropriate ions allowed the measurement of all compounds and internal standard in two runs. Since the 6α- and 6β-hydroxy metabolites were not resolved on OV-225 as TMS derivatives, they were measured as combinations, i.e., 6α,βHC and 6α,βHM. The ions which were scanned were selected from Table III on the basis of their relative abundance, selectivity in the presence of components with similar retention times and commonality with

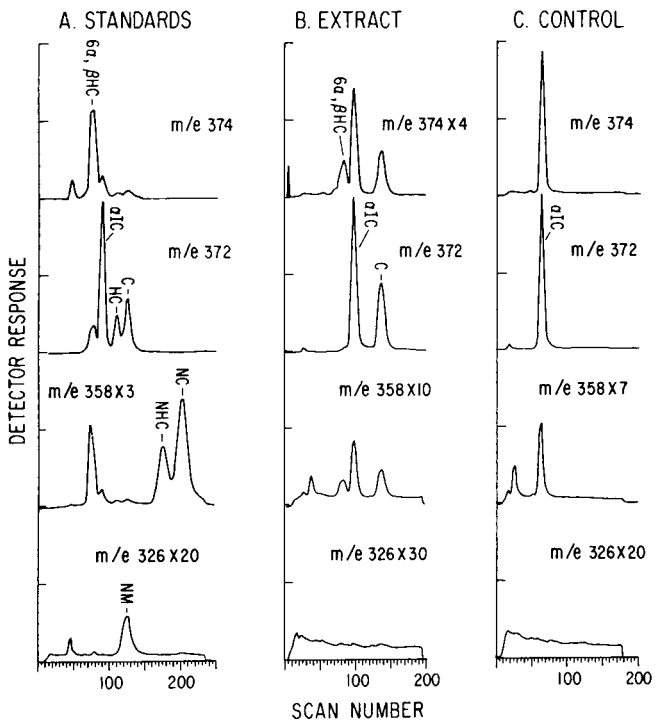

Fig. 3. Mass fragmentograms of extracts (trimethylsilyl derivatives) of guinea pig urine. (A) Control urine with added standards; (B) 24-h sample following subcutaneous administration of codeine (15 mg/kg); (C) control urine collected prior to drug administration. Each run was restricted to four ions as shown.

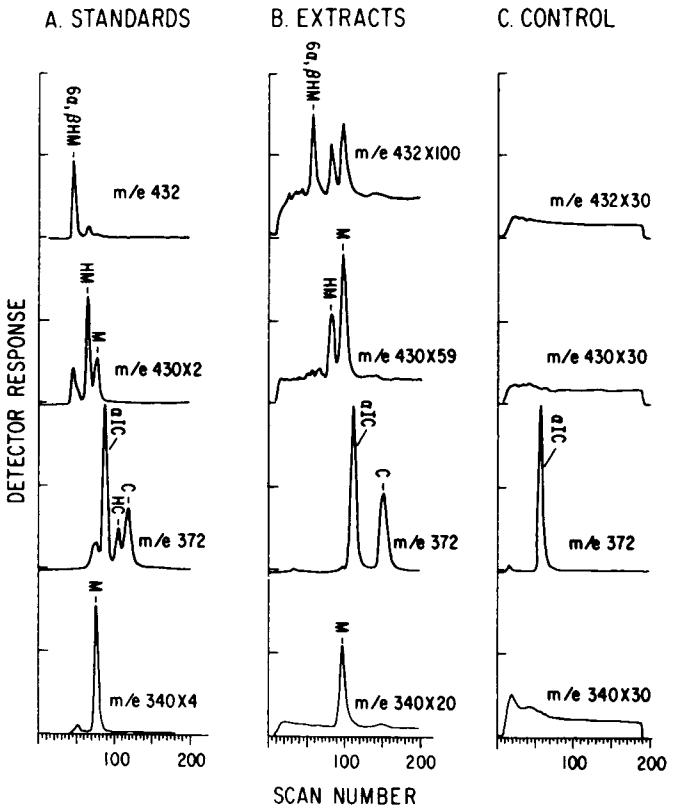

Fig. 4. Mass fragmentograms of extracts (trimethylsilyl derivatives) of guinea pig urine. (A) Control urine with added standards; (B) 24-h sample following subcutaneous administration of codeine (15 mg/kg) (C) control urine collected prior to drug administration. Each run was restricted to four ions as shown.

other resolvable compounds. For instance, ion 430 *m/e* was scanned for M (t_R = 1.76) and HM (t_R = 1.48) which were resolved chromatographically but were not common to the spectra of 6αHC (t_R = 1.83) and 6βHC (t_R = 1.72).

Standard curves were constructed for all components by plotting peak height ratios of compound divided by internal standard versus concentration (0—4 μg/ml for C and NC and 0—2 μg/ml for all other components). Correlation coefficients (r) for all compounds were consistently greater than 0.98. Minimal detectable quantities in urine were ca. 0.01 μg/ml for most compounds with the exception of NM which was ca. 0.10 μg/ml. Typical MF scans of standards extracted from normal urine are shown in Figs. 3A, 4A and 5A.

Urinary analyses for trace metabolites of M and C from guinea pig

Urine was collected from six guinea pigs (male, albino Hartley, weight 305—350 g) following the subcutaneous administration of C (15 mg/kg) or M (8 mg/kg). Samples were hydrolyzed, extracted and analyzed by MF as described. Daily standard curves were also prepared. Following C administration, 6α,βHC (Fig. 3B) was evident in the first analysis and 6α,βHM, HM and M (Fig. 4B) were present in the second run. Control urine from the same animals

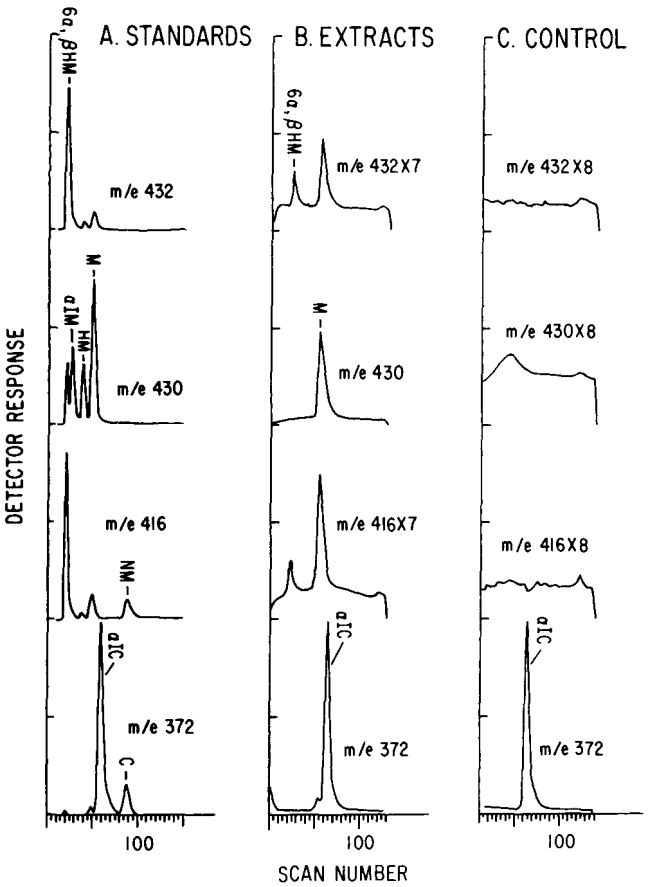

Fig. 5. Mass fragmentograms of extracts (trimethylsilyl derivatives of guinea pig urine. (A) Control urine with added standards; (B) 24-h sample following subcutaneous adminitration of morphine (8 mg/kg); (C) control urine collected prior to drug administration.

was free from interferences at the appropriate retention time (Figs. 3C and 4C). Further characterization of 6α,βHC by GC—MS on 3% Silar-5CP without derivatization revealed that 6αHC and 6βHC were present in nearly equal amounts. Following M administration only 6α,βHM was evident (Fig. 5B). The amount of total metabolite as percent dose for each component was calculated as less than 1%.

The presence of 6α,βHM in the urine of guinea pigs following M administration implies the formation of HM, in agreement with the report by Klutch [1] that HM is a minor metabolite of M. Although HM was not detected in samples of the present study it may be that the 6-keto-reductase activity of these animals is greater than those previously studied resulting in conversion of HM to 6αHM and/or 6βHM. Also, it should be noted that HM was detected in the urine of guinea pigs following C administration. Two sources are possible for production of this metabolite as shown in Fig. 1. C could be converted to HC in an analogous fashion to the transformation of M to HM, followed by O-demethylation of HC to HM. Alternately C could be first O-demethylated to

M followed by conversion of M to HM. The presence of M and 6αHC and/or 6βHC (reduction products of HC) suggests that both pathways are likely to be operative in the formation of HM from C.

The occurrence of these minor metabolites poses interesting pharmacological questions. Although most of these metabolites are equipotent or more potent than the parent drug, their abundance is sufficiently low to obviate significant pharmacological activity. Perhaps the occurrence of these compounds represents a fingerprint of the enzymatic capacity of individual species and perhaps even individual animals. The usefulness of these observations is under investigation.

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DETERMINATION OF A PYRIMIDO-BENZAZEPINE ANXIOLYTIC AGENT AND ITS 5-HYDROXY METABOLITE IN WHOLE BLOOD, PLASMA AND URINE BY GAS—LIQUID CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION AND BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Electron-capture gas—liquid chromatographic and reversed-phase high-performance liquid chromatographic assays are described for the quantitation of the compound, 9-chloro-7-(2-chlorophenyl)-5H-pyrimido[5,4-*d*][2]-benzazepine, [I], a member of the benzazepine class of compounds undergoing clinical evaluation as anxiolytic agents. Studies on the biotransformation of [I] in the rat and dog showed that the compound was metabolized mainly by hydroxylation to yield the 5-hydroxy compound, [II], 9-chloro-7-(2-chlorophenyl)-5H-pyrimido [5,4-*d*][2]-benzazepin-5-ol (major metabolite), along with the formation of lesser amounts of the N-oxide, [III], 9-chloro-7-(2-chlorophenyl)-5H-pyrimido[5,4-*d*][2]-benzazepine 3-oxide, and the phenolic analogue, [IV], 3-chloro-4-(9-chloro-5H pyrimido-[5,4-*d*][2]benzazepin-7-yl)phenol. This report describes the quantitation of [I] and [II] (major metabolite) in plasma using the above analytical techniques, both in preclinical studies in the dog and in clinical pharmacokinetic studies in man.

INTRODUCTION

The compound, 9-chloro-7-(2-chlorophenyl)-5H-pyrimido[5,4-*d*][2]-benzazepine, [I], Fig. 1, is a member of the benzazepine class of compounds undergoing clinical evaluation as anxiolytic agents [1].

In vitro studies on the biotransformation of [I] [2], using a 9000-*g* microsomal fraction from a rat liver homogenate showed that the compound was metabolized mainly by hydroxylation to yield the 5-hydroxy compound, [II], 9-chloro-7-(2-chlorophenyl)-5H-pyrimido[5,4-*d*][2]-benzazepin-5-ol, along with the formation of lesser amounts of the N-oxide, [III], 9-chloro-7-

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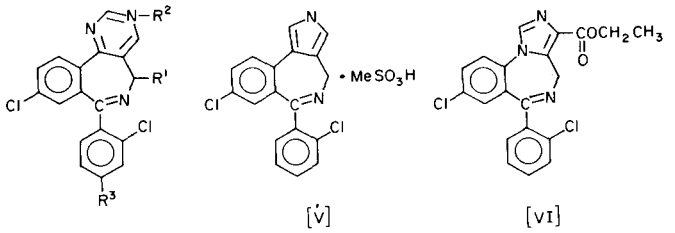

[V] [VI]

Compound	R'	R²	R³
[I]	H	—	H
[II]	OH	—	H
[III]	H	→O	H
[IV]	H	—	OH

Fig. 1. Chemical structures of compounds referred to in the text.

(2-chlorophenyl)-5H-pyrimido[5,4-*d*][2]-benzazepine 3-oxide, and the phenolic analogue, [IV], 3-chloro-4-(9-chloro-5H-pyrimido[5,4-*d*][2] benzazepin-7-yl)phenol. In vivo biotransformation studies in the rat and in the dog [3] confirmed that unconjugated [II] was the major plasma metabolite. The N-oxide metabolite, [III], and the phenol, [IV], were minor metabolites. The synthesis and pharmacological properties of these compounds are reported elsewhere [4]. The primary focus of this report is on the quantitation of [I] and [II] in plasma using different analytical approaches.

Simultaneous quantitation of compounds [I] and [II] necessitated the silylation of [II] to enhance its response to gas—liquid chromatography with electron-capture detection (GLC—ECD), using the compound, 8-chloro-6-(2-chlorophenyl)-2H,4H-pyrrolo[3,4-*d*][2]-benzazepine methanesulfonate, [V], as the internal standard. The sensitivity limit of this assay is 2 ng of [I] and [II] per ml of plasma.

A reversed-phase high-performance liquid chromatographic (HPLC) assay (UV detection at 254 nm) was developed for the determination of [I] and [II] (sensitivity limit: 50 ng/ml). Although it is less sensitive than the GLC—ECD assay, its simplicity and high sample throughput via automation offers advantages during pre-clinical drug development when sensitivity is not critical [5]. A normal-phase HPLC method (UV detection at 254 nm) was also developed (senstivity limit: 25–50 ng/ml) for the determination of compounds [I], [II], [III], and [IV] using 8-chloro-6-(2-chlorophenyl)-4H-imidazo[1,5-*a*][1,4]benzodiazepine-3-carboxylic acid ethyl ester, [VI], as the internal standard, and was used to verify the specificity of the GLC—ECD assay. All assays involve extraction of [I] and [II] into benzene from blood, plasma, or urine buffered to pH 6.1 (saturated potassium chloride solution), the residue of which is analyzed using the appropriate methods described.

EXPERIMENTAL

Reagents

All reagents must be of analytical reagent grade (> 99% purity). Saturated potassium chloride, pH 6.1, approximately 4.8 *M*, is prepared by adding 300

g of potassium chloride (Matheson, Coleman and Bell, Reagent Crystals, Norwood, OH, U.S.A.) to 900 ml of distilled deionized water.

The organic solvents (all analytical-reagent grade) used were as follows: benzene, methanol, acetonitrile, and isopropanol were from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Acetone and *n*-hexane (H-301) were from Fisher Scientific (Springfield, NJ, U.S.A.); ethanol (absolute, U.S.P. grade) was from U.S. Industrial Chemicals (Tuscola, IL, U.S.A.).

Benzene—acetone—methanol (85:10:5), stored over anhydrous sodium sulphate (Mallinckrodt, St. Louis, MO, U.S.A.) is the solvent for GLC—ECD analysis. Methanol—isopropanol (50:50) is the solvent for HPLC analysis. The silylating agent for the derivatization of [II] was N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) from Regis Chemical (Morton Grove, IL, U.S.A.), mixed with acetonitrile in a 1:10 ratio.

GLC—ECD analysis for compounds [I] and [II] in blood or plasma

Column conditions

The column packing was a pretested phase containing 5% OV-1 on 100—120 mesh Gas-Chrom Q (Applied Science Labs., State College, PA, U.S.A.) packed in a U-shaped, 1.2 m or a 1.8 m × 4 mm I.D. borosilicate glass column. The glass column was treated before packing with a 1% solution of Prosil-28 (PCR Research Chemicals, Gainesville, FL, U.S.A.), for 10—15 sec, thoroughly rinsed with distilled water and dried for 1 h at 100°C. The packed column was conditioned at 325°C under "no flow" conditions for 4 h, then at 275°C for at least 18 h with carrier gas flow-rate of 40 ml/min.

Instrumental parameters for GLC—ECD analysis

A Tracor gas chromatograph, Model 222 equipped with a ^{63}Ni electron-capture detector containing a 15-mCi ^{63}Ni β-ionization source, and an auto-sampler (Model 7671A, Hewlett-Packard, Avondale, PA, U.S.A.) was used. Argon—methane (90:10) from Liquid Carbonic (Harrison, NJ, U.S.A.) was used as the carrier gas and the column head pressure was preset at 275 kPa (40 p.s.i.), with a column flow-rate of 80 ml/min and a detector purge of 20 ml/min. The temperature settings were as follows: oven, 230°C or 250°C for the 1.2-m or the 1.8-m columns, respectively; injection port, 275°C; detector, 350°C.

The electron-capture detector linearizer (Model No. 114460) standing current was adjusted to $0.5 \cdot 10^{-9}$ A, the relative pulse width was adjusted to 0.15 which corresponds to 0.75 μsec and the attenuation was set at 16. The chart speed was 1.27 cm/min and the time constant on the 1.0-mV Honeywell recorder (Model No. 194) was 1 sec (full scale deflection). The conditions of flow-rate and column temperature must be adjusted to obtain retention times of 4.2, 5.4, and 6.8 min on the 1.2-m column or 5.7, 7.2, and 9.1 min on the 1.8-m column for [I], [II]-OTMS, and [V], respectively. Under these conditions, 2.4 ng each of [I] and [II]-OTMS, and 20.0 ng of [V] injected on the column give nearly full scale pen response. The minimum detectable amount of [I] and [II] is 2 ng per ml of blood or plasma.

Reversed-phase HPLC analysis of compounds [I] and [II] in blood, plasma or urine

Column

The column used was a 0.30 m × 3.9 mm I.D. stainless-steel column containing μBondapak C_{18}, particle size 10 μm, generating 10,770 plates/m (Waters Assoc., Milford, MA, U.S.A.).

Instrumental parameters

A Waters Model ALC/GPC-204 high-pressure liquid chromatograph equipped with a Model 440 absorbance detector, operated at 254 nm, a Model M6000A solvent delivery system, a U6K injector, or a Waters Intelligent Sample Processor (WISP™) Model 710-B was used. The isocratic mobile phase used was a mixture of methanol—water (75:25) at a pressure of 6 MPa (900 p.s.i.) and a constant flow-rate of 1.3 ml/min. Under these conditions, the retention times of compounds [I], [II], and [V] (internal standard) were 5.3, 4.3, and 6.4 min, respectively, with capacity factors (k') of 3.1, 2.2, and 4.0, respectively. The UV detector sensitivity was $1 \cdot 10^{-2}$ absorbance units full scale deflection (a.u.f.s.) and the chart speed on the 10-mV Hewlett-Packard recorder (Model No. 7132A) was 1.27 cm/min. Under these conditions 50 ng each of [I] and [II] and 140 ng of [V] per 10 μl injected gave nearly full scale pen response. The minimum detectable amount of [I] and [II] is 50 ng/ml of blood, plasma or urine.

Preparation of analytical standards

Compounds [I]: $C_{18}H_{11}Cl_2N_3$, MW = 340.21, m.p. = 122—125°C; compound [II]: $C_{18}H_{11}Cl_2N_3O$, MW = 356.2, m.p. = 174—175°C; compound [III]: $C_{18}H_{11}Cl_2N_3O$, MW = 356.2, m.p. = 189—190°C; compound [IV]: $C_{18}H_{11}Cl_2N_3O$, MW = 356.2, m.p. = 208—210°C; compound [V]: $C_{18}H_{12}Cl_2N_2 \cdot CH_4SO_3$, MW = 423.3, m.p. = 239—241°C; and compound [VI]: $C_{20}H_{15}Cl_2N_3O_2$, MW = 400.3, m.p. = 225–228°C, all of pharmaceutical grade purity (> 99%) were used as analytical standards.

GLC—ECD analysis of parent compound [I] and metabolite [II]

Prepare stock solution of compounds [I], [II], and [V] in separate 10-ml volumetric flasks by dissolving 10.00 mg of each compound in 1 ml of ethanol and 1 ml of methanol. Sonicate in an ultrasonic bath if necessary for 5–10 min for complete solubilization and dilute to volume with acetone—*n*-hexane (1:9). These stock solutions (containing 1 mg/ml of [I], [II], and [V] are used to prepare the following mixed standard solutions by suitable dilutions in acetone—*n*-hexane (1:9) to contain the following concentrations:

Working standard solution	Concentration (ng per 0.1 ml)		
	[I]	[II]	[V]* (internal standard)
1	2	2	—
2	4	4	—
3	8	8	—
4	16	16	—
5	24	24	—
6	48	48	—
7	72	72	—
8	—	—	200

*Internal standard [V]: Standard solution No. 8 is prepared by suitable dilution in benzene—acetone—methanol (85:10:5) and is added as the internal standard to all the derivatized residues, prior to the GLC—ECD analysis of all samples.

Aliquots (0.1 ml) of standard solutions Nos. 1—7 are added to 0.9 ml of the benzene extract of control blood or plasma, derivatized with BSTFA after evaporation (as described under Assay procedure), and are used as the external "matrix" (calibration) standard curve, to verify the linearity and performance of the GLC—ECD system.

Similarly, 0.1-ml aliquots of standard solutions Nos. 1—7 are evaporated to dryness, the residue is reconstituted in 0.5 ml of control blood or plasma, extracted as described under the Assay procedure, the residue of which is derivatized, and used as the processed (recovered) standard calibration curve for the determination of the concentration of [I] and [II]-OTMS in the unknowns.

Calibration of compounds [I], [II]-OTMS, and [V] by GLC—ECD. Calibration (external standard) curves of the peak area ratio of [I] to [V], and of [II]-OTMS to [V] vs. concentration of [I] and [II] added to the extract of control blood or plasma are constructed. Fresh calibration curves of the "matrix" external standards and of the processed (recovered) standards are prepared for each day of analysis to establish the reproducibility of the GLC system.

For reversed-phase HPLC analysis of parent compound [I] and metabolite [II]

Prepare stock solutions of compounds [I], [II], and [V] as previously described for the GLC—ECD assay of compounds [I] and [II]. The stock solutions (containing 1 mg/ml) are used to prepare the following mixed standard solutions by suitable dilutions in acetone—*n*-hexane (1:9) to contain the following concentrations:

Working standard solution	Concentration (ng per 0.1 ml)		
	[I]	[II]	[V] (internal standard)
9	50	50	1400
10	100	100	1400
11	300	300	1400
12	500	500	1400
13	900	900	1400
14	1800	1800	1400
15	2700	2700	1400
16	—	—	1400

Aliquots (0.1 ml) of standard solutions Nos. 9—15 are added to 0.9 ml of the benzene extract of control blood, plasma or urine and evaporated to dryness. The residues are dissolved in 100 μl of methanol—isopropanol (50:50) for reversed-phase HPLC analysis. Appropriate aliquots are injected as the "matrix" external (calibration) standard curve, to establish the parameters for reversed-phase HPLC analysis with UV detection at 254 nm.

Aliquots (0.1 ml) of standard solutions Nos. 9—15 are evaporated to dryness, the residue is reconstituted in 0.5 ml of control blood, plasma or urine, extracted as described and used as the processed (recovered) standard calibration curve for the determination of the concentration of [I] and [II] in the unknowns by reversed-phase HPLC analysis. A separate standard solution, No. 16, containing 1400 ng of [V] per 0.1 ml of acetone—*n*-hexane (1:9) is prepared and added as the internal standard to all unknown samples as per above procedure (i.e., evaporated and unknown sample added), for HPLC analysis.

Calibration of compounds [I] and [II] by HPLC. Calibration (external standard) curves of the peak height ratio of [I] or [II] to [V] vs. concentration of compound added to the extract of control blood, plasma or urine are constructed. Fresh calibration curves of the "matrix" external standards and of the processed (recovered) standards are prepared for each day of analysis to establish the reproducibility of the HPLC system.

Analysis of blood, plasma or urine

GLC—ECD analysis for parent compound [I] and metabolite [II]

The flow diagram of the extraction procedure is shown in Fig. 2. Into a 15-ml conical centrifuge tube (PTFE No. 13 stoppered), add 0.50 ml of heparinized whole blood or plasma, 0.50 ml of pH 6.1, ca. 4.8 *M*, saturated potassium chloride solution (vortex), and extract with 1.0 ml of benzene by shaking for 15 min on a reciprocating shaker (Eberbach, Ann Arbor, MI, U.S.A.) at 80—100 strokes/min. Along with the unknown samples, process nine 0.50-ml specimens of control blood or plasma, two to be used as controls and seven to be used as "matrix" external standards. In addition, process seven 0.50-ml specimens of control blood or plasma containing 0.1 ml of standard solutions Nos. 1—7 (equivalent to 2, 4, 8, 16, 24, 48, and 72 ng of [I] and [II] per 0.50 ml of blood or plasma) as the processed (recovered)

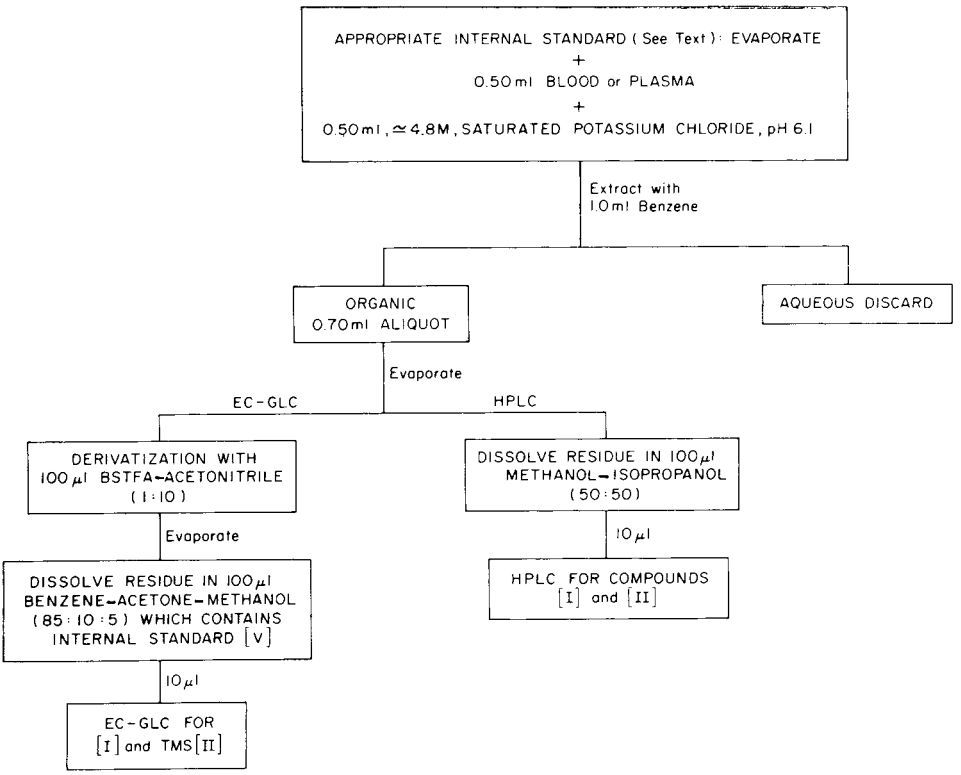

Fig. 2. Flow diagram of the extraction procedure for compounds [I] and [II] from blood or plasma.

standard curve. The solution (100 µl) is evaporated to dryness at 35—40°C in a N-EVAP evaporator (Organomation Assoc., Worcester, MA, U.S.A.) under a stream of clean, dry nitrogen prior to the addition of control blood or plasma. Centrifuge the samples at 1500 *g* in a refrigerated centrifuge (Model PR-J, rotor No. 253, Damon/IEC, Needham, MA, U.S.A.) at 5°C for 10 min. Transfer a suitable aliquot (0.7—0.8 ml) of the upper organic phase into a disposable tube (13 × 100 mm) (which was used in order to eliminate adsorption losses of both compounds) and evaporate the benzene extract to dryness at 35—40°C under nitrogen as before. Immediately add 100 µl of BSTFA—acetonitrile (1:10) to the dry residue, stopper the tubes tightly, vortex, and react at 60°C for 20 min (Multi-Block Heater, Lab-Line Instruments, Melrose Park, IL, U.S.A.), continuing to vortex every 5 min. Evaporate the derivatization mixture to dryness immediately after the reaction and reconstitute the residue in 100 µl of benzene—acetone—methanol (85:10:5) containing 200 ng of the internal standard [V]. Inject a 10-µl aliquot for GLC analysis, either manually or by transferring the reconstituted residue into a 100-µl automatic sampler vial (HP 5080-8779, Hewlett-Packard) for auto-injection.Typical chromatograms of plasma extracts are shown in Fig. 3.

Reversed-phase HPLC analysis of [I] and [II] in blood, plasma or urine

The flow diagram of the extraction procedure is shown in Fig. 2. Follow the

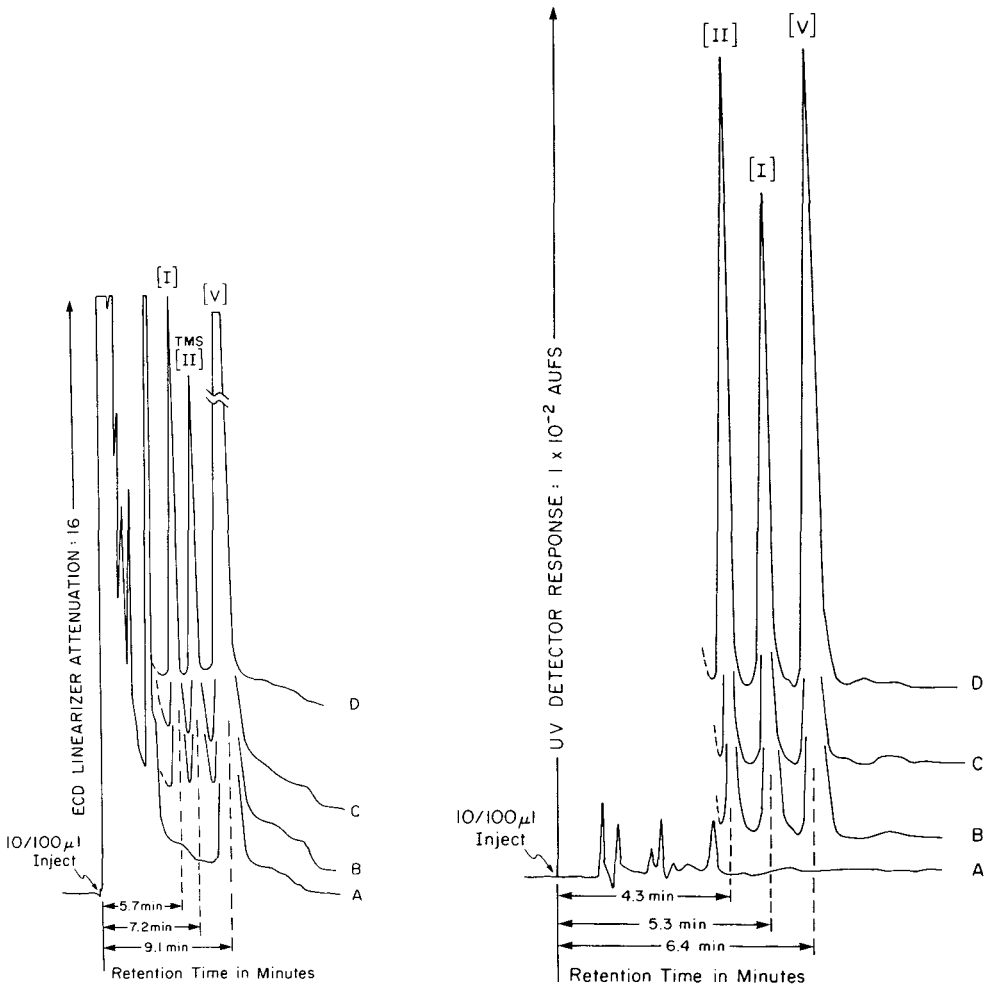

Fig. 3. Chromatograms of the GLC—ECD analysis of derivatized benzene extracts of (A) control human plasma with added internal standard; (B) human plasma following a single 5-mg oral dose of [I]; (C) authentic standards recovered from control human plasma; and (D) authentic standards added to the residue of control human plasma (matrix external standard).

Fig. 4. Chromatograms of the reversed-phase HPLC analysis of benzene extracts of (A) control dog plasma; (b) dog plasma following chronic oral dosing of [I] at 300 mg/kg/day on day 1; (C) authentic standards recovered from control dog plasma; and (D) authentic standards added to the residue of control dog plasma (matrix external standards).

extraction procedure as described for GLC—ECD analysis, adding 100 μl of standard solution No. 16, containing 1400 ng of compound [V], as the internal standard. Along with the unknown samples process nine 0.50-ml specimens of control blood, plasma or urine (as for GLC—ECD), and seven 0.50-ml specimens of control blood, plasma or urine containing 0.10 ml of standard solutions Nos. 9—15 (equivalent to 50, 100, 300, 500, 900, 1800, and 2700 ng of [I] and [II], and 1400 ng of compound [V] (internal standard) per 0.50 ml

of blood, plasma or urine), evaporate to dryness and reconstitute in the appropriate biological specimen. After the extraction step, transfer a suitable aliquot (0.7—0.8 ml) of the upper organic phase into a disposable tube (13 × 100 mm), and evaporate the benzene extract to dryness at 35—40°C under nitrogen. Immediately dissolve the residue in 100 μl of methanol—isopropanol (50:50) and inject a 10-μl aliquot into the liquid chromatograph either manually, or by using the WISP™ auto-injector. A typical chromatogram of plasma extracts is shown in Fig. 4.

Calculations

The concentrations of [I] and [II] in the unknowns were determined by interpolation from a least squares regression equation (power equation: $Y = m\ X^b$) of the calibration data of the recovered standards processed along with the unknowns using peak height or peak area ratios (peak height or peak area of compounds [I] or [II] to peak height or peak area of the internal standard [V] vs. concentration of [I] or [II] per 0.5 ml of plasma, blood or urine). A typical calibration curve as defined by the equation $Y = 0.026X^{0.911}$ is linear from 2 to 72 ng of [I] and [II] per 0.5 ml of plasma, as assayed by the GLC—ECD method for parent compound [I] and metabolite [II]. The correlation coefficient (r) is equal to 0.998 and the average deviation from the line is 6.7%. The same equation is used to define the calibration curves for the reversed-phase HPLC assay. All calibration data were processed by a HP Model 3354B Laboratory Automation System (Hewlett-Packard).

RESULTS AND DISCUSSION

A sensitive and specific GLC—ECD assay was developed for the determination of parent compound [I] and its 5-hydroxy metabolite [II] from 0.50 ml of blood or plasma. The metabolite [II] is converted to its OTMS derivative by reaction with a mixture of BSTFA—acetonitrile (1:10) resulting in a Gaussian shaped symmetrical peak with enhanced ECD response. Although the internal standard, compound [V], has compatible chromatographic behavior to that of [I] and [II]-OTMS, it was introduced in the solvent used to dissolve the sample residue prior to injection, hence, it monitors only the final chromatographic step. This assay was used during preclinical evaluation of the biopharmaceutic/pharmacokinetic profile of [I] in the dog following intravenous and oral administration and during initial clinical evaluation of single 5-mg oral doses of compound [I] in man.

A sensitive and specific reversed-phase HPLC assay was developed for the determination of compounds [I] and [II] from 0.5 ml of blood, plasma or urine using UV detection at 254 nm for quantitation. The major UV absorption bands of both compounds [I] and [II] occur at 235—240 nm and at 270—280 nm, while the major absorption band for the internal standard [V] occurs at 268—276 nm. The Waters Model 440 absorbance detector, used in conjunction with a 254-nm wavelength kit and a medium-pressure mercury lamp, permitted quantitation of [I] and [II] in the nanogram range. Compound [V] was selected as the internal standard because of its compatible extraction and

chromatographic behavior to that of compounds [I] and [II]. This assay was used for biopharmaceutic studies in the dog [5].

Recovery, sensitivity limits, and statistical validation

The determination of percent recovery requires that the recovered standards be compared to the external standard curve comprising authentic standards which have been added to the residue of extracted control blood or plasma. This is necessary due to a chromatographic (GLC—ECD and HPLC) enhancement or "matrix effect" which these compounds exhibit; i.e., the peak response is 10—20% greater when they are chromatographed in the residue of a biological extract, than when chromatographed in organic solvents as pure authentic standards.

GLC—ECD method for parent compound [I] and metabolite [II]

The overall recoveries of parent compound [I] and metabolite [II] are 98.5 ± 8.9% (S.D.) and 99.8 ± 5.2% (S.D.), respectively. The sensitivity limit of detection is 2 ng/ml of plasma for both compounds. The intra-assay precision of [I] and [II] over the concentration range of 2—72 ng/ml of plasma showed mean coefficients of variation of 2.8% and 1.8%, respectively. The inter-assay precision of [I] and [II] over the same concentration range showed mean coefficients of variation of 5.1% and 4.2%, respectively (Table I).

TABLE I

INTER-ASSAY PRECISION OF THE GLC—ECD ASSAY FOR COMPOUNDS [I] AND [II]-OTMS (FOLLOWING DERIVATIZATION), RECOVERED FROM CONTROL DOG PLASMA

Compound	Amount added (ng)	Amount found (ng)	*n*	Coefficient of variation (%)
[I]	2.0	2.2 ± 0.1	3	2.5
	4.0	3.9 ± 0.2	3	5.2
	8.0	7.5 ± 0.5	3	6.1
	16.0	14.7 ± 1.0	3	6.7
	24.0	23.7 ± 0.9	3	3.7
	48.0	49.3 ± 3.6	3	7.4
	72.0	77.0 ± 3.4	3	4.4
			Average =	5.1
[II]	2.0	2.1 ± 0.1	3	2.9
	4.0	3.9 ± 0.2	3	5.3
	8.0	7.8 ± 0.5	3	6.2
	16.0	15.6 ± 0.4	3	2.6
	24.0	24.8 ± 1.2	3	4.8
	48.0	48.5 ± 2.4	3	4.9
	72.0	72.0 ± 2.0	3	2.8
			Average =	4.2

TABLE II

INTER-ASSAY PRECISION OF THE REVERSED-PHASE HPLC ASSAY FOR COMPOUNDS [I] AND [II], RECOVERED FROM CONTROL DOG PLASMA

Compound	Amount added (ng)	Amount found (ng)	*n*	Coefficient of variation (%)
[I]	50.0	50.4 ± 1.4	3	2.8
	100.0	101.0 ± 2.2	3	2.1
	300.0	289.0 ± 8.7	3	3.0
	600.0	611.0 ± 47.5	3	7.8
	900.0	893.0 ± 53.0	3	5.9
	1800.0	1820.0 ± 156.0	3	8.6
	2700.0	2710.0 ± 95.0	3	3.5
			Average =	4.8
[II]	50.0	51.2 ± 2.5	3	4.8
	100.0	101.0 ± 1.7	3	1.7
	300.0	288.0 ± 14.7	3	5.1
	600.0	586.0 ± 23.1	3	3.9
	900.0	888.0 ± 11.4	3	1.3
	1800.0	1880.0 ± 87.2	3	4.6
	2700.0	2710.0 ± 40.4	3	1.5
			Average =	3.3

TABLE III

INTER-ASSAY PRECISION OF THE REVERSED-PHASE HPLC ASSAY FOR UNCONJUGATED COMPOUNDS [I] AND [II], RECOVERED FROM CONTROL DOG URINE

Compound	Amount added (ng)	Amount found (ng)	*n*	Coefficient of variation (%)
[I]	50.0	46.6 ± 5.0	3	10.6
	100.0	97.3 ± 5.6	3	5.7
	200.0	203.0 ± 9.5	3	4.7
	300.0	302.0 ± 7.7	3	2.5
	400.0	396.0 ± 8.3	3	2.1
	1000.0	1010.0 ± 20.6	3	2.0
			Average =	4.6
[II]	50.0	46.6 ± 5.2	3	11.2
	100.0	102.0 ± 7.1	3	7.0
	200.0	200.0 ± 6.2	3	3.1
	300.0	303.0 ± 8.5	3	2.8
	400.0	396.0 ± 6.1	3	1.5
	1000.0	1000.0 ± 6.1	3	0.6
			Average =	4.4

HPLC method (reversed-phase) for parent compound [I] and metabolite [II]

The overall recoveries of compounds [I] and [II] from plasma are 103.6 ± 8.8% (S.D.) and 106.1 ± 11.5% (S.D.), respectively. The sensitivity limit of detection is 50 ng/ml of plasma for both compounds, using UV detection at 254 nm. The intra-assay precision of [I] and [II] over the concentration range of 50--2700 ng/ml of plasma showed mean coefficients of variation of 4.8% and 2.7%, respectively. The inter-assay precision of [I] and [II] over the same concentration range showed mean coefficients of variation of 4.8% and 3.3%, respectively (Table II).

The overall recoveries of unconjugated compounds [I] and [II] from urine are 94.6 ± 8.7% (S.D.) and 101.9 ± 7.7% (S.D.), respectively. The sensitivity limit of detection is 50 ng/ml of urine for both compounds, using UV detection at 254 nm. The intra-assay precision of [I] and [II] over the concentration range of 50—2000 ng/ml of urine showed mean coefficients of variation of 5.3% and 6.0%, respectively. The inter-assay precision of [I] and [II] over the concentration range of 50—1000 ng/ml of urine showed mean coefficients of variation of 4.6% and 4.4%, respectively (Table III).

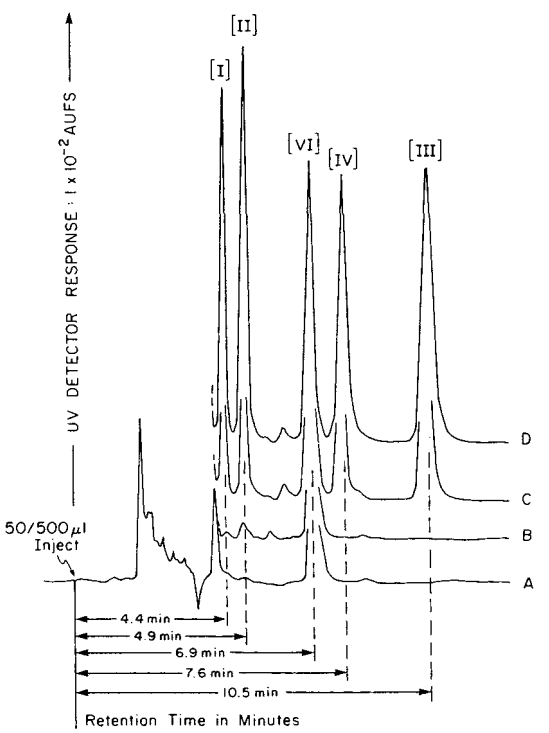

Fig. 5. Chromatograms of the normal-phase HPLC analysis of benzene extracts of (A) control human plasma with added internal standard; (B) human plasma following a single 5-mg oral dose of [I]; (C) authentic standards recovered from control human plasma; and (D) authentic standards added to the residue of control human plasma (matrix external standard).

Normal-phase HPLC analysis of compounds [I], [II], [III], and [IV] in blood or plasma

A sensitive and specific normal-phase HPLC assay was also developed for the determination of compounds [I], [II], [III], and [IV] from 0.5 ml of blood or plasma using UV detection at 254 nm. The column used was a 0.25 m × 4.6 mm I.D. stainless-steel column containing 10-μm Partisil silica, generating 31,000 theoretical plates/m (Whatman, Clifton, NJ, U.S.A.).

The Waters HPLC system was used as before. The isocratic mobile phase used was a mixture of hexane—methanol—tetrahydrofuran—ammonium hydroxide (71.85:8:20:0.15) at a pressure of 4 MPa and a constant flow-rate of 1.5 ml/min.

The retention times of compounds [I], [II], [III], [IV], and internal standard [VI] were 4.4, 4.9, 10.5, 7.6, and 6.9 min, respectively, with capacity factors (k') of 3.23, 3.61, 9.30, 6.46, and 5.77, respectively (Fig. 5). Reversed-phase HPLC was unable to adequately resolve all components of interest.

TABLE IV

PLASMA CONCENTRATIONS OF COMPOUNDS [I] AND [II] IN THE DOG FOLLOWING ORAL AND INTRAVENOUS ADMINISTRATION OF COMPOUND [I]

ND = not detectable, NM = non-measurable (< 2 ng/ml), NST = no sample taken.

