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Chromatographic Reviews			184/1	184/2									
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QUANTITATIVE ANALYSIS OF HISTAMINE IN BIOLOGICAL SAMPLES BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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(Received June 26th, 1979)

SUMMARY

A mass fragmentographic method for the quantitative analysis of histamine in the supernatant from antigen-challenged leukocytes, whole blood, and urine is described. Histamine labeled with two ^{15}N atoms was synthesized and added to the sample as an internal standard. N^{α}-Heptafluorobutyryl-N^{τ}-ethoxycarbonylhistamine was prepared for mass fragmentographic analysis and the molecular ions at *m/z* 379 and 381 were used for monitoring histamine and $^{15}N_2$-labeled histamine, respectively. The quantitation limit of histamine was 2 ng by this method. The experimental error of the method was less than 7% at the level of 5 ng in the supernatant from antigen-challenged leukocytes. The value obtained by this method correlated well with that from radioisotopic enzymatic assay (r=0.990).

INTRODUCTION

It is known that histamine is released from mast cells in tissue, basophils in blood, which is mediated by IgE antibody. A number of studies on histamine have been reported and it is generally accepted that histamine is a mediator of immediate type hypersensitivity [1, 2].

Histamine has been measured by a number of techniques including biological [3], colorimetric [4], and fluorimetric assay [5], and by enzymatic assay using radioisotopes [6]. Biological and colorimetric procedures are laborious and may give rise to the question of specificity. The fluorimetric assay of Shore et al. [5] has been adopted for assaying histamine. However, the butanol extract contained not only histamine but spermidine, which interfered with the measurement of histamine, especially in tissues with a low content of histamine [7]. Recently, the application of high-speed liquid chromatography was introduced to overcome this disadvantage [8]. The radioisotopic enzymatic assay originally described by Snyder et al. [6] is a sensitive and specific method but

it involves the hazards associated with radioactivity and radioactive waste.

It is generally recognized that determination by mass fragmentography is a most specific and sensitive method, and its application for many biologically important compounds has been reported. Nevertheless, the quantitation of histamine by mass fragmentography has not yet been reported. The purpose of this study is to develop a method which allows the quantitation of histamine.

EXPERIMENTAL

Materials

Histamine dihydrochloride was purchased from Merck (Darmstadt, G.F.R.). Heptafluorobutyric anhydride (HFBA) was obtained from Wako Pure Chemical (Osaka, Japan) and ethyl chloroformate (ECF) was from Tokyo Kasei Kogyo (Tokyo, Japan). All organic solvents used were of analytical grade. Ethyl acetate was stored over molecular sieve type 3A (Wako).

$^{15}N_2$-Labeled histamine used as an internal standard was synthesized using potassium [^{15}N]phthalimide (99.6 atom per cent; Prochem, London, Great Britain) as the starting material according to the method of Fraser and Raphael [9]. The labeled histamine was dissolved in 0.01 *N* hydrochloric acid solution to make a concentration of 600 ng/ml and kept at 4°.

The radioisotope-labeled compounds, [2,5-^{3}H]histamine dihydrochloride (specific activity, 7.7 Ci/mmol) and S-adenosyl-L-[methyl-^{14}C]methionine (specific activity, 58 mCi/mmol) used for the enzymatic assay were purchased from the Radiochemical Centre, Amersham, Great Britain.

Preparation of the crude extract from the Japanese cedar pollens

Japanese cedar pollens were collected and defatted with diethyl ether. The defatted pollens were extracted with 0.125 *M* ammonium hydrogen carbonate solution and the extract was dialyzed against 5 m*M* ammonium hydrogen carbonate solution. The dialyzate was lyophilized and kept at 4° until use.

Samples

Supernatant released from antigen-challenged leukocytes. In vitro histamine release reaction from washed leukocytes was carried out as follows. Venous blood of a patient hypersensitive to cedar pollen was drawn into a plastic syringe containing heparin. The leukocyte suspension, obtained by the procedure of Siraganian and Brodsky [10], was incubated with the crude extract of Japanese cedar pollens at 37° for 1 h in a tube. The tubes were centrifuged and the supernatant was assayed for histamine.

Whole blood. The venous blood obtained from a patient was repeatedly frozen and thawed, and used for the determination.

All the samples were stored at −20° until assay.

Analytical procedure

An internal standard solution (30 ng), 0.5 ml of 4 *N* sodium hydroxide solution, and 0.5 g of sodium chloride were added to 0.25 ml of sample. The mixture was shaken with 2 ml of ethyl acetate. The organic layer was discarded and the aqueous phase was extracted with 2 ml of butanol. The extraction step

with ethyl acetate can be omitted. The organic layer was transferred to a tube containing 2 ml of heptane and 1.5 ml of 1 *N* hydrochloric acid solution. The mixture was shaken and centrifuged. The aqueous phase was removed and evaporated to dryness under reduced pressure. The residue was mixed with HFBA—ethyl acetate (1:1, v/v) and heated at 80° for 30 min. After removing excess reagent under a nitrogen stream, the residue was treated with 50 μl of a mixture of ECF—ethyl acetate (1:1, v/v) at room temperature for 30 min to form N^{α}-heptafluorobutyryl-N^{τ}-ethoxycarbonylhistamine (HA-HFB-ETO), to which 1 ml of 10% sodium carbonate and 2 ml of dichloromethane were added. The mixture was shaken, centrifuged and the aqueous phase was discarded. The organic phase was removed to dryness under a nitrogen stream; the residue was dissolved in ethyl acetate, and the solution was analyzed by gas chromatography—mass spectrometry.

Gas chromatography—mass spectrometry

An LKB 2091 mass spectrometer coupled with a Shimadzu 7A Model gas chromatograph and data processing system (Shimadzu PAC500FDG) connected to a minicomputer (Okitac-4300b,Oki Electric Industry Co., Tokyo, Japan) was used. The glass column (2 m × 2.4 mm I.D.) was packed with 5% SE-30 on Supelcoport (80—100 mesh; Supelco, Bellefonte, Pa., U.S.A.). The trap current was 50 μA, electron energy 20 eV, flow-rate of carrier gas (helium) ca. 12 ml/min, column oven temperature 200°, injection temperature 240°, separator temperature 250° and ion source temperature 220°.

Radioisotopic enzymatic assay of histamine

Enzymatic assay was performed by a slight modification [11] of the method of Beaven et al. [12]. Briefly, histamine was converted to [^{14}C]methylhistamine by incubation with S-adenosyl-L-[methyl-^{14}C]methionine and the enzyme histamine N-methyltransferase prepared from pig brain. A tracer amount of [^{3}H]histamine was added to correct the efficiency of the reaction. The [^{14}C,^{3}H]methylhistamine formed was extracted from the mixture with chloroform and its radioactivity assayed by liquid scintillation spectrometry. Determinations were done in duplicate.

RESULTS AND DISCUSSION

Mass spectra of the N^{α}-heptafluorobutyryl-N^{τ}-ethoxycarbonyl derivative of histamine and [$^{15}N_2$]histamine as an internal standard are shown in Fig. 1. The intensity of the molecular ion was about 40% of the most abundant peak at *m/z* 166 which resulted from the elimination of $NHCOC_3F_7$ with hydrogen. The molecular ions at *m/z* 379 and 381 were used for monitoring histamine and [$^{15}N_2$]histamine, respectively.

The response at *m/z* 379 of $^{15}N_2$-labeled HA-HFB-ETO was about 2% of *m/z* 381 which was identical with the peak height ratio at *m/z* 379 and 377 for HA-HFB-ETO (Fig. 2A and B). The intensity ratio of *m/z* 379 and 381 was almost the same as the mixing ratio of histamine and [$^{15}N_2$]histamine. Because the peak of $^{15}N_2$-labeled HA-HFB-ETO at *m/z* 379 was about 0.4% when it was measured with hardware multiple ion detector, the contribution to *m/z* 379 was

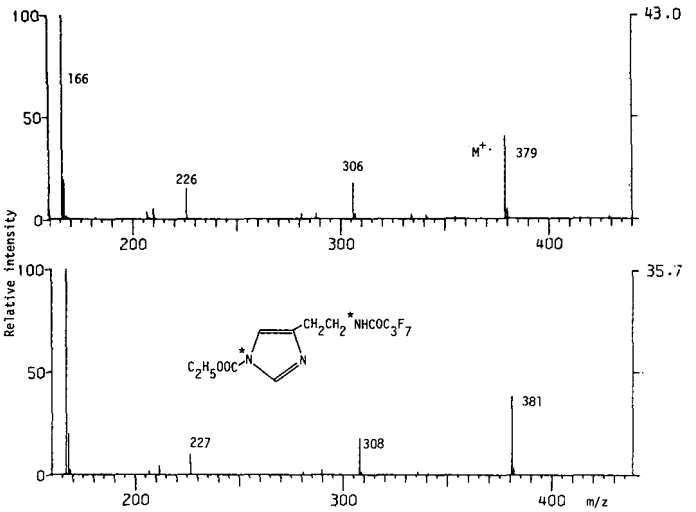

Fig. 1. Mass spectra of N^{α}-heptafluorobutyryl-N^{τ}-ethoxycarbonyl derivative prepared from histamine (upper) and $^{15}N_2$-labeled histamine (lower). Asterisks (*) indicate ^{15}N.

due to the computer error which can not be resolved at the present time. As the contribution to *m/z* 379 was observed in $^{15}N_2$-labeled HA-HFB-ETO, the amount of internal standard added has to be reduced if greater sensitivity is desired, but when 1.9 ng of histamine were mixed with 30 ng of the internal standard an increase in the peak height ratio was easily detected (Fig. 2C).

For gas chromatographic analysis, HA-HFB-ETO is better than the trimethylsilyl and heptafluorobutyryl derivative of histamine, which fact has been reported [13]. In a two-step derivatization procedure, the yield becomes a seri-

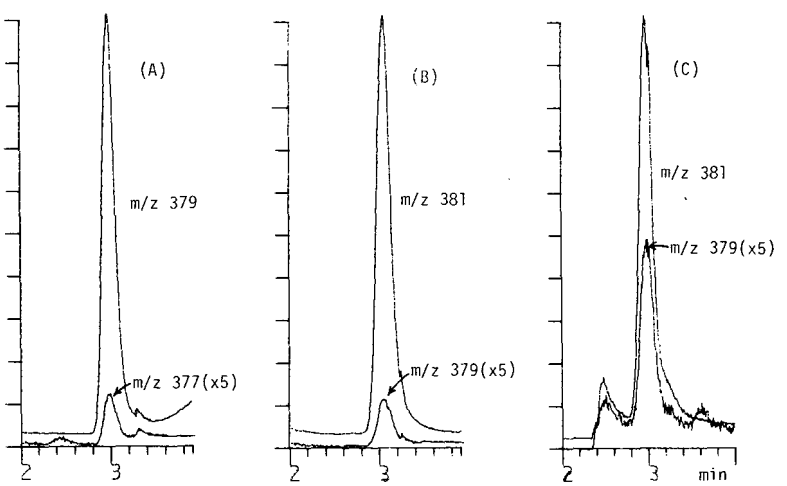

Fig. 2. Mass fragmentograms of the derivative of (A) authentic histamine, (B) $^{15}N_2$-labeled histamine used as an internal standard, and (C) mixture containing histamine (1.9 ng) and $^{15}N_2$-labeled histamine (30 ng).

ous problem. To estimate the recovery of histamine in the present procedure, [^{3}H]histamine was added to the leukocyte suspension. After the sample containing [^{3}H]histamine was extracted and converted to HA-HFB-ETO, the recovery was calculated from the tritium radioactivity. The total recovery, including extraction, clean-up, and two-step derivatization procedures, was about 70%. Hence, this procedure would be applicable for the determination of histamine.

The detection limit was 50 pg of histamine, with a signal-to-noise ratio of 5:1, which permits the quantitative determination of histamine present in whole blood and urine, and in the in vitro histamine release experiment.

In the quantitative determination of histamine, a good linear relationship was obtained between the ratio of the peak heights and amount of histamine in the range of 2—20 ng. A standard solution containing 5.55 ng of histamine was measured by the above method and the value was 5.71 ± 0.17 ng (n=5). This is nearly the theoretical value.

To test the specificity of the determination, the mass spectrometer was focused on the m/z 379, 381 and 306, 308 for a sample of supernatant from antigen-challenged leukocytes. The ratio between the peak heights at both pairs was the same. As shown in Table I, the values obtained were 5.93 ± 0.37 and 5.71 ± 0.29 ng (mean ± S.D.) at m/z 379/381, and 5.59 ± 0.23 at m/z 306/308. This indicates that fragments from compounds other than histamine do not interfere with the quantitation of histamine. Next, this method was compared with the radioisotopic enzymatic assay of histamine. The mass fragmentographic analysis resulted in apparently the same level of histamine measured by radioisotopic enzymatic assay (Table I). The experimental error was less than 7% for mass fragmentography, while it was about 10% for radioisotopic enzymatic assay when five samples were analyzed. The quantitated values obtained by mass fragmentography and radioisotopic enzymatic assay were well correlated (r=0.990), as seen in Fig. 3. It is clear from Fig. 3 and Table I that mass fragmentographic measurement is a specific method for the determination of histamine and more accurate than the radioisotopic enzymatic assay.

TABLE I

COMPARISON OF MASS FRAGMENTOGRAPHY AND RADIOISOTOPIC ENZYMATIC ASSAY

Results are expressed in ng. Samples A, B, C, D, and E were taken from the same supernatant from antigen-challenged leukocytes.

Sample	Mass fragmentography			Radioisotopic enzymatic assay	
	m/z 379 / 381 ($M^{+\cdot}$)		m/z 306/308		
A	5.67	5.78	5.67	4.00	5.13
B	5.58	5.63	5.63	4.16	5.82
C	6.03	5.39	5.26	5.01	5.43
D	5.85	5.59	5.89	5.69	6.13
E	6.51	6.17	5.52	5.10	5.97
Mean	5.93	5.71	5.59	4.79	5.70
S.D.	0.37	0.29	0.23	0.70	0.41
C.V. (%)	6.24	5.08	4.11	14.61	7.19

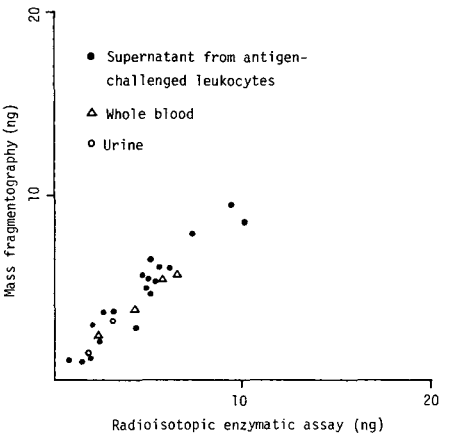

Fig. 3. Correlation between mass fragmentographic measurement and radioisotopic enzymatic assay (r=0.990).

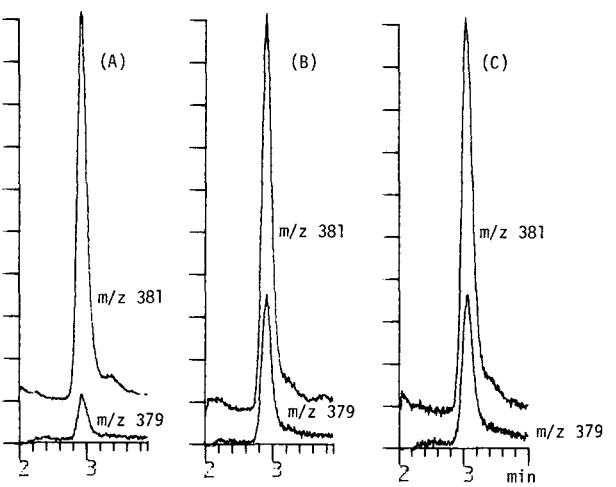

Fig. 4. Mass fragmentograms of histamine extracted from the supernatant from antigen-challenged leukocytes: (A) spontaneous release, (B) 0.01 μg, (C) 0.1 μg antigen challenged. Challenged antigen: crude extract of the Japanese cedar pollens.

Histamine release from leukocytes by antigen challenge has been extensively used for in vitro studies of allergy. After leukocytes were incubated with the crude extract of cedar pollens at 37° for 1 h in a tube, the tube was centrifuged, and the histamine released into the supernatant was determined by mass fragmentography (Fig. 4). When the allergen was challenged at concentrations of 0.01 and 0.1 μg, histamine was released from the leukocytes (basophils), indicating that the patient was hypersensitive to cedar pollens.

The availability of this new method for the analysis of histamine in biological samples will aid clinical research into the action of histamine in allergic patients.

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CHROMBIO. 453

GAS CHROMATOGRAPHIC DETERMINATION OF LOW CONCENTRATIONS OF BENZOIC ACID IN HUMAN PLASMA AND URINE

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(Received June 25th, 1979)

SUMMARY

A method for the determination of benzoic acid down to concentrations of 10 ng/ml in plasma or urine is described. After addition of an internal standard, benzoic acid is extracted at acid pH into diethyl ether. Both compounds are derivatized with pentafluorobenzyl bromide. The derivatives are determined by gas chromatography using a ^{63}Ni electron-capture detector. Hippuric acid is hydrolysed in plasma and urine and total benzoic acid is determined by the same technique.

