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BIOMEDICAL APPLICATIONS

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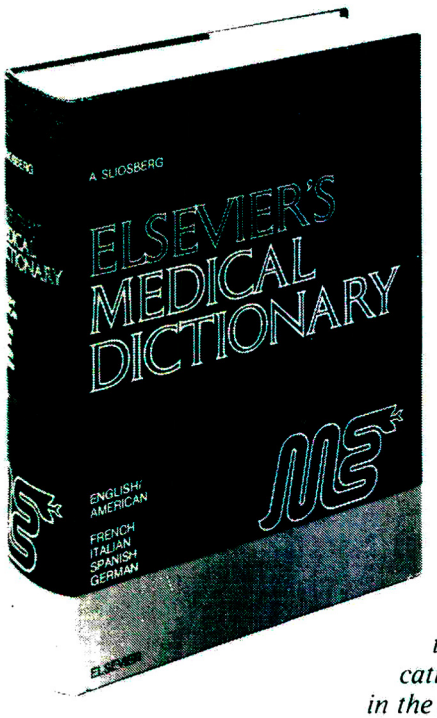

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CHROMBIO. 691

IDENTIFICATION OF SOME ABNORMAL METABOLITES IN PSORIATIC NAIL USING GAS CHROMATOGRAPHY—MASS SPECTROMETRY

KENJI MAEDA* , SHUNSUKE KAWAGUCHI, TOSHIMITSU NIWA, TOYOKAZU OHKI and KAIZO KOBAYASHI

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(First received May 27th, 1980; revised manuscript received July 17th, 1980)

SUMMARY

A gas chromatographic—mass spectrometric analysis was used to separate and identify abnormal compounds in the nail of psoriatic patients. The nail was extracted with heated ethanol, and the extract was analyzed with and without trimethylsilylation. Tetradecanoic acid octadecyl ester, hexadecanoic acid octadecyl ester and octadecanoic acid octadecyl ester were first identified in the psoriatic nail, but were not detected in normal nail.

INTRODUCTION

Psoriasis is a genetic skin disease characterized by glycogen accumulation, excessive cell proliferation, and incomplete differentiation in lesional epidermis. A number of metabolic abnormalities have been noted in the disease [1—4]. However, attention was largely devoted to the analysis of the psoriatic blood, urine and scale. The authors analyzed the affected nail using gas chromatography—mass spectrometry (GC—MS) and detected some previously unidentified compounds, which could not be detected in the normal nail.

MATERIALS AND METHODS

Chemicals

Trimethylsilylating agents, N,O-bis(trimethylsilyl)acetamide and trimethylchlorosilane were purchased from Pierce (Rockford, IL, U.S.A.). Myristic acid, palmitic acid, oleic acid, stearic acid, cholesterol, oxalyl chloride and octadecyl alcohol were the products of Tokyo Kasei (Tokyo, Japan).

Hexadecanoic acid octadecyl ester was synthesized from octadecyl alcohol and palmitoil chloride. Palmitic acid (9 g) and oxalyl chloride (12 ml) were dissolved in benzene (10 ml) and heated at 60°C for 1 h. After concentration with a rotary evaporator, the distillate under reduced pressure (13 mmHg, 188°C)

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was collected, yielding palmitoyl chloride. The latter (4.9 g) and octadecyl alcohol (4.8 g) were dissolved in anhydrous diethyl ether (40 ml) and anhydrous pyridine (3 ml). The mixture was refluxed for 3 h. After washing with hydrogen chloride and concentration with a rotary evaporator, hexadecanoic acid octadecyl ester was purified through a column packed with silica gel 60 F254. The product was confirmed to be pure using ^{1}H NMR spectroscopy.

Sample preparation

Nail samples were obtained from five patients with psoriasis vulgaris and from six healthy adults.

The surface of the nail was discarded and the remainder granulated, 100 mg of the granulated nail was extracted with 10 ml of ethanol at 65°C for 24 h. After concentration with nitrogen gas, the extract was analyzed using GC—MS with and without trimethylsilylation. Trimethylsilylation was performed with 80 μl of N,O-bis(trimethylsilyl)acetamide and 20 μl of trimethylchlorosilane at 30°C for 30 min.

Gas chromatography—mass spectrometry

The instrument used for combined GC—MS consisted of a gas chromatograph, JGC-20K, and a double focusing mass spectrometer, JMS D-300 (JEOL, Tokyo, Japan). The gas chromatograph was equipped with a 3% OV-1 single coiled glass column (2 m × 2 mm I.D.). Carrier gas was helium with a flow-rate of 30 ml/min. The column temperature was programmed from 100°C to 300°C at 4°C/min. Electron impact ionization (EI) mass spectra were recorded under the following conditions: ionizing energy 22 eV, ionization current 300 μA, separator temperature 250°C, ion source temperature 230°C and accelerating voltage 3 kV. Chemical ionization (CI) mass spectra were recorded using methane as a reactant gas, ionizing energy was 250 eV. The other conditions were the same as for EI. High-resolution MS was performed with a data processing system, JMA 2000 (JEOL) with an ionizing energy of 70 eV and a resolution of 5000. Peak matching measurements were performed with perfluorokerosene as a reference compound.

RESULTS

Total ion monitoring chromatograms of the trimethylsilylated extract from psoriatic and normal nail are shown in Figs. 1 and 2, respectively. A number of high peaks were recognized in the psoriatic chromatogram. Peaks 23, 28, 30, 31 and 37 were identified as myristic acid, palmitic acid, oleic acid, stearic acid and cholesterol, respectively. The mass spectra of the peaks were compared with the mass spectra obtained in our laboratory from the derivatives of the authentic compounds.

The EI mass spectrum of peak 29 is shown in Fig. 3. The molecular weight was found to be 342 by recording the CI mass spectrum. The peak was assumed to be octadecanol. The EI mass spectrum of peak 39 is shown in Fig. 4. The ion at *m/e* 480 was confirmed to be the molecular ion by recording the CI mass spectrum. High-resolution MS data indicated that the elemental formulae of the

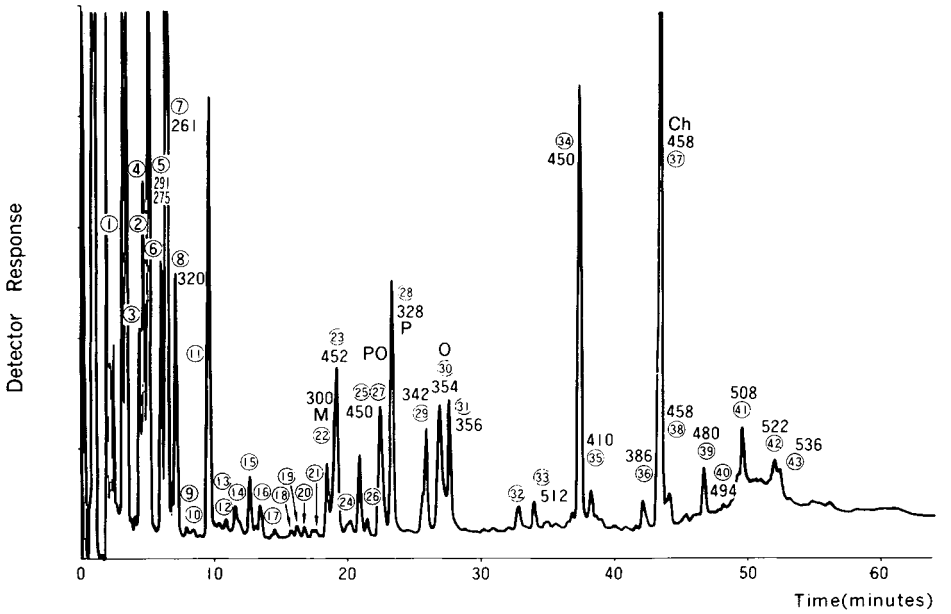

Fig. 1. Total ion chromatogram of the nail extract of psoriasis patients treated (before treatment). Many peaks were seen and they were numbered for easy comparison and evaluation. The figure on each peak indicates the molecular weight of the substance. Peaks: 23, myristic acid; 28, palmitic acid; 30, oleic acid; 31, stearic acid; 37, cholesterol.

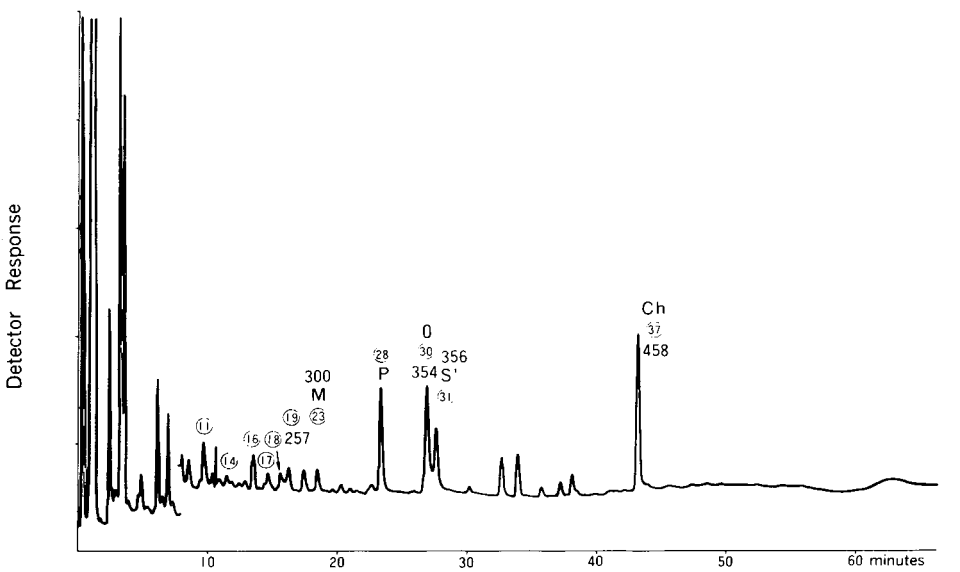

Fig. 2. Total ion chromatogram of a normal nail. Compared to psoriasis the number of peaks is smaller. The number of peaks which also appeared in Fig. 1, and those determined from retention time of the mass chromatogram were identical. Peaks: M, myristic acid; P, palmitic acid; O, oleic acid; S, stearic acid; Ch, cholesterol.

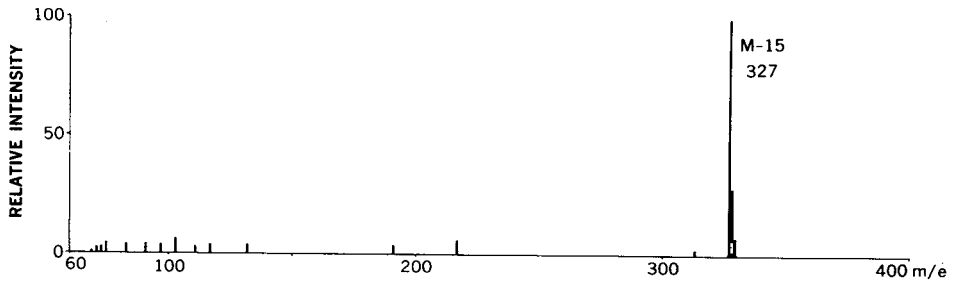

Fig. 3. EI mass spectrum of peak 29. From the determination M = 342, and from retention time of the chromatogram, the peak was identified as octadecanol.

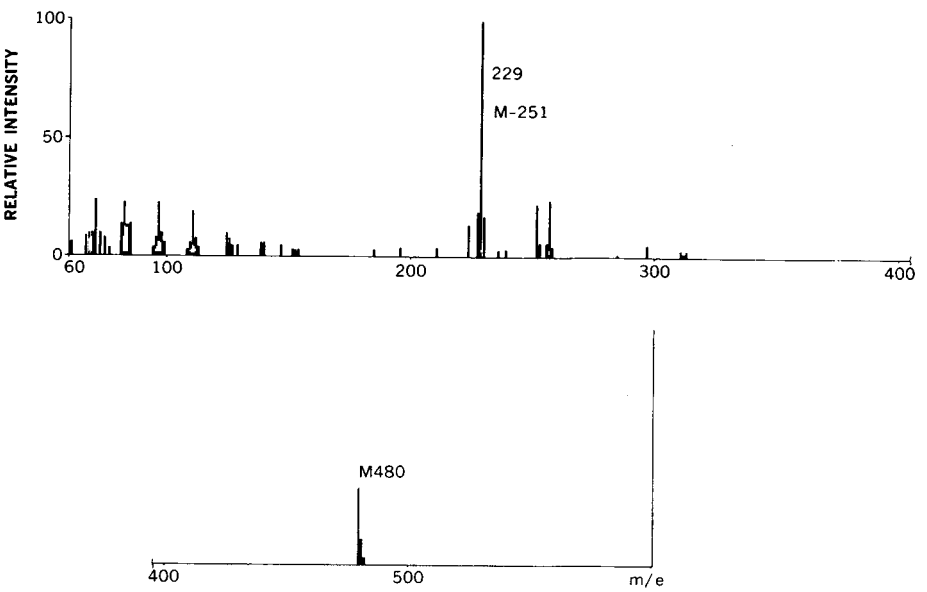

Fig. 4. Mass spectrum of peak 39. M was assumed to be 480. Peaks at *m/e* 229 (M − 251) and 252 are evident.

molecular ion and the ion at *m/e* 229 were $C_{32}H_{64}O_2$ and $C_{14}H_{29}O_2$, respectively. The ion at *m/e* 229 was assumed to be $CH_3(CH_2)_{12}C(OH)O^+H$ formed through double rearrangement of protons. The compound was then identified as tetradecanoic acid octadecyl ester. The EI mass spectrum of peak 41 is shown in Fig. 5. The CI mass spectrum indicated that the molecular ion of the compound was 508. The elemental formula of the compound was $C_{34}H_{68}O_2$, which was derived from high-resolution data. Peak 41 was identified as hexanoic acid octadecyl ester by comparing with the EI mass spectrum of the authentic compound. The EI mass spectrum of peak 43 is presented in Fig. 6. The molecular ion was confirmed to be 536 by recording the CI mass spectrum. The elemental formula of the compound was $C_{36}H_{72}O_2$, identifying peak 43 as octadecanoic acid octadecyl ester. The heights of peak 29 and peaks 39—43 have diminished with improvement of the psoriatic lesions.

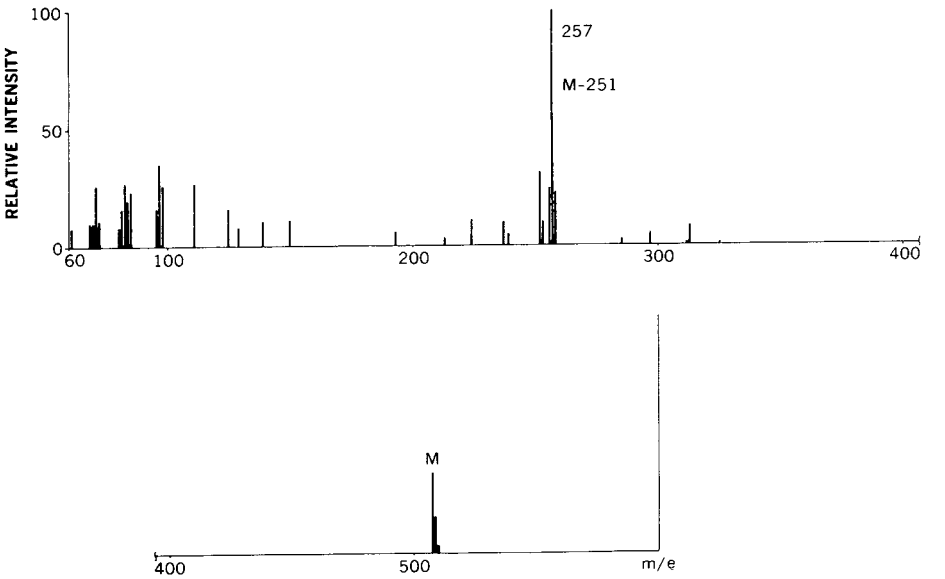

Fig. 5. Mass spectrum of peak 41. *m/e* 508 (M) and 257 (M — 251) were observed and as with peak 39, *m/e* 252 was evident.

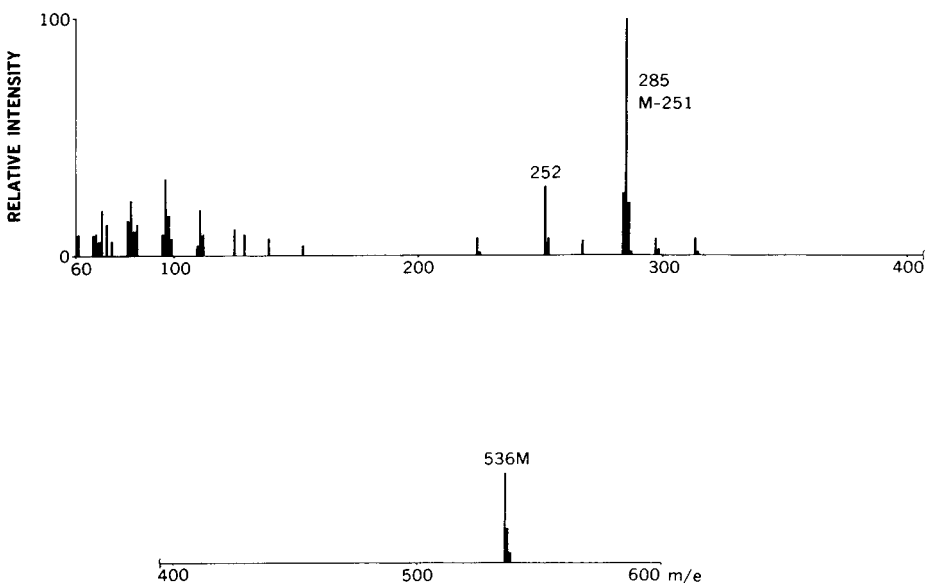

Fig. 6. Mass spectrum of peak 43. *m/e* 536 (M) and 285 (M — 251) were observed. As with peaks 39 and 41, *m/e* 252 was evident.

DISCUSSION

Since the first successful treatment of psoriatic patients with hemodialysis [5], others have also confirmed the effectiveness of the method in a number of retrospective studies on regular hemodialysis patients complicated with the disease [6]. Peritoneal dialysis was also reported to be effective to the psoriatic lesion [7]. However, a recent study reported total ineffectiveness of hemodialysis [8]. In our hospital five out of thirteen patients who were suffering from the disease for a long time and had tried all conventional methods of treatment, remitted completely. These results lead us to a hypothesis that some noxious metabolite(s) accumulate(s) in the blood of psoriasis patients and that the substance is removed by hemodialysis.

The authors detected three abnormal metabolites in the psoriatic nail: tetradecanoic acid octadecyl ester, hexadecanoic acid octadecyl ester and octadecanoic acid octadecyl ester. These esters could not be detected in normal nail nor in psoriatic ultrafiltrate of blood. The origin and its physiological significance are yet unknown.

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CHROMBIO. 674

A MICROLITER METHOD FOR THE GAS CHROMATOGRAPHIC DETERMINATION OF LONG-CHAIN NON-ESTERIFIED FATTY ACIDS IN HUMAN SERUM OR PLASMA

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(First received March 28th, 1980; revised manuscript received July 17th, 1980)

SUMMARY

Non-esterified fatty acids (NEFA) from C_{12} to C_{24} are assayed in human serum or plasma in a four-step procedure: extraction, volume reduction, methylation and gas chromatography. NEFA are extracted with chloroform—heptane—methanol from 50—100 μl of serum or plasma buffered with phosphate. After adding ethyl acetate the volume of the extract is reduced under partial reflux to 5—7 μl. Potassium carbonate, methyl iodide and a crown ether are added to the dry concentrate and the NEFA are selectively methylated with a yield of 100% by heating in a microrefluxer for 10 min. Gas chromatography is carried out with 1 μl of the reaction mixture on a packed column by temperature-programmed operation. Thirteen individual fatty acids are determined in sera of normal adults. The coefficients of variation for 24 determinations of a pooled serum were 2.7% for the total NEFA content and 3—10% for most of the individual NEFA.

INTRODUCTION

Long-chain (C_{12}—C_{24}) non-esterified fatty acids (NEFA) are present in human blood in physiologically varying concentrations [1]. Clinical interest follows from the fact that also certain pathological situations lead to changes in the NEFA pattern; for example, general hyperlipidacidemia in diabetes mellitus or the decrease of the linoleic acid:oleic acid ratio in stroke patients [2].

Therefore assay of NEFA can be useful for diagnosis and in controlling the course of diseases as well as in the search for risk patients in preventive medicine.

A large number of methods for the quantitative determination of total as well as individual NEFA in serum/plasma have been published. The total NEFA content is usually assayed by titration [3—8] or colorimetrically after converting the fatty acids into their copper or cobalt soaps [9—14]. Recently an enzymatic method was described [15]. Individual NEFA are best determined by gas chromatography (GC) [16—22]. However, GC methods with sufficient sensitivity and precision are too laborious and therefore unsatisfactory for clinical use [16—18].

This paper presents a new micromethod for the GC assay of the individual NEFA in serum/plasma, which is based on the prechromatographic microliter techniques described recently [23]. It is as accurate as previous methods but is faster, easier in application and less expensive. In addition to this it is more sensitive so that NEFA such as lauric, myristoleic, linolenic, arachidonic or erucic acid, which are present in serum only in minor amounts, can also be determined.

EXPERIMENTAL

Materials and methods

All reagents and solvents which were of the highest available quality were used without further purification. Undecanoic, lauric, tridecanoic, myristic, myristoleic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, arachic, arachidonic, behenic, erucic, lignoceric acid and their methyl esters were from Sigma (Munich, G.F.R.). 2,6-Di-*tert.*-butyl-*p*-cresol and methyl iodide were purchased from Roth (Karlsruhe, G.F.R.). Potassium carbonate, iodine, potassium dihydrogen phosphate, disodium hydrogen phosphate, dibenzo-18-crown-6, ethyl acetate, propan-2-ol, chloroform, *n*-heptane, and methanol were from E. Merck (Darmstadt, G.F.R.).

For the extraction solution, one part of chloroform is mixed with one part of *n*-heptane and 2% methanol added. The buffer solution is 0.53 *M* potassium dihydrogen phosphate and 0.27 *M* disodium hydrogen phosphate. The methylation solution is a saturated solution of dibenzo-18-crown-6 in methyl iodide prepared at room temperature. The internal standard solution is 2.5 m*M* tridecanoic acid in propan-2-ol.

The calibration mixture consists of 0.2 m*M* each of lauric, tridecanoic, myristic, myristoleic, palmitic, palmitoleic, stearic, oleic, linoleic, linolenic, arachidonic, behenic, erucic and lignoceric acid methyl esters in ethyl acetate.

The microrefluxer [24] and boiling chips suitable for the microvessels were purchased from Forschungsinstitut Berghof (Tübingen, G.F.R.). The microvessels (Fig. 1) were prepared by a glass-blower according to specifications [25]. They were then cleaned adhering strictly to the given instructions [26, 27].

Thin-layer chromatography was performed on 40 mm × 80 mm silica-gel plates without fluorescence indicator (Merck) in a vapour phase saturated flat tank according to the method of Seiler and Knödgen [28]. The plate was

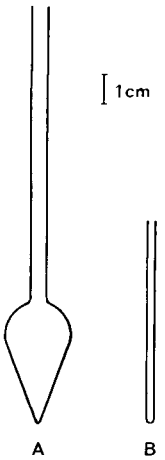

Fig. 1. Special equipment for the microliter technique. (A) Glass vessel of type A for volume reduction from 2000 to 50 μl. (B) Glass vessel of type B for volume reduction from 50 to 5 μl and for the methylation reaction.

developed in a solvent system consisting of *n*-hexane—diethyl ether—glacial acetic acid (90:10:1, v/v; Merck). After drying, the plate was placed in an iodine chamber for visualisation of the lipid and NEFA spots. For the identification of spots the following lipids were run in parallel: dipalmitoyl L-α-phosphatidyl choline, dimyristoyl L-α-phosphatidyl ethanolamine, glycerol tripalmitate, and cholesterol palmitate (Serva, Heidelberg, G.F.R.).

Standard procedure

Extraction. Serum or plasma (50—100 μl) is pipetted into a stoppered glass vessel of about 10 ml volume and 2 μl of the internal standard solution are added. After stirring for 1 min with a Vortex mixer followed by standing for 5 min, 150 μl of buffer solution and 1.25 ml of extraction solution are added. The mixture is vortexed for 2 min and after 15 min standing it is centrifuged at 800 *g* for another 15 min. Most of the upper aqueous layer is then removed to obtain direct access to the lower organic phase.

Concentration. One thousand microliters of the extract are transferred to a microvessel of type A (Fig. 1A) and 1000 μl of ethyl acetate are added. Heating the mixture in a water-bath at 95°C under partial reflux reduces the volume of the extract. Optimum conditions are reached when a condensation zone of some millimeters width can be seen 2—5 mm under the rim of the vessel [25]. This is achieved by varying the immersion depth of the vessel in the water-bath. When the condensation ring begins to drop, another 500 μl of ethyl acetate are added. Heating is continued until only a few microliters of liquid can still be seen in the vessel. Then the vessel is immediately chilled by ice. After cooling for 5 min about 50 μl of the extract have gathered at the bottom of the vessel. The solution is quantitatively transferred to a microvessel of type B (Fig. 1B) by means of a Pasteur pipette. Concentration is then performed as described above at 90°C until about 1 μl of liquid can be recognized. By cooling in the ice-bath a final volume of 5—7 μl of extract is obtained.

Methylation. A 3.5-μl volume of the concentrated extract is transferred to another type B vessel provided with a boiling chip and 3.5 μl of the methylation solution are added. In a controlled-humidity environment [24;29] about 0.5 mg of finely powdered potassium carbonate which had been dried in a drying pistol for 24 h at 250°C over phosphorus pentoxide is added with a special device [30]. The mixture is then refluxed under atmospheric pressure in a microrefluxer for 10 min. Up to twelve reactions can be carried out simultaneously. A sample of the extract can now be directly injected into the gas chromatograph. If the vessels are closed with suitable PTFE caps and kept at about 0°C no loss occurs during days of storage.

Gas chromatography. A Hewlett-Packard Model 5710A double-column gas chromatograph equipped with a flame ionization detector was used. One microliter of the sample was injected on one column while the other was conditioned by several injections of solvent at the same time. GC was carried out on 183 cm × 0.64 cm glass columns packed with 10% Silar 10 CP on Chromosorb W HP, 100—120 mesh (Hewlett-Packard, Frankfurt, G.F.R.), by temperature-programmed operation. The conditions were: oven temperature T_1 130°C, T_2 220°C, ΔT 4°C/min; injection port temperature 250°C; detector temperature 300°C; flow-rates were nitrogen 44 ml/min, hydrogen 60 ml/min, air 240 ml/min; range 10, attenuation 2.

Fatty acid concentrations were calculated electronically with a Hewlett-Packard Model 3380A integrator using the internal standard operation.

RESULTS AND DISCUSSION

Extraction

NEFA are extracted from serum or plasma by a modification of the method of Falholt et al. [14]. When the serum/plasma is buffered at pH 6.4 with potassium dihydrogen phosphate and disodium hydrogen phosphate, chloroform—*n*-heptane—methanol extracts the NEFA without significant co-extraction of other lipids. As shown by thin-layer chromatography, cholesterol esters, triglycerides and phospholipids were present only in trace amounts, whereas the extraction procedures of Dole [3] or of Folch et al. [31] lead to considerable amounts of these lipids in the extract (Fig. 2). As other lipids of serum/plasma can interfere with the analysis of NEFA the extracts obtained by the Dole or Folch procedures have to be purified by thin-layer chromatography [16—18,22]. This laborious step is omitted in our method.

Concentration

The extracts are concentrated to about 1/200 of their original volume under partial reflux in specially designed glass vessels (Fig. 1). Upon addition of ethyl acetate the solvent is rapidly removed in a water-bath at 90—95°C. At the same time the extract is dried azeotropically. The dryness of the concentrates was proved by cooling with acetone—CO_2. No turbidity could be observed, which indicated that the water content was lower than 0.1% [24]. It was demonstrated with serum extracts containing definite amounts of added undecanoic acid and with standard solutions of C_{12}—C_{24} fatty acids that no loss of material occurs with this concentration technique.

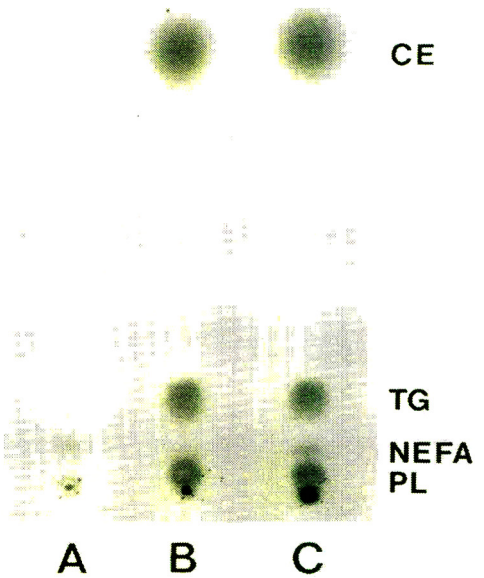

Fig. 2. Thin-layer chromatogram of concentrated serum extracts obtained by different procedures for the extraction of NEFA. (A) Extraction with chloroform—heptane—methanol modified after Falholt et al. [14]. (B) Extraction with propan-2-ol—heptane—HCl according to the method of Dole [3]. (C) Extraction with chloroform—methanol according to the method of Folch et al. [31]. Concentration of the extracts and thin-layer chromatography are described in Experimental. CE = cholesterol esters; TG = triglycerides; PL = phospholipids.

Methylation

The preparation of methyl esters from fatty acids by methyl iodide in the presence of potassium carbonate has already been described [19,32]. However, as the reaction had to be carried out under pressure for 1 h to achieve complete yield it did not seem suitable for a routine assay. The reaction conditions can be mitigated substantially by addition of a crown ether [33]. With dibenzo-18-crown-6, 100% yield of fatty acids from C_6 to C_{24} in the concentration range 5 μM (detection limit under the GC conditions used) to 10 mM is achieved within 10 min of refluxing at atmospheric pressure. The sample is then ready to be injected into the gas chromatograph. Other procedures commonly used to prepare the methyl esters, such as the boron trifluoride—methanol [17,18] or N,N-carbonyldiimidazole—methanol [16] methods, are much more laborious because additional steps of washing, extraction or volume reduction are necessary before chromatography.

The methylation step has also been tested on possible undesired reactions. Neither methylation of the carbon chain of unsaturated fatty acids nor transesterification of esterified fatty acids occurred even under prolonged refluxing [33]. Methylation by the methyl iodide—potassium carbonate method can be carried out in the presence of glyceryl esters in total lipid extracts of serum/plasma. Selective NEFA derivatization is also achieved by methylation with diazomethane [34] and trimethylsilylation [35].

Gas chromatography

With most of the serum or plasma NEFA assays, GC on packed columns is carried out isothermally because bleeding stationary phases such as DEGS or FFAP are used. Isothermal operation, however, means insufficient separation and unequal detection sensitivity for the long-chain NEFA spectrum in serum/plasma. These disadvantages can be avoided with temperature-programmed operation on the low-bleeding stationary phase Silar 10CP. As shown in Fig. 3, the peaks of fifteen equally concentrated fatty acid methyl esters of a standard mixture are clearly separated and equally high.

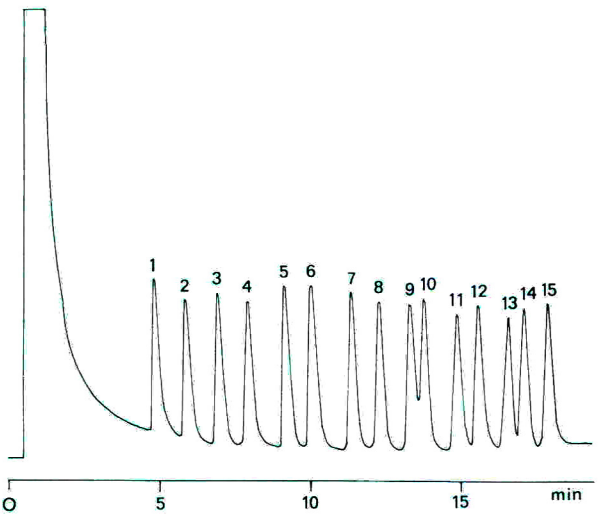

Fig. 3. Gas chromatogram of a standard mixture of the methyl esters of fifteen long-chain fatty acids with carbon chain length C_{12}—C_{24} obtained by temperature-programmed operation on packed Silar 10CP columns. The GC conditions are given in Experimental. Concentration of each fatty acid methyl ester was 0.2 m*M*. 1 = Lauric acid methyl ester; 2 = tridecanoic acid methyl ester; 3 = myristic acid methyl ester; 4 = myristoleic acid methyl ester; 5 = palmitic acid methyl ester; 6 = palmitoleic acid methyl ester; 7 = stearic acid methyl ester; 8 = oleic acid methyl ester; 9 = linoleic acid methyl ester; 10 = arachinic acid methyl ester; 11 = linolenic acid methyl ester; 12 = behenic acid methyl ester; 13 = erucic acid methyl ester; 14 = arachidonic acid methyl ester; 15 = lignoceric acid methyl ester.

In Fig. 4 a chromatogram of a pooled human serum is demonstrated. The corresponding blank chromatogram contains only one prominent peak which derives from an impurity of methyl iodide. This peak is clearly separated from the fatty acid peaks of the serum.

The sample injected into the gas chromatograph contains the methyl esters in about an eightfold concentration compared to the original concentration in the serum/plasma. Therefore, also the fatty acids lauric, myristoleic, linolenic, arachidonic and erucic acid, which are present only in minor amounts, can be precisely determined.

Autoxidation

A series of serum NEFA analyses has been performed in the presence of 0.05% of 2,6-di-*tert.*-butyl-*p*-cresol (BHT) in the unconcentrated extract and

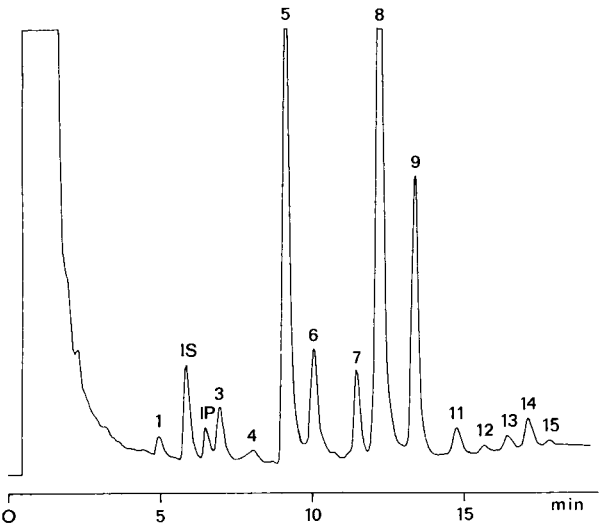

Fig. 4. Gas chromatogram of the NEFA methyl esters from a pooled human serum. The conditions are given in Experimental. IP = impurity from methyl iodide; ISTD = tridecanoic acid methyl ester (from added internal standard). For further designations see legend of Fig. 3.

compared to another series of analyses of the same serum without BHT. No significant difference in the amount of the fatty acids, for example of linolenic and arachidonic acid, was observed. With our technique impairment of the results of NEFA analyses by autoxidation of unsaturated fatty acids in the course of the assay can therefore be excluded [36].

Sample pretreatments

The influence of different pretreatments (coagulation, storage) of a human blood sample has been examined (Table I). NEFA assays were carried out with serum, heparin plasma and EDTA plasma of blood from the same collection. No difference in the NEFA content could be determined between serum and heparin plasma of blood kept for 30 min at room temperature before centrifuging. However, EDTA plasma values were significantly ($p < 0.01$) lower than those of serum. Also in heparin plasma of blood which was allowed to stand at room temperature for 30 min a somewhat higher NEFA content was found compared to heparin plasma of blood which was centrifuged immediately after collection. NEFA analyses of serum or plasma which had been kept at −70°C for several weeks did not differ from those of corresponding samples assayed immediately.

Evaluation of the method

The precision of the method has been evaluated by 24 NEFA assays of a pooled human serum. Mean values, standard deviations and coefficients of variation of thirteen individual long-chain NEFA and of the total fatty acid content are shown in Table II.

Sensitivity has been tested with aliquots of a pooled serum to which decreasing amounts of tridecanoic acid dissolved in propan-2-ol were added. Trideca-

TABLE I

THE EFFECT OF ANTICOAGULANTS AND DIFFERENT STORAGE CONDITIONS ON THE TOTAL* NEFA CONTENT OF A HUMAN BLOOD SAMPLE

The blood obtained by venipuncture from a healthy female adult was collected either in a serum-monovette® (Sarstedt, Nümbrecht, G.F.R.) or in syringes containing heparin (75 U/ml Thrombophob®, Nordmark, Hamburg, G.F.R.) or EDTA (Sarstedt). Centrifugation was carried out at 4°C for 10 min at 1300 *g*.

	Assay immediately after centrifugation (μM; mean ± S.D.; n = 8)	Assay after storage for four weeks at −70°C (μM; mean ± S.D.; n = 8)
Heparin plasma of blood centrifuged immediately after collection	524 ± 43	512 ± 35
Heparin plasma of blood kept 30 min at room temperature before centrifugation	553 ± 37	545 ± 30
EDTA plasma of blood centrifuged immediately after collection	461 ± 28	470 ± 18
EDTA plasma of blood kept 30 min at room temperature before centrifugation	467 ± 33	458 ± 25
Serum of blood kept 30 min at room temperature	550 ± 21	560 ± 19

*The sum of the individual NEFA determined by GC.

TABLE II

MEAN VALUES, STANDARD DEVIATIONS AND COEFFICIENTS OF VARIATION OF 24 DETERMINATIONS OF INDIVIDUAL AND TOTAL NEFA CONCENTRATIONS OF A POOLED HUMAN SERUM

NEFA		NEFA concentration (μM)		Coefficient of variation (%)
		Mean	S.D.	
$C_{12:0}$	lauric acid	8	0.9	11.3
$C_{14:0}$	myristic acid	22	1.8	8.2
$C_{14:1}$	myristoleic acid	8	0.8	10.0
$C_{16:0}$	palmitic acid	242	8.0	3.3
$C_{16:1}$	palmitoleic acid	53	2.6	4.9
$C_{18:0}$	stearic acid	37	3.6	9.7
$C_{18:1}$	oleic acid	287	9.4	3.3
$C_{18:2}$	linoleic acid	121	7.0	5.8
$C_{18:3}$	linolenic acid	12	1.3	10.8
$C_{20:4}$	arachidonic acid	16	1.7	10.6
$C_{22:0}$	behenic acid	1	—	—
$C_{22:1}$	erucic acid	5	0.7	14.0
$C_{24:0}$	lignoceric acid	1	—	—
Total		810	22.0	2.7

noic acid of 0.5 μM could still be clearly determined in a serum with a total NEFA content of about 0.5 mM.

For recovery studies distinct amounts of either tridecanoic, stearic or linoleic acid were added to samples of a serum. The mean recoveries of twelve experiments were 98% for tridecanoic acid, 100% for stearic acid and 97% for linoleic acid (Table III). Advantages of the method for its application as a clinical routine assay are due to the microliter scale. The amount of serum/plasma

TABLE III

RECOVERY EXPERIMENTS

Either tridecanoic, stearic or linoleic acid dissolved in propan-2-ol (2.5 mM) was added to aliquots of a pooled serum and assayed with the standard procedure. In experiments 1—3 oleic acid in the serum was chosen to serve as reference, otherwise the added tridecanoic acid was used as internal standard. The mean values of four assays for each experiment are given.

Exp.	Added fatty acid concentration	Assayed concentration (μM)			Recovery (%)
		Tridecanoic acid	Stearic acid	Linoleic acid	
1	25 μM tridecanoic acid	23	39	114	92
2	50 μM tridecanoic acid	52	40	125	104
3	100 μM tridecanoic acid	98	37	123	93
4	25 μM stearic acid	—	64	120	103
5	50 μM stearic acid	—	88	118	101
6	100 μM stearic acid	—	130	125	95
7	25 μM linoleic acid	—	35	140	96
8	50 μM linoleic acid	—	40	169	99
9	100 μM linoleic acid	—	38	214	97

necessary for the assay is reduced to 50—100 μl. As less solvents and reagents are used the interference from their impurities in the GC analysis is minimized. Furthermore, the costs for chemicals and glassware are lower. The prechromatographic part of the assay can be done with 10—12 samples in parallel and then requires about 3 h.

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HIGH-PERFORMANCE ANION-EXCHANGE CHROMATOGRAPHY OF HUMAN URINE USING PERCHLORATE GRADIENT ELUTION SYSTEMS

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SUMMARY

A 100-μl volume of urine was chromatographed on a 50 × 0.4 cm I.D. column packed with a macroreticular anion-exchange resin. Elution was performed with a concave ammonium perchlorate gradient from 0 to 0.25 *M* at a flow-rate of 0.75 ml/min and a pressure of 7.5—11 MPa. With this perchlorate gradient, no baseline drift occurred in the detection at 254 nm, and even detection at 200 nm was possible. The effect of the addition of ethanol or acetonitrile to the ammonium perchlorate solution was investigated. For the assignment of peaks, ultraviolet spectra of the peaks were measured with stopped-flow scanning spectrophotometry.

INTRODUCTION

Several high-performance liquid chromatographic systems have been developed for the separation of ultraviolet (UV)-absorbing constituents of urine with anion-exchange resins [1—6] or reversed-phase packing material [7]. In these systems, chromatography using macroreticular anion-exchange resins revealed many advantageous characteristics for the separation of constituents of body fluids. For example, body fluids such as urine [4,6], blood plasma or serum [8], hemodialysate [8] and blood cells [9] could be analyzed by a

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standard analytical system using a macroreticular anion-exchange resin and a linear ammonium acetate gradient [6, 9]. However, under this chromatographic condition, the ammonium acetate gradient caused a baseline drift in the detection at 254 nm, and detection at shorter UV wavelengths was impossible because of absorbance of acetate ions. To improve the elution system, Miyagi et al. [5] proposed an elution method based on stepwise elution with ammonium chloride—acetonitrile as the mobile phase. Unfortunately, chloride ions in this mobile phase eroded the steel of the instrument, and the detection was not satisfactory in terms of wavelength limit. Thus, we developed new elution systems to eliminate the baseline drift and to detect the constituents of urine in the short UV-wavelength region.

EXPERIMENTAL

Chemicals

Analytical grade ammonium perchlorate, ethanol and acetonitrile were purchased from Wako (Tokyo, Japan). The reference compounds — creatine, creatinine, pyridoxine, uracil, histidine, theobromine, nicotinamide, hypoxanthine, adenosine, xanthine, phenylalanine, caffeine, tyrosine, tryptophan, theophylline, urocanic acid, uric acid, nicotinic acid, 4-aminohippuric acid, 4-hydroxy-3-methoxymandelic acid, 4-hydroxy-3-methoxyphenylacetic acid, *p*-aminobenzoic acid, hippuric acid, quinaldic acid, *p*-hydroxyphenylacetic acid, vanillic acid, kynurenic acid, *p*-hydroxyphenylpyruvic acid, benzoic acid, *p*-hydroxybenzoic acid, 3-hydroxyanthranilic acid, indoleacetic acid and indoleacrylic acid — were also purchased from Wako.

Resin

The strongly basic anion-exchange resin, Diaion CDR-10, is a macroreticular type having a particle size distribution of 5—7 μm. This resin was obtained from Mitsubishi Chemical Industries (Tokyo, Japan).

Apparatus

An Hitachi Model 634 high-performance liquid chromatograph was used for the urine analysis, and was coupled to a variable-wavelength photometer and 10-mV data recorder. Stopped-flow scanning spectrophotometry was performed with a scan speed of 60 nm/min and a slit width of 4.0 nm for scanning from 340 to 200 nm.

A 50 × 0.4 cm I.D. stainless-steel column was packed with Diaion CDR-10 using a high-pressure slurry technique [10] with 2.0 *M* ammonium perchlorate aqueous solution.

Sample preparation

The urine sample, usually a 24-h collection, was refrigerated until complete, then frozen and stored at −20°C. Before analysis, the sample was thawed and passed through a 0.22-μm Millipore filter to remove particulate matter.

Anion-exchange chromatography

A 100-μl urine sample was introduced onto the column. The urinary con-

stituents were then eluted with a concave ammonium perchlorate gradient at a flow-rate of 0.75 ml/min using the two-chamber gradient generator. The curvature of the gradient could be chosen by the curve coefficient (K) and total gradient volume (V). The curve coefficient is the ratio of the area of upper phase (V_1) against the area of lower phase (V_2) in the area of the rectangle (V_t) divided by the gradient curve. Then $K = V_1/V_2$. The concave perchlorate gradient was formed by placing 20 ml of water in the first chamber, and 20 ml of 0.25 M ammonium perchlorate solution in the second chamber, when $K = 3$ and $V = 20$ were selected. The column temperature was raised from 22 to 70°C over the first 30 min, then maintained at 70°C to the end of the run. Due to the increase in the column temperature, the inlet pressure changed from 11 to 7.5 MPa over the first 30 min. The ammonium perchlorate gradient did not affect the column inlet pressure.

Assignment of the chromatographic peaks

Peak assignments were carried out in three ways: (1) by comparing the retention time of a peak to those of the standard compounds; (2) by injecting the standard compounds along with the sample; and (3) by measuring the UV spectrum of a peak at the peak maximum by stopped-flow scanning spectrophotometry.

RESULTS AND DISCUSSION

Investigation of eluting ion for the separation and detection of UV-absorbing constituents of urine

The first purpose of this study was the elimination of the baseline drift caused by the acetate gradient. Some baseline compensation methods [11], such as series flow, dual columns and chemical compensation, were tested. However, these baseline compensation methods were complicated and not satisfactory for the routine method. Even if compensation is possible, detection at short UV wavelengths could not be performed. Therefore, a gradient elution system using other buffers or salt solutions, which do not absorb in the short UV-wavelength region, was necessary. Anions such as perchlorate, phosphate, sulfate, borate, nitrate, carbonate, sulfamate and chloride were tested. Of these, perchlorate and chloride were satisfactory for both the resolution and the detection of peaks. However, chloride ion was very harmful to the steel of the instrument. Therefore, perchlorate ion was chosen from many advantageous chromatographic results. Similarly, cations such as ammonium, sodium and lithium were tested, and it was found that the species of cation scarcely affected the separation and detection. Consequently, ammonium perchlorate solution was suitable for the separation and detection of UV-absorbing constituents of urine.

Separation of UV-absorbing constituents of urine with a linear or a concave ammonium perchlorate gradient

A series of chromatograms detected at several wavelengths is shown in Fig. 1. These chromatograms were run using the linear ammonium perchlorate gradient. In these chromatograms, baseline drift did not occur during detection at

254 nm, and even detection at 200 nm was possible. The final concentration of ammonium perchlorate solution (0.25 *M*) was sufficient to elute most of the UV-absorbing constituents of urine. However, when the linear ammonium perchlorate gradient was not satisfactory in resolving the peaks eluted at the beginning of the gradient, then the concave ($K = 3$) gradient was employed for high-resolution separation.

UV-absorbance chromatograms measured with the concave perchlorate gradient are shown in Fig. 2. These chromatograms were obtained using the same gradient curve coefficient ($K = 3$) but a different gradient volume (V). The gradient volume for the first (Fig. 2A), second (Fig. 2B) and third (Fig. 2C) chromatogram was 10, 15 and 20 ml, respectively. As Fig. 2C shows, the resolution at $V = 20$ gave excellent results and the resolution of peaks was improved. Also, the shape of the peaks eluted after hippuric acid was sharper than those obtained using the linear gradient elution system.

On the other hand, when the number of detectable peaks on the chromatograms was compared to that obtained with the acetate gradient elution system, the former was lower than the latter. Namely, the constituents strongly re-

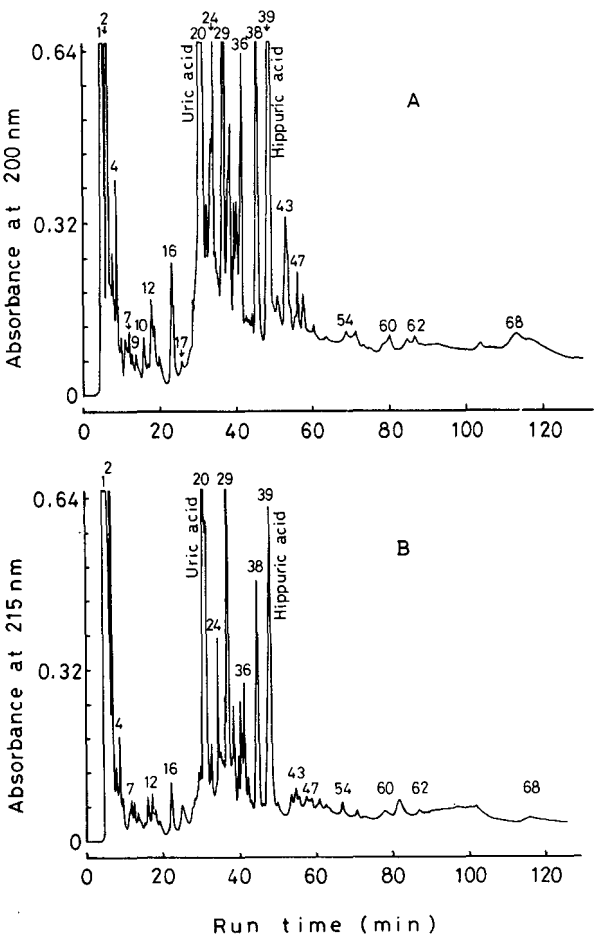

Fig. 1.

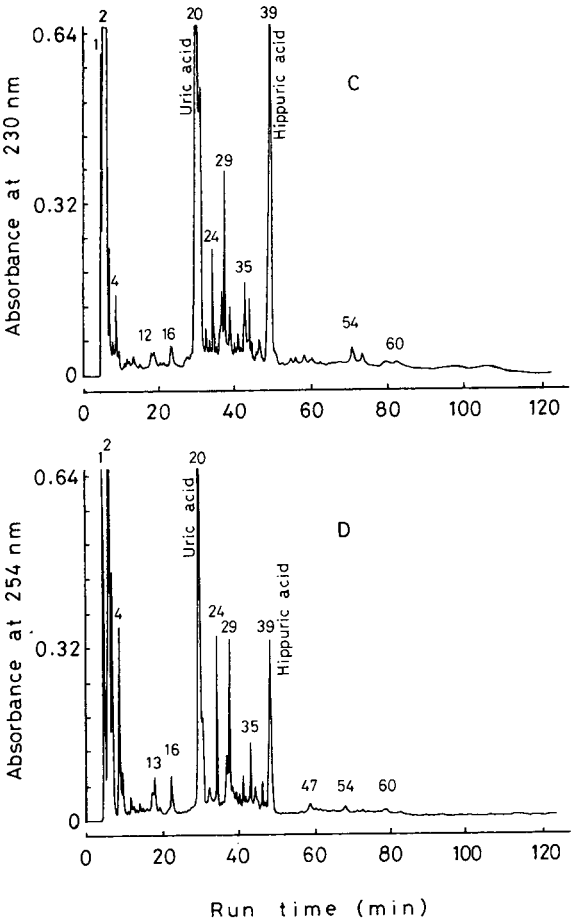

Fig. 1. Chromatograms of human urine detected at (A) 200 nm, (B) 215 nm, (C) 230 nm and (D) 254 nm, by a linear ammonium perchlorate gradient elution system. Conditions: column 50 × 0.4 cm I.D., packed with Diaion CDR-10; eluent, ammonium perchlorate solution (pH 5.5) varying in concentration from 0 to 0.25 *M* in a linear gradient; temperature, increasing from 22 to 70°C over the first 30 min, then 70°C to the end of the run; average flow-rate, 0.75 ml/min; average pressure, 8.5 MPa. For peak assignments, see Table I.

tained on the resin by non-ionic adsorption could not be eluted with the perchlorate gradient.

Concave gradient elution systems with ammonium perchlorate plus organic solvents

To elute the strongly retained constituents, the addition of ethanol or acetonitrile to the ammonium perchlorate solution (0.25 *M*) in the proportion of 15% was used. Two series of chromatograms obtained with ammonium perchlorate—ethanol and with ammonium perchlorate—acetonitrile are shown in Figs. 3 and 4, respectively. The effects of these additives on the retention times of standard compounds and of urinary constituents are seen in Table I. From

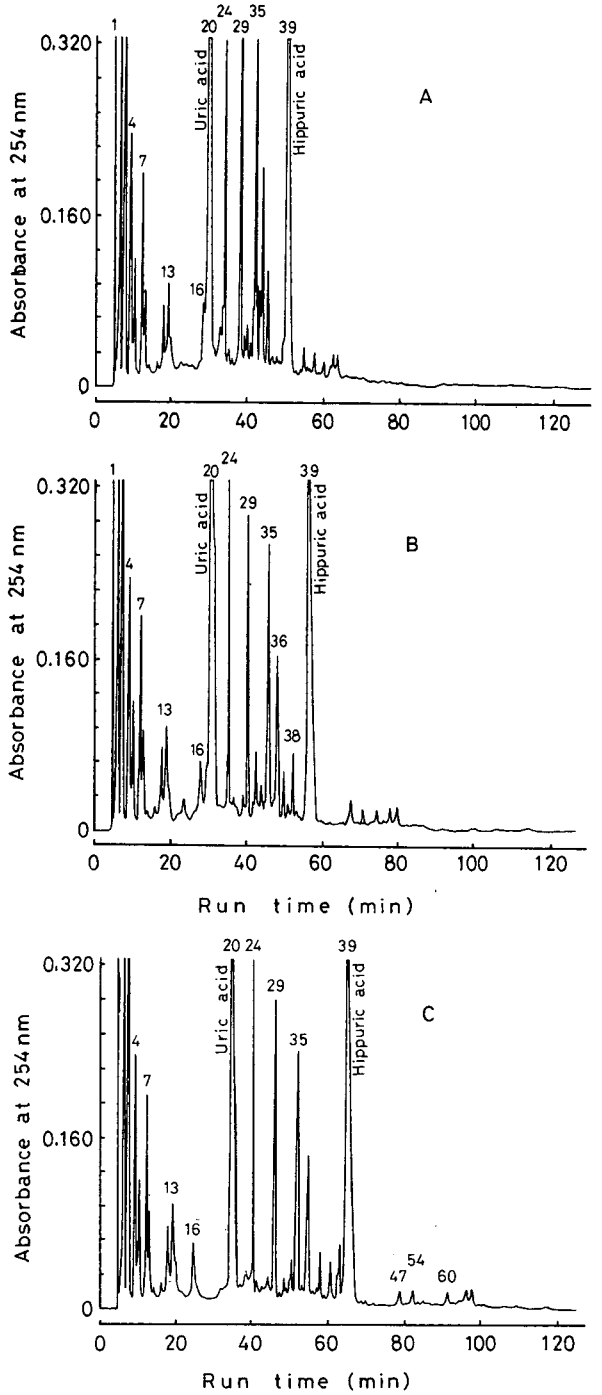

Fig. 2. Chromatograms of human urine measured by a concave ammonium perchlorate gradient elution system. Conditions were the same as in Fig. 1, except for gradient curve coefficient (K=3) and gradient volume (A=10, B=15 and C=20). For peak assignments, see Table I.

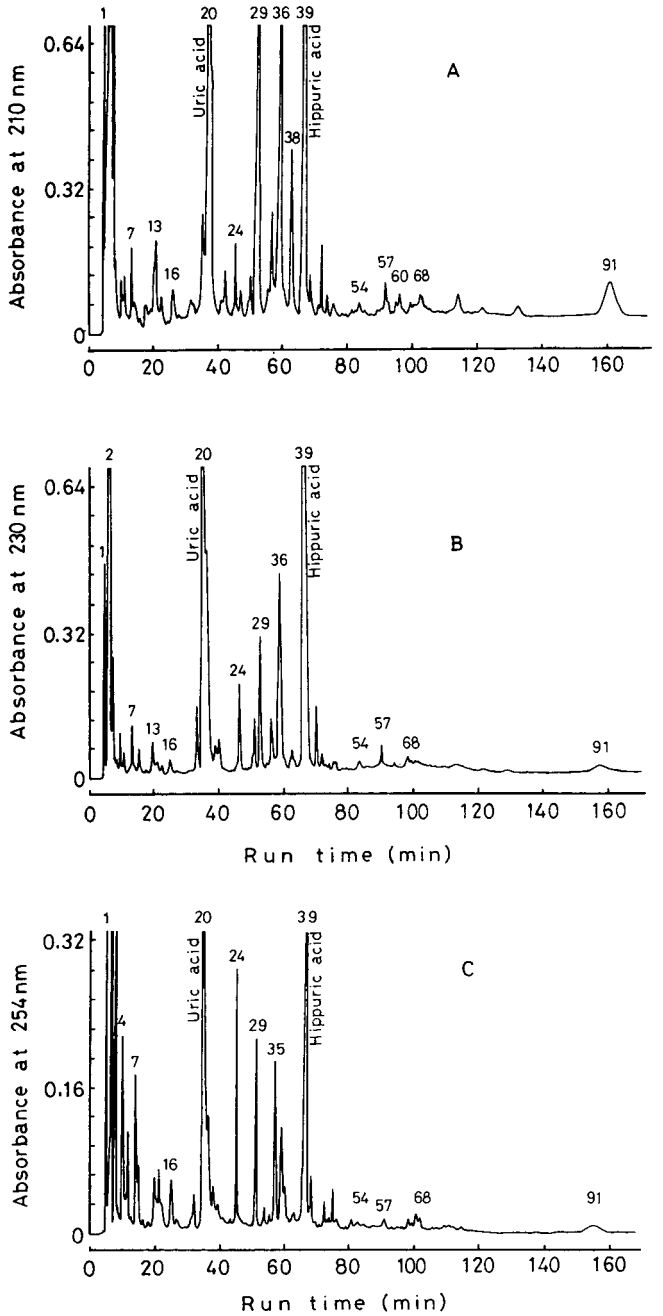

Fig. 3. Chromatograms of human urine measured by a concave gradient elution system with ammonium perchlorate solution plus ethanol. Run conditions were the same as in Fig. 2C. For peak assignments, see Table I.

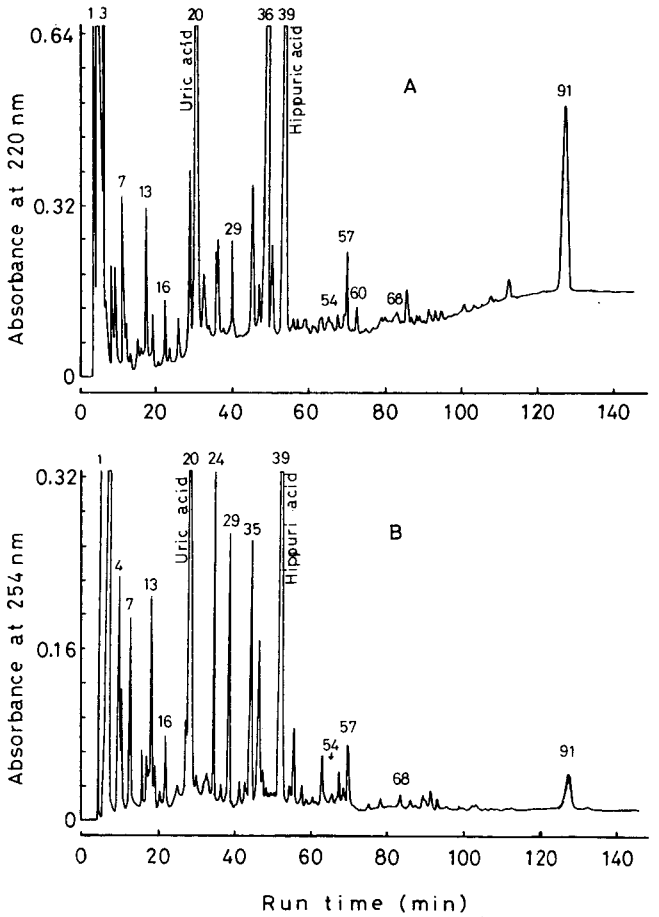

Fig. 4. Chromatograms of human urine measured by a concave gradient elution system with ammonium perchlorate solution and acetonitrile. Run conditions were the same as in Fig. 1 and Fig. 2C. For peak assignments, see Table I.

these chromatograms, it is evident that ethanol and acetonitrile gave good elution for the later-eluting compounds and for the strongly retained compounds on the resin. As a result, the resolution and the number of detectable peaks in these improved elution systems were equal to those of the acetate gradient elution system [4,6].

In these ammonium perchlorate gradient elution systems, the most useful advantage was the detectability of UV-absorbing constituents of urine at the short-wavelength region. As the figures show, each chromatographic peak has a characteristic variation in absorbance at the respective wavelength. Therefore, the assignment of peaks became easier by comparing the UV spectra of peaks to those of standard compounds. Many peaks could be measured in the UV spectra with stopped-flow scanning spectrophotometry. For example, the UV spectra of main peaks on the chromatograms are shown in Fig. 5. The assignment of main peaks was carried out by these methods. The results are listed in Table I. Also, the volume of the urine sample required to detect the main

TABLE I

RETENTION TIMES OF STANDARD COMPOUNDS AND ASSIGNMENT OF MAIN PEAKS ON THE CHROMATOGRAMS

No.	Compound	Peak No.	Retention time (min)			
			A*	B*	C*	D*
1	Creatine	2	6	6	6	6
2	Creatinine	2	6	6	6	6
3	Histidine	3	7	7	7	7
4	Pyridoxine	4	8	8	8	8
5	Uracil	5	9	9	9	9
6	Phenylalanine	6	10	10	10	10
7	Hypoxanthine	13	18	19	18	18
8	Xanthine	16	22	26	22	21
9	Adenosine	17	22	26	23	22
10	Nicotinamide	17	22	26	24	23
11	Caffeine	17	23	26	24	23
12	Theophylline	18	23	28	26	24
13	Tyrosine	18	24	28	26	25
14	Tryptophan	18	25	28	26	26
15	Urocanic acid	19	26	33	33	29
16	Uric acid	20	28	35	35	30
17	Nicotinic acid	23	30	38	36	32
18	4-Hydroxy-3-methoxymandelic acid	26	32	40	39	34
19	Homovanillic acid	27	41	51	49	36
20	*p*-Hydroxyphenylacetic acid	28	43	53	51	38
21	*p*-Aminobenzoic acid	34	45	56	54	42
22	Vanillic acid	36	47	58	56	46
23	Hippuric acid	39	49	65	64	53
24	*p*-Hydroxyphenylpyruvic acid	41	52	75	72	56
25	Kynurenic acid	57	70	85	91	69
26	*p*-Hydroxybenzoic acid	58	75	87	93	72
27	3-Hydroxyanthranilic acid	59	76	89	95	75
28	Quinaldic acid	60	79	92	98	78
29	Indoleacetic acid	68	—	—	101	83
30	Indoleacrylic acid	91	—	—	155	127

*A: Linear perchlorate gradient elution system (Fig. 1). B: Concave perchlorate gradient elution system (Fig. 2C). C: Concave gradient of perchlorate and ethanol (Fig. 3). D: Concave gradient of perchlorate and acetonitrile (Fig. 4).

peaks at short UV wavelengths such as 200 nm was much smaller. Furthermore, not only aromatic compounds, which do not have a high molecular absorption coefficient at 254 nm, but also organic compounds, have more or less absorptivity in the short UV-wavelength region. The possibility of separating and detecting these compounds arose.

CONCLUSION

The purpose of this study was to modify the anion-exchange chromatographic system using the acetate gradient for the detection of UV-absorbing constituents in the short-wavelength region. The benefits of the perchlorate gra-

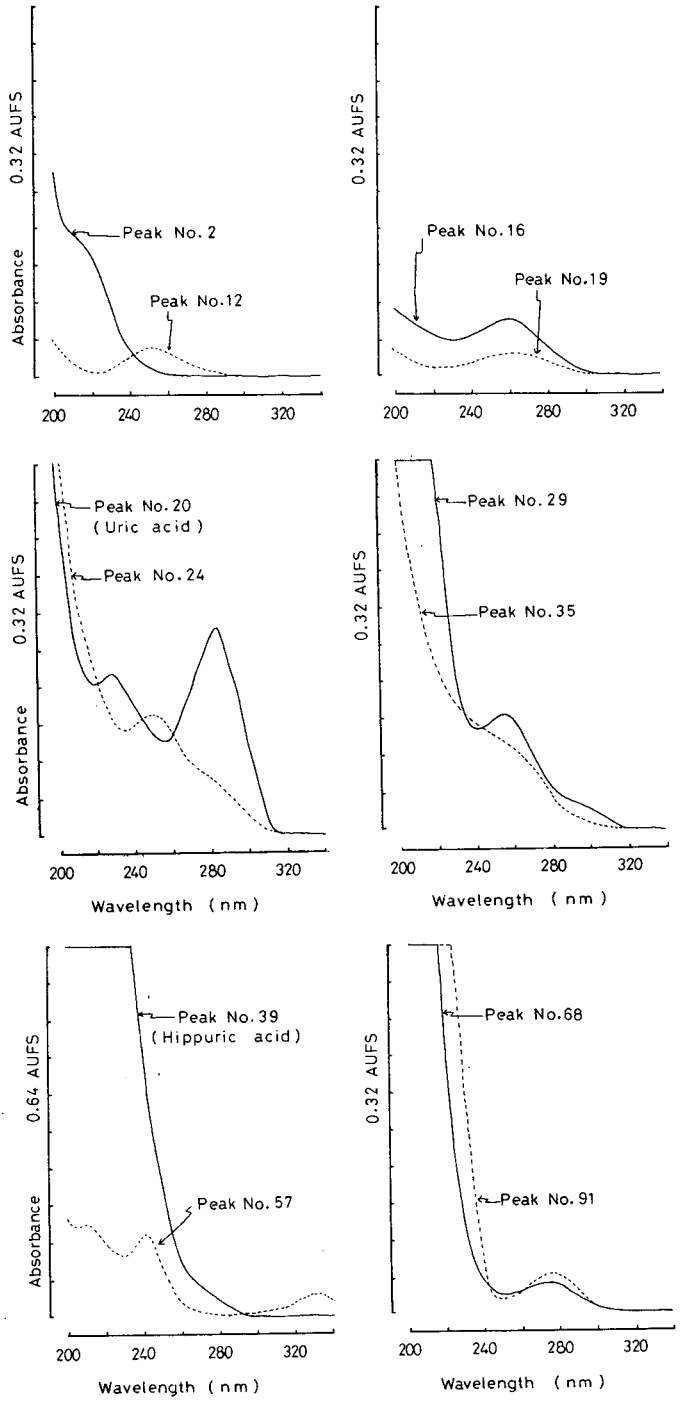

Fig. 5. Ultraviolet spectra of main peaks measured by stopped-flow scanning spectrophotometry. The stopped-flow scanning spectrophotometry was performed under the conditions 60 nm/min scan speed and 4.0 nm of slit width, with scanning from 340 to 200 nm. For peak numbers, see Table I.

dient elution systems include elimination of baseline drift, and an increase in sensitivity of detection. As well as these ammonium perchlorate gradient elution systems being useful as independent systems for the separation and detection of many organic compounds, the systems can supplement the defects of the ammonium acetate gradient elution system. Using these acetate and perchlorate gradient elution systems, the chromatographic investigation of the constituents of body fluids should be facilitated.