Time (h)	Concentration (μg/ml plasma)			
	Intravenous dose (5 mg/kg)		Oral dose (20 mg/kg)	
	[I]	[II]	[I]	[II]
0	ND*	ND	ND	ND
0.017	15.2	ND	NST	NST
0.042	8.7	ND	NST	NST
0.083	7.11	0.18	NST	NST
0.17	6.12	0.26	0.24	0.11
0.25	5.40	0.40	NST	NST
0.33	3.70	0.27	2.52	0.88
0.50	2.56	0.22	2.83	1.21
0.75	1.73	0.15	3.48	1.26
1.0	1.11	0.11	3.20	1.16
1.5	0.64	0.033	4.32	1.33
2	0.35	0.020	3.98	1.12
3	0.20	0.016	3.14	0.72
4	0.11	0.01	1.14	0.28
6	0.07	0.007	0.47	0.10
8	0.04	0.005	0.29	0.04
11.5	0.03	NM	NST	NST
12	NST**	NST	0.15	0.03
24	0.015	NM	0.08	0.006
30	0.009	NM	0.03	NM
48	0.007	ND	0.025	ND
72	NM***	ND	ND	ND

The extraction procedure is the same as that described for reversed-phase HPLC analysis, except that compound [VI] is used as the internal standard and the final residue was dissolved in the mobile phase for adsorption chromatography. This assay was used to monitor the possible presence in human plasma of compounds [III] and [IV], which were reported to be minor metabolites in the urine of rat and in the dog [3]. The N-oxide, [III] (if present), would undergo thermal degradation in the injection port during

TABLE V

MEAN CONCENTRATION OF COMPOUNDS [I] AND [II] IN MAN FOLLOWING THE ORAL ADMINISTRATION OF A SINGLE 5-mg DOSE OF COMPOUND [I]

Time (h)	Mean concentration ± S.D. (ng/ml)			
	[I]	*n*	[II]	*n*
0	N.D.	4	N.D.	4
0.5	5.0 ± 3.1	2	5.2 ± 4.6	2
1.0	15.1 ± 6.9	4	24.4 ± 14.0	4
1.5	15.9 ± 4.4	4	26.4 ± 9.3	4
2.0	17.0 ± 2.8	4	28.9 ± 10.9	4
3.0	15.5 ± 6.7	4	20.2 ± 10.0	4
4.0	12.2 ± 4.6	4	18.3 ± 7.0	4
6.0	8.8 ± 1.8	4	12.8 ± 3.4	4
8.0	6.2 ± 1.3	3	8.5 ± 1.5	3
12.0	4.9 ± 1.0	4	6.2 ± 2.8	4
18.0	2.9 ± 0.5	3	2.8 ± 0.5	4
24.0	2.5 ± 0.7	2	2.0 ± —	1

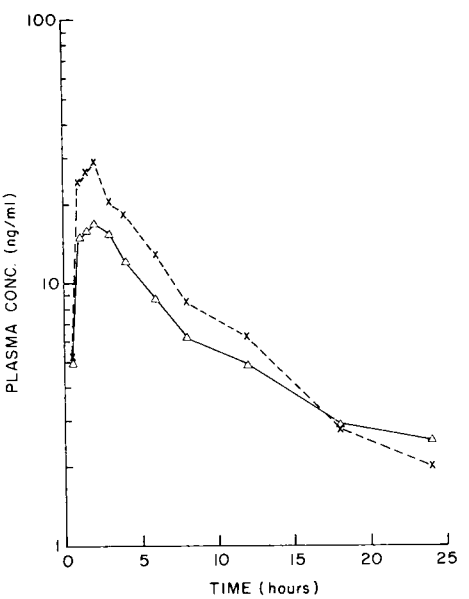

Fig. 6. Mean plasma concentration of compounds [I] (△) and [II] (X) in man following the oral administration of a single 5-mg dose of compound [I].

GLC—ECD analysis to yield [I] thereby impairing the accuracy of its determination.

The overall recoveries of compounds [I], [II], [III], and [IV] from plasma are 99.5 ± 9.8%, 103 ± 12.4%, 102 ± 8.0%, and 58.8 ± 6.6%, respectively. The sensitivity limit of detection is 25 ng/ml of plasma for compounds [I] and [II], and 50 ng/ml of plasma for compounds [III] and [IV].

Application of the methods to biological specimens

The GLC—ECD procedure and the reversed-phase HPLC procedure were used to monitor the plasma concentration—time profiles of compounds [I] and [II] in the dog during biopharmaceutic/pharmacokinetic evaluation following single 5 mg/kg intravenous and 20 mg/kg oral doses of compound [I] (Table IV). The GLC—ECD method was also used to determine the plasma concentrations of compounds [I] and [II] in four human subjects following a single 5-mg oral dose of compound [I] (Table V and Fig. 6).

The reversed-phase HPLC assay was used to determine the bioavailability of oral dosage forms of [I] in the dog [5] and in the evaluation of the stability of compound [I] in dog plasma determined over a 19-month storage interval at −25 to −30°C. The mean change observed (± 5.4%) was within the experimental error of the method, indicating stability of the compound.

The normal-phase HPLC assay for parent compound [I] and metabolites [II], [III], and [IV] was used to monitor the plasma concentration—time profiles of these compounds in two human subjects following a single 5-mg oral dose of compound [I]; however, none of these were detectable. The effluent volume fractions of [III] were collected, evaporated under nitrogen and analyzed by GLC—ECD. No peak for [I] was obtained, indicating the absence of [III] in any significant amounts in human plasma.

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ROUTINE DETERMINATION OF HYDROXYPHENYTOIN IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING AN AUTOMATIC COLUMN-SWITCHING TECHNIQUE

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SUMMARY

A high-performance liquid chromatographic method for the determination of the main phenytoin metabolite, hydroxyphenytoin, in the urine of epileptic patients is described. The use of an automated column-switching technique greatly simplifies the pretreatment steps. Thereby, both time and chemicals are saved. The possibility of error arising during the several pretreatment steps is considerably reduced. Following acid hydrolysis of the hydroxyphenytoin glucuronic acid conjugate the sample is diluted with water and after centrifugation is injected onto the pre-column. After washing for a short time with water, the substances which were absorbed on the head of the pre-column were backflushed with water—acetonitrile as eluent onto the analytical column. Separation is achieved by gradient elution using an ODS reversed-phase column with a particle size of 5 μm.

INTRODUCTION

The determination of 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (p-HPPH) in the urine of patients treated with the anticonvulsant drug phenytoin (5,5-diphenylhydantoin, DPH) offers a means of examining the elimination of this drug as well as assessing whether or not the drug has been taken by the patient.

High-performance liquid chromatography (HPLC) is, next to gas—liquid chromatography, of prime value for carrying out such analyses, because derivatization of drugs can be dispensed with using HPLC. Several HPLC methods for the estimation of p-HPPH in body fluids have, therefore, already been described [1—11]. These methods, however, have some disadvantages.

Using ion-exchange columns the samples (serum) can be injected directly into the column [2]. However, comparing the results using modified silica gel the quality of separation is poor (band broadening) and it takes a very

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long time to carry out a single analysis. The other methods require a number of different steps in preparing the sample. For the determination of p-HPPH in urine these include at least the following steps: (1) Hydrolysis of the sample by heating with concentrated hydrochloric acid or enzymatic cleavage of the p-HPPH glucuronide. (2) Neutralisation of the hydrolysate. (3) Extraction of the drugs and metabolites with an organic solvent. (4) Centrifugation. (5) Evaporating off the organic phase. (6) Taking up the residue with methanol or with the mobile phase of the subsequent chromatographic separation.

METHODS

In recent years it appeared that the use of various column-switching techniques for organic trace analysis is becoming the method of choice in all fields of chemistry [12—14]. In 1981 Roth et al. [15] described a fully automated HPLC method for use in clinical chemistry. Sample pretreatment and chromatographic analysis are combined in one step. Thus, the body fluid (plasma, urine, or saliva) is injected directly onto a pre-column, and protein and other serum constituents are washed through the pre-column with a purge liquid. Following the washing step the retained drugs are carried over with an eluent using the back-flush technique, to the analytical column where the substances are separated.

Time required for analysis can be saved using two pre-columns which are charged alternately. An exact scheme needed for the connection of the valves is given in ref. 15.

Until recently, users of column-switching techniques had to construct their own systems. Since last year systems are commercially available which offer a complete range of possible ways of switching columns, e.g. in the wash-through, pre-separation or back-flush mode, and alternating stages for applying the sample and chromatographic separation. An example of such a construction is given in ref. 16, in which the arrangement is shown in greater detail. This system was used in developing the method as described in this paper.

In previous publications concentrated hydrochloric acid was used for the non-enzymatic hydrolysis of the glucuronide [3, 5, 6, 8—10]. However, to avoid possible corrosive effects of chloride ions in acidic medium on the steel parts of the HPLC apparatus (especially on the inner walls of the analytical columns) a non-oxidizing mineral acid was sought to replace hydrochloric acid.

Sulphuric acid (98%), phosphoric acid (85%) and perchloric acid (70%) were each mixed in a 1:1 ratio with urine and further processed under the conditions given in the experimental section. Only the addition of perchloric acid gave values which were comparable to those obtained with hydrochloric acid. The results of the analyses of 68 patient samples using, in each case, hydrochloric acid and perchloric acid, gave a coefficient of correlation of $r = 0.997$.

A further difficulty in determining p-HPPH in urine is that most of the patients examined here are treated with a mixture of various anticonvulsant drugs which, in the first place, very frequently includes phenobarbital (PB).

Thus a separation column must be found whose specificity enables a sharp separation to be made between p-HPPH, PB and m-HPPH which likewise occurs in significant amounts in the urine of patients. Errors in analysis of up to 40% could otherwise arise, especially with samples containing a low concentration of p-HPPH and a comparatively high concentration of PB.

Attempts using a μBondapak RP-18 column (10 μm), which was used in most of the earlier publications [3—8], gave the desired separation only on gradually increasing the gradient. Consequently the time needed for each analysis (elution of the internal standard MPPH takes about 36 min) is relatively high.

This separation problem could not be solved even using a μBondapak alkylphenyl column (10 μm) with which good separations could be obtained in analyzing flavour components, preservatives and drug residues [17—21].

In further tests only silica gels modified with ODS phases and with particle size of 5 μm were used (Hypersil, Zorbax, Spherisorb, Nucleosil) out of which Zorbax-ODS gave the best separations (see Fig. 1).

In addition, an attempt was made to reduce the time of analysis by substitut-

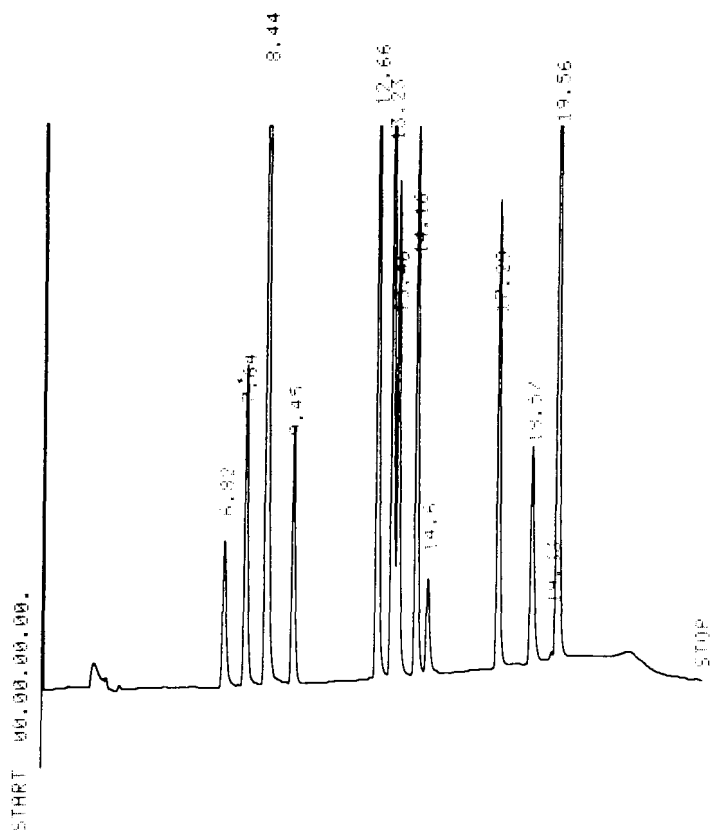

Fig. 1. Chromatogram of a methanolic stock solution of antiepileptic drugs, metabolites and internal standards. Retention times (min): PEMA 6.82, ET 7.64, HPB 8.44, PRI 9.45, p-HPPH 12.66, PB 13.23, m-HPPH 13.46, N-DES 14.16, C-EP 14.60, DPH 17.29, CBZ 18.57, MPPH 19.56.

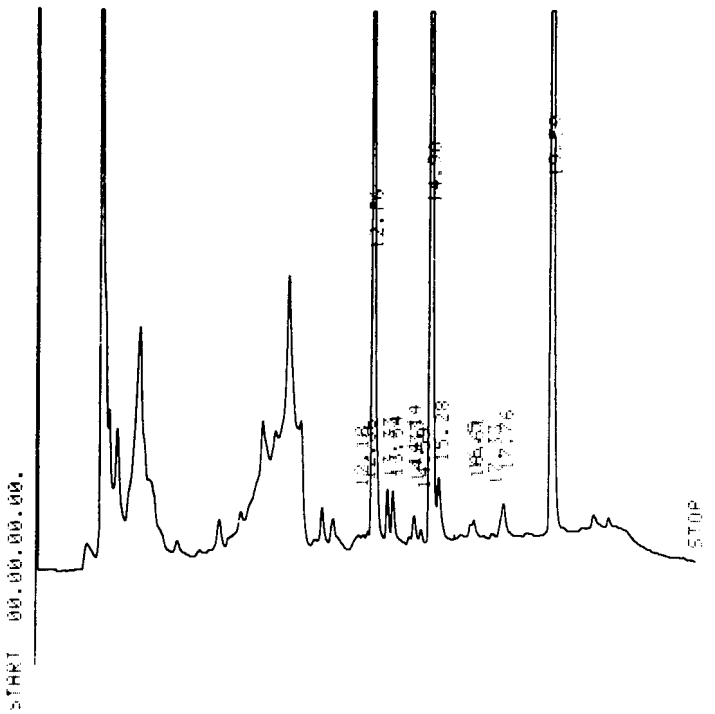

Fig. 2. Chromatogram of a patient sample. Retention times (min): p-HPPH 12.76, PB 13.33, m-HPPH 13.54, HPTH 14.98, MPPH 19.59.

ing the internal standard 5-(*p*-methylphenyl)-5-phenylhydantoin (MPPH) by 5-(*p*-hydroxyphenyl)-5-(*p*-tolyl)-hydantoin (HPTH). The possible reduction in separation time of about 5 min (see Fig. 2) could not be achieved, because in the time range in which HPTH is eluted carbamazepine-10,11-epoxide (C-EP) and other interfering impurities are also eluted.

EXPERIMENTAL SECTION

Chemicals

Chemicals used were obtained from the following firms: p-HPPH, m-HPPH, MPPH, HPTH, 5-ethyl-5-(*p*-hydroxyphenyl)-barbituric acid (HPB), 2-ethyl-2-phenylmalonediamide (PEMA) and N-desmethylmethsuximide (N-DES) from EGA-Chemie, Steinheim, G.F.R. Phenobarbital (PB) from Bayer, Leverkusen, G.F.R. Carbamazepine (CBZ), carbamazepine-10,11-epoxide (C-EP), primidone (PRI), ethosuximide (ET) and diphenylhydantoin (DPH) from Desitin-Werk/ Carl Klinke, Hamburg, G.F.R.

Acetonitrile "for chromatography" (LiChrosolv) was from Merck, Darmstadt, G.F.R. Water "for use in HPLC" was from Baker Chemicals, Deventer, The Netherlands. All other chemicals were of analytical reagent grade and were obtained from Merck.

Apparatus

The equipment used was obtained from the following firms: Automatic pipetter/diluter from Corning/Gilford, Düsseldorf, G.F.R. TCS metal block thermostat (ambient temperature—120°C) from Barkey Labortechnik, Bielefeld, G.F.R. Rotixa/K centrifuge from Hettich, Tuttlingen, G.F.R. HPLC low-pressure gradient-former 2500, two HPLC constant-flow pumps 600/200, sample preparation Model SE-2, spectrophotometer SP-4, Shimadzu printer-plotter-integrator C-R 1B from Gynkotek, Munich, G.F.R. Autosampler WISP 710 B from Waters, Königstein/Taunus, G.F.R. Analytical column Zorbax-ODS (5 μm, 250 mm × 4.6 mm I.D.) from Du Pont, Frankfurt/Main, G.F.R. Pre-columns LiChrosorb RP-18 (10 μm, 40 mm × 4.6 mm I.D.) from Bischoff-Analysentechnik, Leonberg, G.F.R.

Chromatographic parameters

Temperature of the column: ambient temperature. Injection volume: 30 μl. Detection wavelength: 205 nm. Pre-column: eluent water, flow-rate 1.0 ml/min, duration of washing 200 sec. Analytical column: eluent water—acetonitrile (gradient elution), flow-rate 1.5 ml/min. In order to avoid degassing the solvents, water and acetonitrile were pre-mixed in a ratio 9:1 (v/v) for the container A and in a ratio 4:6 (v/v) for the container B.

The gradient programme was as follows:

Step	A (vol. %)	B (vol. %)	Acetonitrile (vol. %)	Time (min)
1	090	010	15	0.00
2	090	010	15	1.00
3	020	080	50	19.00
4	100	000	10	0.00
5	100	000	10	1.00
6	090	010	15	0.00
7	090	010	15	End

Calibration and control samples, internal standard

From a stock solution containing 300 mg of p-HPPH in 100 ml of acetone, a 50-μl quantity was made up each time to 500 μl using blank urine. This corresponds to 300 μg of p-HPPH per ml of urine in each calibration sample. About 1000 calibration samples, and for purposes of internal quality control 0.5-ml portions of a pooled patients urine, were frozen. These samples can be kept unchanged at about —18°C for long periods of time (at least six months).

For preparing the internal standard solution 150 mg of MPPH are dissolved in 100 ml of acetone.

Preparation of the sample

A 500-μl quantity of urine is pipetted each time into a 10-ml centrifuge tube with a screw cap. To each of these patients samples, as well as to the thawed-out samples for calibration, 100 μl of the internal standard solution and 500 μl of perchloric acid (70%) are added.

The samples are well mixed and heated at 100°C (± 0.5°C) for 30 min in a metal block heater. After cooling the samples are diluted with 3 ml of water. The insoluble constituents of the urine are centrifuged off and 30 μl of the supernatant fluid are injected.

A few hours later a small amount of precipitate is observed in the sample vials which, however, does not disturb the determination.

Lifetime of the column

A detectable increase in pressure of the analytical column was noted after about 800 injections. The filter frit at the head of the column was removed and cleaned in an ultrasonic bath. Apart from this 2 mm of the packing material, which had become coloured, was removed and replaced by a suspension of LiChroprep (40—63 μm, Merck) in isopropanol. In addition to the cleaned metal frit, the head of the column was plugged with a cellulose filter (Knauer, Berlin, G.F.R.). The effectiveness of the column has, following about further 300 injections, hitherto remained unchanged.

The pre-columns were, regardless of the pressure, changed after every 400—500 injections in order to keep contamination of the analytical column down to the smallest amount possible.

RESULTS AND DISCUSSION

To check whether the method described in this paper is suitable for routine laboratory purposes the following analytical parameters were examined: (1) Time required for hydrolysis of the samples. (2) Linearity of the determination. (3) Error in analysis.

The following statistical terms were used for the evaluations (abbreviations in parentheses): number of samples, injections or duplicates (n), maximum value (X_{max}), minimum value (X_{min}), mean value ($\bar{X}$), median (m), standard deviation (s), coefficient of variation (C.V. %), coefficient of correlation (r), mean value of the absolute differences of the duplicates $X-Y$ ($\bar{d}$), mean deviation from the mean value expressed as a percentage $100(X-Y)/(X+Y)$, ($\overline{dX}$ %).

Time for hydrolysis of the samples

Whereas 1 h was apparently needed for the complete hydrolysis of urine samples using concentrated hydrochloric acid [5], 30 min was sufficient time for the complete cleavage of the p-HPPH glucuronide using perchloric acid.

To test the stability of free p-HPPH and of the internal standards MPPH and HPTH under the experimental conditions described above, an aqueous—methanolic solution was treated with increasing boiling time. Thereby a slight decrease in concentration of these three substances even from 60 min onwards was observed. The HPTH constituent was changed the most rapidly, whilst MPPH was the most stable compound.

Linearity of the determination

A patient sample containing more than 500 μg/ml p-HPPH was diluted with blank urine to give percentage concentrations of 80, 50, 20, and 10. The

TABLE I

MEASURED AND CALCULATED VALUES OF THE LINEARITY CONTROL

Diluted sample	X (%), Y (μg/ml p-HPPH)				
	100	80	50	20	10
Measured Y	515.5	420.0	255.5	101.5	51.0
Calculated Y	518.5	414.4	258.3	102.2	50.1

original sample and the dilutions were measured in duplicate (Y) (Table I).

The regression equation of these data is $Y = 5.205X - 1.94$ having a correlation coefficient of $r = 0.9998$. It is thence concluded that the p-HPPH analysis, described in this paper, of the urines of patient samples which come within the usual concentration range is linear.

Analytical error

Deviations on multiple analysis of the same sample. To test what differences arise on preparing samples on the same day, 0.5-ml quantities of a homogeneous pool urine A were taken 53 times and analysed as described above. To evaluate the reproducibility of the p-HPPH determination over several days three samples of a pool urine B were each investigated on six consecutive days, and samples of a pool urine C over several months. The results are summarized in Table II.

TABLE II

REPRODUCIBILITY OF THE ANALYSES OF POOL URINES

Pool urine	X (μg/ml p-HPPH)						
	n	X_{max}	X_{min}	$\bar{X}$	m	s	C.V. (%)
A	53	244	226	233.1	232	4.34	1.86
B	18	210	195	202.6	204	5.17	2.55
C	59	319	284	299.8	300	9.90	3.30

Reproducibility of the double determinations. Hitherto only the analytical errors due to the variations in preparation of the same samples have been statistically considered. Moreover, the influences which arise due to different substances contained in each patient sample, and which affect each estimation, have not yet been considered.

It is, however, just the samples examined in the course of routine analyses which, seen apart from their content of DPH metabolites, can have a very different substance composition. For this reason it is of particular interest to the analyst to know the degree of reproducibility of each individual value with which he can reckon.

We obtained a measure of the range of error which is to be expected in

TABLE III

REPRODUCIBILITY OF THE DOUBLE DETERMINATIONS

Patient samples	X (μg/ml p-HPPH)					
	n	$\bar{X}_1$	$\bar{X}_2$	r	$\bar{d}$	$\overline{dX}$ (%)
Within-day	216	225.64	224.83	0.998	6.09	1.41
Day-to-day	315	226.86	224.57	0.992	11.61	2.75

the determination method described here by analysing in duplicate patients urine both during the day as well as after several days up to weeks later (frozen samples) (Table III).

CONCLUSIONS

The use of a column-switching technique which combines the separate steps, namely sample purification, concentration, and chromatographic separation, in one process enables a considerable saving both of chemicals and time in carrying out routine laboratory analyses. Following acid hydrolysis of the p-HPPH glucuronic acid conjugate it sufficed to dilute the sample and inject it after centrifugation directly onto the pre-column. It was thereby possible to reduce considerably the number of pretreatment steps mentioned above. Besides this the time required for splitting the p-HPPH glucuronide conjugate is reduced to 30 min, and possible corrosion of the HPLC apparatus by chloride ions in an acidic medium is avoided by replacing hydrochloric acid with perchloric acid.

With a mean value of 1.41 for the percental deviation from the mean value for the double determination within a single day, and of 2.75 for the double determination within a time interval ranging from several days up to weeks (see Table III), the method described is sufficiently reproducible for purposes of routine analyses.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF GALANTHAMINE, A LONG-ACTING ANTICHOLINESTERASE DRUG, IN SERUM, URINE AND BILE

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SUMMARY

The anticholinesterase drug galanthamine is obtained from alkalinized serum by repeated liquid—liquid extraction. The resulting extract is approximately 100 times concentrated with respect to the original sample. Quantitative determination of galanthamine is performed with normal-phase liquid chromatography using a mixture of dichloromethane—*n*-hexane and ethanolamine as an eluent. Phenacetin is used as internal standard. The absorption of the column effluent is monitored at 235 nm. No endogenous sources of interference have been observed. A galanthamine serum level of 5 ng/ml is found as the minimum detectable concentration; the coefficient of variation at this level is 37.8% (n = 4). For the assay of galanthamine in the concentration range 10—100 ng/ml, standard deviations vary between 18.9 and 2.5% (n = 32).

INTRODUCTION

The alkaloid galanthamine hydrobromide is a tertiary amine belonging to

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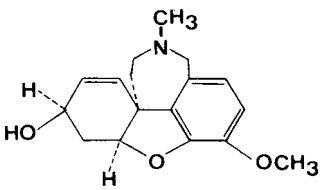

Fig. 1. Structural formula of galanthamine.

the phenanthrene group (see Fig. 1). Since the compound is an anticholinesterase drug, its pharmacological actions are both central and peripheral.

Galanthamine was isolated in 1952 by Proskurnina and Yakovleva [1] from the bulbs of the common snowdrop, *Galanthus woronowi* (Amaryllidaceae family) and in 1956 by Paskow and Iwanova-Bubewa from the bulbs of *Galanthus nivalis* (see ref. 2). It is available for clinical use as Nivalin (Pharmachim, Sophia, Bulgaria) and as Galanthamine (Medexport, Moscow, U.S.S.R.). Until now, galanthamine has mainly been in clinical use for the reversal of the neuromuscular blockade caused by various curare-like agents [2—5].

Antagonism of the respiratory depressant effect of opiates such as morphine, pethidine, dextromoramide, etc., in both animals and man has also been reported [6—9]. Furthermore, galanthamine has been used to reverse the central anticholinergic syndrome caused by scopolamine [10, 11] and certain central effects of droperidol and diazepam [12].

This broad variety of central effects of galanthamine and especially the finding that, in rabbits, the compound reversed opiate-induced respiratory depression, but not the concomitant analgesia [9], makes galanthamine a useful pharmacological tool in the search for better antagonists in anaesthesia.

In further (clinical-)pharmacological studies, concentration—effect relationships will be highly important; therefore, a sensitive and reproducible method for the determination of galanthamine in biological materials has to be developed.

Some work, mostly in the field of thin-layer chromatography, has been done on the assay of galanthamine [13—17]. High-performance liquid chromatographic (HPLC) determination of alkaloids in general has been studied by several authors. Wu and Wittick [18] and Rasmussen et al. [19] describe methods for the separation of alkaloids, using reversed-phase chromatography.

Verpoorte and Baerheim Svendsen [20—22] and Rasmussen et al. [19] report the application of normal-phase chromatography on silica. Also the use of polar bonded phases had been reported [23]. To obtain low detection limits and short analysis times, particle sizes of 5 and 3 μm were selected and the capacity factors (k') of the components of interest were kept below 6.

The development of the method was started using reversed-phase chromatography on octadecyl phases. In spite of the available tools of this technique we failed to develop a method with the required sensitivity. The application of normal-phase chromatography on 3 μm silica gel and a mixture of *n*-hexane—dichloromethane—ethanolamine (500:500:0.25, v/v) as eluent gave good results in terms of detection limit and speed of analysis. The necessary pretreatment of the serum samples was started with extraction columns; however, even extensive experimental work gave no satisfactory results. We found

a repeated liquid—liquid extraction method very selective, but unfortunately it is more time-consuming.

MATERIALS AND METHODS

Chemicals

All chemicals and solvents were of analytical grade (Merck, Darmstadt, G.F.R.). Aqueous solutions were made in double-distilled water.

Dichloromethane and *n*-hexane were both of HPLC grade (Merck) and purified prior to use by filtration through a 0.45-μm HVLP membrane (Millipore Corporation, Bedford, MA, U.S.A.).

Galanthamine HBr and the standard phenacetin were obtained from Koch-Light Labs. (Colnbrook, Great Britain) and Onderlinge Pharmaceutische Groothandel (Utrecht, The Netherlands), respectively.

Apparatus

The experiments were performed on a liquid chromatograph consisting of a reciprocating pump (Model 100-A, Beckman Instruments Inc., Berkeley, CA, U.S.A.) and a variable-wavelength UV detector (type Uvidec 100-III, Jasco, Tokyo, Japan) operating at 235 nm and 2.5×10^{-3} a.u.f.s. The pump delivered a constant flow of 1 ml/min. Injections of 10 μl were made with an injection valve equipped with a 20-μl loop (Model CV-6-UHPa-N60, Valco, Houston, TX, U.S.A.). The reversed-phase columns, 15 × 0.46 cm, using Polygosil 60-C_{18} 5 μm as packing material (Macherey-Nagel, Düren, G.F.R.) were home-made. The columns were constructed of stainless steel tubing (SS-316, Handy and Harman, Norristown, PA, U.S.A.) 15 cm × 6.4 mm O.D. × 4.6 mm I.D.

The normal-phase columns, 10 × 0.46 cm, were pre-packed with CP^{tm} Micro Spher Si 3 μm manufactured by Chrompack (Middelburg, The Netherlands).

To suppress the influence of fluctuations of temperature, tubing and column were suitably isolated. Detector signals were registered with a Yew recorder (Yokogawa Electric Works, Tokyo, Japan).

Galanthamine serum standards

Prepare a stock solution by dissolving 25.64 mg of galanthamine HBr in 200 ml of water. This solution is equivalent to 10^5 μg of galanthamine base per l. Dilute this solution with blank human serum to make serum standards with final galanthamine concentrations of 5, 10, 20, 40, 50, 70, and 100 ng/ml. Serum standard amounts of approximately 5 ml are frozen. There is no degradation of galanthamine after six months of storage at —20°C.

Sample preparation

Pipet 2.0 ml of serum into a 10-ml glass test tube and add 2.0 ml of a 20% (w/v) aqueous solution of trichloroacetic acid as protein-precipitating agent. The tube is then rotated on a Vortex mixer for 10 sec, allowed to stand at room temperature for 5 min, and centrifuged at 1600 *g* for 10 min.

A 3.0-ml aliquot of the supernatant is transferred in a 25-ml glass test tube

with stopper, and alkalinized by adding 0.6 ml of 4 *M* sodium hydroxide and 2.0 ml of buffer (1 *M* ammonia solution titrated with 2 *M* hydrochloric acid to pH 9.0). Extraction of galanthamine base is carried out twice with 5 ml of dichloromethane (DCM) by Vortex mixing for 2 min and centrifuging at 1600 *g* for 5 min. DCM extracts are transferred to a clean 25-ml glass test tube with stopper and back-extracted twice with 5 ml of 0.05 *M* sulphuric acid by Vortex mixing for 2 min and centrifuging at 1600 *g* for 5 min. Sulphuric acid extracts with galanthamine are transferred to a clean 25-ml glass test tube with stopper and alkalinized by adding 0.4 ml of 4 *M* sodium hydroxide, and 2.0 ml of buffer pH 9.0, prepared as described above. Subsequently galanthamine base is extracted twice with 5 ml of DCM by Vortex mixing for 2 min and centrifuging at 1600 *g* for 5 min. The DCM phase with galanthamine base, obtained after each extraction, is transferred to a 10-ml conical test tube, containing 0.5 ml of phenacetin internal standard solution in DCM (12.5 μg/l). The organic phase is evaporated under a gentle stream of nitrogen at 50°C in a heating bath. The inside wall of the test tube is washed with 0.5—1 ml of DCM, and the DCM is then evaporated to dryness as mentioned above. Each extract is dissolved in 25 μl of mobile phase by Vortex mixing for 30 sec. Then a 10-μl aliquot is injected immediately into the chromatograph.

Chromatographic procedures

The silica column was loaded with ethanolamine by pumping an approximately 0.1% solution of ethanolamine in *n*-hexane—dichloromethane (1:1,

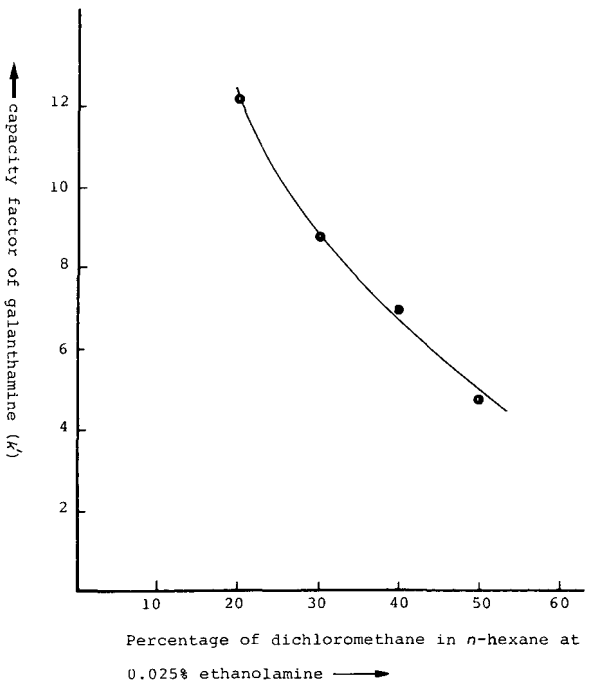

Fig. 2. Influence on the capacity factor (*k'*) of galanthamine by various percentages of dichloromethane in the eluent mixture consisting of dichloromethane, *n*-hexane and a constant amount of 0.025% ethanolamine.

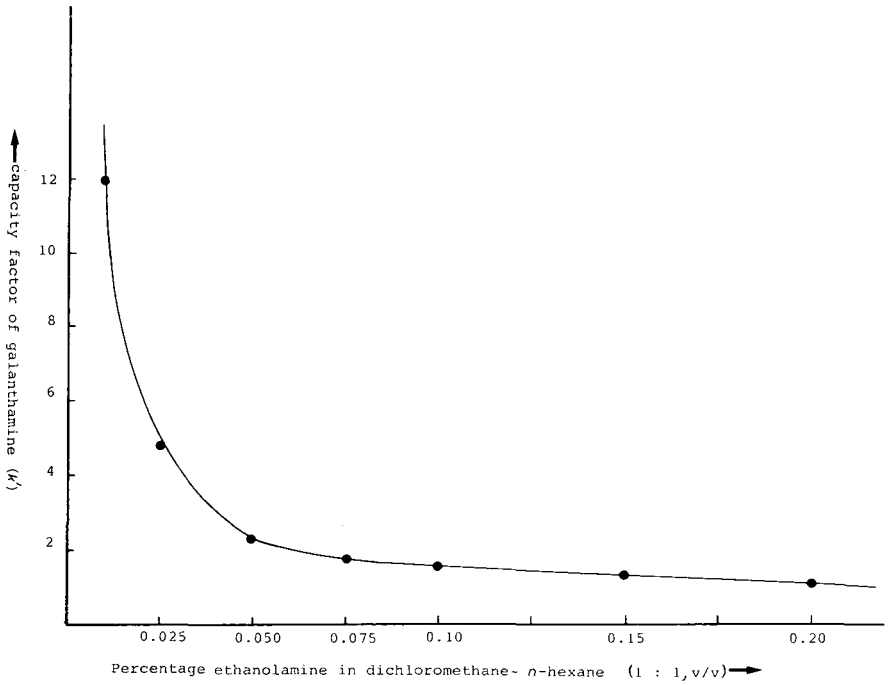

Fig. 3. Influence on the capacity factor (k') of galanthamine by various amounts of ethanolamine in the eluent mixture consisting of *n*-hexane and dichloromethane (1:1, v/v).

v/v) for about 1 h. Then the eluent, dichloromethane—*n*-hexane—ethanolamine (500:500:0.25, v/v), was pumped through the system until equilibrium; preferably this was done overnight. The capacity ratio (k') of galanthamine can be controlled by the content of dichloromethane and ethanolamine as is shown in Figs. 2 and 3, respectively. In practice the control of k' by the ethanolamine content is more easy.

The HPLC equipment and conditions described above permit the detector to operate at 235 nm and 2.5×10^{-3} a.u.f.s. Galanthamine has an absorption maximum at 288 nm, and a molar extinction coefficient of 2500 l mol^{-1} cm^{-1}. At shorter wavelengths the extinction coefficient increases at 235 nm to 7500 l mol^{-1} cm^{-1}, providing a more sensitive measurement. Application of lower wavelengths is limited by the quality and absorption of the eluent. In practice, no problems were encountered working at 235 nm.

High demands are put on the quality of the eluent because of the above-mentioned facts, but also to prevent the appearance of interfering peaks in the chromatograms.

Quantitation

The peak height ratio of galanthamine base to the internal standard phenacetin is used for quantitation. The peak height ratios for the serum standards are plotted against concentration to obtain a standard calibration curve.

RESULTS

Typical chromatograms for serum samples containing 0 and 5 ng/ml galanthamine are presented in Fig. 4a and b, respectively; phenacetin is added as internal standard.

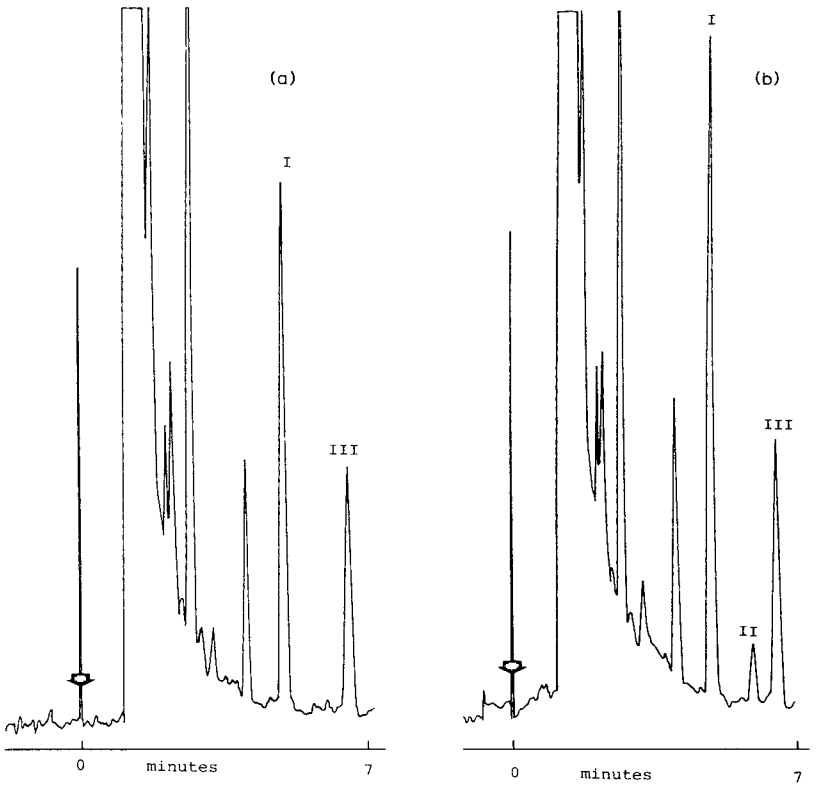

Fig. 4. Chromatograms of serum samples containing 0 ng/ml galanthamine (a) and 5 ng/ml galanthamine (b). Conditions: flow-rate 1 ml/min; eluent *n*-hexane—dichloromethane—ethanolamine (500:500:0.25, v/v); 10 μl injection; UV detection at 235 nm, 2.5 × 10^{-3} a.u.f.s. I = pollution of dichloromethane, II = galanthamine, III = phenacetin.

Calibration curve

The galanthamine/phenacetin ratios, calculated after determination of the serum standards 10, 20, 40, 50, 70, and 100 ng/ml, are used for constructing a calibration curve. The correlation coefficient is calculated by a least-squares procedure and is equal to 0.9996 (n = 32).

Precision and detection limit

The precision of the assay method was assessed by repeated analysis, on different days, of serum samples containing various concentrations of galanthamine. Galanthamine serum levels were calculated by means of a calibration curve. The results are shown in Table I.

The detection limit of galanthamine base in serum is about 5 ng/ml. The coefficient of variation at this level is 37.8% as presented in Table I.

TABLE I

PRECISION DATA

Galanthamine serum standards (ng/ml)	n	Mean galanthamine serum level assayed (ng/ml)	S.D. (ng/ml)	C.V. (%)
5	4	3.20	1.21	37.8
10	5	10.86	2.06	18.9
20	5	20.22	1.93	9.5
40	4	39.48	1.68	4.3
50	8	48.79	2.06	4.2
70	4	69.60	1.75	2.5
100	6	101.0	2.56	2.5

Recovery study

The recovery of galanthamine base was measured by assaying the galanthamine serum standard of 50 ng/ml eight times. Percentage recovery was calculated by comparing the peak height ratio galanthamine/phenacetin with the peak height ratio obtained by directly injecting solutions of the pure compounds. Galanthamine base showed an average recovery of 100.2% with a S.D. of 2.1% (n = 8).

DISCUSSION

To develop a sensitive method, the chromatographic dilution, amongst other factors, should be as small as possible. The use of 3-μm particles and keeping retention times small ($k' < 6$) are necessary to achieve this. The development of the method was started by applying reversed-phase chromatography on octadecyl phases. The influence of methanol content, pH of the eluent and ion-pairing reagents like hexylsulfonate were investigated.

In spite of the available tools of this technique we failed to develop a satisfactory procedure. We observed in these chromatographic systems a decrease in plate number for galanthamine of 75% compared to test components. This might be an indication of non-ideal behaviour of galanthamine under these conditions.

The application of normal-phase chromatography on silica using a mixture of n-hexane—dichloromethane and ethanolamine as an eluent gave much better results. In the latter case we observed a decrease of 25% in the plate number for galanthamine compared to a test mixture, indicating the better thermodynamic conditions in this system.

The influence of the dichloromethane content in the eluent and the addition of ethanolamine are shown in Figs. 2 and 3.

Other amines without a hydroxyl group failed to induce the same effect as ethanolamine. The extraction procedure of galanthamine from serum was started with Sep-Pak (sample enrichment and purification) cartridges (Waters Assoc., Milford, MA, U.S.A.). Sep-Pak cartridges are packed with liquid chromatographic separating materials which retain specific classes of compounds while allowing other materials to pass through. We have done several experi-

ments with Sep-Pak silica and C_{18} cartridges and solvents of different polarity. The extraction yield of galanthamine from Sep-Pak C_{18} after elution with dichloromethane at pH 9 was approximately 100% but HPLC chromatograms showed a high background signal due to co-extracted endogenous serum compounds. This high background of signals did not allow determination of nanogram amounts of galanthamine. From the various attempts that we have made to find an isolation method for galanthamine from serum with low background signals in HPLC chromatograms, the liquid—liquid extraction method presented was derived. The clean-up procedure in the liquid—liquid extraction method, described in this article, is laborious, but makes the assay very specific and sensitive.

The concentration of galanthamine in serum, found by us in preliminary experiments in man, varied between 500 and 60 ng/ml during 3 h, after an intravenous dose of 0.3 mg of galanthamine hydrobromide per kg body weight.

The following drugs, frequently used in patients undergoing anaesthesia, did not interfere with the HPLC determination of galanthamine: morphine, atropine, succinylcholine, thiopental, fentanyl, digoxin, gentamycin and tobramycin. The study of interferences by other components of comedication is continuing.

In our experiments we found that the assay procedure presented is also suitable for the determination of galanthamine in urine and bile.

CONCLUSION

The presented HPLC assay procedure for galanthamine in serum, urine and bile is very sensitive and therefore suitable for use in studies of pharmacokinetics and bioavailability of galanthamine in man.

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DETERMINATION OF NOSCAPINE IN PLASMA BY LIQUID CHROMATOGRAPHY

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SUMMARY

A liquid chromatographic method has been developed for the determination of noscapine in plasma. Noscapine and the internal standard, papaverine, were extracted into methylene chloride by column extraction. The separation was performed on a straight-phase liquid chromatographic system using a mobile phase of hexane—methanol—chloroform—diethylamine. A high detection selectivity was obtained by UV detection at 310 nm. The precision of the method was 3.8% (standard deviation) at a level of 89 ng/ml and 9.5% (standard deviation) at 5.9 ng/ml. The selectivity of the analytical method was evaluated by comparing analytical results after isolation of extracts of plasma samples on reversed- and straight-phase liquid chromatographic systems.