INTRODUCTION

Numerous methods have been already proposed for the quantitative assay of carboxylic acids and particularly benzoic acid in biological fluids. Rowland and Riegelman [1] described a gas—liquid chromatographic (GLC) method using acid pH and diethyl ether for the extraction from plasma, and carbon disulphide as solvent for the preparation of the trimethylsilyl derivatives of submicrogram amounts of carboxylic acids. Coward and Smith [2] reported the gas chromatography of aromatic acids as their trimethylsilyl derivatives, including applications to urine analysis. Sinsheimer and Breault [3] made determinations of various benzoic acid derivatives to test their suitability for metabolism studies. Quantitative determinations of microgram amounts of these derivatives were obtained by gas chromatography, and by reverse thin-layer chromatography—fluorimetry using the quenching of a fluorescent background. Gossele [4] described a gas chromatographic determination of aromatic acids used as preservatives in food. After extraction, these acids were derivatized with N,O-bis(trimethylsilyl)acetamide and detected with a flame ionization

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detector. Benzoic acid was quantitatively determined in this way. Fransson et al. [5] used a liquid—liquid chromatographic system based on ion-pair partition, with silica microparticles as support for the stationary phase, to separate anionic compounds of biochemical and pharmacological interest, including benzoic acid. Amsel and Levy [6] described a pharmacokinetic study of the simultaneous conjugation of benzoic and salicylic acids with glycine, giving hippuric and salicyluric acid, respectively.

All these methods suffer from a lack of sensitivity, as none of them is capable of detecting benzoic acid down to 1 μg/ml. This paper describes the GLC determination of benzoic acid in human plasma and urine at concentrations down to 10 ng/ml using 3-phenylpropionic acid as internal standard. This technique permits the quantitative assay of free benzoic acid in plasma and urine. Acid hydrolysis is used for the determination of total benzoic acid in plasma and urine, thus giving the conjugated benzoic acid (hippuric acid) by difference.

MATERIALS AND METHOD

Reagents

All reagents and solvents are of analytical grade: diethyl ether, sulphuric acid, acetone (Merck, Darmstadt, G.F.R.) and benzene (Mallinckrodt, St. Louis, Mo., U.S.A.). Pentafluorobenzyl bromide (Regis, Morton Grove, Ill., U.S.A.) is stored at 4° in glass containers.

The two methanolic internal standard solutions contain, respectively, 500 ng per 100 μl and 2000 ng per 100 μl of 3-phenylpropionic acid.

Apparatus

Glass tubes are washed with 5 ml of diethyl ether by shaking them mechanically (Infors shaker) for 15 min.

A Hewlett-Packard Model 5710A gas chromatograph equipped with a Hewlett-Packard Model 18713A electron-capture detector is used. The peak areas are given by a Hewlett-Packard Model 3380A electronic integrator. The column is operated at 148°, the injector at 250° and the detector at 300°, with an argon—methane (90:10) flow-rate of 60 ml/min. Glass columns are washed with 1 *M* hydrochloric acid, distilled water, acetone and benzene, and then silanized with a 1% (v/v) solution of hexamethyldisilazane in benzene. After the treatment, the columns are washed again with benzene and dried at 100°.

The column packing is 3% OV-17 on 100—120 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.). The filled columns are flushed with the carrier gas at a flow-rate of 40 ml/min and heated to 300° at a rate of 1°/min. The column temperature is held overnight at 300° and throughout the next day at 270°. The temperature is then repeatedly increased from 150 to 250° for 24 h. During these programmed cycles, the columns are further conditioned by injecting a total of 100 μl of Silyl 8 (Pierce, Rockford, Ill., U.S.A.) by fractions between 150 and 220°. After this treatment, the columns are ready for use.

Acid hydrolysis in plasma and urine

Aliquots (1 ml) of plasma or urine (diluted with water if necessary) and 1 ml of concentrated hydrochloric acid are heated for 16 h at 100° [6]. The extraction is then performed as described below.

Extraction

One hundred microlitres of the internal standard solution are measured into a glass tube, to which 1 ml of the sample, 1 ml of 0.5 *M* sulphuric acid and 5 ml of diethyl ether are then added. The tube is stoppered and shaken mechanically (Infors shaker) for 15 min at 300 rpm and centrifuged at 3900 *g* for 10 min.

An aliquot of the ether phase is transferred to another tube and taken to dryness under vacuum in a rotary evaporator (Büchi) in a water-bath at room temperature.

Derivatization and chromatography

To the dry residue are added 100 mg of anhydrous potassium carbonate and 1 ml of acetone containing 5 mg of pentafluorobenzyl bromide. Each tube is stoppered tightly and put in a dry heating block (Grant Instruments) at 50° for 2 h. After this time, the contents of the tube are evaporated to dryness under a nitrogen stream in a water-bath at room temperature. To the dry residue, 2 ml of water and 1 ml of benzene (4 ml after acid hydrolysis of urine) are added, and the tube is shaken mechanically for 10 min at 300 rpm and centrifuged for 3 min at 2450 *g*.

A 2-μl portion of the benzene layer is injected into the gas chromatograph by the solvent-flush technique.

The benzoic acid content is calculated from the ratio of the peak areas by reference to a calibration curve prepared from a series of methanolic benzoic acid solutions added to water to yield concentrations between 10 and 1000 ng/ml (with 500 ng internal standard), and between 1000 and 6000 ng/ml (with 2000 ng internal standard). This latter range is used after acid hydrolysis of urine.

Collection of samples

Plasma and urine were obtained from three healthy male subjects, who had been instructed not to take any drugs from eight days before and until the end of the study.

Blood samples were drawn at the beginning of the experiment (about 8 a.m.) and at 2, 4, 6, 8, 24, 72 and 120 h thereafter; the blood was transferred to heparinized tubes and centrifuged immediately. Plasma was removed and stored at −20° until analysis.

Urine was collected at the following intervals: 0—4, 4—8, 8—12, 12—24, 24—32, 32—48, 72—80, 80—96, 120—128 and 128—144 h. The volume was measured and an aliquot was stored at −20° until analysis.

RESULTS AND DISCUSSION

Reaction time

The duration of the derivatization reaction was varied from between 30 min and 3 h. A maximum yield of derivative was obtained after 2 h.

Evaporation

After the extraction of benzoic acid with diethyl ether, this solvent is evaporated under vacuum. No reproducible results were obtained when it was taken to dryness under a nitrogen stream at room temperature.

Plasma or urine interference

The chromatograms of blank human plasma and urine extracts showed a peak with the same retention time as the derivative of benzoic acid. Gas chromatographic—mass spectrometric analysis showed the compound corresponding to this peak to be identical with the derivative of benzoic acid: it is known that benzoic acid is normally present in human plasma and urine. For this reason, the calibration curve is obtained using aqueous solutions of benzoic acid. Some chromatograms of urine extracts showed a peak with the same retention time as the internal standard (3-phenylpropionic acid). In these cases, phenylacetic acid was used as internal standard. After ten consecutive injections, a 90-min interval is allowed to wash out non-hydrolysed plasma and urine residues from the column.

Fig. 1 shows the chromatograms of a reagent blank extract and of water containing 400 ng of benzoic acid, 500 ng of 3-phenylpropionic acid and 500 ng of phenylacetic acid. Fig. 2 shows the chromatogram of a blank plasma extract (500 μl of plasma). Fig. 3 shows the chromatogram of a blank urine extract (1 ml from urine diluted 1/100) after acid hydrolysis.

Sensitivity and reproducibility

Table I gives the results obtained when the method is applied to aqueous

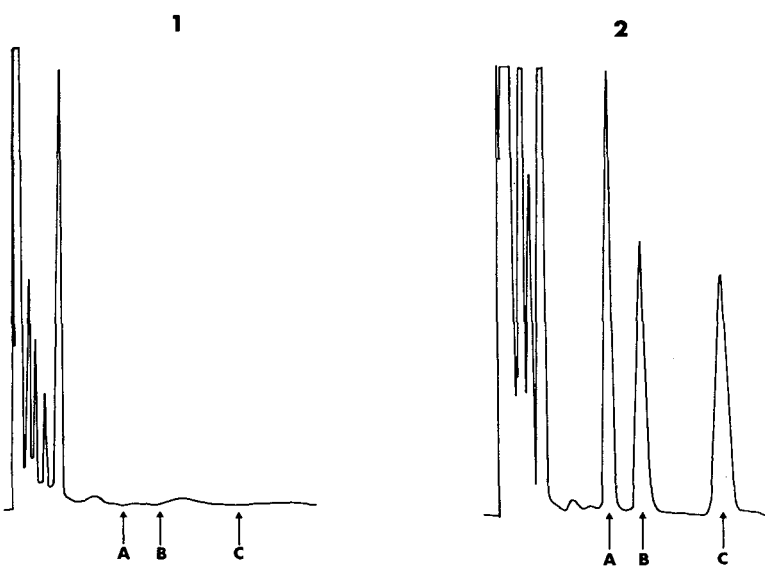

Fig. 1. Examples of chromatograms: (1) reagent blank extract; (2) water containing benzoic acid at 400 ng/ml (A), phenylacetic acid at 500 ng/ml (B) and 3-phenylpropionic acid at 500 ng/ml (C).

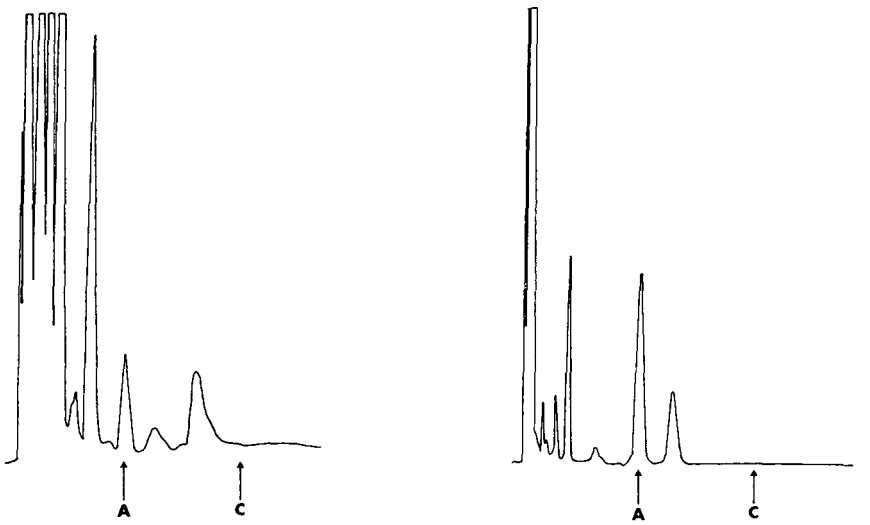

Fig. 2. Blank plasma extract (500 μl of plasma) containing free benzoic acid (A) without any interference at the location of 3-phenylpropionic acid (C).

Fig. 3. Blank urine extract (1 ml from urine diluted 1/100) after acid hydrolysis containing total benzoic acid (A) without any interference at the location of 3-phenylpropionic acid (C).

TABLE I

REPRODUCIBILITY OF THE DETERMINATION OF BENZOIC ACID APPLIED TO AQUEOUS SOLUTIONS

Amount added (ng/ml)	Amount found: average of six assays (ng/ml)	95% confidence interval	Coefficient of variation (S.D. %)
10	11.1	9.5—12.6	13.3
20	21.1	19.0—23.3	9.8
50	48	45—50	4.6
200	197	190—204	3.4
400	394	373—416	5.2
1000	1029	999—1058	2.8
2000	2004	1942—2066	2.9

solutions. The 95% confidence intervals and the coefficients of variation were calculated on the basis of six replicate analyses of each sample. The lower concentration (10 ng/ml) may be taken as the sensitivity limit of the assay, although even lower concentrations could be detected.

Application

The technique was applied to measure the elimination of free and total benzoic acid in non-medicated subjects down to very low concentrations. The plasma and urine concentrations of free and total benzoic acid are given in Tables

II and III, respectively. The differences between total and free benzoic acid were expressed as hippuric acid; they are within the normal range of hippuric acid excretion in human urine, 0.1—1.0 g per 24 h [7]. In addition, small amounts of free benzoic acid were found in both plasma and urine.

Hippuric acid is normally present in human urine as a metabolite of dietary components containing food additives. Lehninger [8] reported that fatty acids with an uneven number of carbon atoms are partly metabolized into benzoic acid, but these fatty acids do not exist in terrestrial animals. The formation of hippuric acid from small doses of benzoic acid is extremely rapid in man, and the renal excretion of hippuric acid is not rate limited by the capacity of the renal tubular transport system, even at the highest excretion rates obtained after administration of benzoic acid [6].

TABLE II

FREE AND TOTAL BENZOIC ACID IN THE PLASMA OF THREE HEALTHY SUBJECTS DURING SIX DAYS, THE DIFFERENCE BETWEEN TOTAL AND FREE BENZOIC ACID BEING EXPRESSED AS HIPPURIC ACID

Subject	Hours	Free benzoic acid (ng/ml)	Total benzoic acid (ng/ml)	Hippuric acid (ng/ml)
1	0	40	1040	1467
	2	26	860	1224
	4	48	790	1089
	6	14	1310	1901
	8	36	680	945
	24	18	1240	1793
	72	36	950	1341
	120	62	1130	1567
2	0	30	1540	2215
	2	52	1160	1626
	4	66	1150	1590
	6	46	1390	1972
	8	52	1380	1948
	24	28	1640	2365
	72	50	2000	2861
	120	36	1990	2867
3	0	164	960	1168
	2	64	1220	1696
	4	136	820	1004
	6	92	1280	1743
	8	82	1160	1582
	24	94	880	1153
	72	86	850	1121
	120	224	930	1036

TABLE III

FREE AND TOTAL BENZOIC ACID IN THE URINE OF THREE HEALTHY SUBJECTS DURING SIX DAYS, THE DIFFERENCE BETWEEN TOTAL AND FREE BENZOIC ACID BEING EXPRESSED AS HIPPURIC ACID

Subject	Hours	Urine volume (ml)	Free benzoic acid (μg)	Total benzoic acid (mg)	Hippuric acid (mg)	Hippuric acid (mg per 24 h)
1	0—4	0	–	–	–	
	4—8	440	510	66	95	
	8—12	0	–	–	–	
	12—24	570	730	450	659	754
	24—32	165	426	166	242	
	32—48	725	1073	435	637	879
	72—80	475	1739	148	214	
	80—96	570	1277	213	311	525
	120—128	266	1229	87	126	
	128—144	580	1032	373	546	672
2	0—4	140	511	125	182	
	4—8	0	–	–	–	
	8—12	560	1764	195	283	
	12—24	300	930	315	461	926
	24—32	420	1638	318	464	
	32—48	320	1024	316	462	926
	72—80	770	2695	323	469	
	80—96	885	1726	279	406	875
	120—128	235	693	175	255	
	128—144	1020	1989	395	577	832
3	0—4	177	404	45	66	
	4—8	168	349	48	70	
	8—12	210	336	85	125	
	12—24	450	765	217	317	578
	24—32	430	568	104	151	
	32—48	500	820	250	365	516
	72—80	165	198	18	26	
	80—96	525	536	190	277	303
	120—128	490	431	102	150	
	128—144	525	567	207	302	452

CONCLUSION

The gas—liquid chromatographic technique described permits the quantitative assay of free benzoic acid and hippuric acid at low concentrations in plasma and urine.

ACKNOWLEDGEMENT

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CHROMBIO. 457

COMPARISON OF HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SERUM PROFILES OF HUMANS AND DOGS

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SUMMARY

The sera of 30 healthy male beagles were analyzed by reversed-phase high-performance liquid chromatography. The profiles were compared with those obtained from the sera of 30 healthy human donors. The chromatograms of each group were very reproducible; however, there were characteristic differences between the two groups. The compounds observed in both the human and canine profiles were identified as creatinine, uric acid, tyrosine, hypoxanthine, xanthine, kynurenine, inosine and tryptophan. Compounds present only in the canine profiles were identified as cytindine, riboflavin and 5-methylcytidine. Compounds present only in the human profiles include uridine, guanosine, hippuric acid and the dietary dependent compounds theobromine and caffeine. The compounds present in both human and canine sera were quantitated and compared statistically. The amounts of these compounds were very similar, except for uric acid.

INTRODUCTION

Biochemical assays of biological samples are useful in characterizing the abnormal processes of physiological disorders. In recent years, alterations in the concentrations of certain nucleosides and bases have been observed in the urine of patients suffering neoplastic diseases [1—5].

Although nucleosides and bases in urine have been studied extensively, their presence in serum has not, owing to the lack of adequately sensitive analytical methods. The development of microparticulate, reversed-phase packings for high-performance liquid chromatography (HPLC) has permitted the accurate, rapid and senstive determination of nucleosides and bases and other low-molecular-weight compounds present in serum at picomole levels [6—11].

Recent efforts in our laboratory have been directed at determining the normal range of values for the low-molecular-weight, UV-absorbing constituents of human sera, in order to evaluate changes that may occur as a result of disease processes [10—12]. We have also investigated the normal range

of low-molecular-weight, UV-absorbing compounds in the sera of male beagle dogs because beagles are used as models in studies of disease processes. Therefore, a comparative study of the constituents in human and the beagle sera was made.

MATERIALS AND METHODS

Instrumentation

A Waters Assoc. (Milford, Mass., U.S.A.) liquid chromatograph equipped with two Model 6000 A solvent delivery systems, a Model 660 solvent programmer, a Model U6K injector and a Model 440 dual-wavelength detector was used in all determinations. An on-line SF 770 Spectroflow Monitor and an FS 970 L.C. Fluorometer (Schoeffel Instruments Div., Kratos Inc., Westwood, N.J., U.S.A.) were also used. A two-channel Omniscribe recorder (Houston Instruments, Austin, Texas, U.S.A.) was used to monitor the 254- and 280-nm responses. Retention times and peak areas were obtained with an HP 3380-A electronic integrator (Hewlett-Packard, Avondale, Pa., U.S.A.).