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PROCEDURE FOR THE SIMULTANEOUS DETERMINATION OF THE NATURAL POLYAMINES AND THEIR MONOACETYL DERIVATIVES

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SUMMARY

The separation of the natural polyamines and their monoacetyl derivatives by high-performance reversed-phase liquid chromatography is reported. Octane sulfonate was used to form ion pairs with the polycations and the *o*-phthalaldehyde method for post-column derivatization. The method allows polyamine and acetylspermidine determinations directly from tissue extracts and body fluids without pre-purification.

INTRODUCTION

In a recent paper we reported a method for the determination of the naturally occurring monoacetyl derivatives of di- and polyamines [1]. This method and the earlier method of Abdel-Monem and Ohno [2] relied on the derivatization of the amines with dansyl chloride and subsequent thin-layer chromatographic separation. Although suitable for urine analyses from the point of view of specificity and rate, the method requires considerable manual work and experimental skill and is not suited for automation.

The *o*-phthalaldehyde—2-mercaptoethanol reagent [3] is well established now for post-column derivatization of the polyamines after ion-exchange column chromatographic separations [4,5]. Therefore, it was decided to work out a separation of the known natural monoacetyl derivatives and of the non-conjugated polyamines to an extent which would allow their direct assay in conjunction with the *o*-phthalaldehyde method in tissues and body fluids without sample pre-purification. Since even very sophisticated cation-exchange

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column chromatographic procedures seemed not to give fully convincing results [6—9], a reversed-phase system was chosen using *n*-octane sulfonate for ion pairing. Separation of ion pairs on reversed-phase columns is now well established [10], but the method has not been employed to our knowledge to polyamine separations.

The new method turned out to be efficient, rapid, reproducible and sufficiently simple as to allow its routine application.

MATERIALS AND METHODS

The chromatographic system

A Varian high-pressure liquid chromatograph (Model 8500) was used, to which a loop injector (Valco valve CV-6-UHPa-N60; Valco Instruments, Houston, TX, U.S.A.) (loop volume 250 μl) was attached. A pre-column (100 mm × 3 mm) was filled with a pellicular silica core with C_{18}-brushes (CO:PELL ^{tm}ODS, Catalog No. M018; Reeve Angel, Clifton, NJ, U.S.A.). The actual separations were performed with a μBondapak C_{18} column (3.9 mm × 300 mm; 10 μm particles) (Waters, Paris, France). Column effluent and *o*-phthalaldehyde reagent were mixed in a T-piece and after flowing through a coil of PTFE tubing (2 m × 0.5 mm I.D.) the effluent—reagent mixture passed through a flow-cell of 1.5 mm optical path length (Hellma, Düsseldorf, G.F.R.). A Perkin Elmer fluorescence spectrometer Model 204 A was used as detector. This was equipped with a 150 W xenon arc lamp. Fluorescence excitation was achieved at 345 nm, and emission was measured at 455 nm. Column, T-piece, and PTFE coil were kept at 35 ± 0.5°C by means of circulating water, which was temperature-controlled by a thermostat. The signal of the fluorescence detector was recorded at two sensitivities using a two channel recorder (Model 1200, W + W Electronic A.G., Basel, Switzerland). The reagent was pumped with a piston pump (Dosapro; Milton Roy, St. Pierre, France). The pulses of the pump were damped by a bulb trap between pump and mixing T-piece.

Chemicals

Sodium acetate ($CH_3COONa{\cdot}3H_2O$) (DAB6-grade), boric acid, potassium hydroxide, 2-mercaptoethanol and the wetting agent Brij-35 (30% solution in water) were from E. Merck (Darmstadt, G.F.R.). *o*-Phthalaldehyde was from C. Roth (Karslruhe, G.F.R.); acetic acid, acetonitrile and other common chemicals were from Baker Chemicals (Deventer, The Netherlands). 1-Octanesulfonic acid (sodium salt) was a product of Eastman Kodak (Rochester, NY, U.S.A.). Putrescine dihydrochloride, cadaverine dihydrochloride, spermidine phosphate, spermine phosphate and carnosine were from Fluka (Buchs, Switzerland); anserine nitrate, homocarnosine sulfate, agmatine sulfate, histamine dihydrochloride, dopamine hydrochloride and serotonin creatinine sulfate were from Sigma (St. Louis, MO, U.S.A.); putreanine was from Cal-Biochem (San Diego, CA, U.S.A.). 1,7-Diaminoheptane (base) was from Aldrich Europe (Beerse, Belgium). The dihydrochloride was prepared by reaction with HCl and recrystallization from ethanol. The acetyl-di- and -polyamine

hydrochlorides were prepared in our laboratory according to published procedures [11].

Elution buffer

The elution system consisted of a gradient which was prepared from two buffer solutions. Buffer A: 0.1 *M* sodium acetate adjusted to pH 4.50 with acetic acid and containing 10 m*M* octane sulfonate. Buffer B: 0.2 *M* sodium acetate (adjusted to pH 4.50 with acetic acid) plus acetonitrile (10: 3, v/v), containing 10 m*M* octane sulfonate. The acetonitrile was distilled over phosphorus pentoxide before use.

o-Phthalaldehyde reagent

This was prepared by dissolving 50 g of boric acid, 44 g of potassium hydroxide and 3 ml of the Brij-35 solution per litre of distilled water. To this solution 2 ml of 2-mercaptoethanol and 400 mg of *o*-phthalaldehyde dissolved in 5 ml of distilled methanol were added before use.

Sample preparation

Tissue samples were freshly prepared from decapitated rats and were immediately homogenized in 0.2 *N* perchloric acid which contained 0.5 μM 1,7-diaminoheptane dihydrochloride as internal standard. The extracts were filtered through a Millipore filter (Millex 0.22 μm) and were applied either directly or after appropriate dilution with 0.1 *M* sodium acetate buffer (pH 4.50) to the column.

In order to avoid bacterial contamination urine samples were collected in polyethylene flasks containing 4 *N* HCl. Samples of 100 μl were mixed with 100 μl of a solution of 500 pmoles of 1,7-diaminoheptane dihydrochloride in 0.2 *N* perchloric acid and were diluted with 800 μl of 0.1 *M* sodium acetate buffer (pH 4.50) immediately before analysis.

Samples and standard solutions were diluted with buffer only in polypropylene tubes, and dilutions were never stored over extended periods in order to avoid spermidine and spermine losses due to adsorption to surfaces.

Chromatographic separation

The buffer flow-rate was 90 ml/h. Before each run the column was equilibrated for 6 min with buffer A. Then the sample was applied to the column and separated as follows. A linear gradient was prepared from buffer A and buffer B with an increment of 2% per min of buffer B for 30 min. At this time the gradient contained 60% buffer B. For the remaining time the increment of buffer B was increased to 4% per min and elution was completed. Resetting to buffer A was usually done at 45 min after commencing the run, if no unusual impurities were eluted from the column. Accordingly one separation required 51 min.

Column eluent and *o*-phthalaldehyde were mixed in a 1:1 ratio and fluores-

cence intensity was continuously recorded at two sensitivities which usually differed by a factor of 10 in the case of urine samples, and by a factor of 20 in the case of tissue samples.

Quantitative evaluation of the chromatograms

Since the eluted zones gave Gaussian signal responses and since the peak width at half height was nearly identical for all compounds, it was possible to evaluate quantitatively simply by peak height measurements. Besides using an internal standard, 1,7-diaminoheptane dihydrochloride, a standard mixture was run after every third sample in order to control instrumental sensitivity, and reproducibility of the separations.

RESULTS AND DISCUSSION

Many reports are available now on various aspects of the *o*-phthalaldehyde procedure as a method of continuous post-column derivatization of polyamines [4—7, 11]. Therefore it seems not necessary to give a detailed account of the experiments which were performed to determine sensitivity, reproducibility and linearity of dose—response curves.

Linear relationships between amine concentration and fluorimeter signal were established with mixtures of the following compounds: monoacetylputrescine, putrescine, histamine, N^1-acetylspermidine, N^8-acetylspermidine, spermidine, monoacetylspermine and spermine, in the concentration range 50 n*M* to 5 μM. The smallest amount applied in these experiments was 125 pmoles. However, the method allows less than 50 pmoles to be measured. In order to establish reproducibility of the method, a perchloric acid liver extract was diluted 1 : 1; 1 : 4; 1 : 9 and 1 : 19. From each dilution three samples were run, and standard deviations were calculated from the twelve determinations. They were as follows: putrescine ± 8.1%; spermidine ± 3.9% and spermine ± 6.4%.

The long-term reproducibility was tested by comparing the peak heights obtained from standard solutions over a period of three months. Only monoacetylputrescine showed an S.D. > ± 10%. This is due to the relative instability of the separation system at the beginning of the run (see below). For the evaluation of the actual concentration measurements the standard samples run after every third tissue samples were used in order to exclude sensitivity changes of the detection system.

So far our results were entirely in agreement with previously published reports. The considerably higher concentration of the borate buffer used for the preparation of the *o*-phthalaldehyde reagent was necessary to ensure alkaline reaction conditions in our chromatographic system. This buffer had no obvious disadvantage over the more dilute buffer used in conjunction with ion-exchange column chromatographic separations [4,6,12].

The main emphasis of this work was on the improvement of polyamine separations. Since these polycations are highly polar, *n*-octane sulfonate was chosen for ion pairing. Preliminary results showed, however, that heptane sulfonate, and even *n*-hexane sulfonate, can give adequate separations in conjunction with a column with C_{18}-brushes.

In order to obtain single ion species even with the polycations spermidine and spermine, a high concentration (10 m*M*) of the pairing anion was required.

Fig. 1 shows a separation of a mixture of some amino acids, the basic dipeptides anserine, carnosine and homocarnosine, and of a number of amines. Fig. 1B shows a typical chromatogram of the standard mixture which was used for external standardization and was usually run after every third tissue or urine sample.

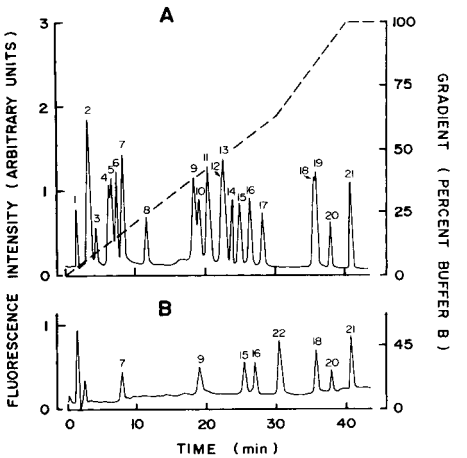

Fig. 1. Separations of mixtures of reference compounds. For details of the separation conditions see the Methods section. (A) Complete amino acid and amine mixture. (B) Standard mixture (5 nmoles/ml). This was normally used for external standardization of polyamine determinations in urine and tissues. Peaks: 1 = glutamic acid; 2 = methylamine and ammonia; 3 = histidine; 4 = anserine; 5 = carnosine; 6 = homocarnosine and putreanine (N-(4-aminobutyl)-3-aminopropionic acid); 7 = monoacetylputrescine; 8 = monoacetylcadaverine; 9 = 1,3-diaminopropane and 1,4-diaminobutane (putrescine); 10 = *p*-tyramine; 11 = 1,5-diaminopentane (cadaverine); 12 = histamine; 13 = serotonin; 14 = N^1-methylhistamine; 15 = N^1-acetylspermidine; 16 = N^8-acetylspermidine; 17 = agmatine; 18 = spermidine; 19 = N^1-(3-aminopropyl)-1,3-diaminopropane; 20 = N^1-acetylspermine; 21 = spermine; 22 = 1,7-diaminoheptane.

As Fig. 1 shows, the two isomeric monoacetylspermidines are completely separated from each other and from other known compounds. Monoacetylspermine is also well separated from both spermidine and spermine and the method should also allow the determination of agmatine.

From separations with a sophisticated cation-exchange method [6] it was presumed that the complete separation of the usual amino acids and the basic dipeptides carnosine and homocarnosine from monoacetylputrescine would allow the unambiguous determination of the latter compound. Indeed, there was only one major inadequacy in the separations of reference compounds, as appears from Fig. 1A: 1,3-diaminopropane co-migrated with 1,4-diaminobutane (putrescine). This seems to limit the usefulness of the method for polyamine analysis. However, 1,3-diaminopropane has not been demonstrated unambiguously in mammalian tissues or urine. The fact that spermidine and 3-aminopropyl-1,3-diaminopropane partially overlapped is also of little practical conse-

quence. The separations shown in Fig. 1 are more complete than any previously reported polyamine separation, and they are, moreover, somewhat faster than some of the ion-exchange column chromatographic procedures [4,7]. Spermine elutes from the column at 41 min and elution is usually stopped at 45 min. An additional 6 min are required for column equilibration with buffer A, before the following sample can be applied; i.e. one needs 51 min for each run.

If the method is to be used for histamine determinations in tissue or urine there are two possibilities to circumvent the problem caused by the overlapping of the histamine and serotonin zones:

(A) Sample pre-separation on small Dowex 50W-X8 columns (H^+) as described previously [1] removes serotonin (and *p*-tyramine) and recovers histamine quantitatively.

(B) An *o*-phthalaldehyde reagent of basically the same composition, however, without 2-mercaptoethanol reacts with histamine and a few other primary amines such as spermidine [13,14] to give fluorescent derivatives. Serotonin and most other amines react to give compounds with negligibly low fluorescence quantum yields.

The method has been used extensively for the determination of free and acetylated polyamines in rat and human urine, and for polyamine determinations in rat liver. Fig. 2 shows the separation of a urine sample of a normal rat. The retention times of the various amines are very reproducible (0.5 min), with the exception of the compounds with retention times of less than 12 min. Among these compounds monoacetylputrescine is the most significant. Its retention time was not only somewhat dependent of the time of equilibration with buffer A, but was also influenced by the size of the urine sample. Moreover, it turned out that urine contains a number of constituents which overlap with the monoacetylputrescine zone under our chromatographic conditions; this is a serious but the only major practical limitation so far known to the present method. Pre-separation of samples on Dowex 50W-X8 columns [1] removes quite a number of obviously lipophilic urinary constituents, as can be seen in Fig. 3. This figure shows the separations of the urine of a melanoma patient (A) without any pre-purification, and (B) after chromatography through the above-mentioned cation-exchange column.

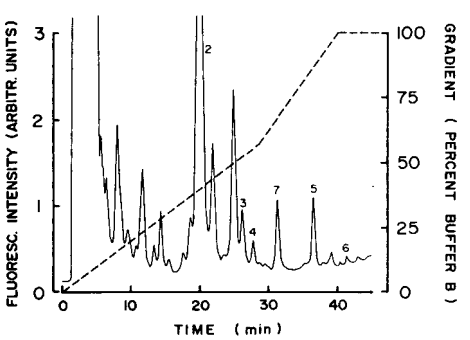

Fig. 2. Separation of a 1:9 dilution of the urine of a normal rat. Peaks: 2 = putrescine; 3 = N^1-acetylspermidine; 4 = N^8-acetylspermidine; 5 = spermidine; 6 = spermine; 7 = 1,7-diaminoheptane (internal standard). For details of the separation conditions see the Methods section.

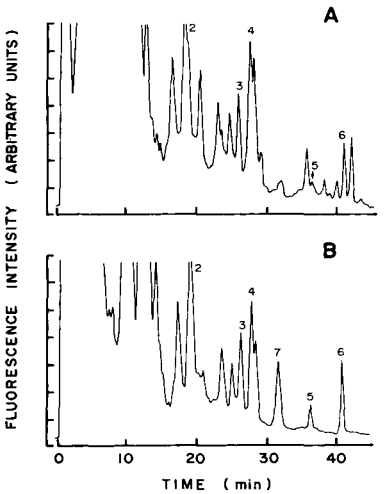

Fig. 3. (A) Separation of a 1:9 dilution of the urine of a male melanoma patient. (B) Separation of the same urine sample after pre-chromatography through a Dowex 50W-X8 column (H^+) [1], addition of internal standard, and appropriate dilution. Peaks: 2 = putrescine; 3 = N^1-acetylspermidine; 4 = N^8-acetylspermidine; 5 = spermidine; 6 = spermine; 7 = 1,7-diaminoheptane (internal standard). For details of the separation conditions see the Methods section.

The recovery of the acetylspermidines from the Dowex columns was quantitative, in agreement with our previous finding [1]. The amounts of free spermidine and spermine seem, however, somewhat increased after column chromatography of human urine. The most likely explanation of this finding is that acid-labile conjugates exist from which the polyamines are liberated. The recently detected polyamine—pyridoxal Schiff bases [15] are likely candidates. Unfortunately, even pre-purification by ion-exchange column chromatography did not remove all interfering compounds, and was therefore not an adequate procedure for monoacetylputrescine determinations in urine. But, disregarding this compound, all other di- and polyamines of biological interest could be determined quantitatively in urine, even without sample pre-purification.

The situation is somewhat more favourable in the case of tissue samples. These can be directly applied on the column as extracts in 0.2 *N* perchloric acid. Fig. 4 shows an example. In normal liver, the monoacetylputrescine concentration is very low. Its concentration is considerably increased, however, together with that of putrescine, after intoxication with thioacetamide. This increase can be clearly demonstrated, although the monoacetylputrescine zone was only incompletely separated from an unidentified compound which is present in liver. From the same figure it appears that neither N^1-acetylspermidine nor N^8-acetylspermidine are present in normal liver in amounts directly detectable with the method under standard separation conditions, i.e. the concentration of these compounds is lower than 5 nmoles/g. In the liver of thioacetamide-treated rats, the N^1-acetylspermidine concentration was markedly enhanced, and could be determined quantitatively.The N^8-acetylspermidine concentration was not increased to a detectable concentration by treatment with thioacetamide.

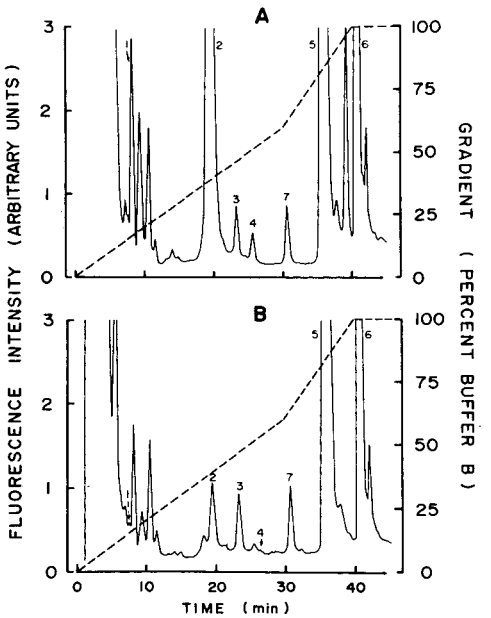

Fig. 4. Separations of perchloric acid extracts of rat liver (1:15 homogenate). (A) Liver of a rat treated with 150 mg/kg thioacetamide 16 h prior to isolation of the liver. (B) Liver of an untreated control animal. Peaks: 1 = monoacetylputrescine; 2 = putrescine; 3 = histamine; 4 = N^1-acetylspermidine; 5 = spermidine; 6 = spermine; 7 = 1,7-diaminoheptane (internal standard). For details of the separation conditions see the Methods section.

Since the concentrations of spermine and spermidine are very high compared with those of putrescine and the acetylated polyamines, fluorescence was recorded at two sensitivities (not shown in the figures), which usually differed by a factor of 20. The data obtained for rat liver putrescine, spermidine and spermine concentrations were identical which previously reported values [16].

It was mentioned above that retention times were constant in repeated runs, and were exactly the same for samples and standards, with the limitation that compounds eluting before 12 min showed slight changes in retention times which depended on various factors. However, after several hundred tissue and urine samples (i.e. after about three months' use of the same column) the average retention time had gradually decreased by 1 to 2 min, depending on the compound. This change, however, did not significantly influence the quality of the separations. Refilling the pre-column had no influence on the changed separation characteristics.

The method described here is still not fully satisfactory, because its application to monoacetylputrescine determination is limited. However, it is the first column chromatographic procedure which allows the direct determination of acetylspermidines and of non-conjugated polyamines in a single run. No method of comparable sensitivity for polyamine derivatives is presently available, disregarding the two methods relying on derivatization with dansyl chloride prior to separation [1,2].

Dansyl derivatives of the non-conjugated polyamines are well separated on

reversed-phase columns [17—20]. However, it was not possible to separate the dansyl derivatives of N^1-acetylspermidine and N^8-acetylspermidine from each other on this type of column [21]. It would be interesting to know, whether other derivatives which have been suggested for polyamine analyses, such as the *o*-phthalaldehyde [22], fluorescamine [23,24], tosyl [25] or benzoyl derivatives [26], are more suitable for reversed-phase liquid chromatography than the dansyl derivatives.

The combination of gas—liquid chromatography and mass spectrometry is in principle an alternative method for the determination of acetyl derivatives of spermidine [27,28]. The usual derivatization with trifluoroacetic anhydride and related reagents is, however, hampered by the possible displacement of acetyl groups.

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CHROMBIO. 672

SIMULTANEOUS DETERMINATION OF DOPAMINE, DOPAC AND HOMOVANILLIC ACID

DIRECT INJECTION OF SUPERNATANTS FROM BRAIN TISSUE HOMOGENATES IN A LIQUID CHROMATOGRAPHY—ELECTROCHEMICAL DETECTION SYSTEM

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SUMMARY

A simple method based on high-performance liquid column chromatography with electrochemical detection is described for the simultaneous determination of dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in discrete brain regions of rats. The supernatant of a tissue homogenate is injected directly onto a liquid chromatograph, thus omitting the commonly adopted adsorption step. Of the four different supports tested Nucleosil C_{18} (5 μm) was found superior with respect to chromatographic performance. The effects of pH, methanol and the ion-pairing agent hexyl sulfate on the retention were studied. The mobile phase used in the final studies consisted of citrate buffer pH 4.25—methanol (92:8, v/v) containing hexyl sulfate ($1.7 \cdot 10^{-3}$ *M*). Standard curves of dopamine, DOPAC and HVA were found linear up to about 600 pmol per injection for each compound. The precisions of the chromatographic step were ($s_{rel.}$ %): 0.72% (dopamine), 1.26% (DOPAC) and 2.69% (HVA).

INTRODUCTION

Dopamine (DA) is an important neurotransmitter in the brain, especially in the limbic system, striatum and hypothalamus. Several different types of centrally active drugs have potent effects on the various dopamine systems in the brain, producing marked changes in the metabolism and functional activity of DA [1]. Methods to measure the endogenous concentrations of DA and its metabolites in discrete brain regions are consequently of great importance in the analysis of DA neurotransmission. Micromethods for determination of catecholamines have been developed based on fluorescence [2], gas chromatography—mass spectrometry [3] and radio-enzymatic methods [4, 5]. An

attractive alternative is the separation of catecholamines by high-performance liquid chromatography combined with electrochemical detection as developed by Kissinger et al. [6] and later applied by e.g. Maruyama and Kusaka [7] and Hefti [8].

The assay presented in this paper permits the simultaneous determination of DA and its acid metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA). It employs liquid chromatography with electrochemical detection and involves a minimum of sample pretreatment, thus making the method highly suitable for routine analysis.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a Constametric I pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.); an injection valve, Model 7120 (Rheodyne, Berkeley, CA, U.S.A.) equipped with a 20-μl loop; a thin-layer amperometric detector, Model LC-2A (Bioanalytical Systems, West Lafayette, IN, U.S.A.) with graphite paste (CPO) as the working electrode operated at 0.60 V vs. an Ag—AgCl reference electrode unless otherwise stated; and a recorder, Model TE 200 (Tekman, Bicester, Great Britain). The supports were packed in glass-lined stainless steel columns (S.G.E., Melbourne, Australia).

A constant-pressure Haskel AO 15 gas amplifier pump was used for column packing.

Decapitation of rats was performed with a guillotine (Labora, Stockholm, Sweden) and the tissue was homogenized by sonification (Branson B 30 Sonifier, Branson Sonic Power, Danbury, CT, U.S.A.). The centrifuge and micro-test-tubes were from Eppendorf (Hamburg, G.F.R.), pH measurements were made with an Orion Ionalyzer Model 801A.

Chemicals and reagents

Five different commercially available supports were used for packing of the columns: LiChrosorb RP-8, 5 μm (Chrompack, Middelburg, The Netherlands), LiChrosorb RP-18, 5 μm (Merck, Darmstadt, G.F.R.), μBondapak C_{18}, 10 μm (Waters Assoc., Milford, MA, U.S.A.), SAS-Hypersil, 6 μm (Shandon Southern Products, Cheshire, Great Britain) and Nucleosil C_{18}, 5 μm (Macherey-Nagel, Düren, G.F.R.).

Methanol (May & Baker, Dagenham, Great Britain), citric acid, sodium chloride, perchloric acid (PCA) (Merck), sodium hydroxide (Eka, Bohus, Sweden), sodium bisulfite (Fisher, Fair Lawn, NJ, U.S.A.), were all of reagent grade. Hexyl sodium sulfate was obtained from Research Plus Laboratories (Denville, NJ, U.S.A.). Dopamine · HCl (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), *l*-epinephrine bitartrate, *l*-norepinephrine bitartrate, *dl*-metanephrine · HCl, *dl*-normetanephrine · HCl, 3-methoxytyramine · HCl, *l*-3,4-dihydroxyphenylalanine (*l*-DOPA); *dl*-2-methyl-3-(3,4-dihydroxyphenyl)alanine (α-methyl-DOPA), 5-hydroxytryptamine oxalate (5-HT), 5-hydroxyindole-3-acetic acid (5-HIAA) and bis(4-hydroxy-3-methoxyphenylglycol)piperazine (MOPEG) were purchased from Sigma (St. Louis, MO, U.S.A.), N-methyldopamine (epinine) was supplied by Serva (Heidelberg, G.F.R.) and haloperidol by Janssen Pharmaceutica (Beerse, Belgium). The water was deionized and then double quartz-distilled.

Chromatographic technique

The columns were packed by the upward slurry packing technique as described by Bristow et al. [9], with methyl isobutyl ketone as the slurry medium and dichloromethane as the follower. All stainless steel column parts were cleaned by treating successively with dichloromethane, acetone, nitric acid (5 *M*), acetone and dichloromethane again in an ultrasonic bath for 5 min. Glass-lined columns were washed with ethanol. The slurry concentration was approximately 3% (w/v) and the applied pressure 350—400 bar. After packing the support was purified by pumping about 100 ml methanol at a flow-rate of 2—3 ml/min through the column. Column performance was tested according to Bristow and Knox [10].

Mobile phases were degassed in vacuum for 10 min, and deaerated by bubbling through oxygen-free nitrogen for 10 min. Column equilibrium was achieved within 1—2 h at a flow-rate of 0.8 ml/min.

Capacity ratios were calculated relative to the first peak in the composition disturbance, and peak symmetries were measured at a distance from the baseline of 10% of the peak height. All chromatographic values are the means of duplicate or triplicate determinations.

Assay of DA, DOPAC and HVA in rat brain regions

The rat brain was removed within 30 sec after decapitation and dissected on ice. The regions were immediately cooled with dry ice and stored at −70°C until analyzed. For analysis the pieces of tissue (4—50 mg) were weighed in conical 1.5-ml test-tubes and a solution (300—500 μl) consisting of 0.1 *M* perchloric acid, $4 \cdot 10^{-5}$ *M* sodium bisulfite (PCA solution) and the internal standard epinine ($2.5 \cdot 10^{-6}$ *M*) was pipetted into the tubes. The mixture was sonicated at 200 W for about 5 sec while kept on ice, and the homogenate was centrifuged for 2—3 min at 8000 *g*. An aliquot of the supernatant was injected onto the chromatographic column (100 × 3 mm glass-lined stainless steel column packed with Nucleosil C_{18}, 5 μm). Mobile phase was citrate buffer pH 4.25 (ionic strength = 0.1)—methanol (92:8, v/v) containing hexyl sulfate ($1.7 \cdot 10^{-3}$ *M*). The sensitivity setting of the detector was optimized for each peak of interest. Typical settings for a sample of striatum were 10 nA/V (DOPAC), 50 nA/V (DA), 20 nA/V (epinine) and 2 nA/V (HVA).

Quantitations were performed from standard curves of peak height ratios relative to the internal standard against concentrations. The compounds were dissolved directly in pure PCA solution since, as demonstrated below, standard curves obtained from spiked homogenates from the biological material were co-linear with those obtained from such solutions. The standard curves were reproducible from day to day, thus making further simplification of the quantitation procedure possible. A large batch of standard solutions containing DA, DOPAC, HVA and epinine, was divided into small portions, and stored together with samples from a completed study. During analysis standard solutions were injected intermittently (on the average every sixth) with the samples and the concentrations (c_i) of DA and metabolites were calculated based on the response of this standard solution (either a mean value or a value from a neighbour standard sample, if a drift in the response of the standards

is noted during the analysis). Calculations are made according to following formula:

$$c_i = \frac{R_i \times (E - 0.7W)}{S \times W \times I} \quad (1)$$

where R_i = ratio of peak heights, DA or metabolite to internal standard; E = volume of added homogenate solution (μl); W = weight of tissue (mg), 70% of the tissue weight is assumed to be water; S = calculated peak height ratio of 1 pmol of DA, or metabolite, respectively and epinine; I = injected volume (μl).

RESULTS AND DISCUSSION

Choice of chromatographic support

Commerical supports may vary in chromatographic performance for different kinds of compounds [11—13]. Commercially available supports were tested (in preliminary studies), in order to find an acceptable material for the compounds of interest. 5-HT was chosen as the test compound (Table I),

TABLE I

CHROMATOGRAPHIC DATA FOR SOME COMMERCIAL SUPPORTS

Test compound: 5-OH-tryptamine. A. Mobile phase: phosphate buffer pH 6.50; flow-rate: 0.8 ml/min; column length: 100 mm. B. Mobile phase: citrate buffer pH 4.50; flow-rate: 0.8 ml/min; column length; 150 mm.

Support	k'	h^*	N	Asymmetry
A. Lichrosorb RP-8, 5 μm	10.3	127	157	2.1
Lichrosorb RP-18, 5 μm	12.8	87	230	1.8
μBondapak C_{18}, 10 μm	8.2	6.7	1500	1.0
SAS-Hypersil, 6 μm	8.1	90	185	2.4
B. μBondapak C_{18}, 10 μm	14.6	5.9	2525	1.06
Nucleosil C_{18}, 5 μm	28.3	5.0	6050	1.25

$^*h = \frac{\text{HETP}}{\text{dp}}$

since amines often tend to give low chromatographic performance. Three of the supports gave very low efficiencies and severely tailing peaks, and only μBondapak C_{18} gave an acceptable performance. Later Nucleosil C_{18} was found to give a good reduced efficiency (h) and although the peak symmetry was slightly inferior to that obtained on μBondapak C_{18}, Nucleosil was chosen for further studies because of a much better absolute efficiency, which mainly depends on the smaller particle size (HETP is 25 μm compared to 59 μm).

Influence of pH on chromatographic performance

The retention of protolytic compounds in reversed-phase systems with neat aqueous mobile phases is governed by hydrophobic forces, pK_a values and adsorption of the ionized compounds as ion-pairs with buffer components [14, 15]. The dependence of the capacity ratios on pH (Fig. 1) shows on the whole the expected performance. The retention of the two acids (DOPAC

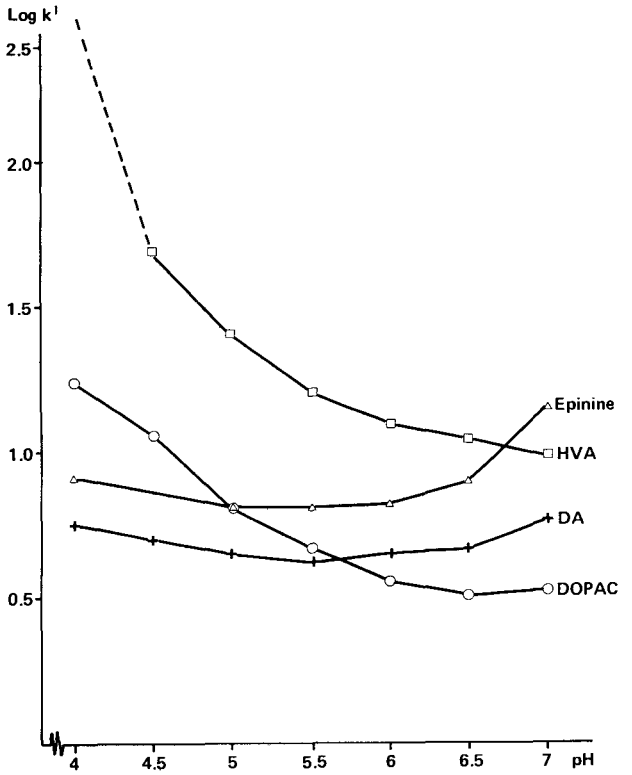

Fig. 1. The effects of pH on the capacity ratios. Support: Nucleosil C_{18} (5 μm). Mobile phase: citrate buffer (ionic strength 0.1).

and HVA) decreased with increasing pH, and the amines tended to be more strongly retained at higher pH. The increasing retention for the amines at pH < 5 is probably a consequence of ion-pair formation with the monovalent citrate anion (pK_2 for citric acid = 4.8). Large selectivity changes occurred with pH; DOPAC, for example, was eluted as third at pH 4 but elutes first at pH 7.

Peak symmetries (Fig. 2) were acceptable at low pH but increased with increasing pH, slightly for the acids but rather drastically for the amines. For further studies a low pH (4.25) was consequently chosen.

Addition of methanol and ion-pairing agent

The rather long retention times with neat aqueous buffer solutions were decreased by the addition of methanol (Fig. 3) with the elution order unchanged and about the same selectivities. Early eluting endogenous peaks will then, however, interfere with DA and epinine. Addition of hexyl sulfate eliminated this problem by increasing the retention times for the amines by an ion-pair effect. Unexpectedly the retention of the acids increased slightly by this addition — a decrease by competition of sites on the support between the two negatively charged species was the expected result [16] as seems to happen with neat aqueous buffer (see Fig. 3) as the mobile phase.

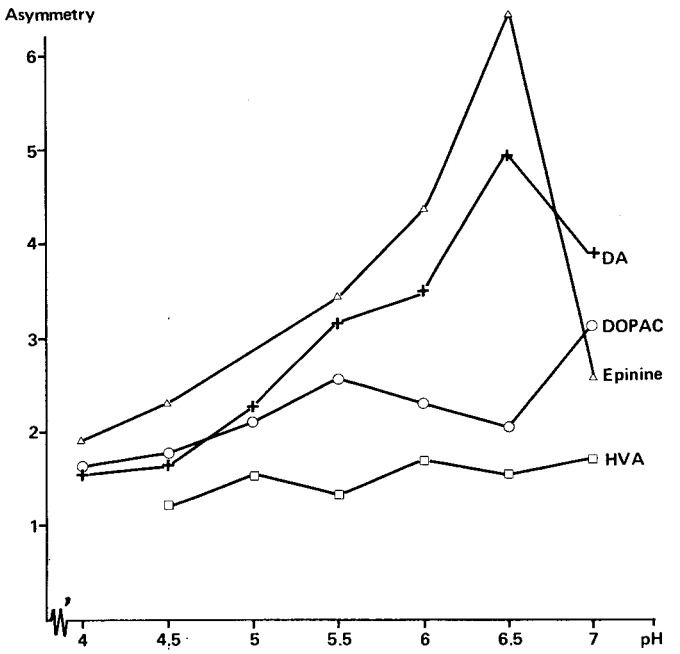

Fig. 2. Effects of pH on peak symmetry. Mobile phase: citrate buffer (ionic strength 0.1). Support: Nucleosil C_{18}, 5 μm. Symmetry = peak asymmetry (back/front) at 10% of top height.

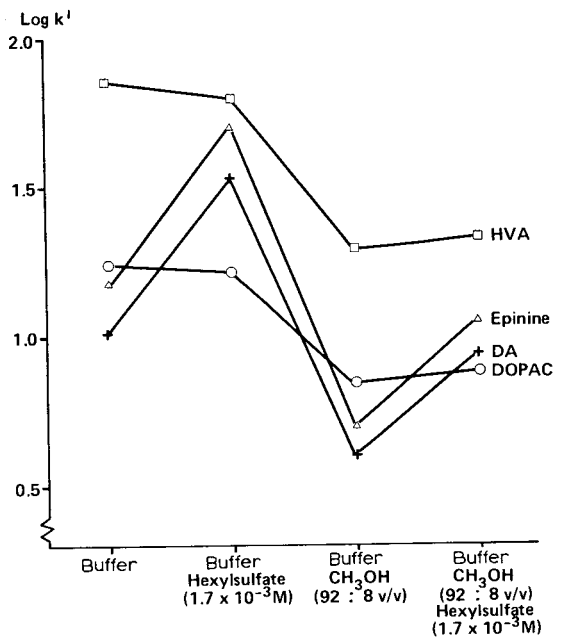

Fig. 3. Effects of methanol and hexyl sulfate on capacity ratios. Support: Nucleosil C_{18}, 5 μm.

Chromatographic selectivity

Capacity ratios of some related compounds which may be present in brain tissue show that no interference may be expected for DA, DOPAC and HVA. Metanephrine elutes just before the internal standard, epinine, but its concentration in brain regions is very low and coupled with low electrochemical response at used potential a significant interference is unlikely and has hitherto not been observed. Some selected selectivity features of the chromatographic system (Table II) demonstrate its capability to separate for example secondary and primary amines, aromatic methoxy- and hydroxy-substituted compounds.

TABLE II

SELECTIVITY OF SELECTED STRUCTURES

Compounds	Selectivity factor
Secondary/primary amine	1.43 ± 0.14 (n = 3)
aromatic OCH_3/OH	2.61 ± 0.47 (n = 4)
aliphatic CH_3/H	1.52
aliphatic H/COOH	3.86
aliphatic NH_3^+/OH	1.20

Selectivity

Typical chromatograms of samples from rat striatum run at two different detector potential settings, 0.60 and 0.80 V, respectively (Fig. 4) show that some compounds give a much higher response at the high potential — HVA, five times; 3-methoxytyramine, ten times; while that of DA, DOPAC and epinine increases only moderately. A drawback of the high potential may be a reduced selectivity as indicated by the larger front (Fig. 4 B) which interferes slightly with the DOPAC peak, and the appearance of additional peaks. For most studies a potential setting of 0.60 V was therefore chosen, but when very low concentrations are expected the higher potential may be used for selected compounds.

Quantitative determinations

Studies on other compounds [17, 18] have indicated an advantage mainly regarding precisions in quantitative determinations by incorporating an internal standard in the method. Epinine, the secondary N-methylamine analogue of DA, that was selected as the internal standard in the present method is here expected mainly to compensate for variations in the performance of the column and working electrode during a working day.

In a possible metabolic pathway for DA conversion to epinephrine, epinine, may be formed as an intermediary compound [19]. The absence of epinine was, however, established in four samples of rat striatum — this region was chosen because of its high content of DA. The detection limit for epinine under the chromatographic conditions used was < 100 fmol, which compared to the amount of epinine used as internal standard, 50 pmol, means that the amount of endogenous epinine was < 0.2% of the added amount. It is improbable that other brain regions contain significantly higher amounts of the compound.

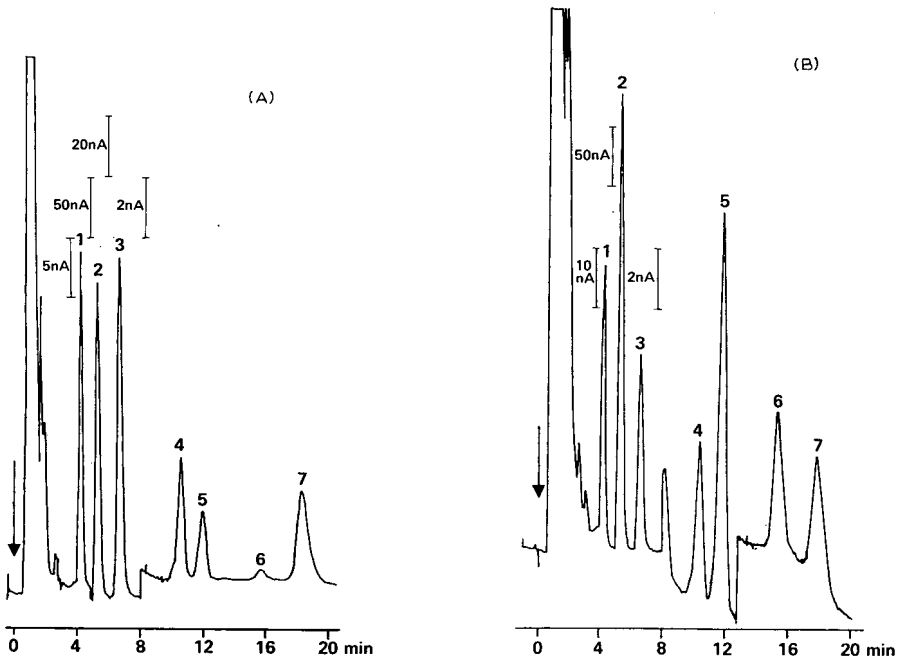

Fig. 4. Chromatograms of rat striata; effect of detector potential, (A) 0.60 V; (B) 0.80 V. Support: Nucleosil C_{18}, 5 μm; mobile phase: citrate buffer pH 4.25 containing methanol (8%) and hexyl sulfate ($1.7 \cdot 10^{-3}$ *M*). Peaks: 1, DOPAC (7.5 pmol); 2, DA (99 pmol); 3, epinine (50 pmol); 4, 5-HIAA; 5, HVA (7.8 pmol); 6, 3-methoxytyramine and 7, 5-HT.

Standard curves of DA, DOPAC and HVA with and without a striatum homogenate were co-linear in all three cases which is illustrated in Table III. Consequently it is not necessary to make standard additions to the rat brain homogenate, but the quantitations can be performed from a simple standard curve made directly in the PCA solution. In practice the standard curves cover a more limited range than those demonstrated here, corresponding more closely to expected endogenous levels. The standard curves were linear up to about 600 pmol per injection for each compound. The amount injected from the

TABLE III

STANDARD CURVES, WITH AND WITHOUT STRIATUM — LINEAR REGRESSION ANALYSIS

Compound	Slope and confidence limits (P = 0.05)	Intercept	Correlation coefficient
DA			
with striatum	0.1364 (0.1355—0.1373)	7.87	1.0000
without striatum	0.1330 (0.1301—0.1358)	0.175	0.9998
DOPAC			
with striatum	0.1371 (0.1357—0.1385)	0.911	1.0000
without striatum	0.1343 (0.1309—0.1378)	0.056	0.9998
HVA			
with striatum	0.03230 (0.03139—0.03321)	0.146	0.9997
without striatum	0.03245 (0.03162—0.03328)	0.026	0.9998

actual brain material normally never exceeds 100 pmol. The limit of detection may vary somewhat from day to day depending on the condition of the chromatographic system and may also differ between different times. This measure of sensitivity is difficult to estimate when the sample contains relatively large amounts of DA and metabolites. In the frontal cortex where the levels are low, the detection limits were approximately 50 fmol (DA and DOPAC) and 150 fmol (HVA).

The precision of the chromatographic step as determined by making 10 consecutive injections of the same sample, a pooled striata homogenate from 10 rats were (s_{rel}%): 0.72% (DA), 1.26% (DOPAC) and 2.69% (HVA).

In the present method the supernatant of the homogenate is directly injected onto the column, thus eliminating the commonly adopted step of adsorption on alumina. This is time-saving and also eliminates a possible source of error and a loss in sensitivity, since several studies on catecholamines (e.g. refs. 20, 21) have reported unsatisfactory recoveries from such adsorptions. As an alternative to adsorption on alumina the extraction of impurities with butanol and heptane has been utilized [22], but the recoveries of DA and 5-HT were only 50%; low recoveries (31—89%) of acid metabolites of DA by extraction with diethyl ether have also recently been reported [8].

A drawback of the direct injection of biological material is a shortened lifetime of the column, especially the column top [23]; a remedy may be the insertion of a pre-column [20, 24]. On average the columns used have been found satisfactory for about 500 injections after which the resolution between DOPAC and DA as well as between HVA and 5-HIAA deteriorates.

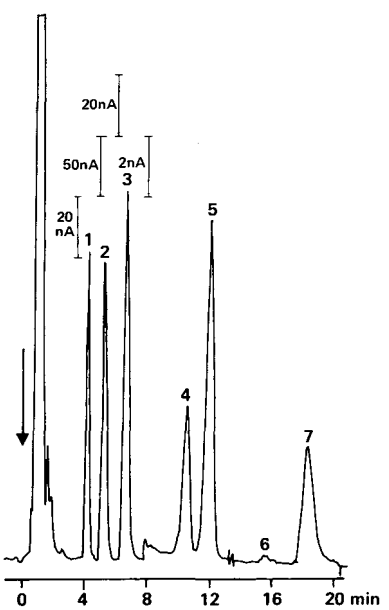

Fig. 5. Chromatograms of striata from rats 2 h after treatment with haloperidol (5 μmol/kg body weight intraperitoneally). Chromatographic conditions: see Fig. 4; potential, 0.60 V. Peaks: 1, DOPAC (22 pmol); 2, DA (67 pmol); 3, epinine (50 pmol); 4, 5-HIAA; 5, HVA (22 pmol); 6, 3-methoxytyramine and 7, 5-HT.

TABLE IV

CAPACITY RATIOS OF SELECTED MONOAMINES AND METABOLITES

Mobile phase: citrate buffer pH 4.25, containing hexyl sulfate ($1.7 \cdot 10^{-3}$ *M*) and methanol (8%); support: Nucleosil C_{18}, 5 μm.

Compound	k'
DA	8.9
DOPAC	7.7
HVA	22.0
Epinine	11.6
DOPA	2.3
Norepinephrine	3.1
α-Methyl-DOPA	3.5
Epinephrine	4.4
MOPEG	5.4
Normetanephrine	6.5
Metanephrine	10.3
5-HIAA	18.8
3-Methoxytyramine	27.8
5-HT	31.8

The method is used for screening of potential neuroleptic drugs. As an example it is demonstrated (Fig. 5) that haloperidol increases the rat striatum levels of DOPAC and HVA significantly (cf. Fig. 4).

The concentrations of DA, DOPAC and HVA have been routinely determined in the following regions of the rat brain: corpus striatum, nucleus accumbens, tuberculum olfactorium, substantia nigra and frontal cortex. In analysis of mesencephalon, however, DOPAC could not be determined due to the interference of an adjacent peak.

The studies presented here have thus led to the development of a simple method for the simultaneous determination of DA, DOPAC and HVA in small samples of brain tissue. However, since several other compounds of importance in psychiatric disorders are well resolved in the chromatographic system (see Table IV), it has a greater unexplored potential for studies in neurochemistry; 5-HIAA, 5-HT and 3-methoxytyramine, for example, appear as resolved peaks with this bioanalytical method.

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CHROMBIO. 689

NOVEL POST-COLUMN DERIVATIZATION METHOD FOR THE FLUORIMETRIC DETERMINATION OF NOREPINEPHRINE AND EPINEPHRINE

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SUMMARY

A novel method is described in which catecholamines are converted into fluorescent products by heating in alkaline borate buffer. The method was applied to the determination of norepinephrine and epinephrine after separation by high-performance liquid chromatography using a pellicular, strong cation exchanger. The new system is simpler than the system based on the trihydroxyindole reaction. It is suitable for the measurement of catecholamines in the range 0.25—20 ng. The assay of catecholamines in human urine is also described.

INTRODUCTION

Among the chromatographic methods for the determination of catecholamines, high-performance liquid chromatography (HPLC) [1—12] has acquired special importance. Several specific and sensitive reactions [13—16] are readily applicable to the determination of these amines in the eluate. The post-column derivatization system using the trihydroxyindole (THI) reaction [1—4,11,12], which has been most widely used for this purpose, is rather complicated. It consists of three steps, namely oxidation, termination of the oxidation reaction, and rearrangement of the oxidation product under alkaline conditions. Several workers [17—23] have proposed pre-column derivatization using fluorescamine [17—20] or dansyl chloride [21—23]. Although these methods require only simple instruments, pre-column reactions can be tedious. Accordingly, a simple and rapid post-column derivatization method is more suitable for routine use.

The present paper describes a novel post-column reaction in which catecholamines are heated in alkaline borate buffer to give intensely fluorescent products. This procedure, which we named the "borate method", was applied to the determination of norepinephrine and epinephrine after separation on a

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simple prototype high-performance liquid chromatograph. The borate method showed a simplicity and speed comparable with that of the electrochemical method.

MATERIALS AND METHODS

Reagents and standards

Norepinephrine, epinephrine, dopamine hydrochloride and DOPA were purchased from Sigma (St. Louis, MO, U.S.A.). All other reagents and alumina (300 mesh) were obtained from Wako (Osaka, Japan). Stock solutions of the catecholamines were prepared by dissolving each amine in 0.01 *M* hydrochloric acid at 100 μg/ml, and appropriate dilution with 0.25 *M* aqueous acetic acid before use.

The mobile phase consisted of 50 m*M* monobasic sodium phosphate solution containing 50 ml of acetonitrile per liter. The reagent for post-column derivatization was 0.5 *M* borate buffer, adjusted to pH 9.7 with sodium hydroxide. All solutions used for the chromatographic studies were filtered through a 0.22-μm micro filter (Fuji Photo Film Co., Tokyo, Japan) and de-gassed.

Separation of the catecholamine fraction from human urine

Daytime specimens of human urine were acidified immediately after collection by adding 1% (v/v) of 6 *M* hydrochloric acid, and stored in a refrigerator [15]. A 5-ml aliquot of the acidified urine was placed in a test-tube and mixed with 1 ml of a 7.5% (w/v) solution of disodium ethylenediaminetetraacetate, and the pH of the mixture was adjusted to 8.5 with 1 *N* sodium hydroxide. To the solution were added 250 mg of alumina, which had been previously treated with 2 *N* hydrochloric acid [24], and the resulting mixture was shaken with an automatic mixer for 5 min. After the mixture had been allowed to stand for 5 min, the supernatant was removed by decantation, and the alumina was washed with three 5-ml portions of water. The washed alumina was then filled into a glass column 4 mm in diameter, and eluted with 0.25 *M* aqueous acetic acid solution; 2.5 ml of the eluate were collected and stored in a refrigerator. A 100-μl aliquot of this solution was injected into the chromatograph.

Chromatographic system

Fig. 1 shows the flow diagram of the chromatographic system. A double plunger pump (Sanuki Industry Co., Tokyo, Japan) was used. Each plunger served independently to deliver the mobile phase and the post-column reagent at constant flow-rates of 0.8 ml/min and 0.4 ml/min, respectively. The mobile phase was pumped through a valve universal injector (Sanuki Industry Co., Tokyo, Japan). A Zipax SCX column (1 m × 2.1 mm I.D.; DuPont Instruments, Wilmington, MA, U.S.A.) was used for the separations. It was operated at 40°C utilizing a Taiyo thermo unit C-600 (Taiyo Scientific Industry Co., Tokyo, Japan). The column eluate was mixed with the reagent in a mixing T-piece and a PTFE-tubing reaction coil (10 m × 0.5 mm I.D.) immersed in a water-bath of 75 ± 0.1°C.

Fluorescence intensity of the effluent was measured at 490 nm; excitation of fluorescence was achieved at 400 nm, using an RF-500 LCA spectrofluoromon-

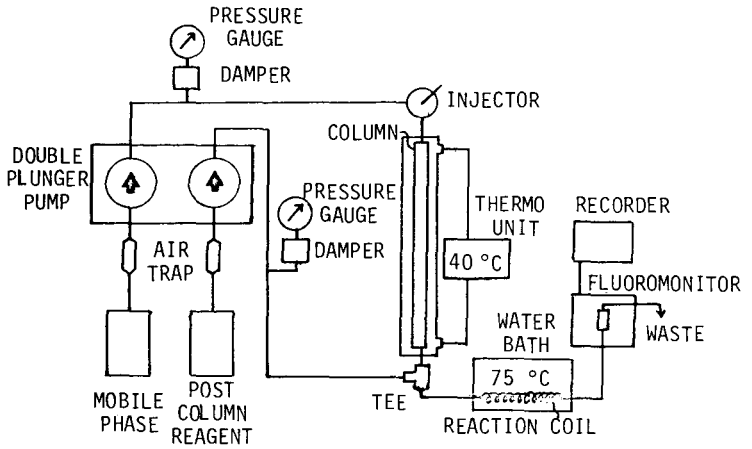

Fig. 1. Flow diagram of the HPLC system used for the separation and fluorescence detection of norepinephrine and epinephrine. Mobile phase, 50 m*M* monobasic sodium phosphate containing 50 ml of acetonitrile per liter (flow-rate, 0.8 ml/min); post-column reagent, 0.5 *M* borate buffer, pH 9.7 (flow-rate, 0.4 ml/min).

itor (Shimadzu Seisakusho, Kyoto, Japan) equipped with a xenon discharge lamp.

Excitation and fluorescence spectra were taken with an RF-510 spectrofluorometer (Shimadzu Seisakusho) which also served for the manual examination of reaction conditions (Fig. 2).

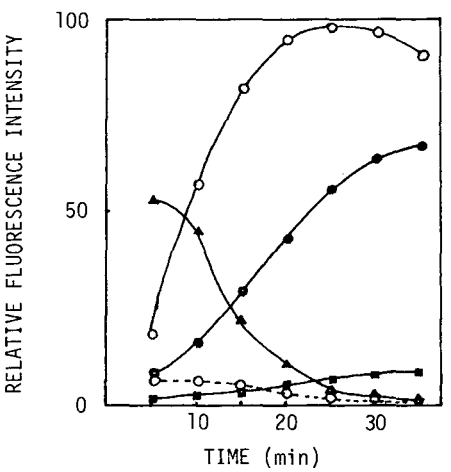

Fig. 2. Reaction conditions for the development of fluorescent products from epinephrine by heating in alkaline buffers. Fluorescence intensity is plotted against reaction time. To 1 ml of 100 ng/ml epinephrine, 3 ml of the buffers listed below were added and the resulting mixture was heated at 60°C for the periods plotted on the abscissa in a thermostatted waterbath. ■—■, 0.5 *M* borate buffer (pH 7.0); ●—●, 0.5 *M* borate buffer (pH 8.0); ○—○, 0.5 *M* borate buffer (pH 9.0); ▲—▲, 0.5 *M* borate buffer (pH 10.0); ○ — — ○, 0.5 *M* phosphate buffer (pH 9.0).

RESULTS AND DISCUSSION

The present study was focused on the development of a simple system for the analysis of norepinephrine and epinephrine, which provide valuable diagnostic information.

Catecholamines show intense fluorescence when heated in alkaline media, but the fluorescence quenches immediately. Addition of boric acid to the reaction mixture prevents quenching and gives stable fluorescence. The conditions of this reaction were examined using epinephrine since its content in biological fluid is normally smaller than that of other clinically important catecholamines such as norepinephrine, dopamine and DOPA. Analytical methods for epinephrine require especially high sensitivity.

Preliminary manual examination of the fluorescence reaction was carried out by heating epinephrine solutions in various buffers. Fig. 2 shows fluorescence intensity plotted against the time of heating at 60°C; this temperature gave the maximum fluorescence intensity in the manual procedure. Borate buffer (pH 9.0) gave the most intense fluorescence, whereas slow development of fluorescence was observed at lower pH and the fluorescence was unstable at higher pH. Heating in phosphate buffer (pH 9.0) yielded only poor fluorescence. Borate seems to stabilize the fluorescent product(s) by formation of complexes. The concentration of borate in the range 0.1—0.5 *M* does not seriously affect fluorescence intensity.

Fig. 3 illustrates the excitation and fluorescence spectra of catecholamines heated in the borate buffer (pH 9.0). The excitation and fluorescence maxima are listed in Table I. The excitation and emission maxima of dopamine and DOPA appear at significantly shorter wavelengths than those of norepinephrine and epinephrine.

HPLC of catecholamines in biological fluids on strong cation-exchange columns such as Zipax SCX has been investigated by several workers [1,3,10, 25]. A recent paper [12] reported the determination of norepinephrine and epinephrine using the trihydroxyindole reaction. Fifteen minutes were needed

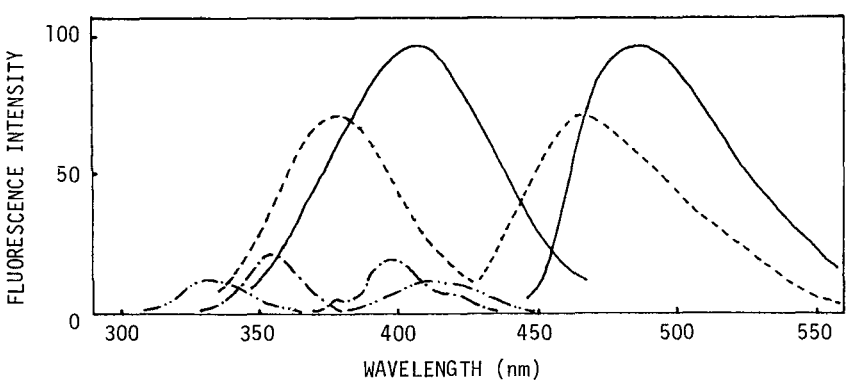

Fig. 3. Excitation and fluorescence spectra of the reaction products of norepinephrine (- - -), epinephrine (——), dopamine (— · —) and DOPA (— · · —). To 1 ml of 100 ng/ml catecholamine, 3 ml of 0.5 *M* borate buffer (pH 9.0) were added and the resulting mixture was heated at 60°C for 25 min in a thermostatted water-bath.

TABLE I

EXCITATION AND EMISSION WAVELENGTHS OF THE REACTION PRODUCTS OF CATECHOLAMINES

Catecholamine	Wavelength (nm)	
	Ex.	Em.
Norepinephrine	380	465
Epinephrine	405	490
Dopamine	353	398
DOPA	330	412

for the elution of a Zipax SCX column with 30 m*M* monobasic sodium phosphate solution containing 60 g of acetonitrile per liter at a flow-rate of 0.8 ml/min. In the present method, the time for the determination was reduced to 10 min using 50 m*M* monobasic sodium phosphate solution containing 50 ml of acetonitrile per liter for the elution.

The post-column reaction conditions were examined using the chromatographic system shown in Fig. 1. Unless otherwise stated, the sample volumes injected were 100 μl and the amount of catecholamines are expressed as weight in a 100-μl sample. The pH of the eluate was maintained constant by delivering 0.5 *M* borate buffer (pH 9.7) to the column effluent. The ratio of flow-rate of mobile phase and borate buffer was 2:1. The reaction mixture passed through the PTFE reaction coil within 1.5 min, the coil being placed in a water-bath (Fig. 1). Fig. 4 shows the fluorescence intensity plotted against the temperature of the water-bath, the maximum fluorescence being at 75°C.

Fig. 5A shows a chromatogram of a standard solution containing 5 ng each of norepinephrine and epinephrine which are clearly separated within 10 min. Fig. 5B shows a chromatogram of the catecholamine fraction from human urine after alumina treatment, giving three peaks. Two of them, the second and

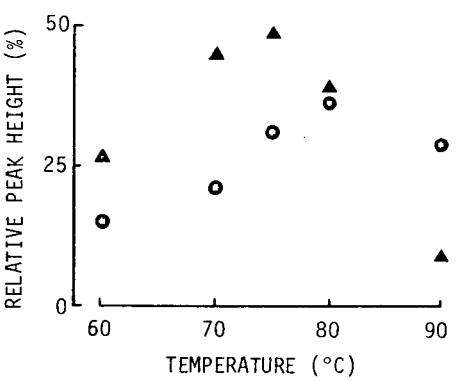

Fig. 4. Fluorescence intensities of the reaction products of catecholamines plotted against the temperature of the water-bath during post-column reaction. Aliquots of 100 μl of a standard solution containing 5 ng of norepinephrine (●) or epinephrine (▲) were injected into the chromatograph.

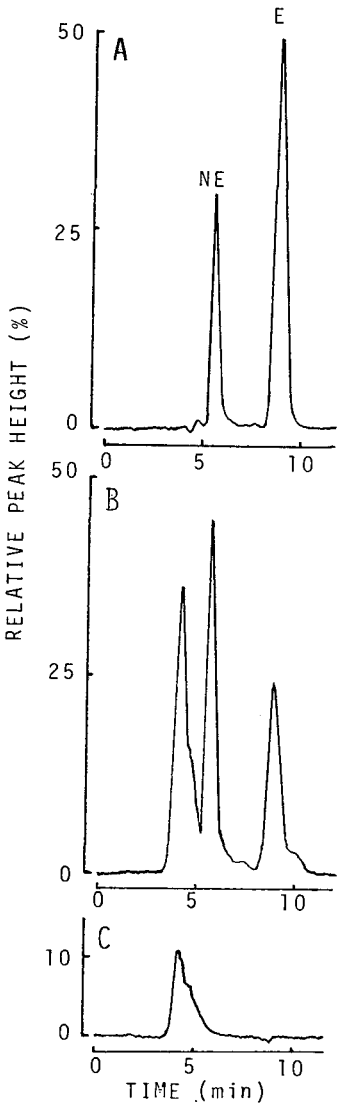

Fig. 5. Chromatographic profile of norepinephrine (NE) and epinephrine (E). (A) Chromatogram obtained by injecting 100 μl of a mixture containing norepinephrine (5 ng) and epinephrine (5 ng). (B) Chromatogram of the catecholamine fraction of human urine under standard conditions. (C) Chromatogram of the catecholamine fraction of human urine. Distilled water was delivered instead of the borate buffer to the column effluent in this experiment.

third peaks, correspond to those of standard norepinephrine and epinephrine. Fig. 5C demonstrates that these two peaks disappear when distilled water is delivered to the chromatograph instead of the alkaline borate buffer. However, the first peak is still observed although its intensity is reduced. This peak has not yet been identified, but it is sufficiently separated from the second and third peaks and does not interfere with the determination of norepinephrine and epinephrine.

There are linear relationships between the peak heights and the amounts of

norepinephrine and epinephrine injected ranging from 0.25 to 20 ng. The coefficients of variation (n=8) for 0.5 ng and 5 ng of standard norepinephrine were 3.3% and 1.9%, respectively, and those for 0.5 ng and 5 ng of epinephrine were 2.4% and 1.6%, respectively.

When the determination was repeated with a 5-ml aliquot of normal human urine, the average norepinephrine (n=5) and epinephrine (n=5) levels were 3.94 μg/dl and 1.19 μg/dl, respectively, with coefficients of variation of 3.6% and 2.2%, respectively. Recoveries of 100 ng of norepinephrine and epinephrine added to 5 ml of human urine were 82% and 88%, respectively.

The "borate method" provides a rapid and reproducible means of determining norepinephrine and epinephrine in human urine. The apparatus for post-column derivatization is simple compared with that necessary for the trihydroxyindole procedure. The present method is expected to be useful in the routine analysis of catecholamines in biochemical or clinical laboratories.

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HIGH-PERFORMANCE AQUEOUS GEL PERMEATION CHROMATOGRAPHY OF HUMAN SERUM LIPOPROTEINS

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SUMMARY

A new application of high-performance aqueous gel permeation chromatography was developed for the analysis of human serum lipoproteins. A good combination of columns (TSK GEL, type PW and type SW) was found for the separation of serum lipoproteins: very low-density lipoprotein, low-density lipoprotein and high-density lipoproteins. Analyses of serum lipoproteins from individual normal subjects and pathological subjects were performed by this combination of columns. The effects of pH and salt concentration of the eluent on the separation of lipoproteins were also investigated.

INTRODUCTION

Gel permeation chromatography (GPC) is a type of liquid chromatography in which the separation is based only on the molecular size. With GPC the experimental procedure is simple and the interpretation of the results is easy, but it takes long time and the resolution is low.

Recently GPC columns packed with microspheres of hydrophilic polymer gels (TSK GEL, type PW; Toyo Soda, Tokyo, Japan) and a new chemically modified silica gel based aqueous support (TSK GEL, type SW; Toyo Soda) have become commercially available. These columns can be used under high pressure in aqueous systems and possess a large number of theoretical plates. Moreover, several grades of columns with different pore sizes are available.

High-performance liquid chromatography with these columns for the analysis of many biological substances, such as polypeptides, proteins, enzymes and polysaccharides, has been studied [1—8]. But these GPC columns have not yet been applied to the analysis of human serum lipoproteins. We were the

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first to establish a method of separation of serum lipoproteins by high-performance GPC using these columns [9,10].