INTRODUCTION

Noscapine is a widely used antitussive agent. Plasma and urine levels of noscapine have been determined fluorimetrically [1]. Even though the selectivity of the fluorimetric method was increased by the modifications suggested by Nayak et al. [2], the sensitivity was not sufficient for pharmaco-

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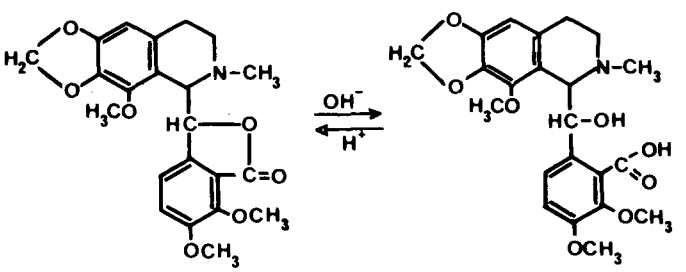
kinetic studies. The present paper gives a liquid chromatographic method for the determination of plasma levels of noscapine suitable for this purpose. Noscapine and the internal standard, papaverine, are extracted from plasma samples by column extraction using methylene chloride—butanol (19:1) as organic phase. The crude extract is evaporated and partly purified before injection into a straight-phase liquid chromatographic column. A high detection selectivity and sensitivity have been obtained by photometric detection of the eluate at 310 nm. Using 2.00 ml of plasma for the analysis, levels of 5 ng/ml can be determined with a precision better than 10% S.D.

The metabolism of noscapine has not been established in detail [3—5]. To confirm that there were no interferences from metabolites, the selectivity of the present method has been evaluated by comparing analytical results from extracts of plasma samples after isolation on reversed- and straight-phase liquid chromatographic systems.

Depending on the pH, the lactone ring on the noscapine molecule can be either opened (noscapine acid) or closed (noscapine), and the equilibrium between these two forms (Fig. 1) has been studied in detail. The apparent first-order rate constants for the process have been determined as well as the equilibrium constants. In buffer solutions the relative amounts of noscapine/noscapine acid are close to unity at physiological pH and increase with decreasing pH. However, in blood and plasma samples no transformation of noscapine into noscapine acid occurred.



Noscapine Noscapine acid

Fig. 1. The equilibrium process between noscapine and noscapine acid.

EXPERIMENTAL

Apparatus

The pump was an LDC 711 solvent delivery system. The chromatographic detector was an LDC Spectromonitor III having a 10.0 mm pathlength and a cell volume of 8 μl. Unless otherwise stated the absorbance of the eluate was measured at 310 nm. A Rheodyne Model 71-20 injection valve with a sample loop of 100 μl or a Waters Model U6K Universal injector was used.

The straight-phase column (250 mm × 4 mm I.D.) was a Hibar® LiChrosorb Si 60, 5 μm (E. Merck, Darmstadt, G.F.R.). The reversed-phase column (150 mm × 4.6 mm I.D.) was home-packed with Spherisorb S 5 ODS (Phase Separations, Queensferry, Great Britain).

The extractions were performed on glass columns (20 mm I.D.) packed with Extrelut® (E. Merck). The Extrelut was purified in a large column (1000 mm × 90 mm I.D.) by ethanol and dried at 100°C.

Chemicals

Noscapine (Bios Courtelier, Brussels, Belgium) and papaverine (E. Merck) were used as bases. 1-Pentanesulfonic acid sodium (Eastman-Kodak, Rochester, NY, U.S.A.) and human albumin (Behring Institut, Behringwerke AG, Marburg, G.F.R.) were used as received. Toluene, methanol and *n*-hexane were of LiChrosolv quality (E. Merck); all other chemicals were of analytical grade and used without further purification.

Mobile phase

The mobile phase for the straight-phase system consisted of hexane—methanol—chloroform—diethylamine (86.5:10.1:3.4:0.034, v/v), which was freshly prepared every day. For the reversed-phase system the mobile phase was composed of 0.005 *M* pentanesulfonic acid in a mixture of methanol—water—acetic acid—triethylamine (40:53:6:1, v/v). The flow-rate was 1.0 ml/min for both systems.

Determination of partition coefficients

The partition experiments were performed in centrifuge tubes by mechanical shaking at 25 ± 1°C (equilibration time 15 min), using equal volumes of organic and aqueous phases. Noscapine and papaverine were initially dissolved in the organic phases (previously equilibrated with the aqueous phase). The concentration of the drugs in the organic phase was determined by straight-phase liquid chromatography after evaporation of an aliquot of the organic phase to dryness with nitrogen and redissolving the residue in the same volume of the mobile phase. The concentration in the aqueous phase was calculated as the difference between the initial concentration in the organic phase and the concentration in the organic phase found at the equilibrium stage.

Determination of apparent first-order rate constants for hydrolysis and lactonization in buffer solutions

The hydrolysis of noscapine was studied at 25.0 ± 0.1°C by mixing 2.00 ml of noscapine (78 μg/ml) in 0.1 *M* phosphoric acid and 20.0 ml of the appropriate buffer (μ = 1.0). The decrease of noscapine and the increase of noscapine acid were monitored by reversed-phase liquid chromatography with photometric detection at 280 nm until the equilibrium stage was reached. Before injection into the liquid chromatograph the buffer solutions were mixed with an equal volume of mobile phase.

The lactonization was studied as described above by mixing 4.00 ml of noscapine acid (39 μg/ml) in 0.1 *M* sodium hydroxide and 20.0 ml of the appropriate buffer (μ = 0.1).

Determination of the degree of hydrolysis and lactonization in blood and 5% (w/v) albumin solution

The degrees of hydrolysis and lactonization were studied at 37.0 ± 0.1°C in

100 ml of blood (containing sodium heparin), 100 ml of 5% (w/v) human serum albumin or 100 ml of phosphate buffer pH 7.4 (μ = 1.0) by adding 14.9 μg of noscapine in 40 μl of 0.1 *M* phosphoric acid or 14.9 μg of noscapine acid in 400 μl of 0.1 *M* sodium hydroxide, respectively.

The total amount of noscapine and noscapine acid was determined by transformation of noscapine acid to noscapine by mixing the sample (2.00 ml) with 1.00 ml of 0.4 *M* phosphoric acid, giving a pH of 2.6, and leaving the solution overnight at room temperature. The solution was analysed according to the analytical method referring to the concentration of noscapine. The amount of noscapine was determined according to the analytical method; however, toluene–butanol (19:1) was used in the initial extraction, since the extraction of noscapine acid was negligible (< 5%) into this organic phase. The amount of noscapine acid was calculated as the difference between the total amount of noscapine and noscapine acid and the amount of noscapine alone.

Analytical method

To the plasma sample (2.00 ml) were added 240 ng of papaverine (internal standard) and 5.0 ml of phosphate buffer pH 4.0 (μ = 1.0). The mixture was poured into the column (packed with 4.0 g of silica) and was allowed to soak for 10 min. Noscapine and papaverine were eluted with 20 ml of methylene chloride–butanol (19:1). The eluate was evaporated to dryness under nitrogen flow at 50°C. The residue was dissolved in 1.0 ml of 0.1 *M* phosphoric acid, extracted with 1 ml of heptane (discarded), neutralized with 1 ml of phosphate buffer pH 6 (μ = 1.0) and extracted with 2.0 ml of toluene–butanol (19:1). After centrifugation the toluene phase was evaporated to dryness with nitrogen flow at 50°C. The residue from the extraction procedure was redissolved in 200 μl of the mobile phase and 50–100 μl were injected into the chromatographic column.

A calibration curve was obtained by analysing five standard samples containing 2.00 ml of blank plasma, 240 ng of internal standard and spiked with noscapine in the concentration range 6–400 ng/ml.

RESULTS AND DISCUSSION

The following symbols are used:

$[\ ]_{org}$, $[\ ]$ = concentrations of ions and molecules in organic and aqueous phase, respectively;

C_{org}, C_{aq} = total concentrations in organic and aqueous phase, respectively;

$k'_{HA} = \frac{a_H + [A]}{[HA^+]}$ = apparent acid dissociation constant of HA^+;

$k_d = \frac{[A]_{org}}{[A]}$ = partition coefficient of A;

$D_A = \frac{C_{A\ org}}{C_{A\ aq}}$ = partition ratio of A.

Extraction

The partition coefficients of noscapine and papaverine were studied using heptane, methylene chloride—butanol (19:1) and toluene—butanol (19:1) as the organic phases and phosphate buffers as the aqueous phases. Constants were evaluated graphically from the following equation by means of the linear plot of D_A^{-1} versus a_{H^+} (ref. 6)

$$\frac{1}{D_A} = \frac{1}{k_d} + \frac{a_{H^+}}{k_d \cdot k'_{HA}}$$

The products $k_d \cdot k'_{HA}$ were calculated from the slopes (Table I). In the analytical method methylene chloride—butanol (19:1) was preferred as initial extraction solvent because of its good extraction properties even at low pH and its high volatility, facilitating subsequent evaporation. Quantitative extraction (> 99%) of noscapine and papaverine into methylene chloride—butanol (19:1) was possible from aqueous buffer solutions pH > 3.8 using equal phase volumes. Extraction of plasma (diluted three times with phosphate buffer, giving a pH of 4.7) with methylene chloride—butanol (19:1), however, produced protein precipitation which reduced the amount of organic phase available for transfer. This fact may explain the low extraction yield (about 80%) obtained in batch extraction experiments from diluted plasma samples.

TABLE I

PARTITION COEFFICIENTS FOR NOSCAPINE AND PAPAVERINE

Aqueous phase: phosphate buffer, μ = 0.1. Temperature: 25°C.

Organic phase	$-\log k_d \cdot k'_{HA}$ ± S.E.	
	Noscapine*	Papaverine**
Methylene chloride—butanol (19:1)	1.43 ± 0.08	1.82 ± 0.04
Toluene—butanol (19:1)	3.02 ± 0.02	3.56 ± 0.02
Heptane	5.24 ± 0.01	5.58 ± 0.01

*pk'_{HA} = 6.8 (ref. 8).
**pk'_{HA} = 6.4 (ref. 11).

To improve the reproducibility and also the extraction yield, column extraction has been used in the present method for quantitative transfer of noscapine and papaverine from buffered plasma (pH 4.7) into the organic phase. The drugs are eluted within the first 15 ml of the organic extractant passing through the column.

After the initial extraction and evaporation of the organic phase, noscapine and papaverine were redissolved in phosphoric acid. Coextracted lipophilic compounds were removed by extraction with heptane. By extraction of the drug and internal standard into toluene—butanol (19:1) the amount of hydrophilic impurities was reduced to a large extent.

Liquid chromatography

Noscapine and papaverine were easily separated on both straight- and reversed-phase systems. The capacity factor (k') for the straight-phase system was 2.8 for noscapine and 3.8 for papaverine, and for the reversed-phase system 3.0 and 3.9, respectively. The straight-phase column had about 9100 theoretical plates and the reversed-phase column about 1200. Moreover, the molar absorptivity of noscapine in the mobile phase suitable for the straight-phase system was about 30% higher than in the mobile phase used in the reversed-phase system. Calculations based on equations given in ref. 7 showed that the straight-phase system should give about three times higher sensitivity than the reversed-phase system (Figs. 2 and 3), and hence the straight-phase system was preferred in the analytical method.

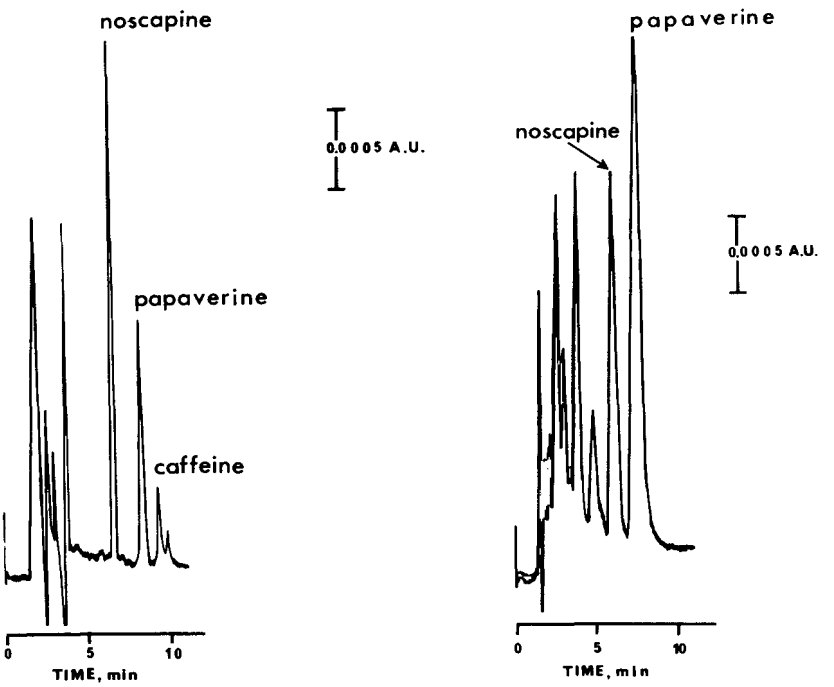


Fig. 2. Straight-phase liquid chromatogram of a plasma sample containing 197 ng/ml noscapine and 240 ng of papaverine. The noscapine peak corresponds to 49 ng.

Fig. 3. Reversed-phase liquid chromatogram from the same plasma sample that is shown in Fig. 2. The noscapine peak corresponds to 98 ng.

When the straight-phase system was used for separation of plasma extracts, the capacity factor (k') for caffeine, which eluted behind papaverine, decreased continuously. To avoid interference from the caffeine peak it was necessary to regenerate the column after about 150 injections of plasma extracts, by running about 100 ml of water through the chromatographic system. By this procedure the retention time for caffeine returned to its original value. With

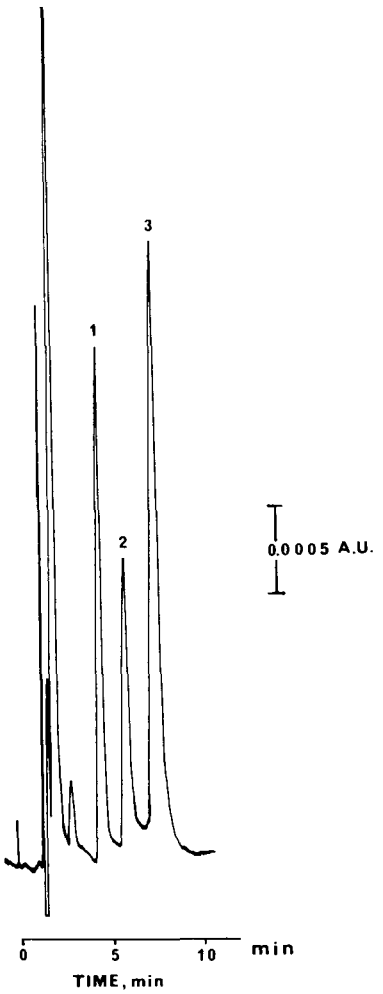


Fig. 4. Reversed-phase liquid chromatogram with chromatographic detection at 280 nm. Injected amounts: 186 ng of noscapine acid (peak 1), 149 ng of noscapine (peak 2), and 119 ng of papaverine (peak 3).

this handling the columns have been used for at least 1500 injections without any signs of deterioration.

Simultaneous determination of noscapine acid, noscapine and papaverine was possible only on the reversed-phase system (Fig. 4). This system was therefore used in the studies of hydrolysis and lactonization of noscapine and noscapine acid, respectively, in buffer solutions.

Internal standard

Papaverine is a suitable internal standard since it eluted just after noscapine in the chromatographic systems used. Papaverine has, like noscapine, a high UV absorbance at 310 nm which enables a high detection selectivity. The extraction properties of papaverine and noscapine are also very similar (Table I).

Hydrolysis and lactonization in aqueous solution

A pH-dependent equilibrium between noscapine and noscapine acid in aqueous solutions (Fig. 1) has previously been described [8]. The apparent first-order rate constants (k) for hydrolysis of noscapine and lactonization of noscapine acid in buffer solutions are given in Table II. The relative amounts of noscapine/noscapine acid at the equilibrium stage at different pH are given in Fig. 5. The data obtained with the selective analytical procedure used in the present paper, with concomitant determination of noscapine and noscapine acid, were of the same magnitude that Pawelczyk and Zajac [8] found by the less-selective photometric technique.

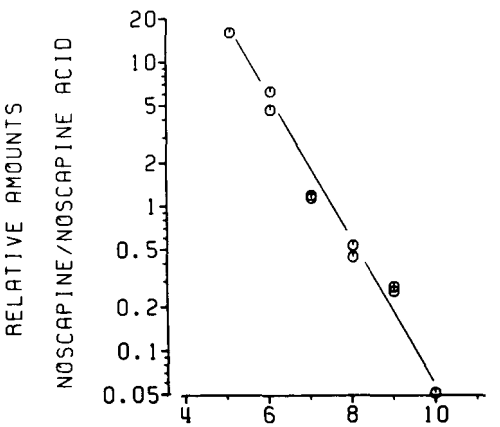


Fig. 5. The relative amounts of noscapine/noscapine acid at equilibrium in buffer solutions at different pH. Temperature 25.0 ± 0.1°C.

TABLE II

APPARENT FIRST-ORDER RATE CONSTANTS (k) FOR THE EQUILIBRATED HYDROLYSIS OF NOSCAPINE AND THE LACTONIZATION OF NOSCAPINE ACID IN BUFFER SOLUTIONS (μ = 1.0) AT DIFFERENT pH

Temperature: 25.0 ± 0.1°C.

pH	$-(k \pm$ S.E.$) \cdot 10^2$ (h^{-1})	
	Hydrolysis	Lactonization
2.0	—	156 ± 7
2.6	—	61 ± 2
3.0	—	29.8 ± 0.3
4.0	—	8.8 ± 0.1
5.0	—	9.5 ± 0.5
6.0	8.5 ± 0.3	8.0 ± 0.3
7.0	13.1 ± 0.3	11.9 ± 0.1
8.0	16.5 ± 0.6	17.3 ± 0.7
9.0	18 ± 1	16.3 ± 0.8
10.0	23.0 ± 0.4	—

Hydrolysis and lactonization in blood and 5% (w/v) albumin solution

The results from the study of the equilibrium between noscapine and noscapine acid in buffer solution indicate that both forms may exist in blood and plasma samples. However, noscapine in blood or 5% (w/v) albumin solution was not transformed into noscapine acid within 24 h. Addition of noscapine acid to blood and 5% (w/v) albumin solution gave an almost complete transformation of noscapine acid to noscapine within 24 h (Fig. 6). The difference in equilibrium stage between buffer, blood and albumin solution may depend on the strong binding of noscapine to proteins. Plasma samples from three patients collected 0–6 h after the intake of 150 mg of noscapine were analyzed for the total amount of noscapine and noscapine acid as well as for noscapine only. The mean value of the quotients (noscapine + noscapine acid)/noscapine was 1.01 with a standard deviation of 0.102 (n = 24). Hence, it can be concluded that it is unlikely that noscapine acid is a metabolite of noscapine in man.

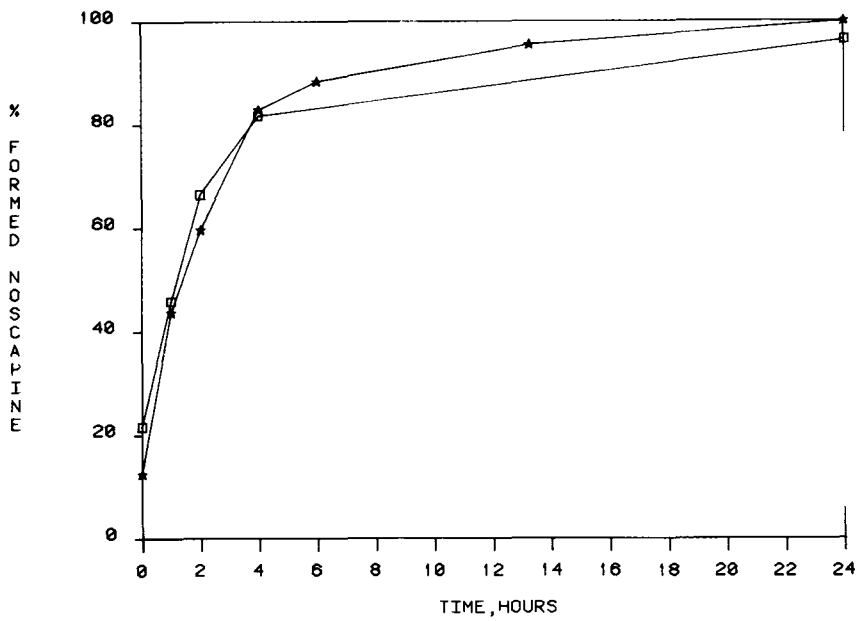


Fig. 6. Transformation of noscapine acid to noscapine in blood (□) and in 5% (w/v) albumin solution (★).

Selectivity of the analytical method

A high detection selectivity towards endogenous compounds and also towards noscapine acid was obtained in the present method using a detector measuring at 310 nm (Fig. 7) (cf. ref. 9). The selectivity of the proposed analytical method against other possible metabolites than noscapine acid was evaluated by comparing the analytical results of noscapine from plasma extracts with reversed- and straight-phase systems. The analytical results from the reversed-phase system/straight-phase system were plotted against the analytical results from the straight-phase system (Fig. 8) [10]. The plot (n =

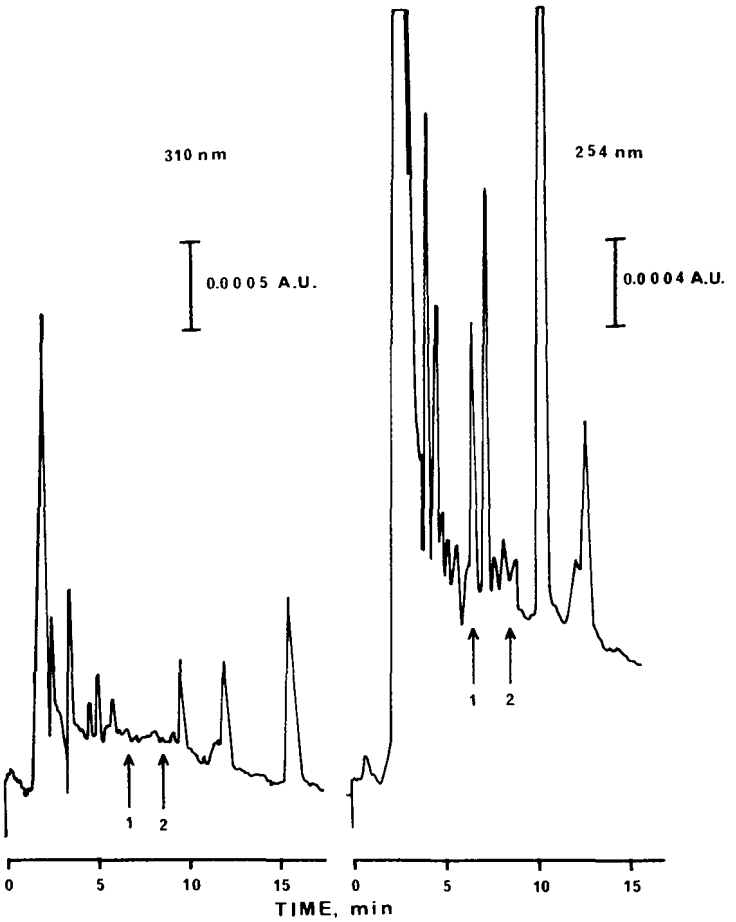


Fig. 7. Detection selectivity. Blank plasma samples were analyzed according to the proposed analytical method with two detectors coupled in series and detection wavelengths of 310 nm and 254 nm, respectively. 1 and 2 indicate the retention times of noscapine and papaverine, respectively.

TABLE III

PRECISION OF THE TWO LIQUID CHROMATOGRAPHIC SYSTEMS AT DIFFERENT LEVELS

Drug level (ng/ml)	Standard deviation (%)	
	Straight-phase	Reversed-phase
89	3.8	6.6
18	7.3	9.3
5.9	9.5	—*

*Signal-to-noise ratio < 2.

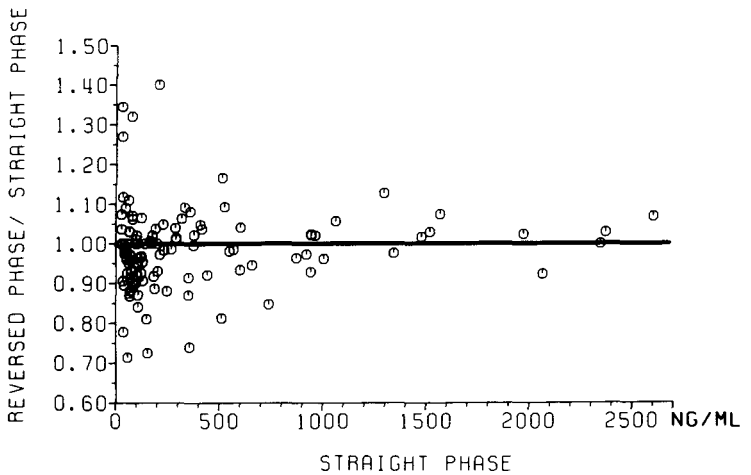


Fig. 8. Comparison of plasma levels of noscapine from the analytical results of the reversed-phase and straight-phase systems.

111) gives a mean value of 0.99 and a standard deviation of 10.6% for plasma levels > 25 ng/ml, i.e. essentially the same results were obtained by the two methods, which implies no codetermination of metabolites in any of the methods.

Recovery, precision and sensitivity

Quantitation was made by construction of a calibration curve by plotting the peak height ratios of noscapine/papaverine against the concentration of noscapine. The linear calibration curve had a correlation coefficient of 0.9999, a slope of $(1.015 \pm 0.001) \cdot 10^{-2}$ and an intercept of 0.02 ± 0.03.

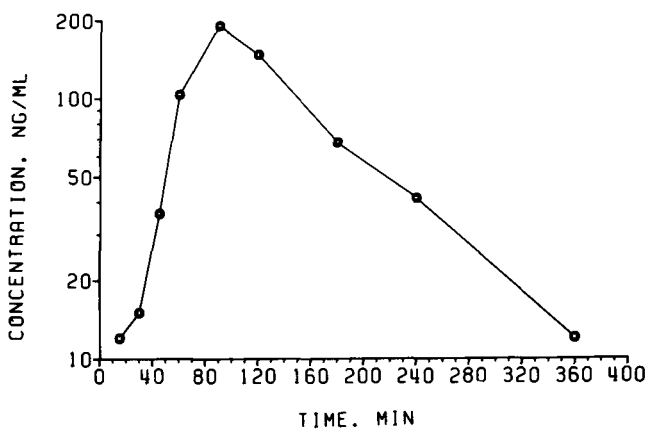


Fig. 9. Plasma levels of noscapine after an oral dose of 150 mg of noscapine.

The absolute recovery from blank plasma samples spiked with 92 ng/ml noscapine, obtained by using aliquots throughout the method, was 81% compared with direct injection of noscapine dissolved in mobile phase.

The precision of the proposed methods is presented in Table III. Using 2.00 ml of plasma for the analysis, the lower limit for determination was 5 ng/ml for the straight-phase system and 15 ng/ml for the reversed-phase system.

Application to biological samples

A chromatogram from 2.00 ml of plasma containing 197 ng/ml noscapine and 240 ng of papaverine is given in Fig. 2. The same plasma sample chromatographed with the reversed-phase liquid chromatographic system is shown in Fig. 3. The plasma levels after an oral dose of 150 mg of noscapine are presented in Fig. 9.

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CHROMBIO. 1693

RAPID HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE MEASUREMENT OF ATENOLOL IN PLASMA USING UV DETECTION

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SUMMARY

A rapid, selective and reproducible high-performance liquid chromatographic method has been developed for the measurement of the β-adrenoceptor blocking drug atenolol in small (400 μl) volumes of plasma. Following solid phase sample preparation using Bond-Elut™ mini-columns the compound is separated by high-performance column liquid chromatography on a microparticulate (6 μm) cyano column using acetonitrile—ammonium dihydrogen phosphate (4:96) containing triethylamine (0.25%, v/v) as the mobile phase, and the absorption of the column effluent is monitored at 224 nm. The practical limit of quantitation, based upon an assay volume of 400 μl, is 25 ng/ml for atenolol. The average coefficient of variation is 3.1%.

INTRODUCTION

Atenolol, 4-(2-hydroxy-3-isopropylaminopropoxy)-phenylacetamide, is a selective β_1-adrenoceptor antagonist recently approved in the United States for the treatment of hypertension [1, 2]. Studies in animals and humans suggest that this drug is free of intrinsic sympathomimetic activity and is devoid of significant membrane-stabilizing activity [3—6].

Previous procedures for the determination of atenolol in plasma and urine employed gas—liquid chromatography (GLC) with electron-capture detection [7, 8], or high-performance liquid chromatography (HPLC) with spectrophotofluorimetry [9—12] or spectrophotometry [13]. The GLC determination of atenolol is specific and sensitive to 10 ng/ml, but is relatively complex and requires lengthy prederivatization steps. The spectrophotofluorimetric method is relatively sensitive but is of questionable specificity and employs an elaborate extraction procedure with internal standards procainamide or metoprolol, drugs which might be administered in combination with atenolol.

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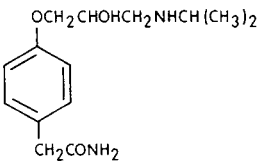
The spectrophotometric method lacks sensitivity at the wavelength chosen requiring a large volume of plasma for the assay [13].

The procedure reported here for the measurement of plasma atenolol concentrations is based upon the principle of rapid sample preparation with Bond-Elut™ columns of a relatively small plasma volume (400 μl), followed by the separative capability of HPLC and the sensitivity of UV detection.

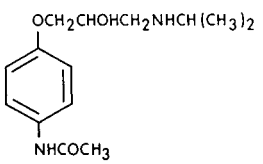
EXPERIMENTAL

Materials and reagents

Atenolol and the internal standard practolol (Fig. 1) were supplied by Stuart Pharmaceuticals (Wilmington, DE, U.S.A.) and by ICI Pharmaceuticals (Macclesfield, Great Britain), respectively. Ammonium dihydrogen phosphate and triethylamine were purchased from Aldrich Chemicals (Milwaukee, WI, U.S.A.). Acetonitrile, acetone and methanol were of HPLC grade and obtained from Fisher Scientific (Fairlawn, NJ, U.S.A.). Bond-Elut™ CN columns (column capacity 1 ml) and Vac-Elut manifold were purchased from Analytichem International (Harbor City, CA, U.S.A.).



ATENOLOL



PRACTOLOL

Fig. 1. Structures of atenolol and practolol.

High-performance column liquid chromatography

The solvent delivery system is a constant-flow reciprocating Constametric III pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.). The analytical column is a prepacked (25 cm × 4.6 mm I.D.) stainless-steel column containing Zorbax™ CN (6 μm) polar bonded-phase packing used in the reversed-phase mode (DuPont, Wilmington, DE, U.S.A.). A six-port rotary valve injector (Model 7125, Rheodyne, Berkeley, CA, U.S.A.) with a 50-μl sample loop is used for sample injection. A cyano guard column cartridge (Brownlee Labs., Santa Clara, CA, U.S.A.) is used between the injector and the analytical column which effectively minimizes the accumulation of particulate matter on the analytical column. The mobile phase is acetonitrile—0.0125 *M* ammonium dihydrogen phosphate—triethylamine (4:96:0.25, v/v), the pH being adjusted to 5.5 with 1.0 *M* phosphoric acid. The solvent flow-rate is 1.5 ml/

min with a column inlet pressure of 103.4 bars. The eluate is monitored continuously for absorbance at 224 nm using a variable-wavelength Spectromonitor III (Laboratory Data Control) and the detector output is displayed on a Linear Instruments Model 858 dual-pen recorder (Irvine, CA, U.S.A.).

Bond Elut™ clean-up procedure

Sample preparation is done with Bond-Elut™ columns containing a sorbent which has been modified by covalently bonding cyanopropyl functional groups to the surface. The Bond-Elut column is positioned in the luer fittings in the Vac-Elut cover. Ten columns can be used at a time. A vacuum of 25—50 cm Hg is applied to the manifold to elute the different washes. The column is activated by passing 2 ml of methanol from a squeeze bottle followed by 2 ml of distilled water. The vacuum is turned off as soon as the water has run through to prevent the tubes from drying out. A 400-μl aliquot of patient's plasma containing the internal standard practolol in a concentration of 500 ng/ml is added to the column. For the calibration curve, pooled plasma containing the appropriate concentration of atenolol and the same concentration of practolol is used. The vacuum is turned on and the sample is drawn into the column. Each column is then filled twice with distilled water from a squeeze bottle, allowing columns to empty between fillings. This is followed by one wash with acetone. Following the last wash the vacuum is maintained until the column is dry. The cover of the manifold is then removed and the stainless-steel needles of the Vac-Elut cover are wiped with a tissue to remove drops of washing solution. Appropriately labelled collection tubes are positioned under each column. A 200-μl aliquot of eluting solvent (10 m*M* acetic acid, 50 m*M* triethylamine in methanol) is added to the column, and the vacuum is renewed. The suction is continued while two more 200-μl aliquots of the eluting solvent are added. The vacuum is turned off and combined eluents are collected. The combined eluents are evaporated under nitrogen, suspended in 80 μl of mobile phase and 50 μl are injected onto the analytical column.

Instrument calibration

Standard solutions containing atenolol at concentrations of 25, 50, 100, 250, 500, 750 and 1000 ng/ml were prepared in heparinised drug-free pooled plasma by dilution of a 1 mg/ml solution of this compound in methanol. The plasma and methanolic solutions were stable for at least one month, if stored at −20°C and in the absence of light. The amounts of drug in the unknown samples were calculated from their peak heights relative to the internal standard by linear regression using the reciprocal of the concentration ($1/C$) as the weighting factor [14]. To quantitate the lower concentrations with better relative accuracy it was necessary to employ $1/C$ as the weighting factor rather than unit weighting which is generally used in linear regression analysis. As an alternative to weighted linear regression two standard curves could be used, one covering the lower range (25—500 ng/ml), another covering the higher range (500—1000 ng/ml) of concentrations. The advantage in choosing the former method lies in its convenience, and the simplicity of having one equation to define the entire concentration range.

The reproducibility of the method was investigated by taking the coefficient of variation of five curves over the entire range of 25—1000 ng/ml. This was calculated from the standard deviation of the values divided by the mean peak height ratio (atenolol/internal standard) for each concentration. Standard curves based upon peak height of drug to the peak height of the internal standard were linearly related to concentration with the correlation coefficient being consistently greater than 0.998.

RESULTS AND DISCUSSION

Choice of separation

GLC with electron-capture detection [7, 8] was previously employed for determination of atenolol in plasma. Although the GLC methods are highly selective and sensitive they require lengthy derivatization steps.

Due to the low volatility of atenolol, HPLC was evaluated as an alternative method of resolving the compound prior to detection. The retention behavior

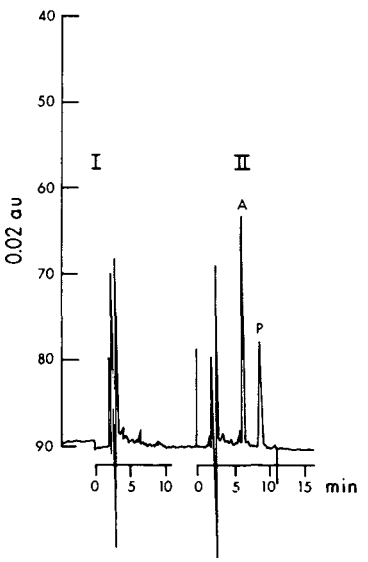


Fig. 2. High-performance liquid chromatograms of extracts from plasma. (I) Control plasma; (II) plasma containing 250 ng/ml of atenolol (A) and internal standard, practolol (P).

of atenolol was compared on three different column sorbents, namely CN (normal- and reversed-phase), C_{18} (reversed-phase) and silica gel absorption columns. Of the microparticulate columns tested the Zorbax CN column (in the reversed-phase mode) exhibited the greatest separation selectivity. The capacity factor (k') values of atenolol and the internal standard practolol were 2.0 and 3.3, respectively. The chromatographic separation using this method was excellent as can be seen from the representive chromatograms of these compounds (Fig. 2). Furthermore the chromatographic time is less than 10 min.

Choice of HPLC detection mechanism

The two most widely used forms of sample detection of HPLC effluents are UV absorption and fluorimetric methods. Electrochemical detection techniques are now being applied to electrochemically active drugs. UV, fluorimetric and electrochemical scans were obtained on atenolol (Fig. 3). Spectral scans were performed by preparing a 250 μM solution of atenolol in the mobile phase. In the case of the UV scan a Beckman Spectrophotometer UV 5230 (Beckman Instruments, Irvine, CA, U.S.A.) was used at a scan speed of 1 nm/sec and chart speed of 10 nm/in. Fluorimetric scans were performed on an Amicon SPF-500 (American Instrument Company, Silverspring, MD, U.S.A.) at a scan speed of 250 nm/sec. The electrochemical scan was performed using a CV-1B cyclic voltammetric apparatus (Bioanalytical Systems, Lafayette, IN, U.S.A.) using a glassy carbon electrode and Ag/AgCl reference electrode at a scan rate of 250 nV/sec. In all situations a blank spectrum on the mobile phase was obtained.

The molar absorptivity at 224 nm (Fig. 3A) is sufficiently intense to permit atenolol detection in a small volume of plasma (400 μl). The drug was found to have intrinsic fluorescence (Fig. 3B) and previous methods have employed spectrophotofluorimetric methods to determine atenolol in plasma [9—11]. Our method, with a smaller sample volume, was able to obtain comparable sensitivity after a much simpler and more specific extraction procedure with UV detection. Since UV detection is the most widely used detection system our method can be widely applied without the need for a spectrophotofluorimeter. Atenolol was found to have no electrochemical activity, and this method of detection is therefore not applicable.

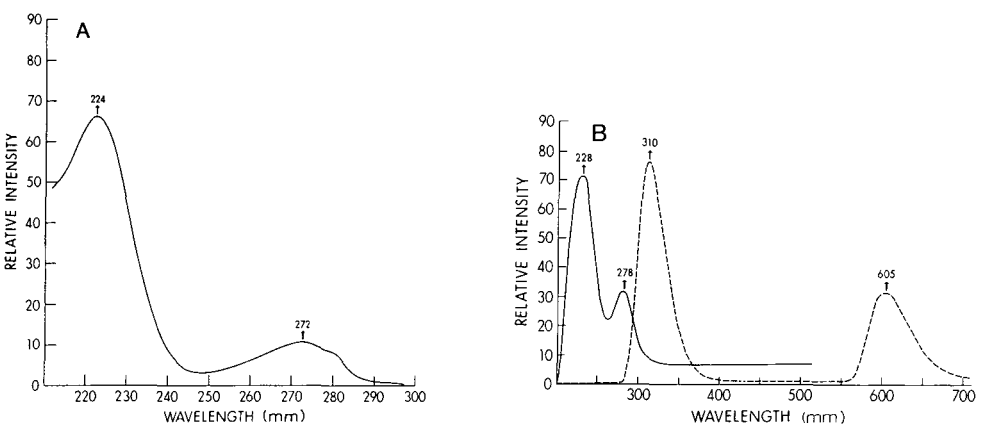


Fig. 3. Spectral scans of atenolol (for conditions see text). (A) Absorption spectrum of atenolol (UV); and (B) excitation (——) and emission (— — —) spectra of atenolol (fluorescence).

Effect of the solvent strength, pH and triethylamine content of the mobile phase on solute retention

The capacity factor (k') of atenolol and practolol was found to be a function of the acetonitrile and triethylamine content of the mobile phase. Changing pH of the mobile phase also substantially altered the retention

time of the drugs. Increasing the solvent strength of the mobile phase by increasing the acetonitrile content produced a concentration-dependent decrease in retention of both atenolol and practolol. The addition of triethylamine to the mobile phase produced a concentration-dependent decrease in the retention of the compounds and sharpened the peaks. These effects most probably result from the ability of triethylamine to cap the free acidic silanol groups on the silica surface. Increasing pH increased the relative retention of atenolol.

Sample preparation procedure

Bond-Elut is a family of disposable, solid-phase sample preparation columns that can process samples in a fraction of the time of traditional methods with good selectivity and reproducibility. The cyanopropyl sorbent was chosen after comparison with a reversed-phase (octadecyl) sorbent. The CN sorbent exhibited greater separation specificity than the hydrophobic phase for the extraction of atenolol. The saving in time for the analysis using Bond-Elut columns is the most important favorable factor for this method. Typically, ten samples can be processed in 5 min, ready for instrumental analysis. Since elution solvent volume is low, evaporation presents little or no problem and selective elution procedures yield final samples with fewer endogenous impurities than are usually obtained in liquid–liquid extraction methods.

Interfering substances

Several drugs which are often concurrently administered to cardiac patients were also examined for their possible interference with the quantification of atenolol (Table I). None of the drugs tested interfered in the assay.

TABLE I

CAPACITY FACTOR (k') OF DRUGS OFTEN ADMINISTERED CONCURRENTLY WITH ATENOLOL

Drug	k'
Atenolol	2.0
Practolol	3.3
Metoprolol	7.8
Quinidine	24.3
Procainamide	3.6
Disopyramide	8.0
Verapamil	3.5
Lidocaine	5.0
Timolol	6.5
Nadolol	4.0

Recovery

Under the extraction conditions chosen the absolute recovery of atenolol from heparinised human plasma was 105% and 107% at concentrations of 100 and 1000 ng/ml, respectively. These findings were obtained by direct comparison of the peak height ratios obtained on analysis of 50-μl portions of

standard to those obtained from spiked plasma extracts. However, the internal standard was added prior to the final analytical procedure to correct for any possible injection volume errors, partial evaporation or spillage, should these have occurred.

Accuracy, reproducibility and sensitivity

The combination of low detector noise following injections of plasma extracts, high extraction efficiencies and the high molar absorptivity (ϵ_{224}) permits the quantitation of atenolol in human plasma at low concentrations. The accuracy and precision of the present method were determined by assaying 400-μl aliquots of plasma containing 25, 50, 250, 750 and 1000 ng/ml of atenolol (Table II). The daily standard curves over a period of two months had an average coefficient of variation of 3.1% for atenolol (Table III). The limit of quantitation, defined as minimum signal-to-noise ratio of 4, is 25 ng/ml for atenolol; percentage difference from theoretical is 15% or less; and the coefficient of variation is less than 10%. The lower limit of detection however, was less than 10 ng/ml. These concentrations generally yielded a percentage difference from theoretical values greater than 15%. Therefore, 25 ng/ml is taken as the lower practical limit of quantitation.

TABLE II

PRECISION AND ACCURACY OF ATENOLOL MEASUREMENT BY HPLC

Atenolol added to plasma (ng/ml)	*n*	Mean of calculated atenolol concentrations (ng/ml)	S.D.	C.V. (%)	Percent difference from theoretical value
25	6	28.0	0.931	3.3	12.0
50	7	52.7	3.42	6.4	5.4
250	6	249	5.52	2.2	0.4
750	6	772	11.32	1.4	2.9
1000	6	1021	10.21	1.0	2.1

TABLE III

REPRODUCIBILITY OF STANDARDS OVER A PERIOD OF TWO MONTHS

n = 6 for each concentration.

Concentration (ng/ml)	Mean of peak height ratios	Standard deviation	Coefficient of variation (%)
25	0.0995	0.0027	2.7
50	0.1742	0.0100	5.8
100	0.2731	0.0203	7.4
250	0.7707	0.0170	2.2
500	1.4057	0.0176	1.2
750	2.349	0.0344	1.4
1000	3.103	0.0319	1.0
		Overall C.V. = 3.1%	

Analysis of five samples of each concentration of 25, 50, 100, 250, 500, 750 and 1000 ng/ml of atenolol gave a linear regression coefficient of 0.9986 for the line $Y = 0.0030258X + 0.014576$ where Y is the peak height ratio and X the concentration in ng/ml of plasma.

Practical application of the method

The current method was used to assess atenolol concentrations and β-blocking effect in subjects participating in an exercise conditioning program. Nine healthy adults (six men and three women), mean age 29 years (range 28—37 years) and mean weight 76 kg (range 53—94 kg) took atenolol, 100 mg once daily (morning) for two months. Symptom-limited maximal exercise tolerance tests were performed 7—11 h after drug ingestion five days after beginning therapy and before discontinuing therapy to assess drug effect. Blood samples were taken immediately prior to treadmill exercise. During the two-month treatment period the subjects participated in an aerobic exercise program (minimum 45 min, four times per week) designed to improve their physical fitness. A mean improvement of 22% increase in work performed to exhaustion was noted. Relevant data concerning drug effect and drug levels are shown in Table IV. The data indicate that neither chronic versus acute dosing (two months versus five days), nor physical conditioning appear to affect the degree of β-blockade or drug level obtained with atenolol.