Materials

All standard reference compounds and high-purity enzymes were purchased from Sigma (St. Louis, Mo., U.S.A.). Solutions of the reference compounds and enzymes were prepared in doubly distilled, de-ionized water and buffered with reagent-grade potassium dihydrogen orthophosphate (Mallinckrodt, St. Louis, Mo., U.S.A.) at pH 7.0. Methanol was purchased from Burdick and Jackson (Muskegon, Mich., U.S.A.).

Chromatographic conditions

Separations were obtained on microparticulate, chemically bonded, reversed-phase columns (300 × 3.9 mm I.D., μBondapak; Waters Assoc.) protected by a pre-column (50 × 4.6 mm I.D.) dry-packed with 25-μm, pellicular, reversed-phase material (Whatman, Clifton, N.J., U.S.A.). A 35-min linear gradient from 100% of the initial eluent (0.02 *F* potassium dihydrogen orthophosphate, pH 5.6) to 40% of the final eluent (60% methanol—water) was used to obtain rapid separations of the serum constituents. The flow-rate was 1.5 ml/min and the temperature was ambient.

Sample preparation

To obtain human serum samples, freshly drawn blood was collected from donors of similar age in Vacutainer tubes without anticoagulant. The donors had no known diseases and were not taking any medication. The blood was allowed to clot spontaneously for 10–15 min at ambient temperature. Then the tubes were centrifuged at 575 relative centrifugal force (RCF) for 10 min and the supernatant was allowed to sit over the clot for 3—4 h [11]. The supernatant was collected and filtered through Amicon membrane cones (Amicon, Lexington, Mass., U.S.A.) to remove protein and other materials with molecular weights greater than 25,000. The protein-free filtrates were transferred into polyethylene vials and stored at −20°.

Canine serum samples were obtained from a closed-colony group of male

beagle dogs of similar age, weight and size maintained at the Borriston Research Labs. (Temple Hills, Md., U.S.A.). Blood was drawn, allowed to clot and centrifuged in the manner described above. The supernatant was collected and transported frozen. Upon receipt, the samples were allowed to sit for 3 h at ambient temperature, then filtered through the membrane cones.

Each serum sample was analyzed in triplicate.

Peak identifications

Tentative identifications of the low-molecular-weight, UV-absorbing compounds present in the serum were based on comparisons of retention times and absorbance ratios ($area_{280}/area_{254}$) with those of reference compounds. Each standard reference compound which had a retention time and absorbance ratio similar to those of a peak in a serum chromatogram was co-chromatographed with the serum sample to determine if the standard co-eluted and if the absorbance ratios were constant.

To further support identifications, enzymatic peak shifts and UV and fluorescence spectra were obtained for the peaks in the chromatograms [10, 11].

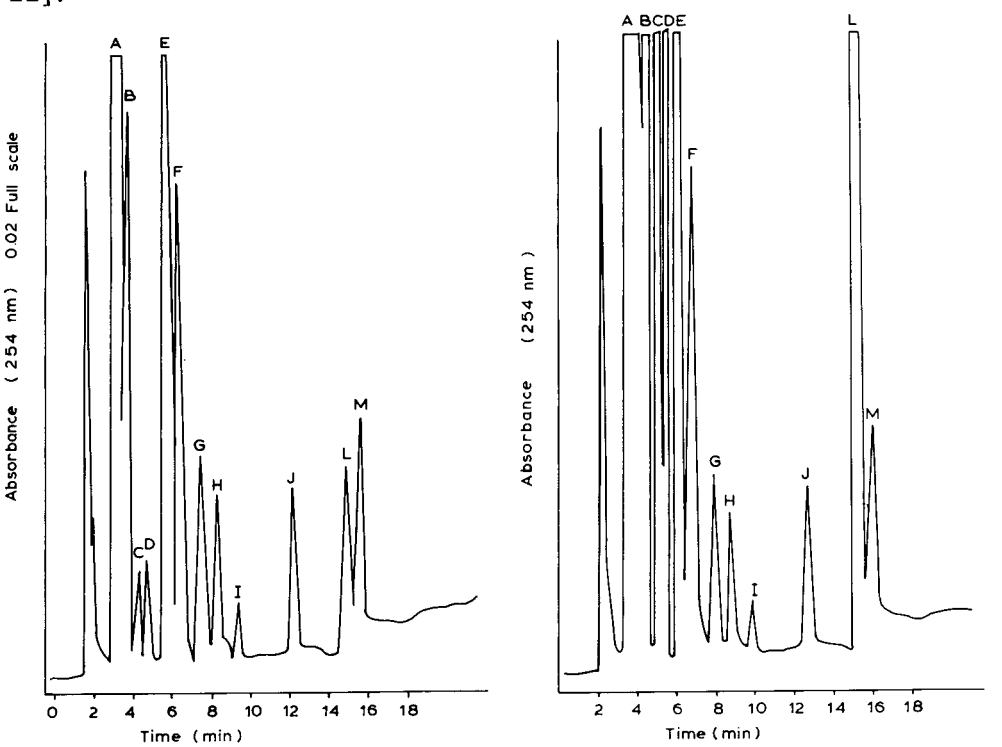

Fig. 1. Chromatogram of beagle serum constituents. Injection volume: 40 μl. Chromatographic conditions listed in text.

Fig. 2. Serum sample illustrated in Fig. 1 co-chromatographed with the standards creatinine (A), uric acid (B), tyrosine (C), cytidine (D) and tryptophan (L).

For the enzymatic peak shift, a 10-μl aliquot of enzyme solution specific to the peak of interest was added to a 50-μl aliquot of serum, the reaction mixture chromatographed and the profile compared with that of the untreated serum sample.

The UV spectra of the peaks of the serum profiles were obtained by a stopped-flow scanning technique [8]. Background interference was stored in the detector memory module, where it is automatically subtracted to give an accurate spectrum of the serum peaks as they eluted from the column.

Fluorescence was used selectively to detect and identify uric acid, tyrosine, kynurenine and tryptophan in the serum profiles [7, 11]. Further, as few naturally occurring serum constituents fluoresce, it is possible to characterize these biologically important compounds without interferences.

RESULTS

The identification techniques used in determining the compounds present in the human and canine HPLC profiles are illustrated with a beagle serum sample (Fig. 1). The peaks of the profile were first identified by co-chromatographing the serum sample with standard reference compounds that had similar retention times and absorbance ratios to those of peaks in the serum profile. Increases in peak areas caused by the co-eluting compounds and constant absorbance ratios (Fig. 2) indicate that peaks with the retention times of creatinine, uric acid, tyrosine, cytidine and tryptophan are present in the sample. Peaks were further characterized by obtaining UV spectra of each peak

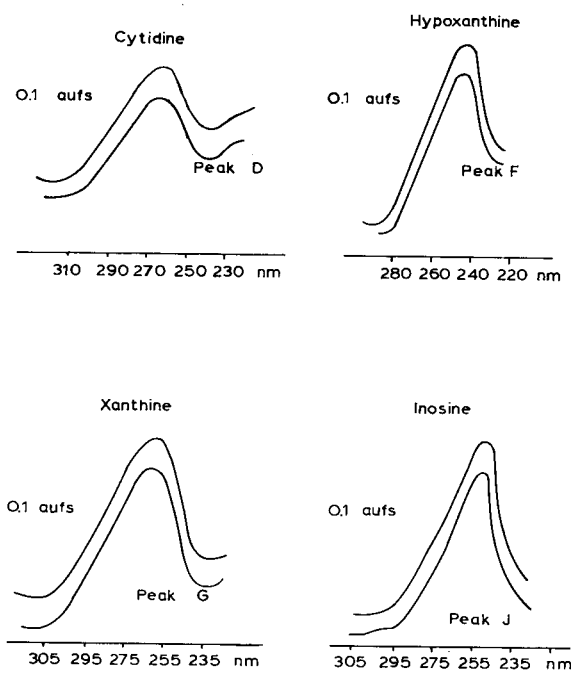

Fig. 3. UV spectra obtained with the stopped-flow technique from canine serum and reference compounds.

in the serum profile. The spectra of the serum peaks were then compared with those of standard reference compounds (Fig. 3). Chromatograms using fluorescence detection were obtained with an excitation wavelength of 285 nm and an emission cut-off filter of 320 nm, which indicated that the three peaks in Fig. 4 were tyrosine, kynurenine and tryptophan. Finally, commercially available enzymes specific to the compound of interest were used to confirm peak identity by reacting an aliquot of enzyme solution with the serum sample. For examlpe, an aliquot of serum that was incubated with a xanthine oxidase solution for 25 min was chromatographed. The chromatogram was then compared with the profile of the untreated serum sample (Fig. 5). The disappearance of two peaks in the profile supports the identification of peaks F and G as hypoxanthine and xanthine. Therefore, using a combination of data obtained with the various techniques, the peaks of the canine profiles were identified as (A) creatinine, (B) uric acid, (C) tyrosine, (D) cytidine, (F) hypoxanthine, (G) xanthine, (J) inosine and (L) tryptophan. Peaks E, H and I were tentatively identified as riboflavin, kynurenine and 5-methylcytidine, respectively. Peak M remained unidentified.

The same identification procedures were used to determine the peaks of the human serum profiles [11]. A comparison of the human and canine profiles is

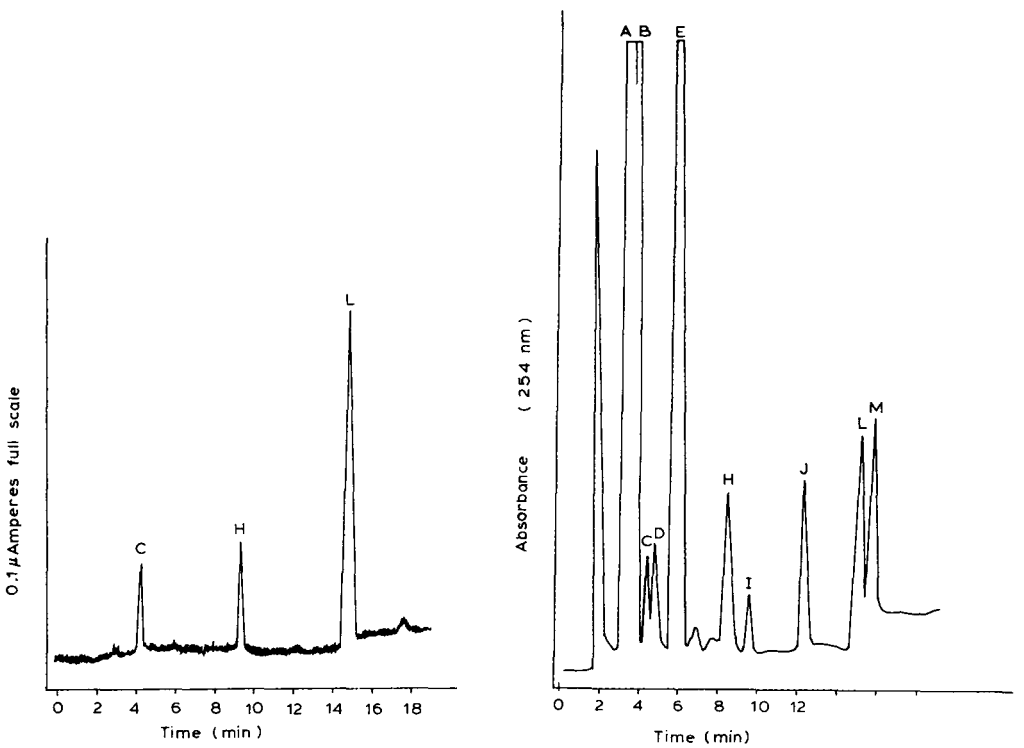

Fig. 4. Canine serum detected with fluorescence. Excitation wavelength: 285 nm. Emission cut-off wavelength: 320 nm. Peaks: C = tyrosine, H = kynurenine, L = tryptophan.

Fig. 5. Canine serum sample reacted with xanthine oxidase. See text for conditions. Note the absence of peaks F (hypoxanthine) and G (xanthine), the increase in peak B (uric acid) and no change in any of the other peaks.

shown in Fig. 6. Creatinine, uric acid, tyrosine, hypoxanthine, xanthine, kynurenine, inosine and tryptophan were found in the sera of both humans and beagle dogs. Uridine, guanosine, hippuric acid, theobromine and caffeine were found only in the human sera, while compounds with the retention times of cytidine, 5-methylcytidine and riboflavin were found only in canine sera.

The compounds present in the serum profiles were quantitated by an external standard method. Response factors for each known peak were determined by chromatographing a known amount of the standard reference compounds. The response factors were linear over the range of concentrations found in serum. An average value of triplicate analyses of the serum

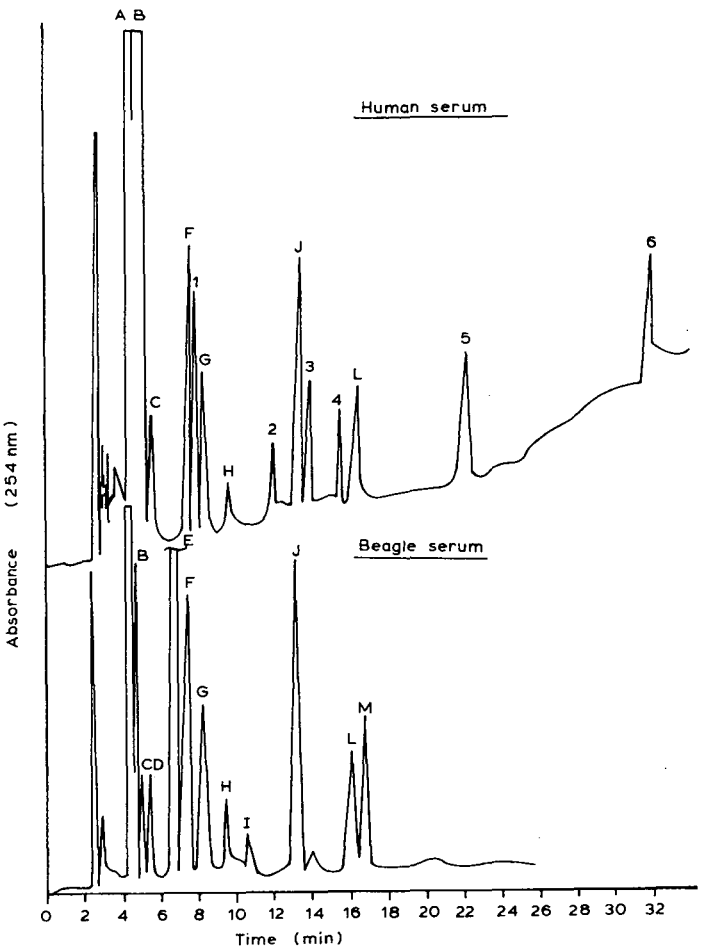

Fig. 6. Comparison of the low-molecular-weight, UV-absorbing serum constituents of humans and beagle dogs. Injection volume: 40 μl. Components of human serum: creatinine (A), uric acid (B), tyrosine (C), hypoxanthine (F), uridine (1), xanthine (G), kynurenine (H), unknown (2), inosine (J), guanosine (3), hippuric acid (4), tryptophan (L), theobromine (5) and caffeine (6). Components of canine serum that differ from those of human serum: cytidine (D), riboflavin (E), 5-methylcytidine (I) and unknown (M). The numbered peaks are those which do appear in the chromatogram of the human serum but not in that of the beagle serum.

TABLE I

CONCENTRATIONS OF LOW-MOLECULAR-WEIGHT UV-ABSORBING COMPOUNDS IN BEAGLE AND HUMAN SERA

Compound	Concentration ± standard deviation (μmole/l)	
	Beagle serum	Human serum
Creatinine (Cre)	89.1 ± 30.6	83.1 ± 11.3
Uric acid (UA)	6.96 ± 10.6	295 ± 39.0
Tyrosine (Tyr)	43.2 ± 12.5	62.2 ± 11.3
Cytidine (Cyd)	1.90 ± 0.82	—
Riboflavin (Rbf)	209 ± 59.0	—
Hypoxanthine (Hyp)	8.95 ± 3.33	7.16 ± 2.81
Uridine (Urd)	—	3.17 ± 1.11
Xanthine (Xan)	8.72 ± 2.25	2.62 ± 1.04
Kynurenine (Kyn)	116 ± 40	103 ± 48
5-Methylcytidine (5mCyd)	2.66 ± 0.84	—
Inosine (Ino)	4.85 ± 1.40	5.62 ± 2.87
Guanosine (Guo)	—	0.881 ± 0.515
Hippuric acid (HA)	—	0.613 ± 0.477
Tryptophan (Trp)	55.4 ± 13.2	13.7 ± 3.57
Theobromine (Thb)	—	0 to 6.35 (dietary)
Caffeine (Caf)	—	0 to 15.6 (dietary)

components was used in the quantitations. Table I lists the average values and normal range of values for the low-molecular-weight, UV-absorbing compounds present in the sera of beagle dogs and humans.

The data from the human serum profiles were compared statistically with the data obtained from the canine serum profiles using a two-group comparison test based on Student's *t*-distribution. The levels of the compounds present in both the human and canine serum were not significantly different (95% confidence limit), except for uric acid, the amount of which was significantly greater in the human sera than in the canine sera.

DISCUSSION

Recently, reports on the identification and quantitation of some low-molecular-weight, UV-absorbing constituents of human sera by HPLC have appeared [10, 11]. Although there are a number of reports on the high-molecular-weight constituents of canine sera [13–16], little has been reported on the low-molecular-weight constituents, such as nucleosides and bases.