In this paper, the study of the combination of these columns for analysis of lipoproteins is described. The effects of pH and salt concentration of the eluent on the separation of lipoproteins are investigated. A few examples of analysis of normal and pathological sera are also reported. The new method of separating serum lipoproteins by high-performance GPC is useful for the study of lipoprotein metabolism and related diseases.

EXPERIMENTAL

Apparatus

High-performance liquid chromatography was carried out using an HLC 805 (Toyo Soda) equipped with a variable-wavelength absorbance detector.

Ultracentrifugal isolation of lipoproteins from human serum was performed using an RP 505 rotor in an Hitachi 55 P-2 ultracentrifuge (Hitachi, Tokyo, Japan).

Materials and methods

Human sera used in these experiments were obtained from normal and pathological subjects after 12—16 h of fasting. Lipoprotein fractions for analysis by high-performance GPC were isolated from human serum by an ultracentrifugal method [11]. Samples of sera were adjusted to $d = 1.21$ with solid sodium bromide and diluted to five volumes with aqueous sodium bromide solution of $d = 1.21$. Aliquots of 5.0 ml of the solution were transferred to 6.0-ml centrifuge tubes and centrifuged at 105,000 g for 24 h at 8°C. After centrifugation, the top fractions of 0.5—1.0 ml containing lipoproteins were collected. The lipoprotein fractions prepared by this procedure did not contain the serum proteins except for a small amount of albumin as demonstrated by immunological assay.

Of these samples 20—40 μl were subjected to high-performance liquid chromatography on the HLC 805 equipped with GPC columns (TSK GEL, type PW and type SW) and eluted with 0.1 M Tris·HCl buffer (pH 7.4) at a flow-rate of 1.0 ml/min. The lipoprotein fractions were monitored by the absorbance at 280 nm. In the combined column system, the columns were connected by stainless-steel tubing with an internal diameter of 0.4 mm.

Ovalbumin dissolved in 0.15 M NaCl at a concentration of 1.0% was used to study the relationship between molecular weight and elution volume for the combination of GPC columns.

RESULTS AND DISCUSSION

Serum lipoproteins are classified according to their densities into very low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoproteins (HDL_2 and HDL_3). The molecular weights and the chemical compositions of each lipoprotein class are summarized in Table I.

For analysis of serum lipoproteins by high-performance GPC we used the lipoprotein fractions ($d < 1.21$) of human serum prepared by the ultracen-

TABLE I

CHEMICAL COMPOSITION BY DRY WEIGHT OF HUMAN SERUM LIPOPROTEINS [12]

Particle*	Molecular weight ($\times 10^{-6}$)	Density (g/cm^3)	Protein (%)	Phospholipid (%)	Triglyceride (%)	Cholesterol (%)
VLDL	19.6	0.97	8	18	50	19
LDL	2.3	1.035	21	22	11	45
HDL_2	0.36	1.09	41	30	4.5	21.4
HDL_3	0.18	1.15	55	23	4.1	14.9

*VLDL = very low-density lipoprotein; LDL = low density lipoprotein; HDL = high-density lipoprotein.

trifugal method as described in the experimental part. These fractions did not contain the serum proteins having a molecular weight similar to those of lipoproteins. Therefore, the peaks of lipoproteins can be monitored by the absorbance at 280 nm. The small amount of albumin remaining in the lipoprotein fractions did not interfere with the detection of lipoproteins by absorbance at 280 nm.

The properties of the GPC columns used in this experiment are shown in Table II. Each column was 600 mm long with an internal diameter of 7.5 mm.

TABLE II

PROPERTIES OF TSK GEL COLUMNS

Grade	Particle size (μm)	Exclusion molecular weight		Theoretical plate number (plates/ft.)
		Protein	Dextran	
G6000PW	17 ± 2	—	3×10^7	3000
G5000PW	17 ± 2	—	7×10^6	3000
G4000SW	13 ± 2	1×10^6	6×10^5	> 5000
G3000SW	10 ± 2	3×10^5	1×10^5	> 5000

A mixture of lipoprotein fractions (sample 1) containing VLDL, LDL, HDL and albumin was examined using a single column (G3000SW, G4000SW, G5000PW and G6000PW), and their separation patterns monitored by absorbance at 280 nm are shown in Fig. 1 a–d, respectively. Though the G3000SW column gave very sharp peaks, only three peaks were observed and VLDL and LDL eluted as one peak at the G3000SW exclusion volume (Fig. 1a). In the case of the G4000SW column, four peaks were obtained (Fig. 1d). The G-5000PW column (Fig. 1c) and the G6000PW column (Fig. 1d) divided the high-molecular-weight fractions into two peaks and were useful for analysis of VLDL and LDL. But in both columns only three peaks were observed, and HDL and albumin eluted as one peak. From these experiments, it is concluded that a combination of GPC columns (G3000SW and G5000PW or G6000PW) was required for analysis of all lipoprotein fractions.

Examination of the analysis of the same sample as in Fig. 1 (sample 1) and the mixed lipoprotein fraction (sample 2) containing VLDL, LDL, HDL_2, HDL_3 and albumin, was carried out using combinations of columns composed

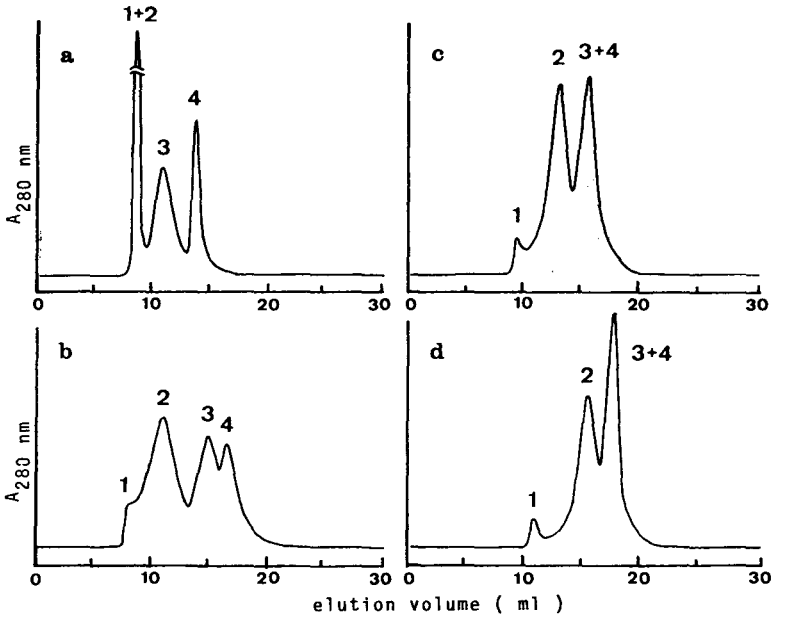

Fig. 1. Elution curves of a mixed lipoprotein fraction (sample 1) by a single column (600 mm × 7.5 mm I.D.). Column: (a) G3000SW; (b) G4000SW; (c) G5000PW; (d) G6000PW. Eluent: 0.1 *M* Tris·HCl buffer (pH 7.4). Flow-rate: 1.0 ml/min. Load volume: 20 µl. Temperature: room temperature. Peaks: 1 = VLDL; 2 = LDL; 3 = HDL; 4 = albumin.

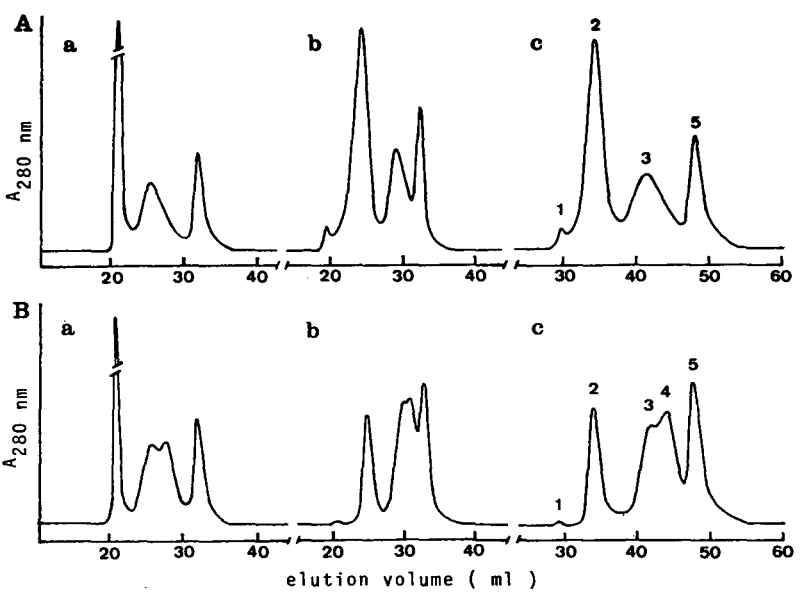

Fig. 2. Elution curves of mixed lipoprotein fractions (A, sample 1; B, sample 2) by combination columns of G3000SW and G5000PW. Column: (a) G3000SW × 2 (G3000SW + G3000SW, 1200 mm × 7.5 mm I.D.); (b) = G5000PW + G3000SW (1200 mm × 7.5 mm I.D.); (c) G5000PW + G3000SW × 2 (G5000PW + G3000SW + G3000SW, 1800 mm × 7.5 mm I.D.). Peaks: 1 = VLDL; 2 = LDL; 3 = HDL_2, 4 = HDL_3; 5 = albumin. Conditions as in Fig. 1.

of G5000PW and G3000SW. Their separating profiles are shown in Fig. 2. The combination columns, composed of G5000PW and G3000SW (G5000PW + G3000SW, G5000PW + G3000SW × 2), separated all lipoprotein fractions, i.e. VLDL, LDL, HDL_2 and HDL_3 (Fig. 2b and c).

The same experiments were performed using combinations of columns composed of G6000PW and G3000SW (G6000PW + G3000SW and G6000PW + G3000SW × 2), and similar elution patterns to those in Fig. 2 were obtained.

In Fig. 3 the results of the examination of GPC columns for the separation of lipoproteins in these experiments are summarized. Fig. 3 shows the relation

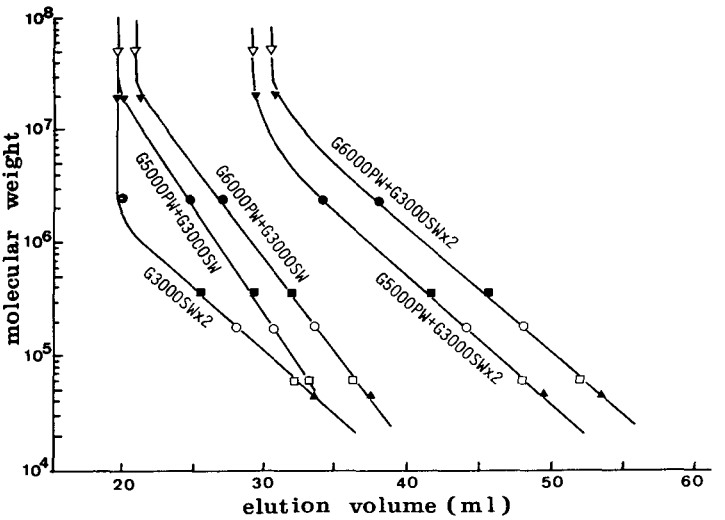

Fig. 3. The relation between molecular weight of lipoproteins and elution volume for the combination GPC columns. Conditions as in Fig. 1. ▽, chylomicron; ▼, VLDL; ●, LDL; ■, HDL_2; ○, HDL_3; □, albumin; ▲, ovalbumin.

between molecular weight of lipoproteins (VLDL, LDL, HDL_2 and HDL_3) and other samples (chylomicron, human serum albumin and ovalbumin) and their elution volume using 0.1 *M* Tris·HCl buffer solution (pH 7.4) as eluent at a flow-rate of 1.0 ml/min. The G3000SW column was useful for the separation of low-molecular-weight fractions less than 5×10^5; namely HDL_2, HDL_3 and albumin. This corresponds to the finding that the G3000SW column is optimum for the separation of most proteins of molecular weight between 4×10^4 and 4×10^5 [6]. The separation of high-molecular-weight fractions (VLDL and LDL) was carried out by the combination columns containing G5000PW or G6000PW. In the case of the combined columns (G5000PW + G3000SW and G6000PW + G3000SW), all of the plots for VLDL, LDL, HDL_2 and HDL_3 were linear and these column systems may be used to determine the molecular weights of lipoproteins.

It is found from Figs. 2 and 3 that the combined columns containing two G3000SW (G3000SW × 2, G5000PW + G3000SW × 2 and G6000PW + G3000SW × 2) are suitable for the separation of the fractions HDL_2 and HDL_3. Subsequently, the combination columns composed of G5000PW and two G3000SW or G6000PW and two G3000SW are good for analysis of all lipoprotein fractions.

The dependence of the separation of lipoproteins on pH and salt concentration of the eluent was examined using the combination of columns (G6000PW+ G3000SW × 2). The elution patterns of a mixed lipoprotein fraction (sample 3) containing VLDL, LDL, HDL_2, HDL_3 and albumin, with various eluents are shown in Fig. 4. The elution profiles with each eluent were similar, but the

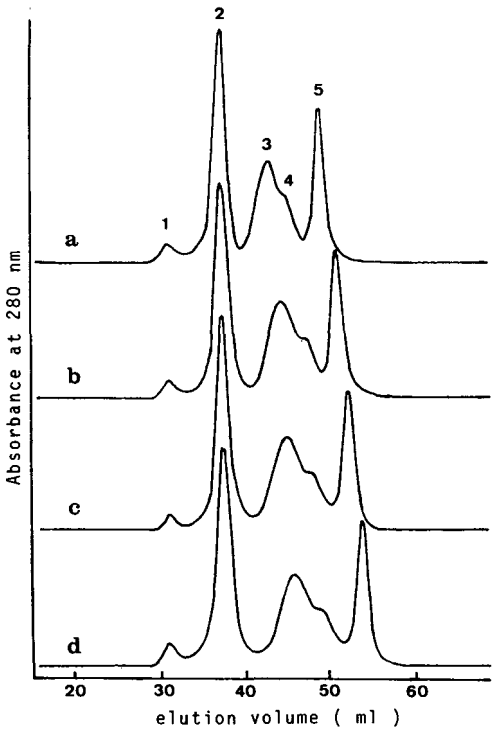

Fig. 4. Elution curves of a mixed lipoprotein fraction (sample 3). Column: G6000PW + G3000SW × 2. Eluent: (a) 0.1 *M* Tris·HCl buffer (pH 8.4); (b) 0.1 *M* Tris·HCl buffer (pH 8.4) containing 0.15 *M* NaCl; (c) 0.1 *M* Tris·HCl buffer (pH 7.4); (d) 0.15 *M* NaCl (pH 6—6.2). Flow-rate: 1.0 ml/min. Load volume: 40 µl. Peaks as in Fig. 2.

elution volumes of HDL fractions and albumin increased with a lower pH and addition of sodium chloride. On the other hand, the elution volumes of VLDL and LDL were not influenced by pH and salt concentration. Fig. 5 shows the relation between molecular weight and elution volume for LDL, HDL_2, HDL_3 and albumin using various kinds of eluents. It was found from Figs. 4 and 5 that each medium examined in this experiment could be used as eluent for analysis of lipoproteins and that lowering the pH and adding salts tended to increase the elution volumes of the fractions HDL_2 and HDL_3.

The analysis of the lipoprotein fractions prepared from the individual subjects by the ultracentrifugal method was achieved using the combination of columns (G5000PW + G3000SW × 2). Results of analysing two samples from normal subjects (subject 1 = young female; subject 2 = old male) and four examples of pathological subjects (subject 3 = coronary heart disease; subject 4 = liver cirrhosis; subjects 5 and 6 = hyperlipidemia) are shown in Fig. 6a—f,

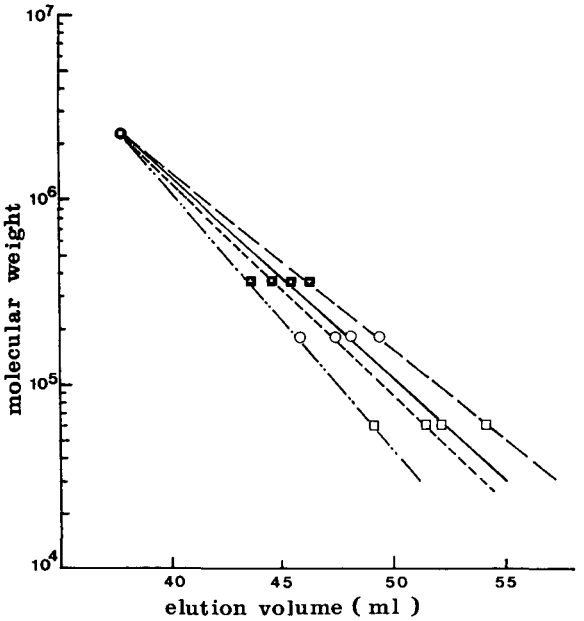

Fig. 5. Effect of pH and salt concentration of eluent on elution volume of each lipoprotein (— ·· — ··, 0.1 *M* Tris·HCl buffer (pH 8.4); - - - - - -, 0.1 *M* Tris·HCl buffer (pH 8.4) containing 0.15 *M* NaCl; ———, 0.1 *M* Tris·HCl buffer (pH 7.4); — — —, 0.15 *M* NaCl (pH 6—6.2). Conditions as in Fig. 4. •, LDL; ▪, HDL_2; ○, HDL_3; □, albumin.

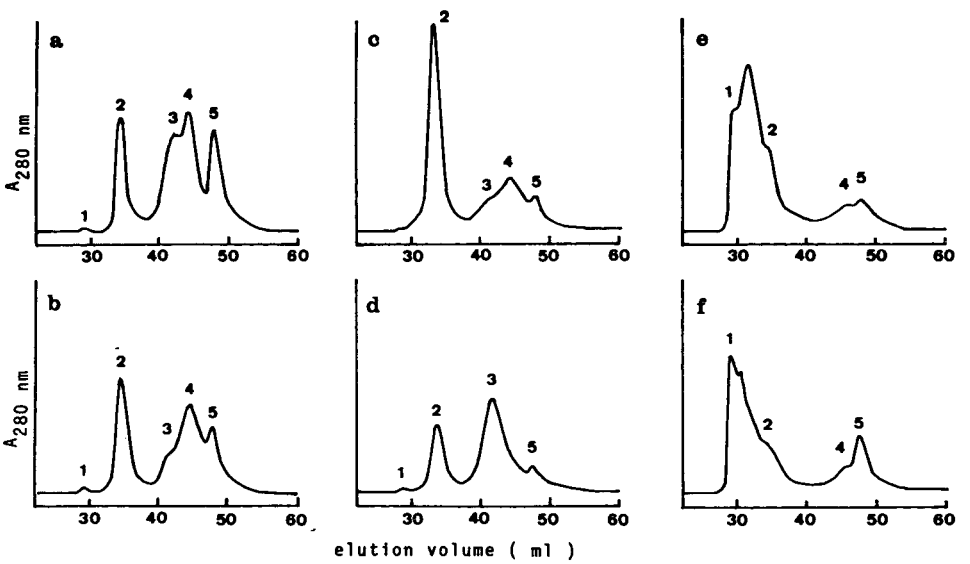

Fig. 6. Analysis of serum lipoproteins for individual subjects. (a) subject 1 (young female); (b) subject 2 (old male); (c) subject 3 (coronary heart disease); (d) subject 4 (liver cirrhosis); (e and f) subjects 5 and 6 (hyperlipidemia). Column: G5000PW + G3000SW × 2. Eluent: 0.1 *M* Tris·HCl buffer (pH 7.4). Flow-rate: 1.0 ml/min. Load volume: 20 μl. Peaks as in Fig. 2.

respectively. The level of each lipoprotein was found to vary with individual subjects. Especially in the case of pathological subjects, there were distinct patterns with respect to each disease. Coronary heart disease (Fig. 6c) had a higher LDL level and lower HDL level than those of normal subjects. In the case of liver cirrhosis, total lipoprotein levels were low and HDL fractions were mainly composed of HDL_2 (Fig. 6d). Hyperlipidemia (Fig. 6e and f) had characteristic patterns with a large amount of high-molecular lipoprotein fractions containing the fractions intermediate between VLDL and LDL.

From these experiments it was found that much information about each lipoprotein level in human serum could be obtained from the lipoprotein fraction ($d < 1.21$) by high-performance GPC.

This method of separation may be very convenient for the preparation and analysis of lipoproteins because of its short experimental time and high resolution. Moreover, analysis of cholesterol, triglyceride and phospholipid in each lipoprotein fraction can be performed by appropriate measurements after the separation by high-performance GPC. This analytical method is useful for the study of lipoprotein metabolism and related diseases.

In our laboratory, the micromethod of rapid analysis of cholesterol in each lipoprotein fraction was established using this method of separation [10], and studies of the relation between cholesterol content of each lipoprotein fraction and related diseases are being continued.

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CHROMBIO. 696

NEW AND HIGHLY SENSITIVE ASSAY FOR L-5-HYDROXYTRYPTOPHAN DECARBOXYLASE ACTIVITY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY—VOLTAMMETRY

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SUMMARY

This paper describes a new, inexpensive and highly sensitive assay for aromatic L-amino acid decarboxylase (AADC) activity, using L-5-hydroxytryptophan (L-5-HTP) as substrate, in rat and human brains and serum by high-performance liquid chromatography (HPLC) with voltammetric detection. L-5-HTP was used as substrate and D-5-HTP for the blank. After isolating serotonin (5-HT) formed enzymatically from L-5-HTP on a small Amberlite CG-50 column, the 5-HT was eluted with hydrochloric acid and assayed by HPLC with a voltammetric detector. N-Methyldopamine was added to each incubation mixture as an internal standard. This method is sensitive enough to measure 5-HT, formed by the enzyme, 100 fmol to 140 pmol or more. An advantage of this method is that one can incubate the enzyme for longer time (up to 150 min), as compared with AADC assay using L-DOPA as substrate, resulting in a very high sensitivity. By using this new method, AADC activity was discovered in rat serum.

INTRODUCTION

Aromatic L-amino acid decarboxylase (AADC, EC 4.1.1.28) [1] is the enzyme which catalyzes the decarboxylation of L-DOPA and L-5-hydroxytryptophan (L-5-HTP) to dopamine and serotonin (5-HT), respectively, in mammalian tissues. The enzyme was homogeneously purified from hog kidney and was shown to decarboxylate various aromatic L-amino acids including L-DOPA and L-5-HTP [2]. Enzymatic decarboxylations of L-DOPA and L-5-HTP lead to the biosynthesis of catecholamines (dopamine, noradrenaline and adrenaline) and indoleamines (serotonin and melatonin), respectively. Since

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monoamines are physiologically very important, the assay of AADC using both L-DOPA and L-5-HTP are frequently required. However, the activity of AADC in some tissues such as human brain is very low, and the presence of AADC in human brain has even been questioned because of the possibility of nonenzymatic decarboxylation [3—5].

In order to resolve the above discrepancies on the presence of AADC in human brain, we had already established a specific method to measure L-DOPA decarboxylase activity by using high-performance liquid chromatography (HPLC) and voltammetry [6]. Since L-5-HTP is also an important substrate for AADC, the assay of the activity using L-5-HTP is also necessary, and it has some advantages over L-DOPA as substrate. We have therefore developed a new, specific and highly sensitive method for the measurement of L-5-HTP decarboxylase activity by using HPLC and voltammetry of 5-HT [7—9], as in our AADC assay using L-DOPA as substrate [6].

EXPERIMENTAL

Materials

L-5-HTP, D-5-HTP, 5-HT, pargyline HCl and N-methyldopamine were obtained from Sigma (St. Louis, MO, U.S.A.); pyridoxal phosphate was from Katayama Chemicals (Osaka, Japan); Amberlite CG-50 was from Rohm and Haas (Philadelphia, PA, U.S.A.). All other chemicals were of analytical grade.

Rats were killed by decapitation. Immediately after the decapitation cerebral cortex was dissected. Rat serum was also collected. Human cerebral cortex was also dissected at autopsy from patients without a history of neurological disorders. The brains were homogenized with 0.32 *M* sucrose solution (1 part tissue plus 9 parts 0.32 *M* sucrose solution) in a Potter glass homogenizer. Amberlite CG-50 (type 1, 100—200 mesh) was activated by washing with 2 *M* HCl, 2 *M* NaOH and finally with water, equilibrated with 1 *M* potassium phosphate buffer (pH 6.5), and stored in the same buffer.

Experimental procedures

The standard incubation mixture for L-5-HTP decarboxylase contained (total volume 400 μl, final pH 8.3) : 30 m*M* sodium phosphate buffer (pH 9.0), 0.01 m*M* pyridoxal phosphate, 1.0 m*M* L-5-HTP (or D-5-HTP for the blank), 0.1 m*M* pargyline HCl, and the enzyme. Incubation was done at 37°C for 20—120 min, and the reaction was stopped by adding 80 μl of 3 *M* trichloroacetic acid. After 10 min 1.82 ml of water and 100 μl of 0.01 *M* HCl containing 100—500 pmol of N-methyldopamine as an internal standard were added, and the mixture was centrifuged at 1600 *g* for 10 min. The supernatant was passed through a column (packed volume 0.5 ml) of Amberlite CG-50 (Na^+) equilibrated with 0.1 *M* potassium phosphate buffer (pH 6.5). The resin was washed twice with 4.5 ml of the buffer and with 200 μl of 1 *M* HCl. The 5-HT adsorbed was eluted with 1.4 ml of 1 *M* HCl. A 100-μl aliquot (or 50 μl) of the eluate was injected into the high-performance liquid chromatograph (Yanaco L-2000) with a Yanaco VMD-101 voltammetric detector and a Yanapak ODS-T reversed-phase column (particle size 10 μm, 25 cm × 0.4 cm I.D.) (Yanagimoto, Kyoto, Japan). The carrier buffer for the liquid chromatography was 0.1 *M* potassium phosphate buffer containing 10% methanol, pH 3.2, with a flow-rate

of 0.5 ml/min. The detector potential was set at 0.8 V against the Ag/AgCl electrode. The peak height of 5-HT was measured and converted to pmol from the peak height of N-methyldopamine added as an internal standard. The retention times under these conditions were: N-methyldopamine, 5.0 min; 5-HT, 9.25 min; and 5-HTP, 10.0 min.

RESULTS

This HPLC—voltammetry system for the measurement of 5-HT and 5-HTP was found to be very sensitive. The standard curves of 5-HT and N-methyldopamine (internal standard) showed linearity from 200 fmol to 70 pmol.

Among the brain regions of rats, cerebral cortex has the lowest enzyme activity. Therefore, for the development of this method, rat cerebral cortex homogenate was used as the enzyme source. Fig. 1 shows the chromatographic pattern of the 5-HTP decarboxylase activity in rat cerebral cortex. The experimental incubation with L-5-HTP (Fig. 1A) showed significant formation of 5-HT in contrast to a small amount in the incubation with D-HTP (Fig. 1B). In this method 5-HTP was completely removed from the Amberlite CG-50 column. Even if very little 5-HTP is present in the Amberlite eluate, it can be

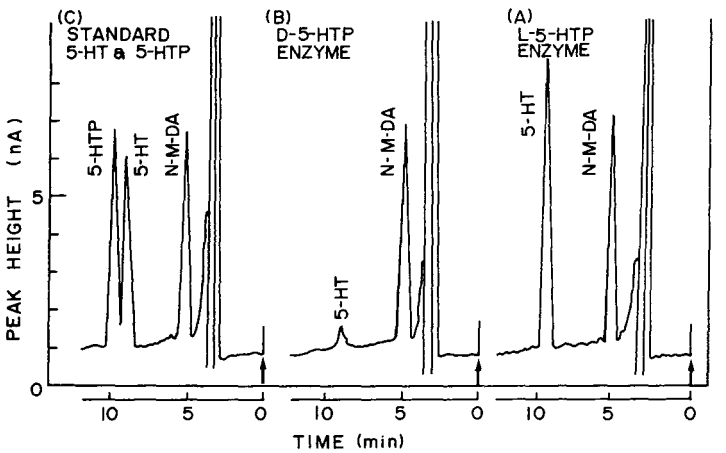

Fig. 1. HPLC elution pattern of L-5-HTP decarboxylase incubation mixtures with homogenate of rat cerebral cortex as enzyme. The conditions were as described under Experimental procedures. The standard incubation mixture contained 5 mg of rat cerebral cortex. (A) Experimental incubation with L-5-HTP; (B) blank incubation with D-5-HTP; 250 pmol of N-methyldopamine (N-M-DA) were added to each sample after incubation. (C) Standard mixture of 50 μl, containing 17.5 pmol, each of L-5-HTP, 5-HT and N-M-DA.

separated from 5-HT by HPLC. Fig. 1C shows the complete separation of the mixture of standard L-5-HTP and 5-HT.

L-5-HTP decarboxylase activity in a homogenate of rat cerebral cortex as a function of enzyme concentration is shown in Fig. 2A. Complete linearity was observed for plots of the amounts of homogenate from 1 to 15 mg tissue against those of 5-HT formed from L-HTP. Fig. 2B shows the rate of formation of 5-HT using rat cerebral cortex homogenate as enzyme. The reaction proceeded linearly up to 2 h.

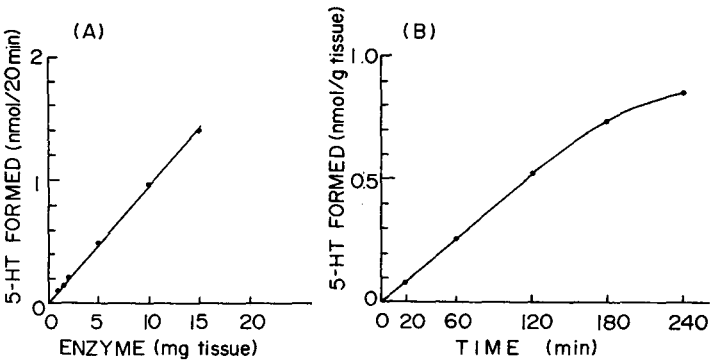

Fig. 2. (A) L-5-HTP decarboxylase activity in the homogenate of rat cerebral cortex as a function of enzyme concentration. The standard incubation mixture was used and incubation was for 20 min at 37°C. (B) Rate of 5-HT formation using the homogenate of rat cerebral cortex as enzyme at 37°C. Standard incubation mixture containing 1 mg of rat cerebral cortex was used as described under Experimental procedures.

Lineweaver—Burk plots illustrating the effect of the concentration of L-5-HTP on the rate of formation of 5-HT by a homogenate of rat cerebral cortex as enzyme are shown in Fig. 3. The Michaelis constant (K_m) toward L-5-HTP and the maximum velocity ($v_{max.}$) values were calculated to be $9.5 \cdot 10^{-6}$ M and 3.27 nmol/min per gram wet tissue, respectively.

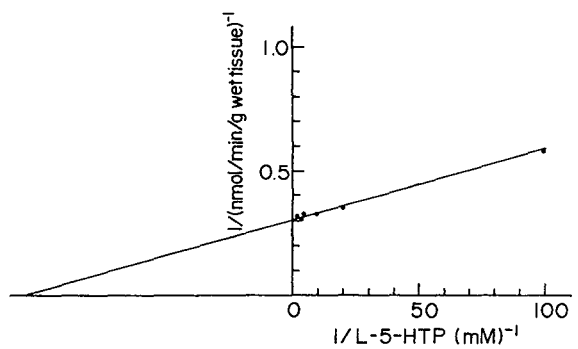

Fig. 3. Lineweaver—Burk plots illustrating the effect of the concentration of L-5-HTP on the rate of 5-HT formation by the homogenate of rat cerebral cortex as enzyme. The K_m and $v_{max.}$ values were calculated to be $9.5 \cdot 10^{-6}$ M and 3.27 nmol/min per gram wet tissue, respectively.

Since 5-HTP decarboxylase activity is found to be very low in human brains and only a small amount of data is available [10], we used this method for the measurement of the enzyme activity in the human cerebral cortex, which has the lowest activity. As shown in Table I, 5-HTP decarboxylase activity in human brains was variable and very low. Rat cerebral cortex had about 40-fold higher activity than human cerebral cortex.

The enzyme activity was discovered in rat serum by our method, and the activity was about 50 pmol/min per millilitre serum. The activity in human serum appears to be very low as compared with rat serum, about 1 pmol/min per millilitre serum, which is close to the limit of sensitivity by this method.

TABLE I

AROMATIC L-AMINO ACID DECARBOXYLASE ACTIVITY WITH L-5-HTP AS SUBSTRATE IN HUMAN AND RAT CEREBRAL CORTEX

Sample	AADC activity* (pmol/min/g wet tissue)	*n*
Rat cerebral cortex	3254 ± 53	4
Rat serum**	48.5 ± 9.4	3
Human cerebral cortex	89.3 ± 89.5	6
Range	21.2 — 246.0	

*The assay was carried out as described under Experimental procedures. Values are given as Mean ± S.E.M.
**pmol/min/ml of serum.

DISCUSSION

Many assay procedures have been reported on the activity of AADC: spectrophotometric [11,12], spectrofluorimetric [1], gas chromatographic [13], and radiometric [2,3,14]. Amongst these methods, the radiometric method using L- or DL-[1-^{14}C]DOPA as substrate to measure $^{14}CO_2$ formed [2,3] may be most widely used, since the method is simple and sensitive. However, as CO_2, not dopamine, is the product measured by this radioassay, non-enzymatic decarboxylation cannot be distinguished from enzymatic decarboxylation. We had established a highly sensitive and specific assay for AADC activity using L-DOPA as substrate and D-DOPA for the blank by HPLC—voltammetry [6]. This method is more sensitive than radioassay and can only measure the enzymatic decarboxylation of L-DOPA.

Since AADC forms not only dopamine from L-DOPA but also 5-HT from L-5-HTP as substrate, we have also tried to establish a new assay for AADC using L-5-HTP as substrate by HPLC—voltammetry. 5-HT was found to be assayed by voltammetry with a high sensitivity (limit of sensitivity, 200 fmol).

The present assay has many advantages. Firstly, it is very sensitive. The limit of sensitivity was about 1 pmol of 5-HT formed enzymatically. This method can measure $v_{max.}$ with saturated substrate concentration. This method is even more sensitive than our HPLC—voltammetry method using L-DOPA [6], because the reaction with L-5-HTP proceeds linearly for longer time (2 h) than that with L-DOPA. The sensitivity of the present AADC assay is determined solely by the blank value using D-5-HTP as substrate. The blank is derived either from 5-HT formed by the non-enzymatic decarboxylation or from 5-HT contained in a crude enzyme preparation. Secondly, this method is specific, because it only measures enzymatically formed 5-HT from L-5-HTP. Thirdly, it is economical. Also the maintenance of the glassy carbon electrode of the electrochemical detector is easy. Fourthly, as N-methyldopamine is used as internal standard in each incubation mixture, this method is very accurate.

It should be noted that AADC activity was for the first time found in rat serum by the present method. This method is considered to be useful to measure AADC activity using L-5-HTP as substrate and a small amount of brain

nucleus as enzyme source. Also, the assay of AADC in serum by this method would be useful for physiological and pathological studies of aromatic amino acid metabolism.

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CHROMBIO. 675

ANALYSIS OF SERUM IRON BY GEL PERMEATION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic procedure for the determination of serum iron is reported. Serum iron extracted with methyl isobutyl ketone was converted to dibenzoylmethane chelate (molecular weight 725), and it was separated from excess dibenzoylmethane (molecular weight 224) by gel permeation chromatography. The chelate was determined by measuring ultraviolet absorption at 280 nm. Good reproducibility, recovery, and correlation with the conventional colorimetric method were observed.

INTRODUCTION

Analysis of serum iron is one of the important clinical tests, and has mostly been performed by a colorimetric method using bathophenanthroline sulfonic acid; however, this method requires a larger quantity of serum compared to other tests. Recently, high-performance liquid chromatography (HPLC) has been introduced into the field of analytical chemistry. Previously, analysis of iron(III) by gel permeation HPLC was reported by Yuki et al. [1]. In this method, iron(III) was extracted into methyl isobutyl ketone (MIBK) from 4—6 *N* hydrochloric acid solution, and was reacted with dibenzoylmethane (DBM), molecular weight 224, to form a chelate compound, iron(III)—$(DBM)_3$, of molecular weight 725. The difference in molecular weights between the chelate and DBM was large enough for the separation of these compounds by gel permeation HPLC. This method, which was simple and sensitive, was successfully applied to the determination of serum iron. Details of these experiments are presented in this article.

EXPERIMENTAL

Reagents and materials

Hydrochloric acid: concentrated hydrochloric acid (reagent grade) was

mixed with the same volume of water and was distilled twice. To the constant boiling fraction collected was added one-tenth volume of MIBK, and the mixture was shaken well in a separating funnel to remove trace iron. After standing, the hydrochloric acid layer was taken and used. The concentration of the hydrochloric acid after MIBK extraction was 95% of the initial concentration. Hereafter, this MIBK-treated hydrochloric acid will be designated simply as MIBK—HCl. MIBK, a commercial product of reagent grade (Wako, Osaka, Japan) was purified by distillation.

Tetrahydrofuran (THF) was a commercial product prepared for HPLC (Ishidzu, Osaka, Japan). DBM, a reagent grade material (Nakarai, Kyoto, Japan) was purified by distillation at reduced pressure.

Octa-*p*-nitrobenzoyl sucrose was synthesized as reported in the previous paper [1], and was used as an internal standard (I.S.) (molecular weight = 1535.14).

DBM solution: 60 mg of DBM and 50 μg of I.S. were dissolved in 10 ml of THF.

n-Butylamine was a reagent grade material (Wako) and was purified by distillation and used as a 5% solution in THF. Ammonium ferric sulfate was reagent grade (Nakarai).

Control serum was Nescol X from Nihon Shoji (Osaka, Japan). Pooled serum and patients' sera were kindly supplied by Y. Aoki, Toho University Hospital.

Deionized distilled water was used throughout these experiments.

Apparatus

A Shimadzu LC-2 liquid chromatograph was used equipped with a sample injection valve of 0.2 ml capacity, a prepacked HSG-15 column (500 × 7.9 mm; particle size 10 μm; polystyrene type), and a spectrophotometric detector SPD-1 (path length 6 mm, volume 6.4 μl).

Standard procedure for the analysis of serum iron

A mixture of 0.2 ml of serum, 0.8 ml of MIBK—HCl, and 1 ml of MIBK was shaken longitudinally in a glass-stoppered test tube (10 ml) placed horizontally on a reciprocating shaker at 100 strokes/min for 15 min. One-half millilitre of the upper layer was mixed with 1 ml of DBM and 0.1 ml of butylamine solution. After standing for 10 min, 0.2 ml of the mixture was injected into the liquid chromatograph. Elution was carried out with THF at a flow-rate of 0.8 ml/min at room temperature. The I.S. and the chelate were eluted at retention times of 18 and 20 min, respectively. Peaks were detected at 280 nm, and iron was determined by measuring its peak height in comparison with that of the I.S. Sensitivity = 0.08 a.u.f.s.

Calibration curve

To 0.2 ml of standard solutions of iron prepared by dissolving ammonium ferric sulfate in water were added 0.8 ml of MIBK—HCl and 1 ml of MIBK; the mixtures were treated in the same way as given under Standard procedure. A straight line was observed for iron concentrations up to 53.7 μmol/l (300 μg per 100 ml).

RESULTS AND DISCUSSION

Since serum iron is usually bound to protein in the form of transferrin, it is necessary to liberate it from the protein prior to analysis. In the colorimetric method, protein-bound iron is liberated by treatment of serum with 1 *N* hydrochloric acid at 100°C for 1—2 min. As reported in the previous paper [1], 4—6 *N* hydrochloric acid was found to be optimum for MIBK extraction. It was found that the protein-bound iron was dissociated by treatment with 4—6 *N* hydrochloric acid without heating, and the liberated iron was conveniently extracted into MIBK.

Fig. 1 shows the effect of the volume of MIBK—HCl on the MIBK extraction of iron from serum. Addition of 0.8 ml of MIBK—HCl to 0.2 ml of serum gave complete extraction of iron into MIBK. Vigorous mixing of the mixture using a thermomixer resulted in the formation of an emulsion from which it was difficult to separate the organic layer. Thus, conditions for extraction were set as follows: the mixtures were shaken longitudinally in glass-stoppered test-tubes, laid horizontally on a reciprocating shaker, at 100 strokes/min for 15 min. The effect of the shaking time on extraction is illustrated in Fig. 2.

After MIBK extraction, DBM was added to form a chelate. However, as the MIBK extract contained a small amount of hydrochloric acid, DBM did not form a chelate with iron due to the strongly acidic conditions. Although, in

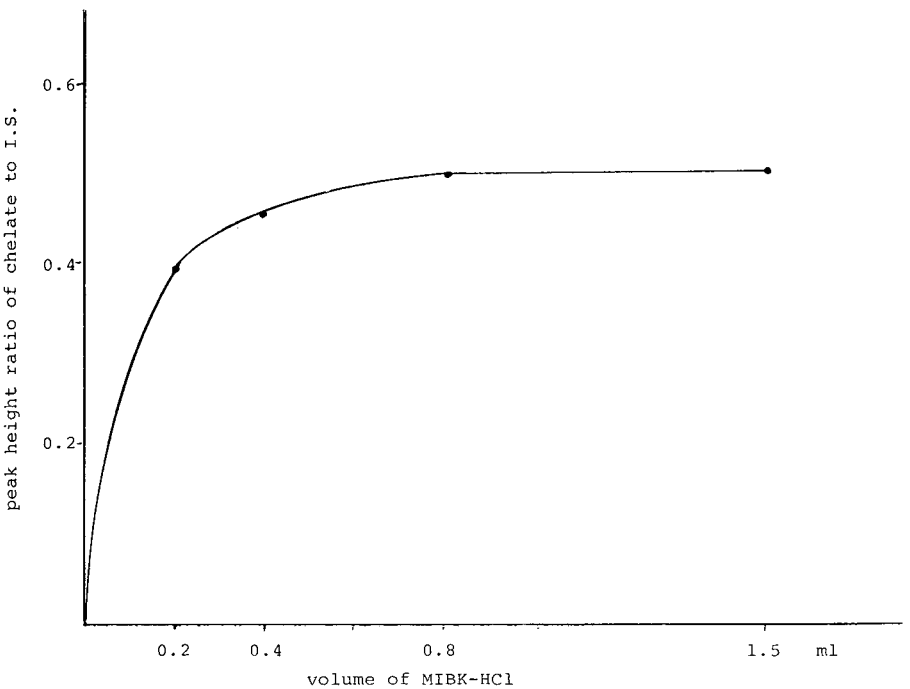

Fig. 1. Effect of volume of MIBK—HCl on extraction of iron(III) into MIBK. To 0.2 ml of serum were added the specified volume of MIBK—HCl and 1 ml of MIBK, and the mixtures were shaken as described in the text. After standing, 0.5 ml of supernatant, 1.0 ml of MIBK, and 0.1 ml of 5% butylamine were mixed, and 0.2 ml of the mixture was injected into the HPLC apparatus. Chromatographic conditions are referred to the text.

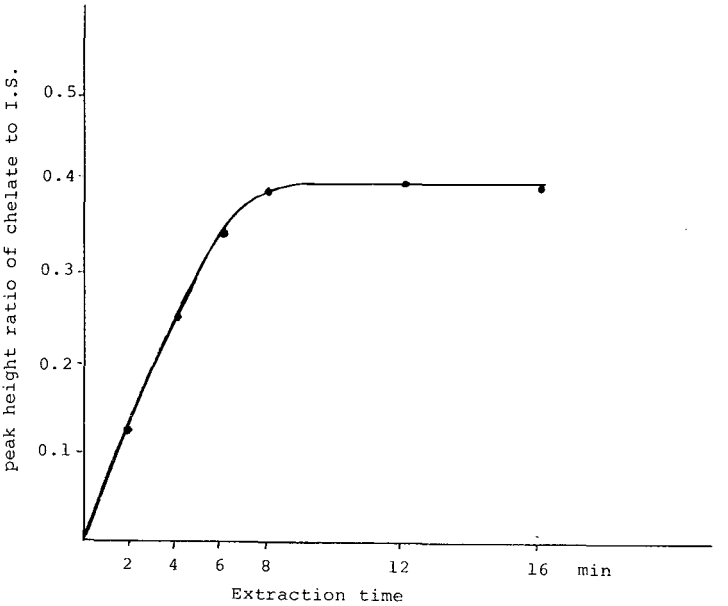

Fig. 2. Relationship between extraction time and extraction efficiency. To 0.2 ml of control serum were added 0.8 ml of MIBK—HCl and 1 ml of MIBK. The mixtures were shaken as described in the text for 2, 4, 6, 8, 12, or 16 min, then treated according to the standard procedure.

the previous study [1], propylamine was added to neutralize the hydrochloric acid extracted, this amine gave rise to a white precipitate, probably propylamine hydrochloride. The formation of a precipitate is undesirable for the subsequent procedures. It was found that addition of butylamine did not give any precipitate, while triethylamine, diisopropylamine, laurylamine, piperidine, and morpholine did. Aromatic amines were not tested because of their ultraviolet absorption.

Absorption at 254 nm was used for the detection of the chelate in the previous experiments [1]. However, with serum samples there was not a good separation between the peaks of the chelate and other substances. As measurements at different wavelengths give different chromatograms, the selection of wavelength is an important factor for quantitative analysis. After a number of experiments, a wavelength of 280 nm was chosen, as it showed the best resolution of the chelate although the sensitivity was slightly lower. Fig. 3 shows chromatograms obtained at 254 and 280 nm for comparison. A straight calibration line was observed up to 53.7 μmol/l using a peak height method measured at 280 nm. A blank test gave a small peak at the same retention time as that of the chelate. Treatment of constant-boiling hydrochloric acid with MIBK made the blank peak smaller. However, in spite of purification of reagents and cleaning of the glassware, complete elimination of the blank peak has so far been unsuccessful. Thus, the peak height of the blank test had to be subtracted from that of the samples run.

Within-day reproducibility was examined with pooled serum. Results obtained with ten runs gave 15.4 ± 0.446 μmol/l (mean ± S.D.) and the coefficient

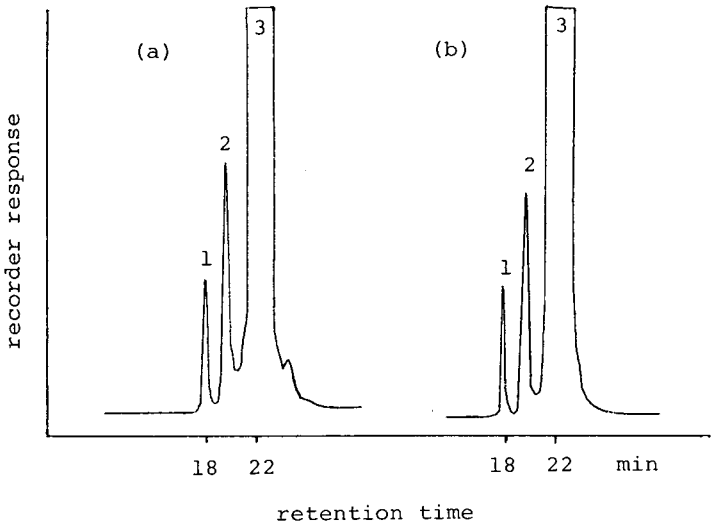

Fig. 3. Difference between chromatograms measured at 254 nm (a) and at 280 nm (b). Control serum (0.2 ml) was treated as described in the text, and chromatograms were prepared at 254 and 280 nm. Peaks: 1 = I.S.; 2 = iron—DBM chelate; 3 = DBM and others.

of variation (C.V.) was 2.83%. Day-to-day reproducibility was examined for ten days with the same serum. Mean ± S.D. was 15.2 ± 0.456 μmol/l, and the C.V. was 2.96%. For the recovery test, dried control serum (17.9 μmol iron per l when dissolved in a specified volume of water) was dissolved by the addition of different concentrations of ammonium ferric sulfate solution, and these sera were analysed by HPLC. Satisfactory recovery was obtained, as shown in Table I.

TABLE I

RECOVERY TEST

Exp. No.	Iron added to control serum (μmol/l)	Iron found (μmol/l)	Recovery (%)
1	0	17.7	
2	0	17.9	
3	0	18.1	
Mean		17.9	
4	9.0	26.3	93.3
5	9.0	26.6	96.7
6	9.0	26.0	90.0
7	9.0	26.8	98.9
Mean			94.7
8	17.9	35.6	98.9
9	17.9	34.3	91.6
10	17.9	35.3	97.2
11	17.9	36.0	101.1
Mean			97.2

Sixty-nine samples were analysed by the proposed HPLC method and a conventional colorimetric method [2], and the two methods were compared. The results are shown in Fig. 4. The regression line obtained was $y = 0.953x + 0.50$ (μmol/l), where y represents the results obtained by the HPLC method and x those obtained by the colorimetric method ($n = 69$). The correlation coefficient (r) was 0.961.

Serum contains some other metals such as calcium, magnesium, copper, zinc, etc. However, MIBK extraction is extremely selective for iron as reported by Goto and Sudo [3] and Tajima and Kurobe [4,5]. This was also stated in the previous paper [1] in which it was demonstrated that a ten-fold concentration by weight of the above ions did not interfere with the determination of iron by gel permeation HPLC. Thus, the values obtained by this method should unambiguously be those of serum iron. This was further evidenced by the correlation coefficient and regression line obtained with patients' sera compared with that obtained with the colorimetric method.

Thus, it was demonstrated that the analysis of serum iron by gel permeation HPLC was selective and sensitive, and the whole procedure could be performed

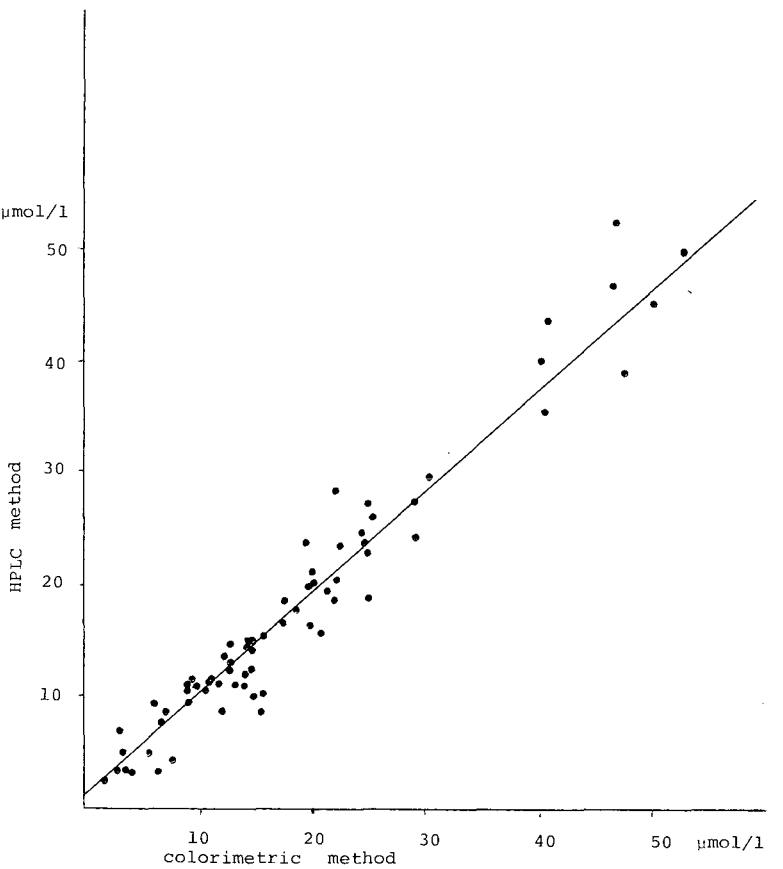

Fig. 4. Sixty-nine patients' sera were analysed by the HPLC method, and the results were compared with those from the colorimetric method. $n = 69$; $y = 0.953x + 0.50$; $r = 0.961$.

within 1 h per sample. Since the first peak appears at 18 min after injection and all UV-absorbing substances are eluted within 30 min, sample injection could be repeated every 15 min. The chelate formed after addition of butylamine is so stable for at least 6 h [1] or more, that a number of samples can be prepared at a time. In the experiments reported here, 0.2 ml of serum was used, but half of this volume was found to be sufficient for routine analysis. Elimination of the blank peak would make the analysis possible with much less serum. It was also expected that elution with a higher flow-rate would reduce the chromatographic time and the interval between sample injections. These possibilities are currently under investigation.

In conclusion, this method is simple since it does not involve a deproteinization step, and can be used in clinical laboratories in its present form. Further improvements suggested above would increase the practical value of the method.

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ISOELECTRIC FOCUSING STUDIES OF HUMAN PANCREATIC SECRETION

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SUMMARY

Pure bile, pancreatic and duodenal human juices have been analyzed by isoelectric focusing, either at rest or upon stimulation with caerulein. In rats, stimulation has also been performed with secretin. Twenty bands have been resolved and quantified in the pancreatic secretion. By developing zymograms, a number of isozymes have been identified: 6 isoamylases [p*I*'s 7.2, 7.1 and 6.6 (major) and p*I*'s 7.4, 6.7 and 5.8 (minor)], 3 lipases [p*I*'s 7.0 and 6.8 (major) and 6.4 (minor)], two major alkaline proteases (p*I*'s 9.8 and 8.4) and one major acidic protease (p*I* 4.3) and one band of RNAase activity (p*I* 8.6). The stimulation kinetics follow a mechanism according to Palade, indicating uniform response to secretogogues, parallel intracellular transport and parallel discharge of pancreatic exocrine proteins.

INTRODUCTION

The pancreas is a suitable organ for studies of mammalian cell function and differentiation at the biochemical level in terms of organ-specific enzymes [1—4]. Its proteolytic enzymes chymotrypsin and trypsin have been used as biochemical indicators for characterization of cellular differentiation and regulation of functional activity [1, 5]. These enzymes are also suitable indicators in studies of molecular evolution [6] or species variation [7].

The exocrine pancreas is also an attractive biochemical model for the study of the induction and modulation of enzymes, since it synthesizes and secretes an impressive variety of proteins which span a broad range of molecular weights, isoelectric points and actual or potential activities [8]. In contrast to this pleotropic function, all of the exocrine cells of the pancreas are similar in appearance, each containing a reasonably homogeneous population of secretion (zymogen) granules [9]. It has been reported that the exocrine pancreas can apparently exert a long-term control over specific enzymes in response to specific diets [10, 11]. At present, there are two major lines of evidence in support of two opposite models: a model of "selective modulation" [12, 13] and a model of "parallel and synchronous discharge" [14, 15]. According to the first model, some stimuli, such as the presence of lysine in the intestine [12] or the injection of chymodenin [13] can elicit a rapid, selective, several-fold increase of some enzymatic activities in the pancreatic discharge granules. According to the second model, irrespective of stimulant (carbamylcholine, caerulein or 75 m*M* potassium chloride) and time of stimulation, the exocrine proteins are discharged in parallel and in constant proportions [14, 15].

The analysis of the pancreas discharge products can be also very useful in medical diagnosis and treatment, since it can be expected that pathological disturbances, such as chronic pancreatitis or carcinoma, can lead to alterations in the pattern of isoenzymes, as resolved by electrophoretic techniques. In order to obtain a deeper insight into these problems, we have applied the technique of isoelectric focusing (IEF) to the analysis of pure pancreatic juice, pure bile and duodenal juice in both rats and humans, in physiological conditions and under hormonal stimulation.

MATERIALS AND METHODS

This study was performed first in rats and subsequently in human patients admitted to a surgical ward for pathological conditions of the main biliary ducts, of the pancreas or of the upper gastrointestinal tract. In rats, pure bile and pure pancreatic juice were obtained by direct double cannulation of the main biliary duct (the pancreatic ducts discharge in its lower portion); attempts to obtain duodenal juice were unsuccessful. In human patients pancreatic secretion was collected by direct cannulation of the Wirsung duct during surgery for pathological conditions of the Vater papilla; pure bile was collected from patients bearing an external drainage (Kehr tube) and duodenal juice by cannulation with a Levine tube.

Stimulation of the pancreatic gland was in two different groups of animals by secretine alone (Booths, 12 U/kg) and by ceruletide alone (ceruleina Farmitalia, 0.33 μg/kg) with a single intravenous administration. Stimulation with a single substance was performed in order to demonstrate any possible difference in the IEF pattern: in view of the results obtained we used ceruletide alone in human patients, always with a single administration by endomuscular route (0.75 μg/kg). Samples were collected every 10 min during a 1-h period in the animals, during 90 min in duodenal cannulation and for approximately 40—50 min during surgery. Samples were immediately frozen in liquid nitrogen. Total protein content was determined by the method of Lowry et al. [16]. IEF

was carried out as previously described [17] using polyacrylamide gel plates containing 2% Ampholine (LKB, Bromma, Sweden) in the pH range 3.5—10. Urea (4 *M*) was added to the gel to avoid macromolecular aggregation. The samples were seeded in pockets precast in the gel [18]. At the end of the run (4 h) the plates were fixed in trichloroacetic acid (TCA) and the proteins stained with Coomassie Brilliant Blue R-250 [18]. Measurements of the isoelectric points of the protein bands were made with an Ir/IrO_2 electrode [19].

The gel scans were performed with an experimental apparatus, built in our laboratory, consisting of a flying spot (a red diode light source) moving over the Coomassie Blue stained gel. The scanner is piloted by a Digital PDP8/e computer, which also stores in a magnetic tape the gel signal, amplified by a solid-state photomultiplier. The data are displayed graphically on an X-Y plotter Plotmatic 815M (M.F.E.). The position of each peak and its relative area is typed on a teletype. This system is linear up to 3 A. In addition to Coomassie Blue staining, zymograms for amylase [20], protease [21], lipase [22] and RNAase [23] activities were developed in the screening of human samples. As we have also recently suggested and described in detail [24], best results were obtained by performing an agarose contact print.

RESULTS

Figs. 1A and B show the stimulation kinetics of rat pancreatic and biliary juices, respectively, upon injection with a single dose of the secretogogue caerulein. The total protein content in pancreatic secretion rises steeply, reaches a peak within 30 min and then declines steadily to basal levels within 50 min. The volume of secretion increases up to five times above the normal levels while its protein content is increased by a factor of three. Quite different kinetics are exhibited by bile juices: their volume increases only by 15%, while their protein concentration gradually decreases three-fold as compared with physiological levels.

Fig. 2A shows the IEF profiles of rat pancreatic juice upon stimulation with two different secretogogues, caerulein and secretin, at different time intervals. To avoid preferential adsorption of some protein components, the samples have been applied to the gel slab in pockets precast in the gel, from the anodic side, after 30 min of prefocusing. More than 20 protein components are resolved by IEF, and no material is precipitated or trapped within the pocket. A very similar protein distribution is observed both in the absence or in the presence of 4 *M* urea, suggesting that the IEF profile is not due to protein aggregates. As readily apparent by a visual inspection of the gel, and as shown by the scans in Fig. 2B, the protein patterns, and their relative distribution, are practically identical under stimulation with either secretogogue.

Figs. 3A and B show the the stimulation kinetics of pancreatic and biliary juices, respectively, from a normal human adult, upon injection of a single dose of secretin. In the case of pancreatic juices, while the overall stimulation pattern is similar to the one obtained in rats, the peak is reached in only 15—20 min, while the increase in protein concentration is only 50% above unstimulated levels. The pattern in the bile is almost identical to the stimulation profile in rats.

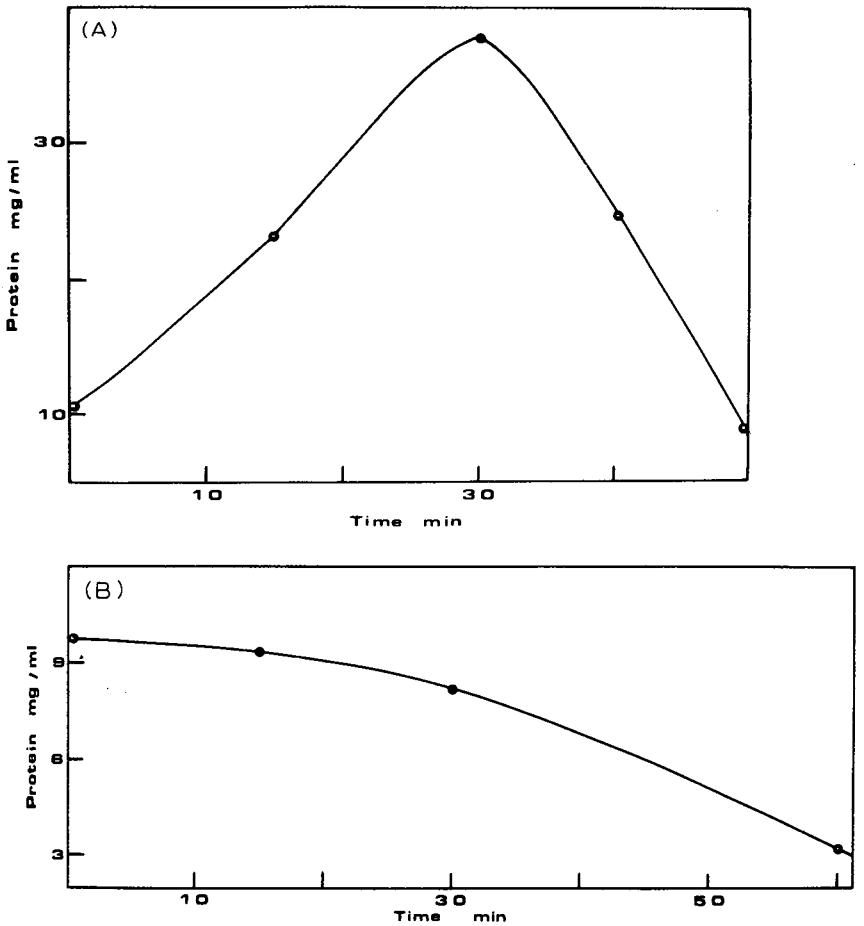
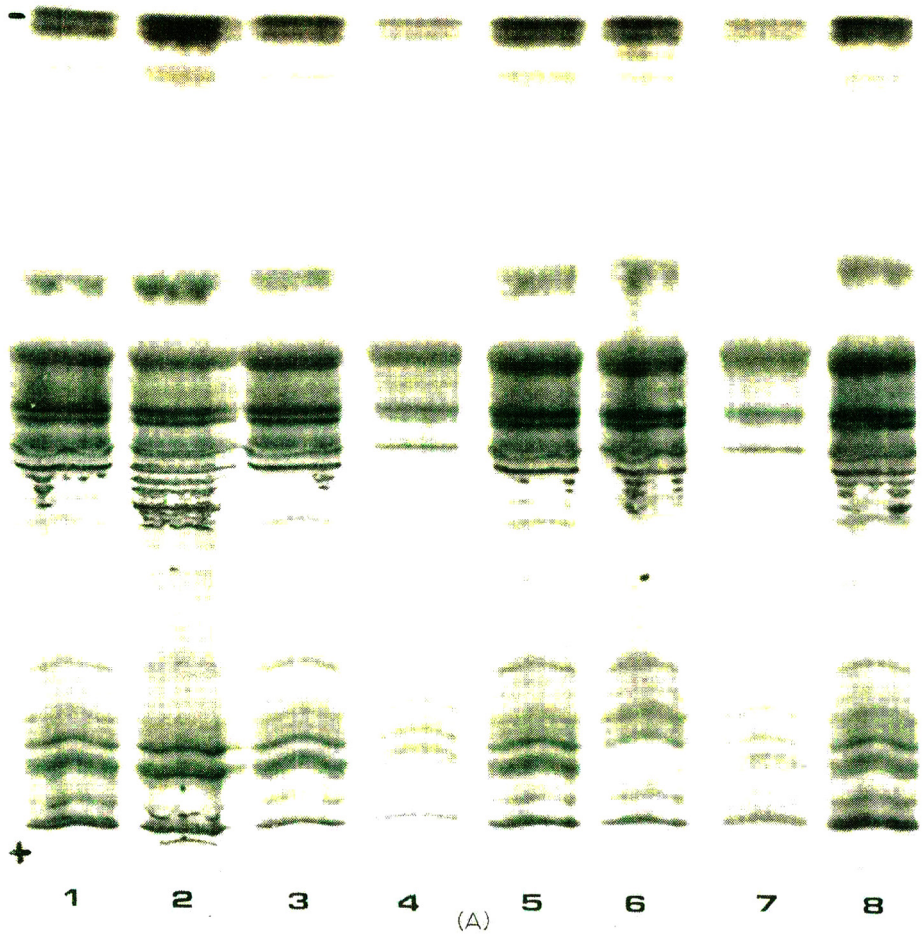
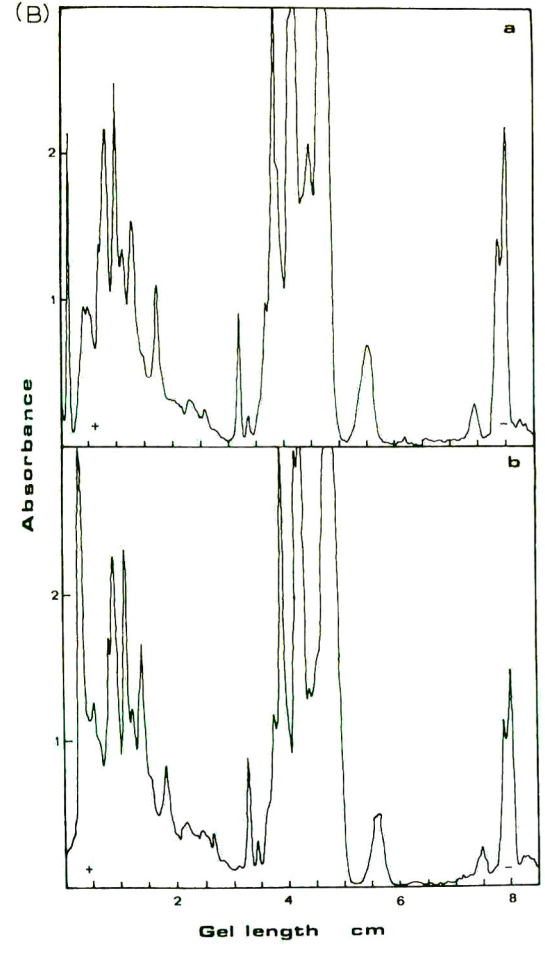


Fig. 1. Kinetics of pancreatic (A) and biliary (B) secretion in rat after caerulein stimulation (330 ng/kg intravenously). The protein concentration was determined according to Lowry et al. [16]; the possible interference by tensioactive substances from the bile was not investigated.