TABLE IV

CONCENTRATION—TIME EFFECT DATA FOR ATENOLOL BEFORE AND AFTER TWO MONTHS DOSING DURING A PHYSICAL TRAINING PROGRAM

	Atenolol level (ng/ml)			Heart rate suppression			Time of treadmill test after dose (h)		
	Mean	S.D.	Range	Mean	S.D.	Range	Mean	S.D.	Range
Before	254	71	140—367	29.8	13.2	13—43	8.9	1.6	7—11
After	263	65	120—334	28.8	11.4	14—55	8.9	1.1	7—11

CONCLUSIONS

The method described here has been found to be useful for the measurement of plasma atenolol concentration during exercise studies and may prove useful in single-dose pharmacokinetic studies. Small sample volume combined with short chromatographic time and minimal source of interference has been achieved in the method described here which thus has considerable advantages over previously published methods.

ACKNOWLEDGEMENTS

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CHROMBIO. 1694

THE ASSAY OF A NOVEL HISTAMINE H_2-RECEPTOR ANTAGONIST, SK&F 93479, IN HUMAN PLASMA BY NORMAL-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A selective assay of a new histamine H_2-receptor antagonist, SK&F 93479, in human plasma has been developed. The method uses liquid—liquid extraction from the biological sample and analysis of the resulting extract by normal-phase high-performance liquid chromatography with UV detection for quantitation of the drug and an added standard. The assay is sufficiently accurate and precise to determine the compound at concentrations as low as 0.025 mg l^{-1}. The coefficient of variation of the assay averages 5.7% at concentrations between 0.1 and 2.0 mg l^{-1}, but increases to 21.8% at 0.02 mg l^{-1}. SK&F 93479 can be determined in spiked plasma samples, at concentrations between 0.05 and 0.80 mg l^{-1} with a bias of between —7.5 and +3.6%, but at 0.02 mg l^{-1} concentrations were underestimated by 15% on average. The assay has been used for pharmacokinetic and bioavailability studies: after a single 0.5 mg kg^{-1} oral dose in man, plasma concentrations can be monitored for up to 70 h after dosing.

INTRODUCTION

SK&F 93479, 2-{2-[5-(dimethylaminomethyl)furan-2-ylmethylthio]ethylamino}-5-(6-methylpyrid-3-ylmethyl)-4-pyrimidin-4-one (I in Fig. 1), is a novel, non-imidazole antagonist of histamine at H_2-receptor sites. This compound was considerably more potent than cimetidine (Tagamet®) as an inhibitor of histamine stimulated gastric acid output in experimental animals [1]. In clinical studies with healthy male subjects [2] SK&F 93479 was found to be up to 20 times more potent than cimetidine, on a molar basis, in reducing gastric acid output stimulated by a test meal. In addition, the maximum antisecretory effect of SK&F 93479 was maintained for longer periods than were seen with cimetidine.

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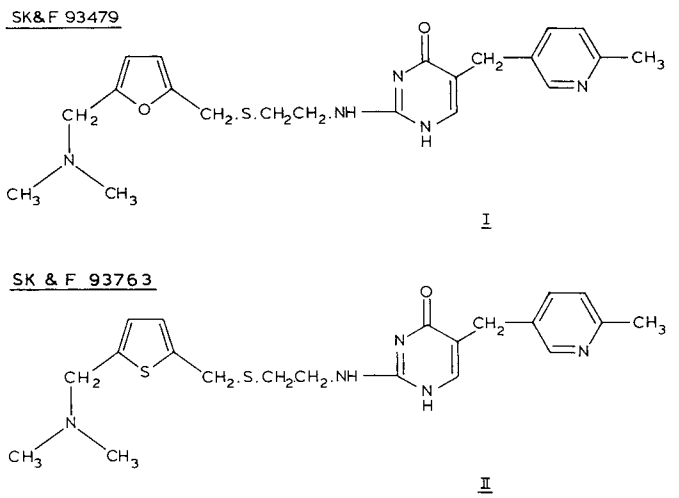

Fig. 1. Structures of SK&F 93479 and SK&F 93763 (internal standard).

Potent drugs, such as SK&F 93479, require a sensitive and specific assay to enable investigators to follow the kinetics over the time-course of pharmacological activity, which in turn permits an optimum dosage regimen to be devised.

Previous chromatography-based assays for histamine H_2-receptor antagonists have used a variety of organic solvents for extraction of the drugs and metabolites from biological fluids. Cimetidine has been extracted using ethyl acetate [3, 4], methylene chloride [5, 6] and octanol [7, 8], which has also been used to extract ranitidine [9] and oxmetidine [10]. The resulting extracts have then been subjected to high-performance liquid chromatography (HPLC) for separation of drug, metabolites and added (internal) standard. Normal-phase silica chromatography has been used for cimetidine assay [6–8] and oxmetidine assay [10]. Reversed-phase (octadecylsilyl-bonded silica) chromatography has been used in the analysis of cimetidine [3, 4] ranitidine [9] and oxmetidine [10] and cyano-bonded silica has been employed in a recently published assay for cimetidine [5].

This paper presents an analytical method for the determination of SK&F 93479 in human plasma by UV absorption after normal-phase HPLC of an extract. The extraction procedure, which precedes chromatography, is similar to that described by Lee and Osborne [7] for cimetidine. The validated method was used to assay plasma samples taken from healthy male subjects to whom the drug was administered.

EXPERIMENTAL

Materials

Analytical grade chemicals were used throughout this study with the following exceptions: 1-octanol (Koch-Light, Colnbrook, Great Britain; puriss), methanol HPLC grade and acetonitrile HPLC grade S from Rathburn (Walkerburn, Great Britain) and 0.88 sp. gr. ammonium hydroxide solution

(May and Baker, Dagenham, Great Britain; reagent grade). The water used was purified by deionisation, then distilled in an all-glass apparatus and stored in glass containers.

All solvents and solutions for HPLC were filtered through 0.5-μm membrane filters (Millipore type HA and FH for aqueous and organic solvents, respectively); prior to use the components of the solvent system, except the ammonia, were mixed and degassed by the application of reduced pressure. The ammonium hydroxide solution was then added.

Solutions of 1 mol/l carbonate buffer (pH 9) were prepared as follows; to 5 l of 1 mol/l sodium bicarbonate was added sufficient 1 mol/l sodium carbonate to adjust the pH value of the solution to 9.0.

The polypropylene centrifuge tubes (12 ml) and stoppers (Type 300 PP and 301 PT, respectively) were obtained from Henleys Medical Supplies (London, Great Britain).

A standard solution of SK&F 93479 (I, Fig. 1) was made by dissolving 1.263 mg of the trihydrochloride salt (equivalent to 1.000 mg base) in 100 ml ethanol; a solution containing 1.000 mg of SK&F 93763 (II, Fig. 1), 2-{2-[5-(dimethylaminomethyl)thien-2-ylmethylthio] ethylamino}-5-(6-methylpyrid-3-ylmethyl)-pyrimidin-4-one, dissolved in 100 ml ethanol was used as an internal standard. Both ethanolic solutions were stored at −20° C until used and were found to be stable for at least six months under these conditions.

Plasma samples

Blood from subjects who were receiving the drug was withdrawn by cannula or syringe into heparinised containers, mixed, centrifuged and the plasma obtained transferred to plain tubes, which were quick frozen over solid carbon dioxide and then kept at −20° C pending analysis.

Extraction of plasma samples

To 2 ml plasma in a polypropylene centrifuge tube (12 ml) were added 1 ml of 1 mol/l (pH 9) carbonate buffer, 50 μl ethanol containing 500 ng SK&F 93763 as an internal standard, and 5 ml 1-octanol. The tubes were stoppered, placed on a blood cell suspension mixer for 15 min, then centrifuged at 2500 *g* for 5 min, after which 4.5 ml of the organic layer were transferred to another tube containing 3 ml of 0.02 mol/l hydrochloric acid.

After stoppering, the tubes were re-extracted by the same technique and centrifuged to separate the two phases; the octanol was removed by aspiration and 2.5 ml of the acid layer were transferred to another centrifuge tube to which 200 μl acetonitrile were added.

The contents of each tube were mixed by vortex; about 5 g of anhydrous potassium carbonate were added and the contents were again mixed by vortex. The effect of this saturation with potassium carbonate was to salt out the acetonitrile, which was then separated cleanly by centrifugation for 2 min at 2500 *g* and transferred to glass vials, which were stored at −20° C to await chromatographic analysis.

Standard curve

The standard curve was prepared using 2-ml aliquots of drug-free human plasma which had been spiked in duplicate, with 0, 10, 20, 50, 100, 150 and

200 μl of the standard ethanolic solution of SK&F 93479, corresponding to 0, 0.05, 0.10, 0.25, 0.50, 0.75 and 1.00 mg l^{-1} of SK&F 93479 in plasma. These tubes were vortex mixed, allowed to equilibrate for 5 min and then extracted at the same time and by the same procedure as the test samples.

High-performance liquid chromatography

The chromatograph consisted of a Perkin-Elmer Series 3B pump and an LC-75 variable-wavelength UV detector with the wavelength and absorbance set at 220 nm and 0.02 a.u.f.s. respectively. The separation of analytes was achieved by a stainless-steel column 250 mm × 4.6 mm I.D. packed with 5-μm silica (Ultrasphere from Altex). The mobile phase consisted of acetonitrile—methanol—water—ammonium hydroxide 0.88 sp. gr. (200:80:10:1.5, v/v) and was pumped at a flow-rate of 2 ml min^{-1}.

Aliquots of the sample extracts (40 μl) were introduced into the chromatograph via either a Rheodyne 7125 valve or Waters WISP automatic injector. Under these conditions the retention times of SK&F 93479 and the internal standard were 3.2 and 2.4 min, respectively.

Quantitation of SK&F 93479 concentrations

The detector output was fed into a Perkin-Elmer Sigma 10 data station which integrated the areas of the peaks corresponding to SK&F 93479 and the internal standard. A plot of the SK&F 93479/internal standard peak area ratios versus drug concentration was drawn for the spiked standards by a program on a Hewlett-Packard 9825 desk top computer. A regression line was drawn through the points; using this regression another program calculated the concentration of SK&F 93479 in the test samples using the peak area ratios.

Radiochemical studies

The efficiency of the extraction process, for various concentrations of SK&F 93479 in plasma, was assessed by the use of [^{14}C]SK&F 93479, labelled in the C-2 position of the pyrimidone ring (Fig. 1) (radiochemical purity: 96.4%, specific activity: 97.1 μCi mg^{-1}). This material was used to spike drug-free human plasma over a concentration range of 0.025—1.00 mg l^{-1}. A 100-μl aliquot of the spiked plasma and the recovered acetonitrile layer from each sample were put into separate plastic scintillation vials together with 5 ml Picofluor scintillant and counted in a Searle Mk. III counter. From the radioactivity data obtained, the overall recovery of the method was calculated.

RESULTS AND DISCUSSION

Chromatography

Typical chromatograms observed after injection of extracts of human plasma are shown in Fig. 2. No endogenous compounds with retention times corresponding to that of SK&F 93479 have been encountered in the predose samples of volunteers who received the drug, nor in the drug-free human plasma used for validation of the assay. The unknown endogenous compounds, giving peaks at 5 min after injection, do not interfere with the automated assay if samples are injected at 4-min intervals.

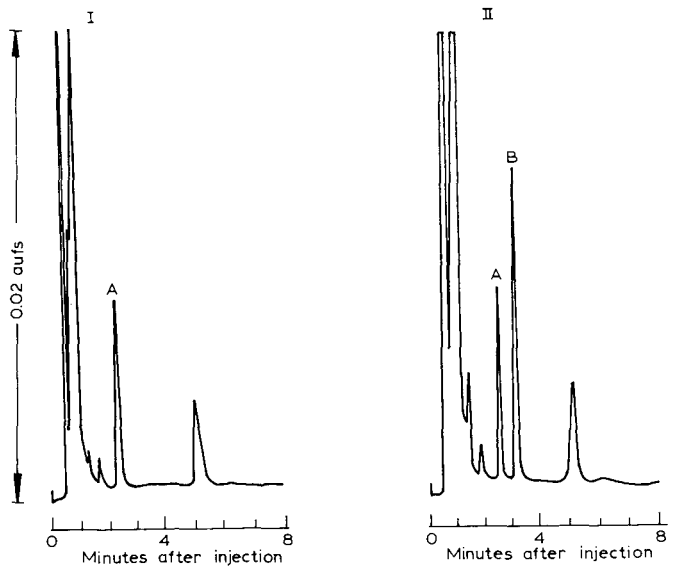

Fig. 2. Normal-phase chromatograms of extracted human plasma taken (I) before and (II) after the administration of SK&F 93479 to the subject. Peaks: A = internal standard, SK&F 93763; B = SK&F 93479.

Co-extraction of putative metabolites such as the N-oxide and S-oxide is unlikely to interfere with the assay since these compounds are considerably more polar than SK&F 93479 and thus would have longer retention times in the normal-phase chromatographic system used. However, if present, such metabolites would restrict the throughput of samples.

Recovery of SK&F 93479 from plasma

The results showing the recovery of [^{14}C]SK&F 93479 are presented in Table I; as can be seen the recovery of the drug in the range 0.025—1.00 mg l^{-1} averaged 29.1%. This fell to 18.6% at 0.025 mg l^{-1}. These low recoveries were partly the result of taking less than total volumes of the octanol and hydrochloric acid extracts;this reduced the possible recovery to 75% of maximum, so that losses due to adsorption or inefficient partition were about 60%. This contrasts with losses for oxmetidine by a similar process [10] of only 25%.

The probable reason for the less efficient extraction of SK&F 93479 at pH 9.0 is the pK_a value of 8.53 associated with this molecule, indicating a substantial ionisation at the selected pH. However, the other pK_a values for SK&F 93479 are 3.03, 6.11 and 10.20 which makes difficult the selection of an appropriate pH for extraction into octanol.

Under the conditions employed (2 ml plasma sample and injection of 40 μl of the final acetonitrile extract) the useful sensitivity of the assay was considered to be 0.025 mg l^{-1}.

TABLE I

RECOVERY OF [^{14}C]SK&F 93479 FROM SPIKED HUMAN PLASMA

In all cases n = 10.

Concentration of SK&F 93479 (mg l^{-1})	Percent mean recovery*	Standard deviation
0.025	18.6	1.67
0.050	19.5	2.19
0.10	23.0	3.41
0.25	27.7	2.16
0.50	31.1	4.34
1.00	28.5	2.95

*Calculated from $\frac{\text{d.p.m. in total recovered acetonitrile} \times 100}{\text{d.p.m. in plasma sample extracted}}$. Having regard to the volumes of octanol and hydrochloric acid taken, the maximum possible recovery was 75%.

TABLE II

BETWEEN-DAY PRECISION OF SK&F 93479 PLASMA ASSAY

Concentration of SK&F 93479 (mg l^{-1})	Mean drug/I.S. ratio*	Standard deviation	Number of determinations	Coefficient of variation (%)
0.020	0.11	0.02	10	21.8
0.050	0.16	0.02	20	11.1
0.10	0.31	0.02	20	7.4
0.25	0.75	0.04	19	5.9
0.50	1.56	0.08	20	4.9
0.75	2.64	0.13	10	4.9
1.00	3.17	0.22	20	6.8
1.50	4.92	0.25	10	5.1

*I.S. = Internal standard.

Precision and accuracy of the assay

The precision and accuracy of the assay were assessed by spiking 2-ml volumes of drug-free plasma with various concentrations of SK&F 93479. The precision of the assay is presented in Table II and is expressed as the coefficient of variation (C.V.). In the range 0.10—2.00 mg l^{-1} the average C.V. was 5.7%, however at lower concentrations the assay became less precise, viz. 11.1% C.V. at 0.05 mg l^{-1} and 21.8% C.V. at 0.02 mg l^{-1}.

The assessment of the accuracy of the method is presented in Table III and shows that the average deviation in the range 0.05—0.80 mg l^{-1} was 4.0% but at 0.02 mg l^{-1} the mean calculated concentration was 15% below the known concentration.

TABLE III

ACCURACY OF SK&F 93479 PLASMA ASSAY

Concentration of SK&F 93479 (mg l^{-1})	Mean calculated concentration	Standard deviation	Number of determinations	Percent deviation of mean from known value
0.02	17	7	10	−15.0
0.05	49	5	10	−2.0
0.10	103	6	8	+3.0
0.20	185	6	9	−7.5
0.40	384	13	9	−4.0
0.80	829	62	8	+3.6

These results supported the decision to set the limit of sensitivity of the assay at 0.025 mg l^{-1}.

Stability of SK&F 93479 in plasma

In order to assess whether there were any potential problems of stability of the drug in deep-frozen plasma samples awaiting analysis, sufficient 2-ml aliquots of drug-free plasma were spiked at 0.10 and 0.50 mg l^{-1} to permit the assay of ten tubes for each concentration at 0, 1, 2, 4, 6 and 10 weeks after freezing and storage at −20° C. When thawed the samples were extracted as described above and freshly spiked plasma samples were also analysed to produce a calibration curve.

The results of the study are presented in Table IV and show that the drug in plasma was stable for approximately four weeks; thereafter there was a 20% fall in drug concentration between weeks 4 and 10. Therefore, it is essential that plasma samples are stored for no longer than four weeks before analysis.

The stability of SK&F 93479 in plasma during the immediate post-sampling period was also determined: forty tubes, each containing 2 ml human plasma spiked at 0.10 mg l^{-1} and a second set containing 0.50 mg l^{-1} were allowed to equilibrate for 10 min after vortex mixing.

TABLE IV

STABILITY OF SK&F 93479 IN PLASMA STORED AT −20° C

Week No.	Calculated concentrations	
	0.100 mg l^{-1}	0.500 mg l^{-1}
	Observed concentrations (mg l^{-1})	
0	0.108 ± 0.003 (n = 9)	0.506 ± 0.021 (n = 10)
1	0.110 ± 0.006 (n = 10)	0.551 ± 0.019 (n = 9)
2	0.101 ± 0.008 (n = 9)	0.501 ± 0.018 (n = 9)
4	0.094 ± 0.008 (n = 10)	0.503 ± 0.026 (n = 8)
6	0.072 ± 0.007 (n = 9)	0.424 ± 0.030 (n = 10)
10	0.081 ± 0.001 (n = 10)	0.437 ± 0.019 (n = 10)

TABLE V

STABILITY OF SK&F 93479 IN PLASMA AT AMBIENT TEMPERATURES

Time (h)	Peak height ratio (SK&F 93479/SK&F 93763)			
	0.10 mg l^{-1} SK&F 93479	Percent of original	0.50 mg l^{-1} SK&F 93479	Percent of original
0	0.39 ± 0.01 (n = 8)	100	1.48 ± 0.06 (n = 10)	100
2	0.34 ± 0.04 (n = 10)	87	1.50 ± 0.06 (n = 10)	101
4	0.33 ± 0.01 (n = 8)	85	1.48 ± 0.10 (n = 10)	100
6	0.32 ± 0.02 (n = 9)	82	1.58 ± 0.04 (n = 10)	107

At time zero ten tubes of each concentration were extracted as described previously, this was repeated after 2, 4 and 6 h during which the tubes remained at ambient temperature. After HPLC analysis the peak height ratios of the drug to internal standard were calculated and the results are presented in Table V.

The drug was stable in plasma for at least 4 h at a concentration of 0.50 mg l^{-1}, but in the 0.10 mg l^{-1} samples there was a drop in concentration over the first 2 h. It is therefore recommended that all plasma samples are deep frozen as soon as possible after separation from blood cells.

Human studies

The assay was used to monitor the concentration of SK&F 93479 in plasma samples from a subject to whom the drug had been administered orally (0.5 mg kg^{-1}) and by intravenous infusion for 30 min (0.2 mg kg^{-1} h^{-1}). The results are presented in Fig. 3.

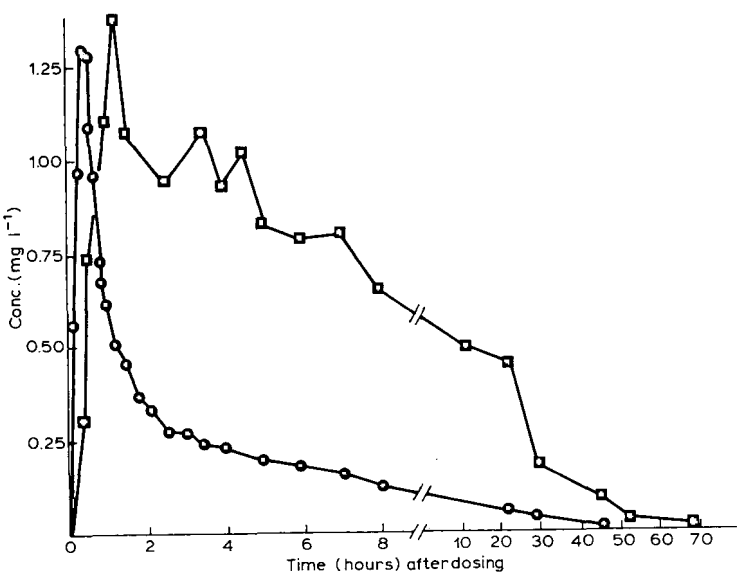

Fig. 3. Concentrations of SK&F 93479 in human plasma after oral or intravenous administration. ▪, 0.5 mg kg^{-1}; •, 0.2 mg kg^{-1} h^{-1} for 30 min.

Plasma concentrations were followed for 48 h after the infusion and for 70 h after the oral dose. Since the major phase of elimination of SK&F 93479 from the plasma has a half-life of about 5 h [11], this analytical method was sensitive enough to follow the elimination through several such half-lives. It may be used with confidence to assess the kinetic parameters applicable to SK&F 93479 in man and to predict the probable dosing regimen necessary to maintain control of gastric acid output.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AFLATOXINS IN HUMAN URINE

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SUMMARY

A method was developed for the extraction and determination of unconjugated aflatoxins in human urine by high-performance liquid chromatography. The analysis is based on the elimination of lipid-soluble constituents other than unconjugated aflatoxins in urine by light petroleum extraction. The unconjugated aflatoxins were subsequently extracted from the aqueous phase with chloroform—acetone. Chromatography was performed isocratically with a silica column at 40°C. The resolved aflatoxins were detected and identified by ultraviolet and fluorometric detectors. The recoveries of aflatoxins B_1 and G_1 added prior to the extraction were 72% and 83%, respectively. This procedure is simple, sensitive and practically useful for epidemiological survey of unconjugated aflatoxins in human urine from areas with a high risk of aflatoxin consumption.

INTRODUCTION

Aflatoxins are major metabolites produced by some strains of *Aspergillus flavus*. Their role as a potent hepatotoxic agent as well as a hepatocarcinogen is evident in a number of animal species [1, 2]. The contamination of human food by these mycotoxins and the detection of aflatoxins in autopsy specimens as reported elsewhere have suggested a role for aflatoxins in human diseases, in particular, acute encephalopathy with fatty degeneration of viscera and hepato-

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cellular carcinoma [2—7]. Nevertheless, none of the reports have provided direct evidence for the existence of aflatoxins in living human tissues or biological fluids.

Data from experimental animals show that the major portion of aflatoxin B_1 consumed is metabolized in the liver. Both native aflatoxins and their metabolic products are excreted mainly in urine and bile [1, 8]. Approximately 0.2% of the oral dose of aflatoxin B_1 was excreted unchanged in the urine of primates within 24 h [9]. The detection of aflatoxins and their metabolic products in human urine will serve as unquestionable proof of the presence of aflatoxin in humans. In addition, the identification of aflatoxin derivatives in urine may help in the understanding of aflatoxin metabolism in man.

In order to detect the presumably small amount of native aflatoxins in human urine, extremely sensitive methods for extraction and detection are required. Gregory and Manley [10] described a procedure for the high-performance liquid chromatographic (HPLC) determination of aflatoxins in animal tissues and products. The recoveries of aflatoxins B_1 and G_1 blended with whole milk before the extraction were 72 and 75% respectively, with a detection limit of 0.05 ± 0.1 ng/g for each aflatoxin. Reversed-phase as well as normal-phase HPLC was used for the analysis of aflatoxins, with the minimum detectable amount of each aflatoxin in the range 1—2 ng using an ultraviolet (UV) detector and 0.3 ng with a fluorescence detector [10—14].

In this paper, we describe a simple method for the extraction and sensitive detection of unconjugated aflatoxins in human urine, based on normal-phase HPLC equipped with both UV and fluorometric detectors.

EXPERIMENTAL

Chemicals

Aflatoxins B_1 and G_1 were purchased from Makor Chemical (Jerusalem, Israel). They were dissolved in an appropriate volume of absolute methanol. Standard solutions containing 0.06 and 0.02 μg of aflatoxins B_1 and G_1, respectively, and 3.3 μg each of aflatoxins B_1 and G_1 per ml of elution solvent were subsequently prepared and used for HPLC analysis.

Extraction of aflatoxins from urine

Five millilitres of human urine were used for each extraction. The urine sample was extracted twice, each time with 50 ml of light petroleum (b.p. 40—60°C), to eliminate lipid- and fat-soluble constituents. Aflatoxins in the aqueous portion were extracted with 50 ml of chloroform. After a second extraction with 50 ml of chloroform—acetone (1:1, v/v), the organic phases were combined, shaken with 10 g of anhydrous sodium sulfate, and filtered through Whatman No. 40 paper under vacuum. The aliquot was evaporated to dryness at approximately 50°C in a rotary evaporator and stored at −20°C in nitrogen atmosphere and protected from exposure to light. Each extracted sample was dissolved in 2 ml of elution solvent and filtered through a Millipore filter (pore size 0.45 μm) before analysis.

Equipment

Normal-phase chromatography was performed with a DuPont Model 870 HPLC system, equipped with a universal septumless injector, temperature-controllable column compartment and a variable-wavelength spectrophotometer. The fluorescence of resolved samples was determined with a connected Jasco Model FP 550 spectrofluorometer. Separations were achieved with a Zorbax Sil column (25 cm × 4.6 mm I.D., particle size 6 μm, DuPont Co., Wilmington, DE, U.S.A.) at an elution rate of 1 ml/min and a nominal pressure of 10.4 MPa. The temperature in the column compartment was maintained at 40°C. Detector wavelength was 362 nm for spectrophotometric absorption. With fluorescence detection, the samples were excited at 365 nm and the fluorescence was recorded at 430 nm. The amount of aflatoxins in the extract was calculated from the known concentration of standard aflatoxins and the area under the UV-absorbing peak measured by a Hruden planimeter (the use of peak height analysis yielded similar results).

Elution solvent system

The elution system consisted of analytical grade toluene—ethyl acetate—absolute methanol—90% formic acid (89:7:2:2, v/v) [13]. Ethyl acetate, absolute methanol and 90% formic acid were mixed prior to the addition of toluene. The solvent was filtered through a Millipore filter (pore size 0.45 μm) and degassed by stirring under vacuum.

RESULTS AND DISCUSSION

A chromatogram of standard aflatoxins B_1 and G_1 is shown in Fig. 1. Aflatoxins B_1 and G_1 were eluted with retention times of 9.5 and 16.5 min, respectively. The extremely high fluorescence of aflatoxins B_1 and G_1 was also demonstrated in the same chromatogram and was used for the identification of aflatoxins. Under the conditions employed in this study, the minimum detectable amounts of aflatoxins B_1 and G_1 were 3 and 1 ng per 50 μl of injected amount, respectively (Fig. 2).

Analysis of aflatoxins in urine

A chromatogram of an extract from normal urine is shown in Fig. 3. Seta et al. [15] observed more than 100 UV-absorbing constituents in human urine by anion-exchange HPLC. Among these, 33 components were identified. In the present report, several lipid- and fat-soluble constituents in urine were removed by extraction with light petroleum and only a few UV-absorbing constituents were left and resolved by normal-phase HPLC; none of them exhibited fluorescence.

Aflatoxins that were added to urine prior to extraction remained in the aqueous phase after light petroleum extraction and were subsequently recovered in the chloroform extract. The chromatogram of an extract from human urine with added aflatoxins (Fig. 4) explicitly shows the UV-absorbing and concomitant fluorescent peaks of aflatoxins B_1 and G_1. The recoveries were 72% and 83% of the added amounts for aflatoxins B_1 and G_1, respectively.

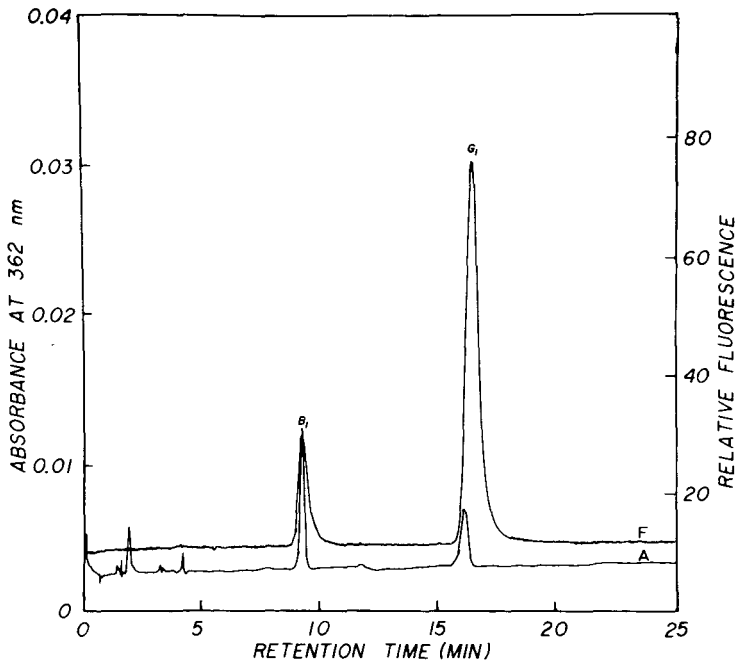

Fig. 1. HPLC resolution of aflatoxins B_1 and G_1 (0.16 μg of each) on Zorbax Sil column. Mobile phase toluene—ethyl acetate—methanol—90% formic acid (89:7:2:2), flow-rate 1.0 ml/min, column temperature 40°C, UV detection at 362 nm (A) at 0.04 a.u.f.s., fluorescence detection (F) as described in the text. The injected amount was 50 μl.

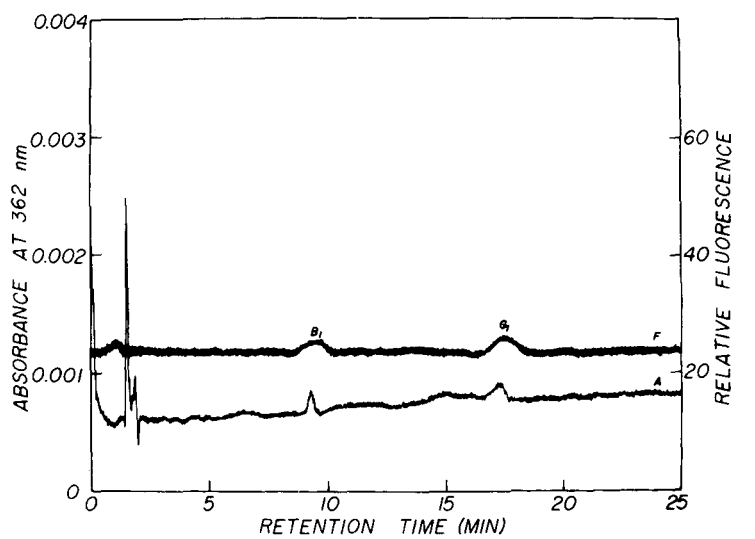

Fig. 2. HPLC resolution of aflatoxins B_1 (3 ng) and G_1 (1 ng). The operating conditions for HPLC were similar to those described in the legend to Fig. 1. The UV chromatogram was recorded at 0.005 a.u.f.s.

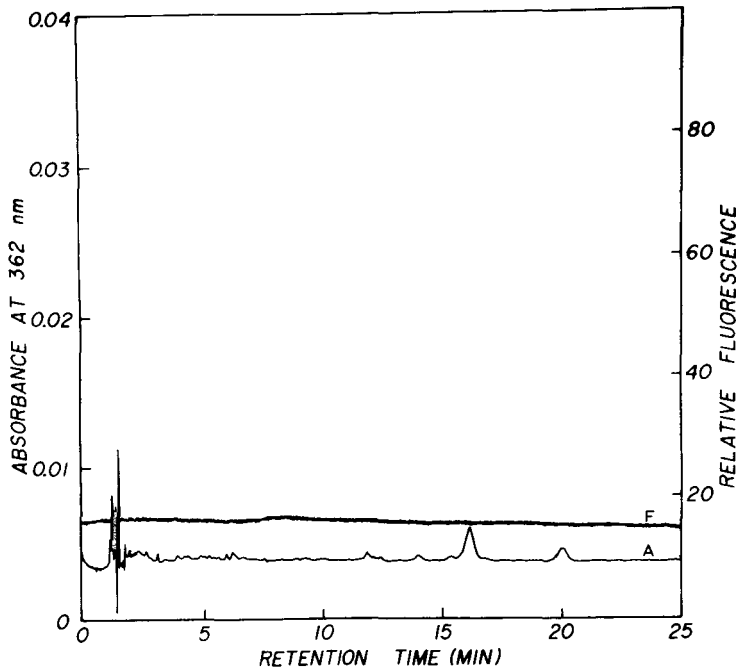

Fig. 3. HPLC separation of an extract from normal human urine. The injected amount was 100 μl, which was equivalent to 250 μl of unextracted urine. The operating conditions for HPLC were similar to those described in the legend to Fig. 1.

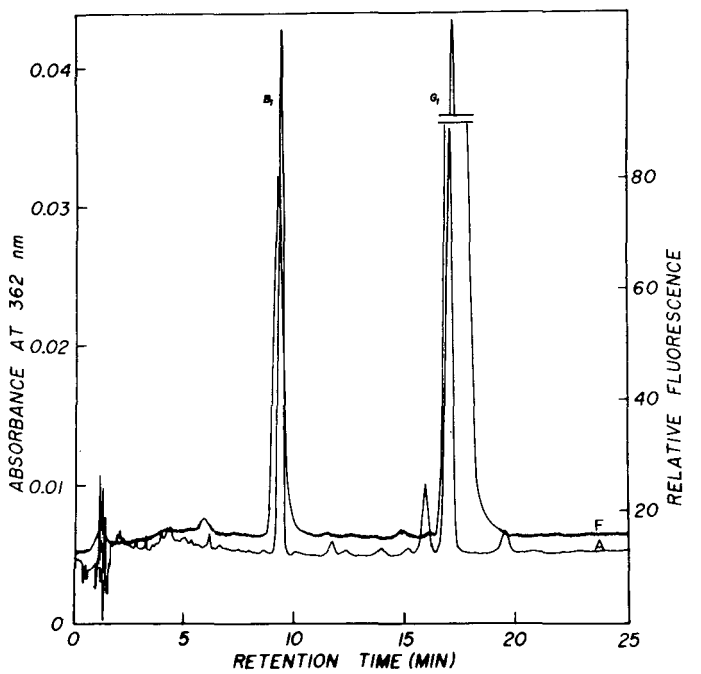

Fig. 4. HPLC separation of extract from human urine with added aflatoxins. Amounts of 6 and 10 μg of aflatoxins B_1 and G_1, respectively, were added to each ml of urine before extraction. The subsequent operating conditions were similar to those in Fig. 3.

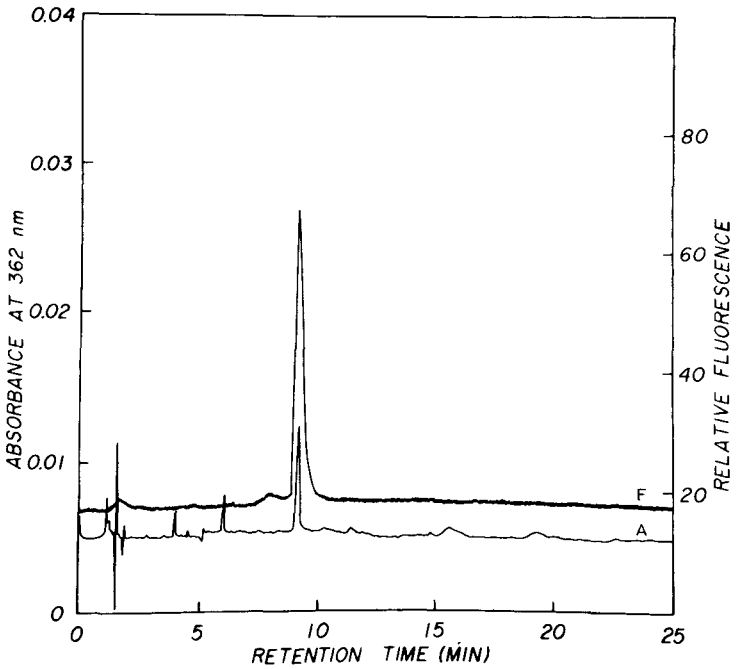

Fig. 5. HPLC separation of an extract from urine of a child in Songkhla province, using the same extraction procedure and HPLC operating conditions as for Fig. 3.

The observation of a UV-absorbing peak in chromatograms of urine extracts from children in Songkhla Province with a retention time in the vicinity of aflatoxin B_1, suggested the presence of aflatoxin B_1 in the urine (Fig. 5). The extremely high fluorescence of this compound served as a convincing proof for aflatoxin B_1. Similar chromatograms were observed in 30 of 106 urine samples from the same area. The finding is consistent with the epidemiological study on aflatoxin contamination of prepared food in the Songkhla area [16].

Since human urine contains several UV-absorbing constituents, the removal of these constituents by light petroleum without disturbing aflatoxins is simple and effective [17]. As a result, interference in aflatoxin separation from constituents with similar retention times during HPLC analysis was clearly reduced. With the presumably small amount of native aflatoxin B_1 excreted in human urine, an extremely sensitive detection device is essential. HPLC equipped with UV and fluorescence detectors offers a system to fit the requirement. The fluorescence of the resolved sample also served as a confirmation for aflatoxins.

Based on data obtained from the study in primates, the major portion of aflatoxins excreted in human urine should be conjugated derivatives rather than native aflatoxins and unconjugated metabolites. The conjugated derivatives are water-soluble compounds which can be converted back to the unconjugated aflatoxins by enzyme digestion or mild hydrolysis [18]. With the presumed large quantity of conjugated aflatoxins in human urine, the subsequent detection and identification can be achieved by conventional thin-layer chromatography. In comparison, the method described in this paper provides a quick, sensitive and simple procedure for the extraction and identification of uncon-

jugated aflatoxins in urine. This procedure can be employed in the study of in vivo metabolism of aflatoxins in humans as well as in the epidemiological study of aflatoxins in human biological fluids.

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Note

Gas chromatographic determination of glucose in serum with glucose oxidase—catalase system

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Glucose in serum has been determined with the help of enzymes. There are several enzymatic methods such as the glucose oxidase—peroxidase method [1—3], the glucose dehydrogenase—NAD^+ method [4, 5] and the hexokinase—glucose-6-phosphate dehydrogenase method [6]. Among these, the hexokinase—glucose-6-phosphate dehydrogenase method reacts with both glucose and glucose-6-phosphate, and insufficient purity of the enzymes results in false values [6]. Glucose dehydrogenase is difficult to obtain in high purity and is very expensive. On the other hand, glucose oxidase is available in high purity, inexpensive and specifically oxidizes β-D-glucose. However, the glucose oxidase—peroxidase method has a disadvantage that peroxidase is unspecific in activity for indicator substrate, so it is susceptible to interference of reducing compounds. In a previous paper [7], a new method for the gas chromatographic (GC) determination of hydrogen peroxide using a methanol—catalase system was reported, which involved the enzymatic conversion of hydrogen peroxide into formaldehyde and derivatization of the formaldehyde with pentafluorobenzyloxylamine (PFBOA). The purpose of this present paper is to develop a new GC method for the assay of glucose in serum with the glucose oxidase—catalase system as an extension of our previous work on the GC determination of hydrogen peroxide using the methanol—catalase system.

EXPERIMENTAL

Reagents

PFBOA hydrochloride (melting point 115°C) was synthesized from pentafluorobenzyl bromide (Aldrich, Milwaukee, WI, U.S.A.) and N-hydroxyphthalimide (Tokyo Kasei, Tokyo, Japan) [8]. Iodobenzene was used as an

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internal standard. An aqueous solution of glucose (60 μg/ml) was prepared by dissolving glucose in water.

Enzymes

Catalase (hydrogen-peroxide:hydrogen-peroxide oxidoreductase, EC 1.11.1.6) (270,000 U/ml) was obtained from Boehringer (Mannheim, G.F.R.). A stock solution (20,000 U/ml) was prepared by diluting it with distilled water.

Glucose oxidase (β-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) (17.8 U/mg) was purchased from Sigma (St. Louis, MO, U.S.A.). A stock solution (5 U/ml) was prepared by dissolving it in distilled water.

Apparatus and conditions

A Shimadzu GC-4CPF gas chromatograph equipped with a hydrogen flame ionization detector (FID) was used. A 2-m glass column packed with 3% XE-60 on 80—100 mesh Celite 545 AW DMCS was used, with a column temperature of 100°C, a detector temperature of 150°C and a chart speed of 0.25 cm/min.

Standard procedure

To the mixture of 1.25 ml of 0.1 *M* phosphate buffer, pH 5.6, 0.15 ml of methanol, 0.1 ml of catalase solution and 0.5 ml of glucose oxidase, were added 0.5 ml of sample solution containing glucose (or 0.5 ml of ten-fold diluted serum with distilled water) and 0.5 ml of aqueous PFBOA solution (1 mg/ml as the hydrochloride) in a 10-ml test-tube. After mixing, the tube was incubated in a water-bath at 37°C for 90 min. After saturation with sodium chloride and acidification with one drop of 18 *N* sulfuric acid, the PFBOA derivative of formaldehyde was extracted with 0.3 ml of *n*-hexane containing iodobenzene (200 μg/ml) as internal standard. Excess sodium chloride and the aqueous layer were removed with the aid of a syringe with a long needle. An aliquot of the extract was applied to the GC column. Quantitation was carried out using calibration graphs obtained from known amounts of glucose. Blank tests were performed using water instead of sample solution.

RESULTS AND DISCUSSION

The factors affecting the reaction in the methanol—catalase system were investigated in detail in the previous paper [7]. In the present paper, the following factors affecting the overall reaction in the glucose oxidase—catalase system were investigated in further detail.

pH of the reaction solution

Using 30 μg of glucose, the optimal pH in the reaction solution was examined in the range 4—8. The pH was adjusted with 0.1 *M* acetate buffer or 0.1 *M* phosphate buffer. The results in Fig. 1 show the overall effects on the glucose oxidase reaction, on the catalase reaction and on the condensation reaction of formaldehyde produced with PFBOA. The optimal pH was between 5 and 6, which agreed with the fact that glucose oxidase is most active at pH 5.6.

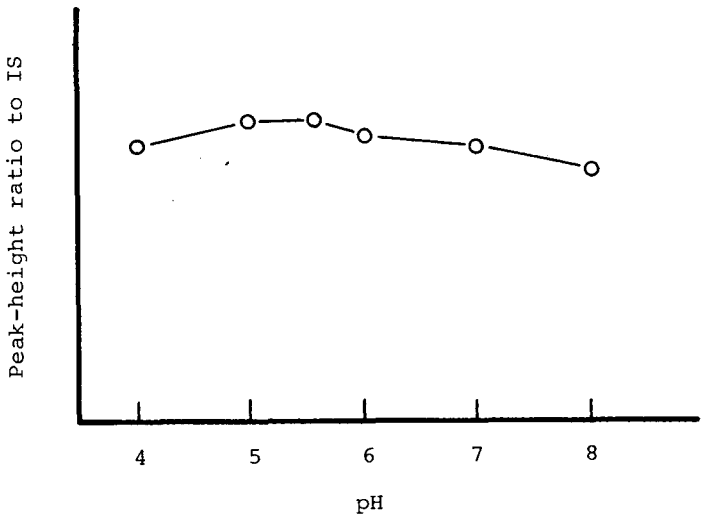

Fig. 1. Effect of pH on the overall reaction in glucose oxidase—catalase system.