However, in the plasma of mongrel dogs, Tseng et al. [17] found that the levels of two compounds, cytidine and uric acid, were 1.89 and 0.367 μM respectively. In addition, uridine was found to be converted rapidly into uracil in the plasma of these dogs by enzymes not present in human plasma. On the other hand, cytidine was converted into uridine in human plasma by enzymes not present in the dogs. Our results support these findings because cytidine was found in the canine sera whereas uridine was not.

In addition, 5-methylcytidine, which has been found in some mammalian

cells [18, 19], appears to be a normally occurring serum constituent in beagle serum although not in human serum.

In man, the end product of purine metabolism is uric acid; however, in many animals and birds the end product is allantoin. The low levels of uric acid in the canine sera support the fact that there is a higher activity of the enzyme uricase in beagles than in humans.

As there are similar concentrations of most nucleosides and bases in the sera of humans and beagles, it appears that the major purine metabolic pathways are similar, even though there are significant differences in the pyrimidine pathways. Therefore, beagles can be used as models of the human system in certain experiments related to purine metabolism, although it remains to be seen whether there are other animal species more closely related to humans in their serum constituents.

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CHROMBIO. 452

RAPID DETERMINATION OF GLYCINE- AND TAURINE-CONJUGATED BILE ACIDS IN HUMAN BILE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

With use of an anion-exchange packing, TSK Gel IEX 540 DEAE, for high-performance liquid column chromatography, glycine- and taurine-conjugated bile acids were separated in 10 min and detected with a differential refractometer. Human bile could be analyzed after a simple pretreatment. The purity of the peaks of glycine- and taurine-conjugated bile acids in human bile was confirmed by enzymatic determination using 3α-hydroxysteroid:NAD oxidoreductase. The molar ratios of the two forms of the conjugates (glycine/taurine ratios) in bile from normal subjects and from patients suffering from various hepatobiliary diseases were measured.

INTRODUCTION

The bile acids are synthesized from cholesterol, metabolized mainly to glycine conjugates (G) and/or taurine conjugates (T) in the liver, stored in the gallbladder, excreted intermittently into the duodenum, partially deconjugated by the anaerobes, reabsorbed from the ileum—proximal colon into the portal vein to reach the liver, and recirculated to reach the enterohepatic circulation.

The G/T ratio in the bile is changed in hepatobiliary diseases and intestinal disorders [1—7]. Patients with ileal disorders showed markedly high G/T ratios from 10 to 20, sometimes nearing 30, while the normal range is between 2 and 5 [3, 8]. This is supposedly due to a more rapid rate of conjugation for glycine than taurine in the liver to compensate for a deficiency in the conjugates caused by malabsorption in the intestine in ileal disorders. On the other hand, the majority of patients with tropical sprue showed

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G/T ratios lower than 1 [2]. These findings suggest the importance of measuring G/T ratios.

The separation of G and T groups has been attained on a column of silicic acid [9], ion-exchange resin [1], for example Dowex-1, and Sephadex PHP-LH-20 [10]. However, these procedures were not sufficiently simple or rapid for routine clinical use.

In this paper, a rapid assay of G and T by high-performance liquid chromatography (HPLC) using an anion-exchange packing, TSK Gel IEX 540 DEAE, was developed and applied to human bile samples.

EXPERIMENTAL

Chemicals

Glycocholic acid (GC), glycodeoxycholic acid (GDC), glycochenodeoxycholic acid (GCDC), taurocholic acid (TC), taurodeoxycholic acid (TDC) and taurochenodeoxycholic acid (TCDC) were of the highest purity obtained from Sigma (St. Louis, Mo., U.S.A.). Glycoursodeoxycholic acid (GUDC), glycolithocholic acid (GLC) and taurolithocholic acid (TLC) were the gift of Dr. Osuga, Faculty of Medicine, Tsukuba University. Cholic acid (C; extra pure reagent) and deoxycholic acid (DC) were obtained from Nakarai (Kyoto, Japan) and lithocholic acid (LC) was from Tokyo Kasei (Tokyo, Japan). Chenodeoxycholic acid (CDC) was kindly supplied by Yamanouchi (Tokyo, Japan) and ursodeoxycholic acid (UDC) from Tokyo Tanabe (Tokyo, Japan). Lithocholic acid-3α-sulfate (LC-3α-S) and glycochenodeoxycholic acid-3α-sulfate (GCDC-3α-S) were synthesized according to the method of Haslewood and Haslewood [11].

Ethanol, methanol, citric acid and sodium citrate were of reagent grade from Kanto Kagaku (Tokyo, Japan). 3α-Hydroxysteroid:NAD oxidoreductase (EC 1.1.1.50; 3α-HSD) from Nyegaard & Co. (Oslo, Norway) and diaphorase (EC 1.6.4.3) from Sigma were products of the highest purity. NAD was obtained from Boehringer (Mannheim, G.F.R.) and resazurin from Daiichi (Tokyo, Japan).

Packing for high-performance liquid chromatography

Recently, an anion-exchange packing, TSK Gel IEX 540 DEAE (Toyo Soda Manufacturing Co., Tokyo, Japan), for HPLC became available. The packing consists of spherical particles of silica gel coated covalently with polyethylene glycol to which diethylaminoethyl residues are attached.

Chromatographic system

The HPLC system used in this study was assembled from modular components and consisted of a Model SM8P10 synchronous motor pump (Japan Servo Co., Tokyo, Japan), a Kyowa KMH-6V sampling valve with a 112-μl loop (Kyowa Seimitsu Co., Tokyo, Japan), a glass column packed with TSK Gel IEX 540 DEAE (5 μm particle diameter, 80 mm × 8 mm I.D.), and a differential refractometer, Shodex RI SE-11 (Showa Denko, Tokyo, Japan). The column temperature was maintained at 45°. The eluent, 0.017 *M* sodium citrate buffer (pH 3.6)—ethanol (3:2, v/v) degassed prior to use by aspiration and ultrasonication, was pumped at a rate of 0.92 ml/min.

Human bile

Human bile samples from normal subjects and from patients suffering from various diseases were collected by intubation using magnesium sulfate mixture as a gallbladder constrictor.

Procedures

The standard bile acids were dissolved in the eluent. The bile was centrifuged at 650 *g* for 20 min and the supernatant was diluted several fold with the eluent. The solution was recentrifuged, if necessary, and was directly applied to HPLC.

For the enzymatic analysis the peak of G or T from HPLC was collected and subjected to enzymatic assay using 3α-HSD according to the method originally reported by Mashige et al. [12] but slightly modified as follows. To each of 0.2-ml portions of the fraction in two separate tubes, A and B, 1.9 ml of 0.05 *M* Tris—HCl buffer (pH 9.0), the mixture of 0.2 ml of resazurin (100 μ*M*)—NAD (6 mg/ml) and 0.5 ml of diaphorase solution (0.5 U/ml) were added. Then, 0.2 ml of 3α-HSD solution (0.024 I.U./ml) was added to tube A and 0.2 ml of 0.05 *M* Tris—HCl buffer (pH 9.0) without 3α-HSD was added to tube B. After incubation at 20° for 60 min, fluorescence was measured at 580 nm (excitation at 560 nm) using an Hitachi MPF-2A fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The net fluorescence was obtained by subtracting the intensity of B from that of A.

RESULTS

Conditions for the separation of G and T groups

The free bile acids (F), G and T were not eluted from the packing with an aqueous solution containing no ethanol. In the presence of 40% ethanol the group separation of G from T was good at pH 3.6 (Table I). F were not retained under these conditions. However, at pH 3.3 and 4.0 separation was observed within the G or T group (Fig. 1). A small change in the ethanol content of the buffer greatly affected the capacity factor (k') of each bile acid (Fig. 2). The more hydrophilic bile acids have a tendency to elute earlier with a lower ethanol content in the buffer. Therefore the ethanol content should be fixed at 40% to allow the bile acids of each group to be eluted simultaneously. There

TABLE I

CAPACITY FACTOR (k')* OF THE FREE (F), GLYCINE (G) AND TAURINE (T) CONJUGATED BILE ACIDS ON TSK GEL IEX 540 DEAE COLUMN

Conditions: TSK Gel IEX 540 DEAE column (5 μm particle size; 80 × 8 mm I.D.); column temperature, 45°; detector, Shodex RI SE-11; eluent, 0.017 *M* sodium citrate buffer (pH 3.6)—ethanol (3:2, v/v); flow-rate, 0.92 ml/min (24—29 kg/cm²).

Bile acid	LC	DC	CDC	UDC	C
F	≈ 0	≈ 0	≈ 0	≈ 0	≈ 0
G	0.83 ±0.00	0.89 ±0.01	0.85 ±0.01	0.90 ±0.00	0.88 ±0.01
T	2.23 ±0.01	2.27 ±0.00	2.34 ±0.01	2.29 ±0.03	2.34 ±0.01

*$k' = (t_R - t_0)/t_0$, where t_R = retention time for solute molecules and t_0 = retention time for solvent molecules.

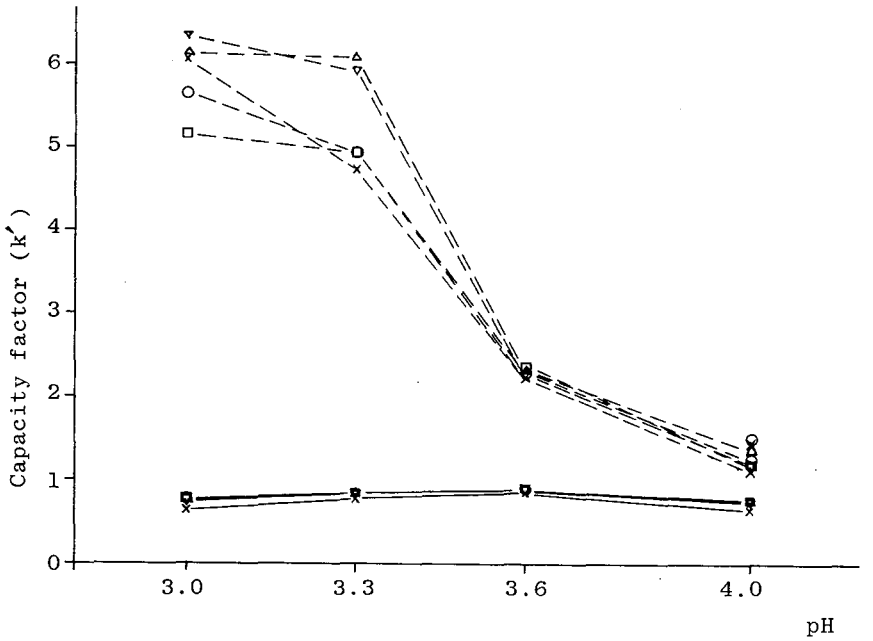

Fig. 1. Effect of buffer pH on the capacity factor (k') of the conjugated bile acids. The HPLC conditions are the same as in Table I except for the pH of the buffer. ×, GLC; •, GDC; ▲, GCDC; ▲, GUDC; ■, GC; ×, TLC; ○, TDC; △, TCDC; ▽, TUDC; □, TC.

were still small differences in the k' values of the bile acids within a group; thus calibration was performed using peak area instead of peak height.

Calibration of G and T

Since the relative peak area (the peak area was calculated by multiplying peak height (h) by peak width measured at 0.607 h) of each bile acid in each group to that of GDC or TDC was almost unity, as shown in Table II, the calibration curves for G and T were prepared using GDC and TDC, respectively, as representatives. The peak areas were proportional to the quantities of bile acids from 0.2 to 4 mM for G and from 0.2 to 2 mM for T.

Recovery and reproducibility of the HPLC method

Various amounts of GDC and TDC were added to a bile sample and analyzed. By the use of the calibration curves mentioned above, the recovery of these added standards was found to be quantitative. The reproducibility of the

TABLE II

RELATIVE RESPONSE OF GLYCINE (G) AND TAURINE (T) CONJUGATED BILE ACIDS

The HPLC conditions are the same as in Table I.

Bile acid	LC	DC	CDC	UDC	C
G	0.97 ±0.02	1	1.02 ±0.01	1.00 ±0.00	0.98 ±0.01
T	1.06 ±0.02	1	1.05 ±0.02	0.97 ±0.01	1.05 ±0.01

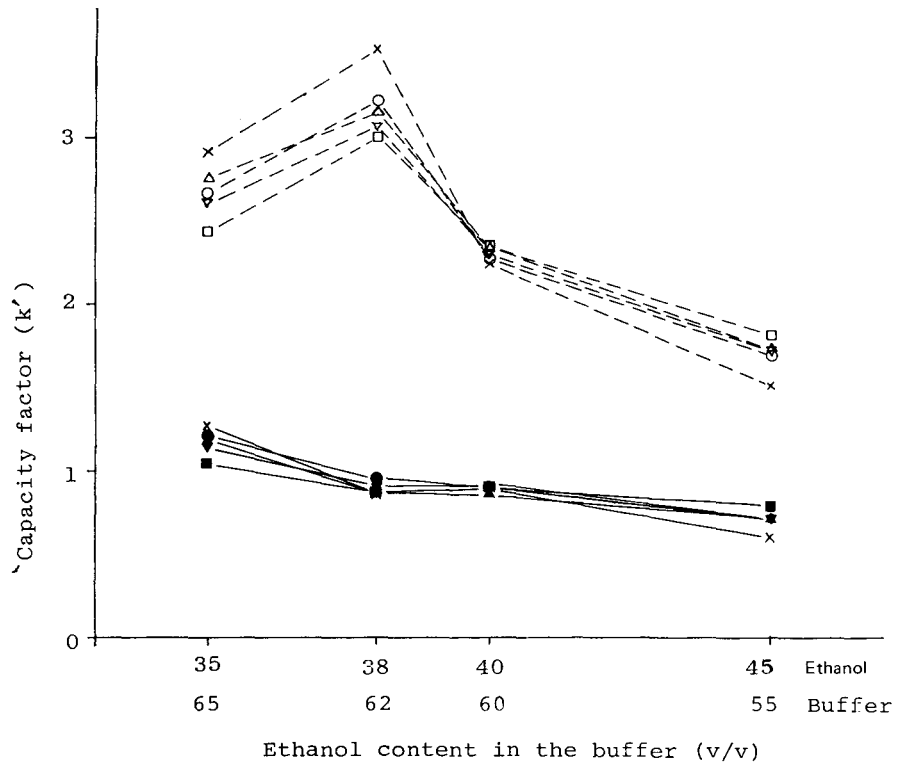

Fig. 2. Effect of the ethanol content of the buffer on the capacity factor (k') of the conjugated bile acids. The HPLC conditions are the same as in Table I except for the ethanol content of the buffer. Symbols as in Fig. 1.

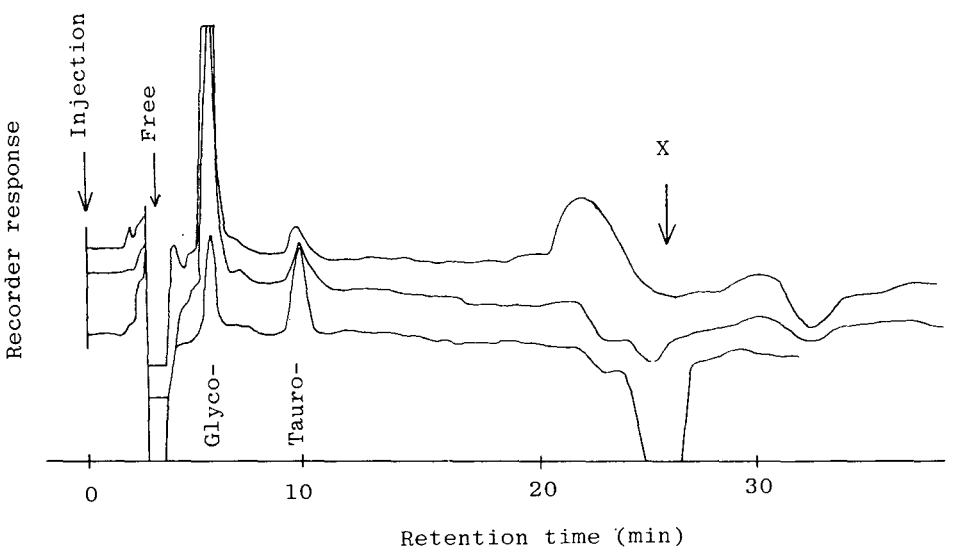

Fig. 3. HPLC of human bile samples from various hepatobiliary diseases. The chromatograms correspond to 28 μl of the original samples. The HPLC conditions are the same as in Table I.