Fig. 2. (A) IEF of rat pancreatic juice. Samples 1—5 obtained by caerulein stimulation: 1, basal and 2—5, after secretogogue administration (2, 0—12 min; 3, 12—25 min; 4, 25—40 min; 5, 40—60 min). Samples 6—8 were collected after secretin stimulation (6, basal; 7, 0—30 min; 8, 30—60 min). Samples 4 and 7 were diluted 5 times, while all the others were diluted 3 times (in all cases, a 30-μl sample was applied in pockets precast in the gel). Experimental: polyacrylamide gel 5%T, 4%C_{Bis}, containing 2% LKB Ampholine pH 3.5—10 and 4 *M* urea; run: 4 h at 15 W constant; temperature, 4°C. Proteins were stained with Coomassie Brillant Blue R-250. (B) Scans at 600 nm of the patterns of samples 4 (a) and 7 (b) in Fig. 2A.



(A)



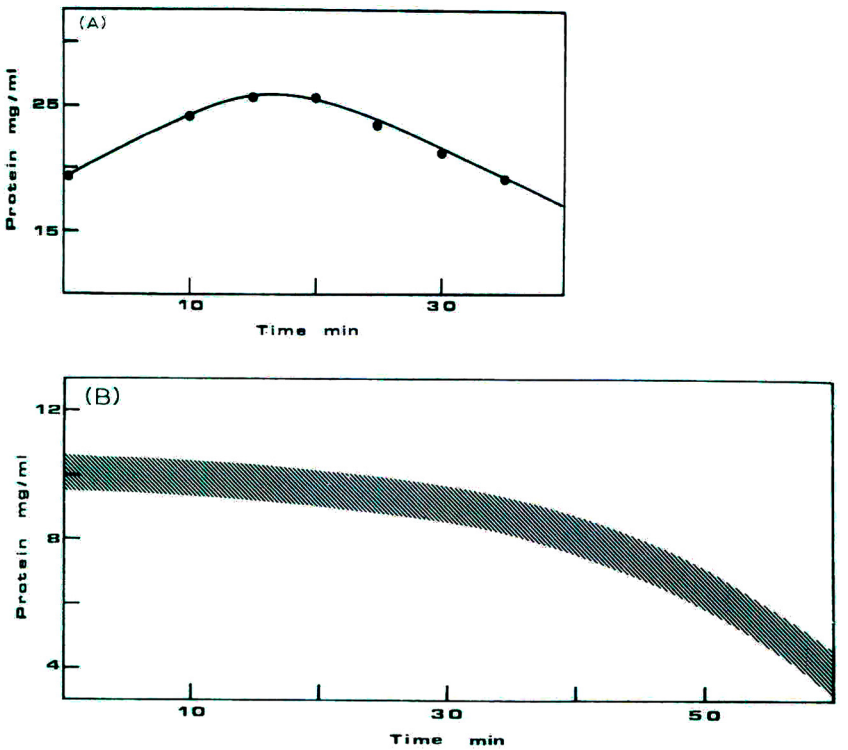


Fig. 3. Kinetics of pancreatic (A) and biliary (B) secretion in humans after caerulein stimulation (0.75 μg/kg). The protein concentration was determined according to Lowry et al. [16].

From a clinical point of view, it is of interest to obtain a reference IEF pattern of pure pancreatic juice from normal individuals, since it can be used as a basic reference for measuring possible qualitative or quantitative variations associated with pathological conditions. Fig. 4 exhibits the IEF profile of normal human adult pancreatic juice, obtained during surgery, upon stimulation with caerulein. A well-resolved pattern of about 20 components, distributed in the pH range 3.5—10, can be appreciated. As in the case of rat pancreatic juice, here too the qualitative and quantitative distributions of the various bands seem to be unaltered during the time of stimulation. This is also confirmed by the four scans of Fig. 5, which show the profiles of the 20 bands resolved by IEF in pancreatic juice. The 20 peaks have been numbered progressively and their relative abundance tabulated in Table I. It can be seen that the area of each peak remains remarkably constant during the period of stimulation, the distribution profile being practically identical to unstimulated samples.

We have also tried to demonstrate if any protein band could be detected in pure bile juice, obtained by cannulating either the choledochus or the cholecystis. Some faint protein bands can indeed be revealed (see Fig. 6), of which a few, indicated by arrows, appear to be in common with proteins of pure pancreatic juice. We cannot exclude, however, a partial contamination of bile secretion with the latter. Focusing of pure bile does not appear to be feasible,

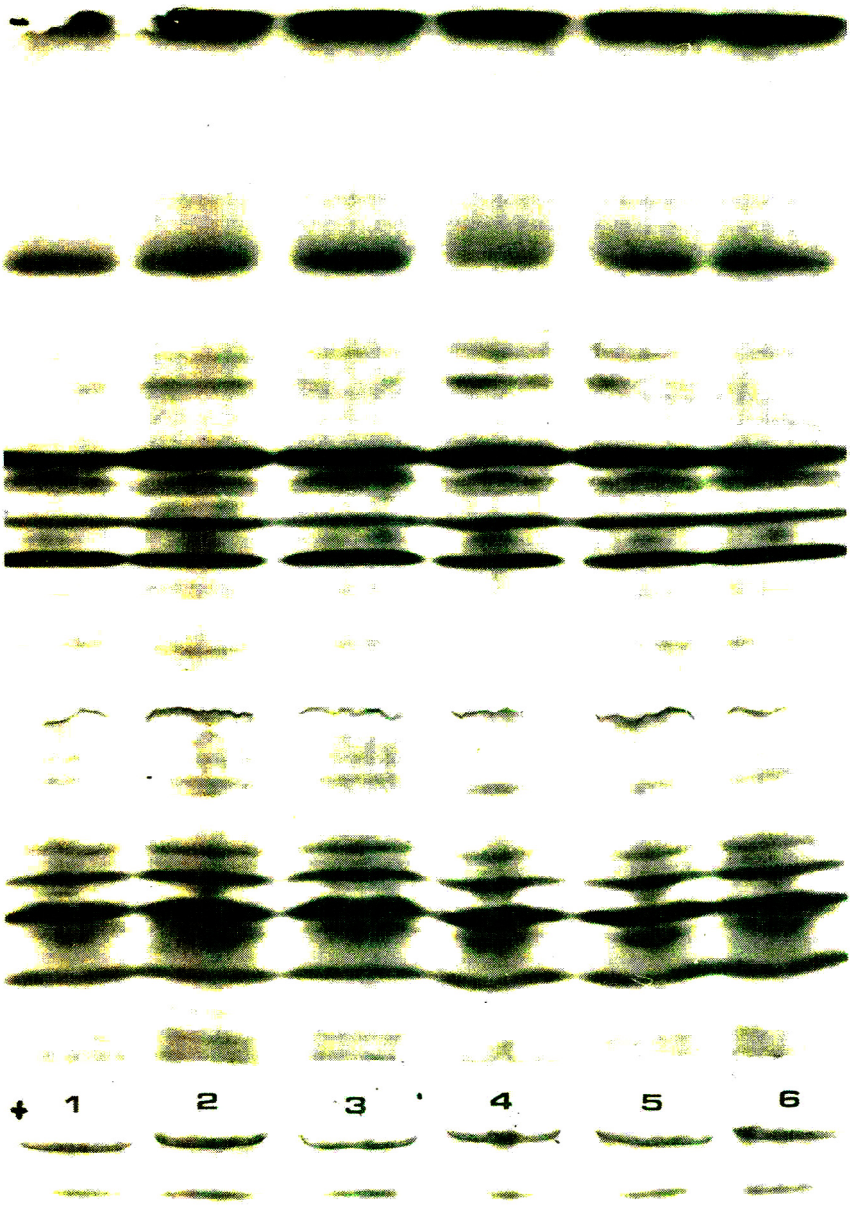


Fig. 4. IEF of human pancreatic juice obtained by surgical cannulation of the Wirsung duct, after caerulein stimulation. Samples: 1, basal secretion; 2, 0—10 min; 3, 10—15 min; 4, 15—20 min; 5, 20—25 min and 6, 25—30 min of discharge after stimulation.

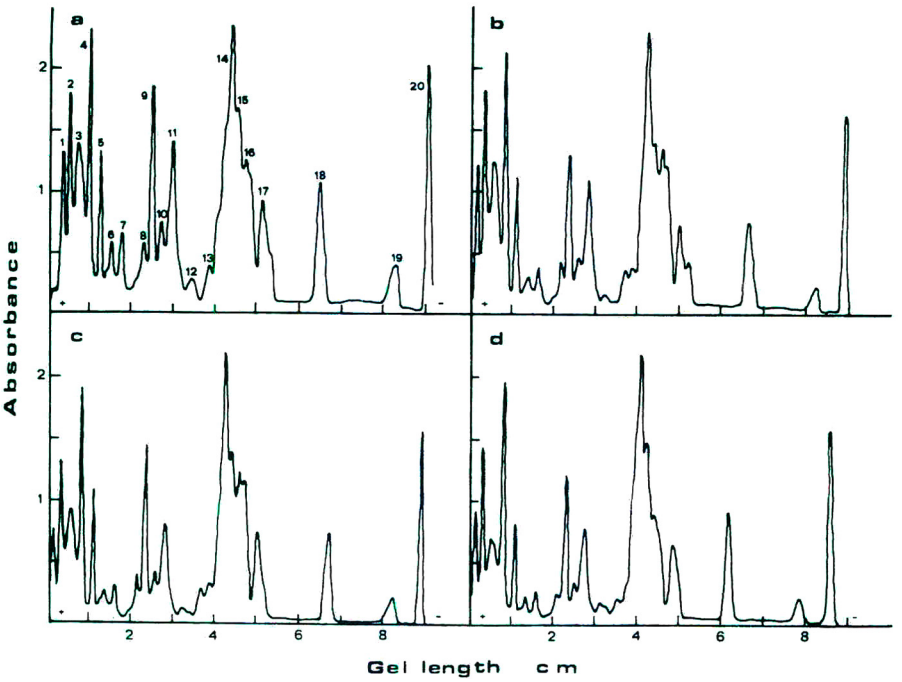


Fig. 5. Scans at 600 nm on IEF patterns from Fig. 4. (a) Sample 1; (b) sample 2; (c) sample 4; (d) sample 6.

TABLE I

RELATIVE PERCENTAGE OF PEAKS 1—20 OF FIG. 5

Data obtained by densitometry at 600 nm of the IEF patterns of human pancreatic secretion after stimulation with caerulein (Fig. 4) (S.D. = standard deviation).

Peak No.	Control	Time (min)				Mean	S.D.
		10	20	30	50		
1	3.7	4.0	3.6	3.5	3.8	3.7	0.172
2	5.7	6.6	5.2	5.5	6.0	5.8	0.477
3	8.0	8.6	8.1	8.3	8.8	8.4	0.300
4	6.5	6.1	7.0	6.8	6.4	6.6	0.314
5	3.4	3.1	3.2	3.0	3.3	3.2	0.140
6	1.1	1.0	1.0	1.1	0.9	1.0	0.075
7	1.6	1.8	1.5	1.5	1.4	1.6	0.136
8	2.4	2.0	2.0	2.2	2.1	2.1	0.163
9	5.1	4.9	4.6	4.8	4.5	4.8	0.213
10	2.4	2.2	2.2	2.3	2.0	2.2	0.132
11	6.0	7.0	5.5	6.5	6.2	6.2	0.500
12	1.9	1.7	1.6	1.8	1.5	1.7	0.141
13	2.0	1.9	1.8	2.0	1.6	1.9	0.150
14	17.6	16.6	18.5	16.6	17.0	17.3	0.720
15	7.6	8.1	8.5	7.4	7.5	7.8	0.417
16	6.6	5.3	7.0	6.5	6.0	6.3	0.585
17	5.3	5.2	6.0	5.8	5.9	5.6	0.326
18	5.1	5.0	5.3	5.6	5.8	5.4	0.300
19	2.9	3.0	2.8	3.1	3.4	3.0	0.206
20	6.2	6.0	5.6	6.4	5.9	6.0	0.271

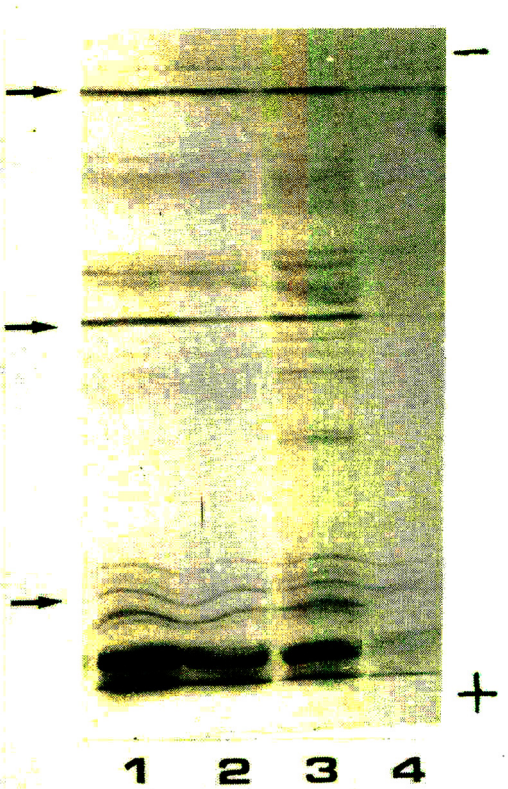


Fig. 6. IEF pattern of human bile: samples 1 and 2, collected from choledochus; 3 and 4 from cholecystis. 1 and 3, untreated, 2 and 4, dialyzed 12 h against Tris—Gly buffer, 10 m*M*, pH 8.2, then 24 h against 5 *M* urea in the above buffer. The arrows refer to protein bands common to pancreatic juice.

since often the IEF pattern is severely disorted by the high levels of biliary pigments and salts, which are not completely eliminated even after extensive dialysis (see samples 2 and 4 of Fig. 6). The biliary pigments collect at the anodic side into two heavy zones, the upper one of yellow colour, the lower one green (see samples 1—3 in Fig. 6), the former being the most abundant.

Besides analyzing pure pancreatic and biliary juices, we have also collected duodenal juice since, from a clinical point of view, this is the sample which can be more conveniently collected as it does not require surgery nor patient hospitalization. Fig. 7 shows the IEF profile of normal human adult duodenal juice taken at different time intervals, up to 60 min after stimulation with secretin. In agreement with what we have described so far, we can detect in this IEF pattern all the components of pancreatic secretion (see Fig. 4) mixed, especially in the acidic portion of the pH gradient with the few, minor protein components found in the bile juice (see Fig. 6). Scans and peak integration have

shown that the ratios of the components from pancreatic juice remain constant while the minor components, ascribed to bile products, in agreement with the stimulation kinetics of Fig. 3B, progressively decrease with time, together with the biliary pigments (1—2 sharp bands focusing below the application pocket; see Fig. 7). Also, in agreement with Fig. 3A, we can see the components from pancreatic juice increase in the duodenal juice, in the typical bell-shaped pattern, reaching a maximum after ca. 35 min of stimulation. The delay on the peak appearance (in pure pancreatic juice the plateau is reached in ca. 20 min) is most probably due to the dilution of the pancreatic juice by the bile secretion, with its considerably lower protein content.

We have also tried a characterization of the various enzyme activities present in the pancreatic secretion by developing a series of zymograms, for amylase, proteases, lipase and RNAase, as described under Materials and methods. The results are summarized in Fig. 8. Six amylase activities have been detected, three major bands having p*I*'s 7.2, 7.1 and 6.6 and three minor components,

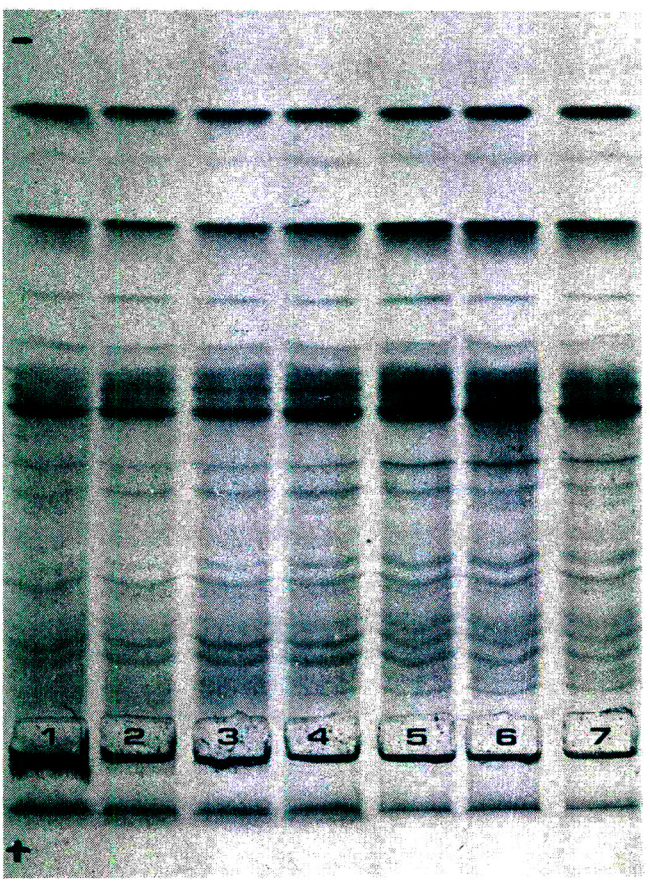


Fig. 7. IEF pattern of duodenal juice from humans after caerulein stimulation. Samples: 1, control; 2, 0—10 min; 3, 10—20 min; 4, 20—30 min; 5, 30—40 min; 6, 40—50 min; 7, 50—60 min after stimulation.

with p*I*'s 7.4, 6.7 and 5.8. These data are in general agreement with those of Berndt et al. [25] and Allan et al. [26]. Three lipase zones are evidenced, two major isozymes with p*I*'s 7.0 and 6.8 and a minor component with p*I* 6.4, in agreement with the IEF spectrum reported by Kurooka and Kitamura in sucrose density gradient columns [27]. Proteases are found with a wide spec-

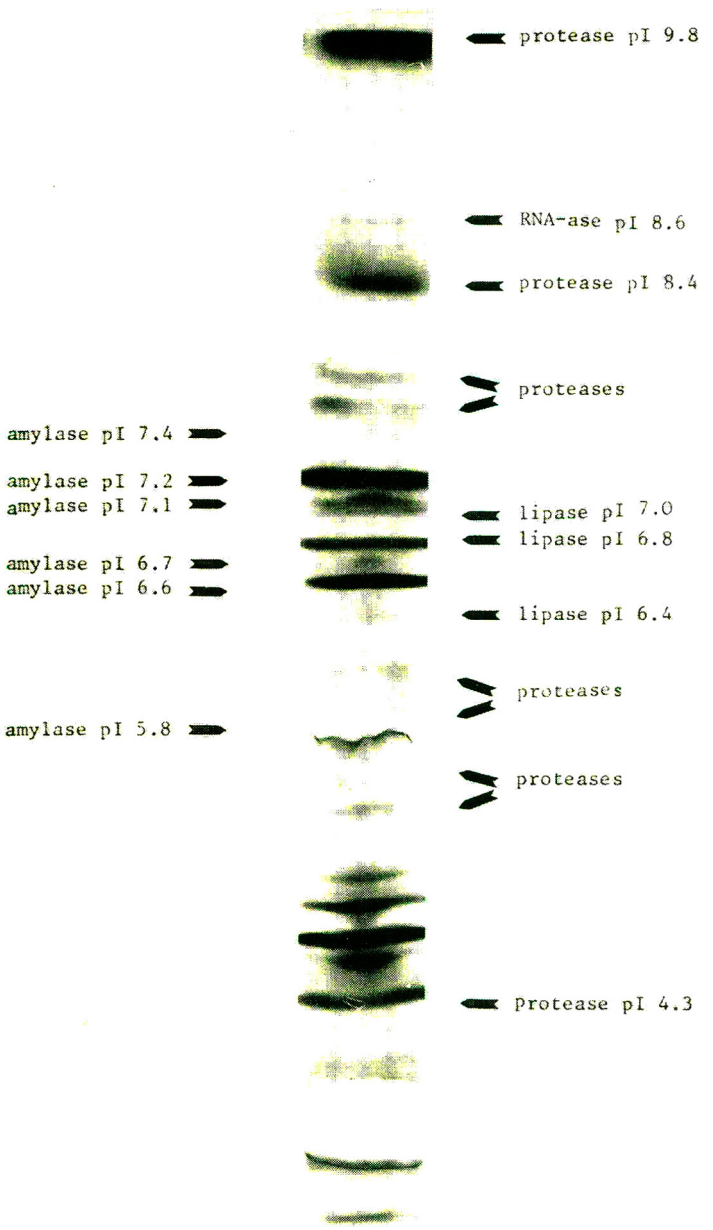


Fig. 8. Summary of the different enzymatic activities in pancreatic secretion as revealed by different zymograms (see Materials and methods).

trum of p*I*'s, in agreement with literature data on rat pancreatic secretion [28]. There are two heavy alkaline bands (p*I* 9.8 and p*I* 8.4) and a major acidic protease (p*I* 4.3). Several minor components are clustered around neutrality. One very sharp band of RNAase activity appears at p*I* 8.6. Some of the bands in the general Coomassie Blue pattern still remain to be identified.

DISCUSSION

In view of a future use of our IEF technique in clinical diagnosis, where the study of pancreatic secretion can be routinely accomplished only by duodenal tubage, it was necessary to study separately pure biliary, pancreatic and duodenal secretions, in order to obtain reference IEF patterns. Our data suggest that duodenal secretions, in which the majority of IEF bands are easily identifiable as pancreatic components, are indeed useful in producing a reference IEF profile of pancreatic juice discharge. Therefore, a future study of pancreatic function and possible alteration during disease can simply be accomplished by sampling and analyzing duodenal juices.

From a biochemical point of view, our data on rat and human pancreatic discharge, in normal conditions or under stimulation, fully support and expand the findings of Palade and co-workers [14, 15] on the synchrony of discharge of actual and potential enzyme activities from guinea pig pancreatic lobules. A parallel discharge also means a parallel intracellular transport. As already pointed out [14, 15], from the beginning of their appearance in condensing vacuoles, the major secretory proteins are already present in the same relative concentrations in which they are found later on in zymogen granules and in the discharge secretion. The fact that this discharge mechanism holds true for guinea pigs, rats and humans, even though the relative stimulation kinetics are somewhat different, suggests that the three major postulates of Palade and co-workers [14, 15]: (a) uniform response to secretogogues; (b) parallel intracellular transport; (c) parallel discharge, are indeed a general mechanism of pancreatic processing of exocrine proteins. However, this should not be taken to mean that the model of "selective modulation" of Rothman and co-workers [11—13] is necessarily wrong. The two models could in fact not be mutually exclusive, but could complement each other, for instance in terms of observation time. Our and Palade's data have only been obtained during a short period of observation or hormonal stimulation. However, over a long term, and in response to specific diets, it appears that the pancreas could alter the ratios among the different enzymes in the discharge granules [29, 30]. This could also happen in case of alteration of pancreatic function due to a disease. Further work is needed to elucidate these aspects.

ACKNOWLEDGEMENTS

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CHROMBIO. 666

DETERMINATION OF ETHAMBUTOL IN PLASMA USING SELECTED ION MONITORING

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SUMMARY

The determination of ethambutol in plasma is described. Using ethambutol-d_4 as an internal standard, ethambutol and the internal standard were extracted with chloroform under alkaline conditions, and converted into their trifluoroacetyl derivatives with trifluoroacetic anhydride in benzene—pyridine (4:1). Selected ion monitoring was carried out by monitoring the peaks at *m/z* 294 and 296 corresponding to the fragment ion $[M/2]^+$ of the derivatives. Ethambutol was determined by use of the peak height ratio of the peak at *m/z* 294 against that at *m/z* 296.

The method was utilized successfully for studying the bioavailability and pharmacokinetics of the drug.

INTRODUCTION

An anti-tuberculosis drug, ethambutol dihydrochloride (EMB·2HCl), *d*-2,2′-(ethylenediimino)-di-1-butanol dihydrochloride, has been widely used in the treatment of tuberculosis. The absorption, metabolism and excretion of the drug have been examined in detail by use of ^{14}C-labelled EMB [1—5]. It is well-known that the therapeutic and toxic responses to EMB relate closely to the plasma levels of the drug, and that plasma levels of 3—5 μg/ml are required for treatment. Therefore it might be significant to determine the characteristics of EMB preparations by measuring the drug in plasma.

Several colorimetric methods [6—8] have been reported for the determination of EMB in biological fluids; however, they were not necessarily sufficiently sensitive or selective to apply to plasma samples. Recently an electron-capture gas chromatographic method [9,10] and selected ion monitoring (SIM) using

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the chemical ionization mode [5,11] were presented to this end.

In order to examine the bioavailability of EMB preparations, a more convenient method was required for the routine determination of EMB in plasma. As a result of some modifications of existing methods [5,9—11], we devised a convenient SIM method for the determination of EMB in plasma using EMB-d_4 as an internal standard. This paper describes the method and the pharmacokinetics of EMB in beagle dogs.

EXPERIMENTAL

Chemicals and reagents

EMB·2HCl was of J.P.IX grade (Lederle Labs., New York, NY, U.S.A.). EMB-d_4·2HCl was synthesized from *dl*-2-aminobutanol (reagent grade; Tokyo-Kasei Kogyo, Tokyo, Japan) and 1,2-dibromoethane-d_4 (isotope purity 99%; Merck Sharp & Dohme, Dorval, Canada) by the method of Wilkinson et al. [5,11,14]. Trifluoroacetic anhydride (TFAA) was purchased from Tokyo-Kasei Kogyo. All other chemicals were of reagent grade (Kishida Chemicals, Tokyo, Japan) and used without further purification. A stock EMB solution was prepared by dissolving 13.5 mg of EMB·2HCl in 100 ml of water; it was stored at 5°C protected from the light. Standard samples were prepared by spiking blank plasma with the stock EMB solution at a concentration of 0.1—5 μg of EMB per ml of plasma; these were stored at −20°C until analyzed. An internal standard solution was prepared by dissolving 13.5 mg of EMB-d_4 · 2HCl in 100 ml of water; this was stored at 5°C. A 1 *M* HCl in methanol solution was prepared by diluting concentrated HCl with methanol.

Plasma samples

Plasma samples were taken at seven intervals during 10 h after a single administration of 125 mg or 250 mg of EMB·2HCl (commercial tablet) to four male beagle dogs together with 10 ml of water. Each dog was fasted for 16 h before and for 3 h after the drug administration. Blood was collected in heparinized syringes by venipuncture, and centrifuged in the usual manner to separate the plasma. The plasma samples were stored at −20°C until analyzed.

Analytical procedure

Sample preparation. To plasma samples of 0.5—1.0 ml corresponding to 0.1—5 μg EMB, in a 12-ml glass-stoppered centrifuge tube were added exactly 0.1 ml of the internal standard solution, 0.5 ml of 4 *N* NaOH and 6 ml of chloroform. The tube was shaken vigorously for 10 min and centrifuged at 2000 *g* for 10 min. The aqueous layer was removed by aspiration. To the organic layer were added 3 drops of 1 *M* HCl in methanol, and then the solvent was evaporated to dryness in vacuo. The residue was dissolved with 50 μl of benzene—pyridine (4:1), and reacted with 50 μl of TFAA for 2 h at room temperature to give sample solutions. The sample solutions were analyzed by SIM.

Conditions for selected ion monitoring. A Hitachi gas chromatograph—mass spectrometer equipped with multi-ion monitor, Model RMU-6MG, was used. The column was a 1 m × 3 mm I.D. glass tube packed with 2% OV-17 coated on 80—100 mesh Gas-Chrom Q. The column was kept isothermally at 150°C,

the injection port and the separator were held at 180°C and 250°C, respectively. Helium was used as a carrier gas at a flow-rate of 40 ml/min. The ionization, acceleration and ion multiplier voltage were set at 30 eV, 3.2 kV and 1.8 kV, respectively. The ionization chamber was held at 160°C. Both the exit and collector slits were set at 0.2 mm. The ions at *m/z* 294 and *m/z* 296 were used for monitoring. The recorder attenuation was chosen in the range 0.05—2.0 V in accordance with the concentration of EMB in the sample solutions. The sample size was 1—3 μl.

Calculations. The concentration of EMB in plasma samples was determined from a calibration curve prepared by using the peak height ratio of *m/z* 294 against *m/z* 296. The calibration curve was obtained with the standard samples treated in the same manner as the plasma samples.

Gas chromatography—mass spectrometry

About 5 ml of the stock EMB solution (0.5 mg EMB) or of the internal standard solution (0.5 mg EMB-d_4) were taken into a glass-stoppered tube, and evaporated to dryness in vacuo. The residue was reacted with TFAA in a manner similar to that described in *Sample preparation* to prepare the injection solution for gas chromatography—mass spectrometry. Mass spectra were obtained by electrographical recording under conditions similar to those described for SIM.

RESULTS AND DISCUSSION

Mass spectra

In order to determine the EMB derivative for SIM analysis, we examined both the trifluoroacetyl (TFA) and the trimethylsilyl (TMS) derivatives. Both TFA EMB and TMS EMB eluted as a single, sharp and symmetric peak as reported by several authors [5,9—13]. After examining in detail the comparative merits of both derivatives, we chose TFA EMB in view of its stability. The stability of TFA EMB is superior to that of TMS EMB as suggested by the referees of this journal.

Fig. 1 shows the mass spectra of TFA EMB and TFA EMB-d_4 together with the assignment of the main peaks. The spectra did not involve the molecular ion peaks $[M]^{\cdot+}$ (*m/z* 588 for TFA EMB and *m/z* 592 for TFA EMB-d_4); however, the peaks at *m/z* 570 of TFA EMB and at *m/z* 574 of TFA EMB-d_4 indicated the respective tetratrifluoroacetyl derivatives. The base peak consisted of the peak corresponding to the fragment ion $[M/2]^+$ (*m/z* 294 for TFA EMB and *m/z* 296 for TFA EMB-d_4). In addition, the fragment ion $[M/2]^+$ had neither the cluster peak at *m/z* 296 arising from TFA EMB nor that at *m/z* 294 arising from TFA EMB-d_4. Taking into account the mass spectral feature of the TFA derivatives, the fragment ion $[M/2]^+$ was preferred for the SIM analysis.

Selected ion monitoring profiles

Fig. 2 shows the SIM profiles of EMB in plasma obtained using the analytical procedure. TFA EMB and TFA EMB-d_4 elute at 2.3 min and 2.0 min, respec-

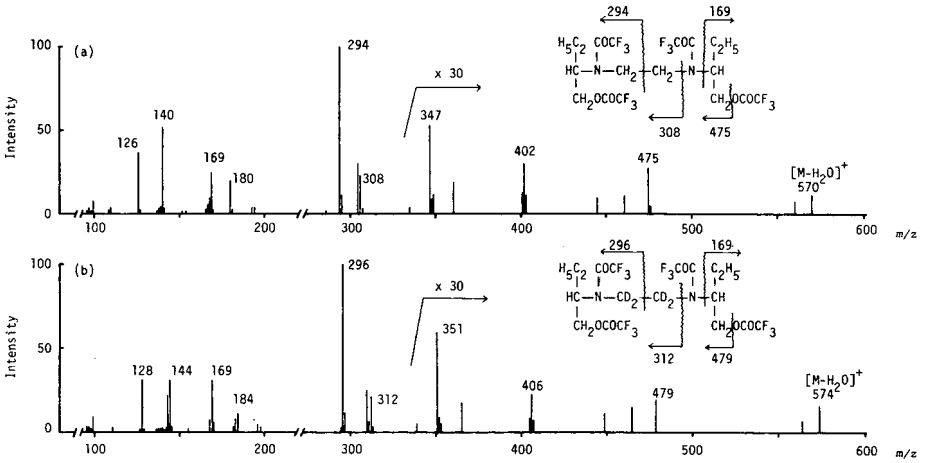

Fig. 1. Mass spectra of TFA derivatives of ethambutol (a) and ethambutol-d_4 (b) obtained by means of GC—EI—MS.

tively, as sharp and symmetric peaks. The difference in their retention times may be due to an isotopic effect.

There were no interferences from plasma components and no overlapping caused by the cluster ions of the monitoring ions (*m/z* 294 and *m/z* 296). The TFA derivatives remained unchanged for several days at room temperature when protected from moisture; the SIM profiles were scarcely changed 3 days after trifluoroacetylation.

Calibration curve and precision

The calibration curve showed good linearity ($r \geqslant 0.998$) between the peak height ratio and EMB concentrations in the range 0.1—5 μg/ml of plasma. The precision decreased somewhat with decreasing EMB concentrations, as seen in Table I; however, EMB could be determined within ± 9% of the coefficient of variation (C.V.) at 0.1 μg/ml of plasma or above. It was considered that such

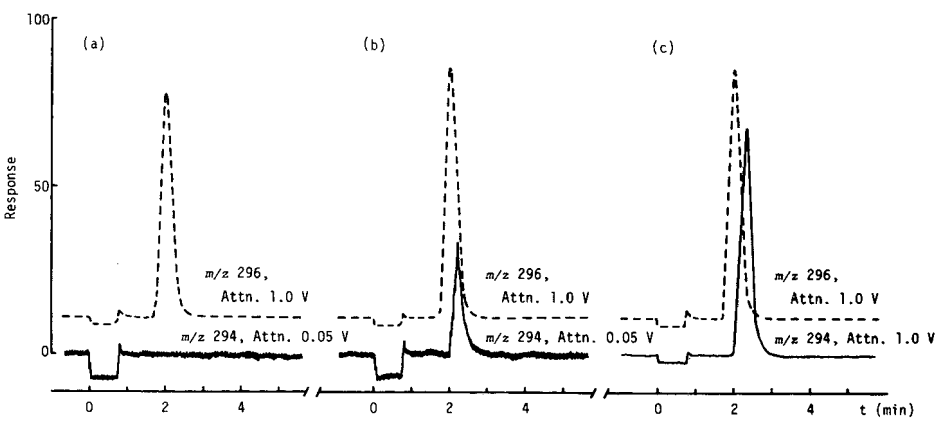

Fig. 2. Selected ion monitoring profiles of blank plasma (a) and standard samples; 0.3 μg ethambutol per ml of plasma (b) and 5.0 μg ethambutol per ml of plasma (c).

TABLE I

PRECISION OF THE SIM METHOD FOR THE DETERMINATION OF ETHAMBUTOL (EMB) IN PLASMA

$n = 7$.

EMB added (μg/ml)	EMB found (μg/ml) Mean ± σ_n	C.V. (%)	Recovery (%)
0.105	0.102 ± 0.0087	8.53	97.1
0.527	0.520 ± 0.0291	5.60	98.7
5.27	5.31 ± 0.116	2.18	100.8

sensitivity and precision would be acceptable for the evaluation of the bioavailability of EMB preparations and the pharmacokinetics of the drug.

The addition of hydrogen chloride to the chloroform extract was necessary to prevent the loss of EMB and the internal standard (EMB-d_4) in the concentration process. When HCl was not added, no quantitative results could be obtained.

On the use of the TMS derivatives

The TMS derivatives of EMB and EMB-d_4 were also applicable to SIM by monitoring the fragment ion $[M—CH_2OSi(CH_3)_3]^+$ (*m/z* 245 of TMS EMB, *m/z* 249 of TMS EMB-d_4) using the same clean-up method and analytical conditions as described in *analytical procedure*. Trimethylsilylation proceeded quantitatively to completion with N-trimethylsilylimidazole in acetonitrile within 10 min at room temperature. The derivatives were stable for 24 h at room temperature; however, they decomposed gradually giving no peaks 3 days after derivatization. We did not use TMS EMB because its stability was inferior to that of TFA EMB.

Application to the bioavailability and pharmacokinetics of EMB

The SIM method was utilized for studying the bioavailability of EMB preparations in dogs. Fig. 3 summarizes the time course of EMB in the plasma of beagle dogs after a single oral administration of 125 mg and/or 250 mg of EMB·2HCl tablets prepared in our laboratory.

There were individual differences in the plasma profiles of EMB, however, the mean values showed good dose-dependence. EMB was absorbed rapidly from the gastrointestinal tract to reach a maximum plasma level at 1 h after oral administration; it was eliminated smoothly, the plasma level becoming one-tenth of the maximum level at 7 h. In addition, the elimination process could be interpreted in terms of a two-compartment open model.

The distribution rate constants, k_{12} and k_{21}, between the central compartment (C_1) and the tissue compartment (C_2), and the elimination rate constant K_{el}, were calculated by computer fitting to give 0.36 h^{-1}, 0.34 h^{-1} and 0.83 h^{-1}, respectively. It was also estimated that EMB in the plasma was distributed

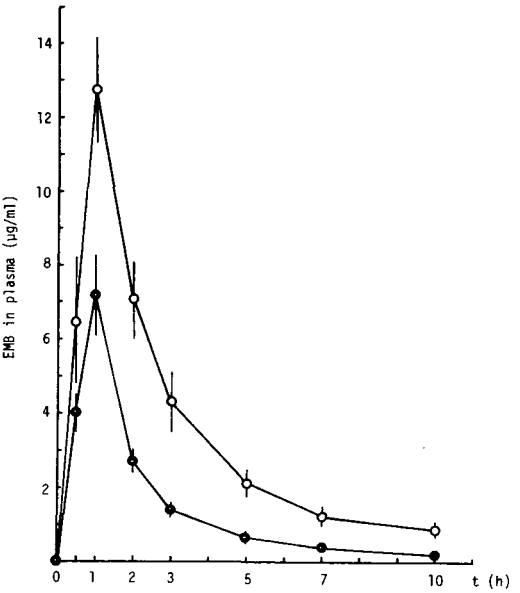

Fig. 3. Time course of ethambutol (EMB) in beagle dogs after the oral administration of 125 mg of EMB·2HCl per dog (•) and of 250 mg of EMB·2HCl per dog (○) in the form of commercial tablets. Each value is the mean ± standard error (n = 4).

into the tissue compartment with a half-life of 0.6 h, and that it was eliminated with a disposition (β) half-life of 3.6 h. It is of interest that the pharmacokinetic pattern of EMB in man [5] is similar to that in dog, and different from that in rhesus monkey [9].

The SIM method was also applicable to urine samples. We believe that this SIM method can be utilized conveniently for the routine analysis of EMB in biological fluids.

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CHROMBIO. 669

DETERMINATION OF PHENELZINE IN HUMAN PLASMA WITH GAS CHROMATOGRAPHY—MASS SPECTROMETRY USING AN ISOTOPE LABELED INTERNAL STANDARD

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SUMMARY

A quantitative gas chromatographic—mass spectrometric assay was developed for the determination of phenelzine in human plasma. Phenelzine, in aqueous solution or in plasma reacts at room temperature with pentafluorobenzaldehyde to form quantitatively a hydrazone derivative. The derivative has good gas chromatographic characteristics. The assay utilizes selected ion monitoring in a gas chromatographic effluent, the molecular ion generated by electron impact ionization of phenelzine derivative. Phenelzine-d_5 was synthesized and used as an internal standard. The assay can measure 2 ng/ml of the drug with about 10% precision.

The method was used for the determination of steady state levels of phenelzine in the plasma of patients taking a therapeutic dose of the drug.

INTRODUCTION

Several aralkyl hydrazines, synthesized by Biel et al. [1], have been shown to be potent central nervous system stimulants. Phenelzine, β-phenylethylhydrazine, is a powerful monoamine oxidase inhibitor [2] and is of considerable value in the treatment of neurotic but not endogenous depression [2, 3]. The drug has been shown to be more effective than amitriptyline in non-endogenous depression [4]. Clinical efficacy and incidence of side effects seem to be related to acetylator status [5—7], obviously acetylation is an important biotransformation pathway for phenelzine.

Phenelzine is readily susceptible to auto-oxidation; decomposition occurs when basified solutions are extracted into organic solvents [8, 9]. Recently Caddy et al. [10] reported a gas chromatographic [GC] method for the analysis of phenelzine in human urine. The analysis is based on the reaction of phenelzine with acetone in basic solution to form phenelzine hydrazone and

the GC quantitation of the resulting hydrazone using a suitable internal standard. The method lacks sensitivity and could not be used for phenelzine assay in the plasma of patients on normal dose of the drug.

This paper describes a gas chromatographic—mass spectrometric (GC—MS) assay of phenelzine in human plasma. Phenelzine in aqueous solution reacts with pentafluorobenzaldehyde to form quantitatively the corresponding hydrazone. The derivative can be extracted with organic solvents from aqueous solutions and has excellent GC and MS characteristics. Selective ion monitoring (SIM), the technique built on combined GC—MS [11,12] was used to develop a sensitive and specific assay for phenelzine in human plasma with site-specific deuterium labeled phenelzine as internal standard. The method was used to measure phenelzine in the plasma of patients receiving 60 mg phenelzine sulphate (30 mg twice daily) for 28 days.

MATERIALS AND METHODS

Reagents

Analytical grade phenelzine sulphate (Warner-Lambert Research Institute, Morris Plains, NJ, U.S.A.), phenylacetic acid-d_7 (Merck, Sharp & Dohme, Montreal, Canada) (96.4 atom % deuterium), lithium aluminium hydride (Pfaltz & Bauer, Flushing, NY, U.S.A.), triphenylphosphine (Aldrich, Milwaukee, WI, U.S.A.), hydrazine monohydrate (64% in water), pentafluorobenzaldehyde (Eastman Organic Chemicals, Rochester, NY, U.S.A.), Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) were used without further purification. All solvents were of analytical grade (Fisher Scientific, Pittsburgh, PA, U.S.A.), silanized tubes (10 ml) with screw caps were used for plasma extraction, final solvent evaporation was performed in 5-ml glass stoppered centrifuge tubes (Kimble, Owens, Illinois, Toledo, OH, U.S.A.). Pasteur pipets with hand drawn constricted tips were utilized for all solution transfers.

All blood samples were collected in ethylenediaminetetraacetic acid using glass free collection, the plasma was frozen and stored till analysis.

Phenelzine-d_7

Phenylacetic acid-d_7 in tetrahydrofuran was reduced with lithium aluminum hydride to give quantitatively β-phenylethanol-d_7 [13]. Equimolar amounts of β-phenylethanol-d_7 and triphenyl phosphine in carbon tetrachloride were heated under reflux for 12 h [14]. The resulting β-phenylethyl chloride-d_7, 65% yield, was chromatographically identical to the authentic unlabeled chloride. The labeled chloride on hydrazinolysis with aqueous hydrazine [1] gave phenelzine-d_7 (Fig. 1). A selected ion detection analysis of phenelzine-d_7 showed the presence of an ion equivalent to 99.1% ± 0.2% (n = 5) phenelzine-d_7 and an ion equivalent to 0.9% ± 0.3% (n = 5) phenelzine-d_0.

Instrumentation

Preliminary GC was performed on a Perkin-Elmer instrument, Model 3920 (Norwalk, CT, U.S.A.) equipped with a silanized 1.8-m column packed with 1% OV-17, maintained at 190°C with a detector temperature of 300°C. The carrier

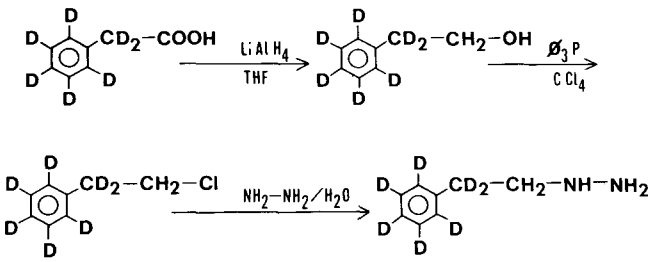

Fig. 1. An outline for the synthesis of phenelzine-d_7.

gas (nitrogen) flow-rate was 30 ml/min. MS was done on LKB-9000 instrument (Stockholm, Sweden) equipped with a multiple ion detector peak matcher accessory (MID/PM) [11, 12]. GC column temperature was 190°C, the flash heater was at 220°C, the separator was at 235°C and the ion source was at 250°C, the helium flow-rate was 20 ml/min.

Derivatization method with pentafluorobenzaldehyde

A 50-µl aliquot of phenelzine stock solution (200 ng/µl in 0.2 *M* aqueous sulphuric acid) was taken in 10-ml centrifuge tubes. To this were added 1 ml of pH 6.7 phosphate buffer and 25 µl of pentafluorobenzaldehyde solution (20 µg/µl in dimethylformamide). The solution was shaken for 5 min, 3 ml of benzene were added and again shaken for 10 min and centrifuged. The organic phase was separated, 50 µl of *n*-tetracosane stock solution (400 ng/µl in benzene) were added and the material was evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was reconstituted in 50 µl of benzene, 1 µl of the solution was injected into the gas chromatograph (Fig. 2A). The electron impact (EI) mass spectrum of the material (Fig. 3) is consistent with the structure of phenylethylhydrazone of pentafluorbenzaldehyde (Fig. 4).

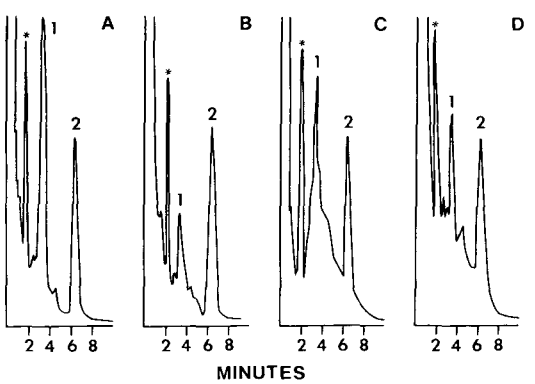

Fig. 2. Gas chromatogram with flame ionization detection of phenelzine derivative (1) with internal standard (2). Phenelzine extracted from (A) water and subsequently derivatized with pentafluorobenzaldehyde; (B) spiked plasma and subsequently derivatized; (C) phenelzine derivatized in spiked plasma and the derivative subsequently extracted; (D) the crude phenelzine derivative extracted and further chromatographed on LH-20 and eluted with benzene. The peak marked with an asterisk arises from excess of pentafluorobenzaldehyde present in the extract.

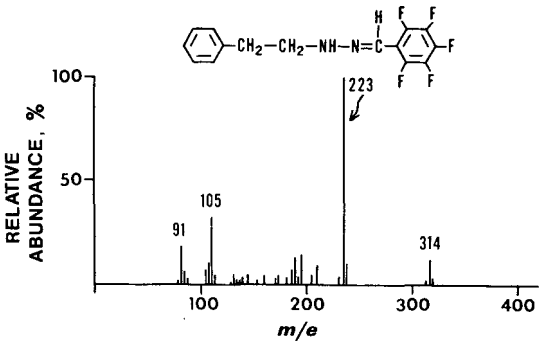

Fig. 3. EI (70 eV) mass spectrum of phenelzine derivative.

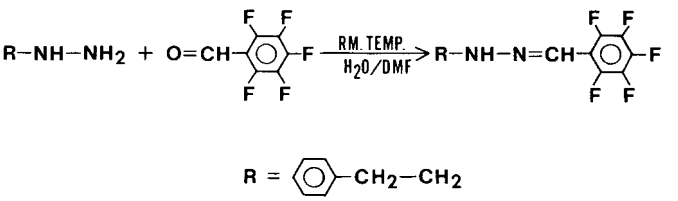

Fig. 4. Derivatization reaction of phenelzine with pentafluorobenzaldehyde.

Extraction and derivatization of phenelzine from plasma

Method A. Drug-free plasma (2 ml) and 50 µl of the phenelzine stock solution were taken in a centrifuge tube. To this were added 2 ml of pH 6.7 phosphate buffer, the solution was thoroughly mixed on a vortex mixer and extracted with 10 ml of benzene—ethyl acetate (4:1, v/v). The organic layer was separated, 1 ml of 0.1 *N* sulphuric acid was added and the mixture was shaken for 15 min. The organic layer was discarded, the aqueous phase was adjusted to pH 6.7 and 25 µl of pentafluorobenzaldehyde solution were added. The material was vigorously shaken, phenelzine derivative was extracted with 3 ml of benzene, 50 µl of the *n*-tetracosane stock solution were added and the material was evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was constituted in 50 µl of benzene, ca. 1 µl of the solution was injected into the gas chromatograph. The gas chromatogram (Fig. 2B) is fairly clean, nevertheless the recovery of phenelzine, compared with the standard (Fig. 2A) is only 20% ± 9% ($n = 4$).

Method B. Drug-free plasma (2 ml) and 50 µl of phenelzine stock solution were taken in a centrifuge tube. To this were added 2 ml of pH 6.7 phosphate buffer, the material was thoroughly mixed and 25 µl of the pentafluorobenzaldehyde solution were added. The solution was vigorously shaken for 15 min and extracted with 10 ml of benzene. The organic layer was separated, 50 µl of the *n*-tetracosane stock solution were added to it and the material was evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was reconstituted in 50 µl of benzene, ca. 1 µl of the solution was injected into the gas chromatograph (Fig. 2C), the chromatogram shows phenelzine overlapping with other extraneous peaks. The crude phenelzine derivative was chromatographed on a 10 cm × 0.5 cm I.D. Sephadex LH-20 column. The

column was eluted with benzene, first 5 ml of the eluate were collected and evaporated to dryness. The residue was reconstituted in 50 μl of benzene, an aliquot (1 μl) of this solution was injected into the gas chromatograph. GC analysis (Fig. 2D) shows phenelzine cleanly separated from contaminants and indicating 65% + 7% (n = 4) recovery of the drug in the spiked plasma.

RESULTS AND DISCUSSION

Extremely poor and variable recovery of phenelzine (in Method A) from spiked plasma is in accord with earlier studies [10] and is attributed to its reported instability. However if it is derivatized first in the plasma and/or aqueous solution, the derivative, unlike native phenelzine, is perfectly stable and can be successfully extracted and purified by conventional techniques (Method B). The mass spectrum of the hydrazone (Fig. 3) shows a molecular ion at m/e 314, base peak at m/e 223 ($M—C_6H_5C^{\cdot}H_2$) and ions of modest intensity at m/e 105 (phenylethyl cation) and at m/e 91 (benzyl cation). The fragmentation pattern depicted in Fig. 5 is readily discernible. The molecular

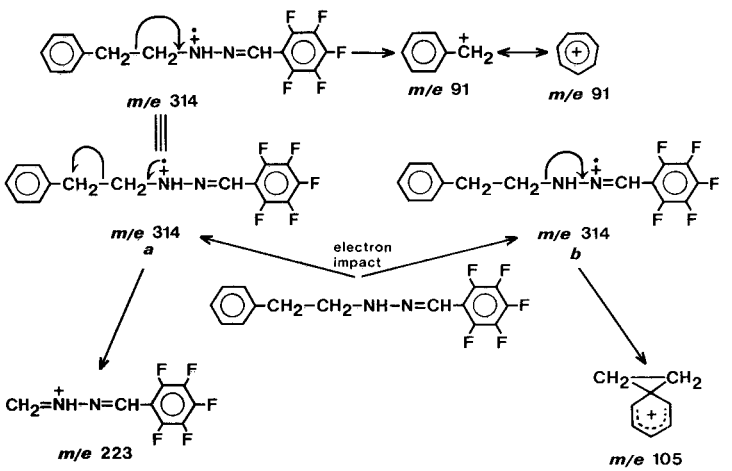

Fig. 5. Proposed fragmentation of phenelzine derivative under electron impact ionization.

ion formed by loss of a lone-pair electron from either nitrogen atom undergoes a typical β-cleavage fragmentation process [15, 16] to give the observed ions. The mass spectrum of phenelzine-d_7 derivative (Fig. 6) shows a molecular ion at m/e 321 and is similar to that of phenelzine derivative. Most ions are shifted to a higher mass by 7 a.m.u., except the ion at m/e 223, which is a common ion from both phenelzine derivative and phenelzine-d_7 derivative.

Selected ion monitoring assay

The ion at m/e 314 is specific for the phenelzine derivative (m/e 321 for phenelzine-d_7 derivative) and is a convenient working mass for SIM assay. Furthermore control human plasma, subjected to the described procedure for phenelzine, showed no significant background ions at m/e 314 and 321. Although the ion intensity at m/e 314 in the spectrum of phenelzine derivative

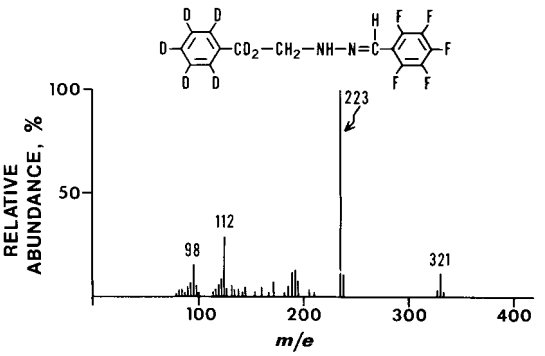

Fig. 6. EI (70 eV) mass spectrum of phenelzine-d_7 derivative.

is 12% of the base peak at 70 eV (Fig. 3), it is increased to 52% of the base peak at 20 eV. Consequently plasma phenelzine extract along with labeled phenelzine-d_7 was processed as described above (Method B), an aliquot of the material was injected into the gas chromatograph—mass spectrometer, phenelzine was quantitated by measuring the ion intensities at *m/e* 314 and 321 respectively. Known amounts of phenelzine along with its isotopic analog in fixed amount, were added to control plasma and processed as described. Phenelzine was quantitated from the ratios of ion intensities at *m/e* 314 and 321. Analysis of the data gave a slope of 0.98 ± 0.01 and an intercept of 0.16 ± 0.2 ng. These data affirm a simple linear relationship between the appropriate ion intensity ratios and concentration of phenelzine and exclude any isotopic exchange or any significant kinetic isotope effect in the fragmentation process.

Recovery and precision

Six samples containing 7 ng/ml of phenelzine were analyzed by Method B using 5.4 ng/ml of phenelzine-d_7 as internal standard. The results for these samples were 6.6 ± 0.34 ng/ml. These samples were assayed in duplicate, in this set exactly the same amounts were taken as above but the internal standard (as hydrazone) was added after the extraction. The recoveries for these samples, based on comparison of the ion intensity ratios of the two sets were 62 ± 7.5%. The wide range of recoveries observed is expected in trace analysis and is attributed to variable glassware, GC column adsorption and to susceptibility of phenelzine to oxidative and thermal decomposition. The sensitivity of the assay, being a function of extraction efficiencies, GC column conditions and the ion source, cannot be quoted in absolute terms. With good mass spectrometer performance, clean and freshly silanized GC column and glassware, and better than 50% recoveries an assay sensitivity of approximately 1—2 ng of phenelzine per ml is possible.

The method described above was used for the analysis of the free drug in the plasma of patients maintained on therapeutic dose of phenelzine. The mass fragmentograms obtained from biological extracts (Fig. 7) are clean and symmetrical peaks. The levels of phenelzine (Table I) in the plasma of 25 patients covered in this study varied from 1 to 10 ng/ml; obviously, most of the drug is either eliminated and/or is extensively metabolised in the biological

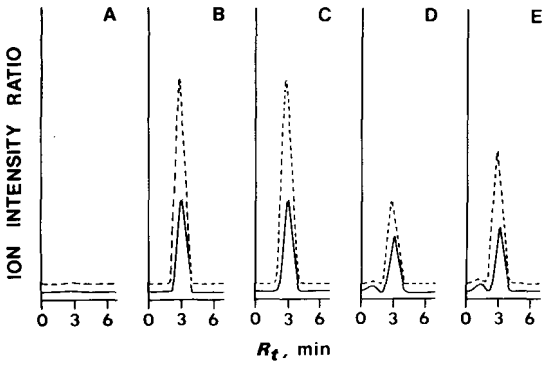

Fig. 7. Selected mass fragmentograms for phenelzine derivative (*m/e* 314) (——) along with phenelzine-d_7 derivative (*m/e* 321) (— — —). (A) Drug-free 2-ml control plasma was processed as in Method B, 2 μl of aliquot (total 10 μl) was injected into the GC—MS system; (B) 2 ml control plasma, spiked with 44 ng/ml of phenelzine-d_7 and 18.3 ng/ml of phenelzine, was processed as in Method B, 1 μl of aliquot (total 25 μl) was injected; (C) 2 ml plasma of a patient, 22 ng/ml of phenelzine-d_7 was added as internal standard and processed as in Method B, 2 μl of aliquot (total 25 μl) was injected and phenelzine concentration was found to be 9.0 ng/ml; (D) 2 ml plasma of a patient, 5.4 ng/ml phenelzine-d_7 was added as an internal standard, and processed as in Method B, 1.5 μl of aliquot (total 15 μl) was injected, phenelzine concentration was found to be 2.9 ng/ml; (E) 2 ml plasma of a patient, 5.4 ng/ml of phenelzine-d_7 was added and processed as in Method B, 1.5 μl of aliquot (total 15 μl) was injected, phenelzine concentration was found to be 2.0 ng/ml.

TABLE I

ANALYSES OF PHENELZINE IN PLASMA OF PATIENTS

Analysis of duplicate runs, using 2 ml of plasma for phenelzine determination; 25 plasma samples were processed as described; 16 samples showed phenelzine concentration 3—6 ng/ml, while 5 samples gave phenelzine concentration 1—2 ng/ml and 4 samples gave phenelzine concentration 9—10 ng/ml.

Subject No.	Phenelzine found (ng/ml)
1	9.6 ± 0.3
2	9.12 ± 0.31
3	5.3 ± 0.29
4	4.9 ± 0.31
5	3.4 ± 0.28
6	1.89 ± 0.2

system. The analytical method described here will permit further detailed assessment of the plasma phenelzine concentration time profiles of the drug and its clinical efficacy.

ACKNOWLEDGEMENT

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ANALYSIS OF PROCARBAZINE AND METABOLITES BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

Twelve compounds representing procarbazine, seven metabolites, and an internal standard were analyzed by gas chromatography—mass spectrometry on a 3% OV-1 column. Procarbazine and four metabolites were derivatized with acetic anhydride.

A sensitive, specific and quantitative assay was established by selected ion monitoring using a synthetic analogue of the drug as an internal standard. The limits of detection were approximately 1 ng/ml of plasma while the limits of quantitation were 10 ng/ml of plasma.

Studies on the degradation of procarbazine · HCl in 0.05 *M* phosphate buffer (pH 7.4) were compared to in vivo studies. At 1 h after incubation of procarbazine · HCl in buffer, the azo and aldehyde metabolites were detected in the highest concentrations representing 27.2% and 20.3% of total drug and metabolites. In the in vivo studies, analyses of rat plasmas indicated that 1 h after an oral dose of procarbazine · HCl, the aldehyde metabolite represented 72% of the total drug and metabolites, and that relatively little of the azo metabolite was present.

INTRODUCTION

Procarbazine · HCl (N-isopropyl-α-(2-methylhydrazino)-*p*-toluamide hydrochloride) is a methylhydrazine derivative which was developed initially as a monoamine oxidase inhibitor [1, 2] and was subsequently shown to be active against a variety of animal tumors. Clinically, it is primarily used as part of the MOPP (mustargen, oncovin, procarbazine, and prednisone) regimen for treatment of Hodgkin's disease and, by itself, is recommended as secondary therapy for treatment of oat cell carcinoma of the lung and primary neoplasms of the brain. Like other cancer chemotherapeutic agents, the clinical use of procarbazine is frequently associated with one or more severe types of toxicity.

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However, the pharmacologic basis for either the toxic or therapeutic effect of the drug has not yet been clearly delineated.

Procarbazine · HCl is stable at room temperature for several years in the dry form if it is not exposed to UV light. In solution or on exposure to UV light, the drug is rapidly converted to numerous by-products. Because the drug is rapidly oxidized in solution and also is metabolized in biological systems, it has been very difficult to determine with specificity, the level of procarbazine and its metabolites in biological tissues after a pharmacologic dose of the drug. In addition, since the drug is also known to be rapidly degraded during assay procedures, it is unlikely that accurate data are currently available on the in vivo levels of the drug and its metabolites.

The recent development of a high-performance liquid chromatographic (HPLC) method for the analysis of eight procarbazine metabolites improved previous assay procedures and permitted their detection in vivo from rat plasma [3]. In this study we have developed a very sensitive and quantitative method based on a derivatization procedure which stabilizes the drug and certain metabolites and allows the detection of procarbazine and seven metabolites in biological fluids using a gas chromatograph—mass spectrometer with a data system.

MATERIALS AND METHODS

Materials

N-Isopropyl-α-(2-methylhydrazino)-*p*-toluamide hydrochloride (procarbazine HCl); N-isopropyl-*p*-formylbenzamide methylhydrazone (hydrazone); N-isopropyl-*p*-formylbenzamide (aldehyde); N-isopropyl-α-(2-methyl-2-azoxy)-*p*-toluamide (azoxy), and N-isopropyl-α-hydroxy-4-toluamide (alcohol) were graciously donated by Hoffmann-LaRoche. N-Isopropyl-*p*-toluamide and N-ethyl-*p*-toluamide were synthesized in this laboratory. All other chemicals used in the syntheses and assay procedure were of certified ACS grade or better or were of the highest purity available.

Synthesis of N-isopropyl-p-toluamide and internal standard

N-Isopropyl-*p*-toluamide was synthesized by a modification of the method of Shriner et al. [4]. A 0.5-ml aliquot of *p*-toluoyl chloride was added dropwise to a solution of 0.5 ml of isopropylamine, 5 ml of dry pyridine and 10 ml of dry benzene. The mixture was heated for 30 min at 60—70°C and then added to 100 ml of water. The organic layer was decanted and the water layer was re-extracted with 10 ml of benzene. The combined organic phases were again extracted with a 5% sodium carbonate solution. The organic phase was dried over magnesium sulfate, filtered, and evaporated until white crystals precipitated. The crystals were then recrystallized from a solution of cyclohexane and ethyl acetate.

N-Ethyl-*p*-toluamide, the internal standard, was prepared from ethylamine and *p*-toluoyl chloride. A commercial 70% aqueous solution of ethylamine was extracted with benzene. The benzene extract was dried over magnesium sulfate and reacted with *p*-toluoyl chloride. Once the dried organic ethylamine solution was obtained, the synthesis of N-ethyl-*p*-toluamide proceeded similarly

to the synthesis of N-isopropyl-*p*-toluamide. For both synthesized compounds, purity was determined by the observance of a single gas chromatographic peak which was characterized by mass spectral analysis.

Extraction and derivatization procedures

Samples to be assayed for drug and metabolites were taken up in 1 ml 0.05 *M* phosphate buffer (pH 7.4) and extracted with 4 ml of toluene by vortex mixing for 1 min. Extraction from buffer was immediately followed by decanting the toluene layer directly into vials containing 0.3 ml of acetic anhydride. However, plasma extracts were centrifuged for 5 min at 2000 *g* to remove remaining solid from the organic phase before decanting. The acetic anhydride reactions were allowed to proceed for 20 min and then air dried. The dried vials were stored at −78° C until the residue was taken up in ethyl acetate for injection into the gas chromatograph.

Gas chromatographic—mass spectrometric assay

A Finnigan 4000 gas chromatograph—mass spectrometer with a 6100 computer data system was utilized for all assays. A 6-ft. glass column with 3% OV-1 on 100—120 mesh Supelcoport packing was employed for all analyses. The following gas chromatographic—mass spectrometric (GC—MS) parameters and conditions were established: injection port temperature, 260° C; oven temperature, 140—270° C at 10° C/min; separator temperature, 265° C; transfer line temperature, 265° C; ionizer temperature, 250° C (electron impact, EI) and 200° C [chemical ionization (CI)—methane and CI—isobutane]; emission current, 4.9 mA; electron multiplier voltage, 1770 V; carrier gas flow-rate, 30 ml/min. Total ion current spectra were obtained in EI, CI—methane, and CI—isobutane. Where possible, peak identification was verified with standards either donated by Hoffmann-LaRoche or synthesized in this laboratory. Once the peak separation, retention time, and identities had been established, all subsequent quantitative work was performed by single ion monitoring (SIM) using CI with isobutane as the reagent gas.

Preparation of standard curves and recovery curves

Recovery curves from rat plasma were established for four procarbazine metabolites (hydrazone, alcohol, aldehyde, and N-isopropyl-*p*-toluamide). Five concentrations ranging from 10 ng/ml to 1 μg/ml were prepared in plasma for each metabolite using N-ethyl-*p*-toluamide as the internal standard. The plasmas were equilibrated for 1 h, extracted and derivatized for injection into the chromatograph. Standard curves were also prepared for the N-isopropyl-*p*-formylbenzamide methylhydrazone and N-isopropyl-*p*-toluamide by direct injection of these compounds in concentrations ranging from 1 ng to 500 ng into the chromatograph along with the internal standard.

Because not all metabolites were available in pure form, several assumptions had to be incorporated into the assay [5]. The levels of procarbazine, azo, and azoxy were all calculated from the hydrazone recovery curve. The levels of N-isopropyl-*p*-ethylbenzamide were calculated from the standard curve established for N-isopropyl-*p*-toluamide.