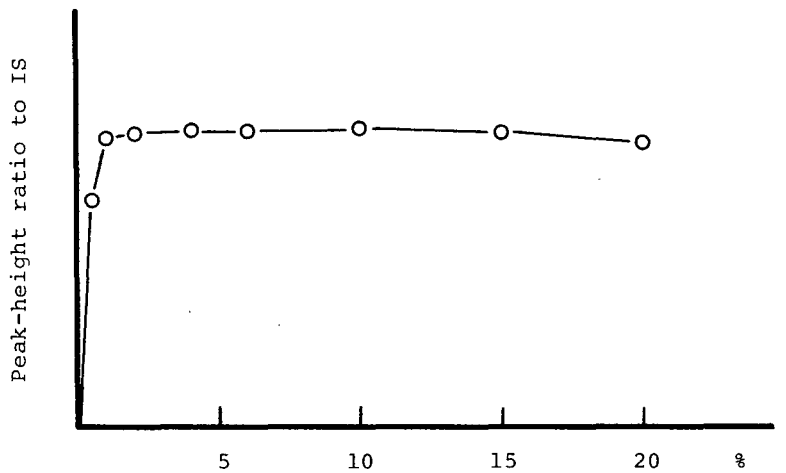

Fig. 2. Effect of methanol concentration.

Methanol concentration

Using 30 μg of glucose, the appropriate concentration of methanol for the glucose oxidase–catalase reaction was examined in the range 1–20% and was found to be between 2 and 10%, as shown in Fig. 2. In the previous paper [7] the suitable concentration of methanol for the determination of hydrogen peroxide using the catalase reaction was found to be between 8.0 and 30%. However, glucose oxidase activity seems to be inhibited in the presence of high concentrations of methanol. Therefore, the measurements in this method were made in the presence of 5% of methanol.

Glucose oxidase concentration

The necessary concentration of glucose oxidase for the determination of

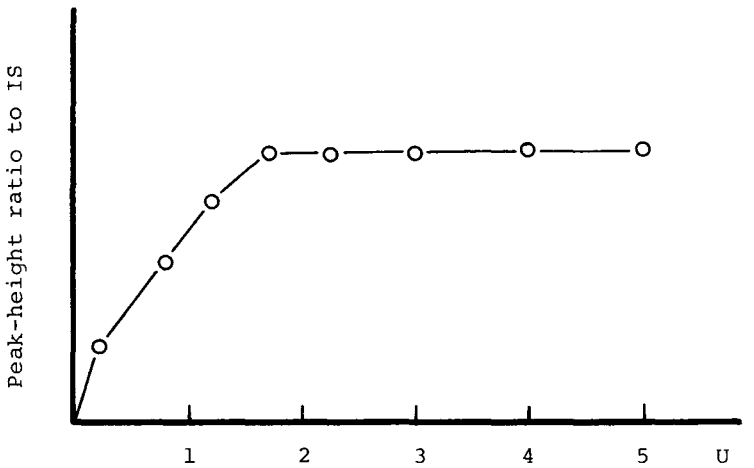

Fig. 3. Effect of glucose oxidase concentration.

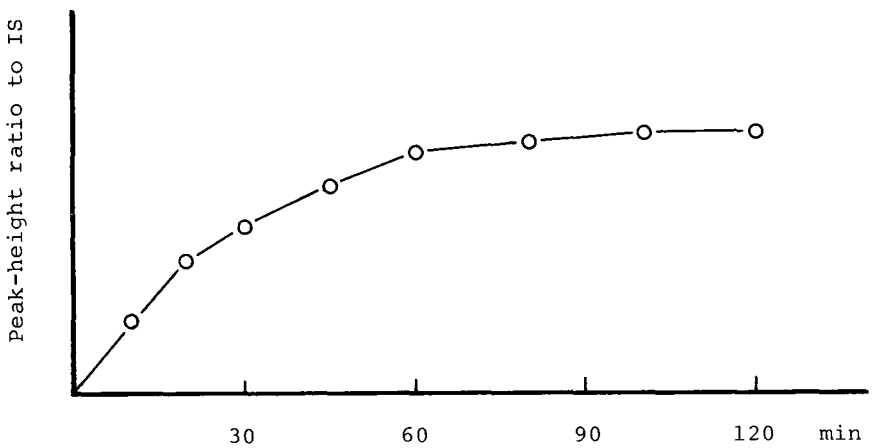

Fig. 4. Effect of reaction period.

glucose was examined. As shown in Fig. 3, it was found that the concentration of glucose oxidase sufficient to obtain a constant value was 2 U for 30 μg of glucose in the reaction system.

Reaction period

Using 30 μg of glucose, the effect of the reaction period was investigated through the entire procedure. It can be seen that the reaction was sufficient in 90 min to obtain a constant glucose value, although after this time the measured values increased slowly with reaction time as shown in Fig. 4.

Influence of reducing compounds

L-Ascorbic acid and uric acid were examined as examples of reducing agents. L-Ascorbic acid (5—20 μg) or uric acid (5—40 μg) was added to 3 ml of the reaction solution with 30 μg of glucose. The results are shown in Fig. 5. Within the range of reducing agent concentrations tested, the measured values were

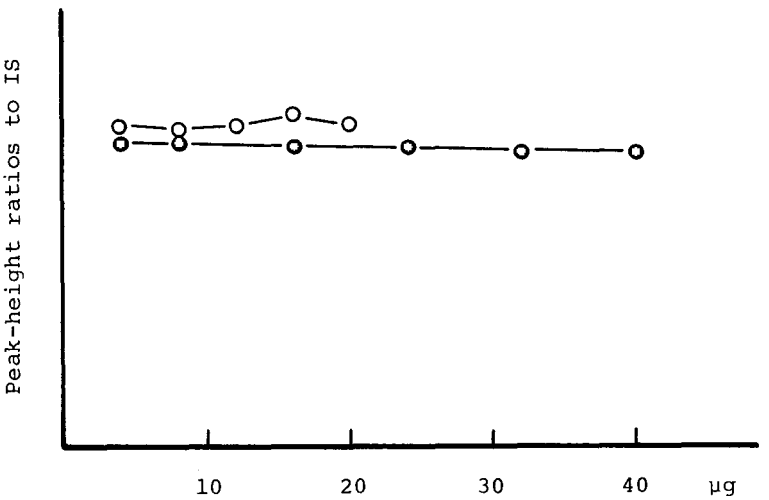

Fig. 5. Effect of coexistence of reducing compounds: ascorbic acid (○) and uric acid (●).

constant. From these results, it can be concluded that the methanol—catalase system is nearly specific for the conversion of hydrogen peroxide into formaldehyde, as expected in the previous paper.

Application

A sample solution containing an individual amount of glucose was measured according to the standard procedure described under Experimental and peak height ratios of formaldehyde formed enzymatically to that of the internal standard were proportional in the range 20—100 μg of glucose in 0.5 ml of sample solution. The coefficient of correlation for the calibration graphs was 0.9991. The reproducibilities of the method were examined with an identical sample solution containing 30 μg of glucose and the coefficient of variation obtained was 2.55% (n = 5).

A typical GC separation of glucose in serum is illustrated in Fig. 6. Iodobenzene was used as internal standard. The peak height ratio of formaldehyde to the internal standard on the chromatogram corresponded to 84 mg/dl glucose in serum. The blank values were found to be negligible towards the FID. A recovery test was carried out on five 0.5-ml portions of an identical ten-fold diluted serum sample spiked with 30 μg of glucose, and the values obtained were calculated to be 102 ± 2.7% (n = 5).

Glucose oxidase is very specific for glucose. However, the glucose oxidase peroxidase method which has widely been used has a disadvantage that it is susceptible to interference from the presence of reducing compounds commonly existing in serum such as ascorbic acid. This important subject remains open. So it is still required to establish a specific method for the assay of glucose in serum.

In this respect, catalase converts methanol specifically to formaldehyde through the action of hydrogen peroxide formed in the glucose oxidation reaction and the catalase reaction is not subject to interference from coexisting reducing compounds. On the other hand, GC is an excellent technique for the

Fig. 6. Gas chromatogram of formaldehyde produced from glucose in serum in glucose oxidase–catalase system on a 2.0 m 3% XE-60 column at 100°C, with FID. Peaks: 1 = formaldehyde PFBOA; 2 = iodobenzene.

determination of formaldehyde. We are not aware of any report dealing with catalase reaction combined with GC for serum glucose determination. The present paper describes a reliable and sensitive method for the determination of glucose by connecting the catalase reaction and the derivatization of formaldehyde with PFBOA.

The proposed method appears to be a little more time-consuming compared with non-chromatographic methods, but a one-step procedure was established to permit the three reactions, glucose oxidase reaction, catalase reaction and condensation reaction with PFBOA, simultaneously. All these things are put together during the incubation; thus, the derivative is formed "on-line", i.e. as the enzyme reacts. In case we need a reliable value of glucose in serum, the method would be useful and helpful.

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Note

Phospholipid analysis and fatty acid content in platelets by the combination of high-performance liquid chromatography and glass capillary gas—liquid chromatography

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(First received December 10th, 1982; revised manuscript received February 23rd, 1983)

Complete separation of phospholipids for preparative purposes usually needs a complex and a multistep procedure including single and two-dimensional thin-layer chromatography (TLC) [1—3]. Column chromatography has also been used with a great variety of stationary phases and solvent mixtures [4]. Recently, high-performance liquid chromatography (HPLC) used under various conditions has been introduced to separate phospholipids [5—17]. However, none of these conditions allows a complete separation of these lipids with sufficient resolution.

On the other hand, the great interest in the polyunsaturated fatty acids as precursors of prostaglandins and/or lipoxygenase products requires high-performance gas—liquid chromatography permitting a baseline separation of fatty acid isomers.

We have modified an HPLC technique previously described [9] in order to obtain a complete separation between the different classes of phospholipids and lysoglycerophospholipids from human platelets. The complete profiling of the fatty acid content of resting platelet phospholipids was then determined using a polar-phase column for the capillary gas—liquid chromatography (GLC).

MATERIAL AND METHODS

Reagents

Organic solvents (Analar grade) were provided by Prolabo (Paris, France) and the purest grade of 2,2,4-trimethylpentane, to solubilize fatty acid methyl esters before analysis by GLC, was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). N-Methyl-N-nitroso-*p*-toluenesulfonamide, silica

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gel G plates and boron trifluoride—methanol were purchased from Merck (Darmstadt, G.F.R.). Butylated hydroxytoluene (BHT) was provided by Fluka (Buchs, Switzerland).

[5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (>90 Ci/mol), labelled standard phospholipids L-α-phosphatidyl-[2-^{14}C]ethan-1-ol-2-amine, dioleoyl (PE, 44 Ci/mol), L-α-phosphatidyl-[U-^{14}C]inositol, 1-pentadecaoyl, 2-nonadecaoyl (PI, 270 Ci/mol), L-α-phosphatidyl-L-[U-^{14}C]serine, dioleoyl (PS, 60 Ci/mol), L-α-phosphatidylcholine, di-[1-^{14}C]palmitoyl (PC, 100 Ci/mol), and [N-*methyl*-^{14}C]sphingomyelin (Sph, 50 Ci/mol) were provided by the Radiochemical Centre, Amersham, Great Britain. [^{14}C]lysoPE (LPE) and [^{14}C]-lysoPC (LPC) were obtained by phospholipase A_2 treatment of PE and PC, respectively. [^{14}C]Phosphatidic acid was produced from PC by phospholipase D treatment. Pancreatic phospholipase A_2 and the cabbage phospholipase D were provided by Boehringer (Mannheim, G.F.R.).

Standard fatty acids were furnished by Supelco (Bellefonte, PA, U.S.A.) or Sigma (St. Louis, MO, U.S.A.). Partisil 5 as stationary phase for HPLC was obtained from Whatman (Clifton, NJ, U.S.A.). Silar 7CP as stationary phase for GLC was provided by Applied Science, (Oud- Beijerland, The Netherlands).

High-performance liquid chromatography

A Chromatem 38 apparatus from Touzart et Matignon (Paris, France) was used; this apparatus was equipped with two pumps and a continuous gradient. A 30-cm long, 6.35 mm (O.D.), 4.6 mm (I.D.) column was filled with Partisil 5 (5 μm) according to a published method [18]. A loop of 50 μl, filled with a slight excess of sample was used for injection. The elution was carried out with a mixture of hexane—isopropanol—acetate buffer 1 m*M*, pH 6, in various proportions (gradient on Fig. 1) at a flow-rate of 1 ml/min. Solvents were de-aerated under vacuum before use. The detection was done by counting 0.5 ml from 1-ml aliquots containing 5×10^{-5} *M* BHT to prevent autooxidation.

Gas—liquid chromatography

An open tubular glass capillary column (70 m × 0.25 mm I.D.) coated with Silar 7CP was used in a Packard Model 427 chromatograph equipped with a solid injector and a flame ionization detector which was connected to a computing integrator. The open tubular glass column was home-made and coated using a static method [19]. Dried helium was used as the carrier gas and the optimal flow-rate was chosen from the Van Deemter curve (arachidonic acid methyl ester at 170°C). Temperature programming was usually 150 to 190°C (1°C/min).

Preparation of biological samples

Human platelets (300,000 per μl) were obtained as previously described [20] and incubated for 1 h at room temperature with 1 μCi/ml [^{3}H]arachidonic acid. Under these conditions, more than 95% of the initial radioactivity was in the phospholipids. Platelets were then extracted twice with 9 volumes of chloroform—ethanol (2:1) containing 5×10^{-5} *M* BHT, and phospho-

lipids were separated from other lipids by TLC on silica gel G. The mixture diethyl ether—methanol—acetic acid (90:1:2) was used for development and the phospholipids remaining at the origin were extracted three times with methanol—water (9:1). They were dried under vacuum and injected onto the HPLC column in solvent A (see Fig. 1).

After their radioactive detection, the glycerophospholipids were dried under vacuum and transmethylated with boron trifluoride—methanol [21]. Fatty acid methyl esters were extracted with 2,2,4-trimethylpentane.

RESULTS AND DISCUSSION

Fig. 1 shows the separation obtained using radioactive standard phospholipids. Amounts of 3—10 nCi of phospholipid (approximately 30 pmol) were injected onto the column. The recovery yield in eluates was higher than 90% for every phospholipid. The use of acetate buffer in the eluent to replace water, as we have done previously [22], gives a better reproducibility of the retention times. Besides, the increase in the column length and the relatively increased proportion of isopropanol in the eluent improved the separation between LPE and PS.

In Fig. 2 one can see a typical radioactivity profile obtained with glycerophospholipids from platelets prelabelled with [^{3}H]arachidonate. Moreover, each phospholipid collected was identified by two-dimensional TLC as we have done previously [23]. The profile was obtained after labelling of only 5×10^8 platelets and the radioactivity detection shows a good sensitivity. The retention times are quite similar to those obtained with tracer amounts of standard phospholipids (Fig. 1) although the quantities obtained from the biological samples were much higher (2—10 nmol, depending on the phospholipid). Using the prelabelling of platelets with [^{3}H]arachidonate we have found a percentage repartition in each glycerophospholipid class of 21.3%,

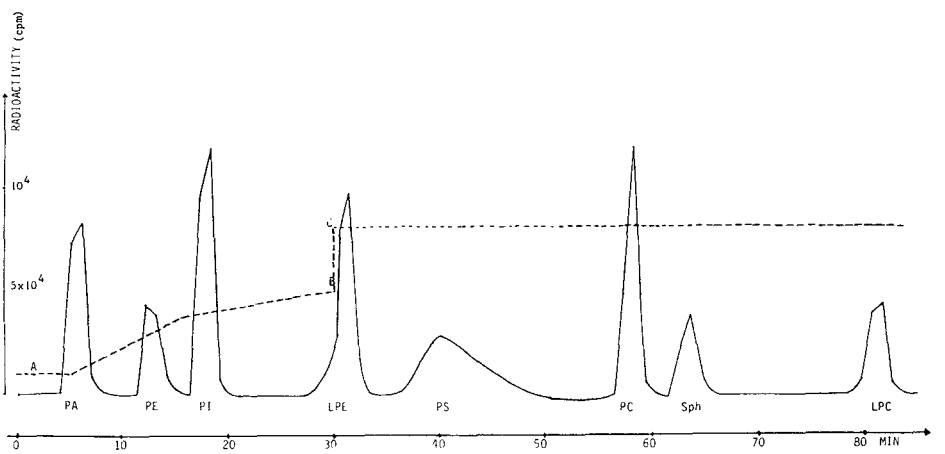

Fig. 1. Profile of radioactive standard phospholipids (3—10 nCi of each). Gradient elution at 1 ml/min on Partisil 5. (———) gradient. (A) Hexane—propanol-2—acetate buffer 1 m*M*, pH 6 (60:120:10). (B) Hexane—propanol-2—acetate buffer 1 m*M*, pH 6 (60:120:16). (C) Hexane—propanol-2—acetate buffer 1 m*M*, pH 6 (60:110:21).

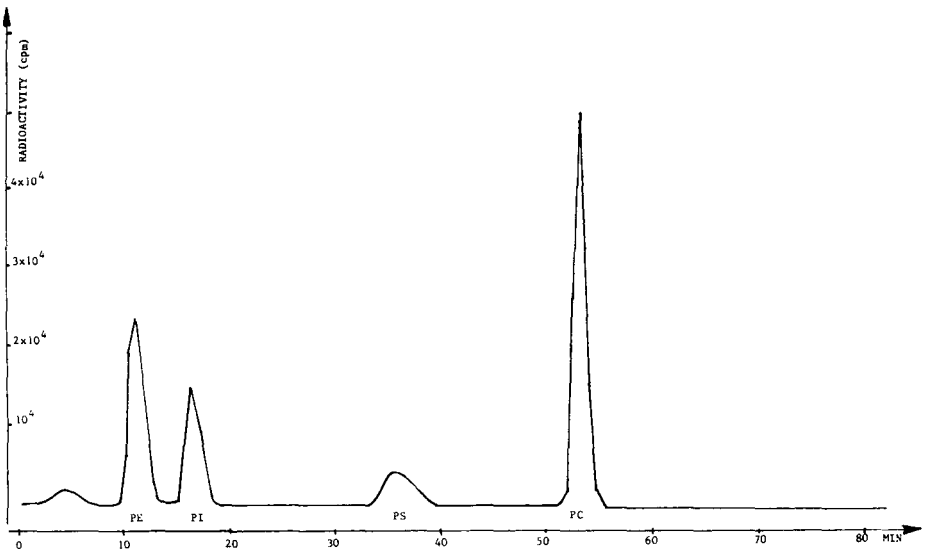

Fig. 2. Typical profile of phospholipids from platelets which were prelabelled with tracer doses of ^{3}H-labelled 20:4ω6 (see Methods).

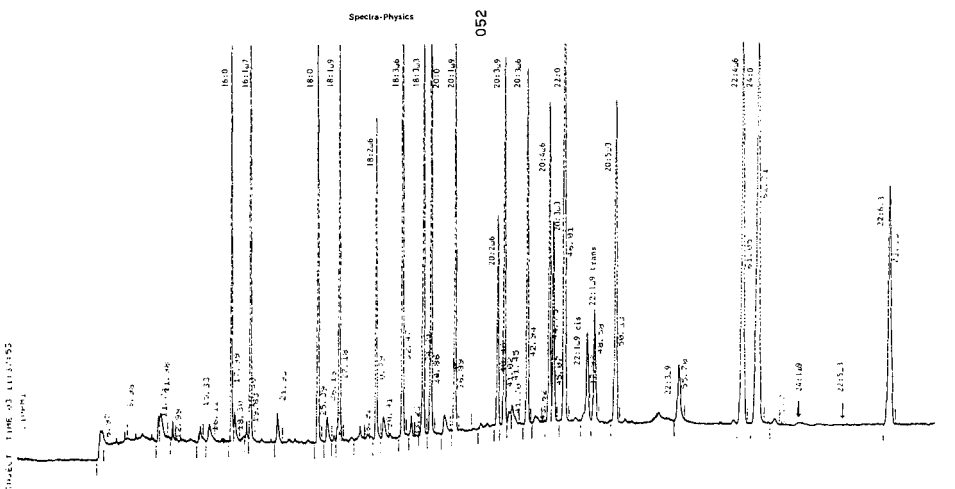

Fig. 3. Profile of standard fatty acid methyl esters (5—15 ng of each). Carrier gas flow: helium 1 ml/min. Temperature gradient: 150—190°C (1°C/min). Approximately 130,000 theoretical plates (calculated with 20:4ω6).

14.8%, 6.9% and 54.9% (mean of five determinations), for PE, PI, PS and PC, respectively. These values are in a good agreement with those reported elsewhere [23, 24]. The method we have used did not allow us to detect lysophospholipids which are not labelled by tracer doses of arachidonate and in any case are present in negligible quantities in resting platelets [25]. A way to detect them, especially after platelet stimulation, would be to prelabel platelets with [^{3}H]glycerol.

The HPLC technique presents two major advantages. Firstly, it takes around

1.5 h for complete separation of phospholipids while about 4 h are usually required for two-dimensional TLC. Secondly, phospholipids and their polyunsaturated fatty acids are protected against oxygen during the separation whereas they are exposed during TLC development and mainly during their localization on the plate, usually by autoradiography. This second point is very important for the further characterisation of the phospholipids and especially for analysis of their fatty acid content. A fatty acid methyl ester separation with capillary GLC (Fig. 3) was obtained with fatty acid standards methylated by ethereal saturated diazomethane treatment [26]. The column we used was very efficient with around 130,000 theoretical plates when calculated using the arachidonate methyl ester (20:4ω6) peak, obtained at 170°C; 5—15 ng of each component were injected. Quantitation of each fatty acid was corrected for its own response factor by the detector. This high sensititivy, which may be extrapolated to a limit of 1 ng, was obtained because of a high column efficiency. The separation was highly resolutive especially for C_{20} polyunsaturated fatty acids which are of great interest for the cell prostaglandin synthetase and lipooxygenase pathways. Particularly, a baseline separation can be observed between 20:2ω6 and 20:3ω9, two fatty acids usually not totally separated using free fatty acid phase of Carbowax columns. This point is very important since 20:3ω9 recently appeared to be of special interest in the regulation of platelet aggregation [27]. Fig. 4 represents a run of fatty acids obtained from platelet PE, showing the retention times of the dimethyl acetals of 16:0, 18:0 and 18:1 contained in that phospholipid.

Finally, using a combination of the two analytical techniques described above, we have determined the fatty acid profile of each glycerophospholipid from unstimulated human platelets. The results are shown in Table I and appear quite similar to those published elsewhere [28, 29] except that ours give more details about the minor fatty acids which are relevant to fatty acid dietary manipulations. The weak standard deviation seen in Table I sug-

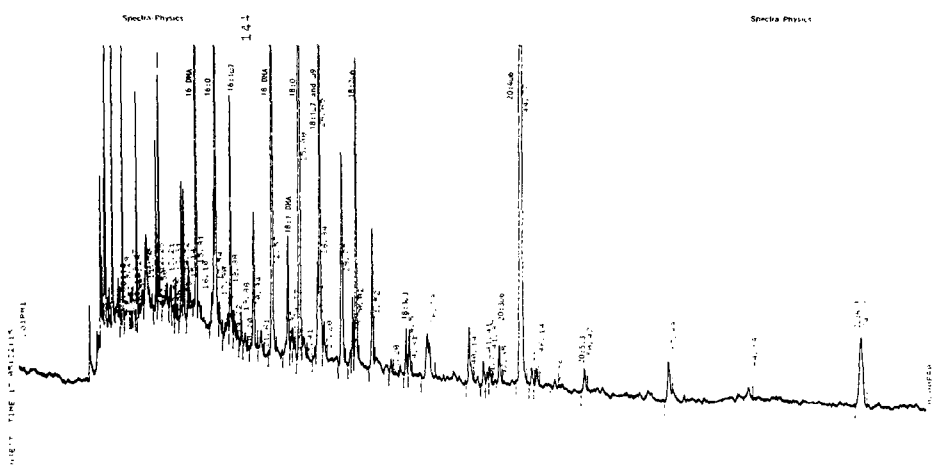

Fig. 4. Typical profile of fatty acid methyl esters obtained from platelet PE. The analytical conditions were those of Fig. 3.

TABLE I

FATTY ACID PROFILES OF THE DIFFERENT PHOSPHOLIPID CLASSES IN HUMAN PLATELETS

Results are given as percentages (mean ± S.D.) of the total fatty acids (n = 5).

	PE	PI	PS	PC
16 DMA	5.70 ± 0.4	—	—	—
18 DMA	9.9 ± 0.6	—	—	—
18:1 DMA	1.1 ± 0.4	—	—	—
16:0	1.9 ± 2.5	11.5 ± 2.3	19.9 ± 6.9	26.0 ± 2.5
16:1ω7	3.4 ± 1.2	4.4 ± 2.2	5.3 ± 2.9	1.9 ± 0.2
18:0	13.8 ± 0.3	29.6 ± 4.1	27.1 ± 5.0	14.7 ± 0.6
18:1ω7 + 18:1ω9 *	6.6 ± 1.2	11.0 ± 3.2	18.5 ± 3.1	18.9 ± 3.0
18:2ω6	3.4 ± 1.0	4.7 ± 2.1	4.6 ± 1.7	12.4 ± 2.6
18:3ω6	0.6 ± 0.8	<0.1	0.1 ± 0.3	<0.1
18:3ω3	0.2 ± 0.3	0.6 ± 0.5	1.2 ± 1.0	0.8 ± 0.5
20:0	0.7 ± 0.2	2.8 ± 1.5	1.7 ± 1.0	1.2 ± 0.7
20:1ω9	0.2 ± 0.2	<0.1	<0.1	0.8 ± 0.7
20:2ω6	0.2 ± 0.2	<0.1	<0.1	0.7 ± 0.1
20:3ω9	0.3 ± 0.3	N.D.	<0.1	0.2 ± 0.2
20:3ω6	0.6 ± 0.1	0.6 ± 0.3	1.0 ± 0.4	2.2 ± 0.2
20:4ω6	42.5 ± 4.6	32.4 ± 3.5	16.7 ± 3.3	15.7 ± 0.4
20:3ω3	N.D.**	N.D.	N.D.	N.D.
22:0	0.1 ± 0.1	0.8 ± 1.0	1.1 ± 0.7	1.7 ± 1.3
22:1ω9(*cis*)	N.D.	N.D.	N.D.	N.D.
22:1ω9(*trans*)	N.D.	N.D.	N.D.	N.D.
20:5ω3	0.5 ± 0.3	<0.1	N.D.	0.2 ± 0.1
22:4ω6	0.7 ± 0.6	<0.1	<0.1	0.9 ± 0.9
24:0	0.1 ± 0.1	N.D.	<0.1	0.3 ± 0.4
24:1	N.D.	N.D.	N.D.	N.D.
22:5ω3	<0.1	N.D.	N.D.	N.D.
22:6ω3	1.8 ± 2.3	N.D.	<0.1	0.7 ± 0.7

*We have summed the 18:1 isomers because they are not completely separated.
**N.D. = not detected.

gests that the combined analysis gives a good reproducibility of the measurements.

We can conclude that the combination of HPLC and capillary GLC used as described above represents a quick, reproducible and very sensitive approach to the study of phospholipids of animal cells and their fatty acid composition, especially with regard to the oxygenated metabolism of polyunsaturated fatty acids.

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Note

Dyes permit immediate evaluation of high-performance liquid chromatographic system performance

Application to high-performance liquid chromatography with radioimmunoassay detection

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Reversed-phase high-performance liquid chromatography (HPLC) is a powerful tool for separating peptides with similar amino acid sequences [1—3]; however, minor changes in either mobile phase composition or flow-rate may affect retention time [4]. When eluted peptides are quantitated by radioimmunoassay (RIA), several days are generally required before HPLC malfunctions become evident. Although it is possible to evaluate column performance with standards supplied by a column manufacturer or with a tryptic digest of a known protein, it is advantageous to evaluate performance with the conditions to be used for experimental samples. Furthermore, it is desirable to have an indicator permitting rapid verification that the HPLC system is functioning properly during each separation. The present investigation characterizes the retention times of several commercially available dyes and demonstrates that phenol red can be used as a preliminary indicator of HPLC performance during HPLC with RIA detection of α-melanocyte stimulating hormone (αMSH)-like peptides in biological samples.

MATERIALS AND METHODS

A Beckman (Berkeley, CA, U.S.A.) Model 334 high-performance liquid chromatograph consisting of two Beckman 110A pumps, a Beckman 421

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controller, an Hitachi 155 variable-wavelength spectrophotometer and a LKB 2111 Multirac fraction collector was used for these studies. For chromatography using an octadecylsilane (ODS) column (Beckman Ultrasphere ODS, 250 × 4.6 mm, 5 μm particle size), the aqueous component of the mobile phase was 0.2 *N* formic acid adjusted to pH 3.2 with triethylamine (TEAF) or 0.2 *N* phosphoric acid adjusted to pH 3.2 with triethylamine (TEAP) [5]. For chromatography using a cyano (CN) column (Waters μBondapak CN, 300 × 3.9 mm, 10 μm particle size) the TEAP buffer was diluted ten-fold before use [6]. Acetonitrile (Burdick and Jackson Labs., Muskegon, MI, U.S.A.) was the organic solvent used for all chromatographic separations.

Dyes were obtained from the following sources: methyl green (Fisher Scientific, Fairlawn, NJ, U.S.A.), phenol red, bromphenol blue, acid red 150, sudan orange G, brilliant green, crystal violet and Coomassie brilliant blue G (Sigma, St. Louis, MO, U.S.A.) and May Grunwald (Allied Chemical, Morristownship, NJ, U.S.A.). All dyes except bromphenol blue and sudan orange G were prepared at a concentration of 0.1 mg/ml in 2 *N* acetic acid. Bromphenol blue and sudan orange G were dissolved in TEAF—acetonitrile (79:21). The dye solutions were centrifuged before being injected into the chromatograph. Phenol red appears to be of sufficiently high quality that additional purification before use is not necessary. Some of the other dyes may require additional purification (i.e. Waters Sep-Pak) before routine use.

Enzymatically dispersed rat intermediate lobe cells were resuspended (100,000 cells/ml) in 1 ml of 2 *N* acetic acid containing the protease inhibitors iodoacetamide and phenylmethylsulphonyl fluoride [5]; this cell suspension was sonicated and diluted 20-fold with 2 *N* acetic acid containing the protease inhibitors and phenol red (0.1 mg/ml). A 100-μl aliquot of this solution was injected into the HPLC system; the gradient profiles and flow-rates are specified in the captions to the figures and tables. The mobile phase in each fraction was evaporated to dryness in a vacuum centrifuge (Savant Instruments, Hicksville, NY, U.S.A.) and the amount of immunoreactive-αMSH (IR-αMSH) was determined as described previously [5]. The presence of phenol red in the eluent from the chromatograph was either monitored spectrophotometrically at 254 nm or visually by the yellow color in some of the HPLC fractions.

RESULTS

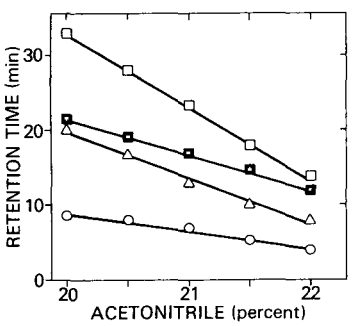
Several dyes eluted with different retention times from either an ODS or CN column (Table I). Using an ODS column, the retention times of the dyes were slightly longer with the TEAF buffer than with the TEAP buffer, however, the rank order of dye retention time remained the same. Using an ODS column with TEAF—acetonitrile as the mobile phase, the retention time of phenol red and the three forms of IR-αMSH were a linear function of acetonitrile concentration (Fig. 1). When the acetonitrile concentration was 21%, the three forms of IR-αMSH occurring in the dispersed intermediate lobe cells (which comigrated with synthetic desacetyl-αMSH, αMSH and N,O-diacetyl-αMSH) were resolved (Fig. 2); the phenol red did not interfere with the RIA (data not shown).

TABLE I

RETENTION TIMES OF DYES IN CHROMATOGRAPHIC SYSTEMS

A linear gradient consisting of the indicated aqueous component and acetonitrile (5% to 100%) was run over 95 min at a flow-rate of 1 ml/min and 1-ml fractions were collected. The data represent the retention time of each dye in the indicated chromatographic system.

Dye	Retention time (min)		
	ODS column		CN column
	TEAF	TEAP	TEAP
Methyl green	26	25	23
Phenol red	30	29	17
May Grunwald	37	32	24
Bromphenol blue	49	46	33
Coomassie brilliant blue G	52	49	40
Sudan orange G	54	50	33
Acid red 150	56	53	38
Crystal violet	67	64	44
Brilliant green	76	71	50



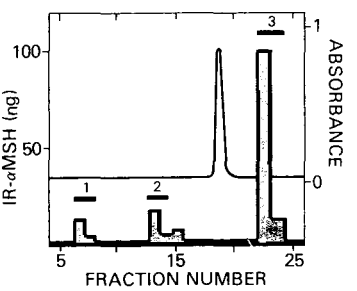


Fig. 1. Changes in retention time of the αMSH-like peptides and phenol red as a function of the acetonitrile concentration. The αMSH-like peptides from rat intermediate lobe cells and phenol red were resolved using an ODS column and a mobile phase consisting of an isocratic solution of acetonitrile and TEAF buffer (as indicated); the flow-rate was 1 ml/min. The presence of desacetyl-αMSH (○—○), αMSH (△—△) and N,O-diacetyl-αMSH (□—□) in the HPLC fractions were determined by RIA. The presence of phenol red (■—■) in the HPLC fractions was determined visually. For each acetonitrile concentration, the peak retention time of each compound is presented.

Fig. 2. Chromatographic resolution of the αMSH-like peptides and phenol red. The αMSH-like peptides from intermediate lobe cells and phenol red were resolved on an ODS column with the mobile phase TEAF—acetonitrile (79:21) at a flow-rate of 1 ml/min. The filled horizontal bars above the chromatographic fractions indicate the retention time of synthetic αMSH-like peptides.

DISCUSSION

Phenol red provides an example of a dye which can be used as an indicator of HPLC performance under specific chromatographic conditions. Routinely,

we determine the retention time of phenol red several times before injecting biological samples. After adequate performance of the HPLC system is verified, biological samples containing phenol red are injected. Using this protocol, HPLC system malfunctions can be determined rapidly. For example, check valve failure, decreased column efficiency or a partially obstructed frit result in phenol red retention time variation or the presence of phenol red in more than two 1-ml fractions. Once a problem is identified, procedures to improve system performance can be immediately initiated. Phenol red may not be the dye of choice for all chromatographic systems, however one or more of the dyes listed in Table I may be suitable as an indicator of HPLC performance in other chromatographic systems.

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Note

Improved method for high energy nucleotide analysis of canine cardiac muscle using reversed-phase high-performance liquid chromatography

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High-energy nucleotide analysis is gaining increased use in laboratories investigating the biochemical manifestations of cardiac ischemia. Many investigators have utilized the depletion of high-energy nucleotides and subsequent accumulation of degradation products associated with ischemia to study changes in cardiac cell metabolism, structure and function [1, 2]. High-performance liquid chromatography (HPLC) enables a sensitive separation of these compounds based on their stoichiometric differences. Unfortunately, the heterogeneity of high-energy nucleotides and their metabolites ordinarily makes their separation in a single isocratic assay difficult and time consuming. The methodology described in the present report provides excellent resolution of high-energy nucleotides and their major degradative products from totally ischemic canine cardiac muscle with minimal assay time using isocratic elution with a very economical buffer system.

MATERIALS AND METHODS

Chromatographic equipment

Sample application and solvent delivery were accomplished by a variable-volume injection system (Model U6K, Waters Assoc., Milford, MA, U.S.A.) in conjunction with a reciprocating pump (Model 6000A, Waters Assoc.). Each sample was manually injected via a 25-μl Hamilton syringe (Hamilton, Reno, NV, U.S.A.). The separation system consisted of an untreated Radial Pak-A reversed-phase column (C_{18}, 10 μm, 8 mm I.D., Waters Assoc.) and a radial compression module (Model RCM 100, Waters Assoc.) operated at 175 bar. A double-beam UV detector (Model 441, Waters Assoc.) monitored the absorbance of the eluents at 254 nm. A data module (Model 730, Waters

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Assoc.) integrated the detected response, providing peak areas and retention times for each sample.

Buffer preparation

An amount of 11.5 g of HPLC grade ammonium dihydrogen phosphate (Fisher Scientific, Fair Lawn, NJ, U.S.A.) weighed on a Cahn TA-450 Balance (Cahn Instruments, Cerritos, CA, U.S.A.) was brought up to 1 l using ultrapure, reagent-grade water (Milli-Q, Millipore, Bedford, MA, U.S.A.). The pH was adjusted to 5.5 (Accument Model 750 selective ion analyzer, Fisher Scientific) with 3 *N* ammonium hydroxide (A.C.S. grade, Mallinckrodt, Paris, KY, U.S.A.) diluted with ultrapure water. The buffer was filtered and degassed using a Millipore Solvent Clarification Kit with a 0.45-μm aqueous filter (Type HA, Millipore). Fresh buffer solution was prepared prior to each group of sample runs.

Sample extraction and preparation

Canine cardiac tissue was divided into subendocardial, midmyocardial and subepicardial slices weighing between 75 and 150 mg. Reliable results, however, have been obtained with samples as small as 25 mg in subsequent studies. Each tissue slice was weighed quickly on a Cahn Model DTL microbalance and placed in 1.5 ml of 3.6% perchloric acid (70%, ACS, Fisher Scientific) at 0.5°C and then immediately homogenized using a Tri-R stirrer. Weighing and transfer to the perchloric acid required 10—15 sec. Following homogenization, tissue was allowed to extract for 30 min at 0.5°C, followed by centrifugation at 850 *g* for 20 min at 0.5°C. The supernatant was neutralized with potassium carbonate—potassium hydroxide to a pH of 5.0—6.0 and frozen until analysis.

Standard preparation

Standards for ATP, ADP, AMP, inosine, hypoxanthine and xanthine were prepared by dissolving high-quality pure standards (Sigma, St. Louis, MO, U.S.A.) in ultrapure water to approximate concentrations. The exact concentration was then determined using a double beam spectrophotometer (Model 250, Gilford Instruments, Oberlin, NJ, U.S.A.).

Column equilibration and rejuvenation

Before assays were performed, the column was flushed at least ten times with 100% methanol, followed by a thorough flushing with 2% methanol in ultrapure water. The column was allowed to equilibrate with ammonium phosphate buffer until response factors, as determined by the Data Module 730, were repeatable.

After sample runs were terminated, the column was flushed with 2% methanol in ultrapure water to prevent build-up of ammonium salts and then flushed with 100% methanol to wash out any remaining organic compounds and to inhibit bacterial growth.

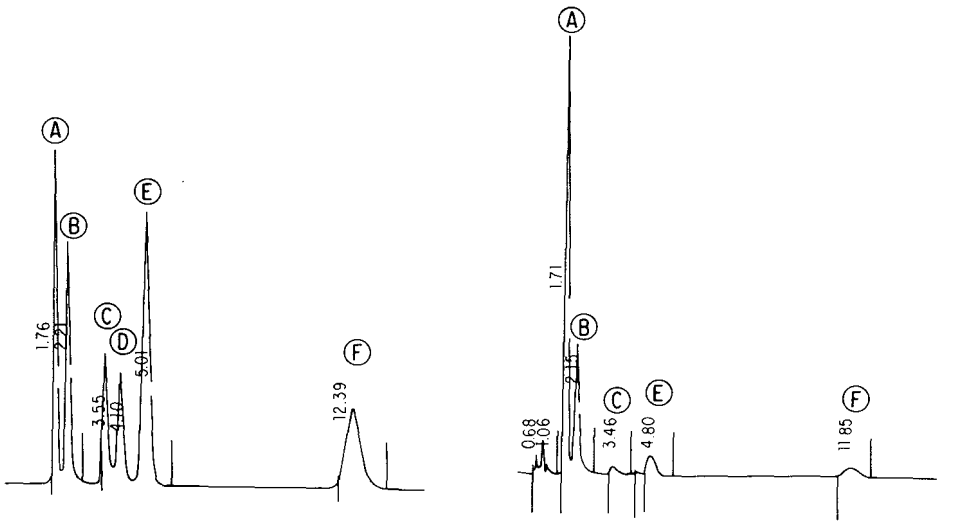

Fig. 1. Chromatogram showing the resolution of standards: A, adenosine 5′-triphosphate (ATP); B, adenosine 5′-diphosphate (ADP); C, hypoxanthine (HYP); D, xanthine (XAN); E, adenosine 5′-monophosphate (AMP); F, inosine (INO). Radial Pak-A column (C_{18}, 10 μm, 8 mm I.D.), ammonium dihydrogen phosphate buffer (0.1 *M*, pH 5.5, ambient temperature), flow-rate 4 ml/min.

Fig. 2. Control canine cardiac tissue. Peaks and chromatographic conditions as given in Fig. 1.

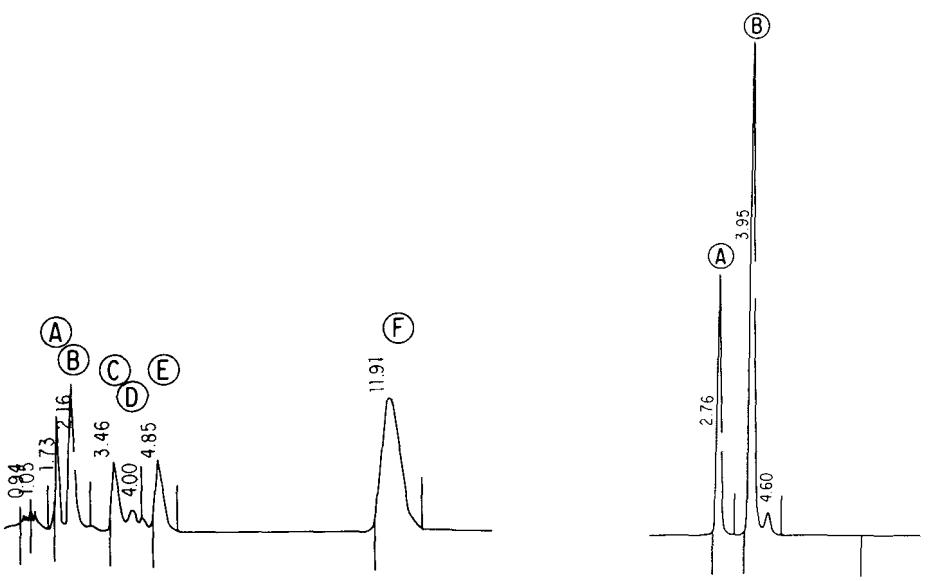

Fig. 3. Canine cardiac tissue after 60 min of total ischemia. Peaks and chromatographic conditions as given in Fig. 1.

Fig. 4. Chromatogram for standards A, phosphocreatine (CP); and B, creatine (C). Flow-rate, 1 ml/min; otherwise chromatographic conditions as given in Fig. 1.

RESULTS

Quantitation

Calibration chromatograms for the standards ATP, ADP, AMP, inosine (INO), hypoxanthine (HYP), and xanthine (XAN) were generated by injecting 20 μl of a standard mixture of known concentration determined as previously described. Fig. 1 is a representative chromatogram showing good resolution of the standards when collectively assayed.

Likewise, frozen tissue samples were thawed, centrifuged at 4000 *g* for 30 min and then 20 μl of the supernatant were injected. Figs. 2 and 3 are chromatograms of canine cardiac tissue obtained at the control period and after 60 min of total in vitro ischemia, respectively, illustrating the metabolic degradation of high-energy phosphates and the subsequent accumulation of metabolites.

TABLE I

SUBENDOCARDIAL, MIDMYOCARDIAL AND SUBEPICARDIAL HIGH-ENERGY NUCLEOTIDE DEPLETION AND DEGRADATIVE METABOLITE ACCUMULATION OVER TIME DURING TOTAL ISCHEMIA IN VITRO, 37°C (n = 10)

Values are given in μmol/g wet weight.