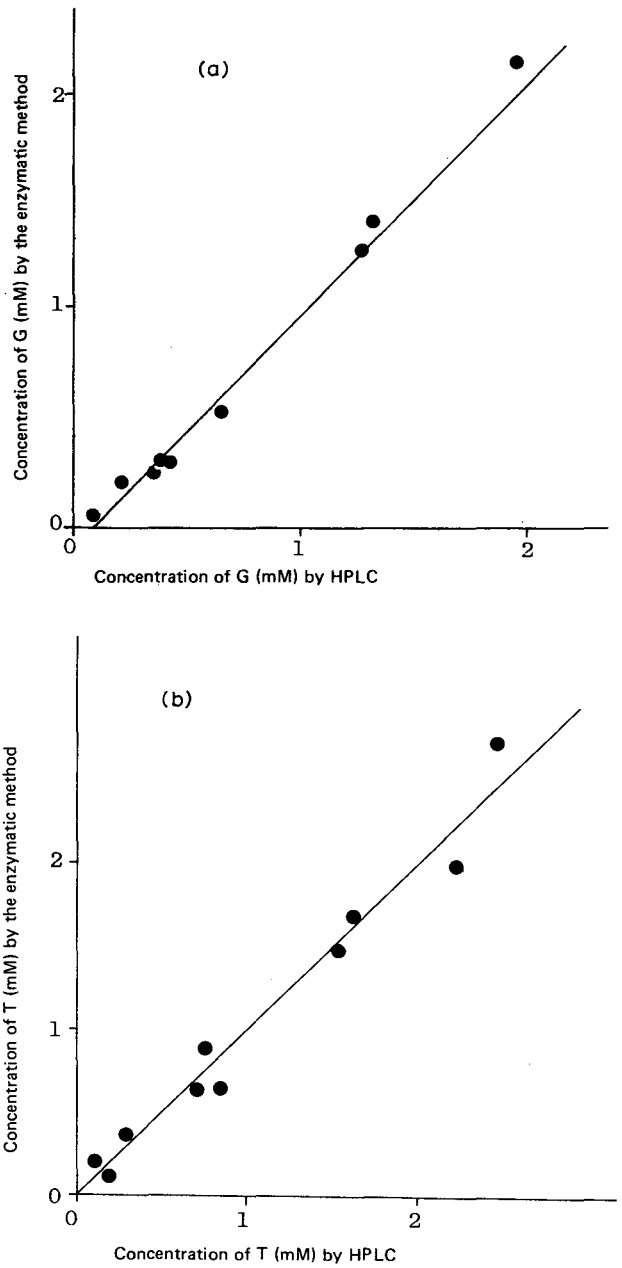

Fig. 4. Correlation of the values of the glycine (a) and taurine (b) conjugated bile acids obtained by the present method with those obtained by the enzymatic method. Peak fractions corresponding to G and T were treated as in the text.

TABLE III

CONCENTRATION AND RATIO OF THE CONJUGATED BILE ACIDS DETERMINED BY HPLC IN HUMAN BILE FROM NORMAL SUBJECTS AND FROM PATIENTS SUFFERING FROM VARIOUS HEPATOBILIARY DISEASES

Sample	Disease***	Concentration (m*M*)		G/T ratio
		G	T	
1*	Obstructive jaundice	0.53	0.20	2.7
2**	Obstructive jaundice[a]	10.72	4.88	2.2
3**	Obstructive jaundice[a]	1.50	2.20	0.7
4**	Cholelithiasis	16.00	3.20	5.0
5**	Cholelithiasis	1.63	0.82	2.0
6**	Cholelithiasis[b]	20.11	3.99	5.0
7*	Choledochus cyst	5.24	1.30	4.0
8*	Choledochus cyst	1.81	3.02	0.6
9**	Dilation of choledochus	10.21	1.51	6.8
10*	Carcinoma of the gallbladder[c]	63.25	8.25	7.7
	Normal subjects (n = 7)	—	—	2.2 ±0.8

*Hepatic bile.
**Gallbladder bile.
***a, During operation; b, under medication with CDC; c, post operation.

analysis was good (4.64 ± 0.13 m*M*, C.V. = 3%, for G and 1.17 ± 0.08 m*M*, C.V. = 4%, for T, n = 6).

Confirmation of the purity of the peaks of G and T

Typical chromatograms obtained with human bile are shown in Fig. 3. The values obtained by the enzymatic determination of bile acids in the fractions corresponding to G and T agreed well with those obtained using the present method ($y = 1.08x - 0.16$, $r = 0.990$ in Fig. 4a, and $y = 1.02x - 0.003$, $r = 0.988$ in Fig. 4b).

Application to human bile

The method was applied to human bile samples from normal subjects and from patients suffering from various hepatobiliary diseases. The values of G, T and the G/T ratio are shown in Table III. The normal value of the G/T ratio agreed with those in the literature; for example, 3.2 ± 1.0 (n = 20; Sjövall, 1960 [8]) and 3.4 ± 0.6 (n = 4; Abaurre et al., 1969 [3]). The values obtained from patients suffering from obstructive jaundice and cholelithiasis also agreed with those in the literature [8, 13, 14].

DISCUSSION

It seems reasonable that F, G and T were eluted in this order using the TSK Gel IEX 540 DEAE anion-exchange column because in the eluting buffer (pH 3.6) F, G and T are scarcely, partially and almost completely dissociated, respectively (pK_a of F, G and T: 5.0—6.5, 3.8—4.8 and 1.8—2.0, respectively [15]). Therefore, the mechanism of the separation by this system is mainly ion exchange. In addition, the fact that only a slight change in the ethanol content

of the buffer affects the k' of each bile acid suggests the secondary contribution of reversed-phase partition.

The group separation of G and T in human bile was possible in about 10 min after simple pretreatment of the bile samples. It would be suggested that each peak of G or T obtained from bile samples contained solely 3α-hydroxysteroids. Therefore, this method is simple, rapid and reliable in obtaining G/T ratios of bile. It is also useful for investigating the conjugation pattern of bile acids in hepatobiliary diseases.

The sensitivity of the method with a differential refractometer is about 0.1 m*M* for G and T. However, by using fluorescence detection with 3α-HSD, NAD and/or diaphorase—resazurin [16, 17], sufficient sensitivity will be obtained to analyze human serum.

The fairly good agreement between the values of the G/T ratios in human bile obtained by the present method and those in the literature would suggest the adequate reliability of this method.

The negative peak X in Fig. 3, which did not react in the enzymatic assay, was subjected to acid hydrolysis [18] and a portion was subjected to HPLC. As the result, new peaks appeared with retention times of 5.5 min and 10 min, which corresponds to those of G and T, respectively. The synthesized LC-3α-S and GCDC-3α-S were injected into the HPLC column and eluted at about 26 min. These data suggest that peak X contained 3α-sulfate(s) of bile acids.

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Biomedical Applications

CHROMBIO. 456

DETERMINATION OF TRYPTOPHAN AND METABOLITES IN RAT BRAIN AND PINEAL TISSUE BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

Tryptophan and many of its indole metabolites were separated using reversed-phase high-performance liquid chromatography (HPLC) and determined using electrochemical detection. This was accomplished isocratically using an acetate—citric acid eluent with various amounts of methanol. Brain and pineal tissue was analyzed for several tryptophan metabolites. Tissue preparation required only homogenization in acidic solution and centrifugation prior to application to the HPLC column. Detection limits in the low picogram range were found for those indoles separated.

INTRODUCTION

The involvement of tryptophan (TRP) and its metabolites in a variety of both normal functions and pathological states has been well established [1]. Tryptophan is metabolized by two major pathways, either the "kynurenine" pathway, leading to the formation of NAD, or through a series of indoles, as shown in Fig. 1.

Various techniques have been employed to determine tryptophan and its metabolites in brain and other tissues, including thin-layer chromatography [2, 3], amino acid analysis [4], gas—liquid chromatography with electron-capture detection [5], UV spectrometry [6], fluorescence spectrometry [7, 8], gas chromatography—mass spectrometry (GC—MS) [9, 10] and radio-immunoassay (RIA) [9, 11, 12]. Each of these has its drawbacks, either in sensitivity, selectivity or versatility. Recently, several analyses employing high-performance liquid chromatography (HPLC) with fluorescence detection of tryptophan metabolites have been described [13—17]. HPLC with electro-

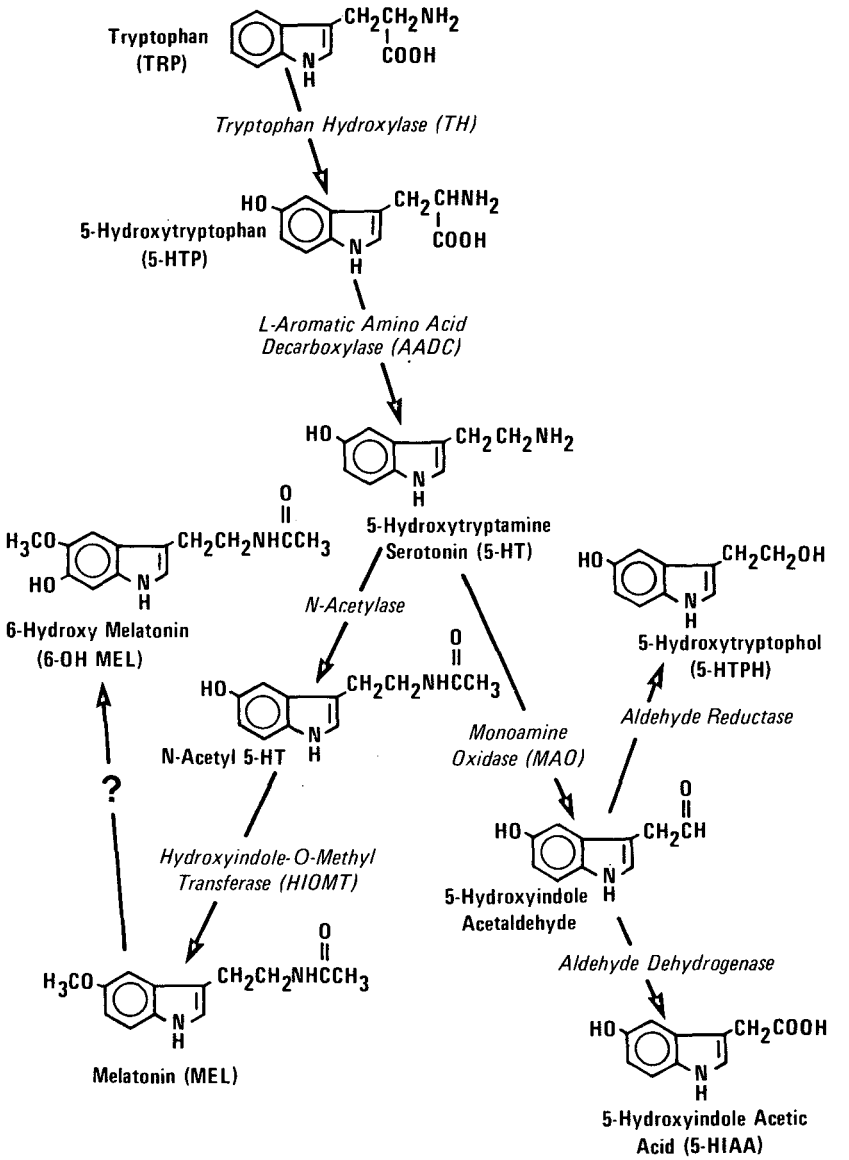

Fig. 1. Proposed synthetic route for tryptophan—indole metabolism in rat brain and pineal.

chemical detection (LCEC) has been employed to determine 5-HT and 5-HIAA in brain tissue [18—20], serum [21] and cerebrospinal fluid [22]. These LCEC methods have employed conventional pellicular (30—50 μm) ion-exchange resins, excluding the simultaneous determination of amine, amino acid and acid metabolites. The use of reversed-phase HPLC allows the simultaneous determination of these with a single injection, but it has not previously been coupled with electrochemical detection for the determination of this series of compounds.

LCEC has been primarily used for the determination of compounds with low oxidation potentials (i.e., <+0.8 V versus Ag/AgCl), such as the catecholamines and their metabolites [23]. However, with proper care in elimination of excess background oxidation current and electrode preparation, compounds of higher oxidation potentials may be determined.

We report here the separation of a variety of tryptophan metabolites [tryptophan, 5-hydroxytryptophan, 5-hydroxytryptamine, 5-hydroxyindoleacetic acid, N-acetyl-5-hydroxytryptamine, melatonin (N-acetyl-5-methoxytryptamine), 5-methoxytryptophan, 5-methoxyindole, 5-hydroxytryptophol and 5-methoxytryptophol] by reversed-phase HPLC and their detection by electrochemical oxidation. These chromatographic separations have been applied to the determination of tryptophan and several metabolites in rat brain and pineal tissue. Detection limits in the low picogram range are reported for all those metabolites analyzed.

EXPERIMENTAL

Apparatus

Chromatography was performed with a Spectraphysics Model 3500 liquid chromatograph equipped with a 25 cm × 3.2 mm I.D. stainless-steel column packed with 10-μm Vydac 201 TP reversed-phase resin (Separations Group, Hesperia, Calif., U.S.A.). Electrochemical detection was accomplished using a Model LC-2A amperometric detector [Bio-Analytical Systems (BAS), West Lafayette, Ind., U.S.A.]. Detector electrodes of two types were employed. For solvents containing methanol, the TL-3 detector electrode (BAS) was packed with a wax—graphite paste, CP-W (BAS). For aqueous solvents containing no methanol, the TL-3 was packed with a silicone grease—graphite (40:60, w/w) paste.

Oxidation potentials of compounds of interest were determined by cyclic voltammetry using the Model CV-1A (BAS) at a scan rate of 100 mV/sec. A carbon paste working electrode and an Ag/AgCl reference electrode were employed.

Reagents

Standards were obtained from Sigma (St. Louis, Mo., U.S.A.). Reagent-grade methanol (J.T. Baker, Phillipsburg, N.J., U.S.A.) was used as obtained. All other chemicals used were of reagent grade. Solvents were filtered through a 0.1-μm Millipore filter and vacuum deaereated. Standard solutions (2 m*M*) were prepared in 0.1 *M* perchloric acid and diluted to appropriate concentrations. Melatonin, N-acetylserotonin, 6-hydroxymelatonin and methoxylated indoles were dissolved in 0.1 *M* perchloric acid—methanol (1:9) and diluted appropriately with 0.1 *M* perchloric acid.

Tissue preparation

Male albino rats of weight ca. 400 g (Sprague-Dawley, Simonsen Labs., Gilroy, Calif., U.S.A.) maintained on a 12-h light—dark cycle were used. The animals were killed at approximately 2400 h under a very dim red light and the pineals and brains removed. The pineals were placed on dry-ice, and the hypo-

thalami, a portion of cerebellum (ca. 50 mg) and a portion of the midbrain containing raphe nuclei [24] were dissected out and also placed on dry-ice. Tissues were stored at −80° until taken for analysis.

Prior to analysis, brain tissues were weighed into 1.5-ml polypropylene tubes and 400 μl of 0.1 *M* perchloric acid added to each. Pineal glands were not weighed, but were placed in a 1.5-ml polypropylene tube and 200 μl of 0.1 *M* perchloric acid added to each. All tissues were thoroughly disrupted by sonication (Sonifier Cell Disruptor Model W 185D, Heat Systems — Ultrasonics Inc., Plainview, N.Y., U.S.A.). The tissue homogenate was then centrifuged at 15,000 rpm for 10 min using a Brinkman Model 3200 microcentrifuge. A 50-μl amount of the clear supernatant was injected into the chromatographic system. Quantitative determinations were made by comparing the peak heights of the samples with those given by known concentrations of standards.

RESULTS

Table I shows the electrochemical characteristics (oxidation potentials), retention times, detection limits and chromatographic conditions used for several indoles and tryptophan metabolites. Although indoles containing phenolic functional groups are considerably more readily oxidized, it is obvious that the lack of the phenolic group does not preclude the determination of many indoles using LCEC. In order to maximize the sensitivity for all indoles chromatographed, the applied potential was maintained at +0.9 V versus Ag/AgCl. Although increasing the potential to +1.0 V increased the sensitivity for tryptophan and some of the methoxylated metabolites, the detection limits were worse, as the baseline noise also increased and electrode lifetime decreased.

TABLE I

CHROMATOGRAPHIC AND ELECTROCHEMICAL PROPERTIES OF INDOLE METABOLITES OF TRYPTOPHAN

Compound	Oxidation potential*	Retention time (min)	Solvent system**	Limit of detection (pg)
Tryptophan	+0.88	8.0	1	15
5-HTP	+0.54	3.2	1	5
5-HT	+0.58	5.2	1	5
5-HIAA	+0.58	10.8	1	10
N-Acetyl-5-HT	+0.48	5.6	2	20
6-Hydroxymelatonin	+0.45	13.6	2	20
Melatonin	+0.70	10.4	3	20
5-Methoxytryptophan	+0.80	4.7	2	20
5-Methoxytryptophol	+0.70	8.8	3	10
5-Methoxyindole	+0.91	17.2	3	25
5-Hydoxytryptophol	+0.52	3.2	1	10

*Determined at a carbon paste electrode by cyclic voltammetry. Scan rate, 100 mV/sec; solvent, 0.1 *M* citric acid—0.1 *M* sodium acetate, pH 4.1. Ag/AgCl reference electrode.

**Flow-rate, 0.7 ml/min. Solvent: (1) 0.1 *M* sodium acetate—0.1 *M* citric acid, pH 4.1; (2) 0.1 *M* sodium acetate—0.1 *M* citric acid—10% methanol, pH 4.1; (3) 0.1 *M* sodium acetate—0.1 *M* citric acid—25% methanol, pH 4.1.

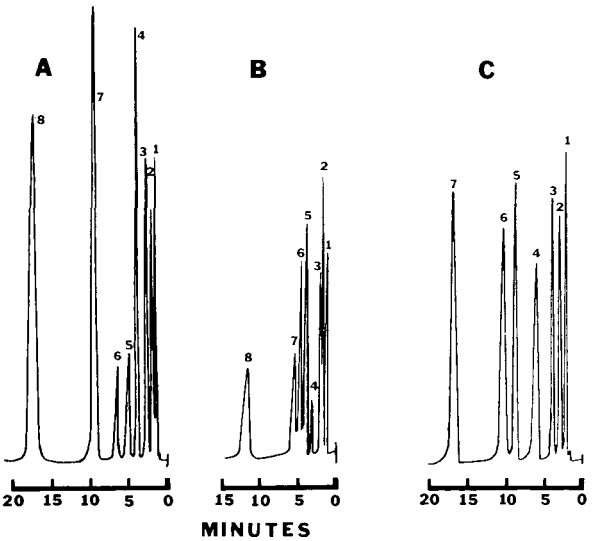

Fig. 2. (A) Solvent system 1 (see Table I). Peaks: 1 = norepinephrine, L-DOPA; 2 = dopamine, epinephrine; 3 = 5-hydroxytryptamine (5-HTP); 4 = serotonin (5-HT); 5 = 3,4,dihydroxyphenylacetic acid (DOPAC); 6 = tryptophan (TRP); 7 = 5-hydroxyindole-3-acetic acid (5-HIAA); 8 = homovanillic acid (HVA). (B) Solvent system 2. Peaks: 1 = solvent; 2 = 5-HTP; 3 = 5-HT; 4 = TRP; 5 = 5-HIAA; 6 = 5-methoxytryptophan; 7 = N-acetylserotonin; 8 = 6-hydroxymelatonin. (C) Solvent system 3. Peaks: 1 = 5-HT; 2 = N-acetylserotonin; 3 = 6-hydroxymelatonin; 4 = tryptophan methyl ester; 5 = 5-methoxytryptophol; 6 = melatonin; 7 = 5-methoxyindole.