TABLE I

CHARACTERIZATION OF PROCARBAZINE AND PRODUCTS ISOLATED BY GC–MS

Peak No.	Compound detected	M.W.	Retention time (min)	EI base peak	CI—Methane M ± 1	CI—Methane base peak	CI—Isobutane M ± 1	CI—Isobutane base peak	Drug or metabolite
1	Methylhydrazine (diacetyl)	130	1.65	88	131	89	131	131	methylhydrazine
2	N-Ethyl-*p*-toluamide	163	3.13	119	164	164	164	164	internal Standard
3	N-Isopropyl-*p*-toluamide	177	3.20	119	178	178	178	178	N-isopropyl-*p*-toluamide
4	N-Isopropyl-*p*-ethylbenzamide	191	4.14	133	192	192	192	192	azo
5	N-Isopropyl-*p*-formylbenzamide	191	4.62	133	192	192	192	192	aldehyde
6	N-Isopropyl-α-(2-methylazo)-*p*-toluamide	219	5.70	90	220	177	220	220	azo
7	N-Isopropyl-α-hydroxy-4-toluamide	193	6.15	135	194	194	194	194	alcohol
8	N-Isopropyl-α-hydroxy-4-toluamide (acetyl)	235	6.80	177	236	57	236	236	alcohol
9	N-Isopropyl-α-(2-methyl-2-azoxy)-*p*-toluamide	235	7.79	90	236	236	236	236	azoxy
10	N-Isopropyl-*p*-formylbenzamide methylhydrazone	219	8.60	161	220	220	220	220	azo
11	N-Isopropyl-*p*-formylbenzamide methylhydrazone (acetyl)	261	11.06	73	262	262	262	262	hydrazone
12	N-Isopropyl-α-(2-methylhydrazino)-*p*-toluamide (diacetyl)	305	11.79	191	306	89	306	306	procarbazine

Degradation study in buffer

In order to study the degradation of drug in buffer, 2 mg of procarbazine HCl were dissolved in 100 ml of 0.05 *M* phosphate buffer (pH 7.4) and allowed to incubate with constant stirring for 24 h at room temperature. One-milliliter aliquots were removed at timed intervals for analysis.

Animal sample preparation and extraction

Normal Sprague-Dawley male rats weighing 180—200 g were housed in plastic cages and fed Purina lab chow and water ad libitum before and during experimentation. The rats were given a single 30 mg/kg oral dose of procarbazine · HCl in deionized water. One hour after administration, the rats were decapitated and trunk blood was collected in heparinized vials. The blood was centrifuged at 2000 *g* for 5 min to obtain plasma and 1-ml aliquots were placed in tubes containing the internal standard, N-ethyl-*p*-toluamide. Each tube was extracted with 4 ml of toluene by mixing for 1 min in a vortex mixer.

RESULTS

The GC assay of a mixture of procarbazine and metabolites as displayed by total ion current (TIC) using N-ethyl-*p*-toluamide as the internal standard is given in Fig. 1. Good separation of all peaks was achieved on the Supelcoport

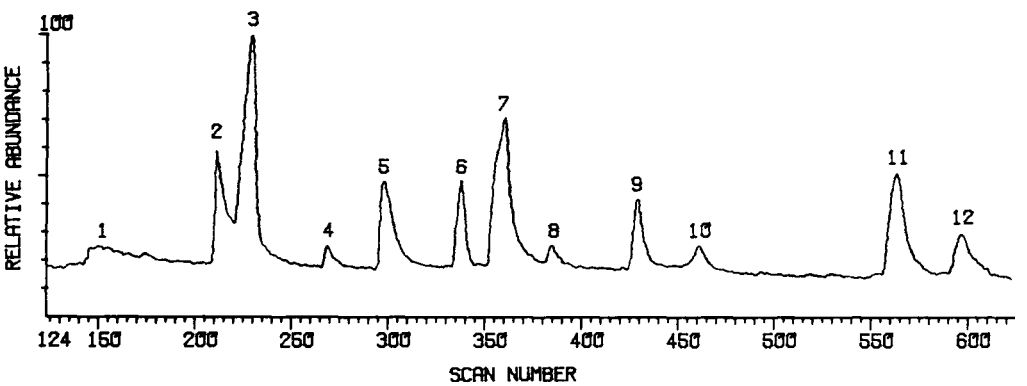

Fig. 1. TIC plot of a mixture of procarbazine, metabolites, and degradation products after derivatization with acetic anhydride. Peak numbers correspond to those identified in Table I.

OV-1 column. Maximum sensitivity for quantitation was achieved by SIM using CI—isobutane by focusing on the base peak for procarbazine and for each metabolite. The data system was programmed to sequentially focus on three sets of ions so that all twelve compounds assayed could be monitored from a single injection. The time interval from elution of the first peak, methylhydrazine, to the elution of the final peak, procarbazine, was 10.4 min. All peaks were initially identified from their EI and CI mass spectra and from their characteristic retention times as compared to that of standard compounds (Table I). Although twelve distinct peaks were eluted from the column, these represented internal standard, drug and only seven metabolites. Direct injection of these products resulted in a sensitivity limit in the picogram range and was linear from 1 ng to at least 500 ng.

TABLE II

DEGRADATION OF PROCARBAZINE · HCl IN 0.05 *M* PHOSPHATE BUFFER (pH 7.4)

Values represent percent of total procarbazine and metabolites analyzed with time in a 1-ml aliquot of a 100 ml 0.05 *M* phosphate buffer (pH 7.4) solution containing 2 mg of procarbazine · HCl.

Time	Procarbazine	Hydrazone	Azo	Azoxy	Alcohol	Aldehyde	N-Isopropyl-*p*-toluamide	Methyl-hydrazine
0 min	58.0	19.8	5.6	N.D.*	N.D.	12.3	4.2	N.D.
5 min	54.5	16.6	9.4	N.D.	N.D.	14.3	5.2	N.D.
45 min	12.0	13.7	26.8	N.D.	14.9	16.5	16.3	N.D.
1 h	8.0	13.6	27.2	N.D.	14.3	20.3	16.4	N.D.
2 h	3.1	11.2	31.2	N.D.	13.4	26.5	14.6	N.D.
4 h	0.6	8.1	25.9	N.D.	10.9	40.3	14.2	N.D.
8 h	N.D.	5.8	23.2	N.D.	6.6	54.0	13.7	N.D.
24 h	N.D.	8.8	14.6	N.D.	6.8	60.4	9.3	N.D.

*N.D. = Not detected.

TABLE III

PER CENT OF PROCARBAZINE AND METABOLITES IN PLASMA

Values obtained from normal male Sprague-Dawley rats decapitated 1 h after a single oral dose (30 mg/kg) of procarbazine · HCl in deionized water.

	Procarbazine	Hydrazone	Azo	Azoxy	Alcohol	Aldehyde	N-Isopropyl-*p*-toluamide	Methyl-hydrazine	Total (μg/ml)
Rat 1	0.2	2.3	4.4	3.3	15.8	72.0	2.1	N.D.*	6.0
Rat 2	0.4	4.5	5.9	2.8	13.4	68.0	4.7	N.D.	7.5

*N.D. = Not detected.

Direct injection of N-isopropyl-α-(2-methylazo)-*p*-toluamide (peak 6) into the gas chromatograph resulted in the elution of two additional peaks (peaks 4 and 10). These two peaks apparently resulted from on-column isomerization and degradation of the N-isopropyl-α-(2-methylazo)-*p*-toluamide. Therefore, the total azo metabolite was estimated by including these two peaks in the azo pool. Since the alcohol metabolite was only partially derivatized (peaks 7 and 8) these two peaks were also added together to determine total alcohol metabolites. All other peaks represent compounds that were stable under the conditions of the assay procedure.

For the purpose of comparison with the in vivo studies, the levels of drug and degradation products were determined after a 1-h incubation period of procarbazine in 0.05 *M* phosphate buffer (pH 7.4) (Table II). The major degradation products were the azo and aldehyde. Small quantities of unchanged procarbazine were also present as were hydrazone, alcohol, and N-isopropyl-*p*-toluamide. Levels of azoxy and methylhydrazine were not detected.

Normal male Sprague-Dawley rats were given a single oral 30 mg/kg dose of procarbazine · HCl and sacrificed 1 h later (Table III). Of the total drug found in plasma, the aldehyde appeared as the major metabolite constituting 72.0% of the total extractable drug and metabolites. No other single metabolite constituted more than 16% of the total. Only 0.2% of unchanged procarbazine was present in the plasma 1 h after administration of the drug.

In order to gain insight into the accuracy and reproducibility of the assay procedure from the initial extraction to the final quantitation, several aliquots of the same plasma sample from a rat given a 30 mg/kg single oral dose of procarbazine were assayed. In Table IV, the data suggest that for the low concentrations being measured, there is acceptable reproducibility from plasma. This indicates that the assay procedure itself is a viable means of determining levels of procarbazine and metabolites from biological fluids.

DISCUSSION

Several derivatizing processes were attempted in order to prevent the oxidation of procarbazine to the azo and hydrazone products. Trimethylphenylammonium hydroxide, several silylating reagents, and trifluoroacetic anhydride reacted with only one of the hydrazine nitrogens. Acetic anhydride and N-acetylimidazole were able to derivatize both active sites on the nitrogens of the hydrazine moiety of procarbazine although N-acetylimidazole produced only a partial acetylation reaction. Acetic anhydride gave a complete conversion of procarbazine to its diacetyl form. It also acetylated the one active site on the nitrogen of the hydrazone. It was necessary to react both nitrogens of the hydrazine in order to distinguish the hydrazine from the hydrazone given the potential ability of the monoacetylhydrazine to oxidize to monoacetylhydrazone. The alcohol with an active alcoholic hydrogen was incompletely acetylated and, therefore, both alcohol and acetylated alcohol were detected. Methylhydrazine, a proposed procarbazine metabolite was acetylated on each of its hydrazine nitrogens to yield a diacetyl product.

The GC—MS assay permitted the detection of procarbazine and seven

TABLE IV

STUDIES ON THE REPRODUCIBILITY OF THE ASSAY PROCEDURE FROM PLASMA

Aliquot	Procarbazine	Hydrazone	Azo	Azoxy	Alcohol	Aldehyde	N-Isopropyl-*p*-toluamide	Methyl-hydrazine	Total (μg/ml)
1	0.4	4.3	4.8	3.8	N.D.	84.0	2.3	N.D.*	4.9
2	0.2	2.3	4.4	3.3	15.8	72.0	2.1	N.D.	6.0
3	0.3	1.3	4.3	3.5	16.0	72.0	2.1	N.D.	6.4
$\overline{X}$ ± S.D.**	0.3 ± 0.1	2.6 ± 1.7	4.5 ± 0.3	3.5 ± 0.3	13.2 ± 2.8	76.0 ± 8.0	2.2 ± 0.1		5.8 ± 0.9

*N.D. = Not detected.

**Mean ($\overline{X}$) = Derived from reported values plus one-half the minimal detectable level (where one value is N.D.).

metabolites from biological systems. Although some variation in sensitivity exists between metabolites, the general limit of sensitivity has been determined to be approximately 1 ng/ml of plasma. The limit of quantitation, based upon linearity of standard curves, is 10 ng/ml of plasma. The HPLC method for the detection of procarbazine and its metabolites as described by Burce and Boehlert [6] reported a sensitivity limit of 20 ng and a limit of quanitation of 200 ng per injection. Because the maximal human dose range of procarbazine is six times lower per kg than the relatively low animal dosage (30 mg/kg) used in the present studies, this increase in sensitivity of the GC—MS method may be crucial for clinical studies with this drug.

The retention time of the final peak of the GC—MS assay was 11.8 min. The HPLC assay of Burce and Boehlert [6] which separated procarbazine and three metabolites had a final peak retention time of 14.2 min. The HPLC assay established by Weinkam and Shiba [3] which separated eight procarbazine metabolites required 90 min for the elution of the final peak. Such long retention times could encourage on-column degradation of procarbazine and metabolites.

Eight percent of the total drug and metabolites present in the buffered solution at the end of 1 h existed as unchanged procarbazine, but the percentage of unchanged drug present in the blood of rats given a single dose of procarbazine at an equivalent period of time ranged from only 0.2 to 0.4%. The hydrazone metabolite was present at 13.6% in the buffer study whereas it was approximately one-fourth lower in plasma at an equivalent period of time. The azoxy metabolite did not form by degradation in buffer but was found in low but significant levels in blood. The majority of the drug was present as the azo metabolite in the in vitro buffer study and as the aldehyde metabolite in plasma.

A very slow conversion of the aldehyde to N-isopropylterphthalamic acid has also been suggested [3]. However, under the conditions employed in the assay procedure used in our study, this metabolite was not detected. Methylhydrazine, a proposed metabolite of procarbazine, was not detected in either the buffer or the in vivo studies by the method used in this work.

As was expected, the in vivo breakdown and metabolism of the drug and its metabolites was even more rapid than the degradation of the drug in buffer. It is possible that the more rapid conversion of procarbazine to its metabolites in vivo was due to microsomal metabolism [3, 7—9]. Since the level of the azo metabolite in blood was markedly lower than that noted in buffer at an equivalent period of time, it would seem likely that this metabolite is a major substrate for enzymatic metabolism.

The GC—MS assay described in this study provides a sensitive, rapid analysis of procarbazine and its major metabolites. The data obtained from the animal studies supports the viability of this assay procedure for the determination of procarbazine and metabolites in biological systems.

ACKNOWLEDGEMENTS

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benzamide methylhydrazone, N-isopropyl-α-(2-methyl-2-azoxy)-*p*-toluamide, N-isopropyl-α-hydroxy-4-toluamide, and N-isopropylterphthalamic acid. This work was supported in part by grants from the W.W. Smith Foundation and the Jean Ann Leslie Memorial Fund.

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CHROMBIO. 677

GAS CHROMATOGRAPHIC ANALYSIS OF THERAPEUTIC CONCENTRATIONS OF MAPROTILINE IN SERUM, USING FLAME-IONIZATION DETECTION

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SUMMARY

For the measurement of the tetracyclic antidepressant maprotiline in human serum, a gas chromatographic method with flame-ionization detection has been developed. The assay specifications obtained are as follows: a precision (C.V.) of 3.5—6.4%, and a relative recovery of 97—109% using amitriptyline as internal standard. The sensitivity of the assay from serum was 40 nmol/l. The applicability of the method has been shown by measuring steady-state serum levels of five inpatients. The steady-state serum levels of maprotiline given at a daily dosage of 75 mg varied from 272 to 570 nmol/l.

INTRODUCTION

Numerous studies have shown that patients receiving the same doses of tricyclic antidepressant drugs exhibit a wide (10—30-fold) inter-individual variation in steady-state plasma or serum concentrations [1—3]. The correlation between the plasma levels of some tricyclic antidepressants and their therapeutic effects found in some [4—7] but not in all [8,9] studies suggests that measurement of antidepressant plasma concentration may provide valuable information for improving clinical management of depressed patients.

Maprotiline is a new antidepressant drug. Its structure differs from conventional tricyclic antidepressants in that it has a hexagonal central ring in the molecule with an ethylene bridge (Fig. 1). Its pharmacological effects have been reviewed recently by Pinder et al. [10]. In the review article of Scoggins et al. [11] there are 138 references describing various methods for the measurement of antidepressant concentration in biological fluids. In only one of these was there an assay technique for maprotiline [12]. In the method described, maprotiline was converted into the heptafluorobutyramide derivative after

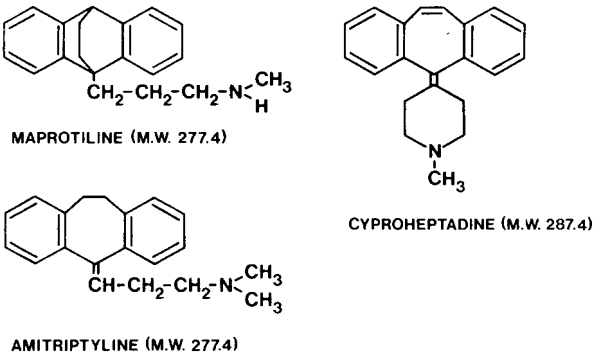

Fig. 1. Structures of maprotiline, cyproheptadine and amitriptyline. Amitriptyline and cyproheptadine were used as internal standards.

separation from biological fluid. The derivative was determined quantitatively by gas—liquid chromatography using an electron-capture detector. Riess [13] used the double radio-isotope derivative technique for the assay of maprotiline.

While mass fragmentographic and gas chromatographic determinations using a nitrogen detector are optimal for the determination of many drugs at low concentrations, the equipment is not available in all laboratories. The method described here is designed for those laboratories that do not have access to more complex and expensive equipment, such as gas—liquid chromatography with a nitrogen detector or high-performance liquid chromatography. Thus we report here a relatively simple gas chromatographic procedure for direct determination of underivatized maprotiline in human serum using flame-ionization detection.

MATERIALS AND METHODS

Reagents

The following reagents were used. Petroleum ether (b.p. 40—60°C, redistilled), isoamyl alcohol and toluene were obtained from E. Merck (Darmstadt, G.F.R.). *n*-Heptane was from J.T. Baker (Deventer, The Netherlands), and diethylamine from Fluka (Buchs, Switzerland). All reagents were of analytical grade. The antidepressant drugs used for calibration were donated by the manufacturers and were of Ph.Nord. grade.

Glassware

All extraction tubes were acid-washed and finally rinsed with distilled water in a dish-washing machine. The small conical test-tubes in the final step were of a disposable type made from new Pasteur pipettes. The extraction tubes were equipped with PTFE-lined screw-caps.

Internal standards

For determination of maprotiline, an aqueous solution of amitriptyline hydrochloride was used as an internal standard. For determination of other

antidepressants, cyproheptadine was used as internal standard.

Amitriptyline hydrochloride was purchased from Oy Star Ab (Tampere, Finland). A stock internal standard, 1 m*M*, was prepared in distilled water; it was shown to be stable for at least a month at +4°C. A working internal standard, 30 μM, was prepared by diluting the stock solution in water.

EXTRACTION PROCEDURE

To 4 ml of serum, 0.1 ml of 30 μM amitriptyline standard was added in a 15-ml PTFE-lined screw-capped tube. The solution was made alkaline with 0.2 ml of 2 *M* sodium hydroxide solution and extracted with 7 ml of *n*-hexane—isoamyl alcohol (100:3) by a rotation mixer for 15 min, followed by centrifugation for 5 min at 1500 *g*. The organic phase was transferred to a clean 10-ml screw-capped tube, then 2 ml of 0.1 *M* hydrochloric acid solution was added and the solution mixed for 15 min. After centrifugation of 5 min at 1500 *g*, the acid phase was transferred to a 5-ml conical test-tube. The aqueous phase was made alkaline with 0.2 ml of 2 *M* sodium hydroxide solution and mixed with 0.6 ml of *n*-hexane—isoamyl alcohol. After phase separation, the organic phase was transferred to a small tube made from a Pasteur pipette and evaporated to dryness using a KOH-containing vacuum desiccator. The residue was redissolved in 10 μl of *n*-heptane—toluene—isoamyl alcohol—diethylamine (80:20:1.5:1), and 1 μl was injected into the gas chromatograph.

Chromatography

The gas chromatograph used was a Varian Model 2100 with a flame-ionization detector. The glass column, 1.80 m × 2 mm I.D., was silanized and packed with 1.4% Carbowax 20M plus 1.4% KOH on Gas-Chrom Q (60—80 mesh). The column temperature was 210°C. The temperatures in the injector and detector were 250°C. The flow-rate for the carrier gas (nitrogen) was set at 30 ml/min; the hydrogen flow-rate was 30 ml/min; the air flow-rate was 300 ml/min.

The peak areas of the drugs were recorded using a Hewlett-Packard integrator 3380S, which also printed out retention times.

Quantitation

Standard samples containing known amounts of the substances to be determined were included in each determination series. The concentrations in the unknown samples were calculated by comparing peak areas to those of the internal standards. All standards and samples were run in duplicate.

Blood samples

Blood samples were obtained from patients undergoing chronic maprotiline therapy. The samples were drawn between 7 and 8 a.m., and maprotiline (75 mg) was given between 7 and 8 p.m. The sera were stored at +4°C until analyzed. The analyses were performed within 24 h.

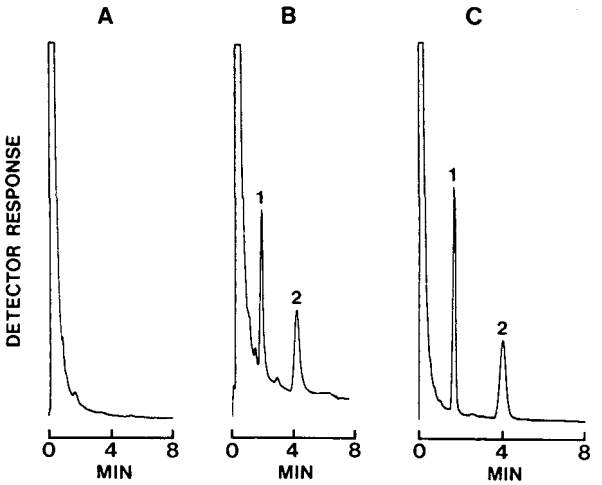

Fig. 2. (A) Chromatogram obtained from an extract of 4 ml of blank serum. (B) Chromatogram obtained from an extract of 4 ml of human serum containing amitriptyline (1) as internal standard and maprotiline (2). (C) The same as (B), but extraction made from water. Amitriptyline and maprotiline peaks correspond to 750 n*M* and 500 n*M*, respectively.

RESULTS

In Fig. 2, chromatograms obtained from an extract of 4 ml of blank human serum (A) are shown, as well as extracts from serum (B) and water (C), spiked with internal standard (amitriptyline) and maprotiline 500 n*M*.

There was a linear correlation between maprotiline serum concentrations and the area ratio between the drug and internal standard over the range measured, 100—1500 n*M*. The calibration curve for maprotiline had a slope of 0.714, an ordinate intercept of −0.048, and a correlation coefficient of 0.999. The lower limit of sensitivity of the assay was 40 n*M* of serum.

Within-run precision (Table I) was determined on eight replicate extractions of 100, 500 and 1000 n*M* maprotiline in serum. The precision of determination [expressed as coefficient of variation (C.V.) %] was between 3.5 and 6.4. In the between-run precision studies, C.V. % were 5.8 and 4.8, respectively, in the pools 500 n*M* and 1000 n*M*.

In the accuracy studies, the recovery of maprotiline was determined. The absolute recovery of maprotiline from serum was measured in the following way. Known amounts of maprotiline were added to blank serum samples, and the extraction procedure was performed without internal standard, which was not added until just before the last evaporation. As shown in Table II, absolute analytical recoveries of the drug ranged from 55 to 71%. Table II shows that the relative recoveries at therapeutic concentrations varied between 97 and 109%. These results confirm that amitriptyline is a suitable internal standard for maprotiline determination in this assay system.

The mean morning serum concentration of five inpatients receiving maprotiline 75 mg on the previous evening was 107 μg/l (385 n*M*). There were inter-individual variations from 75 to 158 μg/l (272—570 n*M*) (Table III).

In Table IV and Fig. 3 we have shown that the method described can be used

TABLE I

PRECISION OF ASSAY OF MAPROTILINE IN HUMAN SERUM

	100 n*M*	500 n*M*	1000 n*M*
Within-run			
n	8	8	8
$\bar{x}$	109	484	1007
S.D.	3.8	22.9	64.0
C.V. (%)	3.5	4.7	6.4
Between-run		Pool A	Pool B
n		6	6
$\bar{x}$		480	986
S.D.		28.1	47.6
C.V. (%)		5.8	4.8

TABLE II

ACCURACY OF THE ASSAY OF MAPROTILINE

n	Amount added (n*M*)	Relative recovery (%)	Absolute recovery (%)
8	100	109	55
8	500	97	70
8	1000	101	71

TABLE III

PLASMA LEVELS OF MAPROTILINE IN FIVE PATIENTS

Patient			Concentration		Maprotiline dose (mg)	Other drugs	Dose
No.	Sex	Age	n*M*	μg/l			
1	F	28	337	93	75	Thioridazine hydrochloride	2× 25 mg + 1× 50 mg
2	M	61	570	158	75	Thioridazine hydrochloride	3× 100 mg
3	F	71	389	108	75	—	
4	F	53	356	99	75	Thioridazine hydrochloride	1× 100 mg
5	M	63	272	75	75	Melperone hydrochloride	2× 25 mg + 1× 100 mg
						Oxazepam	2× 15 mg
						Lactulose	1× 15 mg

TABLE IV

RELATIVE RETENTION TIMES OF SOME ANTIDEPRESSANTS

Drug	Relative to cyproheptadine	Relative to amitriptyline
Trimipramine	0.45	0.99
Amitriptyline	0.46	1.00
Imipramine	0.54	1.22
Mianserine	0.68	1.52
Nortriptyline	0.69	1.53
Desipramine	0.81	1.83
Protriptyline	0.92	2.06
Maprotiline	1.07	2.20
Clomipramine	1.13	2.52

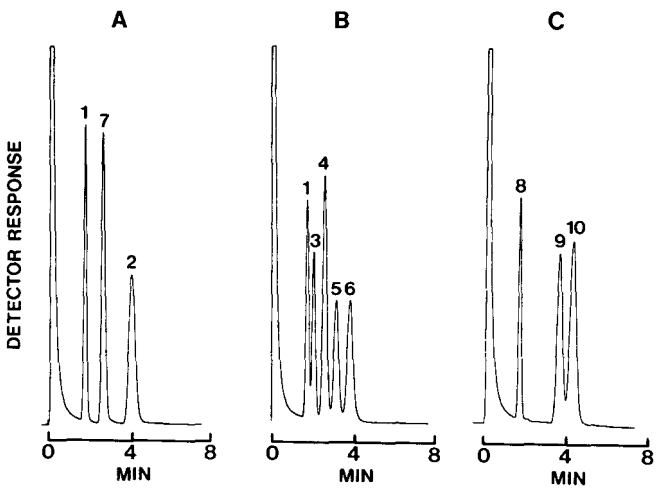

Fig. 3. (A) Chromatogram obtained from the extract of amitriptyline (1), mianserine (7), and maprotiline (2) in aqueous solution. (B) Chromatogram from aqueous extract of amitriptyline (1), imipramine (3), nortriptyline (4), desipramine (5) and cyproheptadine (6). (C) Chromatogram from an extract of trimipramine (8), protriptyline (9) and clomipramine (10) in aqueous solution.

for identification of the main antidepressants. Well-resolved symmetrical peaks were obtained. In the columns packed with supports with Carbowax + KOH as liquid phase it is typical that even after the stabilization of the column the retention times will eventually become shorter. Instead of this the relative retention times remained stabile. Because cyproheptadine is structurally related to tricyclic antidepressants (Fig. 1) and is a compound rarely administered to psychiatric patients, we selected it for the internal standard of the assay.

DISCUSSION

There are two reasons why we consider the determination of maprotiline in the serum of patients to be valuable. (1) There is a growing awareness among

clinical pharmacologists and clinical chemists that the serum levels of antidepressant medication may be important in controlling clinical efficacy, monitoring compliance and preventing side-effects [14]. (2) The potential risk of accidental and suicidal maprotiline overdoses seems to be similar to that of tricyclic antidepressants [10].

In the procedure presented, accuracy and precision values are sufficient for most clinical purposes and are comparable to previously reported values, using gas–liquid chromatography, of tricyclic antidepressants [11]. In the determination of standard curves, extractions made from serum and water gave an identical peak area ratio relationship. We therefore used aqueous standards in day-to-day precision studies.

The relative retention time between amitriptyline as an internal standard and maprotiline was 2.2. Even 40 n*M* maprotiline could be detected. Thus the method has a sensitivity required for the determination of the serum levels of maprotiline in man, both in therapeutic and in toxic concentrations.

Concentrations measured in the morning from the serum of inpatients after chronic medication of maprotiline given at bedtime agree well with those obtained by Riess using a double radio-isotope derivative technique [13]. In his study, the mean steady-state concentrations for daily dose levels of 50, 100 and 150 mg were 67, 143 and 216 μg/l, respectively. Twofold inter-individual variations in the steady-state serum levels of maprotiline found in the present study are smaller than those described earlier [3] for other antidepressants, but the small number of patients in our material might explain this discrepancy.

The main advantage of our method is the possibility for simultaneous identification and quantitation of the major antidepressants. As can be seen from Table IV, all the commonly prescribed antidepressants can be eluted in the system described. On the other hand, mianserine and nortriptyline, as well as trimipramine and amitriptyline, had the same retention times. Probably not in treatment, but theoretically, these compounds could be combined in an overdose situation. Drugs used by our patients (thioridazine, melperone, oxazepam) did not interfere with maprotiline.

There are some important aspects of the technique which should be taken into consideration: to eliminate interfering peaks in chromatograms glassware has to be thoroughly cleaned, solvents have to be very pure, and plastic sealings have to be tested before analyses are carried out.

To summarize — due to the increasing therapeutic value of maprotiline as an antidepressant, the measurement of its serum concentrations has been regarded as important, and a new, sensitive, specific method for this purpose has been described.

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CHROMBIO. 678

DETERMINATION OF BUFURALOL AND ITS MAJOR METABOLITES IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic method for the determination of bufuralol, a benzofuran analogue, in plasma is described.

The unchanged drug, the major metabolites and an internal standard are extracted from plasma, purified by back-extraction steps and thereafter separated using a reversed-phase liquid chromatographic system. The detection is carried out by means of a fluorescence detector and an UV detector connected in series. The sensitivity of the assay for the unchanged drug and the major metabolite is about 1 ng/ml plasma using a 0.5 ml specimen per analysis and the relative standard deviation of the whole assay lies in the range ± 4—5%.

The procedure was successfully used to determine plasma levels in volunteers following a single oral dose of 40 mg of bufuralol. The results obtained using the new high-performance liquid chromatographic method were compared with those determined by another method which combines gas chromatography with mass fragmentography, and it was found that these two sets of results coincided quite well.

INTRODUCTION

The benzofuran derivative bufuralol, 7-ethyl-α[(*tert.*-butylamino)-methyl]-2-benzofuran methanol·HCl (substance I in Table I), a β-adrenergic blocking agent, is under clinical investigation for the treatment of cardiac arrhythmias, angina pectoris and hypertension. Studies in animals and man have shown the drug to be rapidly metabolized [1]. The major metabolites found in human plasma are the carbinol (II), the ketone (III) and the phenol (IV) (Table I). The carbinol and ketone metabolites have been synthesized and show β-blocking activity comparable to the unchanged drug. Therefore, pharmacokinetic studies in man should include the determination of these two metabolites, as well as of the unchanged drug. A spectrofluorimetric assay following thin-layer chromatography for the determination of bufuralol and the carbinol has been published [2]. More recently the determination of bufuralol and its metabolites in plasma by mass fragmentography and by gas chromatography

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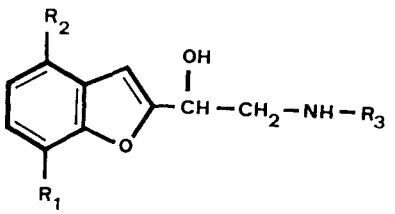
TABLE I

STRUCTURE OF BUFURALOL AND METABOLITES

Substances I—III and the internal standard were used as their racemates.



	R_1	R_2	R_3
Substance I, bufuralol	$CH_2 \cdot CH_3$	H	$C(CH_3)_3$
Substance II, carbinol metabolite	$CHOH \cdot CH_3$	H	$C(CH_3)_3$
Substance III, ketone metabolite	$CO \cdot CH_3$	H	$C(CH_3)_3$
Substance IV, phenol metabolite	$CH_2 \cdot CH_3$	OH	$C(CH_3)_3$
Internal standard (I.S.)	$CH_2 \cdot CH_3$	H	$CH(CH_3)_2$

with electron-capture detection has been described [3]. In the last few years, high-performance liquid chromatography (HPLC) has proved to be a successful method in the analysis of drugs and metabolites in biological fluids. Since the published procedures for the determination of bufuralol and its metabolites demand either a highly specialized equipment or are rather complicated to perform, a new assay using HPLC has been developed.

Bufuralol (I) and the carbinol metabolite (II) show fluorescence in solution, which can be used for their detection in HPLC. The ketone metabolite (III) does not fluoresce, but can be quantitated by monitoring its UV absorbance in the eluent. No reference substance was available for the phenol metabolite (Table I); therefore it was not possible to develop the HPLC method for this substance.

EXPERIMENTAL

Reagents

All reagents were of analytical grade purity. The diethyl ether used for extraction was distilled and stored at 4°C. Methanol for preparation of the mobile phase was of spectro grade quality (Uvasol® ; Merck, Darmstadt, G.F.R.) and distilled prior to use.

Mobile phase for HPLC: 125 ml of an aqueous* 0.02 *M* $(NH_4)_2HPO_4$ solution, (pH adjusted to 9.2 with ammonia) were mixed with 375 ml of methanol. The mobile phase was degassed immediately before use by pulling a vacuum on the reservoir for a few minutes.

*Bidistilled water has to be used.

Standard solutions

Ten milligrams each of bufuralol, the carbinol metabolite, the ketone metabolite and the internal standard (I.S.; Table I) were weighed into separate amberized 10-ml volumetric flasks and dissolved in methanol to give stock solutions containing 1 mg/ml. Starting from these stock solutions separate dilutions, containing 10 μg/ml of substances I, II, and III, or 2.5 μg/ml of the internal standard, were prepared by diluting aliquots of the corresponding stock solutions with methanol. An intermediate mixture containing 1 μg/ml each of bufuralol (I) and carbinol (II) or 2 μg/ml of ketone (III), was made by pipetting 1 ml, or 2 ml, of the diluted standard solutions into a 10-ml amberized volumetric flask and adjusting to volume with methanol. With this mixture (M) and a 2.5 μg/ml internal standard solution, working standards were prepared as given in Table II.

TABLE II

WORKING STANDARDS

Solution No.	Aliquots of solution (ml)		Diluted with methanol to (ml)	Concentration (ng per 40 μl)			
	I.S.	M		Bufuralol (I)	Carbinol (II)	Ketone (III)	Internal standard
1	0.5	5	10	20	20	40	5
2	0.5	2.5	10	10	10	20	5
3	1	2.5	20	5	5	10	5
4	1	1.25	20	2.5	2.5	5	5
5	1	0.5	20	1	1	2	5

Plasma standards

With the diluted stock solutions in methanol containing 10 μg/ml each of substances I, II and III, an intermediate mixed standard solution with water was prepared by pipetting 4 ml of the bufuralol and the carbinol, or 8 ml of the ketone solution, into a 20-ml volumetric flask and adjusting to volume with water. A 2-ml volume of this solution was added to a 20-ml volumetric flask and diluted to volume with blank plasma. Starting from this plasma stock solution further dilutions according to Table III were prepared. The plasma standards were stored in 1—2 ml portions at —20°C.

Instrumentation

The HPLC system consisted of an Altex (Berkeley, CA, U.S.A.) Model 100 controlled reciprocating pump, a Waters (Milford, MA, U.S.A.) U-6K injector, a Uvikon (Kontron, Zürich, Switzerland) LCD 725 UV detector and a Labotron (Kontron) SFM 22 fluorimeter. The two detectors were connected in series directly after the column, first the UV detector and second the fluorimeter. The column (250 mm × 3.2 mm I.D.) was stainless steel and packed with Spherisorb ODS (Phase Separations, Queensferry, Great Britain) 5 μm particle size.

TABLE III

PLASMA STANDARDS

The plasma standards were prepared and stored in 1—2-ml portions at —20°C.

Plasma standard No.	Concentration (ng/ml plasma)		
	Bufuralol (I)	Carbinol (II)	Ketone (III)
1 (stock solution)	200	200	400
2	100	100	200
3	50	50	100
4	25	25	50
5	10	10	20
6	5	5	10*
7	2.5	2.5	5*

* The detection limit of the ketone metabolite is 10 ng/ml of plasma. These concentrations are near or below this limit.

HPLC operating conditions

The solvent flow-rate used was 0.6 ml/min at a pressure of 150 bar. The UV detector was operated at 231 nm, the cell volume was 8 μl, the path length 6 mm, time constant 1. With the sensitivity set at 1×10^{-2} a.u.f.s., 60 ng of the ketone gave nearly a full-scale pen response on a W + W recorder (Kontron) Model 1200 (dual-channel recorder) set at 5 mV. The fluorimeter flow-cell had a volume of 25 μl; the excitation wavelength was 250 nm, the emission wavelength 300 nm. The instrument was operated at high sensitivity. Twenty nanograms of bufuralol, 10 ng of the carbinol metabolite and 20 ng of the internal standard injected in 40 μl gave nearly a full-scale pen response on the W + W recorder Model 1200 set at 100 mV. The chart speed of the dual-channel recorder was 0.5 cm/min. The capacity factors (k') of the substances were bufuralol 4.7, carbinol metabolite 2.0, ketone metabolite 2.9, and internal standard 3.7. k' is defined as $(V_r - V_0)/V_0$, where V_r = retention volume and V_0 = void volume.

Extraction procedure

All glassware used in the assay was acid-washed in potassium dichromate in concentrated sulfuric acid, rinsed with deionized water and ovendried. There was no need of treatment with a siliconizing agent [2,3].

In a 12-ml glass-stoppered centrifuge tube there were added to 0.2 ml plasma (for concentrations below 25 ng/ml, 0.5 ml plasma) 0.2 ml of the following internal standard solution (prepared freshly each day); 200 μl (80 μl for samples with concentrations below 25 ng/ml) of the stock solution in methanol, containing 2.5 μg/ml, were pipetted into a 10-ml volumetric flask and diluted to volume with 2.5% NaOH. After mixing on a Vortex mixer, 5 ml of distilled diethyl ether were added, the tube was stoppered and rotated for 5 min on a rotary tube mixer. After centrifugation at 700 g, 4.5 ml of the organic layer were transferred by a glass pipette to another centrifuge tube, containing 1 ml of 0.1 N HCl. The extraction of the sample was repeated with a second aliquot of diethyl ether as described above. The combined ether extract was rotated for

5 min with the 0.1 *N* HCl. After centrifugation at 700 *g* the organic layer was discarded and 1 ml of diethyl ether added. After short mixing and centrifuging the ether was removed by aspiration. To the aqueous residue 0.2 ml of 1 *N* NaOH and 5 ml of dichloromethane were pipetted. The tube was rotated again for 5 min and centrifuged. The aqueous layer was aspirated and discarded; 4.5 ml of the organic phase were evaporated to dryness at 15—20°C with a vacuum evaporator (Rotavapor, Büchi, Flawil, Switzerland). The residue was dissolved in 100 µl of the mobile phase and an aliquot of 40 µl or 80 µl injected. The sample solutions were stored in a refrigerator. Parallel to the unknown samples, three plasma standards were analysed, according to the expected plasma levels (Table IV).

TABLE IV

AMOUNTS OF PLASMA AND PLASMA STANDARDS TO BE USED FOR THE DETERMINATION OF BUFURALOL

Expected concentration range of bufuralol (ng/ml)	Amount of plasma to be extracted (ml)	Amount of internal standard in 0.2 ml of 2.5% NaOH (ng)	Amount injected (µl)	Plasma standards to be used (No. from Table III)
⩾25	0.2	10	80 40*	2, 3 and 4, if necessary 1 for high concentrations 5, 6, and 7, if necessary 4
<25	0.5	4	80	

*Plasma concentration above 100 ng/ml.

Calculations

The peak heights of the extracted plasma standards were directly proportional to the concentrations.

The plasma levels of the ketone metabolite were determined by calculating the linear regression curve, based on the extracted plasma standards and comparing the peak heights of the unknowns with this calibration curve.

The calculation of bufuralol and the carbinol were performed in two different ways:

(1) Using the peak-height ratio technique (ratio of the peak height of the compound to be determined to the peak height of the internal standard) a calibration curve was calculated as a linear regression with the extracted plasma standards. The unknown plasma levels were determined by interpolation of their peak-height ratio from these calibration curves.

(2) With a Hewlett-Packard data system 3353 connected parallel to the recorder (only the fluorescence measurements were considered), the unknown plasma concentrations were calculated, based on the peak-area ratio technique.

RESULTS AND DISCUSSION

Chromatographic system

The resolution of the chromatographic system was determined daily by injection of 40 µl of the reference mixtures, tabulated in Table I, in accordance

with the concentration range of the unknown plasma samples.

Chromatograms of plasma standards and unknown plasma samples are shown in Figs. 1 and 2. It is obvious that extracts of human blank plasma yielded no interferences from endogenous components in the fluorimetric determination. The UV detection of the ketone metabolite was more critical due to some variation of the baseline. The interpretation of the graphs was sometimes difficult near the limit of detection. Improved sensitivity should be expected with the recently available UV detectors.

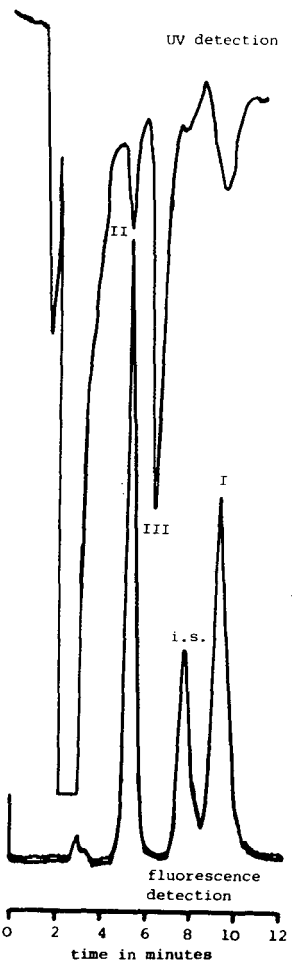


Fig. 1. Chromatograms of spiked plasma standards. Sample volumes of 0.2 ml of plasma were used: 10 ng internal standard in 0.2 ml of 2.5% NaOH were added, and 80 μl of the final solution were injected. Concentrations, in ng/ml plasma, were: bufuralol (I) 100; carbinol metabolite (II) 100; ketone metabolite (III) 200. Column: 250 mm × 3.2 mm, Spherisorb ODS, 5 μm. Mobile phase: methanol—0.02 *M* $(NH_4)_2HPO_4$ (pH 9.2) (75:25, v/v); flow-rate 0.6 ml/min. UV detection at 231 nm; fluorescence detection at 250 nm/300 nm.

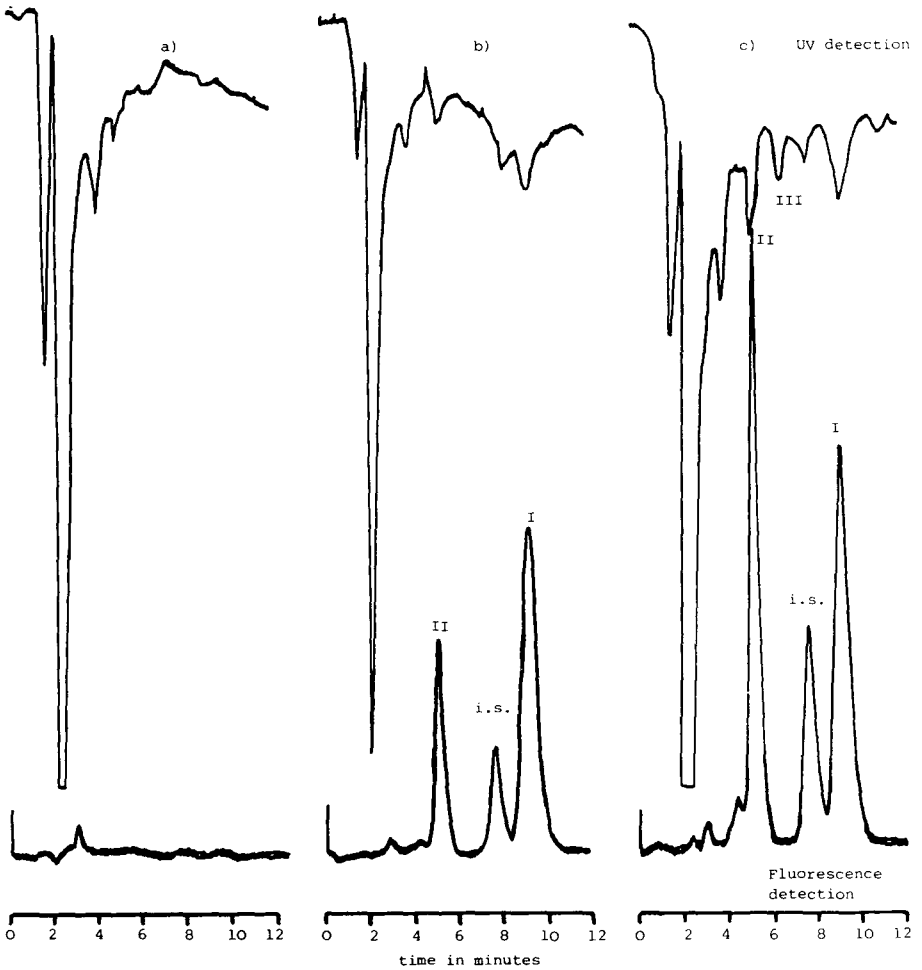


Fig. 2. Chromatograms of plasma extracts of a volunteer, who received an oral dose of 40 mg of bufuralol. Sample volumes of 0.2 ml of plasma were used; 10 ng internal standard in 0.2 ml of 2.5% NaOH were added. (a) Blank plasma, 80 μl injected. (b) 0.75 h after administration, 40 μl injected. (c) 5 h after administration, 80 μl injected. Bufuralol (I), carbinol metabolite (II), ketone metabolite (III), internal standard (i.s.). Test conditions were as given in Fig. 1.

Recovery

With the clean-up procedure described the overall extraction yields were at all concentrations as follows: bufuralol 72% ± 5%, carbinol metabolite 69% ± 6%, ketone metabolite 65% ± 4%, internal standard 68% ± 5%.

Sensitivity

The sensitivity of the HPLC method, using 0.5 ml of plasma, was 1 ng/ml for bufuralol and the carbinol metabolite, and 10 ng/ml for the ketone.

Relative standard deviation

The relative standard deviation of a single determination for the entire procedure, evaluated by analysing the same unknown plasma samples on different days, was found to be about ± 4—5% for bufuralol and the carbinol metabolite down to a concentration of 5 ng/ml. The corresponding relative standard deviation for the ketone metabolite could only be estimated; it was in the range of ± 10—20% at concentrations above 20 ng/ml.

Comparison of the results of the gas chromatography—mass spectrometry method with the HPLC method

In Table V plasma concentrations of the unchanged drug and the carbinol metabolite of a volunteer after a single oral dose of 40 mg of bufuralol determined by gas chromatography—mass spectrometry (GC—MS) and HPLC are compiled. The data show that the two methods give comparable results. Up to now not much information has been collected with respect to the determination of the ketone metabolite. However, it was found that after a single oral dose of 40 mg of bufuralol the concentration of the ketone metabolite is below 50 ng/ml within 24 h after the administration.

TABLE V

COMPARISON OF THE RESULTS OBTAINED WITH THE GC—MS METHOD* AND WITH THE HPLC PROCEDURE

Plasma concentrations are in ng/ml of the unchanged drug and the carbinol metabolite after a single oral dose of 40 mg of bufuralol (volunteer N.G.).

Time after administration (h)	GC—MS method		HPLC method (mean values)	
	Bufuralol	Carbinol metabolite	Bufuralol	Carbinol metabolite
0 (plasma)	<1	<1	<1**	<1**
0.25	23.8	15.9	22.8	15.4
0.50	210	87.7	210	85.6
0.75	210	88.8	200	88.8
1	78.7	55.2	73.2	55.1
1.5	215	99.7	197	100
2	196	103	181	97.8
3	168	101	159	105
4	57.7	57.5	59.6	61.8
5	97.9	100	100	85.7
6	65.7	91.9	64.5	86.2
8	40.2	77.5	33.5	68.4
10	22.2	61.1	—	—
12	14.3	49.5	14.2	42.8
24	2.3	14.9	1.6	12.8

*See ref. 3.

**No interferences.

CONCLUSIONS

The described HPLC method is rapid, sensitive and reproducible for the simultaneous determination of bufuralol and its carbinol metabolite. For pharmacokinetic studies, where large series of samples have to be analysed, this new procedure is easier to perform than the published GC—MS assay.

The ketone metabolite is also detectable, but the sensitivity and the reproducibility are poorer than for the two other substances.

The experience gained with bufuralol shows that, in extracts of plasma, the fluorimetric detection in HPLC is less sensitive to interfering endogenous components than the UV detection. The fluorimetric detection depends on two parameters, the emission and excitation wavelengths, yielding a higher specificity than the UV extinction. Generally, for pharmacokinetic studies of substances with native fluorescence it should be determined whether the fluorimetric detection assay may be more advantageous.

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DETERMINATION OF THE ANTIFUNGAL AGENT, KETOCONAZOLE, IN HUMAN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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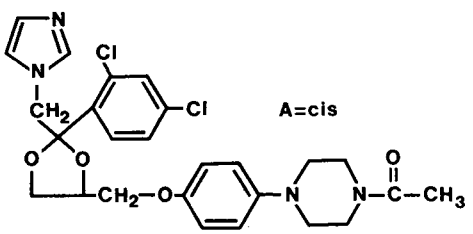
SUMMARY

A rapid and selective high-performance liquid chromatographic (HPLC) assay for the quantitative determination of ketoconazole, an orally active antifungal agent, in human plasma is described. After extraction of the drug from plasma, the compound is separated by HPLC using a reversed-phase column and detected by UV light at 205 nm. Quantitation is accomplished by external standardization and the determination of peak areas is performed with the aid of an integrating computer. The average recovery of ketoconazole over a concentration range of 0.1—20.0 μg/ml was 88.2 ± 4.07% S.D. The maximum sensitivity of the assay is less than 0.1 μg/ml. The assay is suitable for use in pharmacokinetic studies following the administration of therapeutic doses of ketoconazole to humans.

INTRODUCTION

Ketoconazole (I; *cis*-1-acetyl-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazine) (Fig. 1) is an



(I)

Fig. 1. Structure of ketoconazole (I).

orally active broad-spectrum antifungal agent [1] effective in vivo and in vitro against different forms of candidiasis [2], dermatophytosis [3], and coccidioidomycosis [4] in man and other animal species. A microbiological assay [2,4]

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has been employed for the determination of ketoconazole in plasma. The plasma levels, as measured by the bioassay, varied between 1 and 6 μg/ml in man following the oral administration of 200 mg of ketoconazole. The microbiological assay, however, is not specific since it determines the total antifungal activity of the plasma which might also include a contribution due to the presence of active metabolites. This paper describes a new high-performance liquid chromatographic (HPLC) method for the determination of ketoconazole in human plasma which is rapid, selective and suitable for use in pharmacokinetic studies. No interferences from three potential metabolites of ketoconazole nor from miconazole (Monistat I.V.®), which could be administered concomitantly with this drug [5,6], were observed. Although a gas–liquid chromatographic assay has been used for the analysis of biological specimens [7], this represents the first published assay for ketoconazole capable of determining levels of unchanged drug in plasma following the administration of this potent antimycotic imidazole.

EXPERIMENTAL

Apparatus

Analyses were performed on a liquid chromatographic system composed of a Waters Model M6000A pump (Waters Assoc., Milford, MA, U.S.A.) equipped with a LC-55B variable-wavelength UV (205 nm) detector (Perkin-Elmer, Norwalk, CT, U.S.A.). Separations were accomplished at ambient temperature on a 30 cm × 3.9 mm I.D. μBondapak/CN reversed-phase column (Waters Assoc.). Samples were introduced onto the column through a Model U6K septumless injector (Waters Assoc.) with a 25-μl Pressure-Lok syringe (Precision Sampling, Baton Rouge, LA, U.S.A.). Chromatograms were traced on a Linear Model 300 (Linear Instruments, Irvine, CA, U.S.A.) strip-chart recorder (1 mV) and peak area integration was performed by a Hewlett-Packard (Avondale, PA, U.S.A.) 3354B integrating computer interfaced with the detector by employing a Model 18652A (Hewlett-Packard) A/D converter. Analog signal modification to reduce noise from the UV detector to both the strip chart recorder and A/D converter was achieved with the use of a Model 1021A electronic filter and amplifier (Spectrum Scientific, Newark, DE, U.S.A.).

Reagents and solvents

Ketoconazole (I), three potential metabolites of ketoconazole: *cis*-1-acetyl-4-(4-hydroxyphenyl)piperazine (II); *cis*-1-acetyl-4-[4-(1,2-dihydroxyethyl)methoxyphenyl]piperazine (III); and *cis*-1-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxalan-4-yl]methoxy]phenyl]piperazine (IV); three structural analogs of ketoconazole: *cis*-1-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-4-(1-methylethyl)-piperazine trihydrochloride 2-propanolate (V), *cis*-methyl-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]-phenyl]-1-piperazine acetate (VI), *cis*-ethyl-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]-1-piperazine carboxylate dihydrochloride hemihydrate (VII), and miconazole (1-[2,4-dichloro-β-(2,4-dichloro-benzyloxy)phenethyl]imidazole) (VIII), were synthe-

sized and supplied by Janssen Pharmaceutica (Beerse, Belgium). All chemicals and reagents except acetonitrile, methanol, and ethyl acetate (distilled in glass, Burdick & Jackson, Muskegon, MI, U.S.A.) were reagent grade.

Chromatographic conditions

The mobile phase was composed of 0.05 *M* KH_2PO_4—NaOH buffer (pH 6.0)—acetonitrile (65:35, v/v) and the flow-rate adjusted to 2.0 ml/min. The solvent mixture was prepared daily using doubly distilled water and degassed under reduced pressure before use.

Instrument settings

The output voltage (1 V/2 a.u.f.s.) of the LC-55B spectrophotometer was adjusted using a signal attenuator (Perkin Elmer) set at 0.2 a.u.f.s. and the time constant set at the "normal" position. Noise suppression of the spectrophotometric output to both the recorder and the A/D converter was achieved by adjusting the cutoff frequency of the Model 1021A Electronic filter to 0.01 Hz and the gain of the amplifier to ×1. The net output to the recorder (1 mV) under these conditions (i.e. the sensitivity of the detector) was determined to be 0.01 a.u.f.s.

Extraction

An aliquot (2.0 ml) of plasma was transferred to a 15-ml test-tube (15 × 125 mm) fitted with a PTFE-lined screw cap, alkalinized by the addition of ammonium hydroxide (0.25 ml), and then extracted twice with ethyl acetate (5.0 ml) by vigorously shaking the mixture for 30 sec. Separation of the layers was facilitated by centrifugation for 1 min at 1500 *g*. The organic layers were transferred with the aid of a Pasteur pipette and pooled in a clean screw-cap test-tube. Drug was back extracted into sodium chloride-saturated 1 *N* HCl (2.0 ml), the phases separated by centrifugation as before, and the organic layer discarded. The aqueous phase was extracted again with ethyl acetate (5.0 ml) and the organic layer discarded. Final extraction of the drug with ethyl acetate (twice with 5.0 ml) was accomplished after alkalinization of the aqueous phase with ammonium hydroxide (0.5 ml). The phases were separated after each extraction by centrifugation to obtain a clear supernatant, and the organic layers were transferred and pooled in a 20-ml glass vial. An aliquot (1.0 ml) of 1% ammoniacal methanol was added to the ethyl acetate extract before concentrating the sample to dryness under a stream of nitrogen in a water-bath (50°C). The resulting residue was reconstituted and quantitatively transferred with 1% ammoniacal methanol (ca. 2 ml) to a 2.5-ml conical centrifuge tube. The contents of the conical tube were concentrated into the tip by washing the walls of the tube several times with aliquots (ca. 0.2 ml) of 1% amoniacal methanol. The final residue was dissolved in the HPLC mobile phase before separation by HPLC. All samples were analyzed the same day they were extracted.

Extraction efficiency

Blood from several untreated volunteers was drawn into heparinized vacutainers® and centrifuged to generate a plasma pool from which an extraction

efficiency was established using the following procedure. Known amounts of ketoconazole (I), dissolved in methanol (0.01, 0.1 and 1.0 μg/μl), were added to aliquots (2.0 ml) of drug-free plasma, achieving concentrations of 0.1, 0.5, 2.0, 5.0, 10.0 and 20.0 μg/ml. Replicate samples ($n>6$) in each concentration group were thoroughly mixed after fortification with drug, and stored frozen (−20°C) in screw-cap test-tubes for one week until extracted as previously described. All samples at each concentration were analyzed in duplicate. Since the prepared drug concentrations ranged over two orders of magnitude (0.10–20.0 μg/ml), varying volumes of the final residue dissolved in the HPLC mobile phase were injected for analysis by HPLC. Typically, when the drug concentration approached 0.1 μg/ml, one-fifth (20 μl/100 μl) of the dissolved residue volume was analyzed, whereas at a concentration of 20.0 μg/ml only one-four hundredth (5 μl/2000 μl) of the dissolved residue was injected. After separation by HPLC, quantitation of ketoconazole was accomplished by use of the external standard method described below.

Calibration and standard solution preparation

Ketoconazole (25.0 mg) was dissolved in methanol and diluted to volume in a 25-ml volumetric flask to achieve a concentration of 1.0 μg/μl. Solutions containing 0.1 and 0.01 μg/μl of ketoconazole were prepared daily by serial dilution of the 1.0 μg/μl methanolic stock solution with the mobile phase system described earlier. Solutions of compounds II—VIII were prepared by dissolving a quantity (10 mg) of each standard in methanol (10 ml) to achieve a concentration of about 1 μg/μl.

The external standard method, prepared from the software section of the computer, is predicated on a linear relationship between amount injected and peak area (μV-sec as reported by the integrating computer). This relationship was initially evaluated by repeated ($n\geqslant4$) injections of 20, 50, 100, 150 and 200 ng of ketoconazole from the 0.01 μg/μl stock solution prepared with mobile phase. Thereafter, an average calibration response factor was established by the computer following triplicate injections of 100 ng of ketoconazole.

Specificity

Assay specificity was evaluated by injecting aliquots (1 μl) of the prepared methanolic solutions containing either ketoconazole (I), its potential metabolites (II, III, IV), structural derivatives of ketoconazole (V, VI, VII) or miconazole nitrate (VIII) onto the HPLC column to determine their relative separation. In addition, a portion of the extract (20 μl/100 μl) from a drug-free plasma sample was also routinely evaluated at the highest sensitivity (0.01 a.u.f.s.) for the presence of any extractable UV-absorbing (205 nm) material which might interfere with the measurement of ketoconazole.

RESULTS AND DISCUSSION

The average recovery of drug from plasma samples, to which ketoconazole had been added, was determined to be 88.2 ± 4.07% S.D. (Table I) with an average precision (C.V.) of 4.61%. Statistical analysis [8] demonstrated that there were no significant differences ($p > 0.227$) among the mean recoveries

TABLE I

RECOVERY OF KETOCONAZOLE FROM HUMAN PLASMA AT VARIOUS CONCENTRATIONS

Theoretical plasma concentration (μg/ml)	Average observed plasma concentration (μg/ml)	n	Average recovery* (%)
0.1	0.0876	16	87.6 ± 5.06
0.5	0.448	5	89.6 ± 8.52
2.0	1.714	7	85.7 ± 2.89
5.0	4.435	6	88.7 ± 1.24
10.0	8.90	6	89.0 ± 2.13
20.0	17.66	5	88.3 ± 4.57
			$\bar{x}$ 88.2 ± 4.07%**

*$\bar{x}$ ± S.D.
**C.V. = 4.61%.

from each concentration group. These data, therefore, suggest that there is no concentration dependence on extraction efficiency over the range of drug plasma levels which was evaluated.

Linear regression analysis of the curve described by plotting peak area (μV-sec) versus ng injected indicated a linear fit of the data ($p > 0.05$) from 0 to 200 ng with a coefficient of determination (r^2) equal to 0.9975. The slope of the line was calculated to be 1268.5 μV-sec/ng and the intercept, which was not significantly different from zero ($p < 0.05$), determined to be 2962.6 μV-sec. Over a two-month period of analysis, the slope of this line demonstrated little change, with a coefficient of variation equal to 2.6%.

The capacity factors (k') for ketoconazole (I) and compounds II—VIII are listed in Table II. Baseline separation between ketoconazole and the three potential metabolites (compounds II—IV) is achieved within 11 min. Two of the structural analogs (compounds VI and VII) elute with retention times similar to that observed for ketoconazole and one of its potential metabolites (IV) and would not be appropriate as internal standards under the chromatographic conditions described. The third analog, compound V, has a retention time that would require a total analysis time in excess of 20 min. The presence of miconazole (VIII) in a plasma sample at therapeutic concentrations, as a

TABLE II

CAPACITY FACTORS (k') OF KETOCONAZOLE AND COMPOUNDS II—VIII AS DETERMINED BY HPLC

Compound	k'
I	4.18
II	0.85
III	0.53
IV	7.47
V	12.0
VI	4.65
VII	7.23
VIII	10.2

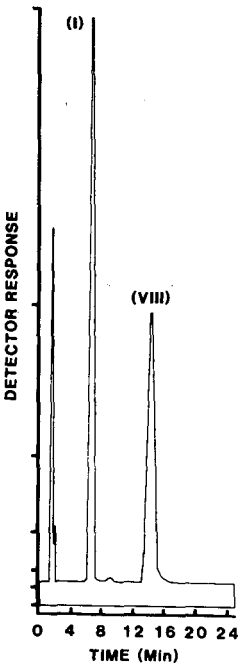

Fig. 2. HPLC chromatogram of ketoconazole (I) and miconazole (VIII). Approximately 1 μg of each compound was injected and detected at 205 nm (0.05 a.u.f.s.).

result of concomitant therapy with Monistat I.V.® [5,6], would not interfere with the measurement of ketoconazole (Fig. 2).

After separation by HPLC, ketoconazole was detected at 205 nm ($\epsilon \approx 24{,}750$ in ethanol). Absolute amounts of 0.01 μg of ketoconazole can be determined at a sensitivity of 0.01 a.u.f.s. However, since there are a large number of potential chromophores such as co-extracted endogenous compounds or other impurities that are active at 205 nm, it is important that the highest quality solvents be utilized for both the extraction of plasma and the preparation of mobile phase. Otherwise, undesirably high detector background might interfere with the measurement of ketoconazole. Ethyl acetate extracts from drug-free plasma were free of interfering UV (205 nm) absorbing peaks (Fig. 3A). On occasion, when a peak with a retention time similar to that of ketoconazole was observed in a drug-free plasma extract, it was found to be equivalent to less than 0.01 μg/ml. A representative chromatogram of extract from plasma containing ketoconazole concentrations of 0.1 μg/ml is shown in Fig. 3B. Samples from quality control pools containing known amounts of ketoconazole have been stored frozen (−20°C) for up to one week without decomposition.

CONCLUSIONS

In summary, an HPLC assay for the determination of ketoconazole in plasma has been developed which is suitable for use in pharmacokinetic studies following the administration of therapeutic doses to humans. The method is rapid and specific for unchanged drug and is capable of measuring plasma levels as low as

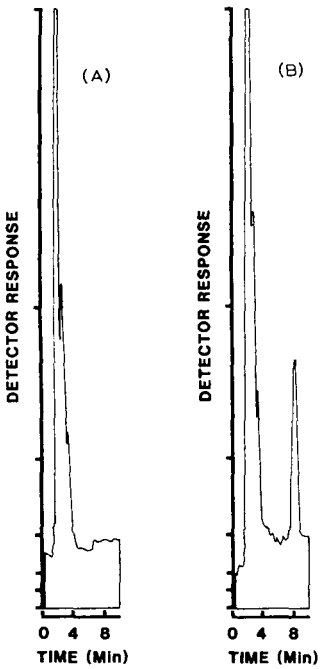

Fig. 3. Representative chromatograms of extracts from (A) drug-free plasma; one-fifth of the total extract volume (100 μl) dissolved in the HPLC mobile phase was injected and analyzed at UV (205 nm); (B) human plasma containing 0.1 μg/ml of ketoconazole. Detector response for both chromatograms is 0.01 a.u.f.s.

0.1 μg/ml. Data from this study have demonstrated that both recovery and detection of ketoconazole are linear over the range of clinically significant concentrations of this drug in human plasma.

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CHROMBIO. 333

DETERMINATION OF PLASMA LEVELS OF TWO AROMATIC RETINOIC ACID ANALOGUES WITH ANTIPSORIATIC ACTIVITY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method is described for the quantitative analysis in plasma of Ro 10-9359, an aromatic retinoic acid analogue, ethyl all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate and its major metabolite Ro 10-1670, all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,3,6,8-nonatetraenoic acid. The compounds are extracted from patient plasma with diethyl ether, separated on a reversed-phase column and detected and quantified by their UV absorption. The experimental error is below 9% in the concentration range 42—445 ng/ml. The detection limit is about 10 ng/ml. The method was applied to the analysis of plasma levels in healthy volunteers, receiving 75 mg orally.

INTRODUCTION

The compound ethyl all-*trans*-9-(4-methoxy-2,3,6-trimethylphenyl)-3,7-dimethyl-2,4,6,8-nonatetraenoate (Ro 10-9359) (Fig. 1) has been shown to have a therapeutic and prophylactic effect against epithelial tumors in mice [1—4]. Later, it has been shown to have an antipsoriatic activity in man [5] and its clinical usefulness for this purpose is currently being investigated. To examine the pharmacokinetics of the compound in man an analytical method with high specificity, sensitivity and capacity is required.

The thermal instability of the retinoids [6, 7] excludes gas chromatographic procedures for the determination of these substances. Previously described methods for the determination of retinol and retinoic acid were based on spectrofluorometry or spectrophotometry [8—11]. However, these methods were not appropriate for the determination of the retinoids in plasma due to

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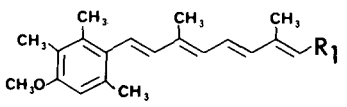
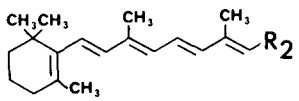


R_1	Compound
$-C(=O)-O-C_2H_5$	Ro 10-9359
$-COOH$	Ro 10-1670
$-C(=O)-O-CH(CH_3)-CH_3$	Ro 11-0249



R_2	
$-CH_2-OH$	Retinol
$-COOH$	Retinoic acid
$-C(=O)-O-NH-C_2H_5$	Ro 8-4968

Fig. 1. Chemical structures of the retinoids (Ro 10-9359, Ro 10-1670 and Ro 11-0249), retinol and retinoic acid.

their low specificity and sensitivity. Liquid—gel partition chromatography [12] could probably solve the problem of specificity and sensitivity but it is a slow and time consuming method. High-performance liquid chromatography (HPLC) has previously been used for the analysis of vitamin A, its acid, its isomers and esters. Liquid—solid adsorption or reversed-phase chromatography [13—23] have been utilized in these cases.