Compound		Control (3—5 min)			15 min			30 min	
		Sub-endo	Mid	Sub-epi	Sub-endo	Mid	Sub-epi	Sub-endo	Mid
ATP	Mean	5.517	5.438	5.458	3.927	4.140	4.271	2.764	3.134
	S.D.	1.018	1.029	0.976	0.570	0.698	0.819	0.447	0.636
	S.E.	0.339	0.343	0.325	0.180	0.221	0.259	0.141	0.201
ADP	Mean	1.282	1.432	1.424	1.136	1.116	1.273	0.986	1.038
	S.D.	0.204	0.232	0.234	0.193	0.174	0.188	0.153	0.161
	S.E.	0.068	0.077	0.078	0.061	0.055	0.059	0.048	0.051
AMP	Mean	0.195	0.239	0.214	0.160	0.144	0.180	0.183	0.192
	S.D.	0.092	0.105	0.121	0.053	0.049	0.069	0.075	0.090
	S.E.	0.029	0.033	0.038	0.017	0.016	0.022	0.024	0.028
HYP	Mean	0.145	0.150	0.124	0.298	0.321	0.279	0.571	0.507
	S.D.	0.025	0.035	0.037	0.096	0.090	0.073	0.160	0.124
	S.E.	0.014	0.020	0.021	0.055	0.052	0.042	0.092	0.071
XAN	Mean	—	—	—	0.115	0.097	0.088	0.123	0.118
	S.D.	—	—	—	0.021	0.030	0.047	0.065	0.049
	S.E.	—	—	—	0.012	0.017	0.027	0.037	0.029
INO	Mean	0.248	0.353	0.270	1.572	1.437	1.125	2.566	2.384
	S.D.	0.127	0.113	0.165	0.646	0.535	0.566	0.725	0.608
	S.E.	0.048	0.043	0.062	0.244	0.202	0.214	0.274	0.230
ΣAd		6.994	7.109	7.096	5.223	5.400	5.724	3.933	4.364
Σ {NS+B}—{ADO}		0.393	0.503	0.394	1.985	1.855	1.492	3.260	3.009
Total		7.387	7.612	7.490	7.208	7.255	7.216	7.193	7.373

Phosphocreatine (CP) and creatine (C), additional indicators of cardiac muscle energy stores, can also be quantitated using this buffer system and a wavelength of 214 nm. Fig. 4 is a calibration chromatogram for the standards CP and C.

Applications

The described method was used in a study to determine whether a transmural progression of metabolic changes occurs during in vitro total ischemia. Following rapid excision of a canine heart, subendo-, midmyo-, and subepicardial samples were obtained at control (3—5 min following excision) and 15-min intervals for determination of high-energy nucleotides and their major degradative metabolites. Data obtained from ten dogs are summarized in Table I and show a progressive depletion of nucleotides with a consequent

45 min			60 min			Rigor initiation			
Sub-epi	Sub-endo	Mid	Sub-epi	Sub-endo	Mid	Sub-epi	Sub-endo	Mid	Sub-epi
3.218	1.923	2.400	2.620	1.492	1.673	1.862	0.950	1.234	1.487
0.364	0.472	0.361	0.452	0.386	0.629	0.529	0.424	0.542	0.657
0.115	0.149	0.114	0.143	0.173	0.281	0.236	0.134	0.171	0.208
1.056	0.937	0.960	1.081	0.810	0.862	1.000	0.793	0.798	0.947
0.138	0.144	0.161	0.244	0.170	0.197	0.219	0.211	0.188	0.190
0.043	0.046	0.051	0.077	0.076	0.088	0.098	0.067	0.059	0.060
0.216	0.217	0.157	0.217	0.224	0.333	0.356	0.383	0.252	0.288
0.157	0.104	0.054	0.102	0.073	0.158	0.165	0.218	0.105	0.148
0.050	0.033	0.017	0.032	0.033	0.071	0.074	0.069	0.033	0.047
0.449	0.777	0.670	0.538	0.820	1.021	0.929	1.194	1.028	0.790
0.088	0.181	0.194	0.178	0.359	0.470	0.465	0.280	0.325	0.147
0.051	0.105	0.112	0.103	0.254	0.332	0.329	0.162	0.188	0.085
0.143	0.136	0.099	0.111	0.297	0.238	0.122	0.243	0.161	0.176
0.050	0.057	0.040	0.031	0.246	0.048	0.069	0.048	0.052	0.042
0.029	0.033	0.023	0.018	0.174	0.034	0.049	0.028	0.030	0.024
1.903	3.281	3.032	2.857	3.283	3.362	2.990	4.095	3.806	3.401
0.520	0.419	0.701	0.396	1.333	0.752	0.567	0.329	0.355	0.645
0.197	0.158	0.265	0.150	0.770	0.434	0.328	0.116	0.125	0.228
4.490	3.077	3.517	3.918	2.526	2.868	3.218	2.126	2.284	2.722
2.495	4.194	3.801	3.506	4.400	4.621	4.041	5.532	4.995	4.367
6.985	7.271	7.318	7.424	6.926	7.489	7.259	7.658	7.279	7.089

accumulation of metabolites during the course of in vitro total ischemia. The results show a statistically significant gradient of ATP depletion, with the subendocardium consistently showing accelerated energy utilization compared to the subepicardium ($P < 0.05$). Ultrastructural evidence of irreversible injury first appeared in the subendocardium at the onset of ischemic contracture and occurred when ATP levels declined to less than 1 μmol/g wet weight. In summary, these data show that during total ischemia in vitro, cell death begins in the subendocardium at the onset of ischemic contracture and progresses towards the subepicardium over time. These changes occurred independent of variations in collateral flow or wall tension [3].

DISCUSSION

Analysis of high-energy nucleotides and their major metabolites is essential for assessing the ability of chemical and mechanical interventions to delay or reverse myocardial ischemic cell injury and for delineating the pathogenesis of ischemic cell death. Precise collective analyses of these compounds can be accomplished using reversed-phase HPLC [4—6].

The myocardial adenine nucleotide content (ΣAd), the sum of ATP, ADP, and AMP, and the nucleoside and base content (ΣNS + B), the sum of adenosine (ADO), INO, HYP, and XAN, represent the major nucleotides and their catabolic byproducts in myocardial ischemia. During ischemia, the fall in ΣAd is accounted for primarily by the marked accumulation of ΣNS + B while the total adenine pool remains essentially unchanged (Table I). The apparent recovery of more than 100% of the initial pool may be partially attributed to the catabolism of guanidine, uridine and cytidine, and late in the ischemic time course, to NAD [7]. However, in totally ischemic cardiac tissue, the contribution of ADO to the adenine pool is very slight. ADO, which is produced by the dephosphorylation of AMP by 5′-nucleotidase in the sarcolemma, can easily diffuse across the cellular membrane and is rapidly deaminated to INO by the large quantities of adenosine deaminase present in the myocardium [8]. We elected not to quantify ADO chromatographically, eliminating the need for gradient elutions and thereby reducing the equilibration delay between sample runs.

The compounds CP and C are of interest occasionally, and as noted, can readily be separated and quantified using a wavelength of 214 nm, although 80% of CP is normally lost in less than 1 min of severe ischemia [7].

These results show that ammonium dihydrogen phosphate buffer in conjunction with a radial compression system performs excellent separation of adenine high-energy nucleotides and their major metabolites. Ammonium phosphate has the advantages of being inexpensive and easy to prepare, with low viscosity, high surface tension, a low UV cutoff point and a large buffering capacity. Given the volume of work inherent in metabolic studies, this method offers efficient, precise and economical determinations.

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Note

High-performance liquid chromatographic determination of 24,25-dihydroxyvitamin D_3 in serum

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The second most abundant vitamin D metabolite in serum is 24,25-dihydroxyvitamin D (see ref. 1). Several procedures have been described in the literature for quantifying 24,25-dihydroxyvitamin D [24,25$(OH)_2$D] in serum [1]. Most of these employ high-performance liquid chromatography (HPLC) for initial purification and competitive protein binding (CPB) as the final step in the analytical procedure. CPB is used because of its sensitivity and because relatively low concentrations of 24,25$(OH)_2$D are found in serum (1—5 ng/ml) [1]. For determination of vitamin D and its hydroxylated metabolites in serum, we have recently reported a multiple assay procedure using HPLC and CPB [2]. Because the CPB technique is sensitive to interfering compounds, we have now modified the procedure so that it allows quantification of 24,25-$(OH)_2$D using direct UV detection. Two forms of 24,25$(OH)_2$D may occur in serum, one derived from vitamin D_3 (cholecalciferol) and the other from vitamin D_2 (ergocalciferol). In this procedure they can be measured separately.

EXPERIMENTAL

Chemicals and instruments

Crystalline 25-hydroxyvitamin D_3, 24,25-dihydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 were generous gifts from F. Hoffmann-La Roche (Basel, Switzerland). Radioactive 24,25$(OH)_2$-[23,24(n)-^{3}H]D_3 (82 Ci/mmol) and 25,26$(OH)_2$-[23,24(n)-^{3}H]D_3 (gift) were obtained from The Radiochemical Centre (Amersham, Great Britain). All solvents and analytical grade chemicals were supplied by E. Merck (Darmstadt, G.F.R.). Hexane and propan-2-ol were dried for HPLC by molecular sieving and filtered under vacuum. 2,5-Diphenyloxazole (PPO) and *p*-bis-2(5-phenyl-oxazolyl)-benzene (POPOP) were

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purchased from NEN Chemicals (Frankfurt am Main, G.F.R.). Sephadex LH-20 was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden).

The HPLC system consisted of an Altex high-performance liquid chromatograph (Altex Scientific, Berkeley, CA, U.S.A.) equipped with a Model 110A pump, a pressure filter, a Model 153 UV detector with 8-μl flow-through cell, and a Rheodyne Model 7125 20-μl-loop injector. A stainless steel column (20 cm × 4.0 mm I.D.) prepacked with 7-μm spherical microparticulate silica (Nucleosil 50-7) and equipped with a precolumn (5 cm × 4.0 mm I.D.) packed with Polygosil 60-30 precolumn packing was obtained from Macherey-Nagel (Düren, G.F.R.). A stainless steel column (20 cm × 4.0 mm I.D.) prepacked with 7-μm microparticulate octadodecyl silica (Nucleosil 7-C_{18}) was also supplied by Macherey-Nagel and was equipped with a precolumn (3 cm × 4.0 mm I.D.) packed with Bondapak C_{18}/Corasil (Waters Assoc., Milford, MA, U.S.A.). A Model Ultrobeta 1210 liquid scintillation counter (LKB Wallac, Turku, Finland) was used at 15°C for scintillation counting. ^{3}H-labeled fractions were counted for recovery estimations with about 40% counting efficiency in a toluene scintillant containing 4 g of PPO and 50 mg of POPOP per liter of toluene.

Serum extraction

Serum samples were collected from students and healthy adult laboratory workers in September 1982. To each aliquot of serum (2—5 ml) 4500 dpm of 24,25$(OH)_2[^3H]D_3$ was added as an internal standard for calculating the final metabolite recovery. After vortex mixing, the sample was allowed to equilibrate for 30 min and it was then extracted with chloroform—methanol using a modification of the method of Bligh and Dyer [3], as described previously [2]. Briefly, 3.75 volumes of chloroform—methanol (1:2, v/v) were added, and after shaking the mixture was allowed to stand for 30 min. The phases were separated by adding 1.25 volumes of chloroform. The lower chloroform layer was collected and the upper aqueous layer was re-extracted twice with an additional 1.25 volumes of chloroform. The combined chloroform layers were then washed with an equal volume of saturated aqueous sodium chloride solution and evaporated on a rotary evaporator at reduced pressure.

Sephadex LH-20 and HPLC purification of 24,25$(OH)_2$D

The serum extract was then chromatographed at 20°C on a column (10 × 1 cm) containing 2.5 g of Sephadex LH-20 in a solvent system of hexane—chloroform—methanol (9:1:1, v/v). The extract was applied in 0.5 ml of the column solvent and rinsed with an additional 0.5 ml of solvent. The 15—25 ml fraction containing 24,25$(OH)_2$D and a number of other dihydroxylated vitamin D metabolites was collected and dried under nitrogen. HPLC on a Nucleosil 50-7 column equilibrated in propan-2-ol—hexane (1:9, v/v) and eluted at a constant flow-rate of 1.0 ml/min was used for further purification of 24,25$(OH)_2$D. Absorbance of the eluate was monitored continuously at 254 nm. Standards of nonradioactive 25$(OH)D_3$, 24,25-$(OH)_2D_3$, and 1,25-$(OH)_2D_3$ were injected to determine their elution positions. The 24,25$(OH)_2$-$[^3H]D_3$ eluted identically with nonradioactive 24,25$(OH)_2D_3$. The 24,25-

$(OH)_2D$ fraction (5—8 min) was collected and dried under nitrogen.

HPLC quantitation of 24,25$(OH)_2D_3$

The dried extract was redissolved in 25 μl of the eluent, and in the final step serum 24,25$(OH)_2D_3$ was purified by reversed-phase HPLC on a Nucleosil 7-C_{18} column equilibrated in water—methanol (1:9, v/v) at a constant flow-rate of 0.7 ml/min. Absorbance of the eluate was monitored continuously at 254 nm. The 25$(OH)D_3$, 24,25$(OH)_2D_3$, and 1,25$(OH)_2D_3$ were injected to determine their elution positions. The 24,25$(OH)_2[^3H]D_3$ eluted identically with the nonradioactive standard. The 24,25$(OH)_2D$ fraction (7—10 min) was collected into a counting vial, evaporated, and counted in the toluene scintillant along with the initial portion of 24,25$(OH)_2[^3H]D_3$. The height of the 24,25$(OH)_2D_3$ peak was divided by the percentage recovery to yield a corrected peak height, which was related to peak heights of 24,25$(OH)_2D_3$ standards to calculate the amount of 24,25$(OH)_2D_3$ in the original sample. Dividing by the sample volume gave the concentration in ng/ml.

RESULTS

A typical standard curve obtained after injection of 1—32 ng of 24,25-$(OH)_2D_3$ into a Nucleosil 7-C_{18} column and elution as described above was linear (r = 0.999). When the injection of 24,25$(OH)_2D_3$ was repeated, the

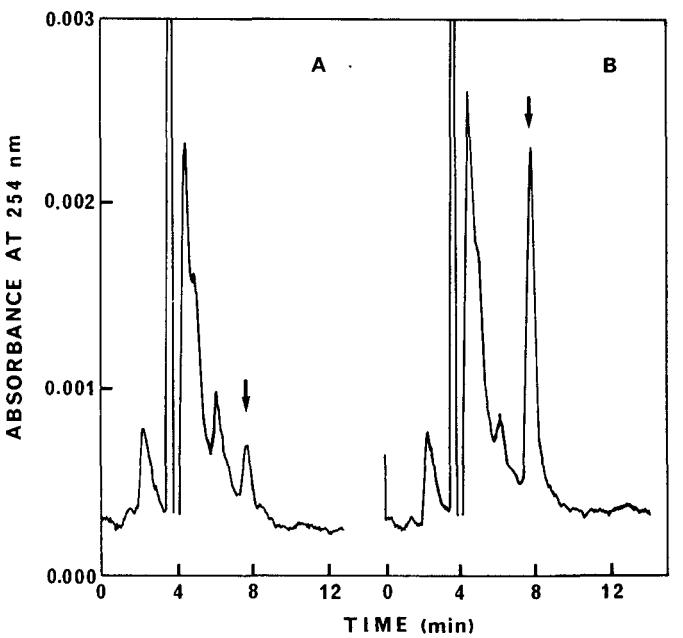

Fig. 1. (A) UV-absorption profile of extract of human serum containing 1.7 ng/ml 24,25-$(OH)_2D_3$. The UV detector was set at 0.005 absorbance units full scale. A reversed-phase column was employed with water—methanol (1:9, v/v) as eluent. The flow-rate was 0.7 ml/min. The 24,25$(OH)_2D_3$ peak elutes about 8 min after injection (arrow). (B) (UV-absorption profile of a similar serum sample to which 8.2 ng/ml 24,25$(OH)_2D_3$ had been added before extraction (100.1% recovery). Conditions for chromatography were as in A.

response was highly reproducible (16.4 ng corresponded to 9.3 ± 0.1 cm S.D., n = 7, coefficient of variation 1.3%). One nanogram of $24,25(OH)_2D_3$ was readily detected using UV detection set at 0.005 absorbance units full scale.

The UV-absorption profile observed after injection and elution of a serum extract is shown in Fig. 1. The $24,25(OH)_2D_3$ was eluted at about 8 min. When standard $24,25(OH)_2D_3$ was added to the serum before extraction, more than 95% of the added amount was measured in the final UV-absorption profile when the peak height was corrected for ^3H-recovery. Addition of $25(OH)D_3$ or $1,25(OH)_2D_3$ did not alter the determination of $24,25(OH)_2D_3$. The recovery of $24,25(OH)_2[^3H]D_3$ was 41.7 ± 7.0% (S.D., n = 10) with a range of 31.8—50.7%. $25,26(OH)_2[^3H]D_3$ was well resolved from $24,25(OH)_2[^3H]D_3$ (not shown). Intra-assay variability was 5.5% (10.0 ng/ml, n = 6) and inter-assay variability 9.3% (10.0 ng/ml, n = 6). In normal adults (age 20—24 years) the $24,25(OH)_2D_3$ concentration was 2.3 ± 0.9 ng/ml (5.5 ± 2.2 nmol/l, mean ± S.D., n = 10) with a range of 0.9—4.2 ng/ml. This was 9.1% of the concentration of $25(OH)D_3$ in the same samples (25.2 ± 6.5 ng/ml). Analysis of serum from rats treated previously with large amounts of vitamin D_2 indicated that $24,25(OH)_2D_2$ is eluted about 1 min later than $24,25(OH)_2D_3$. However, the UV method is not sensitive enough to measure $24,25(OH)_2D_2$ (if present) in serum samples from normal adults.

DISCUSSION

The 25-hydroxylation of vitamin D_3 in the liver is only partially regulated by feedback, whereas further hydroxylations of $25(OH)D_3$ to $24,25(OH)_2D_3$ or $1,25(OH)_2D_3$ in the kidneys are strictly regulated by the concentrations of serum calcium, phosphate and parathyroid hormone [4]. The $24,25(OH)_2D_3$ may play a role in bone mineralization [5]. In various physiological and pathological states and during treatment with vitamin D or its metabolites, disturbances may occur in the metabolic system of vitamin D, which requires that reliable assay techniques are available. The present study describes a reproducible and sensitive technique using UV quantitation for estimating $24,25(OH)_2D_3$ in serum. This technique avoids the variability and interference of unknown UV-absorbing and binding compounds associated with the CPB method [1, 6]. The assay is easily combined with currently used HPLC and CPB techniques for the assay of other vitamin D metabolites in serum [1].

The procedure described above enables assay of serum $24,25(OH)_2D_3$ using UV detection with a coefficient of variation of 9%. All major metabolites of vitamin D are separated from $24,25(OH)_2D$ during the purification and final quantitation steps. If present in high amounts, $24,25(OH)_2D_2$ can be quantitated separately from $24,25(OH)_2D_3$, but with physiological levels the UV method is not sensitive enough to measure $24,25(OH)_2D_2$ (if present). The assay is sufficiently sensitive for use in some clinical studies. With 5 ml of serum, 1.0 ng/ml $24,25(OH)_2D_3$ can be detected (normal range 1—5 ng/ml) based on an average recovery of 42%. The mean concentration of $24,25(OH)_2D_3$ found in serum in this study is in agreement with values obtained with techniques where HPLC has been used for purification [6—11]. A recently published spectrophotometric assay for plasma $24,25(OH)_2D_3$ is similar to

the present method, except that a different extraction procedure and two sequential straight-phase HPLC columns were used [12]. The advantages of final quantitation on a reversed-phase column are that less UV-absorbing material is eluted at the elution position of $24,25(OH)_2D_3$ and that the elution time is shortened with increased peak sharpening.

ACKNOWLEDGEMENTS

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Note

Separation of porcine zona pellucida components by high-performance liquid chromatography on styro-gel protein columns

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The zona pellucida is an acellular glycoprotein-like structure surrounding the mammalian oocyte. The chemical and physical properties of the zona have been the subject of intensive research during the last ten years. Because of the immunological similarities between human and pig zona, research has focused on this particular topic [1—4]. Despite this intensive effort little is known about the molecular structure of the zona because of the difficulties involved in obtaining adequate separation of the individual components.

Since it has been shown that some of these components might play a role in immunological fertility [5—7], it is imperative that a method is developed to separate the solubilized zonae into individual fractions in order to characterize the biochemical properties of each component.

Using purification methods such as gel filtration or sodium dodecyl sulphate—polyacrylamide gel electrophoresis (PAGE-SDS), only one to four fractions could be obtained by various investigators [2, 8, 9]. In this paper, we report the solubilization of porcine zona pellucida glycoproteins by lithium-3,5-diiodosalicylate and subsequent purification of the components on two styro-gel protein columns by high-performance liquid chromatography (HPLC). The solubilization procedure is similar to the one described by Dietl et al. [10], who showed that four glycoproteins react with specific anti-porcine zona serum. By this procedure it was possible to separate up to eleven individual glycoprotein fractions, as opposed to the conventional techniques that have previously been used.

EXPERIMENTAL

Isolation of zonae pellucidae

Porcine zonae, approx. 50,000, were isolated according to the modified

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method of Gwatkin et al. [8] and Dunbar et al. [2]. The procedure was briefly as follows. Fresh pig ovaries were obtained from the local slaughterhouse, chopped up and the suspension was filtered through a 210-μm nylon screen followed by a 75-μm nylon screen. The cell debris was digested with collagenase (Sigma, St. Louis, MO, U.S.A.) at a concentration of 1 mg/ml for 30 min at 37°C. Following incubation with the collagenase the suspension was filtered as described above.

The isolated zonae remaining on the nylon screen were checked under a microscope for purity and only batches with a contamination of less than 1% oocytes were used for the solubilization process.

Solubilization of zonae

The solubilization procedure was basically the same as described by Dietl et al. [10]. Approx. 50,000 zonae were suspended in 1 ml of 0.3 *M* lithium-3,5-diiodosalicylate in 0.05 *M* Tris—HCl buffer, pH 7.5. The suspension was stirred for 15 min at room temperature; the volume was doubled with bi-distilled water and the mixture was reincubated for 10 min.

The mixture was then centrifuged at 45,000 *g* for 90 min at 4°C. The sediment was discarded and the supernatant mixed with a 50% phenol—water solution (1:1, v/v) to eliminate possible contamination by unspecific proteins in the glycoprotein mixture. The suspension was then stirred for another 50 min at 4°C, centrifuged at 4000 *g* for 60 min (4°C) and the aqueous phase separated. The latter was then dialysed for 48 h against several changes of bidistilled water at 4°C. The dialysate was lyophilized and the glycoproteins were precipitated in absolute ethanol. The precipitate was then collected by centrifugation at 4000 *g* for 60 min at 4°C, and dissolved in 1 ml of phosphate-buffered saline (0.05 *M*, pH 7.3).

High-performance liquid chromatography

The high performance liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) was equipped with a M 6000 pump (420 bar, 0.1—9.9 ml), a septumless sample injector U6K (injector loop capacity 1 μl to 2 ml), and an integrator. The absorption was monitored by a constant-wavelength detector (M 440) at 254 nm. Styro-gel protein columns (Waters) of I-125 and I-60 were used with dimensions of 30 × 0.78 cm and with pore sizes of 125 Å and 60 Å, respectively. The molecular weight separation range for the I-60 column is given by the manufacturers as 1000—20,000 for globular proteins and 600--8000 for proteins of random coil configuration. For the I-125 column the molecular weight ranges were indicated as 2000—80,000 for native globular configurations and 1000—30,000 for random coils. For mobile phases either 0.06 *M* phosphate buffer, pH 7.4, or 0.2 *M* formic acid in 6 *M* urea was used at a flow-rate of 2.5 ml/min. Chromatography was carried out either with the I-125 column alone or with the I-60 used as a precolumn.

The following molecular-weight markers were included: ribonuclease M_r = 13,700, chymotrypsinogen A M_r = 25,000, ovalbumin M_r = 43,000, and bovine serum albumin M_r = 67,000 (Pharmacia, Freiburg, G.F.R.).

For qualitative analyses approx. 100 μg of protein were injected at a time, for preparative HPLC up to 1 mg of protein, and the various fractions

collected. For purification purposes, repeated injections of the same fractions were made until homogeneity was obtained.

Determination of the N-terminal amino acids

The N-terminal amino acids were detected according to the Dns chloride method. The purified HPLC fractions were lyophilized and dissolved in 10 μl of 0.2 *M* $NaHCO_3$. This mixture was then incubated with 10 μl of a 9.27 μM Dns chloride solution in acetone. This was followed by a 60-min incubation at 37°C. The mixture was then dried over sodium hydroxide and hydrolysed in 50 μl of 6 *M* hydrochloric acid for 24 h at 110°C. After hydrolysis the reaction mixture was dried again, dissolved in 50% pyridine and the individual Dns amino acids were identified by thin-layer chromatography (TLC) on polyamide sheets (Nano-plates F 1700, Schleicher und Schüll, Dassel, G.F.R.), in three different systems: 1.5% (v/v) formic acid, benzene—acetic acid (9:1, v/v), and ethyl acetate—methanol—acetic acid (20:1:1, v/v), where the first solvent was used in one direction and the last two successively in the second direction. Dns derivatives of the 21 amino acids (Sigma) served as standards.

RESULTS AND DISCUSSION

A typical HPLC elution profile of the solubilized zonae is shown in Fig. 1. As can be seen, a clear-cut separation of at least nine individual components with molecular weights between > 100,000 and 8200 could be obtained compared to molecular weight standards of ribonuclease (M_r = 13,700), chymotrypsinogen A (M_r = 25,000), ovalbumin (M_r = 43,000), and albumin (M_r = 67,000). These were found to be: M_r > 100,000 (1), = 78,000 (2), = 40,000 (3), = 30,000 (4), = 27,000 (5), = 25,000 (6), = 18,000 (7), = 16,000 (8), = 8200 (9). For calibration of the molecular weights the data module was set to calculate the linear least-square fit for each of the standards, according to the

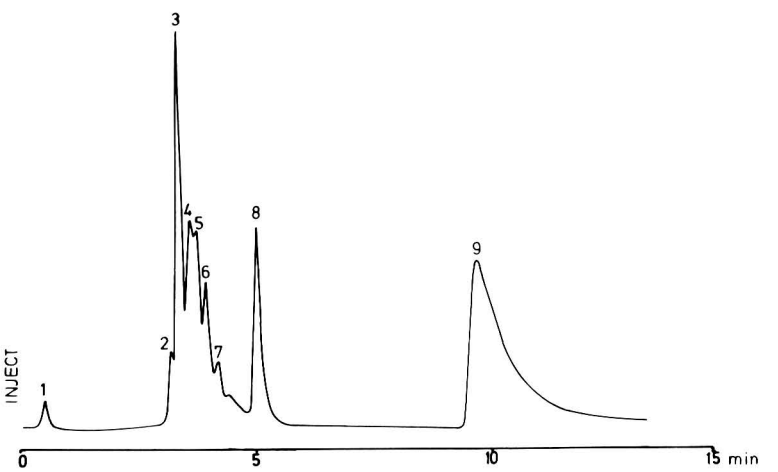

Fig. 1. Elution profile of 50,000 solubilized zonae pellucidae by HPLC using the I-125 protein column. Molecular weights are: 1, > 100,000; 2, 78,000; 3, 40,000; 4, 30,000; 5, 27,000; 6, 25,000; 7, 18,000; 8, 16,000; 9, 8200 (mobile phase 0.06 *M* phosphate buffer, pH 7.4; flow-rate 2.5 ml/min; wavelength 254 nm).

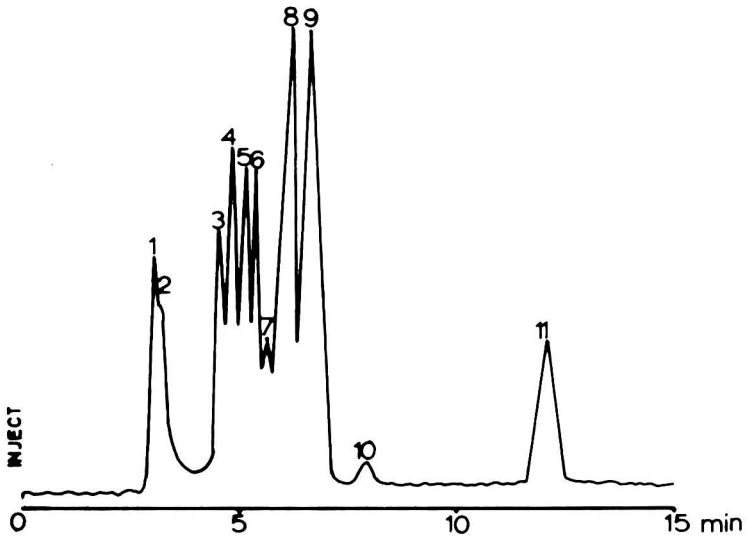

Fig. 2. Elution profile of the solubilized glycoproteins from 50,000 zonae pellucidae by HPLC, using the I-125 column and the I-60 precolumn (mobile phase 0.2 *M* formic acid in 6 *M* urea; flow-rate 2.5 ml/min; wavelength 254 nm).

equation $M_r = D_0 + D_1 (t_R)$, where the calibration coefficients D_0, and D_1 were computed for each experiment and t_R is the retention time of the various standards. Standard errors of estimation ranged from 0.072 to 0.076 and correlation coefficients equalled 0.99.

Fractions 2, 3, 4 and 8 isolated here showed molecular weights similar to those found earlier using immunoaffinity chromatography [8]. In addition, one component with a molecular weight of 40,000 and N-terminal residue of alanine (Ala) has been found to possess boar spermatozoal receptor properties [11].

As can be seen from Fig. 2, it is possible to isolate up to eleven components using the I-60 precolumn and 6 *M* urea in the mobile phase, thus obtaining an even better separation of the individual components (additional peaks 9 and 10). By repeated injections of the four fractions mentioned above, each of these fractions could be purified to homogeneity using the I-125 column alone. This was further confirmed by the fact that only one N-terminal amino acid was found in each of these fractions. The N-terminal amino acids were Arg for M_r = 78,000, Ala for M_r = 40,000, Ala for M_r = 30,000, and H is for M_r = 16,000. This indicates that with this purification procedure it is possible to obtain the various components in a pure enough form that biochemical analysis of the molecules can be carried out.

This seems a much better separation procedure than the various other methods that have been used to date. Gwatkin et al. [8] and Dunbar et al. [2] could only demonstrate ill-defined fractions by PAGE-SDS. Dependent on the solubilization procedure the most homogeneous fractions that have been found up to now were reported by Menino and Wright [9]. They isolated four individual fractions by PAGE-SDS.

Depending on the pH and/or ionic strength of the various mobile phases that we have tried besides 0.2 *M* formic acid and 6 *M* urea, we observed slight varia-

tions in retention times for the same compounds. However, retention times of the same components relative to the molecular-weight markers remained unchanged in a particular mobile phase. Therefore we assume that factors other than merely gel exclusion, i.e. pH, hydrophobic interactions with the solid phase and ionic strength, affect partition coefficients for a given compound.

Purification of zona components is time-consuming and difficult because of the possibility of denaturation of these molecules during prolonged purification procedures. In addition, common methods like gel-filtration chromatography and PAGE-SDS do not give satisfactory separation of the various zona components. Separation of the zona components by HPLC as described here requires 15 min, and collection of the various fractions makes it possible to carry out biochemical analyses in a relatively short time.

Since it was shown previously that porcine zona pellucida antigens cross-react with anti-human zona sera [1], the method presented here could enable the development of an immunological test as a diagnostic aid in immunological sterility cases.

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Note

Protein titration curves using modified cellulose acetate membranes

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Righetti et al. [1] first introduced titration curves in polyacrylamide gel slabs for the study of proteins and their genetic variants. We have modified the technique for use with cellulose acetate membranes and have thereby made more economical use of the carrier ampholyte. An essential requirement of such a modification is the formation of a suitable pH gradient and its subsequent stability during electrophoresis in the second dimension. Methylated cellulose acetate as described by Ambler and Walker [2] provides a suitable alternative to polyacrylamide and has been used in this work to investigate haemoglobin variants.

MATERIALS AND METHODS

Membrane preparation

Sepraphore III cellulose acetate membranes (Gelman, Hawksley Ltd., Northampton, Great Britain) were methylated according to the method of Ambler and Walker [2]. The membranes (12.5 × 14.5 cm) were taken through increasing concentrations of methanol until they were equilibrated with absolute methanol, after which they were transferred to methylating reagent, 4% boron trifluoride in methanol, and incubated in this solution for 45 min at 46°C. The treated membranes were rinsed in three washes of methanol and stored in methanol. They were drained of excess solvent before being placed in ampholyte solution: 7.5% Ampholine (LKB) pH range 3.5—10, glycerol 6%, 6 m*M* each of aspartic acid (Asp), glutamic acid (Glu), lysine (Lys) and arginine (Arg). An 8-ml volume of solution was sufficient for two 12.5 × 14.5 cm membranes. Equilibration was allowed to take place for at least 8 h, occasionally agitating the membranes.

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Isoelectric focusing and electrophoresis

A membrane was removed from the ampholyte solution, held vertically for 10–20 sec to drain and placed on a 12.5 × 26 cm LKB glass plate. Care was taken not to allow air bubbles to enter between the membrane and the plate. A 14.5-cm length of electrode wick, soaked in 0.3 *M* ethanolamine, was placed at the cathode and another, soaked in 0.2 *M* citric acid, was placed at the anode. To establish the pH gradient isoelectric focusing (IEF) was carried out in the first dimension using the LKB Multiphore 2117 apparatus with a 2103 power supply and maintaining a constant 3.5-W power at 3–6°C for ca. 1.75 h. The voltage across the membrane rose from an initial ca. 350 V to ca. 700 V on completion of focusing, with the degree of wetness of the membrane and electrode wicks causing some variation. A marker haemolysate (2 μl) was applied close to the anode and another close to the cathode at one edge of the membrane; the movement of the two markers into one set of focused zones signalled the completion of IEF.

The electrode wicks and 1.5 × 14.5 cm strips of membrane were then cut from the anodal and cathodal edges and discarded. A vertical section 2 × 9.5 cm containing the focused marker was also removed, leaving a membrane measuring 9.5 × 12.5 cm. This was lifted in one quick movement from the glass plate, moved through a 90° angle and carefully positioned, again in one movement, on to a second glass plate. New electrode wicks, 9.5 cm in length and soaked in the same solutions as for IEF, were applied to the membrane. Approximately 2 μl of the haemoglobin solution (25 g/l) under investigation were applied across the membrane in the direction of the pH gradient. Electrophoresis in the second dimension was carried out at a constant 300 V for 30 min at 3–6° C. The pH gradient existing at the end of this procedure was determined by cutting 5 × 10 mm sections across the membrane and eluting the carrier ampholytes into 200 μl of 10 m*M* potassium chloride and measuring the pH of the resulting solutions.

Fixing and staining were carried out simultaneously for 15 min in the following solution: Coomassie Brilliant Blue R 250 2.5 g, methanol 455 ml, deionised water 455 ml, glacial acetic acid 90 ml. Destaining was carried out in a solution containing methanol 500 ml, deionised water 500 ml, glacial acetic acid 100 ml, until the background was clear. The membrane was then transferred to 0.83 *M* acetic acid; it could then be photographed in reflected light without drying.

RESULTS AND DISCUSSION

Fig. 1 shows the pH measurements taken across the membrane after electrophoresis. The gradient is not linear and shallows out in the pH range 7–8.5. This has the effect of enhancing separation of proteins with isoelectric points (p*I*) in that range. Ampholyte mixtures can be altered to create optimum separations over different pH ranges. Haemoglobin A (p*I* 7.0) and C (p*I* 7.4) form two well separated curves as shown in Fig. 2. Other faint titration curves can be seen and represent other proteins present in the haemolysate; the more pronounced of these lines is found halfway between the two haemoglobin curves and is probably the hybrid molecule described by Righetti's group.

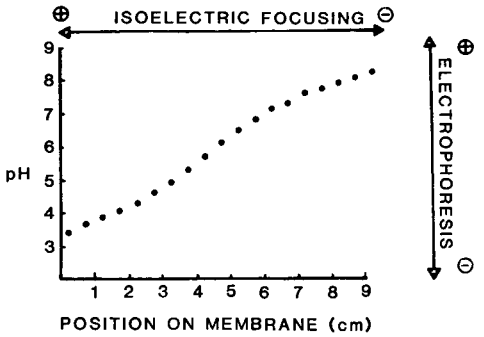

Fig. 1. pH gradient established in Sepraphore III membranes after isoelectric focusing followed by electrophoresis.

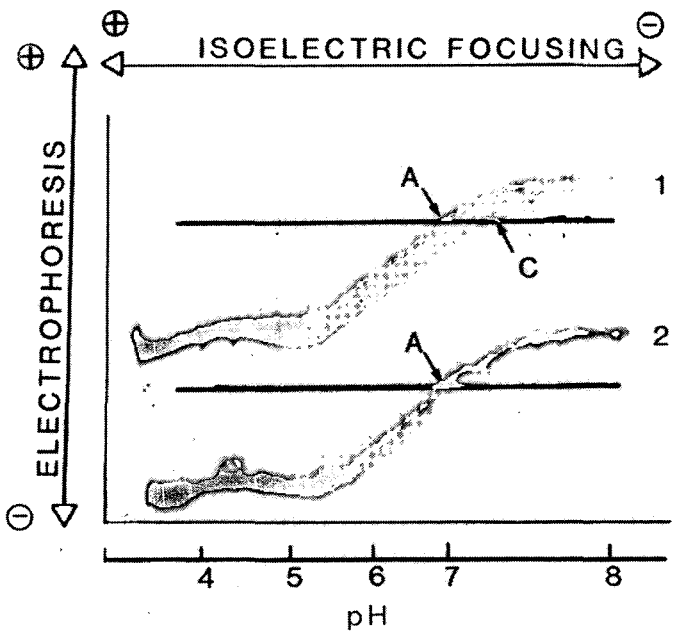

Fig. 2. Titration curves of haemoglobin A + C (1) and haemoglobin A (2) on Sepraphore III membranes. The arrows indicate the p*I* values as points of intersection with the application lines.

Instability of both haemoglobin species is demonstrated by the blurring of the curve in the acid regions of the membrane.

The proposed support medium has a number of advantages over polyacrylamide gel. It is non-toxic, easier to handle and prepare and, once methylated, membranes can be stored in methanol for up to four weeks. Staining and destaining times are short. Smaller quantities of protein can be analysed and the problems associated with molecular sieving are reduced; the medium should therefore be more suitable when large protein molecules are under investigation. Two membranes take up half the amount of carrier ampholyte used by

the original method [1] and slightly less than the reduced LKB modification [3], with the added saving that two haemolysates instead of one can be run on a single membrane.

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CHROMBIO. 1685

Note

Electron-capture—gas chromatographic analysis of ifosfamide in human plasma and urine

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(First received November 24th, 1982; revised manuscript received February 23rd, 1983)

Ifosfamide (IFA) is an anticancer agent that has recently been approved for use in the United States for treatment of non-oat cell bronchogenic carcinoma [1—4]. This drug has also been indicated for use in the treatment of a wide range of human cancers such as lymphoma, ovarian and testicular cancers [5]. Few analytical procedures have been developed for quantitation of this drug following human administration. Pharmacokinetics have been studied by radiochemical analysis of [^{14}C]ifosfamide [6] and by gas chromatography (GC) [4, 7]. This study describes the use of an electron-capture—gas chromatographic (EC—GC) method for IFA determination using heptafluorobutyric anhydride (HFBA) for derivatization.

EXPERIMENTAL

Chemicals

Ifosfamide and cyclophosphamide (CPA) (internal standard) were a gift from Mead Johnson (Evansville, IN, U.S.A.). The derivatizing agent heptafluorobutyric anhydride (HFBA) was obtained from Pierce (Rockford, IL, U.S.A.) and reagent-grade methylene chloride and pyridine were purchased from Mallinckrodt (St. Louis, MO, U.S.A.).

Instrumentation

A Hewlett-Packard 5730A gas chromatograph with a ^{63}Ni (15 mCi) electron-capture detector and 3390A integrator were used in all experiments. Chromatography was performed on a 1.8 m × 4 mm I.D. glass column with 3% SE-30 on Gas-Chrom Q, 100—120 mesh. The column temperature was maintained at 170°C, injection port at 250°C, EC detector at 300°C with a methane—argon (5:95) carrier gas flow-rate of 35 ml/min.

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Sample collection and extraction

Informed consent was obtained from patients with documented non-oat cell bronchogenic carcinoma. IFA doses of 1.2 g/m^2 body surface area were given intravenously (dissolved in 1 l of 5% dextrose in water). Blood samples were drawn in heparinized tubes and plasma was used for IFA analysis. Urine samples were collected periodically throughout the procedure. Plasma or urine (500 μl) was added to a 20-ml screw-capped glass tube containing 10 μg of internal standard dissolved in water (10 μg CPA per ml). To the tubes were also added 1.0 ml of 10% sodium bicarbonate and 10 ml methylene chloride. The samples were shaken for 10 min and centrifuged at 6300 *g* for 10 min. The aqueous layer was aspirated and 9.5 ml of the organic layer removed and added to a new screw-capped tube and dried under a stream of dry air. To the dried residue were added 200 μl of HFBA and the samples were heated at 80°C for 20 min. After derivatization the samples were again dried with a stream of dry air and 200 μl of pyridine added to each sample before injection.

Calculations

Calibration curves were constructed by plotting the peak area ratio IFA/CPA by the ratio of molar quantities of IFA and CPA. After determining the molar quantity of drug in a sample this was multiplied by the molecular weight of IFA (260 g/mol) and divided by the ml of sample to obtain final values in μg/ml plasma or urine.

RESULTS AND DISCUSSION

Fig. 1a and b presents typical chromatograms of normal blank plasma and a plasma sample 1 h following intravenous (i.v.) administration of IFA (internal standard included), respectively. The retention times for IFA and CPA are 4.84 and 6.67 min. Identification is achieved via retention time and peak superimposition, i.e., by injection of IFA and CPA standards (20—50 ng) along with previously extracted samples and observing the increased peak area and height at the corresponding retention times.

The limit of detection (signal-to-noise ratio of 2:1) of this assay was 1 ng/ml plasma or urine and the extraction recovery was 85 ± 5% (n = 25). Repetitive injections of standards and samples gave good reproducibility of retention times (coefficients of variation, C.V. ± 2.5% and 3.1%, respectively). Standard curves were linear in the range of 1—25 μg/ml media and day-to-day reproducibility varied less than 3.7% C.V. Standard stock solutions of the drugs were not stable when stored at —20°C for two days. Variable losses of 5—20% during this time were observed; therefore, fresh samples should be prepared daily. Samples extracted from biological media, derivatized, and stored at —70°C overnight were noted to have variable losses of up to 15%. All samples reported in this paper were analyzed within 4—6 h following extraction and derivatization.

A difference in the response of the electron-capture detector was noted by the use of methane—argon (5:95) as opposed to 100% nitrogen carrier gas. The methane—argon mixture produced approximately a 12% increase in signal

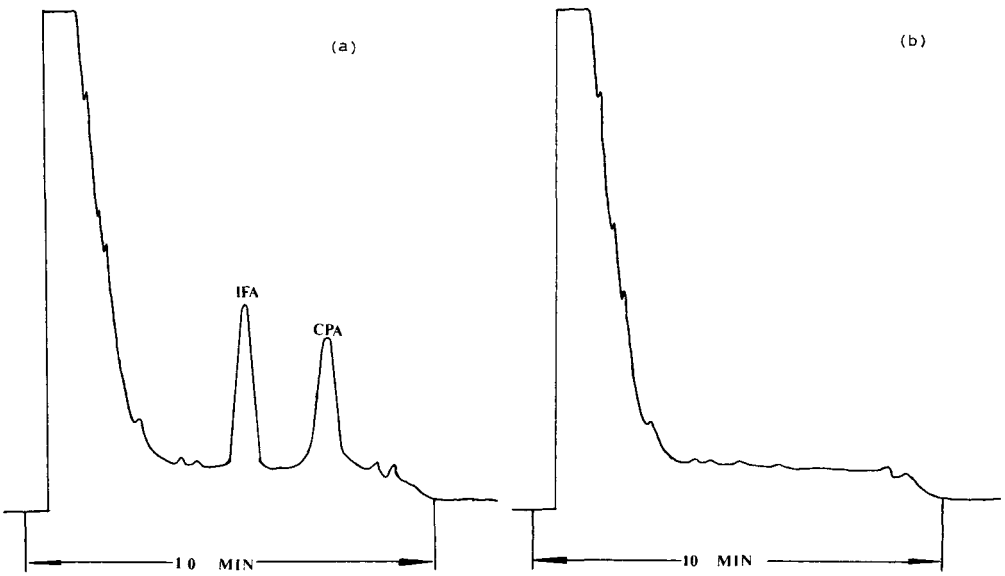

Fig. 1. (a) EC—GC chromatograms of IFA and CPA (internal standard) with retention times of 4.84 and 6.67 min, respectively, extracted from human plasma 2 h following i.v. administration. (b) EC—GC chromatogram of blank human plasma.