Fig. 2 demonstrates the resolution obtained for the tryptophan metabolites and the various chromatographic conditions. Catecholamines and other very polar compounds elute very quickly and do not interfere in the determination of the most polar of the indole metabolites. By increasing the methanol content, one is able to elute the entire series of indole metabolites. Although gradient elution might enable one simultaneously to determine the entire series, this is not yet compatible with electrochemical detection.

Fig. 3 gives examples of the signal-to-noise ratio obtained for the low-level analysis of several of the tryptophan metabolites under the conditions described. The detection limits thus obtained are readily comparable to or better than existing GC—MS [9, 10] and HPLC—fluorescence [13—17] or RIA techniques [9, 11, 12]. Fig. 4 shows sample chromatograms obtained from injection of tissue supernatants. The limits of detection are not approached in the analysis of these samples (ca. 50 mg). This method should therefore be readily applicable to the determination of these metabolites in individual nuclei and tissue samples of the order of 1—5 mg.

Table II shows the results obtained for determination of tryptophan and its metabolites in rat brain and pineal gland. These values compare favorably with values obtained for the same regions by other assay methods.

In summary, we have described the separation of tryptophan and a variety of hydroxylated and methoxylated indole metabolites, N-acetyl-5-hydroxytryptamine, melatonin and 6-hydroxymelatonin. The relative ease of determination of several of these compounds (TRP, 5-HTP, 5-HT, 5-HIAA and melatonin) has been demonstrated in rat brain and pineal tissue. The method as

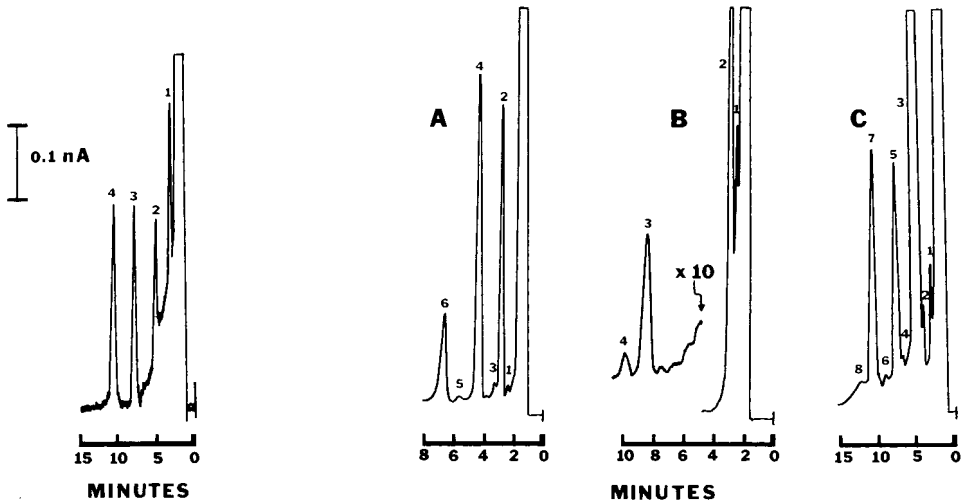

Fig. 3. Chromatogram obtained using solvent system 1 (see Table I). Peaks: 1 = 5-HTP, 39 pg; 2 = 5-HT, 33 pg; 3 = TRP, 105 pg; 4 = 5-HIAA, 93 pg.

Fig. 4. Chromatograms from tissue supernatants. (A) Rat brain cerebellum, solvent system 1 (see Table I). Peaks: 1 = 5-HTP; 2 = 5-HT; 3 = unidentified; 4 = TRP; 5 = unidentified; 6 = 5-HIAA, (B) Rat brain pineal, solvent system 3. Peaks: 1 = TRP; 2 = 5-HIAA; 3 = melatonin; 4 = unidentified. (C) Rat brain pineal, solvent system 1. Peaks: 1 = 5-HTP; 2 = unidentified; 3 = 5-HT; 4 = unidentified; 5 = TRP; 6 = unidentified; 7 = 5-HIAA; 8 = unidentified.

TABLE II

LEVELS OF TRYPTOPHAN AND MAJOR INDOLE METABOLITES IN RAT BRAIN AND PINEAL TISSUE

Values are ng/g wet weight. TRP values are expressed as μg/g.

Region	TRP	5-HTP	5-HT	5-HIAA	Melatonin
Hypothalamus	2.52 ± 0.21	6.5 ± 1.9	841.0 ± 59.0	514.0 ± 57.0	
Raphe	2.09 ± 0.05	2.3 ± 0.5	606.0 ± 14.0	442.0 ± 16.0	
Cerebellum	2.62 ± 0.18	3.5 ± 0.6	70.2 ± 9.1	54.8 ± 6.6	
Pineal*	16.10 ± 2.5	1.02 ± 0.4	82.9 ± 6.7	7.64 ± 0.24	1.35 ± 0.06

*Pineal values are expressed as ng per pineal (n = 3); all others, n = 4.

described is simple, requiring minimal sample handling and preparation. Although several authors have discussed the instability of tryptophan and metabolites, they appear to be stable at low pH. Storage at —80° for several weeks in 0.1 *M* perchloric acid did not lead to degradation.

Preliminary experiments indicate that the chromatographic conditions described are applicable to the determination of 5-HT, TRP, 5-HTP and 5-HIAA in saliva, serum and urine with only minimal sample pre-treatment to precipitate proteins. Such work is now in progress. Pharmacological manipulations of these systems are also being examined.

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MASS SPECTROMETRIC TECHNIQUE FOR THE DETERMINATION OF N-PHOSPHONOACETYL-L-ASPARTIC ACID IN SERUM

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SUMMARY

N-Phosphonoacetyl-L-aspartic acid (PALA), a potent inhibitor of aspartic acid transcarbamylase, is now undergoing Phase I clinical trials. Initial experiments revealed that PALA is not metabolized to phosphonoacetic acid (PAA) in humans. Thus PALA may be quantified in serum after in vitro conversion to PAA. Serum is deproteinized with perchloric acid, lipid extracted with methylene chloride, hydrolyzed with 8 *N* hydrochloric acid at 100° for 3 h, and evaporated to dryness with nitrogen. The residue is silylated, and PAA is quantified by monitoring the $(M+1)^{+\cdot}$ ions of the protonated molecular ions of trimethylsilyl derivatives of PAA and phosphonopropionic acid (internal standard) obtained in chemical ionization with methane. Limit of detection is 0.5 μM (150 ng/ml) PALA using 1 ml serum. PALA was given by continuous infusion to cancer patients at various doses. Maximum levels of PALA (50—500 μM range) were obtained at the end of infusion, followed in most cases by biexponential decay. Persistent residual PALA levels (5 μM for 48 h after infusion) correlated with increased toxicity.

INTRODUCTION

Drugs that interfere with the de novo biosynthesis of pyrimidine nucleotides have proved useful in the treatment of a number of human tumor types. For example, orotidylate decarboxylase is a target of azapyrimidine action, thymidilate synthetase is the target of fluoropyrimidine action, and DNA polymerase is inhibited by cytosine arabinoside. The first committed reaction in the pathway of pyrimidine biosynthesis is the irreversible carbamylation of L-aspartate by carbamylphosphate to form carbamylaspartate (Fig. 1). This reaction is catalyzed by the enzyme aspartate transcarbamylase (ATCase). In 1971, Collins and Stark [1] synthesized N-phosphonoacetyl-L-aspartic acid (PALA, NSC 224131) which acts as a transition-state inhibitor of ATCase, either competitively with carbamylphosphate or noncompetitively with L-aspartate [2].

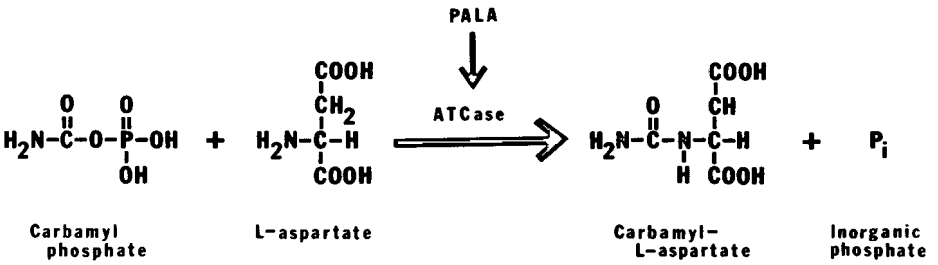

Fig. 1. N-Phosphonoacetyl-L-aspartic acid (PALA) is an inhibitor of aspartate transcarbamylase (ATCase) catalyzed formation of carbamyl-L-aspartate from carbamylphosphate and L-aspartate.

PALA was next shown to be cytotoxic to cultured mammalian cells, and reversal experiments with exogenous uridine or carbamyl-DL-aspartate proved that the cytotoxicity was indeed caused by interference with ATCase [3, 4]. The spectrum of antitumor activity of PALA in mice appears quite different from that of other antimetabolites: there is strong chemotherapeutic activity against several slow growing rodent tumors in contrast to inactivity to the fast-growing experimental leukemias [5].

With the beginning of Phase I clinical trials with PALA at several institutions, there have been concurrent attempts to develop techniques for quantification. In one technique [6] PALA is detached from ATCase by heating, proteins are removed, and PALA is quantified in terms of newly formed [^{14}C]-carbamyl-L-aspartic acid after incubation with intact splenic ATCase, L-[4-^{14}C]aspartic acid, and carbamylphosphate and enzymatic removal of unreacted aspartic acid. From the percentage inhibition of ATCase PALA may be assayed down to 0.1 μM concentration. In another enzymatic assay [7] the inhibition of partially purified aspartate carbamyltransferase from rat liver is utilized; detection limit is 0.1 μg/ml (0.4 μM). These techniques are relatively simple and quite sensitive and certainly adequate in many applications. They do suffer from problems involving enzyme purifications, certain interferences, and a need to run several replicates to obtain averages for a linear calibration curve; similarly, several runs must be made on each patient sample for adequate precision.

The tetramethyl ester of PALA can be formed with diazomethane but the electron impact mass spectrum of the compound exhibits only a weak molecular ion. Based on initial work on the chromatographic and mass spectrometric properties of permethylated PALA [8] a technique was described for quantification utilizing certain fragment peaks and ^{13}C-labeled PALA as the internal standard [9]. The limit of detection of this technique is 2 μg/ml (6.6 μM) which is inadequate to quantify PALA during the decay phase after terminating drug infusion. In addition, monitoring fragment peaks only at the relatively low mass of *m/e* 220, where endogenous interferences often occur, does not provide specificity.

Our initial experimentation with permethylation gave results similar to those described above. Direct trimethylsilylation of PALA appeared promising at the beginning when chemical ionization was employed to obtain the protonated molecular ion; however, when selected ion monitoring was attempted to in-

crease sensitivity, interferences occurred. Initial experiments with high-performance liquid chromatography revealed difficulties in providing a specific and sensitive technique. In connection with a preclinical toxicological study on phosphonoacetic acid (PAA) we have developed a technique for the quantification of PAA based on selected ion monitoring [10]. After establishing that PAA is not an in vivo metabolite of PALA (see Results and discussion), we have developed a technique for the quantification of PALA in human serum in terms of PAA which is a product of the in vitro hydrolysis of PALA. This paper describes the details of the methodology and illustrates applications.

EXPERIMENTAL

Drugs and reagents

Pure PALA (NSC 224131) as both the disodium and tetrasodium salt, was provided by the National Cancer Institute, Bethesda, Md., U.S.A. Most work was carried out with the disodium salt; the tetrasodium salt gave identical results. For clinical work PALA was also supplied by the National Cancer Institute. Ampoules containing 1000 mg PALA in 10 ml normal saline (100 mg/ml PALA) were used. The pH was adjusted to 6.5—7.5 with sodium hydroxide. Intact ampoules were kept refrigerated at 2—8° and unused portions were discarded. Pure phosphonoacetic acid was purchased from Richmond Organic (Richmond, Va., U.S.A.); pure phosphonopropionic acid was purchased from K & K Rare and Fine Chemicals (ICN Pharmaceuticals, Plainview, N.Y., U.S.A.). N,O-Bis(trimethylsilyl)trifluoroacetamide and trimethylchlorosilane (BSTFA + 1% TMCS), silylation grade pyridine and all gas chromatographic column materials were purchased from Pierce (Rockford, Ill., U.S.A.). All solvents used were of "distilled in glass" quality (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.). All other chemicals were of highest purity commercially available and were used without further purification. Gas chromatographic carrier gases and reagent gases for chemical ionization mass spectrometry were of high purity grade from Matheson (Rutherford, N.J., U.S.A.).

Instrumentation

The instrument used was a combined gas chromatograph—mass spectrometer (quadrupole-type mass analyzer, Finnigan Model 3300)—computer (Finnigan Model 6000) system equipped with a chemical ionization source and capability for selected ion monitoring (mass fragmentography).

Internal standard

Phosphonopropionic acid (PPA) was used as the internal standard. The pure compound was dissolved in distilled water to provide solutions containing either 500 ng/μl or 50 ng/μl PPA. In all cases, an adequate amount of internal standard was added at the beginning of an analysis to yield a final concentration approximately 75% of the expected concentration range of PALA.

Standards and calibration samples

Pure PAA and PPA samples were dissolved in a mixture of BSTFA+1% TMCS and pyridine (3:1, v/v) and derivatized as described in Preparation of

serum samples. These samples were used to establish authentic mass spectra under various experimental conditions, to determine detection limits for the pure compounds, to establish sensitivities (in terms of computer-generated peak areas per unit sample quantity introduced) and also for daily routine mass range calibration.

To establish optimal analytical conditions both for sample preparation and also for gas chromatographic—mass spectrometric analysis, normal pooled serum and also normal individual serum samples were spiked with known quantities of PALA and PPA. To obtain calibration curves, samples were prepared containing increasing quantities of PALA and a fixed amount of internal standard. The concentration range of the calibration samples covered the entire range of concentrations expected in the patient samples. The amount of internal standard added was the same for both calibration and patient samples. Blank samples (i.e., no PALA added but PPA included) were also included in every set of calibration runs. A full set of calibration samples was analyzed with every set of samples from patients to compensate for irreproducible experimental errors.

Preparation of serum samples

Blood samples for PALA were drawn from the contralateral arm at various intervals according to the clinical protocol followed in this investigation.

Serum was obtained by letting whole blood clot at room temperature for 20—25 min followed by centrifugation at approx. 500 *g* at room temperature for 10 min. Serum samples were stored at −80° until used. Samples from mice were obtained the same way, pooling the serum of 2—5 animals treated with the same dose of PALA.

To a 0.5-ml serum sample the internal standard was added, followed by vortexing (fast) for 15 sec. Next, 25 μl of perchloric acid (70%) was added and again vortexed (fast) for 15 sec. The fine protein precipitate became tightly packed after centrifuging at 30,000 *g* at 10° for 15 min. The clear supernatant (approx. 0.4 ml) was transferred into a small tube, acidified by adding 0.5 ml concentrated hydrochloric acid, and lipid extracted by adding 1.0 ml methylene chloride and vortexing. For best separation of the aqueous and organic layers, the mixture was centrifuged at 2000 *g* at room temperature for 5 min. (A small layer of emulsion at the interface is acceptable.) The clear upper layer was next pipetted into a 6-ml PTFE-capped silylation vial and heated in an aluminum heating block at 100° for 3 h. At the end of the hydrolysis, the sample became amber colored with occasional black specs which were ignored. Next, the sample was evaporated to dryness with dry nitrogen in a water bath at 50—60°. The remaining residue was a somewhat crusty brown solid. The evaporated samples were kept overnight in a vacuum desiccator filled with solid potassium hydroxide and Drierite. Prior to mass spectrometric analysis, the samples were silylated by adding 200 μl of BSTFA + 1% TMCS—pyridine (3:1, v/v) and refluxing in a dry heating block kept at 100° for 5—8 min. After derivatization some solids did remain at the bottom of the silylation vial; the liquid phase was brown but clear.

When the lowest limit of detection was attempted, a 1.0-ml starting sample size was used and the following changes were made in the procedure described.

Proteins were precipitated with 50 μl perchloric acid, hydrolysis was done with 0.8 ml concentrated hydrochloric acid, lipids were extracted with 3 ml methylene chloride and 600 μl derivatization reagent were added. After silylation the supernatant was decanted into a clean vial, the excess reagent was evaporated with nitrogen, and the residue was reconstituted with 100 μl of reagent; an aliquot of this was introduced into the gas chromatograph. It is noted that the final samples appeared darker in this procedure than in the one commonly used; this did not alter results.