In this paper it was examined whether HPLC could be used also for the simultaneous analysis in plasma of Ro 10-9359 and its major metabolite, the corresponding free acid Ro 10-1670. In the preliminary studies reversed-phase chromatography was found to be most appropriate to the problem and the method was based on this principle. In order to optimize the chromatographic separation the retention properties of the retinoids and some related compounds were studied. The UV absorption and fluorescence characteristics of the drug and its metabolite were also studied to achieve highest specificity and sensitivity of the detection system.

EXPERIMENTAL

Equipment

The HPLC system consisted of a Spectra-Physics Model 3500B high-pressure pump, a Valco sample injector equipped with a 50-μl injection loop and a Laboratory Data Control Spectromonitor II UV detector, operated at 350 nm. The columns used for reversed-phase chromatography were μBondapak C_{18} (300 × 4 mm I.D.) (Waters Assoc., Milford, MA, U.S.A.) and a Hibar LiChrosorb RP-18, particle size 7 μm (250 × 3 mm I.D.) (Merck, Darmstadt, G.F.R.). For liquid—solid adsorption chromatography a Hibar LiChrosorb Si-60, particle size 7 μm (300 × 3 mm I.D.) column was used.

Chemicals

The water was taken from a Milli-Q™ Reagent-Grade Water System (Milli-

pore, Bedford, MA, U.S.A.). Ro 10-9359, Ro 10-1670, retinol and retinoic acid were supplied by Hoffmann-La Roche (Basle, Switzerland). All solvents and chemicals used were of p.a. quality (Merck). Acetonitrile, HPLC-grade, was purchased from Rathburn Chemicals (Walkerburn, Great Britain), and 2,2-di-*tert.*-butyl-*p*-cresol (BHT) from Sigma (St. Lous, MO, U.S.A.).

Solutions

The retinoids easily undergo photoisomerization and oxidation, which makes it necessary to handle and store all substances and solutions in yellow dark-room light. In order to prevent oxidation BHT was added in an amount of about 50 μg/ml [12] to all solutions and all bottles and tubes were flushed with nitrogen when stored and when the plasma samples were extracted.

Solutions of retinol, retinoic acid and the retinoids were prepared by dissolving about 10 mg of the substance in 100 ml of acetonitrile. For the determination of Ro 10-9359 and Ro 10-1670 in plasma, standard plasma samples were prepared by serial dilutions in plasma to give ten concentrations in the range of 5—1000 ng/ml. Solutions of the internal standard, Ro 11-0249, were instable and prepared just before use. All buffer solutions used were prepared from Na_2HPO_4 and NaH_2PO_4 to the desired pH value in a concentration of 0.1 *M*. For routine analysis the mobile phase was a mixture of acetonitrile—water (80:20, v/v) and 1% of acetic acid.

Procedure

Plasma samples were stored under nitrogen at —70°C pending analysis. Into a 15-ml tube, 1 ml of plasma, 3 ml of buffer solution (pH 6) and 40 μl of internal standard solution were introduced. The tubes were flushed with nitrogen and the mixture was extracted with 6 ml of diethyl ether on a reciprocating shaker for 15 min. After centrifugation at 2000 *g* for 10 min, the supernatant was transferred into another 15-ml tube with a conical bottom and evaporated under a stream of nitrogen. The residue was dissolved in 100 μl of acetonitrile and 10—20 μl were injected into the chromatographic system.

A calibration curve was prepared by taking the standard samples through the procedure (Fig. 2). The peak heights were used for the quantitation.

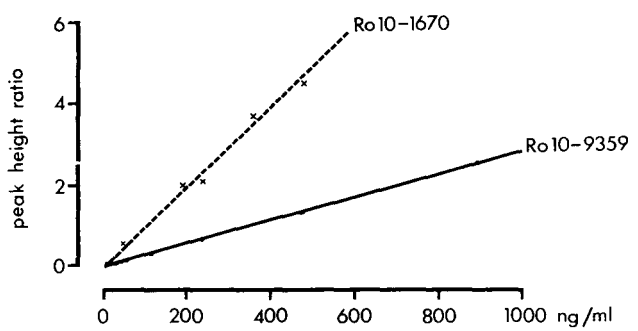


Fig. 2. Standard curves for the quantitation of Ro 10-9359 and Ro 10-1670 in plasma. Peak height ratios of Ro 10-9359 and Ro 10-1670 to Ro 11-0249 versus concentrations of Ro 10-9359 and Ro 10-1670 added to plasma.

RESULTS AND DISCUSSION

Liquid—solid adsorption HPLC systems have been reported to separate isomers and esters of vitamin A with high selectivity [6, 13, 14]. In this study it was found that this approach was successful also for the separation of the retinoids [19]. A typical chromatogram from a plasma extract is shown in Fig. 3A. The separation was achieved using a mixture of hexane—tetrahydrofuran (95:5, v/v). One per cent of acetic acid was used as deactivator of the column as otherwise severe peak tailing occurred with the carboxylic acids. Due to unstable retention times for the most retained peaks after injection of a series of plasma samples, a reversed-phase chromatographic system was studied. The chromatographic properties of the retinoids on a reversed-phase system were investigated as regards the pH and the per cent organic modifier of the mobile phase. First the retention, in terms of capacity factor (k'), was

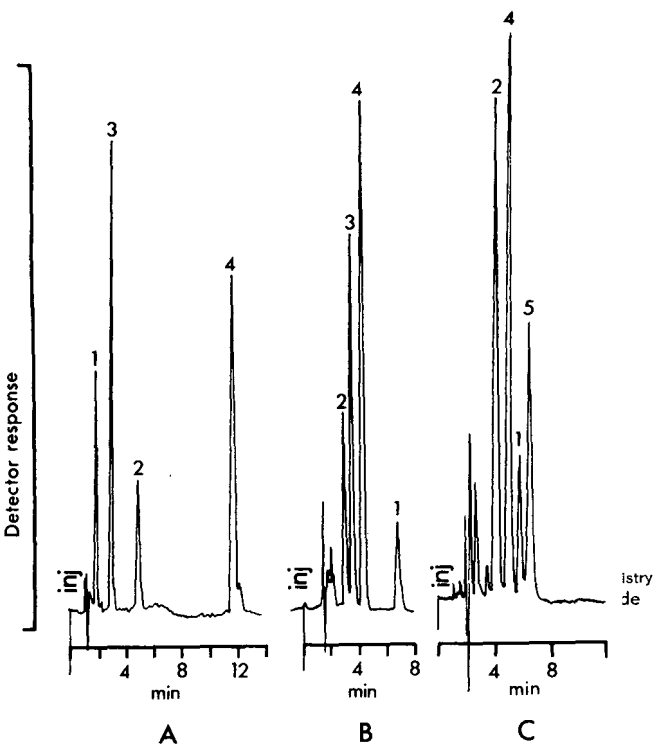


Fig. 3. Chromatograms from the analysis of plasma samples. (A) Liquid—solid adsorption chromatography. Mobile phase: hexane—tetrahydrofuran (95:5, v/v) containing 1% acetic acid. Flow-rate: 1.5 ml/min. Column: Hibar LiChrosorb Si-60. Peaks: 1, Ro 10-9359 (280 ng/ml); 2, Ro 10-1670 (224 ng/ml); 3, retinoic acid (internal standard, 250 ng/ml); 4, endogenous retinol. (B) Reversed-phase chromatography. Mobile phase: methanol—water (90:10, v/v) containing 1% acetic acid. Flow-rate: 2 ml/min. Column: μBondapak C_{18}. Samples are the same as in (A). (C) Reversed-phase chromatography. Column: Hibar LiChrosorb RP-8. Mobile phase: acetonitrile—water (80:20, v/v) containing 1% acetic acid. Flow-rate: 1 ml/min. Plasma sample from a patient 2.5 h after administration of a 75-mg oral dose of Ro 10-9359. Peaks: 1, Ro 10-9359 (174 ng/ml); 2, Ro 10-1670 (112 ng/ml); 4, endogenous retinol; 5, Ro 11-0249 (internal standard, approx. 400 ng/ml).

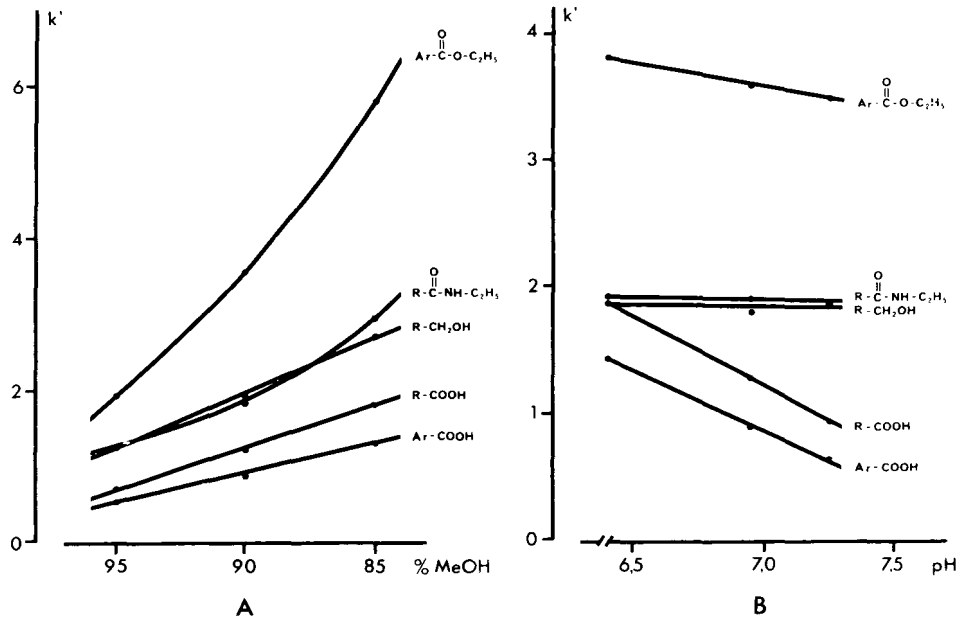


Fig. 4. (A) Effect of concentration of organic modifier on k' values. Mobile phase methanol—water, pH = 7.0. Stationary phase μBondapak C_{18}. (B) Effect of pH of mobile phase on k' values. Mobile phase: methanol—water (90:10, v/v) containing 1% acetic acid, pH = 6.40—7.37. Stationary phase μBondapak C_{18}.

measured for the retinoids as a function of the amount of methanol in the mobile phase at pH 7.0. k' decreased for all substances as the proportion of methanol was increased (Fig. 4A). The influence of pH on the retention at constant methanol—water composition was also studied. As expected the degree of ionization of the carboxylic acids greatly influenced the retention (Fig. 4B). A chromatogram from a plasma sample analysed at pH 7.0 and methanol—water (90:10, v/v) is shown in Fig. 3B. Acetonitrile as organic modifier had a peak-sharpening effect and was used in the routine analysis. A typical chromatogram from the routine analysis, when Ro 11-0249 was used as internal standard, is shown in Fig. 3C.

Ro 11-0249 was found to be the most suitable internal standard to obtain correction for losses during the analytical procedure. However, the internal standard could not eliminate the extraction procedure as a source of error, probably due to exposure of the substances to air and light during the work. The standard deviation was in the range of 7—14% when Ro 10-9359 and Ro 10-1670 were added to drug-free plasma (Table I). The extraction recoveries for Ro 10-9359 and Ro 11-0249 are shown in Table II. The error of the method, determined from 16 samples with concentrations in the range of 42—445 ng/ml, was below 9% (Table III).

The fluorescence properties of the retinoids were investigated for HPLC fluorescence detection, but the relatively weak fluorescence was not preferable to the highly selective and sensitive UV detection obtained at 350 nm. The detection limit in plasma was about 10 ng/ml for Ro 10-9359.

TABLE I

ASSAY REPRODUCIBILITY

Data represent mean ± S.D. from 5 measurements, when Ro 10-9359 and Ro 10-1670 were added to drug-free plasma.

Amount added (ng/ml)		Amount found (ng/ml)	
Ro 10-9359	Ro 10-1670	Ro 10-9359	Ro 10-1670
103	113	107 ± 7.9	117 ± 4.6
206	226	198 ± 21	217 ± 20
310	339	314 ± 45	342 ± 40

TABLE II

EXTRACTION RECOVERIES FROM PLASMA

The internal standard was added after the extraction procedure. Recovery was determined by comparison with a non-extracted methanol standard series. Each figure represents the mean of two observations.

Amount of Ro 10-9359 (ng/ml)			Amount of Ro 11-0249 (ng/ml)		
Added	Found	Recovery (%)	Added	Found	Recovery (%)
35	26	74	36	25	69
70	46	66	72	40	57
104	58	56	89	46	52
174	102	59	143	81	56
348	188	54	268	125	47

TABLE III

EXPERIMENTAL ERROR FOR THE DETERMINATION OF Ro 10-9359 IN PLASMA

Compound I in plasma was determined with duplicate analysis. S.D. = $\sqrt{(2d^2)/2n}$, where d is the difference between duplicate analyses.

Range (ng/ml)	n	mean	S.D.	% of mean
42—445	16	227	19.7	8.7

The time course for the amount of the drug and the major metabolite in plasma was examined after oral administration of 75 mg of Ro 10-9359 to a volunteer (Fig. 5). The peak levels were reached after about 5 h. The plasma levels can readily be detected 24 h after administration which indicates that the method will be appropriate for systematic pharmacokinetic studies in man.

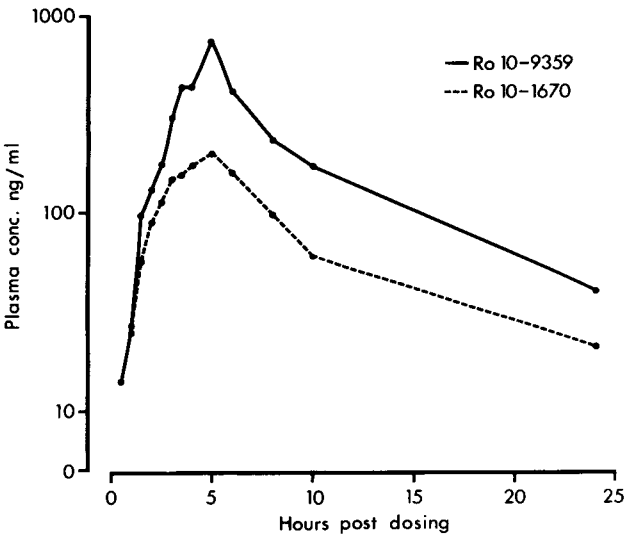


Fig. 5. Plasma levels of Ro 10-9359 and Ro 10-1670 in a patient after oral administration of 75 mg of Ro 10-9359.

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QUANTITATION OF THE ANTICONVULSANT CINROMIDE (3-BROMO-N-ETHYLCINNAMAMIDE) AND ITS MAJOR PLASMA METABOLITES BY THIN-LAYER CHROMATOGRAPHY

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SUMMARY

A quantitative thin-layer chromatography (TLC) procedure is described for the analysis of cinromide (3-bromo-N-ethylcinnamamide) and its two major metabolites, 3-bromocinnamamide and 3-bromocinnamic acid in plasma of the dog. These compounds were recovered from acidified plasma by extraction into benzene with a recovery of 95 ± 5%. All three compounds were quantitated directly on a TLC plate by ultraviolet absorbance densitometry at 270 nm. The linear dynamic range for the quantitation of the compounds on a TLC plate ranged between 10 and 1000 ng. The complete procedure is useful in the working range of 50 ng/ml to 100 μg/ml of plasma with a coefficient of variability of about 10%. Specificity of the method for parent drug and each of its plasma metabolites was confirmed by high-performance liquid chromatography. The method was used to determine the pharmacokinetics of cinromide and its two major plasma metabolites in dogs following a single oral dose of the drug.

INTRODUCTION

Structure activity studies on ring substituted cinnamamides have shown that these compounds have central nervous system stimulant and depressant effects in mice [1]. More recently N-ethyl-3-bromocinnamamide (cinromide) has been shown to have anticonvulsant properties in rodents [2,3] and primates [4] and is currently under clinical evaluation for the treatment of epileptic seizures in patients [5].

As with most anticonvulsant drugs, the basic pharmacokinetics, metabolism and the need for monitoring plasma levels of the drug in patients during clinical testing are important parameters in the development of this type of pharmacologic agent [6]. These factors were important considerations in developing a sensitive but rapid method for measuring cinromide in biological fluids of

animals and man. Since cinnamamides possess strong ultraviolet absorption properties through their conjugated double bond system, cinromide and its two major plasma metabolites can be quantitated by thin-layer spectrodensitometry. The present work describes a quantitative thin-layer chromatography (TLC) method for measuring cinromide and its metabolites, 3-bromocinnamic acid and 3-bromocinnamamide in plasma of animals and man. The procedure is sensitive, specific and rapid enough to accommodate multiple sample analysis. In the present report the procedure was applied to the disposition of cinromide and its metabolites in plasma of dogs.

MATERIALS AND METHODS

Preservative-free chloroform was obtained from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.) and high purity glacial acetic acid was obtained from Aldrich (Milwaukee, WI, U.S.A.). All other solvents were high-performance liquid chromatography (HPLC) grade (Burdick and Jackson Labs.). Silica gel 60 plates (20 × 20 cm, EM Laboratories, Cincinnati, OH, U.S.A.) were scored into 20 × 1 cm channels and the solvent applied mechanically to each channel using 100-μl gas—liquid syringes (Unimetrics, Anaheim, CA, U.S.A.) and an A.I.S. multispotter (Analytical Instrument Specialties, Libertyville, IL, U.S.A.). Rectangular chromatography tanks (23 × 29 cm, Brinkmann Instruments, Westbury, NJ, U.S.A.) lined with Whatman 3-mm chroma-pads (Whatman, Clifton, NJ, U.S.A.) were used for development of the plates. A standard solution for spotting contained 30 μg/ml each of cinromide and 3-bromocinnamamide (BCAM) dissolved in a chloroform–methanol (85:15) solution. A separate standard of 3-bromocinnamic acid (BCA) was prepared at 50 μg/ml in a similar manner. It is important to note that this ratio of chloroform—methanol (85:15) facilitates the competitive binding of the solvent molecules to the silica gel, thereby deactivating the silica at the point of application. This allows the total amount of solute to be chromatographed by preventing its irreversible adsorption to the silica at the origin [7,8].

Animals

Three purebred beagle dogs received cinromide orally in soft gelatin capsules containing 100 mg/kg of cinromide dissolved in Tween 80. Blood was drawn at intervals of 0.5, 1, 2, 4, 8, 12 and 24 h after the dose. Plasma was analyzed for cinromide and its metabolites as described in the text.

TLC procedure

Apparatus. Ultraviolet absorbance measurements were made by scanning the TLC plates with a single beam from a Schoeffel SD 3000 spectrodensitometer (Schoeffel Instruments, Westwood, NJ, U.S.A.) at a wavelength of 270 nm. The total, unfiltered emission from the surface of the plate was determined with a reflectance mode assembly and a Schoeffel SD 300 density computer. Peak areas for sample and reference compounds were simultaneously recorded on a Honeywell Electronic 124 recorder (Honeywell, Minneapolis, MN, U.S.A.) and integrated with an Autolab minigrator (Spectra Physics, Santa Clara, CA, U.S.A.).

Plasma extraction. Following acidification of plasma (1 ml) with 0.5 ml of 1 *N* hydrochloric acid (pH < 2), cinromide and its metabolites (BCAM and BCA) were extracted into 6 ml of benzene by shaking for 10 min. After extraction and centrifugation (1500 *g*) to separate the phases, 100 μl of the benzene layer were spotted directly onto a silica-gel plate as described below. When low amounts (<1.0 μg) of these drugs were present in plasma, an appropriate amount of the organic phase (0.5—2.0 ml) was placed into a disposable glass tube (Kimble, 12 × 75 mm) and evaporated to dryness (N-Evap, Worcester, MA, U.S.A.) under a gentle stream of nitrogen. This residue was redissolved in an appropriate volume of solvent and spotted on a TLC plate as described below. It should be noted that the extraction procedure may be applied equally well to plasma from either rat, dog or man without interference from endogenous substances.

Chromatography. The extraction residue from evaporation was redissolved in 80 μl of chloroform—methanol (85:15) solution and the entire volume drawn into a 100-μl syringe and spotted on a silica-gel plate with the aid of a TLC multispotter (Analytical Instrument Specialties, Anaheim, CA, U.S.A.). During the spotting procedure a gentle stream of warm air was blown across the surface of the plate to increase solvent evaporation. Two standard concentrations of each drug were always processed through the method to verify recovery. In addition, several standards (30—150 ng) were spotted manually on

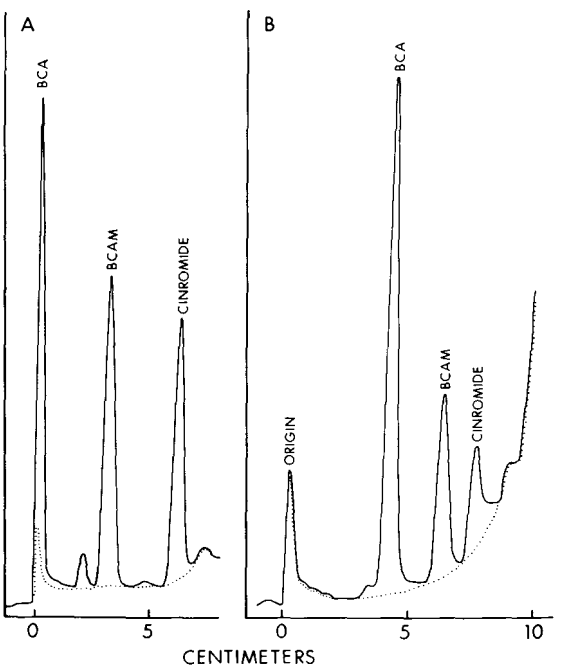

Fig. 1. Thin-layer chromatograms of dog plasma showing cinromide and its metabolites, 3-bromocinnamamide (BCAM) and 3-bromocinnamic acid (BCA), at 1 h (A) and 2 h (B) after an oral dose of 100 mg/kg of cinromide. For the quantitation of cinromide and BCAM (A) the solvent system was ethyl acetate—chloroform—ammonium hydroxide (84:15:1); quantitation of BCA (B) was accomplished in a solvent system of chloroform—methanol (85:15). The dashed line represents the dog plasma blank.

separate channels with a 10-μl Hamilton syringe. Actual chromatograms of cinromide and its metabolites extracted from plasma of a dog following an oral dose (100 mg/kg) of cinromide are shown in Fig. 1. For quantitation of cinromide and its N-dealkylated metabolite BCAM, the plate was developed to 15 cm in ethyl acetate—chloroform—ammonium hydroxide (84:15:1). In this solvent system cinromide and BCAM had R_F values of 0.4 and 0.26, respectively, which provided sufficient separation for quantitation of these two compounds (Fig. 1A). However, since BCA remained at the origin in this system, this metabolite was quantitated separately in a solvent system of chloroform—methanol (85:15) (Fig. 1B).

HPLC procedure

Apparatus. A Waters Assoc. (Milford, MA, U.S.A.) ALC/GPC-204 high-pressure liquid chromatograph equipped with a Model 440 UV detector and M6000A pump was used. A stainless-steel column (25 cm × 3.9 mm I.D.) packed with Partisil PAC (particle size 10 μm) was obtained from Whatman and fitted with a 5-cm precolumn packed with Co:Pell ODS (Whatman). Injections were made through a Waters Model U6K injection system. Peaks were recorded on a 10-mV recorder (Houston Instruments, Austin, TX, U.S.A.) and areas were quantitated with an Autolab System I integrator (Spectra Physics).

Chromatography. The extraction procedure was identical to that described for the TLC procedure. Residues from solvent evaporation were redissolved in 100 μl of chloroform and 50 μl injected onto the column. Analysis of cinromide and its metabolites (BCAM and BCA) were determined at a wavelength of 254 nm in a mobile phase of chloroform—acetic acid (95:5, v/v), at a flow-rate of 60 ml/h at 8.2 MPa (1200 p.s.i.). The column and precolumn were both operated at ambient temperature. A standard stock solution containing 100 μg/ml of cinromide, BCAM and BCA was prepared in the mobile phase and diluted as needed.

RESULTS AND DISCUSSION

Recovery of cinromide and its metabolites

Recoveries of cinromide, BCAM and BCA from human plasma, taken from several analyses over a period of several weeks, are shown in Table I. These compounds were extracted from plasma with recoveries of 96%, 88%, and 100%, respectively. The standard deviation (S.D.) for any individual assay ranged between 5—10% for each of the compounds, reflecting good reproducibility of the procedure. Essentially identical recoveries were obtained from dog plasma. Although the overall recoveries are good and reproducible above 50 ng/ml of plasma, the low polarity of the benzene extraction solvent caused the recovery to decline at low plasma levels so that at 25 ng/ml the recovery was reduced to 25%. The lowest amount of each drug which can be chromatographed and detected on a silica-gel TLC plate was 10 ng, while the upper limit of each compound which could be chromatographed and still retain linearity was about 1 μg.

TABLE I

RECOVERY OF CINROMIDE AND ITS METABOLITES FROM HUMAN PLASMA BY QUANTITATIVE TLC ANALYSIS

Various concentrations of cinromide, 3-bromocinnamamide (BCAM) and 3-bromocinnamic acid (BCA) were added to 1 ml of human plasma and analyzed as indicated in the text.

	Drug added (μg)	Drug found (μg)	S.D. (%)	Recovery (%)	Samples (n)
Cinromide	0.12	0.11	16.4	92	10
	0.24	0.22	6.8	92	9
	0.60	0.57	8.4	95	23
	1.2	1.16	7.1	97	23
	3.0	3.06	10.7	102	11
	6.0	6.01	5.6	100	5
BCAM	0.12	0.10	20.0	83	10
	0.24	0.20	9.0	83	10
	0.60	0.51	9.8	85	26
	1.2	1.00	7.1	83	25
	3.0	2.78	9.4	93	10
	6.0	5.56	4.4	93	11
BCA	1.0	0.95	2.1	95	2
	2.0	2.14	1.9	107	2
	4.0	4.02	15.9	100	4
	5.0	5.18	1.7	104	2
	8.0	8.33	8.8	104	4
	10.0	10.24	4.9	102	6
	20.0	21.02	5.9	105	4
	30.0	29.09	4.0	97	3
	40.0	37.94	3.1	95	2
	50.0	49.90	2.8	100	4

Specificity of the procedure

To verify the specificity of the TLC method, an HPLC procedure was developed to analyze for cinromide and its two plasma metabolites. Various amounts of cinromide, BCAM and BCA were extracted from plasma as described above and a portion of the extract was analyzed for the above compounds by HPLC as described under Materials and methods. Cinromide, BCAM and BCA were recovered through the method to the extent of 84.7%, 76.0% and 78.4%, respectively. It is not clear why these recoveries are lower than those achieved by the TLC method; perhaps this is a result of non-specific column adsorption. It is of interest to note that in the HPLC assay reported by Perchalski et al. [9], only a 61% recovery was obtained for cinromide and its metabolites. All experimental data were corrected for recoveries. Chromatographic profiles of cinromide and its metabolites from a standard solution containing all three compounds and from an extract of plasma containing these compounds are shown in Fig. 2. Fig. 2A shows the retention times and relative peak heights at 254 nm when a mixture containing 100 ng of each compound is separated by HPLC. Fig. 2B shows that cinromide and its two major plasma metabolites were sufficiently separated for quantitation without significant interference from substances normally present in dog plasma.

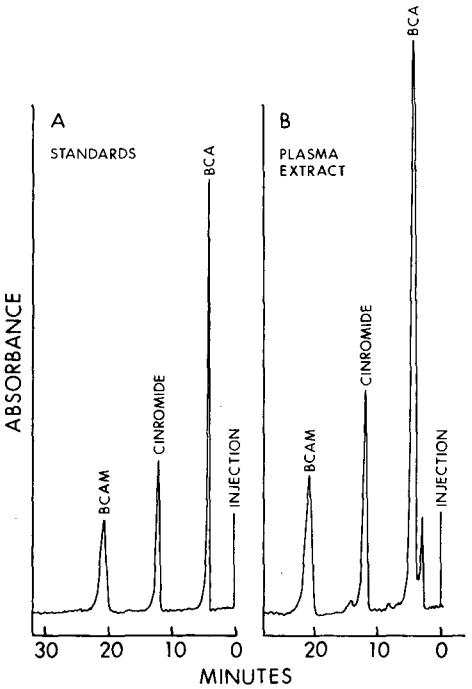

Fig. 2. HPLC chromatogram showing the separation of a standard solution of cinromide, 3-bromocinnamamide (BCAM) and 3-bromocinnamic acid (BCA); each peak represents 100 ng of compound. (B) HPLC chromatogram of a dog plasma extract at 1 h following the oral administration of 100 mg/kg of cinromide. Solvent system: chloroform-acetic acid (95:5). Column: Partisil PAC (25 cm).

TABLE II

COMPARATIVE ANALYSIS OF CINROMIDE AND ITS METABOLITES IN HUMAN PLASMA BY QUANTITATIVE TLC AND HPLC

Human subjects received 300 mg of cinromide and selected plasma samples were assayed for parent drug and metabolites by HPLC and quantitative TLC. Plasma samples selected covered the usual range of plasma concentrations normally found following a therapeutic dose of the drug. The correlation coefficient (r) for the analysis of each compound by the two methods is shown in the last column.

Compound	Plasma concentration (μg/ml)		
	HPLC	TLC	
Cinromide	0.09	0.11	
	0.44	0.52	0.91
	1.10	0.98	
BCAM	0.46	0.59	
	1.93	1.95	0.96
	4.91	5.10	
BCA	1.10	0.96	
	4.90	5.10	0.89
	9.93	9.97	

The data in Table II show the comparative analysis of cinromide and its metabolites in human plasma by quantitative TLC and HPLC. Human subjects received 300 mg of cinromide and selected plasma samples were assayed by both procedures. The plasma samples selected covered the usual range experienced clinically following a therapeutic dose of the drug. The concentrations found for each compound, assayed by both methods, agreed well with correlation coefficients in the range of 0.89—0.96.

Recently an HPLC method has been reported for quantitating cinromide, BCAM and BCA in a subject treated with cinromide (Perchalski et al. [9]). This ion-pairing procedure requires a heated column system and a mobile phase containing a buffering system. Although this procedure is useful in quantitating cinromide and its major metabolites, the TLC procedure has a distinct advantage regarding assay time and perhaps sensitivity, since no standard curve was included in their report. Quantitative TLC allows for multiple assays of up to 36 plasma samples a day where two of three compounds can be quantitated simultaneously. In addition, the TLC method has been applied to an oral pharmacokinetic evaluation of cinromide in the dog.

Pharmacokinetics of cinromide in the dog

The quantitative TLC procedure was used to determine the pharmacokinetic profile of cinromide and its two major plasma metabolites in beagle dogs (Fig. 3). The results represent the mean curves over 24 h from three dogs following

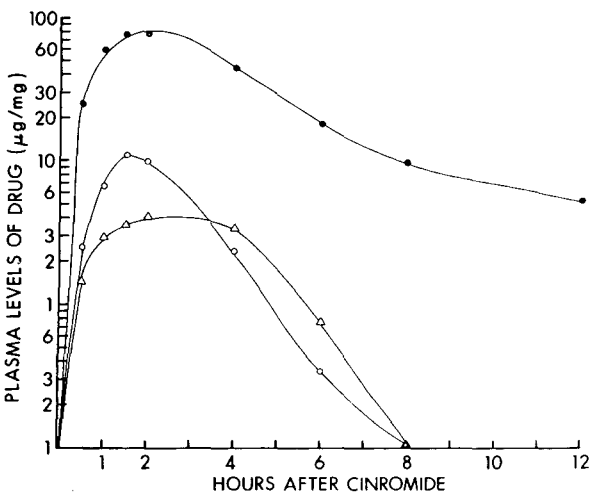

Fig. 3. Plasma levels of cinromide (○) and its metabolites, 3-bromocinnamamide (△) and 3-bromocinnamic acid (●) in beagle dogs following the oral administration of 100 mg/kg of cinromide. The data represent the mean curves from three dogs.

the oral administration of 100 mg/kg of cinromide. The drug was administered as soft gelatin capsules containing drug dissolved in Tween 80. As shown in Fig. 3 and Table III, this formulation provided rapid absorption of cinromide with peak levels of 11.0 μg/ml being achieved in 90 min. The N-dealkylated metabolite 3-bromocinnamamide, and 3-bromocinnamic acid were formed immediately, achieving peak plasma levels of 4.1 and 78.9 μg/ml respectively at

TABLE III

PHARMACOKINETIC PARAMETERS OF CINROMIDE AND ITS METABOLITES IN BEAGLE DOGS FOLLOWING THE ORAL ADMINISTRATION OF 100 mg/kg OF CINROMIDE

Three beagle dogs received 100 mg/kg of cinromide by capsule dissolved in Tween 80. The area under the plasma concentration curve (AUC) was determined by the trapezoidal rule. The results represent the mean ± S.E.

Compound	Peak level (μg/ml)	AUC after 12 h (μg/ml·h)	Half-life (min)
Cinromide	11.0 ± 3.3	29 ± 10	66
3-Bromocinnamamide	4.1 ± 0.6	18 ± 3	60
3-Bromocinnamic acid	78.9 ± 7.0	347 ± 47	132

2 h after the dose. It is of interest that the concentrations of the two plasma metabolites of cinromide are quite substantial; in fact, the area under the plasma curve for 3-bromocinnamic acid is twelve times greater than that observed for the parent drug. The rapid biotransformation of cinromide in the dog was immediately apparent by the obvious appearance of these metabolites on a TLC plate following exposure to ultraviolet light.

In the present study the development of a quantitative TLC method during the initial phase of drug development served as a very useful means for the early detection of significant plasma metabolites. In this respect the procedure not only provided a rapid quantitative tool but also allowed for the early detection and identification of major plasma metabolites of a new pharmacologic agent.

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Note

Quantitative determination of 6-keto prostaglandin $F_{1\alpha}$ in biological fluids by capillary gas chromatography—chemical ionization mass spectrometry

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Prostaglandin I_2 (PGI_2) was shown by Moncada et al. [1] to be a labile substance generated by arterial walls. The prostaglandin is a potent inhibitor of platelet aggregation and plays an important role in the processes of inflammation [2, 3]. This compound is unstable and has been shown to be rapidly decomposed non-enzymatically into 6-keto prostaglandin $F_{1\alpha}$ (6-keto $PGF_{1\alpha}$) in biological fluids [4]. Therefore, it is important and necessary for the study of physiological and pathological roles of PGI_2 in tissues to establish a precise method for quantitative analysis of 6-keto $PGF_{1\alpha}$ in biological fluids.

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Many assay methods for the measurement of 6-keto $PGF_{1\alpha}$ and PGI_2 have been developed to date; for example, radioimmunoassay, bioassay using human platelet aggregation, and gas chromatography—electron impact mass spectrometry [5]. Of these methods, gas chromatography—electron impact mass spectrometry was the most accurate and reliable method for the determination of 6-keto $PGF_{1\alpha}$. However, the mass fragmentograms were easily interfered with by fragment ions from substances in biological samples.

Chemical ionization (CI) mass spectrometry has shown a great potential advantage in the determination of molecular weight and molecular structure of labile compounds [6, 7]. Because the CI mass spectra are much simpler, interfering substances in the samples could be reduced in the mass fragmentograms of prostaglandins. Recently we reported the qualitative analysis of 6-keto $PGF_{1\alpha}$ by CI mass spectrometry and showed the superiority of ammonia gas as a reagent gas [8].

In this paper, we will demonstrate the successful measurement of 6-keto $PGF_{1\alpha}$ in biological fluids by capillary gas chromatography—CI mass spectrometry.

MATERIALS AND METHODS

The following were obtained from commercial sources: N-methyl-N′-nitro-N-nitrosoguanidine (Aldrich, Milwaukee, WI, U.S.A.); O-methylhydroxylamine hydrochloride (Wako, Tokyo, Japan); trimethylsilyl (TMS) imidazole (Pierce, Rockford, IL, U.S.A.); bis-TMS-trifluoroacetamide (Applied Science Labs., State College, PA, U.S.A.); [^{3}H]6-keto $PGF_{1\alpha}$, 150 Ci/mmol (The Radiochemical Centre, Amersham, Great Britain).

6-Keto $PGF_{1\alpha}$ and 6-keto [5,8,9,11,12,14,15-$^2H_7$]$PGF_{1\alpha}$ (6-keto $PGF_{1\alpha}$-d_7) were kindly supplied by Ono Pharmaceutical Co., Osaka, Japan, and Toray Industries, Kamakura, Japan, respectively.

Preparation of sample from carrageenin-induced granuloma containing 6-keto $PGF_{1\alpha}$

Granuloma was induced in male Sprague—Dawley strain rats weighing 150—180 g by the method described in a previous paper [9]. Exudate of 4-day-old granuloma (4—8 ml) was collected in a glass tube containing indomethacin to give a final concentration of 2×10^{-5} *M* and 200 ng of 6-keto $PGF_{1\alpha}$-d_7. The sample was acidified to pH 3.0 with 1 *N* HCl and was extracted with 8 volumes of ethyl acetate twice. The resulting organic phase was evaporated to dryness under reduced pressure. Residues were dissolved in a small amount of ethanol and applied quantitatively to thin-layer chromatography (TLC) plates (HPTLC plates, silica gel 60; E. Merck, Darmstadt, G.F.R.). The plates were developed first with a solvent system of methylene chloride—diethyl ether—petroleum ether (b.p. 30—60°C) (1:1:1, v/v), to remove neutral fatty acid, and then developed successively with solvent C which was the organic phase of ethyl acetate—2,2,4-trimethylpentane—acetic acid—water (11:5:2:10, v/v). The silica gel corresponding to migrated 6-keto $PGF_{1\alpha}$ was scraped off and the prostaglandin was extracted with methanol—chloroform (1:1, v/v). The extract was applied to a TLC plate and the plate was developed

again with solvent C. The silica gel corresponding to 6-keto $PGF_{1\alpha}$ was scraped off and the prostaglandin was extracted with methanol. Recovery of 6-keto $PGF_{1\alpha}$ throughout the entire procedure was estimated by using parallel exudate samples to which 0.1 μCi of [^{3}H]6-keto $PGF_{1\alpha}$ had been added at the beginning of the procedure. The overall recovery of 6-keto $PGF_{1\alpha}$ was 82%.

Preparation of the derivatives for gas chromatography—mass spectrometry

The samples were methylated with diazomethane which was freshly prepared from N-methyl-N'-nitro-N-nitrosoguanidine by the method of Fales et al. [10], converted to methoxime derivatives with O-methylhydroxylamine hydrochloride and finally trimethylsilylated with a mixture of TMS imidazole and bis-TMS-trifluoroacetamide as described previously [8].

Gas chromatography—mass spectrometry

A Shimadzu-LKB Model 9000A equipped with a CI source was used. The data processing system included a GC-MS-PAC 300 DG consisting of an Okitac 4300 mini-computer with 16 K core and a magnetic disk.

A glass capillary column (30 m × 0.3 mm I.D.) coated with SE-30 was used. The column with a solventless injection device [11] was connected to the gas chromatograph—mass spectrometer. The temperatures of column, injection port and ionization chamber were kept at 270°C, 300°C and 270°C, respectively. The flow-rate of helium gas was 30 ml/min. The CI mass spectra were obtained at an electron energy of 500 eV, an emission current of 400 μA and an accelerating voltage of 3.5 kV. Ammonia was used as a reagent gas at 0.8 Torr.

RESULTS AND DISCUSSION

The CI mass spectrum of 6-keto $PGF_{1\alpha}$, using ammonia as a reagent gas, is shown in Fig. 1A. The quasi-molecular ion (QM^+) was recorded at *m/e* 630 with weak intensity. The fragment ion at *m/e* 598 was presumably due to [QM^+—CH_3OH]. The ions at *m/e* 540, 450 and 360 were formed by successive eliminations of trimethylsilanol from QM^+ (*m/e* 630). The base peak was the ion at *m/e* 540. As demonstrated in Fig. 1B, the ion at *m/e* 547 was the base peak in the CI mass spectrum of 6-keto $PGF_{1\alpha}$-d_7. Therefore, quantification of 6-keto $PGF_{1\alpha}$ by mass fragmentography was done to trace the peaks of *m/e* 540 and 547. The quantitative analysis was made by measuring the peak height ratio of the ions at *m/e* 540 and 547. The calibration curve was linear from 40 to 400 pg of 6-keto $PGF_{1\alpha}$.

Fig. 2 shows mass fragmentograms of 6-keto $PGF_{1\alpha}$ in the exudate of rat carrageenin-induced granuloma and human plasma. In both samples there was practically no interference from endogenous substances in the biological fluids. The content of 6-keto $PGF_{1\alpha}$ in exudate of rat carrageenin-induced granuloma was 17.3 ± 6.5 ng/ml (n = 5). For human plasma, the study was made in triplicate, 6-keto $PGF_{1\alpha}$ being extracted from 50 ml of plasma each time (taken from a healthy male volunteer aged 25 years) using the same procedures as for the granuloma exudate. The content of 6-keto $PGF_{1\alpha}$ in human plasma was 400 ± 85 pg/ml (n = 3).

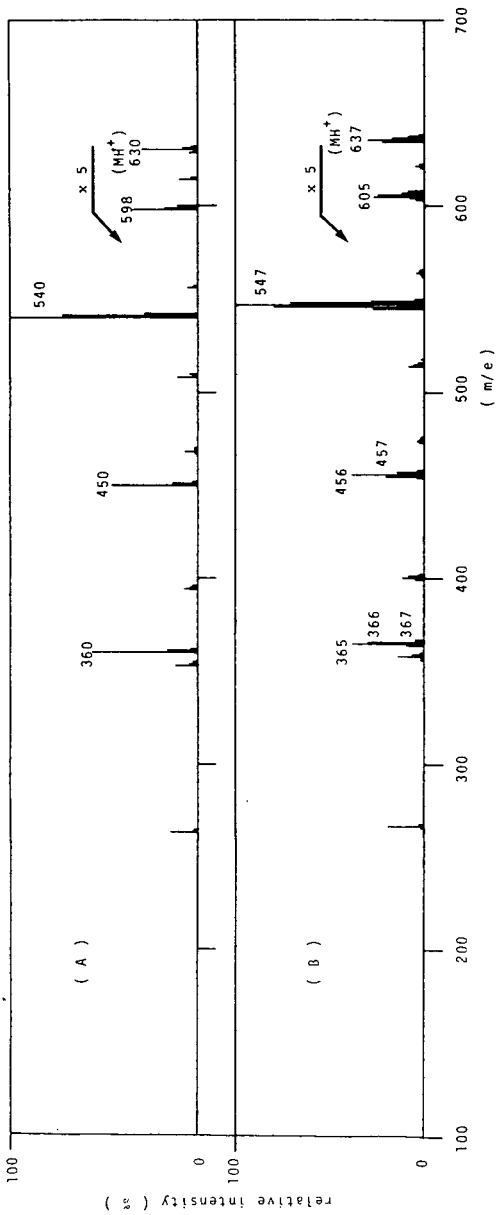

Fig. 1. Ammonia CI mass spectra of the methyl ester—methoxime—TMS derivatives of 6-keto $PGF_{1\alpha}$ (A) and 6-keto $PGF_{1\alpha}$-d_7 (B).

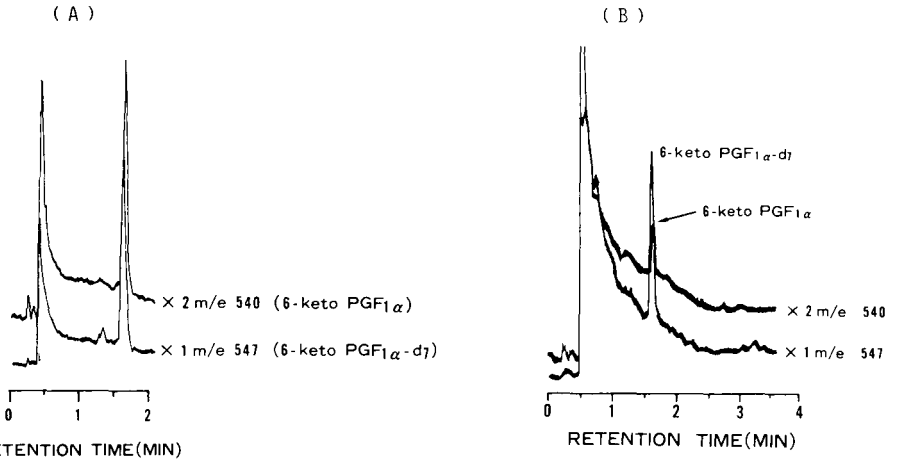

Fig. 2. Mass fragmentograms of the methyl ester—methoxime—TMS derivatives of 6-keto $PGF_{1\alpha}$ from the exudate of rat carrageenin-induced granuloma (A) and human plasma (B).

Further efforts have been made in our laboratory to reduce interfering substances and to increase the recovery of 6-keto $PGF_{1\alpha}$ by using high-performance liquid chromatography. The procedure will contribute to minimizing the starting volume of plasma. The level of 6-keto $PGF_{1\alpha}$ in carrageenin-induced granuloma was similar to the data of Ohuchi et al. [12], measured by radioimmunoassay.

CI mass spectrometry has recently been applied to the structural elucidation and quantitative determination of various biological compounds in terms of increased specificity and sensitivity [6, 7]. Especially ammonia CI mass fragmentography should be a most specific method for the determination of prostaglandins because ammonia CI mass spectra are most simple [6] and interference from other biological compounds could be minimized. Recently, we reported the CI mass spectrum of the methoxime—TMS derivative of 6-keto $PGF_{1\alpha}$ and obtained the molecular ion as the base peak [8]. However, the detection limit with this derivative was found to be approximately 100 times higher than with the methyl ester—methoxime—TMS derivative.

Fitzpatrick [13] reported that glass capillary columns could be used for prostaglandin separation and that the detection limit using such columns was about ten times lower than that obtained with packed columns. For this reason we successfully devised an assay method for 6-keto $PGF_{1\alpha}$ by capillary gas chromatography—ammonia CI mass fragmentography as its methyl ester—methoxime—trimethylsilyl derivative. As demonstrated by the results, the detection limit was decreased to 40 pg.

The work to minimize plasma sample volume is currently under way in our laboratory with the aim of studying plasma 6-keto $PGF_{1\alpha}$ content in cerebrovascular and cardiovascular diseases.

ACKNOWLEDGEMENT

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Note

Simultaneous measurement of sterol and fatty acid composition in small samples

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(First received April 15th, 1980; revised manuscript received July 17th, 1980)

A number of methods is commonly used to prepare fatty acid methyl esters for gas chromatography (GC): the sulphuric acid, toluene—benzene, methanol method [1]; the boron trifluoride method [2, 3]; and the sodium methoxide method [4]. The subject in general is reviewed by Christie [5]. This communication is concerned with the preparation and the hazards of analysis of fatty acid methyl esters and sterols from extracts of lipids and sterols, particularly where sample sizes are too small to permit separation of sterols prior to methylation. This is of particular importance in the analysis of lipids of erythrocyte and platelet membranes (which contain up to 50% sterol) when large blood samples are unobtainable (see e.g. Dyerberg and Bang [6]).

EXPERIMENTAL

In the experiments described here, lipid samples were refluxed with methanol—toluene—sulphuric acid (20:10:1, v/v) for 90 min at 90°C using up to a maximum of 10 mg of lipid sample for a 2-ml reaction mixture. The resulting fatty acid methyl esters and sterol products were extracted with hexane before concentrating for chromatography. Separation of methyl esters and sterol products was achieved using a Pye 104 gas chromatograph and flame ionisation detector fitted with a 5 ft. × 4 mm I.D. glass column packed with Silar 10C on 100—120 mesh Chromosorb Q (Supelco, Bellefonte, PA, U.S.A.). The output data were collected and processed using a Colombia Scientific

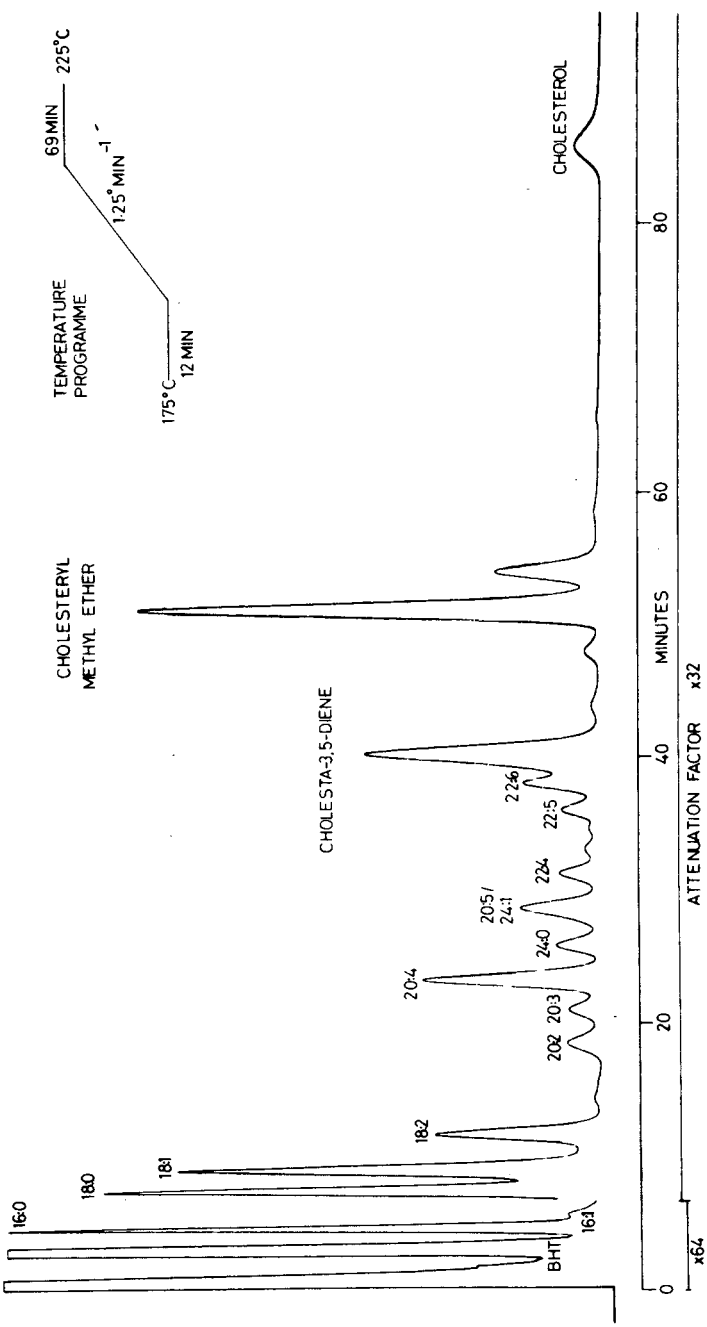

Fig. 1. Fatty acid methyl esters and sterol products of total human erythrocyte lipids from a 5-ml blood sample separated on a 5ft. × 4 mm I.D. glass column packed with Silar 10C on Chromosorb Q. The nitrogen carrier gas flow-rate was 40 ml/min. The temperature programme and attenuations used are indicated on the figure.

Instruments Supergrator 3 programmable computing integrator. The components thus separated were identified by the use of authentic standards [fatty acid methyl esters from Supelco; steroids from the Medical Research Council, Steroid Reference Collection (London, Great Britain)] and characterised by mass spectroscopy (Kratos DS-50SM mass spectrometry data system).

RESULTS AND DISCUSSION

Treatment of cholesterol alone under the above conditions results in the formation of two principal products, cholesta-3,5-diene and cholesteryl methyl ether. GC on Silar 10C shows that cholesterol and its products, including minor ones, all run after carbon No. 22:6, the first being cholesta-3,5-diene and the last, cholesterol, well separated from the others. The mass spectra for the three substances cholesta-3,5-diene, cholesterol methyl ether and cholesterol were characterized by the presence of their parent ions, *m/e* 368, 400 and 386 respectively and their expected major fragment ions, both in the case of the reaction mixture samples and in the reference samples run under the same conditions. Variation of the treatment time of sterol with methanol—toluene—sulphuric acid from 30 min to 4 h alters the relative yield of products as estimated from the gas chromatograms, but the sum of the products remains constant. This is exploited in the analysis of erythrocyte, platelet and serum samples where fatty acid composition and sterol content are determined from the same chromatogram, the latter as the sum of cholesterol and its components (Fig. 1).

Although the reports of shortcomings and artefacts of the different methods of preparation of fatty acid methyl esters number many, there are relatively few reports of these methods involving reactions with cholesterol producing cholesta-3,5-diene and cholesteryl methyl ether. Nonetheless, Morrison and Smith [3] reported the formation of these substances during treatment of sterol esters with boron trifluoride—methanol, and Kawamura and Taketomi [7] using a hydrochloric acid—methanol mixture. It seems not at all surprising therefore to find cholesta-3,5-diene and cholesteryl methyl ether produced from cholesterol using the sulphuric acid—methanol—toluene method. What may be a matter of some importance is that whilst the retention time of cholesterol on many polar GC columns is very long compared with those of the fatty acid methyl esters, and might thus be safely ignored in the analysis of fatty acids of lipid mixtures containing cholesterol, the retention time of cholesteryl methyl ether is shorter and that of cholesta-3,5-diene shorter still, raising the possibility of artefacts in measurements of fatty acid composition.

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Note

High-performance pyrolysis—gas chromatography: potential for differentiating cystic fibrosis cells

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(First received May 6th, 1980; revised manuscript received July 17th, 1980)

Cystic fibrosis (CF) is the most prevalent biochemical genetic disease in Caucasian populations. Approximately one in a $2 \cdot 10^3$ live births is afflicted with this autosomal recessive disorder.

Despite a vast amount of research, the basic defect associated with CF has not been explained. Research directed toward elucidation of the defect has encompassed several diverse areas: metachromasia in cultivated skin fibroblasts, decrease in specific enzyme activity, increase in quantity of mucopolysaccaride or glycogen content, abnormality in collagen metabolism, accumulations of calcium ion, fucose uptake, and many more. Results of these investigations have been reviewed recently by Milunsky [1] and Nadler et al. [2].

Previously, by means of pyrolysis—gas chromatography (Py—GC) [3] we had investigated several genetic biochemical disorders by analyzing cultured skin fibroblasts. Despite the use of columns with low resolving power, one could clearly observe repeatable differences in the chromatographic profile of the cells.

More recently, we reported on Py—GC mammalian cell studies which incorporated both primary human tissues and cultured skin fibroblasts [4]. In this study, use of a microprocessor-controlled gas chromatograph equipped with a moderately efficient packed column rendered pyrochromatograms with much improved definition, compared to the 1972 study [3].

In the present paper, we have used a 43-m SCOT column with high resolving power in an attempt to delineate a cystic fibrosis "factor".

MATERIALS AND METHODS

Nine cultures were purchased from the Human Genetic Mutant Cell Repository (Institute for Medical Research, Camden, NJ, U.S.A.). Pertinent information is presented in Table I. Cells were cultured according to directions of the

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TABLE I

DERIVATIONS OF HUMAN SKIN FIBROBLASTS

GM No.*	Age**	Passage***	Sex	Race	Genetic status	
1011	7	11	M	W	CF	Son
1012	8	9	M	W	CF	Son
1009	34	10	M	W	Heterozygous	Father
1957	11	7	M	W	CF	Brothers
1959	10	5	M	W	CF	
668	10	12	M	W	CF	
38	9	6	F	B.	Normal	

*Genetic Mutant Repository Catalog Number ascribed to a specific cell line from the Human Genetic Mutant Cell Repository, Camden, NJ, U.S.A.
**Age refers to the age of the patient from which the skin fibroblasts were derived.
***Passage indicates the number of times the culture was reinoculated and regrown.

suppliers. In brief outline, the method of culturing consisted of transferring cells to small tissue culture flasks with growth media containing 5% fetal calf serum. After a monolayer was formed, cells were removed from the glass surface by trypsinizing; they were then washed with sterile physiological saline, divided into two portions, and resuspended in fresh growth media. This procedure was continued until approximately 0.1 ml of packed cells were obtained. At that point, we washed the cells in glass-distilled water twice by simply centrifuging at 2000 *g* for 5 min and decanting the supernatant. Pellets were resuspended in glass-distilled water and lyophilized. Lyophilization was carried out in a Vertis Unit. Fine, filamentous, dry particles were the resultant product.

Pyrolysis—gas chromatography of skin fibroblasts

Small microgram quantities of sample were weighed on a Cahn Model G Electrobalance and transferred to a quartz boat (0.9 cm long, 0.24 cm wide, 0.14 cm deep with a 0.025-cm wall thickness). Each sample was placed in the platinum coil of a pyrolysis probe (Chemical Data Systems, Oxford, PA, U.S.A.), the latter being inserted into a quartz-lined interface connected to the gas chromatograph inlet. On pyrolyzing the sample in a stream of helium, thermal degradation products were swept into the gas chromatograph, separated, and detected. The resulting series of peaks, known as a pyrochromatogram, pyrogram (sometimes, as signature or fingerprint) were recorded, and results later compared.

Experimental details

Pyroprobe. Pyrolysis temperature, 801°C; duration of pyrolysis, 10 sec; pyrolysis temperature rise time, 12 msec; interface temperature, 200°C.

Gas chromatography. Varian Model 3700 gas chromatograph with CDS 111 data system. Temperature program, 65°C (4 min hold) then 6°/min up to 165°C (hold for 45—50 min); Carbowax 20M, 43 m × 0.5 mm I.D. SCOT column; $k' = 8.85$, $H_{Eff} = 37{,}600$ (using *n*-butylbenzene); helium carrier gas, flow-rate 39.8 cm/sec; detector, flame ionization (FID), $1 \cdot 10^{-12}$ a.f.s.

RESULTS

From a number of studies carried out in our laboratory on both primary and cultured cells of human, mouse, rat, hamster, as well as cells of microbial origin [3—6], we have found the Py—GC technique to be extremely reproducible in terms of retention time, detector response, and overall shape of profiles. For example, the first peak in a profile invariably emerged at 1.54 min. This fact is also borne out in the following illustrations, taken from duplicate analyses.

In Fig. 1 are depicted four chromatograms. The top three are of patients afflicted with the CF malady; the bottom is that of a normal subject. In comparing the two siblings, ages 11 and 10, we see only minor quantitative differences in areas designated A, B, C, D, E, out of a total number of peaks approximating 135. This number is about double that obtained from a good packed column. The unrelated CF pyrochromatogram differs from the two above in peak size in the areas generally designated B, a small additional peak at B1 and a somewhat larger peak at B2. However, on comparing the CF profiles to that of the normal, the latter shows distinct differences in two areas, 1 and 2. In the bracketed region designated Area 1, the normal profile has two peaks, the first of which is three times the amplitude of the second; in the CF profiles, however, these peaks have similar amplitudes. In Area 2, indicated by arrows, is the portion of the profile of a normal individual which distinguishes it from the CF. In the normal profile the first peak is higher in amplitude than the second. A reverse ratio is observed in CF profiles. Moreover, this Area 2 relationship is consistently reproduced in the three pyrochromatograms seen in Fig. 2.

In Fig. 2 are shown from the top, the Py—GC finterprints of two brothers, GM 1011 (age 7), and GM 1012 (age 8), both homozygous for CF. Their heterozygous father, GM 1009, has the profile shown at the bottom. Once again we see that the siblings' profiles are very similar with only minor differences. The greatest disparity between profiles of the brothers on one hand and the father on the other is indicated at peaks labelled 4 and 5. One observes in the profiles that ratios of principal peak amplitudes reflect a very high order of quantitative reproducibility.

DISCUSSION

One notable characteristic of the Py—GC technique is to give simple information in the form of a fingerprint or pyrochromatogram from biological material which originally existed in a highly complex matrix [6]. This characteristic has also been observed in Py—GC studies of man-made materials with intractable matrices [7].

In the present context, the precise qualitative and quantitative data obtained with the use of microprocessor-controlled instrumentation enable the investigator to differentiate and even to identify cells of biomedical importance.

Stored cells, grown two years earlier, gave essentially the same profiles as seen in the present study. Details were exactly repeated with one exception:

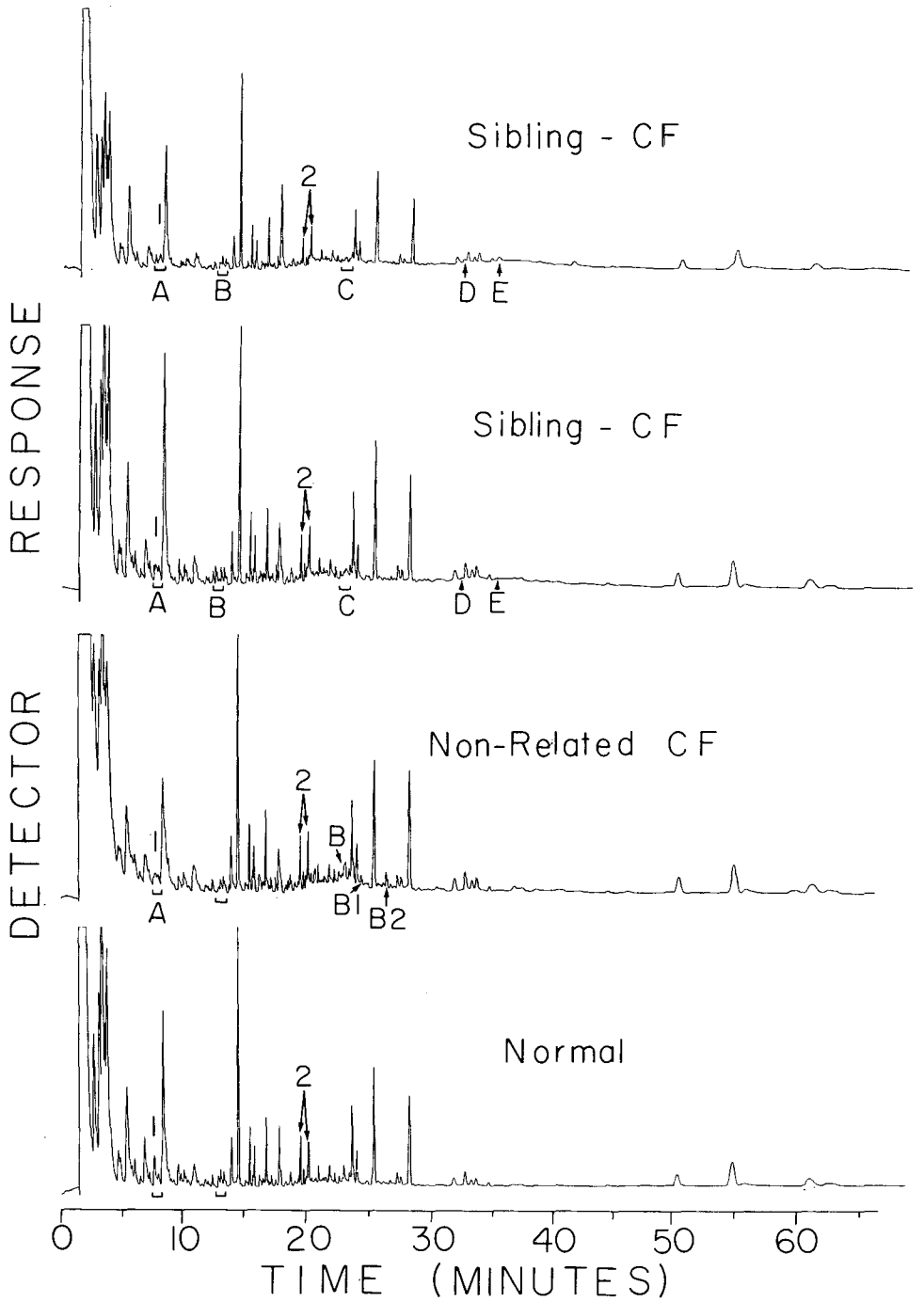

Fig. 1. Pyrolysis—gas chromatograms of human skin fibroblasts from cultured cells of various individuals from top to bottom; two brothers afflicted with cystic fibrosis (GM 1957, GM 1959), a non-related CF, GM 668 and a normal human diploid fibroblast GM 38. All profiles are displayed with a common elution time scale, e.g., bracketed area A appears at 8 min retention time on each chromatogram.