TABLE I

CONCENTRATION OF UNMETABOLIZED IFOSFAMIDE (IFA) IN HUMAN PLASMA AND URINE

Time* (h)	IFA**	
	Plasma	Urine
0	—	—
0.5	22.10 ± 1.35	2.41 ± 0.99
1	7.05 ± 0.97	6.02 ± 1.07
1.5	—	14.46 ± 1.39
2	5.36 ± 0.91	50.60 ± 1.72
7	4.16 ± 0.85	—
8	—	36.75 ± 1.60

*Time after an i.v. dose of 2.0 g IFA.
**Levels expressed in μg drug per ml media (average of three determinations ± S.D.).

intensity of the HFBA-derivatized drug. This was probably due to the greater amount of scavenger methane gas in the carrier gas that traps more thermal electrons [8]. Regardless, the nitrogen carrier gas gave an acceptable baseline for EC—GS analysis. Other derivatizing agents [trifluoroacetic anhydride (TFAA) and pentafluoropropionic anhydride (PFPA)] were evaluated for use in this procedure (obtained from Pierce). Following the derivatization procedure described in the Experimental section it was found that the HFBA derivative gave the greatest detector response. The HFBA response was 9% better than PFPA and 20% more intense than that of TFAA. It was also noted that the HFBA and PFPA derivatives were more stable upon standing at room

temperature for 6 h as opposed to TFAA (variable losses up to 15%). For these reasons, HFBA was chosen as the derivatization agent of choice.

In Table I are given the levels of μg/ml plasma or urine of IFA after an i.v. administration of 2.0 g IFA. The unmetabolized drug peaks in about 2—3 h in urine samples. It is still detectable in samples up to 24 h later. These data are within agreement of previously reported data from this laboratory using a flame ionization GC procedure for analysis [4].

In conclusion, this EC—GC method provides a specific and sensitive determination of IFA in human plasma and urine samples following therapeutic dosing with the drug. A number of improvements have been made in the analytical methodology (such as derivatization agent, carrier gas composition, extraction solvent, and stability studies) over the previously reported procedures [4, 7] that greatly increase this assay's general utility for clinical analysis.

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Note

High-performance liquid chromatographic analysis of tamoxifen and major metabolites in human plasma

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Tamoxifen (ICI.46,474, Nolvadex), a synthetic non-steroidal antiestrogenic compound, is currently being used in metastatic breast cancer therapy [1]. The primary metabolic route involves ring hydroxylation yielding the 4-OH derivative (Ib) and hepatic demethylation to Ic. Both compounds Ib and Ic have some antitumor activity [2—5].

The analytical methods proposed to determine tamoxifen (Ia) and metabolites in biological fluids are high-performance liquid chromatography (HPLC) (or thin-layer chromatography) with fluorimetric detection after photocyclisation to phenanthrenes [6—9], and gas chromatography—mass spectrometry with high-resolution, single ion monitoring [10].

We found several problems in the routine use of the analytical methods reported in the literature, and therefore modified the HPLC analysis proposed by Golander and Sternson [6] in the following way: (1) internal standard (Id) added to the plasma samples; (2) extraction of Ia—d by Sep-Pak cartridges; (3) post-column on-line photocyclisation to the corresponding phenanthrenes (IIa—d).

MATERIALS AND METHODS

Analytical standards of Ia—c were kindly supplied by Imperial Chemical Industries (Macclesfield, Great Britain) and used without further purification. The internal standard (Id) was synthesized by acetylation of Ic with acetic anhydride. Other chemicals were supplied by E. Merck (Darmstadt, G.F.R.) or Carlo Erba (Milan, Italy) and were of the highest analytical grade.

Sample preparation

Plasma samples were obtained by adding to the blood bank plasma known amounts of Ia—c, or by centrifugation of heparinised venous blood samples from advanced cancer patients undergoing tamoxifen therapy.

To the plasma samples (1 ml) were added 100 μl of a stock solution of the internal standard (Id) in methanol, and 2 ml of water—methanol (1:1). After vortexing (20 sec), the mixture was centrifuged at 5000 *g* for 5 min and the supernatant was filtered through a Sep-Pak C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.) previously washed with methanol (5 ml) and distilled water (5 ml).

The cartridge was then eluted with water (5 ml), water—acetonitrile (1:1) (1 ml) and acetonitrile (0.5 ml). These eluates did not contain Ia—d and were discarded.

The cartridge was finally eluted with 5 ml of 0.3 *M* phosphoric acid in acetonitrile. The eluate was concentrated under vacuum to 0.5 ml in a Vortex evaporator (Buchler Instrument, Fort Lee, NJ, U.S.A.). After the addition of 0.5 ml of 10 m*M* KH_2PO_4, 100 μl of this sample were injected into the chromatographic system.

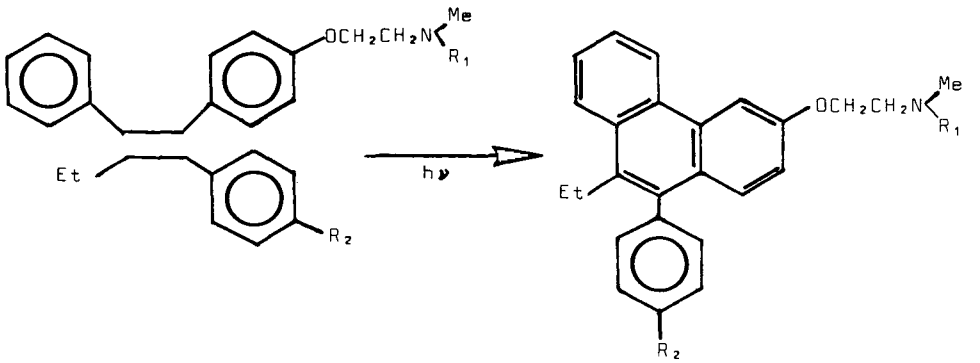
During the preliminary study of the photolysis conditions, a known amount of desipramine (10,11-dihydro-N-methyl-5H-dibenz[*b*,*f*]azepine-5-propanamine), which is naturally fluorescent in the detector conditions, was added to the plasma samples as an additional internal reference.

Chromatography and photocyclisation

The chromatographic system consisted of a Rheodyne Model 7105 injector or a Perkin-Elmer Model 420 Autosampler, a Perkin-Elmer series 3B pump, a Model 650-10S spectrofluorimeter and a Sigma-10 data system.

To the end of the chromatographic column (μBondapak CN, reversed-phase, 10 μm, 30 cm × 3.9 mm, Waters Assoc.) was connected a PTFE capillary tube (Angst+Pfister AGW-28, 0.3 mm I.D.) 5 m long, wound 10 cm away from a Philips HPK-125W high-pressure mercury lamp, mounted inside a 28 × 22 × 22 cm housing. The outlet was directly connected to the fluorescence detector set at λ_{ex} = 260 nm (slit = 10 nm), λ_{em} = 375 nm (slit = 10 nm). This setting guarantees a more favourable signal-to-noise ratio; under these conditions the minimum detectable quantity of 2a—d is less than 0.1 ng.

Photocyclisation of tamoxifen and metabolites (Ia—c), as well as of internal standard (Id), to the corresponding phenanthrene derivatives (IIa—d) (Fig. 1) and complete chromatographic separation of the fluorescent products was achieved with a mobile phase of acetonitrile—0.3 *M* H_3PO_4—10 m*M* KH_2PO_4 (190:50:280) at a flow-rate of 1.5 ml/min.



R_1	R_2	
CH_3	H	Ia, IIa
CH_3	OH	Ib, IIb
H	H	Ic, IIc
$COCH_3$	H	Id, IId

Fig. 1. Photocyclisation of tamoxifen, its metabolites and the internal standard.

Alternatively, the photocyclisation to the phenanthrenes (IIa—d) was carried out by irradiation of the samples obtained in the extraction procedure directly in the Autosampler mini-vials (borosilicate glass, Supelco). In this case, a photochemical reactor was used, consisting of a Philips HPK-125W lamp (placed in the middle of a housing, 80 × 80 × 100 mm, with a 10 × 20 mm slit) and a rotating sample holder (5 cm diameter, 6 vial holders, 60 rpm) with the centre placed 15 cm away from the slit.

RESULTS

Figs. 2a—d are typical chromatograms obtained under different photolysis conditions. Without previous photolysis and with the on-line photoreactor switched off, no peaks were observed, with the exception of desipramine (Fig. 2a), naturally fluorescent and added as internal reference. With 60 min of off-line photolysis and injection of the photocyclisation products, chromatogram 2b was obtained; retention times corresponded to the phenanthrenes (IIa—d). With no previous off-line photolysis and the on-line photoreactor on, chromatogram 2c was obtained. Chromatogram 2d corresponds to the analysis of a sample only partially photolysed off-line (20 min photolysis) and injected into the chromatographic system with the on-line photoreactor switched on. In this case, both phenanthrenes (II) and stilbenes (I) were injected.

The on-line photoreactor gives only partial conversion of Ia—d to phenanthrenes (Ia → IIa, 81.2%, S.D. = 0.45; Ib → IIb, 95.7%, S.D. = 1.8; Ic → IIc, 89.9%, S.D. = 1.3; Id → IId, 81.2%, S.D. = 0.7; mean of five determinations calculated using desipramine as the internal standard). This notwithstanding,

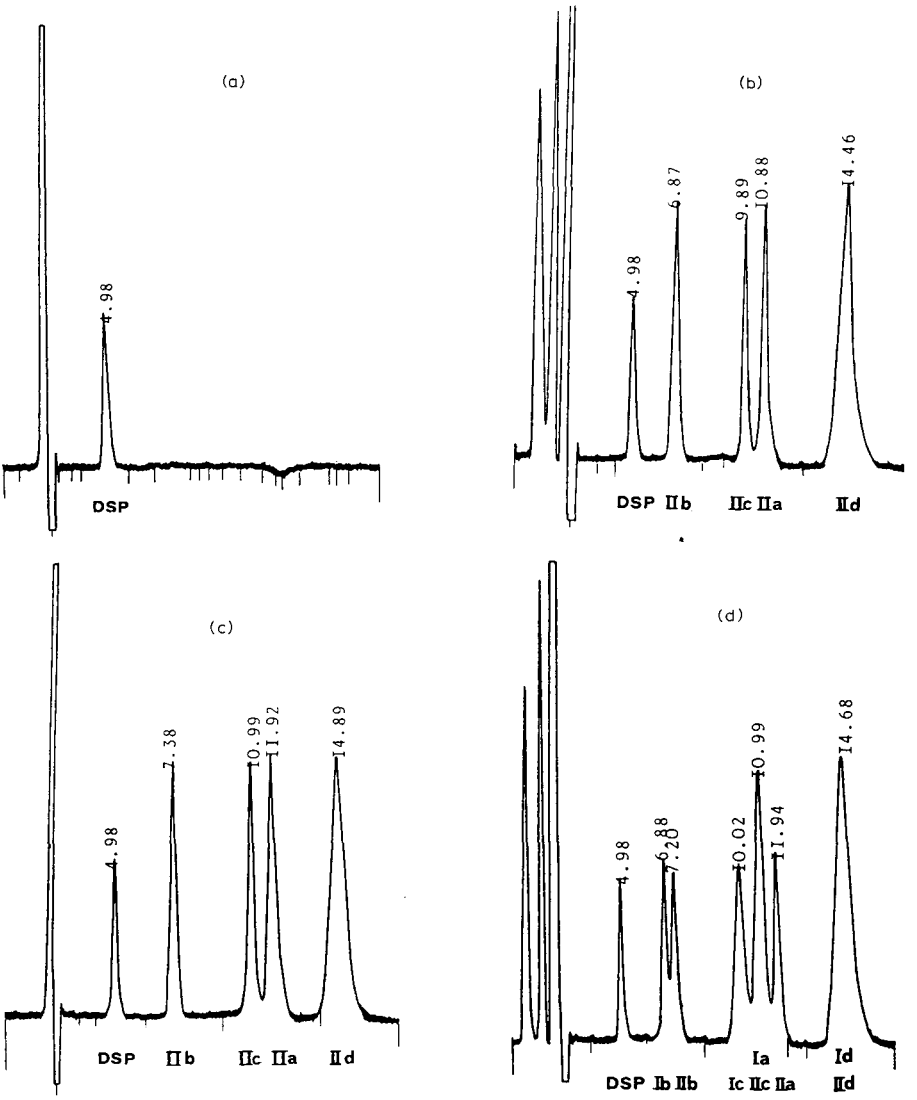


Fig. 2. HPLC analysis of tamoxifen and major metabolites: (a) without on-line photolysis; (b) without on-line photolysis (the sample was previously photolysed off-line); (c) with on-line photolysis; (d) with on-line photolysis and partial off-line photolysis. DSP = desipramine.

the reproducibility is quite satisfactory (Table I) and the calibration factor does not depend on the flow-rate within range 0.7—2.5 ml/min.

Fig. 3 shows the effect of the pH of the mobile phase on the capacity factor k' of tamoxifen (Ia), its metabolites (Ib and Ic), and internal standard (Id). As expected, an increase in pH leads to an increase in the retention time of the basic products (Ia—c), whereas k' for the neutral internal standard (Id) slightly decreases. The optimum operating pH range was, in our experience, 2.0—2.5. It is to be noted that by using reversed-phase C_{18}

TABLE I

REPRODUCIBILITY OF THE ANALYTICAL METHOD

Ia			Ib			Ic		
Added (ng)	Found (ng)	Recovery (%)	Added (ng)	Found (ng)	Recovery (%)	Added (ng)	Found (ng)	Recovery (%)
0.91	1.08	118.7	1.00	1.25	125.0	1.05	0.95	90.5
9.14	10.10	110.5	10.04	10.40	103.6	10.49	12.10	115.3
18.28	17.40	89.7	20.10	20.60	102.5	20.98	18.30	87.2
45.70	43.20	94.6	50.20	52.10	103.8	52.45	52.70	100.5
91.39	91.90	100.6	100.40	93.70	93.3	104.90	107.10	102.1
182.80	172.80	94.5	200.80	208.40	103.7	209.80	210.80	100.4
Mean		102.8			105.6			99.1
S.D.		11.8			11.7			11.1
Mean coefficient of variation (%)		11.5			11.0			11.0

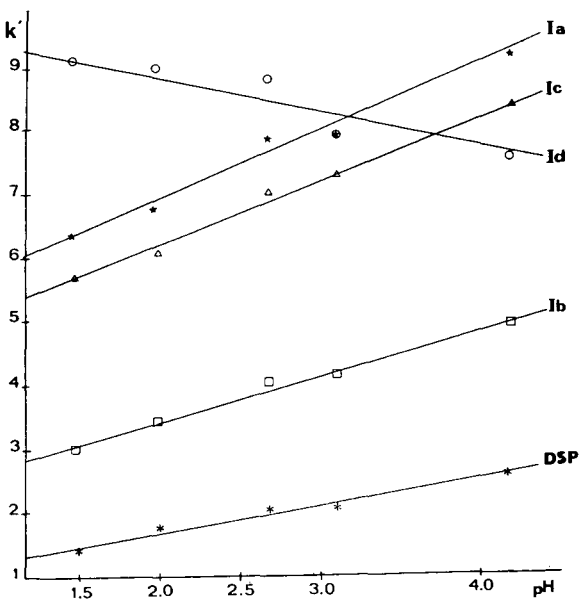


Fig. 3. Influence of the pH of the mobile phase on the capacity factor k'.

or C_8 columns, Id is not a good choice as internal standard because of its rather low retention time.

Fig. 4 reports the plasma levels of tamoxifen and its metabolites determined in one patient treated with a single oral dose of 20 mg. This is the daily dosage currently used in breast cancer management; as can be seen, plasma levels of tamoxifen and metabolites are easily monitored, and well above the sensitivity limit of the analytical method (< 1 ng/ml).

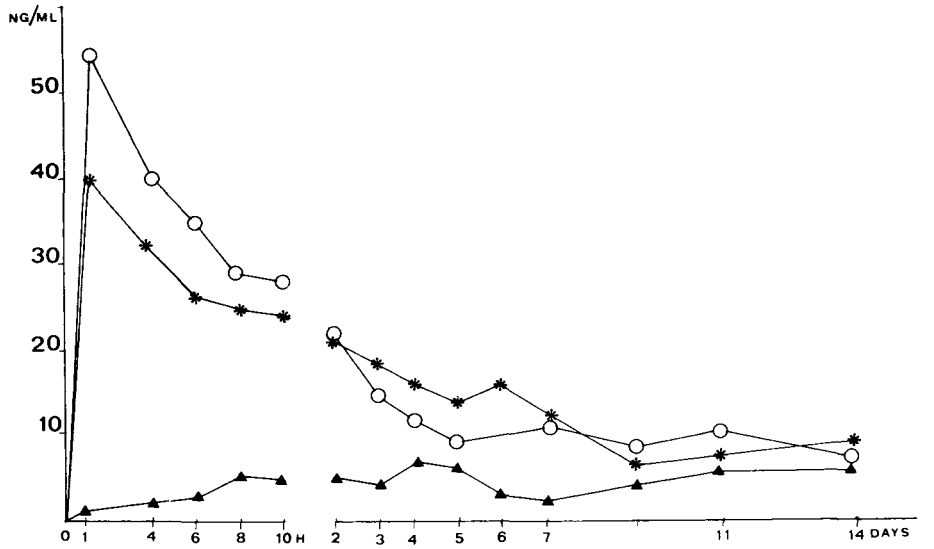


Fig. 4. Plasma levels of tamoxifen (○) and metabolites Ib (▲) and Ic (∗) after a single oral 40-mg dose. Patient D.M.G., normal renal and liver functions.

DISCUSSION

Diethyl ether extraction of the plasma samples, redissolving in the HPLC mobile phase after evaporation of the organic solvent, and photocyclisation — as suggested in the literature — led to erratic results when applied to plasma routinely obtained from advanced cancer patients, who were rather heavily treated with antineoplastic therapies. In addition, under the conditions reported in the literature, in our hands, only the hydroxylated derivative (II) had an acceptable chromatographic behaviour.

Surprisingly enough, with two commercially available reversed-phase columns (Supelcosil LC_{18}, 15 cm × 4.6 mm I.D., 5 μm particle size, and the Merck LiChrosorb RP-18, 25 cm × 4.6 mm I.D., 5 μm particle size) we were not able to elute tamoxifen (or its corresponding phenanthrene derivative) even by using 95% methanol plus 2.5% acetic acid and 2 m*M* sodium pentanesulphonate as the mobile phase.

With these stationary phases, the analysis was successful only by using the stronger acid trifluoroacetic acid, instead of acetic acid. Better results were obtained with a Supelcosil LC-8 column [mobile phase acetonitrile—water (55:45) plus 2.5 m*M* sodium pentanesulphonate and 0.1% trifluoroacetic acid) but the column life was too short to allow routine use of the method.

Solid-phase extraction with C_{18} reversed-phase cartridges, on-line photolysis and use of internal standard, led to a drastic improvement in the reliability of the analysis. In addition, the reduction of the work-up time allows a trained operator to process a batch of 20 samples in about 1 h.

This method is currently used in our institute for the routine analysis of plasma levels of tamoxifen and metabolites in advanced cancer patients.

About 400 samples have been processed up to the present without any analytical troubles.

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Note

A sensitive assay method for pimozide in human plasma by high-performance liquid chromatography with fluorescence detection

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(First received December 14th, 1982; revised manuscript received February 9th, 1983)

Pimozide (Orap®) is a highly potent, long-acting neuroleptic, belonging to the diphenylbutylamine series [1]. The drug is administered orally to psychiatric patients in daily doses of 2—8 mg [2]. Since plasma levels are expected to be very low, a highly sensitive assay method was required.

Recently immunological assay [3] and a high-performance liquid chromatographic (HPLC) method with UV detection [4] were reported. However, the latter method was not sensitive enough for use with human samples and the former involved complicated protein-conjugation steps for antibody preparation.

This paper describes a rapid, sensitive and selective HPLC—fluorescence method for the determination of pimozide in human plasma. The method was used to determine plasma concentrations in pimozide in patients given a single oral dose of 3 mg.

EXPERIMENTAL

Chemicals and reagents

Pimozide (I) and the internal standard (II) (Fig. 1) were synthesized at the Research Laboratories of Fujisawa Pharmaceutical Co. Ltd. The internal standard was obtained by the methylation of pimozide with sodium hydride and methyl iodide and purified by HPLC under analytical conditions.

All inorganic reagents were analytical grade. Aqueous solutions were prepared with deionized water purified by a Millipore Milli-Q® water purification system.

n-Hexane and isoamyl alcohol were analytical grade and used without further purification. Acetonitrile (chromatographic grade) was purchased from Katayama Chemical Industries Ltd., Osaka, Japan.

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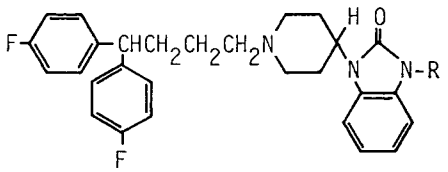

R=H : Pimozide

$R=CH_3$: Internal standard

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

Apparatus

An HPLC system, including a Model 6000A pump and a U6K universal injector (Waters Assoc., Milford, MA, U.S.A.) equipped with a Model FS-970 fluorescence detector (Schoeffel, Westwood, NJ, U.S.A.), was used.

A Varian Model 5000 liquid chromatograph equipped with a Valco loop injector and a Model UV-5 fixed-wavelength detector (Varian, Walnut Creek, CA, U.S.A.) was used for UV detection.

Chromatographic conditions

A 15 cm × 4 mm I.D. stainless-steel column was packed with TSK-GEL LS-410 ODS SIL (particle size 5 μm, Toyo Soda Industries Co. Ltd., Tokyo, Japan) for the analytical column and a 1 cm × 4 mm I.D. stainless-steel column, packed with the same material, was used for the guard column.

Chromatography was performed in a reversed-phase mode using a mobile phase of 48% (v/v) acetonitrile in 20 m*M* KH_2PO_4 adjusted to pH 2.5 with 20 m*M* phosphoric acid. The operation temperature was ambient and the flow-rate was 1.0 ml/min.

The column eluate was monitored by fluorescence detection with excitation at 210 nm and emission above 320 nm or by UV at 280 nm (0.005 a.u.f.s.). The fluorescence detector range was 0.2 μA full scale and the time constant value was 6 sec.

Extraction procedure

To 1.0 ml of plasma in a round-bottomed glass tube, 0.1 ml of the internal standard solution (containing 20 ng of internal standard), 1.0 ml of 1 *N* sodium hydroxide and 5 ml of *n*-hexane—isoamyl alcohol (98:2, v/v) were added and the mixture was shaken reciprocally for 10 min. After centrifugation at 1900 *g* for 5 min, the organic layer was transferred to a conical-bottomed glass tube for re-extraction into 0.1 or 0.2 ml of aqueous acid. After the mixture was shaken and centrifuged, the organic layer was aspirated off and discarded. Almost all of the aqueous layer was injected into the high-performance liquid chromatograph.

All glass tubes were silanized before use.

Standard solution

Stock solutions of pimozide and the internal standard were prepared by dissolving 10 mg of material in 100 ml of 1% phosphoric acid. The standard

solutions for the calibration curve were made by diluting the stock solutions with 1% phosphoric acid. These solutions were stored at 4°C until analyzed.

Human study

Plasma samples were obtained from three male in-patients (age 28—37 years, body weight 61—82 kg) at 1, 4 and 24 h after oral dosing with a 3-mg tablet of pimozide. Blank plasma was obtained from healthy volunteers. The samples were stored at −20°C until analyzed.

RESULTS AND DISCUSSION

Recovery

The effect of kind and volume of inorganic acid for back-extraction was investigated. The results are given in Table I. The recoveries at the 100 ng/ml level of pimozide and the internal standard were, respectively, 62.3% and 21.7% when 0.1 ml of hydrochloric acid was used. The recovery became poorer as the concentration of hydrochloric acid was increased. However, in the case of phosphoric and sulfuric acid, the recoveries were better than 80%. The recovery was also improved by increasing the volume of acid to 0.2 ml.

TABLE I

EXTRACTION RECOVERY OF PIMOZIDE AND INTERNAL STANDARD FROM SPIKED *n*-HEXANE—ISOAMYL ALCOHOL (98:2)

A 5-ml volume of *n*-hexane—isoamyl alcohol, containing 100 ng/ml pimozide and internal standard (I.S.), was extracted with inorganic acid. Aqueous layers were analyzed by HPLC.

Extraction solvent	Volume (μl)	Recovery (%)	
		Pimozide	I.S.
0.1 *M* HCl	100	62.3	21.7
	200	79.2	34.3
0.2 *M* HCl	100	47.2	10.4
	200	66.0	20.7
0.5 *M* HCl	100	44.2	10.7
	200	59.8	17.4
0.1 *M* H_3PO_4	100	81.8	59.9
	200	90.1	69.8
0.2 *M* H_2SO_4	100	84.1	67.3
	200	93.6	80.1

The poor recovery of the internal standard compared with pimozide, especially with hydrochloric acid, may be due to its high lipophilic character. When phosphoric acid or sulfuric acid was used as the solvent for back-extraction, however, the ratios of pimozide against the internal standard were almost constant.

From these results and on account of column life, phosphoric acid was used for back-extraction.

Detector study

First, a UV detection method was investigated. The maximum detection sensitivity was 215 nm; however, many of the background peaks could not be separated from the peak of pimozide. At 280 nm, the maximum absorbance wavelength of pimozide, no interference peak was found at retention times of pimozide or internal standard.

The lower limit of detection was 5 ng/ml for this method. After a single oral dose of 4 mg of radioactive pimozide to humans, plasma levels of pimozide were found to be lower than 2 ng/ml [5].

Consequently, a more sensitive assay method was required and a fluorescence detection method was examined. Since pimozide has no UV absorption

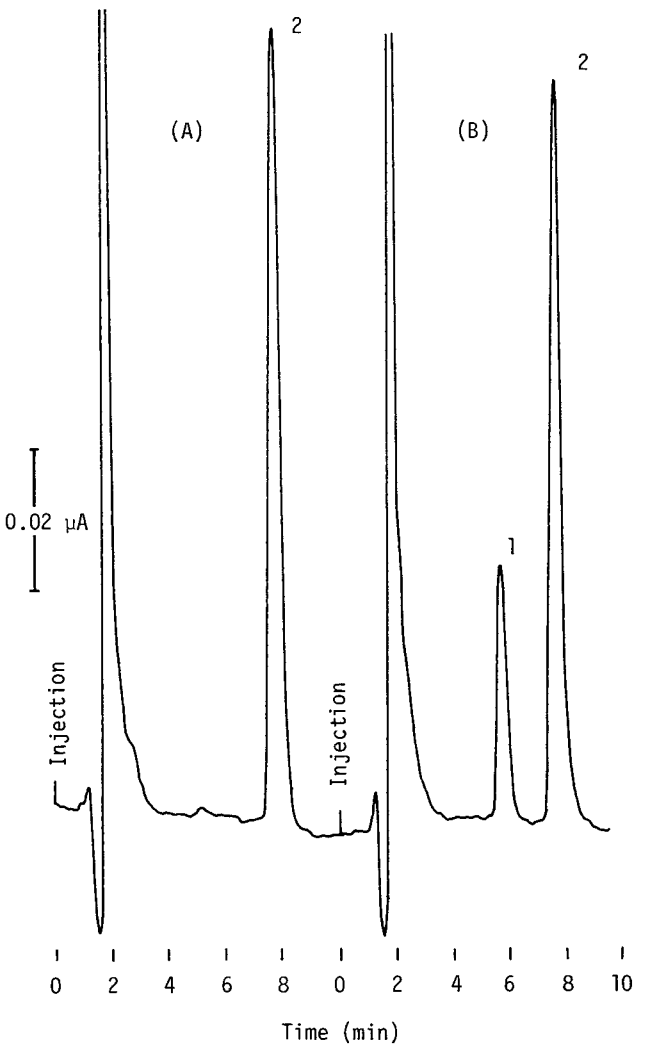

Fig. 2. Chromatograms of (A) blank plasma treated according to the method described under Experimental, and (B) blank plasma sample spiked with 5 ng/ml pimozide. Peaks: 1 = pimozide, 2 = internal standard.

above 300 nm, a variable-wavelength fluorescence detector, equipped with a deuterium lamp (FS-970), was used. The lower limit of detection by this method could be lowered to 0.3 ng/ml.

Chromatograms

Fig. 2 shows chromatograms of pimozide and the internal standard from spiked human plasma. As shown in Fig. 2, there is no background peak at the retention time of pimozide. The retention times of pimozide and internal standard were 6 min and 8 min, respectively.

Calibration curve

The calibration curve for human plasma level of pimozide was obtained as follows. Samples of blank plasma were spiked with pimozide at concentrations of 0.3, 0.5, 1, 2, and 5 ng/ml and the internal standard at a fixed concentration of 20 ng/ml.

The samples were taken through the extraction procedure described under Experimental and were injected into the liquid chromatograph. The ratio of peak height of pimozide to the internal standard was calculated for each chromatogram. A linear regression analysis of these data at the five concentrations of pimozide gave the slope, intercept and correlation coefficient for the human plasma calibration curve. The equation of the curve was $Y = 0.0687X + 0.0004$, and the correlation coefficient was 0.9998.

The lower limit of detection was 0.3 ng/ml at a signal-to-noise ratio of 3 and a sample volume of 1.0 ml.

Reproducibility

Reproducibility was obtained by adding known amounts of pimozide to the plasma and comparing the five samples with a single calibration curve. The results are given in Table II. The coefficients of variation were 4.4% and 2.8% at concentrations of 0.3 ng/ml and 2 ng/ml, respectively. Even at a concentration at the lower limit of detection, good accuracy and precision were obtained.

TABLE II

PRECISION AND ACCURACY IN THE DETERMINATION OF PIMOZIDE IN HUMAN PLASMA

$n = 5$.

Added (ng/ml)	Found (ng/ml) (mean ± S.D.)	C.V. (%)
0.3	0.32 ± 0.01	4.4
2.0	1.86 ± 0.05	2.8

Concentration of pimozide in human plasma

Chromatograms obtained from the plasma of subject A.K. are shown in Fig. 3. Generally, a psychotic patient requires several drugs (i.e. haloperidol, chlorpromazine, etc.) in combination. This subject (A.K.) was also given

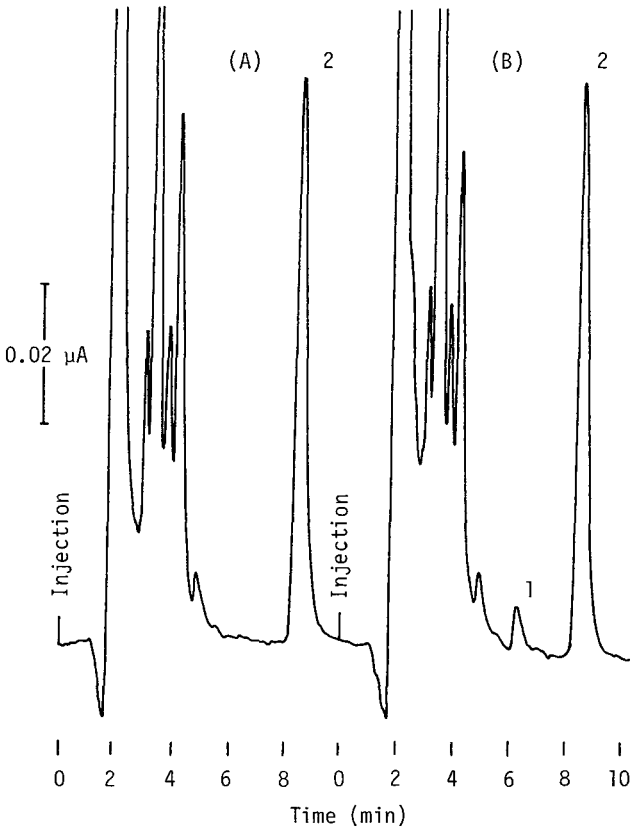

Fig. 3. Chromatograms of clinical plasma extracts: (A) before dosing; (B) 4 h after dosing (containing 0.9 ng/ml pimozide). Peaks: 1 = pimozide, 2 = internal standard. Plasma extracts were obtained from subject A.K. who was given a 3-mg tablet of pimozide in combination with haloperidol, sulpiride, levomepromazine, thiothixene and thioridazine.

TABLE III

PLASMA LEVELS OF PIMOZIDE AFTER A SINGLE ORAL DOSE OF A 3-mg TABLET

Time after dose (h)	Subject			Mean ± S.E.
	K.H.	O.K.	A.K.	
1	n.d.*	0.5	n.d.*	0.2 ± 0.2
4	0.9	3.3	0.9	1.7 ± 0.8
24	1.7	2.2	2.6	2.1 ± 0.2

*n.d. = not detected.

haloperidol, sulpiride, levomepromazine, thiothixene and thioridazine daily; however, no interfering peak was found in the chromatogram obtained from the plasma before dosing with pimozide (Fig. 3A). It can be considered that the differences in extraction recovery, sensitivity to fluorescence detection and plasma levels between other drugs and pimozide cause no interference.

These results, therefore, show that the present method is suitable for monitoring drug levels of pimozide in patients.

After a single oral dose of a 3-mg tablet of pimozide, plasma concentrations were below 3.3 ng/ml at all times of observation. Even at 24 h after dosing, a level of 1.7—2.6 ng/ml of pimozide was found.

McCreadie et al. [6] reported plasma levels of pimozide after a single 24-mg dose by the immunological assay method. They found 15 ng/ml of pimozide at 24 h after dosing. The present results agree with McCreadie's findings on the long-acting character of pimozide.

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Note

Improved liquid chromatographic analysis of phenytoin and salicylate using radial compression separation

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High-performance liquid chromatography (HPLC) is successfully used for quantitation of the antiepileptic agent phenytoin and the antipyretic—analgesic compound salicylate in biological fluids [1—14]. This paper describes an improved HPLC analytic technique for these two compounds, using a newly developed liquid chromatography separation system. The method is sensitive enough for single-dose pharmacokinetic studies of phenytoin and salicylate in humans or animals, and can easily be modified for therapeutic monitoring.

EXPERIMENTAL

Materials

Pure samples of phenytoin, tolylbarbital, salicyclic acid, and 3,4-dimethoxybenzoic acid (DMBA) were obtained from Aldrich (Milwaukee, WI, U.S.A.). All other reagents, analytical grade or better, were purchased from commercial sources and used without further purification. Mobile phase components (water, aqueous acetic acid, and acetonitrile) were separately filtered prior to mixing, then degased after mixing.

Apparatus and chromatographic conditions

A Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph was used. The instrument was equipped with a Model 4000A solvent delivery system, a

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Model 480 variable-wavelength spectrophotometer, and a Model 710B (WISP) automatic sample processor. Detector output (peak height) was quantitated using a Model 730 data module. The separation system was a reversed-phase C-18 radial compression cartridge (spherical 10-μm nonpost-silanized), 10 cm × 5 mm I.D., which was housed in an RCM-100 radial compression module.

For analysis of phenytoin, the mobile phase consisted of water—acetonitrile (70:30). The spectrophotometer was operated at 195 nm, and the mobile phase flow-rate was 0.8 ml/min. For analysis of salicylate, the mobile phase was acetonitrile—water (20:80), to which were added 10 ml of acetic acid per l. The mobile phase flow-rate was 1.0 ml/min, and detector output was quantitated at 305 nm. All analyses were performed at room temperature.

Stock solutions

Standard solutions of phenytoin, tolylbarbital, salicyclic acid, and DMBA each were prepared by dissolving 100 mg in 100 ml of pure methanol. Working solutions were prepared by appropriate dilution with methanol. Solutions are stable for at least one year when stored at 4°C.

Preparation of samples

For phenytoin analysis, tolylbarbital served as internal standard [4]. A fixed amount (2.5 μg) of tolylbarbital was added to a series of 13-ml round-bottom culture tubes equipped with PTFE-lined screw-top caps. To a series of calibration tubes were then added variable amounts of phenytoin ranging from 0.1 to 10.0 μg. Drug-free control plasma (0.5 ml) was added to each of the calibration tubes; 0.1—1.0 ml of unknown plasma or serum was added to all other tubes. Four ml of benzene—isoamyl alcohol (98.5:1.5) were added to all tubes, and the samples were gently agitated in the upright position on a Vortex mixer for 30—60 sec. After centrifugation at 400 *g*, an aliquot (approximately 3.5 ml) of the organic phase was transferred to a 13-ml tapered glass centrifuge tube. The organic solvent was evaporated to dryness at 40—50°C under mildly reduced pressure. The residue was redissolved in 200 μl of methanol, which was then trasferred to an automatic sampling vial equipped with a limited volume insert. The automatic sampler was programmed to inject 20 μl of each sample.

For analysis of salicylate, DMBA served as internal standard. A constant amount (20 μg) of DMBA was added to a series of tubes. Calibration tubes were prepared by addition of variable amounts of salicylic acid, ranging from 1 to 50 μg, to a series of these tubes. Drug-free control plasma (0.5—1 ml) was added to the calibration tubes, and 0.1—1.0 ml of unknown plasma was added to all other tubes. Samples were acidified by addition of one drop of concentrated phosphoric acid, and then extracted with 4 ml of ethyl acetate—benzene (1:1). The samples were agitated by Vortex mixing, centrifuged, and an aliquot of the organic phase separated to a tapered glass centrifuge tube. The organic phase was evaporated to dryness under mildly reduced pressure, but all samples were placed on a bed of ice to avoid sublimation of salicylate. The residue was redissolved in 200 μl of methanol and prepared for injection by the automatic sampler as described above.

Clinical studies

A healthy male volunteer participated in a pharmacokinetic study of intravenous phenytoin after giving informed consent. The subject received a single 300-mg dose of phenytoin sodium (Parke Davis, Ann Arbor, MI, U.S.A.), equivalent to 275 mg of free phenytoin, by infusion into an antecubital vein over a period of 10 min. Multiple venous blood samples were drawn into heparinized tubes during the 72 h after the dose. Concentrations of phenytoin in all samples were determined by the method described above. Using standard pharmacokinetic techniques [15], plasma phenytoin concentrations were used to determine volume of distribution, elimination half-life, and total metabolic clearance.

Another healthy volunteer participated in a two-way crossover study of salicylate pharmacokinetics after giving informed consent. On one occasion, 650 mg of acetylsalicylic acid (aspirin), prepared as the lysine salt (Bayer, Köln, G.F.R.), was infused into an antecubital vein over a period of 5 min. After a washout period of one week, the subject received the same dose of aspirin as two commercially available aspirin tablets (Bayer, New York, NY, U.S.A.) together with 100—200 ml of tap water after an overnight fast. For both studies, multiple venous blood samples were drawn in the 12 h after the dose. Salicylate concentrations in all plasma samples were determined by the method described above. No attempt was made to quantitate the low concentrations of intact aspirin or other metabolites that might have been present.

Using standard kinetic methods [15], salicylate volume of distribution, elimination half-life, and total clearance were determined after the intravenous dose, assuming that the entire 650-mg dose of aspirin was converted to the molar equivalent of salicylate (498 mg). After oral aspirin administration, absolute systemic availability of salicylate was determined by comparison of the total area under the plasma concentration curve with that observed in the same subject after intravenous dosage.

Experimental study

An anesthetized adult mongrel dog received a single 500-mg intravenous dose of aspirin as the lysine salt using an experimental preparation described in detail previously [16, 17]. Multiple blood samples were drawn during the 8 h after the dose, and cisteral cerebrospinal (CSF) samples were drawn from an indwelling cannula. Serum and CSF salicylate concentrations were determined as described above.

RESULTS

Evaluation of the method

Under the described chromatographic conditions, phenytoin and tolylbarbital gave two symmetric well-resolved chromatographic peaks (Fig. 1A). Drug-free blank plasma samples were consistently free of endogenous contaminants at the retention times corresponding to the two compounds. The relation of plasma phenytoin concentration to the phenytoin:tolylbarbital peak height ratio was linear to at least 10.0 μg/ml. The equation of a typical

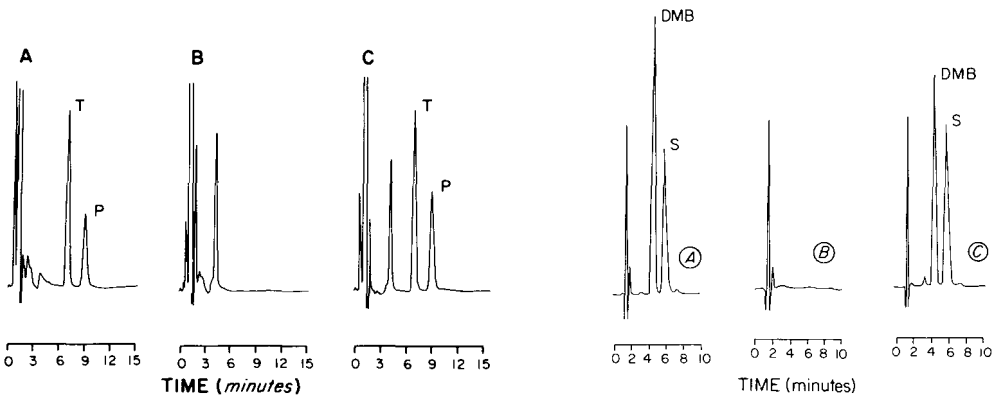


Fig. 1. (A) Chromatogram of a calibration standard containing 1.0 μg/ml of phenytoin (P) and 2.5 μg/ml of tolylbarbital (the internal standard) (T). (B) Chromatogram of a drug-free control plasma extract. (C) Chromatogram of a sample from a subject who had received 300 mg of phenytoin sodium intravenously 12 h previously.

Fig. 2. (A) Chromatogram of a calibration standard containing 10 μg/ml of salicylate (S) and 20 μg/ml of 3,4-dimethoxybenzoate (DMB), the internal standard. (B) Chromatogram of a drug-free control plasma extract. (C) Chromatogram of a sample from a subject who had received a single 650-mg dose of aspirin intravenously 12 h previously.

TABLE I

REPLICABILITY OF IDENTICAL SAMPLES

n = 5 at concentration.

	Plasma concentration (μg/ml)	Coefficient of variation* (%)
Phenytoin	0.1	7.2
	0.25	5.0
	0.5	4.0
	0.75	4.6
	1.0	1.7
	2.5	3.1
	5.0	3.6
Salicylate	2.5	1.9
	5.0	3.8
	10.0	9.3
	25.0	3.1
	50.0	5.8

*Standard deviation divided by mean.

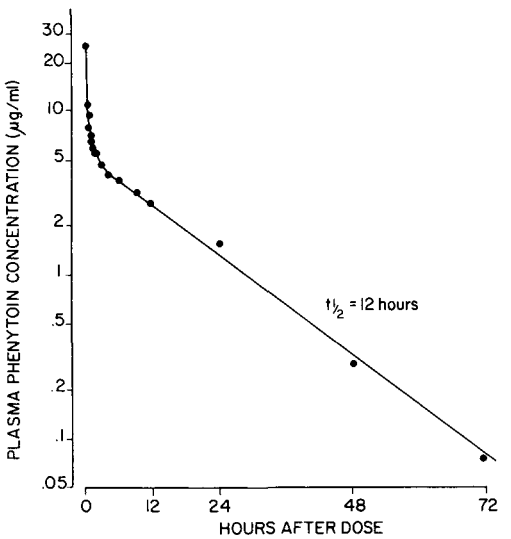
regression line was $y = 0.373x - 0.031$ ($r = 0.999$), where y is peak height ratio and x is plasma phenytoin concentration. Table I show the coefficients of variation for identical samples at various concentrations. The sensitivity limits are approximately 0.05 μg of phenytoin per ml of original sample.

Salicylate and the internal standard dimethoxybenzoate (DMB) likewise gave two well-resolved symmetric chromatographic peaks (Fig. 2A). No interfering endogenous contaminants were observed. The relation of plasma salicylate concentration to the salicylate:DMB peak height ratio was linear to at least 50 μg/ml. The equation of a typical regression line was: $y = 0.051x - 0.02$ ($r = 0.999$), where y is peak height ratio and x is plasma salicylate concentration. Table I shows the replicability of identical samples at various concentrations. The sensitivity limits are approximately 0.5 μg per ml of sample, and can be extended by minor modifications such as reduction of the amount of internal standard added or increasing the injected volume of the final residue.