Patient samples

Blood samples were obtained from cancer patients participating in a Phase I clinical study. Patient selection, the mechanism of the study, dose escalations, consent forms, methods of evaluation, etc. were according to a clinical protocol approved by the Institution and submitted to the National Cancer Institute. The primary objective of the study was to establish the maximum tolerated dose of PALA in patients with cancers not amenable to conventional treatment methods. In a coordinated effort with other institutions, emphasis was placed upon the study of the effects of 24-h and 5-day continuous infusion. In the former case dose levels were escalated from 0.5 to 10.5 $g/m^2/24$ h; in the latter case dose levels were escalated from 4 to 8.7 $g/m^2/5$ days.

Gas chromatography—mass spectrometry

For gas chromatographic separation a glass column (1 m × 2 mm I.D.) filled with 3% OV-17 on Chromosorb W HP (80—100 mesh) was employed. The column was operated isothermally at 150°; injector temperature was kept at 250°. Endogenous constituents trapped by the column at the low operating temperature were removed by periodically heating the column to 250° and keeping it at that temperature until no more eluent could be detected. The sample port was cleaned after every 20—25 analyses by removing 1—2 cm of column material (dark deposits) from the top of the column.

Methane gas was used both as the gas chromatographic carrier gas and as the reagent gas in the chemical ionization source of the mass spectrometer. There was no separator between the gas chromatograph and the ion source; the connecting tube was kept at 240°. The pressure of methane in the chemical ionization source was kept at approximately 1.3 mbar (uncorrected). Operational parameters of the mass spectrometer were adjusted daily for maximum sensitivity (at a resolution of about 400) using pure PAA samples. When extreme adjustments of the ion source became necessary to maintain desired sensitivity, the ion source was cleaned.

Full chemical ionization mass spectra for identification were obtained by operating the instrument in the "full scanning" mode. The "selected ion monitoring" mode was used for quantification in patient samples. Normally, 4-μl sample aliquots were injected into the gas chromatograph. The effluent was vented for a period of 30 sec to avoid contamination of the ion source by the excess silylation reagent and pyridine. Next, the effluent entered the ion source and ions at *m/e* 371 and *m/e* 357 were monitored; for additional confirmation of identity, peaks at *m/e* 355 and *m/e* 341 were also monitored occasionally (see Results and discussion).

RESULTS AND DISCUSSION

Hydrolysis of PALA

The basis of the present technique is the in vitro hydrolysis of PALA in serum to yield PAA. Because it is conceivable that PALA might hydrolyze to PAA in vivo, i.e., that PAA might be a metabolite of PALA, a search was made to detect PAA in the serum of patients receiving PALA at various doses. No PAA was detected in the blood of any patient. The limit of detection of PAA in serum is 20 ng/ml using a 0.2-ml sample size [10]. A series of experiments was also carried out with mice injected with various doses of PALA; no PAA was detected. It is concluded that PAA is not an in vivo metabolite of PALA. Other workers also failed to detect metabolites of PALA using ^{14}C-labeled drug [11, 12].

When heated with 8 *N* hydrochloric acid at 100° PALA hydrolyzes to yield PAA and aspartic acid (Fig. 2). This was confirmed by obtaining the trimethylsilyl (TMS) derivatives of the hydrolysis products. The protonated molecular ion of PAA appeared at *m/e* 357 (see later). The protonated molecular ion of aspartic acid appeared at *m/e* 350 (Fig. 3); as expected, three TMS groups were taken up by the molecule.

The aspartic acid obtained in the hydrolysis of PALA is not suitable for monitoring since there is a considerable amount of endogenous aspartic acid present in human blood. Fig. 4A illustrates the monitoring of the protonated

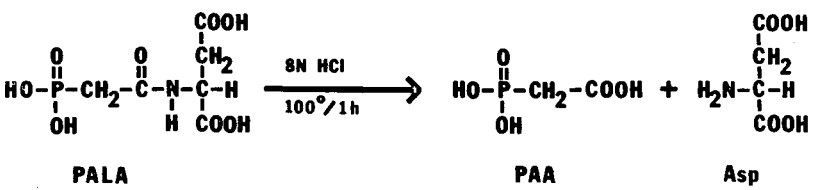

Fig. 2. In vitro hydrolysis of N-phosphonoacetyl-L-aspartic acid (PALA) to phosphonoacetic acid (PAA) and aspartic acid (Asp).

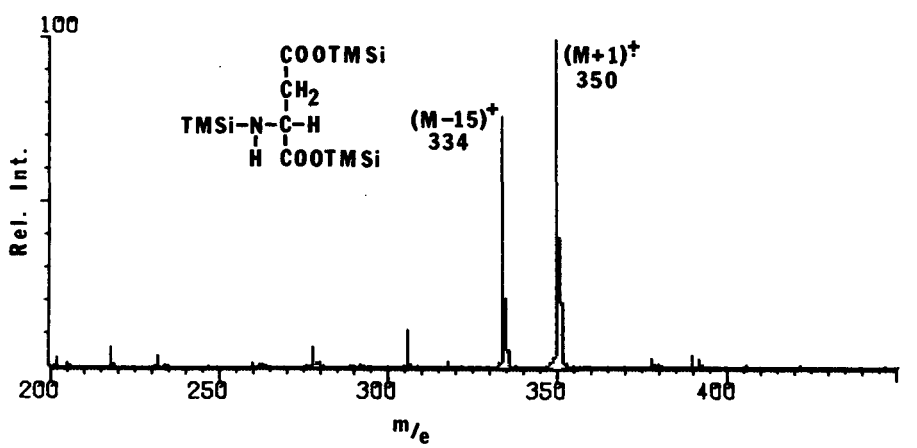

Fig. 3. Chemical ionization (methane) mass spectrum of the trimethylsilyl derivative of aspartic acid.

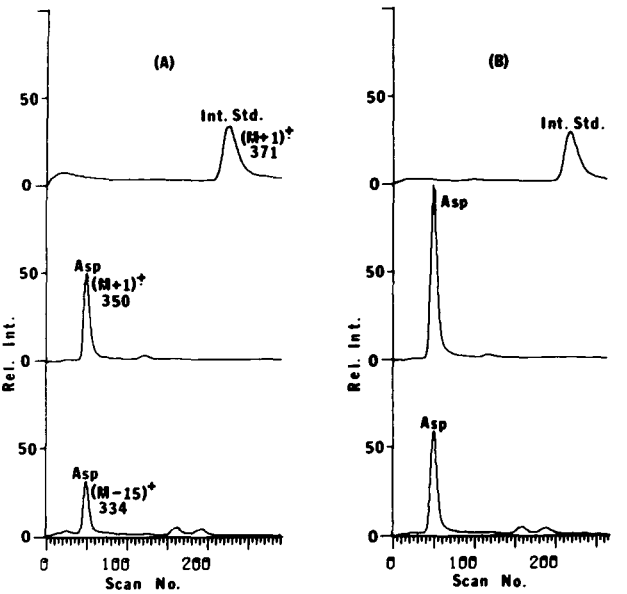

Fig. 4. Selected ion monitoring of the trimethylsilyl derivatives of aspartic acid (Asp) and internal standard (phosphonopropionic acid) in normal serum (A) and in serum spiked with PALA (B).

molecular ion of aspartic acid in normal blood. The $(M-15)^+$ ion, corresponding to the loss of a methyl group, was also monitored for confirmation. The area under the $(M+1)^{+\cdot}$ peak corresponds to approximately 50 μg/ml endogenous aspartic acid. When the same sample was spiked with 100 μg/ml PALA and hydrolyzed, the level of aspartic acid increased (Fig. 4B). Endogenous aspartic acid is a limiting factor in this approach for two reasons: endogenous aspartic acid is variable from patient to patient, and the limit of detection would be poor because of high blank values. In contrast, the zero-time or blank level of the peak at *m/e* 357 was found either below detection limit or at the few ng/ml level in all patient and normal samples analyzed. It was thus concluded that monitoring PAA in hydrolyzed serum samples can be utilized to quantify PALA.

Mass spectra of PAA and PPA

The chemical ionization (methane) mass spectra of pure silylated PAA (Fig. 5) and PPA (Fig. 6) reveal that the base peak in both cases corresponds to the protonated molecular ions with three trimethylsilyl groups taken up. The $(M+1)^{+\cdot}$ ions, at *m/e* 357 for PAA and *m/e* 371 for PPA, are thus well suited for selected ion monitoring. This technique increases sensitivity while retaining a high degree of specificity. As shown in the figures, the $(M-15)^+$ ions, corresponding to the loss of a methyl group, are also present in considerable abundance. These peaks may also be monitored, and the ratio of the $(M+1)^{+\cdot}$ to $(M-15)^+$ peaks may be used as further proof of specificity. Since there are no interfering peaks in this technique, such monitoring was not needed routinely.

As shown in Figs. 5 and 6, no other ions of appreciable abundance appear in

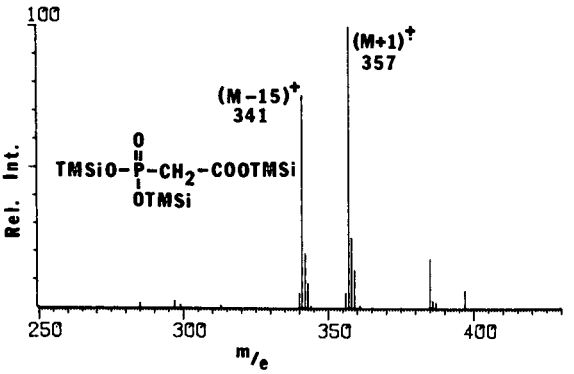

Fig. 5. Chemical ionization (methane) mass spectrum of the trimethylsilyl derivative of phosphonoacetic acid (PAA).

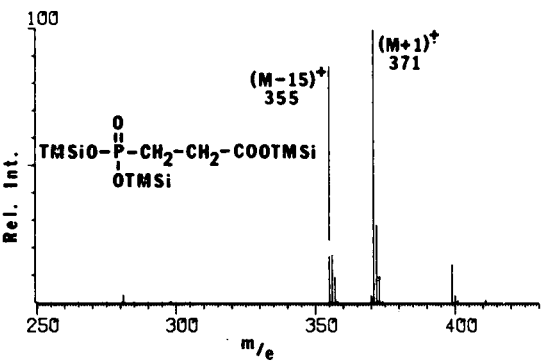

Fig. 6. Chemical ionization (methane) mass spectrum of the trimethylsilyl derivative of phosphonopropionic acid (PPA) used as internal standard.

the mass spectra of PAA and PPA. Ions with *m/e* higher than that of the protonated molecular ion represent the results of addition reactions by the methane reagent gas; such ions are customary in chemical ionization and are of no consequence in quantification.

Detection limits, quantification

The limit of detection of pure phosphonoacetic acid (as the TMS derivative) is 50 pg ($0.5 \cdot 10^{-12}$ mole) injected. The limit of detection of PAA in serum is 20 ng/ml using 0.2 ml sample size. When PALA is hydrolyzed to PAA, the detection limit for PALA is either 300 ng/ml (1.0 μM) when 0.5 ml initial sample is used, or 150 ng/ml (0.5 μM) when 1.0 ml initial sample is used (see Preparation of serum samples). The limit of detection is defined as the amount of substance needed to produce a peak/internal standard area ratio twice that of the blank. For reproducible quantification one needs to inject at least twice the amount corresponding to the limit of detection so that the computer could determine peak areas after appropriate background correction.

Quantification of PALA was accomplished with the aid of calibration curves. Normal serum samples spiked with an increasing amount of PALA (and a fixed amount of internal standard) were run and the ratios of the areas of PAA (from

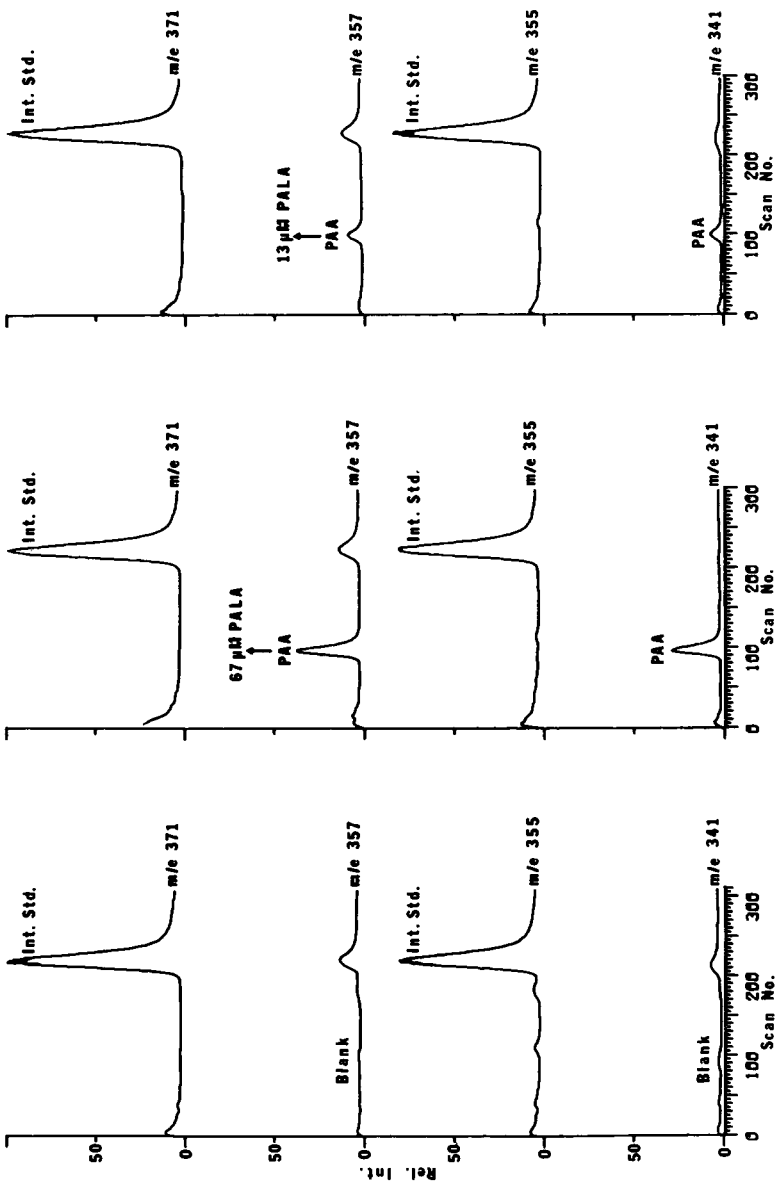

Fig. 7. Selected ion monitoring of phosphonoacetic acid (PAA) hydrolyzed from PALA in serum of patient receiving 1 g/m^2/24 h PALA by continuous infusion. (A) Zero time (pre-infusion); (B) end of infusion; (C) 4 h post infusion. Internal standard: phosphonopropionic acid (PPA).

PALA) and PPA, as determined by the computer, were plotted against the known amount of PALA initially added to a particular calibration sample. The calibration curves thus obtained were straight lines within a PALA concentration range of a factor of 100, and intercepted the Y-axis at or very near the origin. A new calibration curve was obtained for every set of patient samples analyzed.

Reproducibility measurements were made (n = 5) for each concentration point on the calibration curves. The coefficients of variation (defined as standard deviation divided by the mean × 100) were 5—10% in the 2.5—25 μg/ml PALA range, and 8—15% in the 0.5—5.0 μg/ml PALA range.

Concerning the analytical methodology, it is noted that very high levels of PAA may result in a "memory" effect, i.e., some material remains on the gas chromatographic column. Residual PAA can be removed with repeated flushings with the silylating reagent and concurrent increase of the temperature to 250°. Also, for best quantification the first few runs should be ignored when a new gas chromatographic column is employed.

Patient monitoring

Figs. 7 and 8 illustrate selected ion monitoring in patients. In Fig. 7 both the $(M+1)^{+\cdot}$ and $(M-15)^{+}$ peaks of PAA and PPA, respectively, were monitored. The gain in Fig. 8 is ten times that of Fig. 7; this is employed for low levels of PALA. (The computer normalizes to the internal standard.) The "zero time" or blank values represent samples taken prior to drug administration. The area of the blank which is seen only when high gain is used (Fig. 8A) corresponds to approximately 300 computer counts which is about the smallest area that could be quantified.

Fig. 7B shows the level of PALA at the end of a 24-h infusion period during which 1 g/m^2 of the drug was administered. The area of PAA corresponds to a concentration of 67 μM (20 μg/ml) of PALA which was the highest concentra-

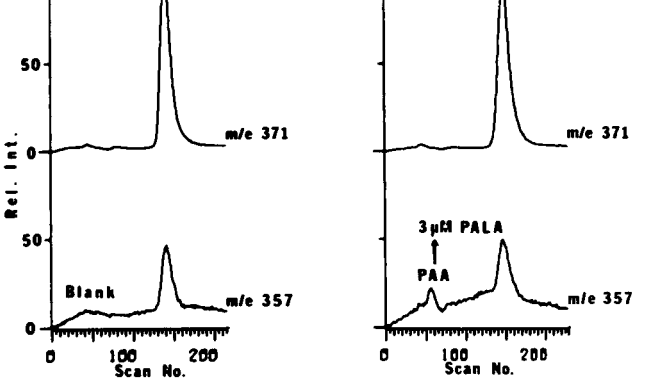

Fig. 8. Selected ion monitoring of phosphonoacetic acid (PAA) hydrolyzed from PALA in serum of patient receiving 4 g/m^2/5 days PALA by continous infusion. (A) Zero time (pre-infusion); (B) 12 h post infusion. Internal standard: phosphonopropionic acid (PPA). Gain is 10 times that of Fig. 7 for low-level PALA determinations.

tion measured during the infusion period. Decay of PALA levels commenced immediately upon termination of infustion. For example, 4 h later the concentration of PALA was 13 μM (Fig. 7C), and by 8 h the level of PALA was below detection limit. In contrast, when the same patient was given a dose of 4 g/m^2 for a 5-day period of infusion, at 8 h after infusion there was 5.3 μM PALA present, and 12 h after infusion there was still 3 μM present (Fig. 8B); it took 24 h to reach the limit of detection.