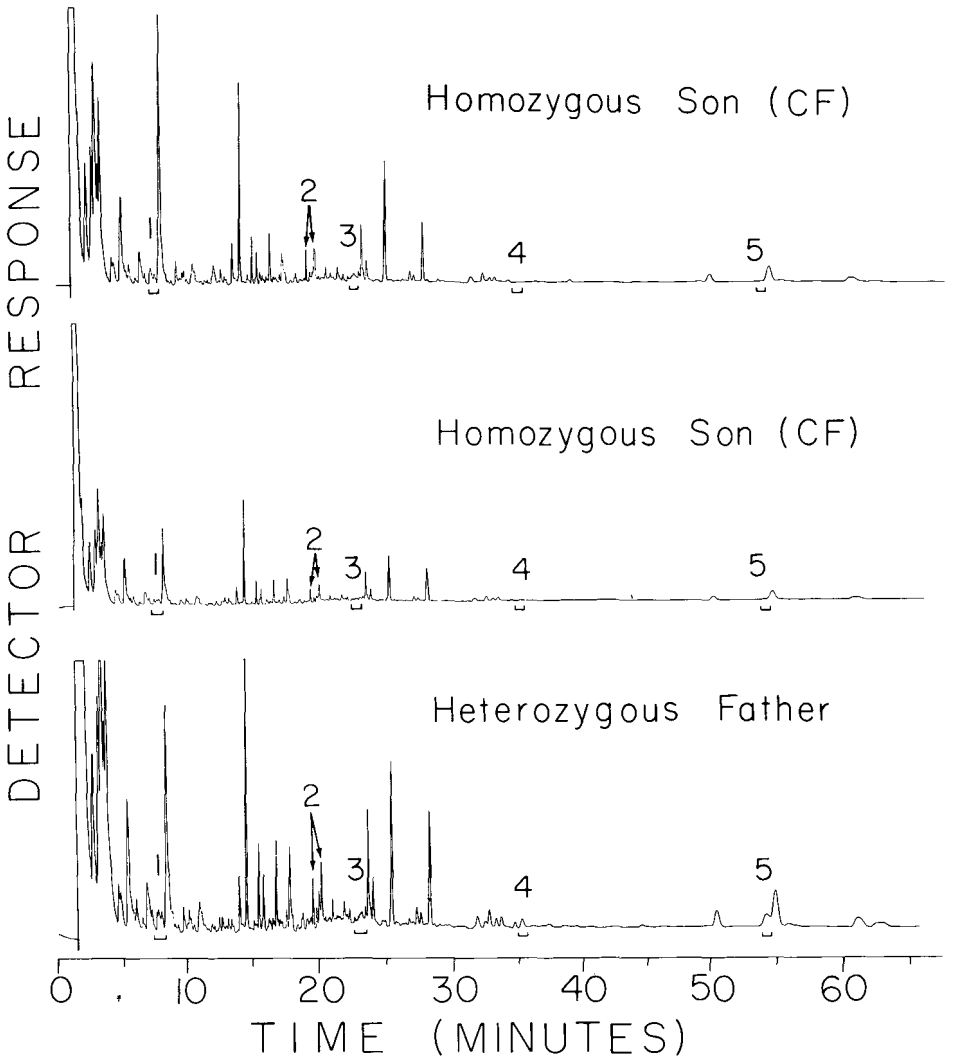

Fig. 2. Pyrolysis—gas chromatograms of human skin fibroblasts from cultured cells of homozygous son, GM 1011 (top), his younger brother, GM 1012 (middle) and their heterozygous father, GM 1009 (bottom). All profiles are displayed with a common time scale, e.g., area 3 covers the 22—23-min retention time period in the three profiles.

the two-year-old cultures always gave an extra peak at 28.50 min. It is possible that the extra peak could be ascribed to a change in pH, media formulation, e.g., use of a different calf serum, or that vital nutrients were present in excessive amounts [8]. The relative quantity of internally bound water in samples might also be a factor. In general, we have observed a certain immutability in pyrochromatograms of mammalian, as well as microbial cells.

The work described here is significant for a number of reasons. First, it paves the way for CF studies on the molecular level, such as those carried out in this institution on normal, primary mammalian cells derived from various human tissues [6]. Second, the pyrochromatograms are reproducible in all aspects, in-

dicating the possibility of delineating directly a cystic fibrosis "factor".

The congruency shown in the two sets of siblings' profiles as contrasted with the normal and heterozygous father may indicate a possible means of identifying heterozygous carriers of the disease.

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We are grateful to Dorothy Caplin, Special Viral Diagnostic Branch, Center for Disease Control, for her expert assistance in growing the cells and to the National Institutes of Health for partial support of this investigation.

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CHROMBIO. 667

Note

Gas chromatographic determination of N-desmethyl-propyphenazone, a metabolite of propyphenazone, in human urine

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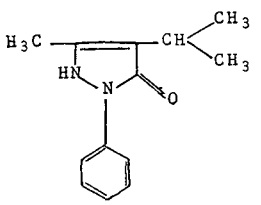
Tateishi and Shimizu [1] have reported that the major urinary metabolite of propyphenazone in man is the enol-glucuronide of N-desmethyl-propyphenazone, accounting for some 80% of the total urinary metabolites, but they did not report the total amount of metabolites as a percentage of the administered dose.



4-isopropyl-3-methyl-1-phenyl-3-pyrazolin-5-one
(N-desmethyl-propyphenazone)

This paper describes a simple gas chromatographic technique for the assay of N-desmethyl-propyphenazone in human urine after hydrolysis of its conjugate.

EXPERIMENTAL

Chemicals and reagents

N-Desmethyl-propyphenazone was supplied by Ciba-Geigy (Basle, Switzerland) and lindane by Applied Science Labs. (State College, PA, U.S.A.). Sulphuric acid (Titrisol; E. Merck, Darmstadt, G.F.R.) and toluene (Nanograde; Mallinckrodt, St. Louis, MO, U.S.A.) were of analytical grade.

Acetate buffer (pH 5.5) was prepared with 4.8 ml of 0.2 *M* acetic acid

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and 35.2 ml of 0.2 *M* sodium acetate. β-Glucuronidase (Type 2; Sigma, St. Louis, MO, U.S.A.) is a bacterial β-glucuronidase containing about 61,500 units/g. The methanolic solution of internal standard contained 25 ng of lindane per 100 μl.

Equipment

A gas chromatograph (Hewlett-Packard, Model 5713 A) equipped with an electron-capture detector (Hewlett-Packard, Model 18713 A) was used. The peak areas were given by an electronic integrator (Hewlett-Packard, Model 3380 A).

The column was operated at 186°C with argon—methane (90:10) at a flow-rate of 60 ml/min. The injector temperature was 250°C and the detector set at 300°C.

The glass column was washed successively with 1 *M* hydrochloric acid, distilled water, acetone and benzene, and silanized with a 1% (v/v) solution of hexamethyldisilazane in benzene. It was then washed again with benzene and dried at 100°C.

The column packing was 5% SP 2100 on 100—120 mesh Chromosorb W HP (Touzart et Matignon, Vitry-sur-Seine, France). The packed column (2 m × 3 mm I.D.) was flushed with the carrier gas at a flow-rate of 40 ml/min and heated to 260°C at 1°C/min. The column temperature was held at 260°C for five days. The column was conditioned by the injection of 100 μl of a silylating agent (Silyl 8; Pierce, Rockford, IL, U.S.A.) in portions between 150°C and 220°C. After this procedure it is ready for use at 186°C.

Enzymatic hydrolysis in urine

One millilitre of urine (diluted with water if necessary), 1 ml of acetate buffer (pH 5.5) and 10 mg of β-glucuronidase were heated for 24 h at 37°C. The extraction was then performed as described below.

Extraction

One hundred microlitres of internal standard solution were measured into a 5-ml glass centrifuge tube, to which 250 μl of $5 \cdot 10^{-3}$ *M* sulphuric acid and 500 μl of toluene were added. The tube was shaken mechanically (Infors-shaker) for 10 min at 250 rpm and centrifuged at 2450 *g*.

Gas chromatography

A 2-μl aliquot of the organic layer was injected into the gas chromatograph. The N-desmethyl-propyphenazone content was calculated from the peak-area ratio by reference to a calibration curve. This curve was obtained by extraction of urine spiked with increasing amounts of N-desmethyl-propyphenazone from 10 to 1000 ng/ml and a constant amount of internal standard (25 ng/ml urine).

Study in man

Eight healthy subjects, who were advised to take no drugs for 8 days before the beginning of the experiment and none besides propyphenazone throughout the duration of the study, each received 250 mg of propyphenazone as two tablets of 125 mg.

Urine was collected over a period of 24 h. The volume was measured and samples were stored at −20°C until analysis.

RESULTS AND DISCUSSION

Precision and recovery

Table I gives the results obtained when the described procedure was applied to urine samples spiked with N-desmethyl-propyphenazone. As seen in the table, a good reproducibility was obtained with concentrations down to 10 ng/ml N-desmethyl-propyphenazone. This low concentration (10 ng/ml) may be taken as the sensitivity limit of the method. Although still lower concentrations can be detected, the peak height or the peak area is so small that the precision of the determination will be unsatisfactory.

TABLE I

PRECISION AND RECOVERY IN THE DETERMINATION OF N-DESMETHYL-PROPYPHENAZONE IN SPIKED HUMAN URINE SAMPLES

Amount added (ng/ml)	Mean amount found (ng/ml) (n=6)	Precision (± S.D.)	Recovery ± C.V. (%)
10	9.9	0.1	99.0
25	24.0	1.5	96.0
50	52	0.7	104.0
100	104	2.3	104.0
150	146	3.7	97.3
200	189	2.9	94.5
			99 ± 4.1

Urine interference

Fig. 1 shows chromatograms of an extract of 1 ml of human urine and of an aliquot (100 µl) of a 24-h urine of a healthy subject treated with 250 mg of propyphenazone. There is no interference from the normal components of the urine extract.

Urine hydrolysis

Preliminary experiments were carried out to determine the best conditions for the hydrolysis of the enol-glucuronide of N-desmethyl-propyphenazone in human urine.

Stability tests showed that N-desmethyl-propyphenazone is stable in urine for 1 h at 70°C or 100°C, and for 48 h at 37°C.

Samples of the same urine, containing N-desmethyl-propyphenazone glucuronide, from subjects treated with propyphenazone were then subjected to both enzymatic and acid hydrolysis.

Enzymatic hydrolysis was studied in urine samples incubated with 10 mg of β-glucuronidase and acetate buffer (pH 5.5) for 1, 4, 16, 24 and 48 h at

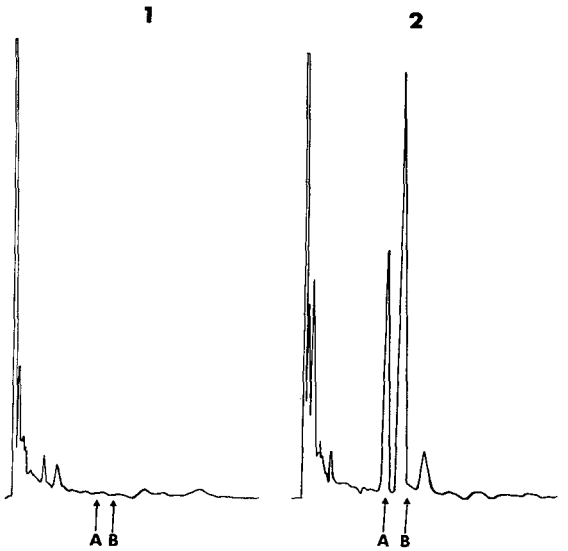

Fig. 1. Examples of chromatograms. (1) Human urine blank (1 ml of urine). (2) 24-h urine (100-µl aliquot) of a healthy volunteer (who had received 250 mg of propyphenazone) after enzymatic hydrolysis with 25 ng of internal standard (A) or 167 ng of N-desmethyl-propyphenazone (B) added.

37°C. A maximum yield of N-desmethyl-propyphenazone was obtained after incubation for 24 h. Identical results were obtained after another 24-h incubation during which fresh enzyme (10 mg) was added every 6 h instead of only at the beginning.

Acid hydrolysis of urine incubated with 1 ml of concentrated HCl for 1 h at 70°C produced no detectable amounts of N-desmethyl-propyphenazone. When the reaction temperature was increased to 100°C, however, N-desmethyl-propyphenazone was found, but in a yield ten to twenty times less than that obtained after enzymatic hydrolysis.

Enzymatic hydrolysis was therefore chosen as the more suitable procedure for the hydrolysis of the propyphenazone metabolite.

Application

The described technique was used for the determination of the enol-glucuronide of N-desmethyl-propyphenazone as a metabolite of propyphenazone in 24-h urine samples of eight healthy volunteers who had received 250 mg of propyphenazone. The average amount of the metabolite found in the urine corresponded to 0.8% of the administered dose of propyphenazone.

This result appears to be at variance with the statement [1] that in man the major portion of a given dose of propyphenazone is metabolized to the demethylated product, which subsequently, and almost quantitatively, undergoes glucuronide conjugation. Actually the authors have reported that the glucuronide of desmethyl-propyphenazone recovered in the first 24-h urines of the rat corresponds to 40—60% of the administered dose, which was very

high (15 mg/kg per os). In man given only 50 mg per os, the glucuronide accounted for 80% of the total urinary metabolite, as in the rat, but no figure has been given for the amount excreted in the urine as a percentage of the dose.

Besides the determination of the enol-glucuronide of N-desmethyl-propyphenazone, propyphenazone was also determined [2] in the same 24-h urine samples of the eight healthy volunteers who had received 250 mg of propyphenazone. As a mean, about 0.04% of the administered dose was excreted as the unmetabolized drug.

CONCLUSION

The proposed gas chromatographic technique permits the quantitative determination of N-desmethyl-propyphenazone in human urine at concentrations down to 10 ng/ml. This method can be applied to the determination of the enol-glucuronide of N-desmethyl-propyphenazone as a metabolite of propyphenazone in urine.

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CHROMBIO. 673

Note

Determination of 5-fluorouracil and pyrimidine bases in plasma by gas chromatography—chemical ionization-mass fragmentography

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1-(Tetrahydro-2-furanyl)-5-fluorouracil (FT), named Ftorafur or FT-207, has been widely used in cancer chemotherapy. 1,3-Bis(tetrahydro-2-furanyl)-5-fluorouracil (FD-1) [1,2], and uracil plus FT (UFT; 4:1, mol/mol) [3—7] have been recently developed as an antitumor agent with more effective activity. We have reported the determination of 5-fluorouracil (5-FU) as an active metabolite of FT and FD-1 in plasma and visceral tissues by gas chromatography—electron impact-mass fragmentography (GC—EI-MF) [8,9], and further the simultaneous determination of 5-FU and uracil in plasma and visceral tissues after administration of UFT by a combination of GC—EI-MF and gas chromatography—electron impact-mass spectrometry for total ion monitoring (GC—EI-MS) [10]. Furthermore, a method for the simultaneous assay of uracil, thymine and cytosine present in biological materials as pyrimidine bases by a combination of GC—EI-MF and GC—EI-MS has been reported [11]. Pantarotto et al. [12] and Min and Garland [13] determined 5-FU as its methylated derivative after administration of 5-FU in plasma or serum by gas chromatography—chemical ionization-mass fragmentography (GC—CI-MF). This paper describes the use of GC—CI-MF in determining the plasma levels of 5-FU and pyrimidine bases, uracil, thymine and cytosine, as their trimethylsilylated (TMS) derivatives.

EXPERIMENTAL

Materials

5-FU, uracil, thymine and cytosine were obtained from Sigma (St. Louis, MO, U.S.A.). 5-Chlorouracil was synthesized in our laboratory. [1,3-$^{15}N_2$]-5-FU and [1,3-$^{15}N_2$]uracil (each 95% enrichment) were purchased from PCR (Gainesville, FL, U.S.A.). N,O-Bis(trimethylsilyl)acetamide (BSA), tri-

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methylchlorosilane (TMCS) and pyridine for the silylating agents and solvent were from Pierce (Rockford, IL, U.S.A.). The other chemicals used were liquid-chromatographic and analytical grade materials.

Instrumentation

A JEOL JMS D300 mass spectrometer (EI/CI ion source) connected to a JEOL JGC-20KP gas chromatograph (Tokyo, Japan) was used.

The coiled glass column (1 m × 2 mm I.D.) of the gas chromatograph was packed with 3% OV-17 on Chromosorb W AW (80—100 mesh) (Gaschro Kogyo, Osaka, Japan). The temperatures of the injector and ion source were 230°C and 160°C, respectively, and analyses were carried out with an initial column temperature of 150°C and a temperature rise of 10°C/min. The carrier gas was helium and the flow-rate 30 ml/min. The mass spectrometer was operated under the following conditions: ionization energy, 190 eV; ionization current, 300 μA; accelerating voltage, 3.0 kV; ion multiplier voltage, 1.8 kV; reagent gas, ammonia; and the pressure of reagent gas, 1.0 Torr. The fragment ions detected were the protonated molecular ion peaks, $(M+1)^+$, of *m/z* 275, 257, 271, 256, 277, 259 and 291 for the TMS derivatives of 5-FU, uracil, thymine, cytosine, [1,3-$^{15}N_2$]-5-FU, [1,3-$^{15}N_2$]uracil and 5-chlorouracil.

Analytical procedure

The analytical procedure for GC—CI-MF was as described previously [8—11] except that for silylation a freshly prepared solution of 100 μl of pyridine containing 25% BSA and 1% TMCS was added to the residue extracted with ethyl acetate, and this solution was kept at 80°C for 20 min to allow silylation to occur.

[1,3-$^{15}N_2$]-5-FU and [1,3-$^{15}N_2$]uracil were used as an internal standard for determination of 5-FU and uracil; 5-chlorouracil was the internal standard for thymine and cytosine. The calibration curves were prepared by plotting the ratio of the peak height of each TMS derivative to that of the TMS derivative of the internal standard against the concentration (1.0, 0.5, 0.25, 0.10, 0.05, 0.01 and 0.005 μg/ml for each compound was added to 1.0 ml of plasma).

RESULTS AND DISCUSSION

The CI mass spectra of 5-FU, pyrimidine bases and their internal standards using ammonia as a reagent gas are shown in Fig. 1. The mass spectra of 5-FU, uracil, thymine, [1,3-$^{15}N_2$]-5-FU, [1,3-$^{15}N_2$]uracil and 5-chlorouracil showed an $(M+1)^+$ ion but no other fragment ions, while the spectrum of cytosine showed an $(M+1)^+$ ion and one fragment ion. The CI mass spectra of these compounds using isobutane as a reagent gas, on the other hand, showed an $(M+1)^+$ ion and many fragment ions.

To obtain suitable GC—CI-MF conditions for determination of 5-FU and pyrimidine bases in plasma after administration of FT or FD-1 plus pyrimidine base, the derivation procedure, the reagent gas and the ions detected by the mass spectrometer were investigated. The silylation procedure with pyridine

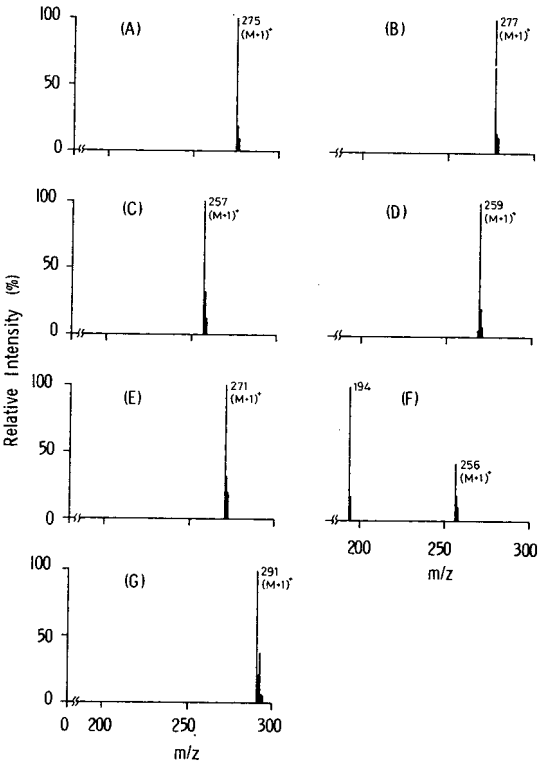

Fig. 1. CI mass spectra of the TMS derivatives of (A) 5-FU, (B) [1,3-$^{15}N_2$]-5-FU, (C) uracil, (D) [1,3-$^{15}N_2$]uracil, (E) thymine, (F) cytosine and (G) 5-chlorouracil using ammonia as a reagent gas.

containing BSA and TMCS, the use of ammonia as a reagent gas and the detection of an $(M+1)^+$ ion were found to result in better separation of 5-FU and pyrimidine bases from plasma constituents and a higher detection sensitivity.

The GC—CI-MF separation pattern of authentic samples of 5-FU and uracil, and of these compounds extracted from plasma after the administration of UFT, using the respective stable isotope-labeled compounds as internal standard, are shown in Fig. 2. The retention times of the TMS derivatives of 5-FU and uracil were 1.5 and 1.7 min, respectively, and the detection limit was 0.001 μg/ml of plasma for each compound.

The GC—CI-MS separation of human plasma extracted with known amounts of added 5-FU and thymine, and 5-FU and cytosine, using 5-chlorouracil as an internal standard, are shown in Fig. 3. The retention times were 2.1, 3.7 and 2.5 min for the TMS derivatives of thymine, cytosine and 5-chlorouracil, respectively. The detection limits for thymine and cytosine in plasma were 0.001 μg/ml and 0.050 μg/ml, respectively.

The precision of the method was ± 3.1%. This assay method appears to be satisfactory for the determination of these compounds in plasma.

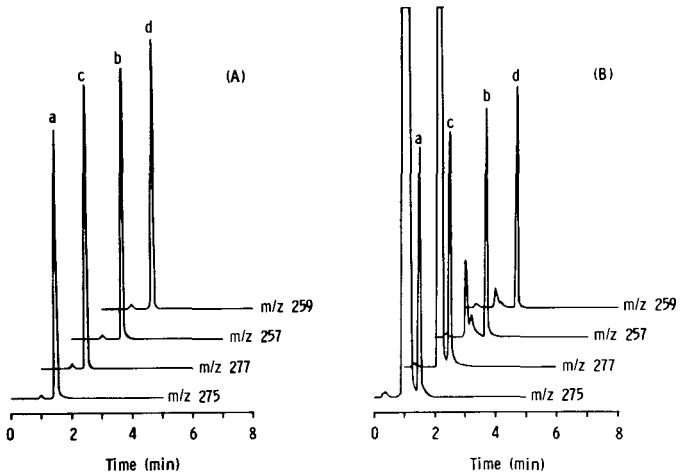

Fig. 2. Separation by GC—CI-MF of (A) authentic samples of 5-FU (a), uracil (b) and the internal standards, [1,3-$^{15}N_2$]-5-FU (c) and [1,3-$^{15}N_2$]uracil (d), and (B) these compounds extracted from human plasma. Results are for the TMS derivatives.

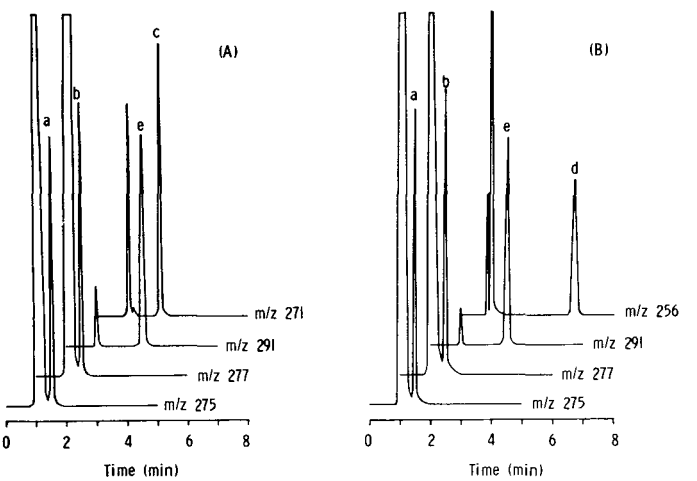

Fig. 3. Separation by GC—CI-MF of (A) 5-FU (a) and thymine (c), and (B) 5-FU (a) cytosine (d), and the internal standards, [1,3-$^{15}N_2$]-5-FU (b) and 5-chlorouracil (e), extracted from human plasma. Results are for the TMS derivatives.

The precision and sensitivity of GC—CI-MF for determination of 5-FU and pyrimidine bases were compared with those of GC—EI-MF reported previously [8—11]. The results obtained by GC—CI-MF were no less than those obtained by GC—EI-MF except for the sensitivity of cytosine. The GC—EI-MF procedure is useful for the simultaneous assay of both plasma and visceral tissue levels of 5-FU and pyrimidine bases; however, the GC—CI-MF method described in this paper could be applied to plasma but not to visceral tissues because of poor separation from visceral tissue constituents.

Thus, the present method should be useful as well as GC—EI-MF for measuring the plasma levels of 5-FU and pyrimidine bases after administration of FT or FD-1 plus uracil, thymine or cytosine.

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Note

Determination of pindolol in biological fluids by an electron-capture gas—liquid chromatographic method on a wall-coated open tubular column

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Pindolol, 4-(2-hydroxy-3-isopropylaminopropoxy)indole, a beta-blocking drug, has until now been determined in plasma by the fluorimetric method described by Pacha [1]. Recently, an electron-capture gas—liquid chromatographic (GLC) procedure using a trifluoroacetyl derivative of pindolol was proposed [2]. On some occasions, these two methods demonstrate, however, a lack of specificity. In the fluorimetric assay, interfering fluorescent material can be coextracted from biological fluids. For the GLC determinations, impurities present in some batches of the reagent may lead to insufficient specificity. A rapid and highly specific electron-capture GLC method using a wall-coated open tubular column is described in this paper.

EXPERIMENTAL

Instrumentation

A Hewlett-Packard gas chromatograph Model 5713A was equipped with a ^{63}Ni electron-capture detector and a capillary injection system 18740B, and was connected to a Sefram P.E. recorder with a scale range of 1 mV. A Hewlett-Packard integrator system 3352C was used for integration and quantitation.

The OV-1 coated open-tubular glass capillary column (25 m × 0.3 mm I.D.) was pretreated and tested before use.

The splitless injection mode was used, a 2-μl sample being injected. The injection period was 30 sec.

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The column temperature was isothermal at 80°C for 2 min, and then programmed from 80°C to 170°C at a rate of 10°C/min. Detector and injector temperatures were 300°C and 250°C, respectively.

The make-up gas (argon with 10% methane) had a flow-rate of 35 ml/min. The flow-rate of the carrier gas (helium) was 3 ml/min. Under these conditions the retention times of propranolol and pindolol were 13 min 5 sec, and 13 min 30 sec, respectively.

Standard solutions

The standard stock solutions (0.1 mg/ml) of pindolol and propranolol were prepared by dissolving these compounds in methanol. The solutions were stored at 4°C and dilutions were made just before use.

Analytical procedure

Pindolol with propranolol as internal standard (20 ng/ml) were extracted from plasma as described previously [2]. The dry extract was dissolved in 100 µl of a 4 *M* trimethylamine solution in iso-octane and derivatized with 10 µl of trifluoroacetylimidazole for 30 min at room temperature. The reaction mixture was shaken vigorously for 5 sec with 1 ml of 0.5 *M* phosphate buffer (pH 5.4) and centrifuged. A 2-µl aliquot of the iso-octane phase was taken for electron-capture GLC analysis.

Standard curves

Under these conditions the calibration curve of pindolol, with propranolol as the internal standard, was linear over the range of 2.5—30 ng/ml (Fig. 1).

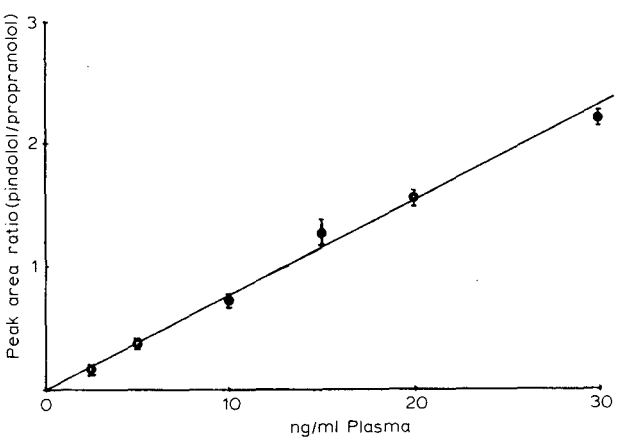

Fig. 1. Calibration curve for the described method.

RESULTS AND DISCUSSION

In the previous paper [2] the method described involved the use of a glass

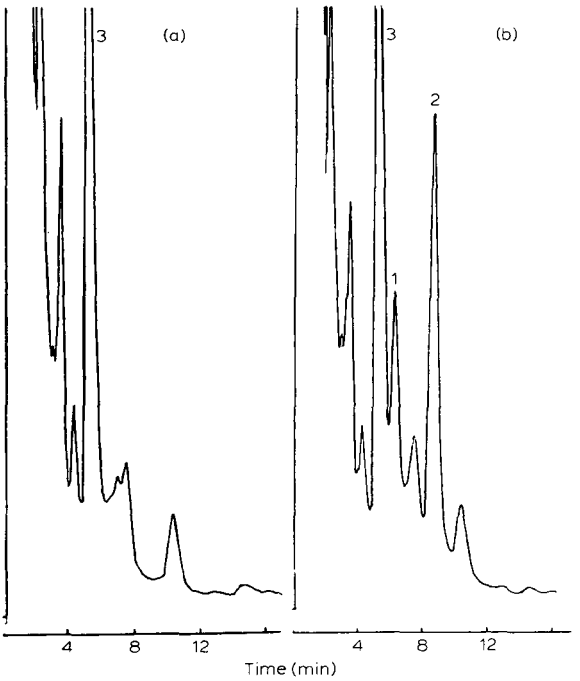

Fig. 2. Gas chromatograms obtained with a 2% OV-17 packed column from (a) a blank human plasma sample and (b) a human plasma spiked with 10 ng/ml pindolol (peak 1) and 50 ng/ml propranolol (peak 2) as internal standard (peak 3 results from the reagent).

column packed with 2% OV-17 on Gas-Chrom Q (100—120 mesh). Pindolol and propranolol were determined with good sensitivity and specificity. Unfortunately, the acetylating reagent trifluoroacetylimidazole had not always the same batch-to-batch purity. This resulted in an interfering peak appearing sometimes on the chromatogram with a retention time slightly shorter than that of pindolol. Fig. 2 demonstrates the poor separation of the two peaks of the "interfering substance" and pindolol. Moreover, if no precautions were taken in the washing procedure for the glassware, interfering peaks appeared on the chromatogram with one of them having nearly the same retention time as pindolol. To avoid this potential source of error in the accuracy and reproducibility of the method, elaborate and time-consuming washing of the glassware was necessary.

The use of a wall-coated OV-1 capillary column with a high efficiency overcame these problems. In a test assay during which no precautions were taken during either the washing of glassware or in the quality of the reagent, the method was validated. This has since been confirmed by numerous analyses. As demonstrated in Fig. 3, under these conditions, interfering substances were completely absent from drug peaks after extraction of human plasma spiked with 10 ng of pindolol and 20 ng of propranolol per ml. The two peaks are well-separated.

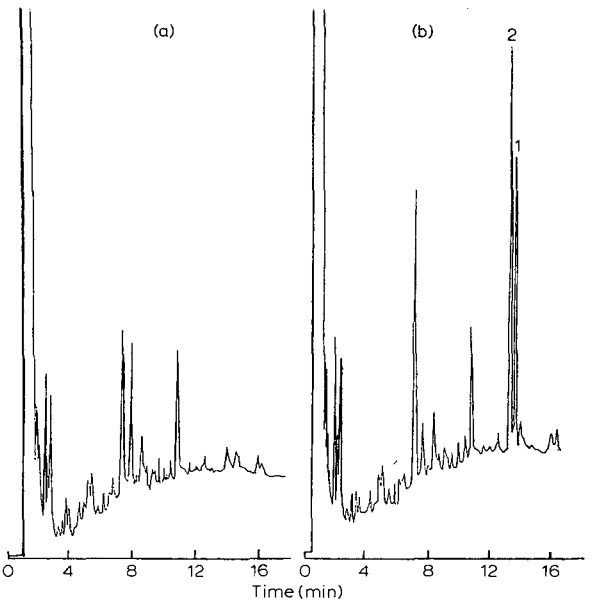

Fig. 3. Gas chromatograms obtained with an OV-1 wall-coated open tubular column from (a) a blank human plasma sample, and (b) a human plasma spiked with 10 ng/ml pindolol (peak 1) and 20 ng/ml propranolol (peak 2) as internal standard.

TABLE I

REPRODUCIBILITY AND ACCURACY OF THE METHOD

Added to plasma (ng/ml)	Recovered from plasma (ng/ml; mean ± S.E.M. $n = 3$)	Coefficient of variation (%)	Accuracy (mean ± S.E.M.)
1	1.05 ± 0.05	8.2	8.0 ± 2.7
2.5	2.66 ± 0.14	9.3	9.7 ± 2.6
5	4.74 ± 0.12	4.3	5.2 ± 2.4
10	10.14 ± 0.20	3.4	2.9 ± 0.9
15	14.94 ± 0.19	2.3	1.8 ± 0.2
20	20.90 ± 0.75	3.0	2.2 ± 0.6
30	29.82 ± 0.60	3.5	2.7 ± 0.7

The experimental procedure, described above, guarantees a better quality of data.

The results of the reproducibility study to validate the method are shown in Table I. The coefficient of variation ranged from 2.3 to 9.3%. The average reproducibility of an assay over the concentration range 2.5—30 ng/ml is ± 4.9%. The accuracy of the method is given by the mean percentage deviation of all concentrations from the theoretical value (see Table I). This value ranged from 1.8 to 9.7 with a mean value of 4.6.

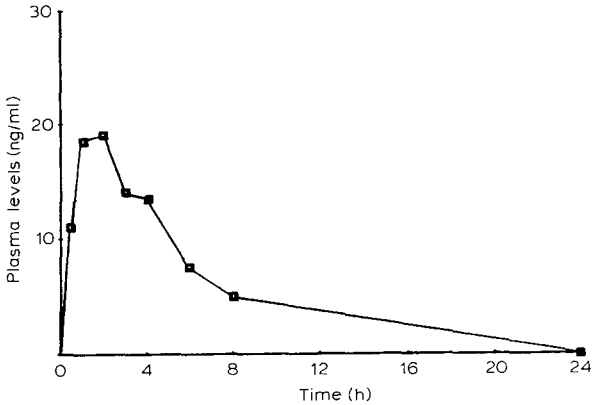

Fig. 4. Plasma levels of pindolol in one subject after a single oral dose of 5 mg of drug administered orally in tablet form.

Fig. 3a demonstrates that in a plasma blank there is no interference with the pindolol peak. The sensitivity of this chromatographic determination is about 5 pg injected on to the column. It is therefore possible to detect with good precision 500 pg of pindolol per ml of plasma using a 2-ml sample. If only 1 ml of plasma is available, the iso-octane phase can be concentrated before injection on to the column to maintain the same limits of sensitivity, but this additional step is time-consuming.

A plasma concentration curve obtained using this method in one volunteer who took 5 mg of pindolol orally is displayed in Fig. 4. The procedure can also be applied to the determination of propranolol with pindolol as internal standard.

CONCLUSIONS

The high resolution and excellent sensitivity obtained by the use of a wall-coated open tubular column compared to the conventional packed column led to the development of an accurate and convenient electron-capture GLC method for pindolol in plasma. This method allows the determination of pindolol in plasma samples from clinical investigations of patients to whom other medications are administered, by minimizing the potential analytical interference.

In addition, the time for sample preparation is reduced, due to simplification of the washing procedure for the glassware. Moreover, the automatization of data processing is reliable and easier, and facilitates the collection of data for population characteristics of pharmacokinetic parameters in different disease states and under various therapeutic regimens.

Because of these advantages, the described analytical method offers a worthwhile alternative to the conventional and currently used procedure.

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Note

Gas chromatographic determination of maprotiline and its N-desmethyl metabolite in human blood using nitrogen detection

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Maprotiline (Ludiomil®) is an antidepressive agent that has been in use for several years. It is found in erythrocytes in concentrations about 2.5 times the plasma concentration [1]. Desmethyl-maprotiline is one of its metabolites.

A double radioisotope derivative (DRID) assay of maprotiline in blood has been described [2] and its clinical applications reported [3]. Electron-capture gas—liquid chromatography (GLC—ECD) using the heptafluorobutyryl derivative of maprotiline was applied to the determination of maprotiline in plasma, blood and urine [4, 5]. Gas chromatography—mass spectrometry (GC—MS) was also used to assay maprotiline from post mortem material [6]. Tricyclic antidepressants and their metabolites were determined in serum by high-performance liquid chromatgraphy (HPLC) [7].

Antipsychotic drugs containing either a secondary or a tertiary amine were determined by gas chromatography using thermionic nitrogen-selective detectors (GLC N-FID) [8—14]. Maprotiline and desmethyl-maprotiline were estimated in serum by GLC N-FID [15]. A comparison between GLC N-FID and DRID for the determination of maprotiline in human plasma was reported [9, 16].

In order to allow the quantitative assay of maprotiline in whole blood by laboratories not equipped with the instrumentation necessary for the DRID method, we describe here a GLC N-FID method for the determination of maprotiline and desmethyl-maprotiline in blood.

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EXPERIMENTAL

Chemicals and reagents

Maprotiline hydrochloride, desmethyl-maprotiline hydrochloride and desipramine hydrochloride were supplied by Ciba-Geigy (Basle, Switzerland). All reagents and solvents are of analytical grade.

The content of four vials of pH 10 Titrisol (Merck 9890; E. Merck, Darmstadt, G.F.R.) is diluted to 500 ml with distilled water to yield the pH 10 buffer used as the extraction buffer.

The solutions of maprotiline hydrochloride, desmethyl-maprotiline hydrochloride and desipramine hydrochloride are prepared in 0.01 *N* sulphuric acid. The aqueous solution of desipramine hydrochloride (internal standard) contains 1 ng/μl.

Equipment

A Hewlett-Packard Model 5710A gas chromatograph equipped with a nitrogen detector (Model 18789A) is used. The column is operated at 245°C, the injector and the detector at 300°C, with a nitrogen flow-rate of 40 ml/min. The flow-rates of hydrogen and air are, respectively, 3 and 30 ml/min. The heating pulse of the cell is 14 V adjusted as recommended by Hewlett-Packard.

The column packing is 3% HI-EFF-8BP on Chromosorb W HP 100—120 mesh (Applied Science Labs., State College, PA, U.S.A.). The packed column (0.30 m × 3 mm I.D.) is flushed with the carrier gas at a flow-rate of 20 ml/min and heated to 250°C at a rate of 20°C per 15 min. The column temperature is held overnight at 250°C.

Extraction and derivatization

Two hundred microlitres of the internal standard solution are measured into a stoppered glass tube. Then, 1 ml of the sample, 2 ml of pH 10 buffer and 5 ml of heptane containing 3% isoamyl alcohol are added. The tube is shaken mechanically (Infors shaker) for 15 min at 300 rpm and centrifuged at 2000 *g*.

An aliquot volume of the organic phase is transferred to another tube and 2 ml of 0.1 *N* sulphuric acid is added. The tube is shaken mechanically for 15 min at 300 rpm and centrifuged at 2400 *g*. The interface between the two layers must be well defined.

The organic phase is discarded, 1 ml of 0.2 *N* sodium hydroxide, 2 ml of pH 10 buffer and 4 ml of hexane containing 5% ethyl acetate are added. The tube is shaken mechanically for 15 min at 300 rpm and centrifuged at 2000 *g*. A maximum volume of the organic phase is transferred to another tube (care being taken not to withdraw any of the aqueous phase), 50 μl of anhydrous pyridine, 20 μl of acetic anhydride are added and the stoppered tube is heated at 60°C for 1 h. After cooling to room temperature, 1 ml ethyl acetate and 2 ml of 1 *N* sodium hydroxide are added. The tube is shaken mechanically for 15 min at 300 rpm and centrifuged at 2000 *g*.

A maximum volume of the organic phase is transferred to a conical tube and taken to dryness under a nitrogen stream at 40°C. If the GC run cannot be made until the next day, the organic phase should be stored at −20°C without taking to dryness.

Gas chromatography

Just before injection onto the column, 50 μl of ethyl acetate are added. The tube is shaken on a Vortex mixer and a 2-μl portion of the ethyl acetate solution is injected into the gas chromatograph.

The maprotiline (or desmethyl-maprotiline) content is calculated from the peak-area ratio by reference to a calibration curve. This curve is obtained by extraction of blood spiked with increasing amounts of maprotiline (20—400 ng/ml) or increasing amounts of desmethyl-maprotiline (50—400 ng/ml) and a constant amount of internal standard (200 ng/ml).

The calibration curve is stable for 24 h and valid for one week. It must be checked every week to assess its reproducibility.

The authenticity of the chromatographic peaks obtained with the monoacetylated derivatives of maprotiline, desmethyl-maprotiline and desipramine was verified by injecting solutions of the pure synthetic monoacetylated derivatives.

RESULTS AND DISCUSSION

Precision and recovery

Tables I and II give the results obtained when the described procedure is applied to blood samples spiked with maprotiline hydrochloride and desmethyl-maprotiline hydrochloride, respectively. Gupta et al. [15] obtained the same results for the estimation of the precision of their method.

TABLE I

PRECISION AND RECOVERY FOR THE DETERMINATION OF MAPROTILINE APPLIED TO SPIKED HUMAN BLOOD SAMPLES

Amount added (ng/ml)	Mean amount found (ng/ml; n = 6)	Precision Reproducibility (± S.D.)	Recovery Accuracy (mean ± C.V.%)
20	23.3	± 1.2	117.0 ± 5.2
50	47.7	± 9.4	95.3 ± 4.7
100	90.7	± 4.0	90.7 ± 4.4
200	205.0	± 8.6	102.7 ± 4.4
400	392.7	± 30.4	98.0 ± 7.4

TABLE II

PRECISION AND RECOVERY FOR THE DETERMINATION OF DESMETHYL-MAPROTILINE APPLIED TO SPIKED HUMAN BLOOD SAMPLES

Amount added (ng/ml)	Mean amount found (ng/ml; n = 6)	Precision Reproducibility (± S.D.)	Recovery Accuracy (mean ± C.V.%)
50	51.0	± 1.7	103.0 ± 3.1
100	104.0	± 9.6	104.0 ± 9.6
200	198.5	± 6.0	99.0 ± 3.0
400	363.4	± 33.0	91.0 ± 8.2

Sensitivity

Concentrations down to 50 ng of maprotiline and desmethyl-maprotiline per ml of blood can be determined accurately. Attempts to use 2 ml of blood instead of 1 ml to improve sensitivity were unsuccessful, because they resulted in a high chemical background.

Plasma interference and selectivity

Fig. 1 shows that there is no interference from the normal constituents of the blood. Known metabolites of maprotiline, other than desmethyl-maprotiline, are hydroxylated or conjugated compounds [1] and they cannot be extracted or detected by our method. A re-extraction step is involved in the extraction procedure. This step is used to purify the extract, thus avoiding some interfering peaks obtained after derivatization.

GLC N-FID and DRID methods are suitable for maprotiline estimation in patient plasma and their precisions are comparable [9, 16], but DRID is a technique not readily accessible to standard laboratories. GLC—ECD using the heptafluorobutyryl derivative of maprotiline [4, 5] was very sensitive when applied to the pure compound. Its application to biological extracts yields a high chemical background.

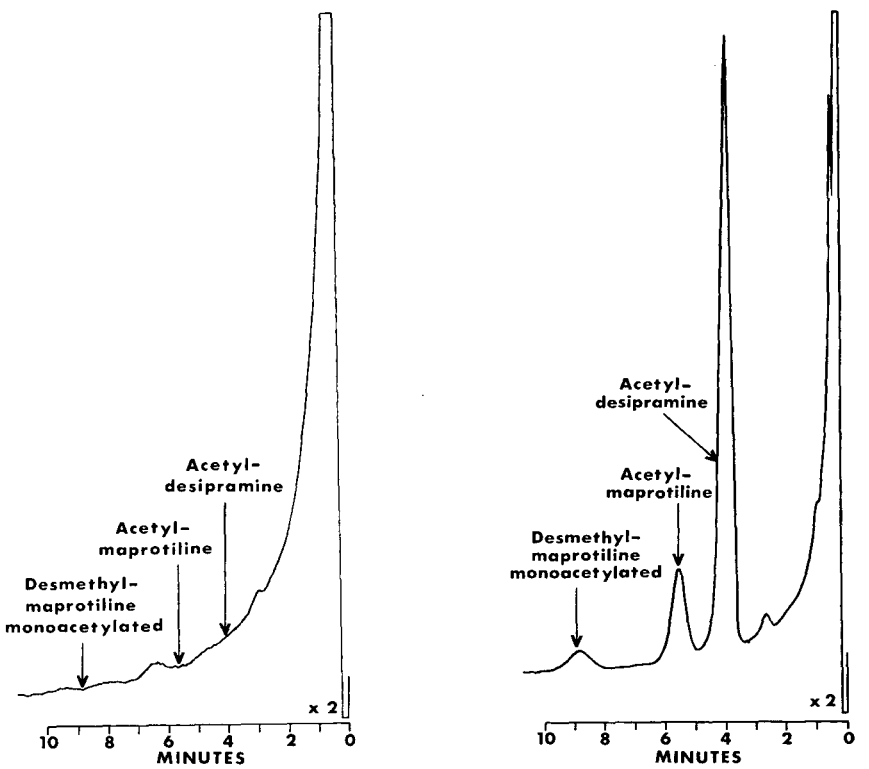

Fig. 1. Example of a chromatogram of a 1-ml human blood blank (the arrows indicate the location of the derivatized amines).

Fig. 2. Example of a chromatogram of an actual blood sample containing (see text) 200 ng of desipramine, 70 ng of maprotiline, and 51 ng of desmethyl-maprotiline.

Our results are comparable to those of Gupta et al. [15], but our method offers the advantage of determining maprotiline and desmethyl- maprotiline in whole blood.

Fig. 2 shows the chromatogram of a blood sample withdrawn on the 19th day from a subject who received one 25-mg Ludiomil® tablet twice a day (50 mg of maprotiline hydrochloride per day for a period of 19 days).

Chromatographic conditions

Silicone stationary phases such as OV-17 (50—50 Me—Ph silicone), SP 2250 DB (50—50 Me—Ph silicone modified for use with basic compounds), OV-25 (25—75 Me—Ph silicone) or SP 2401 DB (trifluoropropyl silicone modified for use with basic compounds) are not suitable for the GLC of acetyl-maprotiline. Acetylated maprotiline and desmethyl-maprotiline either do not separate from each other or are not separated from interference peaks.

More polar stationary phases such as DEGS (diethylene glycol succinate, polyester) or SP 1000 (Carbowax + substituted terephthalic acid) are not suitable for temperatures higher than 220°C.

High-temperature stationary phases such as Poly-S-179 (polyphenyl ether sulfone) and Poly-MPE (polymetaphenoxylene) give peaks with considerable tailing.

The cyclohexanedimethanol succinate (HI-EFF-8BP) has proved very satisfactory for the GLC of acetylated maprotiline and its metabolite; peaks due to extractable impurities elute early, while the desired peaks appear well separated and symmetrical.

CONCLUSION

The described GLC N-FID method makes it possible to determine maprotiline and desmethyl-maprotiline quantitatively in whole blood. In laboratories that are not equipped with the instrumentation necessary for the DRID method [2, 3], this method can be used to monitor maprotiline levels during chronic treatment.

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Note

Gas chromatographic determination of clonazepam

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Clonazepam is an anti-epileptic drug for which therapeutic monitoring of plasma levels is frequently requested. Because the therapeutic concentration is very low (30—60 ng/ml) [1] drug analysis has been limited to gas chromatography (GC) with electron-capture detection. Clonazepam has been determined without derivatisation [2], as the ethylated derivative [3], and, most commonly by hydrolysis in acid to 2-amino-2′-chloro-5-nitrobenzophenone (ACNB) [4]. ACNB has the advantage of being a stable derivative which gives excellent peak profiles and quantitation. The main disadvantages of the latter method are the prolonged acid hydrolysis step requiring a minimum of 20 min, and difficulty in separating the product from plasma constituents and contaminants by GC. This paper reports a study to improve the yield and rate of hydrolysis of clonazepam to ACNB and other minor improvements to the assay.

MATERIALS AND METHODS

Chemicals

Borate buffer, pH 9.0 [4] (contaminants if present may be removed by extracting with toluene) and 2 *M* sodium hydroxide were used. Concentrated hydrochloric and sulphuric acids from J.T. Baker (Phillipsburg, NJ, U.S.A.) were diluted as required. It is essential that the concentrated hydrochloric acid be extracted with toluene. The solvents diethyl ether, heptane, methanol obtained from May and Baker, Dagenham, Great Britain and toluene from Baker were distilled before use.

The standard, clonazepam (Roche, Dee Why, Australia) was 0.015 g in 100 ml of ethanol. Dilute 100 μl to 40 ml (375 ng/ml) with acetone—heptane (1:4, v/v) [4]. The internal standard was desmethylflunitrazepam (Roche) 700 ng/ml in acetone—heptane (1:4, v/v).

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Gas chromatography

A Pye-Unicam 204 equipped with a ^{63}Ni electron-capture detector was used. The temperature settings were: detector 350°C; column 255°C and injector 300°C. The flow-rate was 120 ml/min of oxygen-free nitrogen. No purge gas was used. Glass columns (1.5 m × 4 mm I.D.) were packed with 3% OV-17 or 3% OV-225 on 100—120 mesh Gas-Chrom Q (Applied Science Labs., State College PA, U.S.A.). For the routine method a column packed with a mixture of equal parts by weight of these two phases is used.

Standard method

Internal standard (50 μl), borate buffer (1 ml), and diethyl ether—heptane (40:60, v/v, 2 ml) were added to 0.5 ml of plasma. After shaking and centrifuging, the upper phase was back-extracted into concentrated hydrochloric acid (0.5 ml) in Kimax screw-cap tubes.

After centrifuging, the upper phase was aspirated off and methanol (1.5 ml) and diethyl ether (0.5 ml) were added. The tubes were capped and heated in a boiling water bath for 10 min, then cooled and 3 ml of 2 *M* sodium hydroxide and 2 ml of diethyl ether added. After extraction the ether phase was recovered, evaporated in a stream of air and the residue taken up in 50 μl of toluene. An aliquot of 1—2 μl was injected into the chromatograph.

Hydrolysis experiments

Experiments were done to test the effect of various acids with or without the addition of solvents on the yield of ACNB from clonazapam. Aliquots of clonazepam and desmethylflunitrazepam standards were placed in tubes and the solvent evaporated off. The required mixture of acid and solvent was then added and the tubes heated in a boiling water bath for the required time. The tubes were then cooled as quickly as possible and the hydrolysate prepared for GC as previously described. Yield of ACNB was assessed solely in terms of peak height of replicate 1-μl injections.

RESULTS

The effect of various acids and solvent mixtures on the conversion of clonazepam to ACNB when hydrolysis time is restricted to 15 min or less, is depicted in Fig. 1. Hydrolysis with concentrated hydrochloric acid gives the lowest yield of ACNB. Yield is greatly increased by the addition of methanol to the concentrated hydrochloric acid, the maximum yield being obtained with a ratio of concentrated hydrochloric acid—methanol of 0.5:1.5, v/v.

Greater amounts of methanol depress the amount of ACNB formed. Addition of diethyl ether to concentrated hydrochloric acid—methanol (0.5:1.5) further increases the yield at 10 min and is optimal with the mixture concentrated hydrochloric acid—methanol—ether (0.5:1.5:0.5). Further small increases in the proportion of diethyl ether has no additional effect on the yield, while excess diethyl ether results in phase separation. Addition of diethyl ether to concentrated hydrochloric acid without methanol depresses the yield.

With the mixture concentrated hydrochloric acid—methanol—diethyl ether (0.5:1.5:0.5), the maximum yield is obtained by boiling the tubes for 10 min.

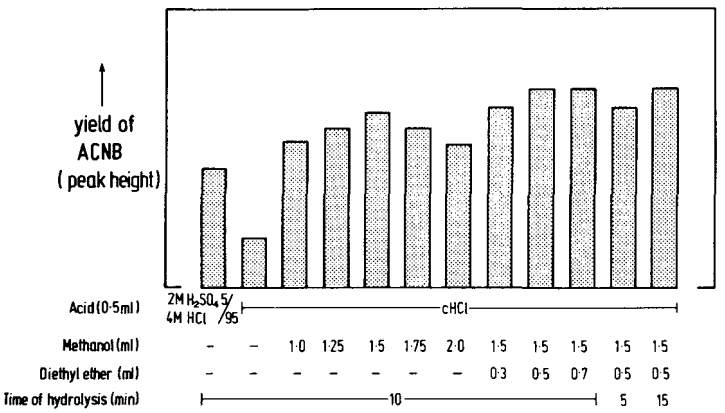

Fig. 1. Yield of ACNB from the hydrolysis of clonazepam at 100° C in various acid and solvent conditions.

After 5 min of heating the yield is approximately 90% of maximum and heating for 15 min gives a yield identical to that obtained at 10 min. Compared to the method presented in this paper the widely used mixture of 2 *M* sulphuric acid—4 *M* hydrochloric acid (5:95) produces less ACNB (93%) and requires a much longer period of heating (30 min).

The within-run coefficient of variation (C.V.) at a clonazepam concentration of 39.2 ng/ml for this method is 3.7% and the between-run C.V. is 6.7%. The detector response is linear over a plasma clonazepam concentration of 5—100 ng/ml and the minimum detectable concentration of clonazepam is less than 2 ng/ml. Typical chromatograms are shown in Fig. 2.

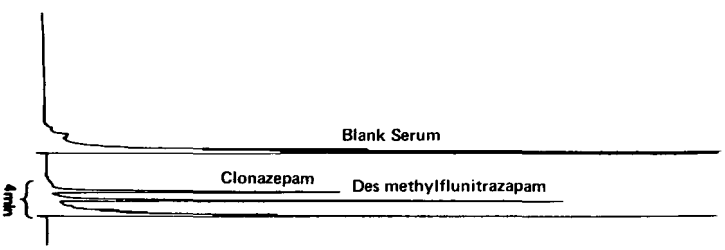

Fig. 2. Chromatograms, after extraction and hydrolysis of a serum containing clonazepam (40 ng/ml) and desmethylflunitrazepam, and of a blank serum.

DISCUSSION

Hydrolysis reactions are commonly solvent dependent. These experiments show that clonazepam can be hydrolysed to ACNB with optimum yield, within 10 min by using a mixture of concentrated hydrochloric acid—methanol—diethyl ether (0.5:1.5:0.5). Not only is the assay time shortened but yields are improved. Also side-reactions and contaminants encountered in other methods we have tried have been minimized. Experiments not reported here have shown that desmethylflunitrazepam and nitrazepam are hydrolysed faster than clonaz-

epam. Nitrazepam can be used as an alternative internal standard to desmethylflunitrazepam.

Diethyl ether is commonly used as the extraction solvent for clonazepam. In these studies it was found to co-extract unwanted plasma constituents. This was overcome by using heptane—diethyl ether (60:40, v/v). Failure to use freshly redistilled diethyl ether gives rise to side-reactions which affect quantitation. Similarly, concentrated hydrochloric acid also may contain products which give side-reactions [4]. These may be either removed or destroyed by washing the acid with toluene. Occasionally concentrated hydrochloric acid so treated discolours on standing, but this has no discernable effect on the method. The extraction of concentrated hydrochloric acid was a crucial step in obtaining a clean chromatogram.

On OV-17 co-extracted plasma constituents are not well separated from the internal standard while with OV-225, analysis time is excessive. A liquid phase of intermediate polarity would be ideal but we have found a mixture of equal parts by weight of the two phases suitable for routine work.

No other anticonvulsants are known to interfere with the assay. However, nitrazepam is only partly resolved from the internal standard on the mixed phase column at 255° C. In such a case OV-225 would be the phase of choice.

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CHROMBIO. 685

Note

Gas chromatographic method for the determination of flumecinol in biological fluids

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Flumecinol (Zixoryn®*; RGH-3332; 3-trifluoromethyl-α-ethyl-benzhydrol) synthesized by Tóth et al. [1] is a new enzyme inducer that induces — on the basis of animal and human experiments — the endoplasmic reticulum-bound mixed-function oxidase system in the liver [2—4].

In order to study the role of flumecinol in humans and dogs it was necessary to elaborate a specific and sensitive method for the quantitative determination of flumecinol in biological samples. Our method is based on a simple extraction and gas—liquid chromatography (GLC) of flumecinol-containing samples.

EXPERIMENTAL

Materials

Flumecinol and internal standard (3-trifluoromethyl-benzhydrol) were the products of G. Richter Ltd. (Budapest, Hungary). Chloroform, potassium hydroxide and sodium citrate were the products of Reanal (Budapest, Hungary) and were of analytical grade. Diethyl ether puriss. (Ferak, Berlin, G.F.R.) and chloroform were carefully purified by distillation before use.

The specific activity of [1-^{14}C]flumecinol was 10.92×10^7 Bq/mmol (2.95 mCi/mmol).

Preparation of calibration curves

To 2 ml of human plasma or 1 ml of dog plasma, 600 ng of 3-trifluoromethyl-benzhydrol (internal standard) and flumecinol (5—300 ng for human

*Manufacturer: Chemical Works of Gedeon Richter Ltd., Budapest, Hungary.

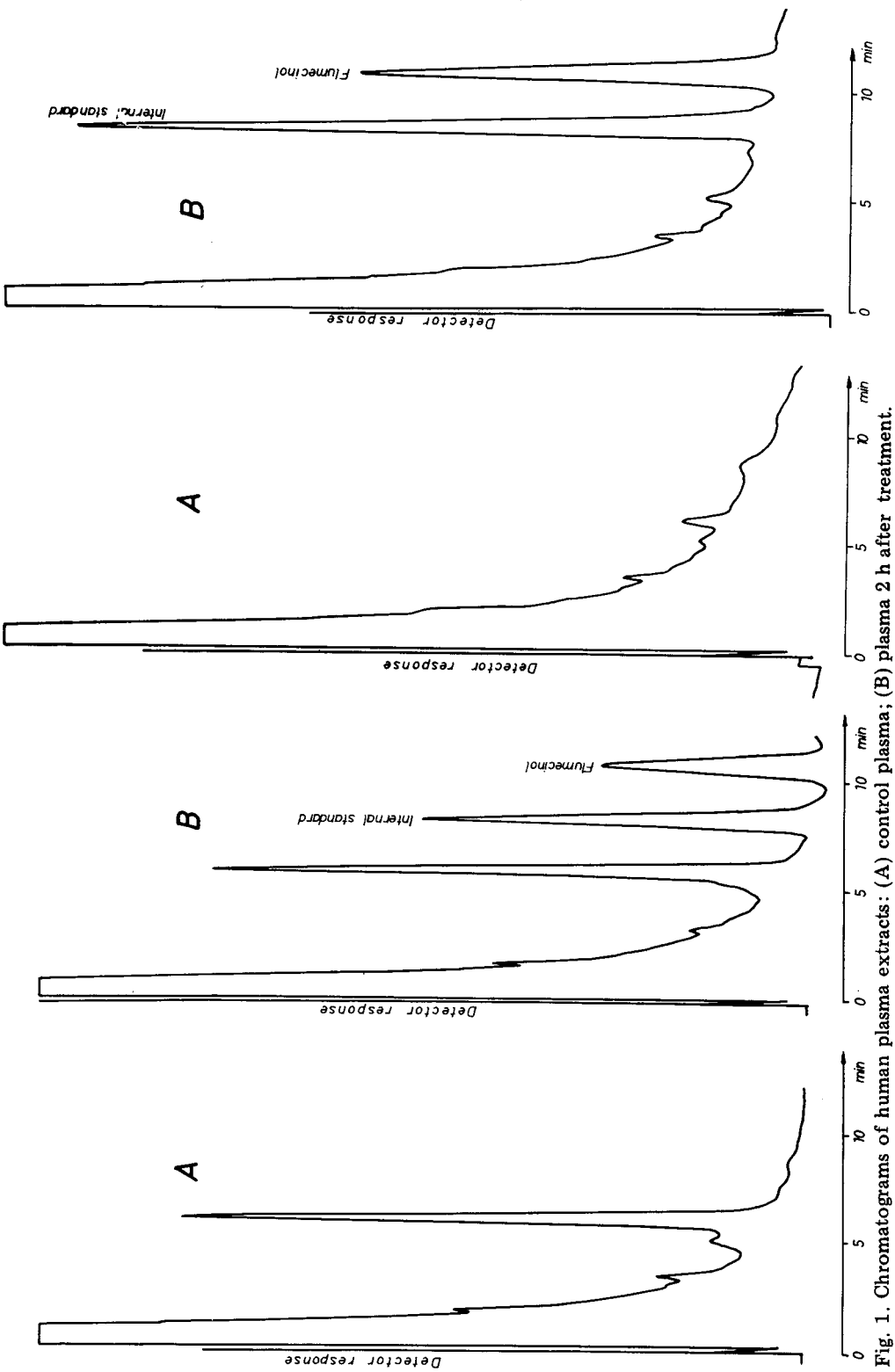


Fig. 1. Chromatograms of human plasma extracts: (A) control plasma; (B) plasma 2 h after treatment.

Fig. 2. Chromatograms of urine extracts: (A) control urine; (B) urine containing flumecinol and internal standard.

samples, and 100—800 ng for dog samples) were added in chloroform. After the addition of 0.6 ml of 2 *N* KOH the samples were extracted with 4 ml of diethyl ether by shaking for 15 min and centrifugation at 3000 *g* for 15 min. The diethyl ether was transferred to test-tubes and evaporated at room temperature. The dry residue was dissolved in 30 μl of chloroform and 1 μl was injected into the gas chromatograph.

For quantification the ratio of peak area of drug to that of the internal standard was used. The recovery of flumecinol was detected by using [^{14}C] flumecinol.

Gas—liquid chromatography

A Hewlett-Packard Model 5736A gas chromatograph with flame-ionization detector was used. The electronic parameters were: range 1, attenuation 2.

Nitrogen of high purity was used as carrier gas. The column packing was a phase containing 10% OV-1 on 80—100 mesh Chromosorb G AW DMCS (Applied Science Labs., State College, PA, U.S.A.) packed in a 0.9 m × 4 mm I.D. glass tube. The column was conditioned at 240°C for 24 h under a gas flow of 30 ml/min. The column was operated at 185°C with a nitrogen flow-rate of 20 ml/min. The temperature settings were injector port 300°C, detector 300°C.

Determination of radioactivity

Radioactivity of liquid samples was counted in a Packard Tri-Carb liquid scintillation spectrometer Model 3310. The aqueous samples were counted in Insta-Gel^{R} (Packard, Downers Grove, IL, U.S.A.) and the diethyl ether samples in a toluene-based liquid scintillation solution (5 g of PPO, 0.1 g of dimethyl POPOP, 100 ml of toluene; Reanal). For the determination of the absolute activity the external standard—channel ratio method was used.

In vivo experiments

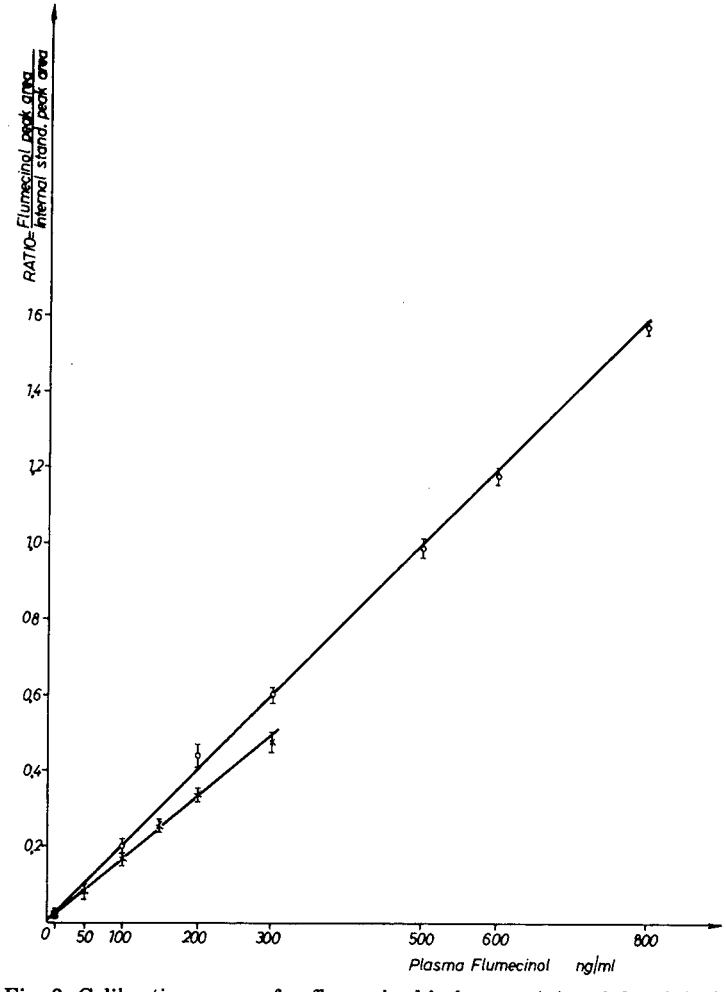
Flumecinol was administered in a single oral dose for the pharmacokinetic examination of the drug. The human dose was 100 mg and that for dog 40 mg/kg.

Sodium citrate (3.8%, w/v) was added to the human or dog blood samples in a ratio of 1:9, to prevent coagulation. To 2 ml of human plasma, 1 ml of dog plasma, or 1 ml human urine, 600 ng of 3-trifluoromethyl-benzhydrol (internal standard) were added in 60 μl of chloroform and the samples were processed as above.

RESULTS AND DISCUSSION

Flumecinol is a compound specifically developed for inducing the mixed-function oxidases in liver and thus increasing the metabolic elimination of endogenous and exogenous compounds. As flumecinol influences the pharmacokinetics of many drugs and possibly even the elimination of the drug itself, it seemed rather important to have a specific and sensitive method for the quantitative determination of flumecinol in biological fluids.