Pharmacokinetic results

Fig. 3 shows plasma phenytoin concentrations in the volunteer subject. The peak concentration measured immediately after the dose was 24.7 μg/ml. Concentrations then fell in biphasic fashion, with an apparent elimination half-life of 12 h. The apparent volume of distribution was 39 l, and the total metabolic clearance 37 ml/min.

Fig. 4 shows plasma salicylate concentrations after intravenous injection of aspirin. The peak concentration was 55 μg/ml, reached immediately after the dose. Thereafter, concentrations declined biphasically, with an elimination half-life of 1.9 h. The calculated volume of distribution was 10.2 l, and the total metabolic clearance 61 ml/min. After oral aspirin administration, the peak concentration was 33.6 μg/ml, reached at 1.0 h after dosage (Fig. 4). The half-life of elimination following oral administration was essentially identical to that observed after the intravenous dose. Based on comparison



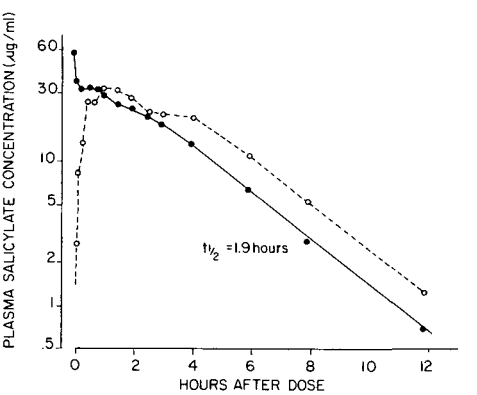


Fig. 3. Plasma phenytoin concentrations in the volunteer subject following a single 300-mg intravenous dose of phenytoin sodium.

Fig. 4. Plasma salicylate concentrations in a volunteer subject after 650-mg intravenous (•——•) and oral (○— — —○) doses of aspirin administered on two occasions.

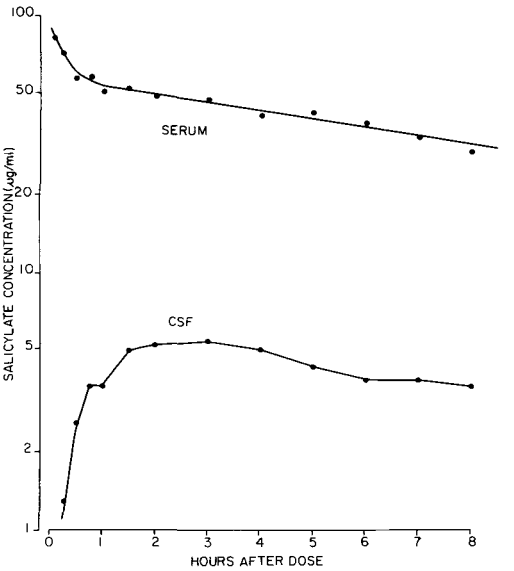


Fig. 5. Serum and CSF salicylate concentrations in the experimental study as described in the text.

of areas under the plasma concentration curve by the two modes of administration, absolute systemic availability of salicylate after oral aspirin was calculated to be 100%.

After intravenous aspirin administration in the experimental study, the peak serum salicylate level was 84 μg/ml, reached immediately after the dose (Fig. 5). Salicylate thereafter disappeared biphasically with a terminal elimination half-life of 8.6 h. Salicylate entered CSF slowly with peak concentrations not reached until 3 h after the intravenous dose. CSF concentrations were considerably lower than those in serum. The slow CSF entry probably reflects the relatively poor lipid solubility of salicylate, with correspondingly slow diffusion across the lipoidal blood brain barrier [18]. The incomplete entry reflects the extensive serum protein binding of salicylate, with only the unbound or free component present in serum being available for diffusion into CSF [16, 17].

DISCUSSION

This paper describes a rapid and sensitive HPLC method for quantitation of phenytoin and salicylate in biologic fluids. The method has advantages over previously described techniques. The same column can be used for analysis of both compounds, so that changing from one assay to the other requires only a shift of mobile phase composition and absorbance wavelength. Second, the use of the automatic injection system allows analysis of up to 100 samples per 24 h by one person working a standard 8-h day, since sample preparation can be done during the work day while chromatography can proceed overnight. Finally, sensitivity and stability of the assay system are enhanced and cost is reduced by use of the radial compression separation system. The

radial compression module applies pressure uniformly to the outside of a flexible-walled cartridge which contains the actual chromatographic material. The external radially-applied pressure forces the wall of the cartridge to conform to the packing material and prevents channel formation, dead spaces (voids), or shifting of the packing. The radial compression cartridges are considerably less expensive than most other HPLC column systems, have a longer life, and will perform well with low mobile phase flow-rates thereby reducing the cost of solvent consumption.

The approach to quantitation of phenytoin and salicylate utilizes internal standardization with structurally related compounds. Biologic samples are extracted into an organic solvent and chromatographed after evaporation and reconstitution. The sensitivity limits of the method are more than sufficient for essentially any type of clinical or basic pharmacokinetic study of these compounds, as demonstrated by the studies described above. During usual therapeutic use, steady-state plasma concentrations of phenytoin and salicylate are considerably higher than those encountered in single-dose pharmacokinetic studies. Thus the present methodology can easily be adapted to therapeutic monitoring either by using small aliquots of plasma (0.1 ml) or by addition of larger amounts of internal standard with a wider calibration range.

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Note

Determination of mexiletine and its metabolites in serum by liquid chromatography with fluorescence detection

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Mexiletine [1-(2′,6′-dimethylphenoxy)-2-aminopropane] (I), Fig. 1, is a new antiarrhythmic drug. While its structure and electrophysiological properties resemble those of lidocaine, I has the advantages of being effective when given orally and having a longer half-life [1]. The pharmacological properties of I have been reviewed recently [1]. The effective therapeutic concentration of I in blood was reported to be 0.5—2.0 μg/ml [2]. However, side effects were noted in some cases with blood concentrations as low as 0.8 μg/ml and severe side effects with concentrations above 2 μg/ml [3]. Prescott et al. [4] reported that the plasma half-life of I was much longer in patients (12—17 h) than in healthy volunteers (9—10 h), and that the half-life was also affected by urinary pH and coadministration of other drugs. This information suggested that therapeutic drug monitoring of blood levels of I may be of value in some patients.

Earlier studies have shown that the major metabolites of I are hydroxymethyl-mexiletine (II) and *p*-hydroxy-mexiletine (III), Fig. 1, and that I, II, and III were further conjugated to form their corresponding glucuronides and also the aryl sulfate of III [4—6].

Several gas chromatographic procedures have been described for the determination of I in biological fluids utilizing a variety of detection systems [7—18]. For the determination of II or III, two different chromatographic systems were required [5]. Two high-performance liquid chromatographic methods

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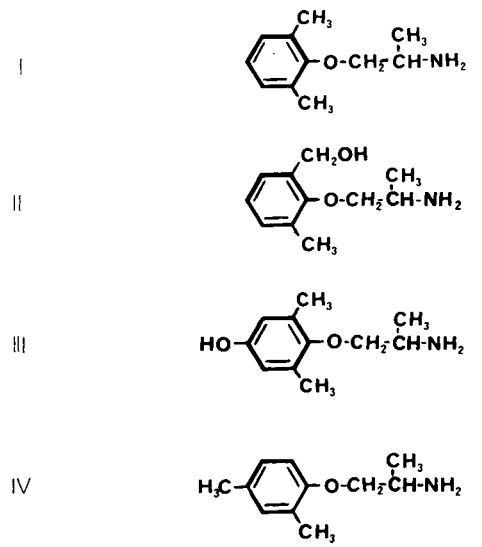

Fig. 1. Structure of mexiletine, its metabolites, and the internal standard. I = mexiletine [1-(2′,6′-dimethylphenoxy)-2-aminopropane], II = hydroxymethyl-mexiletine [1-(2′-hydroxymethyl-6′-methylphenoxy)-2-aminopropane], III = *p*-hydroxy-mexiletine [1-(4′-hydroxy-2′,6′-dimethylphenoxy)-2-aminopropane], and IV = internal standard [1-(2′,4′-dimethylphenoxy)-2-aminopropane].

for the determination of I were published recently utilizing either the UV absorbance of I [19] or of its 2,4-dinitrobenzene derivative [20]. Neither of these methods offers the simultaneous determination of I, II, and III. Precolumn derivatization of primary amines to facilitate their selective detection at low concentrations is a useful technique in liquid chromatography [21, 22]. We report a selective and sensitive method for the simultaneous analysis of I, II, and III following their derivatization with 5-(dimethylamino)-1-naphthalenesulfonyl chloride (Dns chloride). Dns chloride reacts with primary and secondary amine groups, and with phenols at high pH values, yielding highly fluorescent derivatives [21]. This assay was developed to allow the study of the pharmacokinetics of I and its metabolites in patients with renal failure.

EXPERIMENTAL

Reagents and materials

Mexiletine, its metabolites II and III, and the internal standard 1-(2′,4′-dimethylphenoxy)-2-aminopropane (IV), Fig. 1, were gifts from Boehringer Ingelheim (Ridgefield, CT, U.S.A.). Dns chloride was obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals were analytical grade and the organic solvents for liquid chromatography were distilled in glass.

Individual solutions containing 0.1—3.0 mg (as the free base) of each of I, II, and III per liter of 0.05 *M* hydrochloric acid were prepared. Similar concentrations of these compounds in human serum were also prepared. These serum standards were divided into small aliquots and frozen until needed

for assay. The stock internal standard solution was prepared by dissolving 2 mg of IV in 100 ml of 0.05 *M* hydrochloric acid. The diluted internal standard solution contained 0.2 mg of IV in 100 ml of 0.05 *M* hydrochloric acid.

Procedure

A 100-μl aliquot of the diluted internal standard solution was added to 100 μl of serum, followed by 500 μl of 0.5 *M* carbonate—bicarbonate buffer, pH 9.5. The contents were mixed and extracted with 5 ml of diethyl ether—*n*-butanol (9:1). The organic layer was transferred to another tube and evaporated to dryness under nitrogen. To the residue were added 25 μl of 0.1 *M* sodium bicarbonate solution and 100 μl of a Dns chloride solution in acetone (5 mg per 10 ml). The tube was capped and heated at 70°C for 25 min. The tube was allowed to cool to room temperature, then 100 μl of acetone were added. A 10-μl aliquot of this solution was chromatographed.

Liquid chromatography

A modular liquid chromatograph consisted of a Waters Model 271 microprocessor-controlled gradient system coupled to a filter fluorometer (American Instrument Company, Silver Springs, MD, U.S.A.). The fluorometer had a Corning No. 7-51 primary filter, a Wratten No. 8 secondary filter, and a quartz flow cell, with the relative intensity scale set at 0—10. The stainless-steel column was 250 mm × 4.6 mm I.D., packed with spherical 6-μm Zorbax C8 particles (DuPont Instruments, Wilmington, DE, U.S.A.). The column temperature was ambient. The flow-rate of the mobile phase was 2.8 ml/min. A linear gradient was established over 18 min, starting with 72% methanol in 0.04 *M* ammonium acetate (pH 7.0) and ending with 85% methanol in 0.04 *M* ammonium acetate as the eluent. The concentrations of the compounds in the samples were determined from their peak height ratios relative to the internal standard.

RESULTS AND DISCUSSION

The derivatization of I, II, III, and IV was optimal and reproducible under the conditions described above. The Dns derivatives formed were stable for at least 16 h. The composition and molarity of the eluent were also established. Under these conditions, the retention times for I, II, III, and IV were 9.9, 4.8, 15.9, and 10.9 min, respectively. Fig. 2 shows typical chromatograms obtained from serum samples. Recovery of known concentrations of the three compounds (I, II, and III) in serum ranging from 0.1 to 3.0 mg/l was studied using IV as a reference standard and comparing peak height ratios to those obtained with aqueous standards of I, II, and III. The recovery (mean ± S.D.; $n = 6$) for the concentration range specified was 80.7 ± 3.7% for I (range 76.8—84.2), 85.1 ± 3.7% for II (range 79.9—90.0), and 88.1 ± 3.7% for III (range 84.1—93.8).

Samples of serum standards (100 μl) containing 0.1—3.0 mg of each of I, II, and III per l were analyzed. The relationship between the concentration of each compound and its peak height ratio to IV was found to be linear over the concentration range studied. As little as 100 pg of either compound

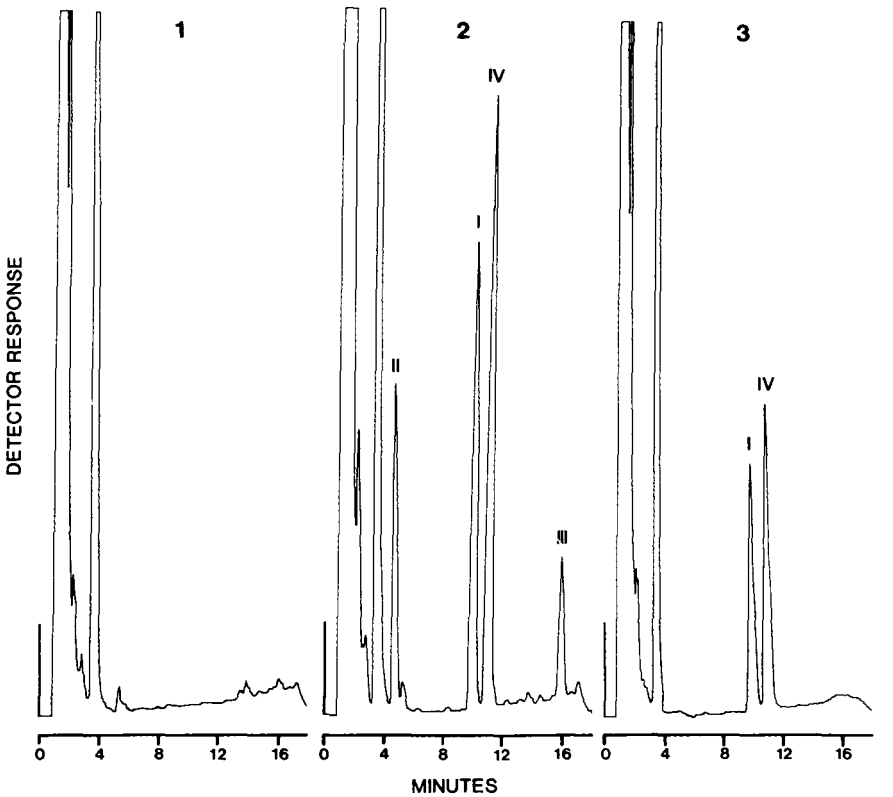

Fig. 2. Chromatograms of serum samples. (1) Patient not receiving mexiletine; (2) standard serum sample containing 2 mg/l of mexiletine (I), 2 mg/l of hydroxymethyl-mexiletine (II) and 1 mg/l of *p*-hydroxy-mexiletine (III); (3) patient receiving mexiletine. The internal standard is IV. Operating conditions: column, Zorbax C8, 250 mm × 4.6 mm I.D.; flow-rate, 2.8 ml/min; eluent A, methanol—0.04 *M* ammonium acetate solution, pH 7.0 (72:28); eluent B, methanol—0.04 *M* ammonium acetate solution (85:15); linear gradient program from 100% A to 100% B over 18 min.

injected on the column could be detected using this procedure. The limit of detection of these compounds was determined after extraction from plasma and was found to be 5 ng/ml.

The precision of this method was determined over several days by analyzing 0.1-ml aliquots of a serum sample containing 2 mg of I and II and 1 mg of III per l. The mean (± S.D.; n = 11) was found to be 2.02 ± 0.15 mg/l for I, 2.11 ± 0.19 mg/l for II, and 1.04 ± 0.11 mg/l for III. These results show a day-to-day precision of 7.4% for I, 9.0% for II, and 10.4% for III.

The proposed method allows for the simultaneous determination of mexiletine and its two major metabolites. If the determination of I alone is desired, an isocratic system can be easily adopted using methanol—0.04 *M* ammonium acetate (80:20) as the eluent, at a flow-rate of 2.5 ml/min where the retention times of I and IV would be 7.9 and 8.9 min, respectively. The use of the gradient was necessary only to reduce the retention time of III. This method also offers the selectivity and sensitivity needed for the determination of I and its metabolites in serum and can be extended to the analysis of these compounds in urine, using the same extraction and derivatization procedures.

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Note

Fluorimetrische Bestimmung von Naproxen im Serum durch direkte quantitative Hochleistungs-Dünnschichtchromatographie

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Naproxen [(+)-6-Methoxy-α-methyl-2-naphthalinessigsäure] wird auf Grund seiner antiphlogistischen und analgetischen Wirkung zur Behandlung von Erkrankungen des rheumatischen Formenkreises eingesetzt [1, 2].

Die in der Literatur am häufigsten beschriebenen Methoden zur Bestimmung von Naproxen in biologischem Material basieren auf gaschromatographischen (GC) oder hochleistungsflüssigkeitschromatographischen (HPLC) Verfahren [3—19]. Die hier beschriebene Bestimmungsmethode beruht auf der Beobachtung, dass Naproxen nach der dünnschichtchromatographischen Trennung durch Bestrahlen mit UV-Licht auf der Hochleistungs-dünnschicht-chromatographie (HPTLC) Platte ein blau fluoreszierendes Photoprodukt bildet, das dann fluorimetrisch gemessen wird.

METHODEN

Geräte

Die Probenauftragung wurde mit dem Linomat III (Camag, Berlin, B.R.D.) durchgeführt. Die Entwicklung der HPTLC-Platten erfolgte in Camag Doppeltrog Kammern. Die Bestrahlung der HPTLC-Platten mit UV-Licht (254 nm) erfolgte im Fluotest Universal (Original Hanau, Hanau, B.R.D.). Die fluorimetrische Auswertung der Chromatogramme erfolgte mit einem Camag TLC-Scanner mit angeschlossenem Varian CDS 111 A Integrator (Varian, Darmstadt, B.R.D.) und W+W Recorder 1100 (W+W Electronic AG, Basel-Münchenstein, Schweiz).

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Chemikalien und Materialien

Naproxen wurde freundlicherweise von der Fa. Syntex (Palo Alto, CA, U.S.A.) zur Verfügung gestellt. Die TLC Trennung erfolgte auf HPTLC-Fertigplatten Kieselgel 60 o.F. 10 × 20 cm (Merck, Darmstadt, B.R.D.). Alle anderen verwendeten Chemikalien hatten den Reinheitsgrad p.a.

Aufarbeitung der Serumproben

In einem verschliessbaren Zentrifugenglas wurden 0.1 ml Serum mit 1.0 ml Methanol versetzt und 2 min gründlich gemischt (Vortex-Genie). Anschliessend wurde 5 min lang zentrifugiert, um das gefällte Eiweiss abzutrennen. Vom Überstand wurden 20 μl mit dem Linomaten auf die HPTLC-Platte aufgetragen.

Test- und Standardseren

Zur Ermittlung von Eichkurven, Wiederfindungsrate und Reproduzierbarkeit sowie zur quantitativen Bestimmung von Serumproben mit unbekanntem Naproxengehalt wurden Test- und Standardseren mit bekanntem Gehalt an Naproxen hergestellt.

Hierzu wurden entsprechende Volumina einer Stammlösung von Naproxen in Ethylacetat (10 mg/100 ml) bei Raumtemperatur durch Einleiten von Stickstoff zur Trockene eingeengt und der Rückstand in 1.0 ml gepooltem Human-Serum aufgenommen.

Chromatographie der HPTLC-Platten

Die Probenauftragung erfolgte strichförmig (5 mm) bei einem Abstand von 15 mm der äusseren Proben vom Plattenrand und einem Abstand der Proben von 6 mm untereinander. Der Abstand der Startlinie vom unteren Plattenrand betrug 15 mm. Jede Probe bzw. jeder Standard wurde zweimal pro Platte nach der Data Pair Methode [20] aufgetragen. Die Entwicklung der Platten erfolgte mit dem Fliessmittel Chloroform—Methanol—Ameisensäure (95:7:7, v/v) (Kammersättigung) bei einer Trennstrecke von 5 cm (R_F = 0.45); Entwicklungszeit ca. 8 min. Hierbei wird Naproxen auch von seinem Metaboliten 6-Desmethyl-naproxen (R_F 0.37) abgetrennt. Nach sorgfältigem Trocknen der Platten im Warmluftstrom wurden sie 1.5 h mit UV-Licht (254 nm) bestrahlt. Nach dieser Zeit hat die Fluoreszenzintensität ihren höchsten Wert erreicht und bleibt mindestens 120 min stabil. Das bei der UV-Bestrahlung entstehende blau fluoreszierende Hauptprodukt (Fig. 1) konnte auf Grund seiner UV-, IR-, MS- und ^{1}H-NMR-Spektren als das 6-Methoxy-2-acetonaphthon identifiziert werden.

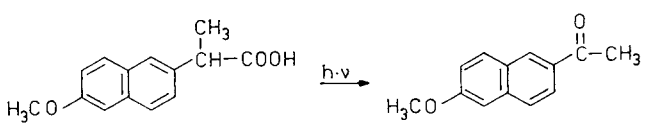

Fig. 1. Photoreaktion (Hauptprodukt) von Naproxen auf HPTLC-Kieselgel 60 Platten (λ = 254 nm).

Fluoreszenzmessung

Die in-situ quantitative Auswertung der Chromatogramme erfolgte bei λ_{ex} 313 nm, 30 nm Bandbreite und einer Spaltbreite von 6 mm × 0.3 mm. Als

Sekundärfilter diente der Kantenfilter 400 nm. Die Scan-Geschwindigkeit betrug 0.5 mm/sec, die Photomultiplierempfindlichkeit Sens 10. Die Fluoreszenz—Ortskurven wurden vom Schreiber bei einem Papiervorschub von 3 cm/min aufgezeichnet. Jeder Fleck wurde dreimal gemessen und der Mittelwert der Integratorwerte gebildet.

ERGEBNISSE UND DISKUSSION

Eichgeraden, die bei der Bestimmung von Serumproben mit bekanntem Gehalt an Naproxen erhalten wurden, zeigten für den Bereich von 5—80 μg Naproxen pro ml Serum eine lineare Beziehung zwischen der Peakfläche und der Serumkonzentration (Korrelationskoeffizient $r \geqslant 0.999$). Da die Eichgerade durch den Nullpunkt geht, reicht eine Standardkonzentration bei der Bestimmung der unbekannten Serumproben aus.

Testseren mit 10, 20, 40 und 60 μg Naproxen pro ml Serum dienten zur Bestimmung der Wiederfindungsrate und Präzision. Jede Serumkonzentration wurde achtmal an einem Tag bestimmt. Die Ergebnisse sind in Tabelle I zusammengefasst. Die Untersuchung derselben Serumprobe mit einem Gehalt von 50 μg Naproxen pro ml Serum gegen einen Standard derselben Konzentration an fünf verschiedenen Tagen ergab einen Mittelwert von 50.4 μg/ml Serum und eine relative Standardabweichung von 2.0%.

Bei den Werten der Tabelle I wurde die Volumenverringerung durch ausgefälltes Eiweiss und die Volumenkontraktion bei der Zugabe von Methanol zum Serum bei der Berechnung der Wiederfindungsrate berücksichtigt. Diese Volumenänderung brauchte bei den eigentlichen pharmakokinetischen Untersuchungen nicht berücksichtigt zu werden, da bei diesen Messungen für jeden Probanden aus dessen Leerserum ein eigener Standard (50 μg/ml Serum) hergestellt wurde. Die Verwendung von probandeneigenem Serum zur Herstellung des Standards hat im Vergleich zu gepooltem Serum den Vorteil, dass individuelle Schwankungen im Proteingehalt des Serums das Ergebnis nicht beeinflussen.

TABELLE I

WIEDERFINDUNG UND REPRODUZIERBARKEIT

Zugesetzte Menge Naproxen pro ml Serum (μg/ml)	Anzahl der Bestimmungen	Wiedergefundene Menge		
		%	μg/ml	S_{rel} (%)*
10	8	99.6 ± 3.2	9.96 ± 0.32	3.2
20	8	99.4 ± 2.3	19.88 ± 0.46	2.3
40	8	95.2 ± 2.8	38.07 ± 1.12	2.9
60	8	93.0 ± 1.0	55.77 ± 0.60	1.1

*S_{rel} = relative Standardabweichung.

Fig. 2 zeigt die Fluoreszenz—Ortskurve einer Serumprobe mit Naproxen (b) und des entsprechenden Leerserums (a). Die Serumprobe stammt von einem Probanden zur Zeit des maximalen Serumspiegels nach der Applikation

eines Suppositoriums mit 500 mg Naproxen und entspricht einer Serumkonzentration von 56.6 μg/ml. Es sind keine störenden Begleitfluoreszenzen beim Leerserum zu erkennen.

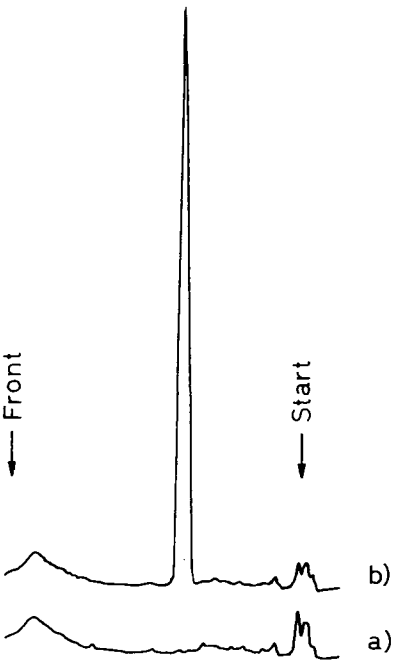

Fig. 2. Fluoreszenzintensitäts—Ortskurven der Chromatogramme eines Leerserums (a) und eines naproxenhaltigen Serums (b). Die Serumprobe (a) wurde einem Probanden vor der Applikation eines 500-mg Naproxen-Suppositoriums, die Probe (b) zur Zeit des max. Serumspiegels (≙ 56.6 μg/ml) entnommen.

Die Vorteile der hier beschriebenen Methode zur Bestimmung von Naproxen im Serum liegen einmal in der geringen benötigten Probenmenge (0.1 ml Serum) und der hohen Empfindlichkeit fluorimetrischer Messungen. Die Nachweisgrenze liegt unter 1 μg/ml Serum (2 ng pro Fleck), die Erfassungsgrenze liegt bei ca. 1—2 μg Naproxen pro ml Serum. Mit dieser Methode gelang es erstmals, Naproxen im Serum mit genügender Empfindlichkeit fluorimetrisch durch in-situ quantitative TLC zu bestimmen. Zum anderen ist das Analysenverfahren gering im Arbeitsaufwand und gut reproduzierbar.

Gegenüber den HPLC- und GC-Verfahren liegt der Vorteil der beschriebenen Methode darin, dass sie durch die Kombination von chromatographischer Trennung und anschliessender Bildung eines fluoreszierenden Photoproduktes sehr spezifisch ist. Ausserdem entfallen arbeitsaufwendige Extraktions- und Derivatisierungsschritte, die bei den GC-Verfahren in jedem Fall und bei den HPLC-Verfahren zumindest teilweise nötig sind.

Im Rahmen biopharmazeutischer Untersuchungen zur Absorption von Naproxen aus Suppositorien wurde die hier beschriebene Methode mit Erfolg zur Bestimmung von Naproxen im Serum eingesetzt [21].

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CHROMBIO. 1688

Letter to the Editor

Rapid extraction of leukotrienes from biologic fluids and quantitation by high-performance liquid chromatography

Sir,

We wish to point out a potential problem in our method using EDTA to improve the liquid chromatography of leukotrienes [1]. We failed to emphasize that EDTA is very poorly soluble in methanol, and that washing of high-performance liquid chromatographic columns equilibrated in methanol with EDTA will lead to precipitation of the EDTA and, potentially, clogging of the column. This problem is totally obviated by first equilibrating the column in water, washing with EDTA, and then totally washing the column again with water prior to equilibrating with methanol-containing mobile phases.

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1 S.A. Metz, M.E. Hall, T.W. Harper and R.C. Murphy, J. Chromatogr., 233 (1982) 193—201.

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Book Review

Electrophoresis. A survey of techniques and applications. Part B: Applications, edited by Z. Deyl, co-edited by A. Chrambach, F.M. Everaerts and Z. Prusík, Journal of Chromatography Library, Volume 18B, Elsevier, Amsterdam, 1983, XIV + 462 pp., price Dfl. 225.00, US$104.75, ISBN 0-444-42114-9.

This is a worthy companion to the impressive Part A on Techniques which this reviewer had the privilege of assessing three years ago [see Analyst (London), 195 (1980) 1007]. The editorial team is only slightly changed from that of Part A by A. Chrambach having taken the place of P.J. Svendsen as a co-editor, essentially for editing the extensive Chapter 9 (110 pages) on the gel electrophoresis and electrofocusing of proteins.

Chapter 9 is almost a monograph in its own right and is essentially the heart of this volume, with the Editor, Chrambach, having skillfully blended the contributions of 24 of the 30 authors of the complete work. This has been helped by the very useful introduction by Chrambach on the parameters and usefulness of second generation polyacrylamide gel electrophoresis (PAGE) complemented by use of sodium dodecyl sulphate (SDS-PAGE), and gel electrofocusing as a prologue to the individual discussions on the PAGE and electrofocusing of membrane proteins, receptors, cell surface antigens, lysosomal glycosidases and sulphatases, haemocyanins, haemoglobins, immunoglobulins, various contractile, cytoskeletal, connective tissue, microtubular, hormone and plasma proteins, and allergens. It is an impossible task to survey each and every contribution in a review of this kind, but suffice it to state that there is within each contribution a wealth of detailed, well-presented information. Among these is the fascinating way in which R.A. Reisfeld and M.A. Pellegrino relate the impact made by PAGE on the understanding of the structure and function of cell surface macromolecules, and especially of the combination of SDS-PAGE with indirect immunoprecipitation (IP) of antigens with specific antibody (IP—SDS-PAGE) in relation to cell surface antigens. The copious list of references (up to ca. 1980/81) serves to help the reader delve for detail.

The first 8 chapters (108 pages in all) of which 6 are by Editor Deyl and one each by co-editor Everaerts (on carboxylic acids) and co-editor Prusik (on peptides and structural analysis of proteins) emphasise the filling-in editors have to do in order to produce systematic and rounded volumes. Deyl covers alcohols and phenols, aldehydes and ketones, carbohydrates, steroids, amines and amino acids, according to the principles of editorial conscientiousness.

The various contributions in this part of the book, and for that matter in the whole book, are well laced with illustrative mobility and related data. Details of complexation reactions and derivatisation procedures are given for dealing with absence of net charges in molecules and for improving separations.

Going forward from Chapter 9 is the application area of glycoproteins and glycopeptides (Chapter 10), by T.C. Bøg-Hansen and J. Hau, for illustrating affinity electrophoresis which is based on reactions between interacting components during electrophoresis. The copious use of extremely good quality illustrative photographs and clear line diagrams of the chapter add credit to the high quality of this volume. Lipoproteins (H. Peeters) and the short chapter on lipopolysaccharides (P.F. Coleman and O. Gabriel) are followed by Chapter 13 on enzymes, that is, the second longest chapter (53 pages) of this 20-chapter volume. S. Zadražil tackles his brief from the standpoint of general electrophoretic separations, preparative procedures, analytical separations and the role of electrophoresis for elucidating various physicochemical properties of enzymes. Regard is paid to the need for keeping pH near the optimum value for enzyme activity.

The last 6 chapters, namely, Chapter 14 on nucleotides, nucleosides, etc. and Chapter 15 on nucleic acids by S. Zadražil, the short Chapters 16 (alkaloids) and 17 (vitamins) by Z. Deyl, Chapter 18 on antibiotics by V. Betina, Chapter 19 on dyes and pigments by Z. Deyl, and a short Chapter 20 on inorganic compounds by F.M. Everaerts and Th.P.E.M. Verheggen, also show a degree of editorial infilling in order to complete the full coverage of this book. Between them, the 101 pages of these chapters also show how the generally available apparatus and techniques for electrophoresis can be used. They bring out the usefulness of mixed solvent media, as for vitamins, and there is a small section on the possible use of non-aqueous media with narrow-bore tubes.

Finally, the good general subject index is supported by an indexed list of compounds separated, thus completing a volume that truly earns its place alongside its companion Part A on the shelves of all those interested in and working on electrophoresis.

UWIST, Cardiff (Great Britain) J.D.R. THOMAS

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Book Review

Advances in steroid analysis, Proc. Symp. Analysis of Steroids, Eger, Hungary, May 20—22, 1981 (Analytical Chemistry Symposia Series, Vol. 10), edited by S. Görög, Elsevier, Amsterdam, Oxford, New York, and Akadémiai Kiadó, Budapest, 1982, XI + 552 pp., price Dfl. 225.00, US$ 104.75, ISBN 0-444-99711-3.

Steroid analysis is one of the major fields of chromatography; consequently more than half of the 69 papers collected in this volume apply chromatographic methods, often combined with other techniques, mainly mass spectroscopy and competitive-binding assays. The programme of the Symposium on the Analysis of Steroids, Eger, Hungary, May 20—22, 1981, with its main two streams (pharmaceutical and clinico-biochemical), was not followed in the lay-out of the book. The subject matter was logically classified according to the methods predominating in the various papers. The division is by itself an asset, as it illustrates the relative importance of the methods. The organizers succeeded in attracting authorities from sixteen countries and from various fields, and this is reflected in the broad scope of the volume. Great variety reigns not only with respect to the techniques, but also regarding the steroids under analysis (hormones, bile acids, plant glycosides and their genins, various drugs, sterols, etc.; traditionally calciferols are also included) and to the areas of application (e.g. pharmaceutical intermediates and products, endocrinology, obstetrics, prenatal diagnosis, oncology, cardiology, rheumatology, doping control, nutrition science).

The first section (*General*) includes six papers which did not fit exclusively into any of the special sections. The exposition of the current state of the art in the analysis of estrogens by Adlercreutz et al. (259 references), based on extensive personal experience, discusses the individual stages of the analysis and the snags involved. It shows the advantages of radioimmunoassay (RIA) methods for routine diagnostics and of chromatographic methods [especially capillary gas chromatography (GC), GC—mass spectrometry (MS) and, after preliminary purification, high-performance liquid chromatography (HPLC)] for very low levels and experimental studies. Görög et al. review recent trends in pharmaceutical steroid analysis. In their opinion, immunoassay for drug analysis does not have such a wide application as in clinical biochemistry, and some "classical" methods, such as titrimetry, are not yet completely out of date. Factors that make the control (mainly by RIA and GC—MS) of anabolic-steroid doping so difficult are explained in the excellent review by Stárka et al. (48 references).

Two special sections are devoted to competitive protein binding: *Steroid—protein interactions* and *Immunological methods* (RIA, enzymeimmunoassay). A fascinating subpicogram method for anabolic steroids is expounded by Exley and Shrimanker. It makes use of avidin immobilized on the walls of test-tubes, and of competition between the biotinyl conjugate of the steroid in question (isolated by means of antibodies against the steroid) and a biotinyl conjugate of E. coli β-D-galactosidase. The sensitivity is based on the high equilibrium constant (K_a) between avidin and biotin. A brilliant overview on advances in steroid immunoassay is presented by Jeffcoate. The whole Proceedings seem to document a stage when the decision between radio-, enzyme- and fluorescence immunoassay ceases to be a subject for speculation and becomes a matter of experimentation and experience. It might well be that all three techniques will flourish side by side, according to circumstances. The last two sections are devoted to *Spectroscopic methods* — UV—visible (including a paper by Fell on higher-derivative spectroscopy), NMR, spectropolarimetry — and to *Polarography*.

The remaining four sections (nearly half of the volume) deal explicitly with chromatography — *Gas chromatography*, *Gas chromatography—mass spectrometry*, *High-performance liquid chromatography* and *Thin-layer chromatography*. Brooks et al. discuss derivatization methods which supplement GC—MS in cases where neither retention time nor fragmentation pattern distinguishes isomers unequivocally; e.g. cholesterol oxidase or formation of cyclic boronates are exploited. Björkhem explains the principle and scope of isotope dilution—mass spectrometry (with deuterium-labelled standards) which may serve as the "definitive" (reference) method for other techniques. Several improvements were introduced into the GC—MS technique by Gleispach et al. (pentafluorophenyl derivatives, direct introduction of a quartz tube into the ion source). The paper by Kutner et al. on GC, LC and MS of bile acids in antarctic seals stands out not only by its exotic subject, but also by the judicious combination of techniques. Three papers from Hungarian authors show that the technique of "overpressured thin-layer chromatography" using a pressurized ultramicrochamber is not just a freak but can improve separation, as exemplified in the realms of cardiac glycosides, bile acids and steroidal quaternary ammonium salts. In the latter study and in some other cases, reversed-phase thin-layer chromatography has been used. The videodensitometer "Telechrom", mentioned in the paper by Tasi-Tóth et al. (without proper quotation, unfortunately), seems to offer great promise in detection and quantification on thin-layer chromatograms.

An author index is not provided, but the subject index has been very thoroughly compiled. For instance, one can find fifteen scattered pages on which protein—steroid conjugates with a carboxymethyl oxime bridge are mentioned.

As in most symposium volumes, experimental papers of the type which might be expected to appear in a respectable journal prevail, but several excellent state-of-the-art reviews may serve as signposts to both the steroid experts and to newcomers to the field. The discussion of the papers is not reported in the proceedings.

The book was produced from camera-ready copies, but the combination of good English and frequent (though not annoying) misprints suggests that the manuscripts have undergone editorial revision and occasional retyping. The print is not always black, yet it is clear.

The binding of the book is good, the paper, sometimes semitransparent, is tolerable.

Hradec Králové (Czechoslovakia) I.M. HAIS

Related articles published in *Journal of Chromatography*, Vols. 260 and 261

Volume 260

Volume 261

Author Index

Subject Index

Errata

In the symposium issue (Vol. 273 (1983) No. 1) a note has been omitted, acknowledging the courtesy of the Regional Museum of Eastern Bohemia in making available from their files the view of Hradec Králové based on a woodcut which appeared in "London News Illustrated" in 1866.

J. Chromatogr., 274 (1983) 299—304
Page 299: Third author's name should read "TYGE GREIBROKK".

PUBLICATION SCHEDULE FOR 1983

Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

MONTH	1982	J	F	M	A	M	J	J	A	
Journal of Chromatography	252 253/1 253/2	254 255 256/1	256/2 256/3 257/1	257/2 258 259/1	259/2 259/3 260/1	260/2 261/1 261/2	261/3 262 263	264/1 264/2 264/3 265/1	265/2 266	The publication schedule for further issues will be published later.
Chromatographic Reviews					271/1		271/2		271/3	
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INFORMATION FOR AUTHORS

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ELECTROPHORESIS

A Survey of Techniques and Applications

edited by Z. DEYL, *Czechoslovak Academy of Sciences, Prague*

JOURNAL OF CHROMATOGRAPHY LIBRARY, 18

PART A: TECHNIQUES

Z. DEYL *(editor)*
F.M. EVERAERTS, Z. PRUSÍK *and*
P.J. SVENDSEN *(co-editors)*

"... provides a sound, state-of-the-art survey of its subject".

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"... the editors have set out to bring everything together into a coherent whole... they have succeeded remarkably well... the book is bound to be well liked and appreciated by readers".

– Journal of Chromatography

This first part deals with the principles, theory and instrumentation of modern electromigration methods. Both standard procedures and newer developments are discussed and hints are included to help the reader overcome difficulties frequently arising from the lack of suitable equipment. Adequate theoretical background of the individual techniques is given and a theoretical approach to the deteriorative processes is presented to facilitate further development of a particular technique and its application to a special problem. In each chapter practical realisations of different techniques are described and examples are presented to demonstrate the limits of each method.

CONTENTS:
Introduction. Chapters: 1. Theory of electromigration processes *(J. Vacik)*. 2. Classification of electromigration methods *(J. Vacik)*. 3. Evaluation of the results of electrophoretic separations *(J. Vacik)*. 4. Molecular size and shape in electrophoresis *(Z. Deyl)*. 5. Zone electrophoresis (except gel-type techniques and immunoelectrophoresis) *(W. Ostrowski)*. 6. Gel-type techniques *(Z. Hrkal)*. 7. Quantitative immunoelectrophoresis *(P.J. Svendsen)*. 8. Moving boundary electrophoresis in narrow-bore tubes *(F.M. Everaerts and J.L. Beckers)*. 9. Isoelectric focusing *(N. Catsimpoolas)*. 10. Analytical isotachophoresis *(J. Vacik and F.M. Everaerts)*. 11. Continuous flow-through electrophoresis *(Z. Prusík)*. 12. Continuous flow deviation electrophoresis *(A. Kolin)*. 13. Preparative electrophoresis in gel media *(Z. Hrkal)*. 14. Preparative electrophoresis in columns *(P.J. Svendsen)*. 15. Preparative isoelectric focusing *(P. Blanický)*. 16. Preparative isotachophoresis *(P.J. Svendsen)*. 17. Preparative isotachophoresis on the micro scale *(L. Arlinger)*. List of frequently occurring symbols. Subject Index.

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PART B: APPLICATIONS

Z. DEYL *(editor)*
A. CHRAMBACH, F.M. EVERAERTS *and*
Z. PRUSÍK *(co-editors)*

Part B is an exhaustive survey of the present status of the application of electrophoretic techniques to many diverse compounds. Those categories of compounds most suited to these separations, such as proteins and peptides, are dealt with in detail, while the perspectives of the applications of these techniques to other categories of compounds less commonly electrophoresed are given. Special attention is paid to naturally occurring mixtures of compounds and their treatment. This is the first attempt to cover the field on such a broad scale and the book will be valuable to separation chemists, pharmacologists, organic chemists and those involved in biomedical research.

CONTENTS: 1. Alcohols and phenolic compounds *(Z. Deyl)*. 2. Aldehydes and ketones *(Z. Deyl)*. 3. Carbohydrates *(Z. Deyl)*. 4. Carboxylic acids *(F.M. Everaerts)*. 5. Steroids and steroid conjugates *(Z. Deyl)*. 6. Amines *(Z. Deyl)*. 7. Amino acids and their derivatives *(Z. Deyl)*. 8. Peptides and structural analysis of proteins *(Z. Prusík)*. 9. Gel electrophoresis and electrofocusing of proteins *(edited by A. Chrambach)*. Usefulness of second-generation gel electrophoretic tools in protein fractionation *(A. Chrambach)*. Membrane proteins, native *(L.M. Hjelmeland)*. Membrane proteins, denatured *(H. Baumann, D. Doyle)*. Protein membrane receptors *(U. Lang)*. Steroid receptors *(S. Ben-Or)*. Cell surface antigens *(R.A. Reisfeld, M.A. Pellegrino)*. Lysosomal glycosidases and sulphatases *(A.L. Fluharty)*. Heamocyanins *(M. Brenowitz et al.)*. Human haemoglobins *(A.B. Schneider, A.N. Schechter)*. Isoelectric focusing of immunoglobulins *(M.H. Freedman)*. Contractile and cytoskeletal proteins *(P. Rubenstein)*. Proteins of connective tissue *(Z. Deyl, M. Horáková)*. Microtubular proteins *(K.F. Sullivan, L. Wilson)*. Protein hormones *(A.D. Rogol)*. Electrophoresis of plasma proteins: a contemporary clinical approach *(M. Engliš)*. Allergens *(H. Baer, M.C. Anderson)*. 10. Glycoproteins and glycopeptides (affinity electrophoresis) *(T.C. Bøg-Hansen, J. Hau,)*. 11. Lipoproteins *(H. Peeters)*. 12. Lipopolysaccharides *(P.F. Coleman, O. Gabriel)*. 13. Electrophoretic examination of enzymes *(W. Ostrowski)*. 14. Nucleotides, nucleosides, nitrogenous constituents of nucleic acids *(S. Zadražil)*. 15. Nucleic acids *(S. Zadražil)*. 16. Alkaloids *(Z. Deyl)*. 17. Vitamins *(Z. Deyl)*. 18. Antibiotics *(V. Betina)*. 19. Dyes and pigments *(Z. Deyl)*. 20. Inorganic compounds *(F.M. Everaerts, Th. P.E.M. Verheggen)*. Contents of "Electrophoresis, Part A: Techniques". Subject Index. Index of compounds separated.

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