Earlier experiments [5] have shown that the application of electron-capture detection for the sensitive detection of the compound failed, most probably



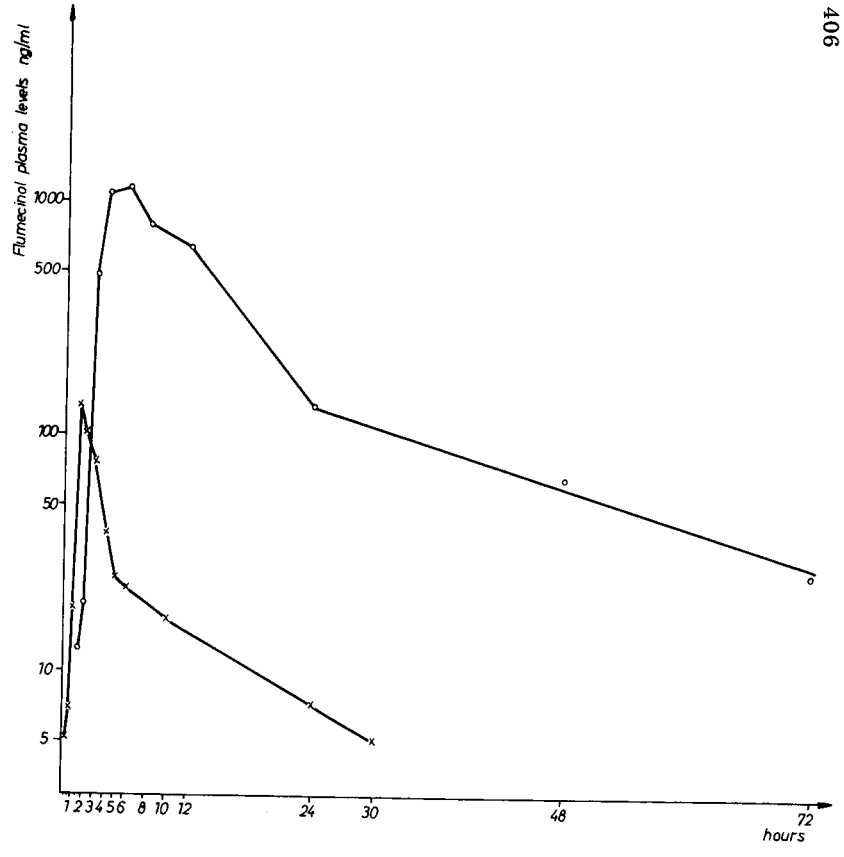


Fig. 3. Calibration curves for flumecinol in human (×) and dog (○) plasma.

Fig. 4. Human (×) and dog (○) plasma flumecinol concentration—time curves after oral administration.

due to steric effects. Therefore, we turned our attention to the less-sophisticated flame-ionization detection.

As flumecinol does not contain any ionizable group it was not possible to purify samples prior to GLC analysis by the acid—base washing technique generally used. We have found that samples extracted under basic conditions (as described in Methods) with diethyl ether gave samples pure enough for the GLC analysis (Fig. 1). The retention times of flumecinol and the internal standard (3-trifluoromethyl-benzhydrol) were 12 and 9 min, respectively.

The method can also be applied to dog plasma and to urine (Fig. 2).

The calibration curves obtained with human and dog plasma show a good linearity between 5 and 800 ng/ml flumecinol. The standard deviation (S.D.) for the calibration curve of human and dog samples was found to be 7.13% and 5.76%, respectively (Fig. 3). Recovery of flumecinol from plasma samples was checked by using radiolabelled drug and was found to be 75.02 ± 1.847% (S.D.).

In Fig. 4 the plasma concentration—time curves are shown for human and dog experiments, demonstrating that the method is sensitive enough for monitoring plasma concentrations after the administration of therapeutic doses.

CONCLUSIONS

A method for determining flumecinol (Zixoryn®) concentrations in biological fluids (human plasma and urine, and dog plasma) at minimum levels of 5 ng/ml has been elaborated. The simple and rapid method is appropriate for routine analysis and pharmacokinetic studies.

ACKNOWLEDGEMENTS

Thanks are due to G. Zólyomi (Institute for Pharmaceutical Research, Budapest) for synthesis of the labelled flumecinol and to Mr. G. Trafikánt for his excellent technical assistance.

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Note

Analysis of procyclidine in human plasma and urine by gas—liquid chromatography

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Procyclidine [1-cyclohexyl-1-phenyl-3-(pyrrolidin-1-yl)-propan-1-ol hydrochloride; Kemadrin, Fig. 1a] is a synthetic anticholinergic compound therapeutically useful in the treatment of idiopathic and neuroleptic induced Parkinson's disease as shown in controlled trials [1,2]. The only existing method [3] for the quantitative determination of procyclidine in either plasma or urine is based on a gas—liquid chromatographic (GLC) procedure which includes an inconvenient initial isolation step and lacks adequate sensitivity for detailed pharmacokinetic studies. The GLC method described here has adequate sensitivity for bioavailability and pharmacokinetic studies. It involves a solvent extraction under alkaline conditions with imipramine (Fig. 1b) as internal standard, followed by quantitation on a gas—liquid chromatograph fitted with a nitrogen—phosphorus detector.

EXPERIMENTAL

Reagents

Procyclidine·HCl was obtained from the Wellcome Foundation (Dartford, Great Britain) and imipramine·HCl from Sigma (Poole, Great Britain). Cyclohexane and toluene, both of Analar grade (Fisons, Loughborough, Great Britain), were glass distilled before use. Freshly deionised water was used for rinsing glassware and in all aqueous solutions.

Glassware

Stoppered test tubes (20 ml, Sovirel type 611-03; V.A. Howe, London, Great Britain) were used for the extraction and autosampler microtubes (tube 834078-902; Du Pont (U.K.), Hitchin, Great Britain) were used in the drying

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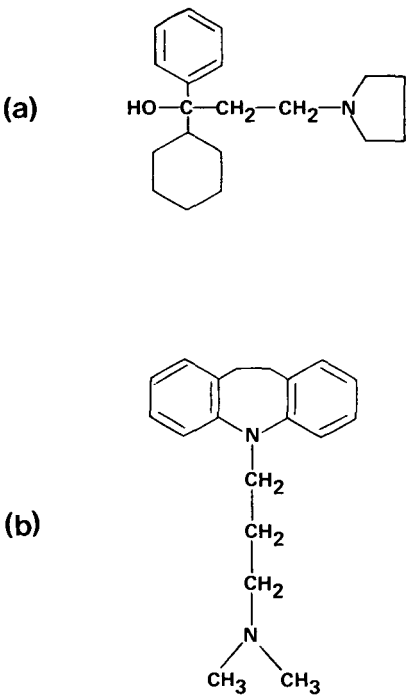

Fig. 1. Molecular structures of procyclidine (a) and imipramine (b).

stage. All glassware was washed with 2 *M* hydrochloric acid and rinsed with water before use. The microtubes were dried in a vacuum oven before use (210°C, <80 mm Hg vacuum for 1 h minimum).

Method

Standard solutions of procyclidine were prepared by dilution of a 1 mg/ml aqueous stock solution with either plasma or urine, depending on the nature of the samples to be assayed. The standards in plasma and urine covered the ranges 0—1 μg/ml and 0—2 μg/ml respectively.

A 1-ml aliquot of standard or sample was placed into an extraction tube and to it were added 40 μl of a 10 μg/ml aqueous solution of imipramine (as internal standard), 1 ml of 1 *M* sodium hydroxide and 4 ml cyclohexane. The tube was then tightly stoppered and mixed for 20 min along its long axis at 25 oscillations per min. The liquid phases were then separated by centrifugation at 1200 *g* for 10 min. A 3-ml aliquot of the cyclohexane layer (top) was then transferred to a microtube and the cyclohexane evaporated at room temperature under a stream of nitrogen.

A further 3 ml of cyclohexane were added to the aqueous layer and the extraction procedure repeated. Cyclohexane (3 ml) from the second extraction was then added to the residue in the corresponding microtube and dried as before. The resultant, combined residue was taken up in 50 μl glass-redistilled toluene about 10 min before injection into the gas chromatograph. All samples were analysed in duplicate.

Gas—liquid chromatographic conditions

The gas chromatograph used was a Model F30 Perkin-Elmer (Beaconsfield, Great Britain) equipped with a nitrogen—phosphorus detector (Perkin-Elmer).

A 1.8 m × 4 mm I.D. glass column was hand packed with 5% OV-17 on Chromosorb W HP (100—120 mesh) and conditioned at 310°C with a 50 ml/min helium carrier gas flow for 24 h before use.

The detector was used in the nitrogen mode under the following conditions: gas flow-rates, hydrogen (8.5 ml/min); air (92 ml/min); carrier gas (helium, 50 ml/min). Manifold, oven and injection port temperature were 321°C, 246°C and 310°C respectively.

Under these conditions procyclidine was well resolved from imipramine, these two components having retention times of 5.85 min and 7.10 min respectively. No interfering peaks were seen in either human plasma or urine.

RESULTS

Calculation of results

A Hewlett-Packard Model 3352 data system was used to calculate the peak areas of procyclidine and the internal standard and their ratios. A known mass of the internal standard (0.4 μg of imipramine) was added to a range of procyclidine standard solutions. A calibration curve was constructed by plotting the concentrations of procyclidine on the abcissa against the ratio of the peak area of procyclidine to that of imipramine on the ordinate. Since the same known mass of internal standard was added to the unknown samples (urine or

TABLE I

METHOD PRECISION AND REPRODUCIBILITY WHEN KNOWN AMOUNTS OF PROCYCLIDINE WERE ADDED TO HUMAN PLASMA OR URINE AND REPEATEDLY ANALYSED

$n = 6$.

Standard concentration of procyclidine (ng/ml)	Ratio of peak area of procyclidine to peak area of internal standard		
	Mean	±S.D.	S.D. of mean (%)
Plasma			
20	0.021	0.0025	11.9
50	0.059	0.0034	5.8
100	0.129	0.0062	4.8
200	0.260	0.0140	5.4
500	0.651	0.0282	4.3
1000	1.298	0.0739	5.7
Urine			
50	0.0613	0.0048	7.83
100	0.1362	0.0034	2.52
200	0.2743	0.02295	8.37
500	0.6940	0.01691	2.44
1000	1.4473	0.1063	7.34
2000	2.971	0.2178	7.33

plasma) the amount of procyclidine in the samples could be calculated from the calibration curve.

Validation

The method was validated by analysing samples of plasma to which known quantities of procyclidine had been added. Six determinations were made for each sample and the precision obtained for concentrations of procyclidine between 50 and 2000 ng/ml is shown in Table I; percentage mean S.D. ranged from 2.5 to 8.4%. At maximum sensitivity (20 ng/ml) the percentage mean S.D. was 11.9. The results show that the recovery of procyclidine added was

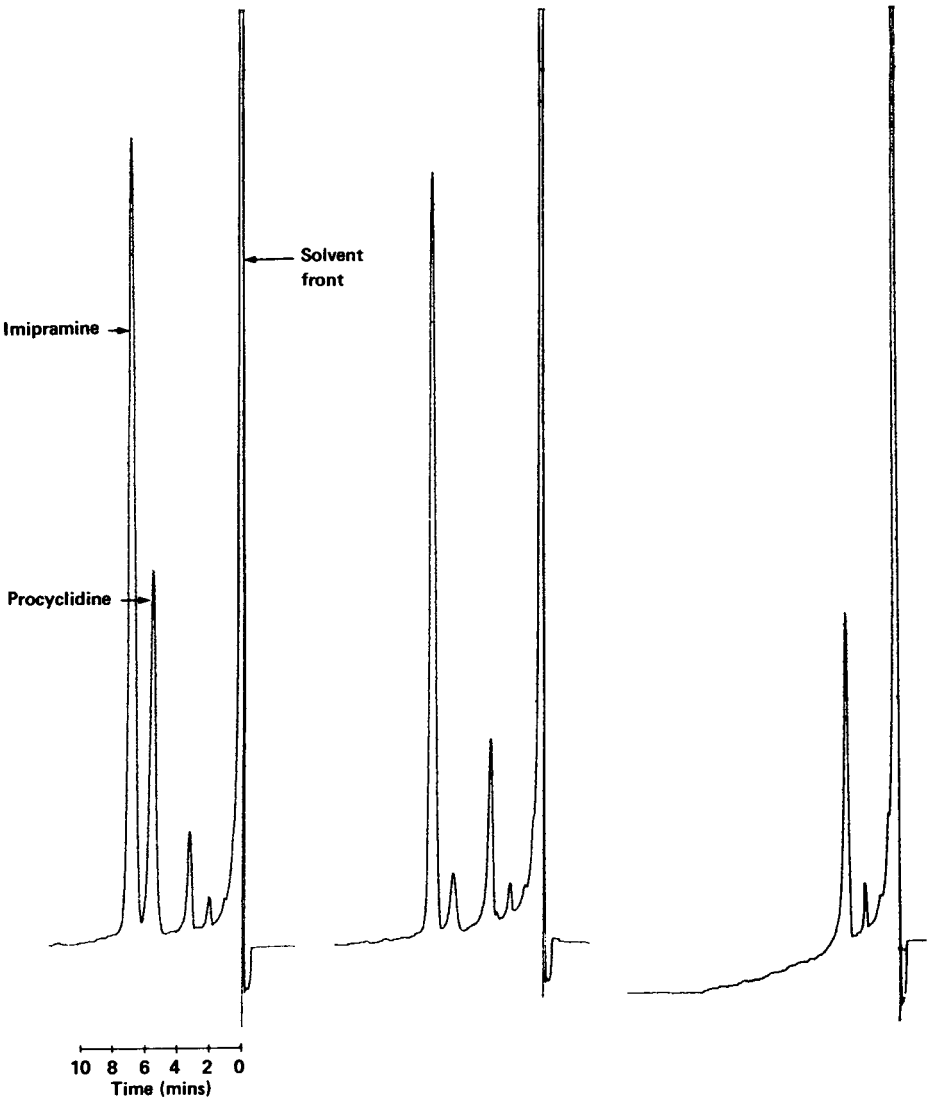

Fig. 2. Chromatograms produced from urine containing (left) 500 and (middle) 100 ng/ml procyclidine with internal standard and (right) a blank urine extract.

linear relative to the drug standards. The calibration curve was described by a straight line using a linear regression programme, Texas TI-51 (III) calculator. For plasma $y = 0.00126x - 0.0018$; for urine $y = 0.00149x - 0.0266$ with correlation coefficients of 0.9979 and 0.9960 respectively, where y = ratio of peak areas of procyclidine and imipramine and x = plasma concentration of procyclidine (ng/ml).

The detector response was linear from 1.6 to 160 ng procyclidine injected onto the column. The lower limit of sensitivity was 1—2 ng injected on column under the standard conditions. No interfering peaks were seen (Figs. 2 and 3) even at high sensitivity. Under the operating conditions described the method is capable of detecting as little as 20 ng/ml of procyclidine in plasma or urine.

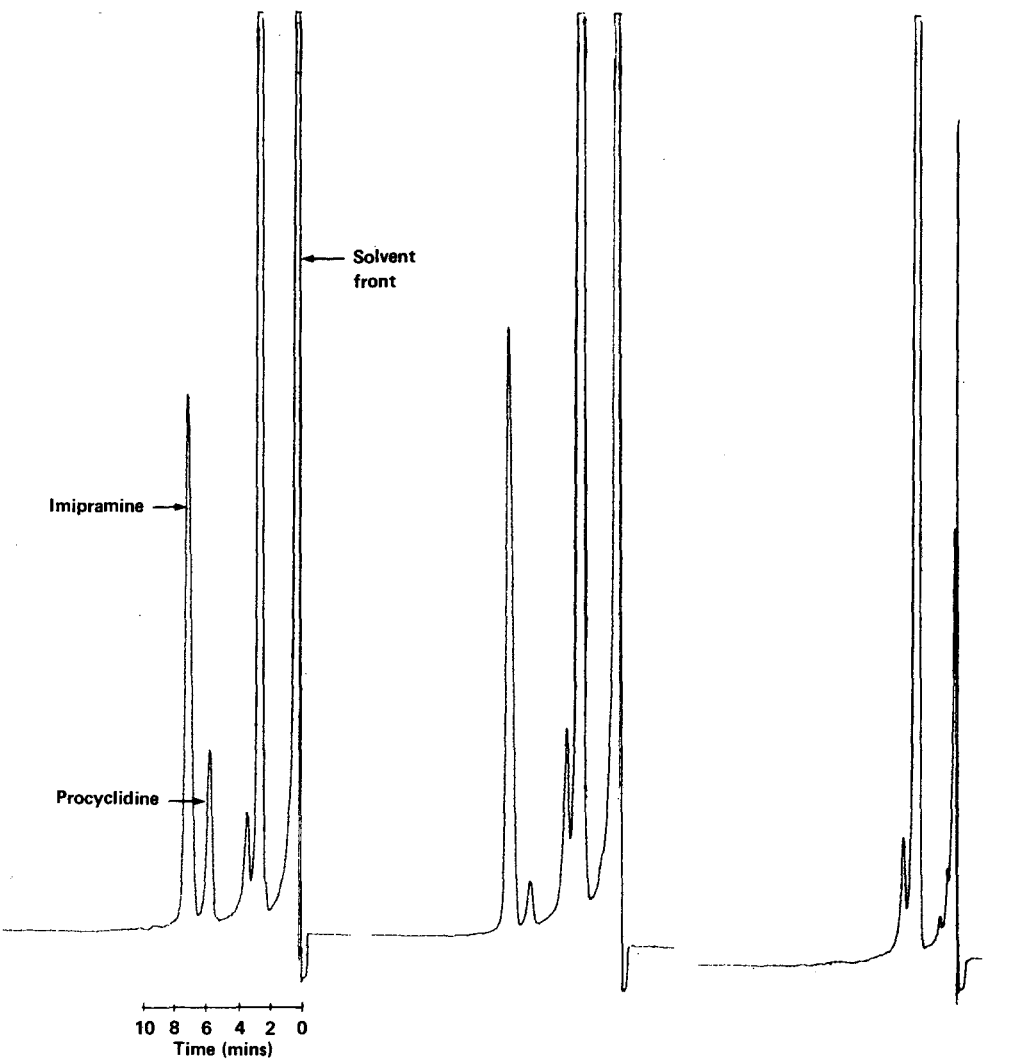

Fig. 3. Chromatograms produced from plasma containing (left) 200 and (middle) 50 ng/ml procyclidine with internal standard and (right) a blank plasma extract.

An example of the plasma concentration curve from a healthy male volunteer after receiving 10 mg of procyclidine orally is shown in Fig. 4.

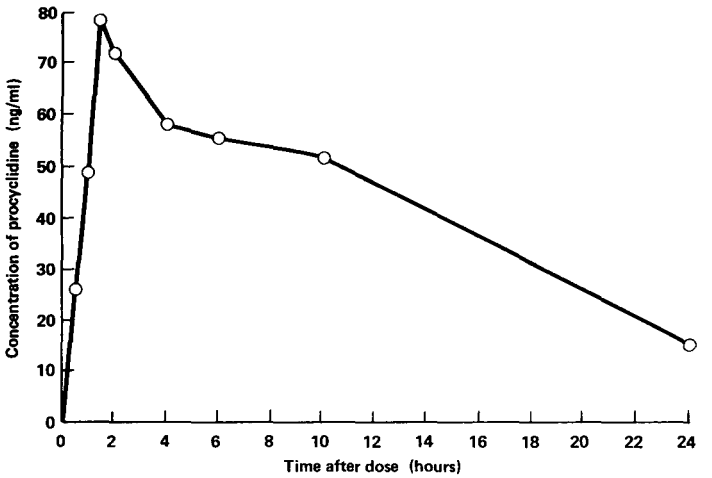

Fig. 4. Plasma profile from a healthy adult male volunteer after 10 mg of procyclidine was given orally.

DISCUSSION

The GLC method described had good precision, was specific for unchanged procyclidine and was also extremely sensitive, 1.6 ng of procyclidine injected onto the column was readily detected. Several precautions were necessary to ensure reproducible results. Some procyclidine is adsorbed onto glass; this causes carry-over from the glass syringe used to inject the samples. This problem can be overcome by washing the syringe in chloroform between injections, as previously reported for the analysis of trimethoprim [4]. Also the extraction tubes and the microtubes used during the extraction procedure should always be soaked in HCl between assays in order to prevent any carry-over of procyclidine. The use of an internal standard reduces error from transfer losses.

The method described is used routinely for the analysis of procyclidine in human plasma and urine and is sufficiently sensitive for bioavailability and pharmacokinetic studies.

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Note

Rapid gas chromatographic method for emergency determination of paracetamol in human serum

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(First received May 28th, 1980; revised manuscript received July 25th, 1980)

The increasing use of paracetamol as an analgesic has resulted in an increase in the number of acute cases of poisoning admitted to hospitals. Clinicians have therefore expressed the desire to have this drug analysed quantitatively by the clinical chemical laboratory on demand, 24 h a day. This desire is well motivated as overdoses of paracetamol are strongly hepatotoxic. The measured serum concentration can determine whether treatment with an antidote, in itself a somewhat critical treatment, should be initiated [1].

An analytical method that is to be used for acute determination of paracetamol must be both rapid and specific, but the sensitivity required in pharmacokinetic studies is not required in cases of poisoning.

Many methods have been described for quantitative determination of paracetamol in serum. Wiener [2] has recently given a review of methods for paracetamol estimation.

Spectrophotometric methods, which up to now have been widely used for the quantitative determination of paracetamol in serum, have not all been specific [3]. Without prior extraction the methods in which paracetamol is measured colorimetrically, following acid hydrolysis to *p*-aminophenol, will also measure inactive glucuronide and sulphate metabolites of paracetamol. The measured concentration will then be falsely elevated, perhaps leading to a misguided decision to start treatment with an antidote.

Gas chromatography (GC) is a more specific analytical method for paracetamol than spectrophotometry. Several GC methods have been described in the literature [2,4—9]. The differences between these methods are in the more or less time-consuming extraction procedures and in the use of different internal standards and different derivatising reagents. In some methods the speed

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of analysis is compromised by the occurrence of unknown peaks with long retention times [6,9].

None of the methods described combine speed of sample preparation with a rapid chromatographic analysis and none of the methods use the structural analogue 2-acetaminophenol as an internal standard. The present method for GC determination of paracetamol is characterised by a simple, rapid extraction procedure, the use of a structural analogue as internal standard and a simple, rapid procedure for derivative formation. Chromatograph settings are chosen so that samples can be injected every 3 min. There are no interfering peaks. The speed and specificity of this method make it suitable for the acute determination of serum paracetamol concentrations.

EXPERIMENTAL

Apparatus

A Pye Unicam GCD gas chromatograph equipped with flame ionisation detector was used. The 90 cm × 2 mm I.D. glass column was packed with 2.8% OV-210 and 3.2% OV-1 on Chromosorb W HP (80—100 mesh). The instrument settings were as follows: temperatures: column, 120°C; injection port, 200°C; detector, 250°C; flow-rates: nitrogen carrier, 40 ml/min; hydrogen, 40 ml/min; air, 400 ml/min; range 10 × 32 and recorder speed 10 mm/min.

Materials

The following reagents were used: ethyl acetate and toluene (analytical grade; Merck, Darmstadt, G.F.R.) trifluoroacetic anhydride (Pierce, Rockford, IL, U.S.A.), paracetamol (Sterling Winthrop, New York, NY, U.S.A.), 2-acetaminophenol (pract.; Fluka, Buchs, Switzerland).

A working solution of internal standard contained 15 mg/l 2-acetaminophenol in ethyl acetate and a stock solution of 60 mmol/l paracetamol in ethanol which was stable for at least 1 year at +4°C.

Paracetamol standards containing 600, 1200 and 1800 μmol/l were prepared by adding drug-free human serum to 50-, 100- and 150-μl aliquots, respectively, of paracetamol stock solution (60 mmol/l) and making up to a total volume of 5.00 ml. The standards were stable for at least 18 months at −20°C.

Sample preparation

Duplicate samples (500 μl) of standards or patient sera were pipetted into conical centrifuge tubes (100 × 16—17 mm) and 2.5 ml ethyl acetate containing the internal standard, 2-acetaminophenol (15 mg/l), added. The samples were rotated for 5 min on a blood mixer (25 r.p.m.) and centrifuged for 5 min at 600 *g*. An amount of 1.00 ml of the ethyl acetate phase was transferred to a conical tube and evaporated in a water bath at a maximum of 50°C in a stream of nitrogen. Twenty-five μl toluene and 25 μl trifluoroacetic anhydride were added to the dry residue. After vigorous mixing on a vortex mixer for 5 sec, approximately 1 μl was injected into the gas chromatograph.

RESULTS AND DISCUSSION

The retention time for the internal standard, 2-acetaminophenol was 1.0 min under the conditions described. The retention time for paracetamol was 1.9 min (Fig. 1B). Thus a new extract could be injected every third minute.

Peak heights for standards and samples were measured. Standard concentrations (600, 1200 and 1800 μmol/l) were plotted against the ratio of paracetamol peak height to internal standard peak height. Paracetamol concentrations in patient samples were estimated from the standard curve obtained. The standard curve for paracetamol was linear up to 4000 μmol/l ($y = 0.00084x - 0.00116$; $r^2 = 0.9987$) using standards prepared from the stock solution as described in the Materials section.

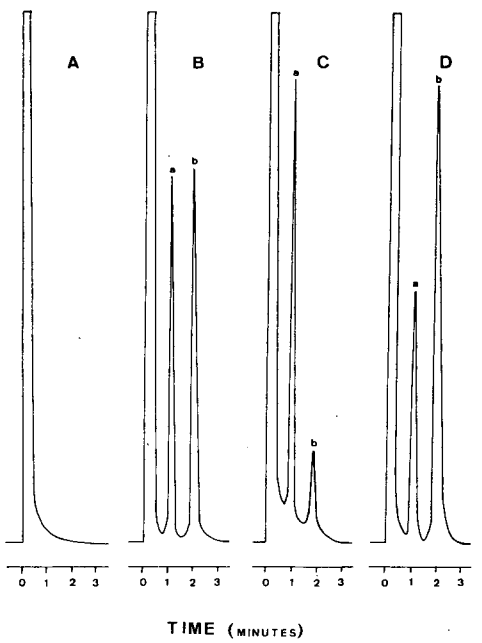

Fig. 1. GC determination of paracetamol in serum. Chromatograms of: (A) blank serum extract containing no internal standard; (B) standard extract containing paracetamol at a level of 1200 μmol/l; (C) serum extract from a patient receiving therapeutic doses of paracetamol corresponding to a serum concentration of 220 μmol/l; (D) serum extract from a poisoned patient with a serum concentration of paracetamol of 2160 μmol/l. Peaks: (a) 2-acetaminophenol (internal standard); (b) paracetamol.

The lower detection limit for the method was about 50 μmol/l. Fig. 1C shows a chromatogram of a serum extract from a patient receiving therapeutical doses of paracetamol (1 g three times a day) corresponding to a serum concentration of 220 μmol/l. Fig. 1D shows a chromatogram of a serum extract from a poisoned patient with a serum concentration of 2160 μmol/l.

The precision of the method was acceptable. The day-to-day coefficients of variation for the three standard levels (600, 1200 and 1800 μmol/l) were 4.5%, 6.6% and 4.5%, respectively ($n = 14$ at all levels). The within-run coefficient of

variation for a single extraction of 1200 μmol/l standard was 4.3% (n = 8). Thus most of the method variation probably occurs during GC.

Sample preparation was a modification of the method described by Alvän et al. [4]. The sample volume and the extraction time were reduced by a factor of four. It was not necessary to extract samples for more than 5 min as longer extraction times did not improve the recovery of paracetamol.

The ratio of toluene to trifluoroacetic anhydride was 1:1 in the present method, compared with 20:1 in the method of Alvän et al. [4]. Acetylated extracts were stable at room temperature for at least 24 h.

Fig. 1A shows the chromatogram of a blank serum extract containing no internal standard. No interfering peaks were observed. The following drugs frequently found in cases of poisoning did not give interfering peaks: barbitone, phenobarbitone, morphine, codeine, ketobemidone, dextropropoxyphene, diazepam, nitrazepam, amitriptyline and nortriptyline.

Fig. 2 shows that salicylic acid, salicylamide and phenacetin gave distinct

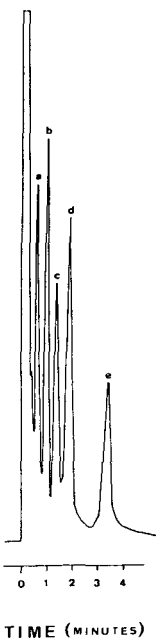

Fig. 2. Chromatogram of trifluoroacetic anhydride derivatives of (a) salicylic acid, (b) 2-acetaminophenol, (c) salicylamide, (d) paracetamol and (e) phenacetin.

peaks in the chromatogram. Furthermore these peaks are clearly separated from those of paracetamol and 2-acetaminophenol. The retention times were as follows: salicylic acid 0.6 min, salicylamide 1.4 min, phenacetin 3.4 min.

Using the structural analogue 2-acetaminophenol as internal standard it is possible to achieve a compensation for the variation in the preparation procedure. 2-Acetaminophenol has the advantage over 3-acetaminophenol used by Alvän et al. [4] in that its chromatographic peak is better separated from the paracetamol peak and that the salicylamide peak does not coincide with the internal standard peak.

A GC emergency procedure requires a gas chromatograph to be on stand-by at all times. The chromatograph used in this laboratory is also used during the day for routine samples and is thus always ready for use in an emergency situation. The column used for paracetamol analysis is also used for the determination of morphine and codeine.

Laboratories with GC equipment should use a GC method for the determination of paracetamol in serum. The present method is rapid and specific and is therefore well suited to the emergency determination of paracetamol in cases of poisoning.

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CHROMBIO. 668

Note

High-performance liquid chromatographic determination of sulphathiazole in human plasma and urine

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Gas chromatographic procedures [1, 2] that have been described for the assay of sulphonamides in body fluids were found to be cumbersome for the assay of sulphathiazole as they require a derivatization step. High-performance liquid chromatographic (HPLC) methods have been also described [3—8]. Sharma et al. [3] described a method for the analysis of sulphathiazole in pure solutions as well as in cattle urine, but as the retention time depends on the nature of the sample (water or urine), it was not applied to plasma. Vree et al. [6] described chromatographic conditions suitable for sulphathiazole but the dilution of the plasma in the sample preparation step limits the sensitivity. A range of sulphonamides in pure solutions are well separated under the conditions described by Cobb and Hill [8] but sulphathiazole is eluted within 20 min.

This paper describes a simple HPLC method for the assay of sulphathiazole in plasma and urine. Plasma samples are injected after protein precipitation by acetonitrile, urine samples after dilution with water.

EXPERIMENTAL

Chemicals

Sulphathiazole, sulphadiazine, sulphamerazine, sulphapyridine, acetonitrile, and pH 4 and 5 buffers (Titrisol) were purchased from Merck (Darmstadt, G.F.R.). Standard solutions of sulphathiazole were made up in distilled water alkalinized with a few drops of 0.1 *N* sodium hydroxide. These solutions were stable for more than two months at 4°C.

The acetyl derivative of sulphathiazole was prepared by acetylation with acetic anhydride—pyridine (1:0.1, v/v) and recrystallization in dioxane—water (1:1, v/v).

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Chromatographic instrumentation and conditions

Chromatography was performed on a Hewlett-Packard 1084A high-performance liquid chromatograph equipped with a fixed-wavelength (254 nm) UV detector. Stainless-steel columns were used; a 12 cm × 7.5 mm I.D. column filled with LiChrosorb RP-8 (5 μm) for plasma, and a 25 cm × 4.7 mm I.D. column filled with LiChrosorb RP-8 (10 μm) for urine. They were packed using the balanced-density-slurry packing technique. The column compartment was at room temperature.

Analytical procedure for plasma and urine

Precipitation of proteins from plasma

In a 10-ml glass centrifuge tube were introduced 500 μl of plasma, 1500 μl of acetonitrile, and 50 μl of distilled water (or calibration solutions). The tube was stoppered and shaken for 30 sec on a Vortex mixer. After a 15-min centrifugation at 2100 *g*, 20 μl were injected onto the column.

Urine dilution

In a 10-ml stoppered glass centrifuge tube were mixed 50 μl of urine, 2400 μl of distilled water, and 50 μl of distilled water or calibration solution; 20 μl of this solution were injected.

Chromatography

Plasma. A precolumn filled with Whatman Co:Pell ODS (10 cm × 4.7 mm I.D.) was used to protect the separation column. The degassed mobile phase, pH 5 buffer—acetonitrile (80:16, v/v), thermostated at 40°C, was used at a flow-rate of 2 ml/min. The column-top pressure was about 80 bars.

Urine. The degassed mobile phase, pH 4 buffer—acetonitrile (92:8, v/v), thermostated at 40°C, was used at a flow-rate of 2.5 ml/min. The column-top pressure was about 100 bars.

Calibration curves. Aliquots of 50 μl of different aqueous solutions of sulphathiazole were added to 500 μl of plasma to produce reference samples in the range of concentrations 250—20,000 ng/ml of plasma. Urine calibration samples were obtained by adding 50 μl of different aqueous solutions of sulphathiazole to 50 μl of urine and 2400 μl of distilled water. The range of concentrations was 2.5—100 μg/ml of urine. The calibration solutions were stable for one week at 4°C.

RESULTS AND DISCUSSION

Sensitivity, reproducibility and accuracy

Various spiked plasma and urine solutions were prepared and analysed several times. The results, summarized in Tables I and II, show that the proposed procedure permits the accurate determination of sulphathiazole at concentrations down to 250 ng/ml of plasma and 2.5 μg/ml of urine. Lower concentrations could be determined with a lower accuracy.

TABLE I

PRECISION AND RECOVERY OF THE HPLC DETERMINATION OF SULPHATHIAZOLE IN SPIKED HUMAN PLASMA SAMPLES

Amount added (ng/ml)	Mean amount found (n = 6) (ng/ml)	Precision C.V. (%)	Recovery (%)
250	255	4.8	102
500	515	2.1	103
1000	908	6.2	91
1600	1546	1.0	97
5000	4961	2.0	99
10000	10302	1.1	103
			99 ± 5.5

TABLE II

PRECISION AND RECOVERY OF THE HPLC DETERMINATION OF SULPHATHIAZOLE IN SPIKED HUMAN URINE SAMPLES

Amount added (ng/ml)	Mean amount found (n = 6) (ng/ml)	Precision C.V. (%)	Recovery (%)
2.5	2.7	5.8	107
10.0	10.1	1.8	101
20.0	20.8	2.2	104
50.0	51.6	1.5	103
			104 ± 3.7

Stability of sulphathiazole

Sulphathiazole remains stable for at least one month in human plasma if the samples are stored at −20° C.

Specificity

Using different columns for plasma and urine, the components of these biological materials do not interfere in the assay of sulphathiazole (Fig. 1 and 2). The method is specific in presence of sulphadiazine, sulphamerazine and sulphapyridine. The metabolite acetyl-sulphathiazole is suitably separated from the parent drug (Fig. 3). However, the method is not reliable for the simultaneous determination of sulphathiazole and its acetylated metabolite.

Application

The method described above was applied to plasma and urine samples from two subjects given orally one experimental tablet of Formo-Cibazol® containing 1 g of formo-sulphathiazole. The results are shown in Table III.

CONCLUSION

The sensitivity of the method permits the determination of sulphathiazole in plasma and urine at the concentrations generally found in pharmacokinetic studies in animals [9, 10] and man.

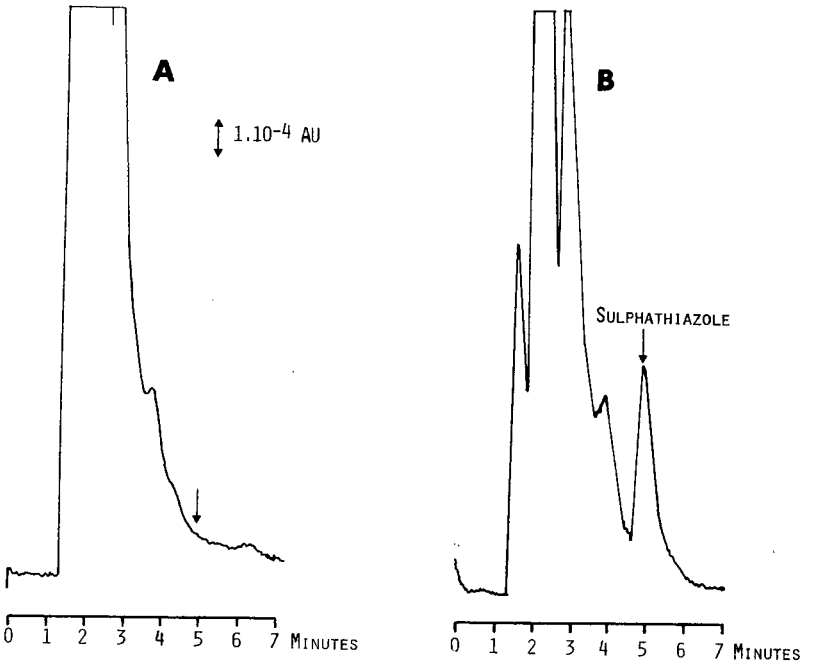

Fig. 1. Chromatograms of blank plasma (A) and human plasma containing 1600 ng/ml sulphathiazole (B).

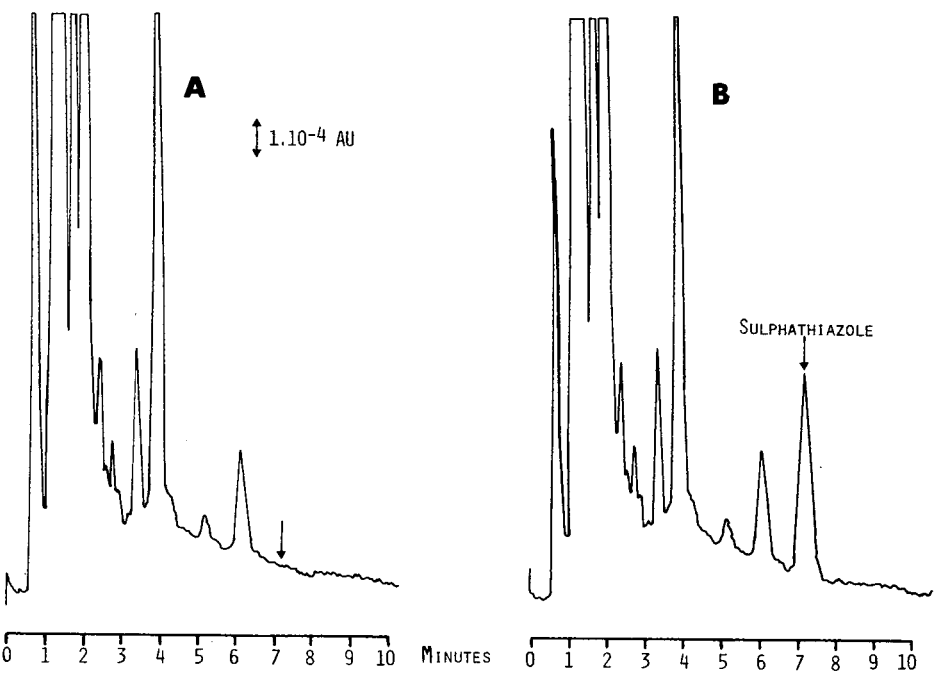

Fig. 2. Chromatograms of blank human urine (A) and human urine containing 20 μg/ml sulphathiazole (B).

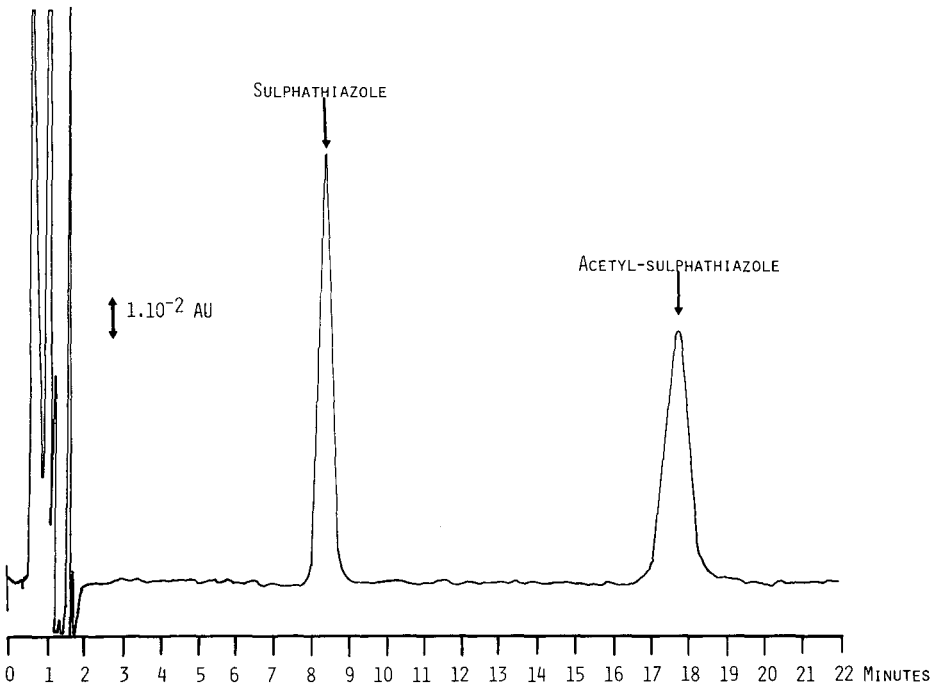

Fig. 3. Chromatogram of an aqueous solution of sulphathiazole and acetyl-sulphathiazole (chromatographic conditions described for urine). Each peak represents 20 μg of each synthetic compound.

TABLE III

PLASMA CONCENTRATIONS AND URINARY EXCRETION OF SULPHATHIAZOLE IN TWO SUBJECTS AFTER THE ORAL ADMINISTRATION OF ONE 1-g TABLET OF FORMO-SULPHATHIAZOLE

Time (h)	Sulphathiazole plasma concentrations (ng/ml)		Intervals of time (h)	Sulphathiazole cumulative urinary excretion (% of the dose)	
	Subject 1	Subject 2		Subject 1	Subject 2
0	ND*	ND*	0—8	0.18	0.42
0.5	122	129	8—24	0.41	0.27
1	223	251			
2	252	342	0—24	0.59	0.69
4	266	357			
6	216	114			
24	346	198			

*ND = Not detected.

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Note

Simultaneous determination of 1-β-D-arabinofuranosylcytosine 5′-triphosphate and 3-deazauridine 5′-triphosphate in human leukemia cells by high-performance liquid chromatography

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A firm knowledge of the pharmacologic behavior of the active metabolite of a drug is critical to the evaluation of the biochemical basis of its therapeutic efficacy. Antileukemic drugs such as 1-β-D-arabinofuranosylcytosine (ara-C) [1] and 3-deazauridine (deazaUrd) [2] require intracellular phosphorylation to their respective 5′-triphosphates, ara-CTP and deazaUTP, for activity. Thus, determination of the active nucleotide forms of these nucleoside analogs in tumor and host tissues is crucial to the biochemical appraisal of such clinical parameters as tumor reduction and resistance to therapy.

We have described sensitive chromatographic methods for the detection of ara-CTP and deazaUTP in bone marrow and tissue samples obtained from patients receiving therapy with either ara-C [3] or deazaUrd [4]. Recent observations in preclinical systems [5—7] suggest that these drugs may express synergistic activity when used together in the treatment of acute leukemia. However, it has been our experience that bone marrow aspirates from patients with leukemia may contain too few cells to permit the performance of multiple determinations of the metabolites of different drugs. This report describes a high-performance liquid chromatographic (HPLC) method for the simultaneous detection and quantitation of ara-CTP, deazaUTP, and CTP, the normal cellular nucleotide affected by the action of deazaUTP, in leukemia cells in vitro and in the leukemic cells of patients being treated with a combination of ara-C and deazaUrd. The assay has the same sensitivity as those previously described for each nucleotide analog alone and represents an improvement in that it is readily adapted to automated analysis.

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MATERIALS AND METHODS

Chemicals

Reagent grade $NH_4H_2PO_4$ was purchased from Fisher Scientific (Fairlawn, NJ, U.S.A.) and used without purification. Both ara-C (Upjohn, Kalamazoo, MI, U.S.A.) and deazaUrd (Division of Cancer Treatment, National Cancer Institute) were provided by the pharmacy of this institution. Normal nucleotides, used as chromatographic standards, were purchased from Sigma (St. Louis, MO, U.S.A.), as was ara-CTP. The [6-^{14}C]deazaUrd, used as a marker for deazaUTP after metabolism by CCRF-CEM cells, was kindly supplied by Dr. A. Bloch and Dr. P. Creaven of Roswell Park Memorial Institute.

Cell culture

CCRF-CEM cells, originally cultured from the peripheral blood of a patient with acute lymphocytic leukemia [8], were grown in agitated suspension culture in RPMI-1640 medium supplemented with 10% fetal calf serum (Grand Island Biological, Grand Island, NY, U.S.A.).

Bone marrow specimens

The treatment protocol (77—73) involving the combination of ara-C and deazaUrd treatment in patients with acute myelogenous leukemia in relapse was approved by the office of Research and the Surveillance Committee of this institution. One goal of this regimen was to evaluate the hypothesis that prior treatment with deazaUrd promotes sensitivty to ara-C in previously resistant cell populations. All patients volunteered informed consent for treatment and for bone marrow aspiration for diagnostic and laboratory investigation. Bone marrow aspirated from patients treated with ara-C and deazaUrd were drawn into syringes containing heparin as an anticoagulant and transported to the laboratory in an ice bath. Nucleated bone marrow cells were separated by standard Ficoll—Hypaque gradient centrifugation procedures [9].

Nucleotide extraction

Single cell suspensions of either CCRF-CEM cells or leukemic bone marrow cells were counted and their mean volumes were determined by an electronic particle counter (Coulter Electronics, Hialeah, FL, U.S.A.). Perchloric acid-soluble material containing cellular nucleotides was extracted and neutralized by standard procedures [3, 4] and stored at −20°C prior to chromatographic analysis.

High-performance liquid chromatography

A Waters Assoc. (Milford, MA, U.S.A.) high-pressure liquid chromatograph Model ALC-204 equipped with two Model 6000A pumps and a Model 660 gradient programmer was used to analyze nucleotides in perchloric acid-soluble cell extracts. Samples of 0.01—2.0 ml were injected onto a column of Partisil-10 SAX anion-exchange resin (Whatman, Clifton, NJ, U.S.A.) with a Model U6K injection system. Optimal separation of ara-CTP and deazaUTP from normal cellular constituents was obtained by the following gradient scheme.

Starting with the initial conditions of 65% Buffer A, 0.005 *M* $NH_4H_2PO_4$, pH 2.8, and 35% Buffer B, 0.75 *M* $NH_4H_2PO_4$, pH 3.7, a concave gradient described by curve 9 on the gradient programmer was run at 3.0 ml/min for 30 min to the final condition of 100% Buffer B. The eluted compounds were detected by their absorbance at 280 nm by the Model 440 monitor or a Schoeffel Instruments (Westwood, NJ, U.S.A.) Model 770 Spectroflow monitor. Absorbance was recorded by a Model 9126 strip chart recorder (Varian, Palo Alto, CA, U.S.A.) attenuated to 10 mV, and peak areas were quantitated with a Model CDS-111 electronic integrator (Varian). Peak areas were converted to absolute quantities using predetermined calibration curves. Baseline drift during gradient elution was minimized if the Partisil column was purged before use with 50 ml 2.0 *M* $NH_4H_2PO_4$, pH 4.2, equilibrated with 60% Buffer A and 40% Buffer B, and treated with 0.10 ml 0.1 *M* disodium EDTA. Following nucleotide analysis, the column was purged with 20 ml of the initial conditions mixture before injection of a subsequent sample.

When desired, this elution scheme and the subsequent analysis was directed by an automatic injector system (Waters Assoc. Model 710A automatic sample processor). The elution scheme described represents an improvement in this respect since schema previously reported for the separation of either ara-CTP [3] or deazaUTP [4] from the cellular ribonucleoside triphosphates consisted of isocratic elution then gradient elution steps in series. These requirements exceeded the capabilities of the automatic injector.

RESULTS AND DISCUSSION

The ability of this elution scheme to separate the major ribonucleoside 5′-triphosphates extracted from CCRF-CEM cells is illustrated in Fig. 1. A parallel culture was incubated with 100 μM deazaUrd for 4 h before addition of 2.0 μM ara-C for one additional hour (Fig. 2). Three major changes are evident in the chromatogram of nucleotides extracted from drug treated cells. First, a new peak eluting between CTP and UTP has been identified as ara-CTP by its UV absorbance characteristics, radioactivity after incubating cells with [^{3}H] ara-C, resistance to oxidation with $NaIO_4$, and cochromatography with authentic ara-CTP. Standards indicate that the incubation procedure resulted in the accumulation of 0.59 nmol of ara-CTP per $2 \cdot 10^6$ cells. A cellular concentration of 292 μM ara-CTP was calculated after the average cell volume (1010 μm^3) was considered and uniform cellular distribution of the nucleotide was assumed. These calculations indicate the ability of cultured human leukemia cells to accumulate ara-CTP to cellular concentrations over 100 times that of the exogenous nucleoside. Second, a new peak eluting between ATP and GTP has been identified as deazaUTP by its UV absorbance characteristics, cochromatography with the majority of radioactivity in extracts after incubating cells with [^{14}C] deazaUrd and sensitivity to incubation with both phosphatase and $NaIO_4$. DeazaUTP was present in drug-treated cells at a concentration of 2.33 nmol per $2 \cdot 10^6$ cells, or 1150 μM. Finally, the CTP peak in drug-treated cells is only 12% that of controls, 0.23 nmol per $2 \cdot 10^6$ cells versus 1.90 nmol per $2 \cdot 10^6$ cells. This suggests the inhibitory action of deazaUTP on the CTP syn-

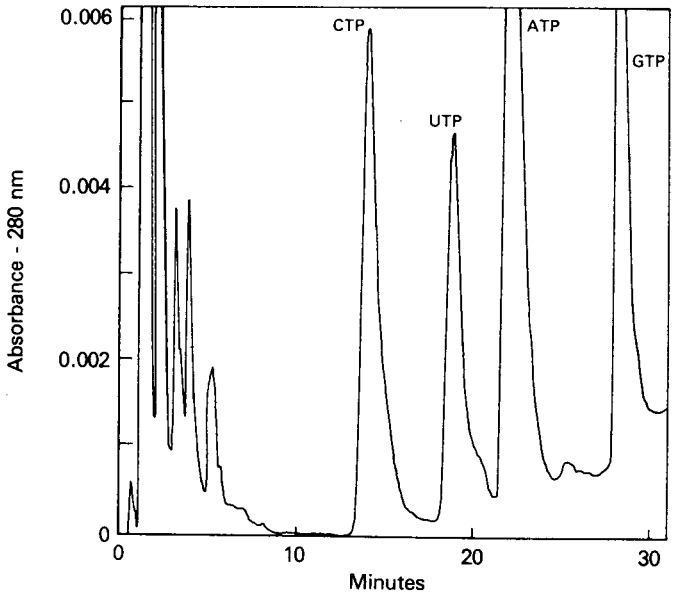

Fig. 1. Separation of perchloric acid-soluble material extracted from the equivalent of $2 \cdot 10^6$ CCRF-CEM cells by HPLC.

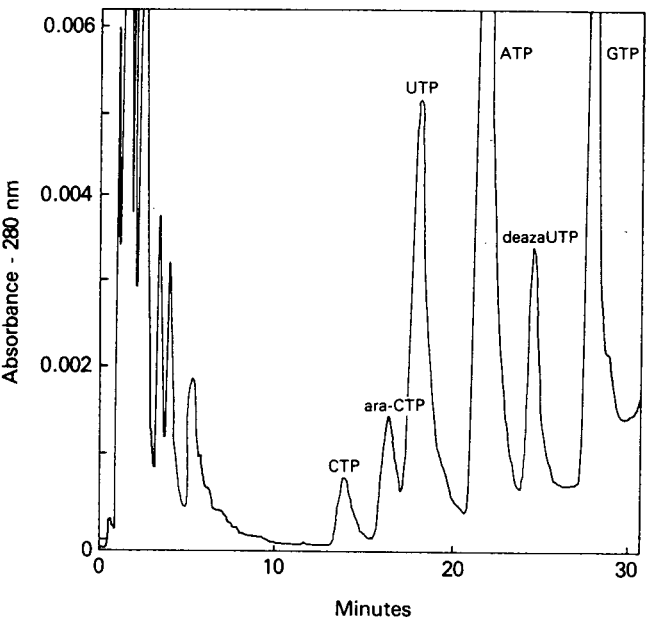

Fig. 2. Separation of perchloric acid-soluble material extracted from the equivalent of $2 \cdot 10^6$ CCRF-CEM cells that had been incubated 4 h with 100 μM deazaUrd before the addition of 2 μM ara-C for one additional hour.

thetase [10] of these cells, leading to a predictable decrease in CTP [4]. Serial dilutions of cell extracts containing deazaUTP and addition of known amounts of ara-CTP to cell extracts indicate the lower limits for quantitation by electronic integration of the peak areas of deazaUTP and ara-CTP to be 250 and 25 pmol, respectively, values similar to those obtained in assays for each nucleotide separately [3, 4] in cell extracts.

The reproducibility of the retention time of each nucleotide separated in this manner is shown in Table I. The maximum variation in the elution times

TABLE I

REPRODUCIBILITY OF RETENTION TIME AND QUANTITATION OF NUCLEOTIDES EXTRACTED FROM CCRF-CEM CELLS

Cells were incubated with deazaUrd and ara-C as described in the legend for Fig. 2. Samples from the equivalent of $1 \cdot 10^6$ cells were injected and analyzed under the direction of the Model 710A automatic sample processor ($n = 8$).

Nucleotide	Retention time (min)	Quantity (nmol/1 · 10^6 cells ± S.E.M.)
CTP	13.50 ± 0.05	0.17 ± 0.005
ara-CTP	16.14 ± 0.09	0.36 ± 0.01
UTP	18.35 ± 0.04	3.78 ± 0.07
ATP	20.90 ± 0.03	6.05 ± 0.11
deazaUTP	25.35 ± 0.02	1.45 ± 0.05
GTP	28.21 ± 0.03	2.04 ± 0.06

was 0.6% (ara-CTP). In addition, the maximum variation in quantitation when the six nucleotides in this cell extract were analyzed was less than 3% (CTP).

This chromatographic technique for the simultaneous detection and quantitation of ara-CTP, deazaUTP, and the affected normal nucleotide, CTP, is directly applicable to biochemical pharmacology studies in the cells of patients receiving ara-C and deazaUrd chemotherapy. Chromatographic analysis of a perchloric acid-soluble extract from nucleated bone marrow cells from a patient who had been treated with ara-C (50 mg/m^2/day) for 2 days then simultaneously with deazaUrd (1000 mg/m^2/day) for 1 day is shown in Fig. 3. Both ara-CTP and deazaUTP are clearly present in addition to the four ribonucleoside 5′-triphosphates. Similar peaks have never been observed in extracts from lymphocytes, leukemic cells from the peripheral blood, red blood cells or solid tumors from patients who had not been treated with either ara-C or deazaUrd. Using the determined mean cell volume of 283 μm^3, the nucleotide concentration in these cells was calculated as follows: CTP, 280 μM;UTP, 1560 μM; ATP, 2230 μM; GTP, 476 μM; ara-CTP, 15 μM; deazaUTP, 150 μM. The last two figures indicate that the nucleoside analogs entered the bone marrow cells, were phosphorylated by the active triphosphates, and accumulated to values considerably in excess of the K_i value for the respective target enzymes [10, 11]. This is significant for the interpretation of the therapeutic effect of these drugs, since this patient's disease had previously failed to respond to treatment regimens containing ara-C or to deazaUrd alone. In a separate publication [12] we present the results of serial determinations on the leukemic cells of patients treated on this protocol that indicate this assay may be useful in establishing the biochemical basis for drug scheduling, interaction, and resistance.

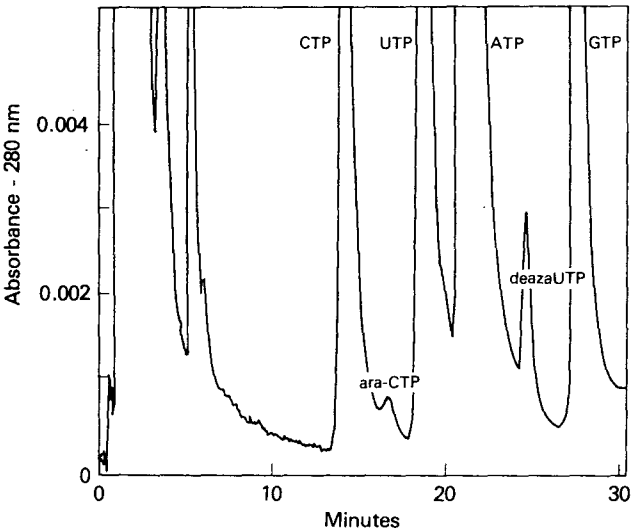

Fig. 3. HPLC separation of perchloric acid-soluble material extracted from the equivalent of $3 \cdot 10^7$ nucleated bone marrow cells from a patient receiving combination chemotherapy with ara-C and deazaUrd.

This rapid and sensitive assay for the simultaneous detection of ara-CTP, deazaUTP, and the affected nucleotide, CTP, should be useful for evaluating the biochemical and pharmacological bases of the therapeutic efficacy of treatment regimens that combine ara-C and deazaUrd. The elution scheme required to achieve this separation is within the capability of automated injection systems currently available.

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Note

Determination of isoxepac in plasma by high-performance liquid chromatography

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Isoxepac (6,11-dihydro-11-oxodibenz[*b*,*e*]oxepin-2-acetic acid) is a non-steroidal anti-inflammatory agent under investigation for the treatment of rheumatoid arthritis. The metabolism [1] and the gas—liquid chromatographic (GLC) determination [2] of isoxepac have previously been described. A high-performance liquid chromatographic (HPLC) analysis was developed to meet the need for a facile and rapid method to determine the pharmacokinetics of isoxepac in patients with rheumatoid arthritis.

EXPERIMENTAL

Materials

Isoxepac and the propionic acid analog (6,11-dihydro-11-oxodibenz[*b*,*e*]-oxepin-2-propionic acid) [2] were obtained from Hoechst UK, Pharmaceuticals Division (Hounslow, Great Britain) and naproxen from Syntex (Palo Alto, CA, U.S.A.). Diethyl ether was glass distilled and methanol was HPLC grade. All other chemicals were AR grade and were used without further treatment.

Extraction

Plasma samples were stored at −20°C till required. In order to minimise the photodecomposition of isoxepac [2] plasma samples were allowed to thaw in the dark and care was taken to avoid direct sunlight during the extraction procedure. A 20-μl aliquot of the internal standard (isoxepac analog 500 μg ml^{-1} in methanol), was added to a 10-ml test tube followed by 0.5 ml plasma and 0.15 ml 2 *N* hydrochloric acid. Diethyl ether (4 ml) was added, the contents mixed by vortexing and the layers separated by centrifugation (5 min at 1000 *g*). A 3.5-ml aliquot of the organic layer was removed and evaporated to

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dryness using a stream of nitrogen. The residue was dissolved in 0.3 ml methanol and stored in the dark at 4°C till analysed.

A calibration curve was constructed over the range 1—32 $\mu g\ ml^{-1}$ by addition of isoxepac in methanol to test tubes and evaporating to dryness with a stream of nitrogen. The procedure was then as described above.

If naproxen was utilised as the internal standard 20 μl of a 250-$\mu g\ ml^{-1}$ methanol solution were added.

Chromatography

A 100 × 5 mm Shandon column (Shandon Southern Products, Runcorn, Great Britain) was slurry packed with LiChrosorb RP-18, particle size 5 μm (BDH Chemicals, Poole, Great Britain) using a constant-pressure pump (Haskel MCP-71). The isocratic mobile phase consisted of methanol—5% glacial acetic acid in water (6:4) and was pumped at a constant flow-rate of 1.3 ml min^{-1} using a Waters 6000A solvent delivery system (Waters Assoc., Northwich, Great Britain). A Rheodyne 7120 valve injector (HPLC Technology, Wilmslow, Great Britain) was fitted with a 10-μl loop and detection was at 254 nm using a Waters 440 absorbance detector.

RESULTS AND DISCUSSION

Isoxepac was quantified by HPLC following diethyl ether extraction of acidified plasma. The extraction efficiencies of various solvents for isoxepac have previously been discussed [2]. Calibration curves were linear over the range 1—32 $\mu g\ ml^{-1}$ with correlation coefficients greater than 0.993 and linear regression analysis gave $y = 0.047x + 0.089$. The coefficient of variation for replicate extractions ($n = 7$) was 1.6% and for replicate injections ($n = 7$) was less than 1%. The capacity factors for isoxepac, isoxepac propionic acid analog and naproxen were 3.0, 4.9 and 5.5 respectively. Typical chromatograms are given in Fig. 1.

Plasma samples were taken from 15 patients undergoing oral isoxepac therapy, 150 mg twice daily and 300 mg at night, 2—6 h following the morning dose. The mean concentration was found to be 24 $\mu g\ ml^{-1}$ (S.D. = 15 $\mu g\ ml^{-1}$) and the range 7—61 $\mu g\ ml^{-1}$. When the concentration exceeded 32 $\mu g\ ml^{-1}$ the plasma samples were suitably diluted and reanalysed. The sensitivity of the method was 1 $\mu g\ ml^{-1}$ of isoxepac in plasma.

The use of the isoxepac analog as an internal standard is preferable as it exhibits similar extraction properties to isoxepac [2] but it has the disadvantage of limited availability. Naproxen is readily available and has a similar capacity factor to the isoxepac analog but care must be taken that patients have not been treated with naproxen for a period of less than two weeks prior to study.

The possibility exists that rheumatoid arthritis patients undergoing isoxepac therapy might also be taking other non-steroidal anti-inflammatory drugs which could interfere in the analysis of isoxepac. Table I gives the retention data for 18 other non-steroidal anti-inflammatory drugs and 5 of these, i.e. indomethacin, ketoprofen, salsalate, sulindac and tolmetin, would interfere. The only identified metabolite of isoxepac is the glucuronide ester [1] and this would not interfere in the assay for the parent drug.

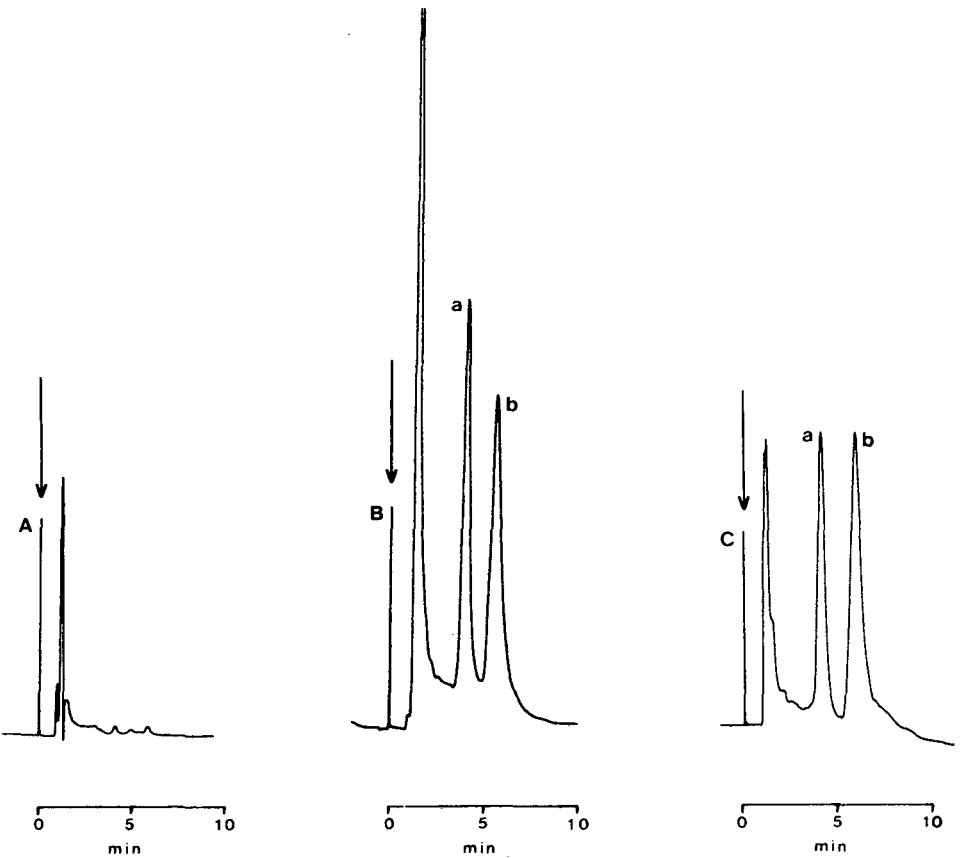

Fig. 1. Chromatograms of extracts of (A) blank plasma; (B) plasma spiked with isoxepac (a) and internal standard (b); (C) plasma from patient underoing therapy with isoxepac (150 mg twice daily and 300 mg at night); this sample corresponds to an isoxepac concentration of 19 $\mu g\ ml^{-1}$.

TABLE I

HPLC RETENTION DATA FOR 19 NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

HPLC conditions: column: 100 × 5 mm packed with 5-μm RP-18; mobile phase:methanol—5% acetic acid in water (6:4).

Drug	Capacity factor	Drug	Capacity factor
Diclofenac	12.1	Naproxen	5.5
Fenbufen	5.0	Oxyphenylbutazone	2.3
Fenclofenac	14.1	Paracetamol	0.2
Fenoprofen	7.7	Penicillamine	0.4
Feprazone	7.4	Phenylbutazone	7.2
Flurbiprofen	9.7	Piroxicam	2.3
Ibuprofen	14.3	Salicylic acid	1.4
Indomethacin*	3.2	Salsalate*	3.8
Isoxepac	3.0	Sulindac*	3.4
Ketoprofen*	3.4	Tolmetin*	3.2

*Drugs which may interfere in the analysis of isoxepac.

HPLC was preferable to GLC [2] because no derivatisation was required and the analysis time was not prolonged by the elution of cholesterol. The use of a diethyl ether extract gave prolonged column life and the method should also be suitable for the quantitative analysis of the glucuronide conjugate in urine [1] following hydrolysis.

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Journal of Chromatography (incorporating *Chromatographic Reviews*) and *Journal of Chromatography, Biomedical Applications*

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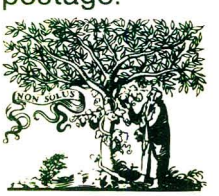

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