Details of the application of this technique to obtain pharmacokinetic data and correlations with clinical observations in Phase I trials in 37 patients were presented [13]; only a few relevant results are summarized here. In infusion studies the highest PALA levels (50—500 μM range) were observed at the end of the infusion period. Higher doses and/or longer infusion periods resulted in higher levels of PALA and longer decay curves. Serum drug levels decreased biexponentially in most cases, with an average first half-life of 100 min and an average second half-life of 8 h. There were at least two cases where PALA appeared to remain in the serum (at the 5 μM level) for at least 48 h after the infusion was terminated; this was accompanied by increased clinical toxicity.

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SIMPLE AND SELECTIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR ESTIMATING PLASMA QUINIDINE LEVELS

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SUMMARY

A reversed-phase, high-performance liquid chromatographic method employing fluorescence detection is described for the rapid quantification of plasma levels of quinidine, dihydroquinidine and 3-hydroxyquinidine. It involves protein precipitation with acetonitrile followed by direct injection of the supernatant into the chromatograph. For the preparation of plasma standards, pure 3-hydroxyquinidine was isolated from human urine by a simplified thin-layer chromatographic procedure. The mobile phase for the chromatography was a mixture of 1.5 m*M* aqueous phosphoric acid and acetonitrile (90:10) at a flow-rate of 2 ml/min. The intra-assay coefficient of variation for the assay of quinidine and 3-hydroxyquinidine over the concentration range 2.5—20 μmole/l was < 1% for both. Interassay coefficients of variation for quinidine (10 μmole/l) and 3-hydroxyquinidine (5 μmole/l) were 3.5% and 4.0% with detection limits of 50 and 25 μmole/l respectively. The method correlated well (r^2 = 0.96) with an independently developed gas—liquid chromatographic—nitrogen detection assay for quinidine which also possessed a high degree of precision. (Intra-assay coefficient of variation 3.6% at 20 μmole/l). As expected, comparison of the high-performance liquid chromatographic assay with a published protein precipitation—fluorescence assay showed poor correlation (r^2 = 0.78).

INTRODUCTION

Quinidine remains an important agent for the treatment of arrhythmias. A relationship between quinidine plasma levels and efficacy has been demonstrated [1] and hence the monitoring of plasma levels has become an important adjunct in the management of patients administered the drug. There are a number of published methods available for measuring quinidine levels with varying degrees of specificity. The original protein precipitation—fluorescence method [2] is still widely used but both that method and extraction—fluorescence

methods have been demonstrated to measure a number of metabolites in addition to quinidine [3, 4].

Recently, a summary of the available quinidine assay methods and their limitations together with a normal-phase high-performance liquid chromatographic (HPLC) assay were reported [4]. Although the reported method possessed high selectivity it lacked the convenience required for rapid emergency assays. A published reversed-phase HPLC assay [5] involving protein precipitation, direct injection and ultraviolet detection offered the advantage of considerably shorter assay time but had limited sensitivity. A recently reported HPLC assay employing extraction and post-column acidification with fluorescence detection [6] did not use internal standardization.

In the present report, a simple reversed-phase HPLC assay for quinidine, 3-hydroxyquinidine and dihydroquinidine employing protein precipitation with direct injection and fluorescence detection is described. Comparisons with a gas chromatographic—nitrogen detection method and a published protein precipitation—fluorescence method are also described.

MATERIALS AND METHODS

Reagents

All reagents were analytical grade and aqueous solutions were prepared using glass distilled water. Specially purified acetonitrile (Unichrom, Ajax Chemicals, Sydney, Australia) was used for high-performance liquid chromatography. The chloroform was nanograde from Mallinckrodt (St. Louis, Mo., U.S.A.) and the methanol, spectrofluorescence grade (Uvasol, Merck, Darmstadt, G.F.R.). Quinidine sulphate was obtained from Burroughs Wellcome (Detroit, Mich., U.S.A.) and contained 9% dihydroquinidine. Commercially available cinchonidine from Koch-Light (Colnbrook, Great Britain) contained traces of quinidine and was recrystallized four times from methanol—water (1 : 1) before use. The cinchonidine also contained traces of dihydrocinchonidine in unknown quantity. Dihydroquinidine was obtained from ICN, Irvine, Calif., U.S.A.

Standards

Stock solutions of quinidine sulphate and 3-hydroxyquinidine were prepared in glass distilled water at concentrations of 200 and 100 μmole/l, respectively. These solutions were stable for at least 2 months at 4°. For the HPLC assay a solution of recrystallized cinchonidine (internal standard) was prepared in acetonitrile (170 μmole/l). For the gas—liquid chromatographic (GLC) assay the cinchonidine solution (68 μmole/l) was prepared in water. These solutions were also stable for at least 2 months at 4°. Plasma standards containing the required amounts of quinidine and 3-hydroxyquinidine were prepared and stored at 4° for no longer than 2 weeks. Peak area ratios of the drug and metabolites to the internal standard were determined for plasma standards and unknowns and quantification performed by reading unknown values from a plotted standard curve.

Isolation of 3-hydroxyquinidine from urine

Urine (20 ml) was collected from a patient taking oral quinidine at steady-

state. It was basified (pH 14) with 1 *N* aqueous sodium hydroxide and extracted twice with chloroform (20 ml). The phases were separated by centrifugation and the aqueous layer discarded. The chloroform extracts were pooled and evaporated under nitrogen (45°) and the residue reconstituted in methanol (1 ml). A 250-μl amount of the methanol solution was chromatographed on each of 4 preparative thin-layer plates (silica gel 60 (20 × 20 cm), Merck) using methanol as the solvent. The fluorescent bands were identified under ultraviolet light and those for quinidine and dihydroquinidine selected by comparison of the R_F values with those of authentic samples of the compounds. Two other bands with larger R_F values (0.8, 0.65) were scraped from each of the plates, separately pooled and extracted with methanol (three times). The extracts were evaporated and the residue treated with diethyl ether (0.5 ml twice) and separated by centrifugation. The diethyl ether was evaporated and a small portion of each residue reconstituted in mobile phase and injected into the high-performance liquid chromatograph. In this way each band was assigned to a peak in the HPLC chromatogram.

The major metabolite isolated (210 μg) (R_F = 0.65) was recrystallized once from methanol—diethyl ether by adding diethyl ether dropwise to seed crystallization. It had a melting point (210—211°) and mass spectrum (*m/e* 340, 267, 189, 173, 152; determined at 20 eV, probe temperature 170° using a Model AEI MS-30 mass spectrometer) identical with that published for 3-hydroxyquinidine [7] and different from that of the recently identified N-oxide metabolite of quinidine [8] which in contrast showed the characteristic fragmentation of an N-oxide. There was insufficient material to obtain a meaningful proton magnetic resonance spectrum. The isolated 3-hydroxyquinidine was used to prepare plasma standards for the estimation of plasma levels in patient samples. Insufficient quantities of the second metabolite were obtained to allow satisfactory recrystallization.

High-performance liquid chromatographic assay

An aliquot of the internal standard solution (cinchonidine in acetonitrile, 170 μmole/l) was added to an equal volume of patient plasma or plasma standard (20—200 μl) and vortexed at high speed for 30 sec. After standing at room temperature for 15 min the mixture was centrifuged and 10 μl of the supernatant injected into the high-performance liquid chromatograph using a 10-μl injector loop.

The chromatograph used was a Spectra-Physics Model SP8000 equipped with a ternary solvent system, helium degassing and automatic data reduction facilities. A 10-μm alkyl phenyl reversed-phase column (μBondapak/Phenyl from Waters Assoc., Milford, Mass., U.S.A.) was used at a column temperature of 50°. The mobile phase was a mixture of 1.5 m*M* aqueous phosphoric acid and acetonitrile (90 : 10) at a flow-rate of 2 ml/min and all solvent lines from the column to the detector were carefully thermally insulated. The effluent was monitored using a fluorescence detector (Schoeffel Model 970) at an excitation wavelength of 320 nm with an emission cut-off filter allowing 90% transmission at 418 nm. The fluorimeter sensitivity setting was 3.5, range 0.4 μA full-scale and time constant 5.0 sec. On completion of an assay run, the column was automatically washed with water for 30 min at a flow-rate of 2 ml/min, fol-

lowed by a 5-h acetonitrile wash at a flow-rate of 0.2 ml/min.

The levels of quinidine and 3-hydroxyquinidine in unkowns were determined from plotted standard curves. Dihydroquinidine had identical fluorescence to quinidine and was determined using the quinidine standard curve. For comparison with the published precipitation fluorescence method [2], other fluorescent quinidine metabolites eluted were assumed to have fluorescence equivalent to quinidine and quantitated as such. This provided a quinidine plus metabolites level which included 3-hydroxyquinidine and dihydroquinidine.

Gas—liquid chromatographic assay

500 μl of the internal standard solution (cinchonidine 68 μmole/l) was added to 1 ml of patient plasma and plasma standards; 2 ml of 0.1 *N* aqueous sodium hydroxide were added followed by 5 ml of chloroform and the mixture was shaken for 10 min at 100 r.p.m. After separation of the phases by centrifugation the aqueous layer was removed by vacuum aspiration and the organic layer transferred to a conical centrifuge tube. The solvent was evaporated under a flow of nitrogen at 45° and the residue reconstituted in 100 μl of methanol. A 2-μl aliquot of the methanol solution was injected into the gas chromatograph (Packard Model 419) equipped with a 1 m × 2 mm I.D. column containing 3% OV-17 on Gas-chrom Q (Supelco, Bellefonte, Pa., U.S.A.). Injector, column and detector temperatures were 280°, 270° and 300°, respectively. An alkali-flame detector (Packard Model 713) was used with the following flow-rates: nitrogen, 30 ml/min; hydrogen, 40 ml/min; air, 260 ml/min. Peak area ratios of quinidine to the internal standard were determined using an integrator (Spectra-Physics, Autolab System IVB).

Spectrofluorometric assay

The method of Brodie and Udenfriend was used [2] with 0.1 ml of plasma. Plasma proteins were precipitated with a solution of metaphosphoric acid and the fluorescence of the supernatant determined using an Aminco-Bowman Ratio Photometer Spectrofluorimeter. The excitation wavelength used was 320 nm and emission monitored at 418 nm. Quantification was performed by comparison of the relative fluorescence with that of plasma standards containing added amounts of quinidine.

Reproducibility and recovery

The intra-assay reproducibility for the HPLC method was determined by assaying five plasma samples containing added amounts of quinidine and 3-hydroxyquinidine (2.5, 5.0, 10 and 20 μmole/l) at each concentration. The inter-assay reproducibility was determined by assaying a previously prepared plasma sample containing quinidine (10 μmole/l) and 3-hydroxyquinidine (5 μmole/l) in each daily run (20 determinations). The intra-assay reproducibility of the GLC method was determined by assaying five plasma samples containing added quinidine (20 μmole/l). The recovery of the two methods was determined by injecting known amounts of quinidine and 3-hydroxyquinidine into the chromatograph and comparing the peak areas with those obtained for an extracted plasma standard.

Comparison of HPLC and GLC assays

Eighteen plasma samples taken from patients on oral quinidine were assayed for quinidine by both the HPLC and GLC assay methods.

Comparison of HPLC and precipitation—fluorescence assays

Twenty patient plasma samples were assayed by HPLC and by the precipitation method. Two scattergrams were plotted: (1) the HPLC quinidine level against the quinidine level obtained by the precipitation method; (2) quinidine plus metabolite levels determined by assuming that unknown metabolite peaks had fluorescence equivalent to quinidine, that is, quinidine + 3-hydroxyquinidine + dihydroquinidine + unknown metabolites plotted against the quinidine level obtained by the precipitation method.

RESULTS AND DISCCUSION

The HPLC assay for quinidine, 3-hydroxyquinidine and dihydroquinidine was sensitive and selective and sufficiently rapid for use in emergency situations. Preparation time for standards and patient samples was approximately 20—30 min and the chromatographic run time 18 min for each sample. If required, as little as 20 μl of plasma could be used for the assay and by injecting 10 μl of the resultant supernatant levels of 50 and 25 nmole/l (16 and 8.5 ng/ml) of quinidine and 3-hydroxyquinidine were detectable at peak height twice noise. The sensitivity could be enhanced by injecting a larger volume onto the column. Improved peak resolution and reproducibility of retention times was obtained using a column oven temperature of 50° rather than ambient temperature. By washing the column carefully with water and acetonitrile after each assay run several hundred injections have been made without any noticeable loss in performance.

Dihydroquinidine had fluorescence equivalent to quinidine and the ratio of the fluorescence intensity of 3-hydroxyquinidine to quinidine was 1.1. The internal standard, cinchonidine, had considerably less intense fluorescence at the excitation wavelength used for the assay (320 nm), hence a relatively high concentration was required to obtain a peak of suitable area. Under these conditions the traces of quinidine in the commercial preparation became an interference and the material required recrystallization four times before use.

Attempts to obtain samples of the metabolites from all sources was unsuccessful. However, a simplified thin-layer chromatographic procedure for the isolation of 3-hydroxyquinidine from human urine in sufficient quantities for the preparation of plasma standards was developed. The isolated material had a melting point and mass spectrum identical with those published for 3-hydroxyquinidine. Since there were no extraction losses in the HPLC assay method reported (linear recovery of 100% over the concentration 2.5—20 μmole/l), it is valid to use the fluorescence ratio of 1.1 (3-hydroxyquinidine:quinidine) to estimate plasma levels of this metabolite.

The chromatograms obtained for the assay of blank plasma, a plasma standard containing 10 μmole/l (3.2 μg/ml) of quinidine and 5 μmole/1 (1.7 μg/ml) of 3-hydroxyquinidine and a patient sample are shown in Fig. 1. Three important metabolite peaks were found in all patient samples, that is, peak 1

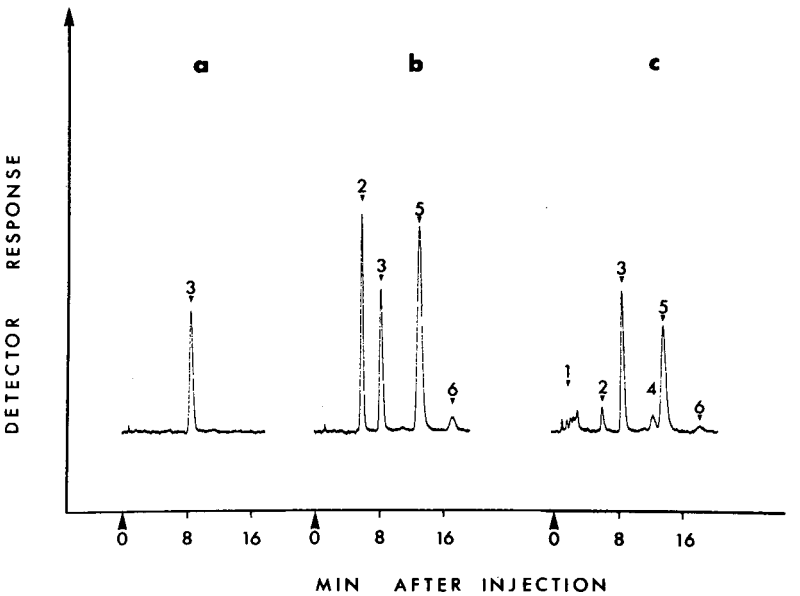

Fig. 1. Chromatograms obtained for the HPLC quinidine assay of (a) blank plasma, (b) plasma standard containing 10 μmole/l quinidine and 5.0 μmole/l 3-hydroxyquinidine and (c) plasma from a patient on chronic oral quinidine therapy containing 0.6 μmole/l 3-hydroxyquinidine, 5.2 μmole/l quinidine and 0.3 μmole/l dihydroquinidine. Deproteinated plasma (10 μl) was injected onto column. Peaks: 1 = polar metabolites; 2 = 3-hydroxyquinidine; 3 = internal standard (cinchonidine); 4 = unidentified metabolite; 5 = quinidine; 6 = dihydroquinidine.

(polar metabolites), peak 2 which was assigned to 3-hydroxyquinidine on the basis of the mass spectrum and melting point of the isolated metabolite and peak 4 which was tentatively assigned to the recently identified N-oxide metabolite [4]. Plasma levels of O-desmethylquinidine and 2′-oxoquinidine have been reported to be very low [9] and their relative fluorescence intensity under the conditions used presently has been reported to be only 0 and 15% of that quinidine [10]. Hence these metabolites will not be detected in the present method and both the major metabolites have been separated from quinidine allowing selective quantification of quinidine plasma levels.

The intra-assay coefficient of variation for the HPLC assay of quinidine and 3-hydroxyquinidine over the concentration range 2.5—20 μmole/l was < 1% in each case. Inter-assay coefficients of variation for quinidine (10 μmole/l) and 3-hydroxyquinidine (5 μmole/l) were 3.5% and 4.5%, respectively. No interference was observed from hydralazine and its metabolites, and propranolol, 4-hydroxypropranolol, imipramine, desipramine, procainamide, N-acetylprocainamide, disopyramide and tocainide in the assays for quinidine, dihydroquinidine and 3-hydroxyquinidine. However, N-desisopropylpropranolol and quinine had retention times identical to that of quinidine.

Chromatograms obtained for the GLC assay of quinidine are shown in Fig. 2. There was a greater detector response to dihydroquinidine than quinidine in this assay. Excess internal standard was used to improve the precision of quan-

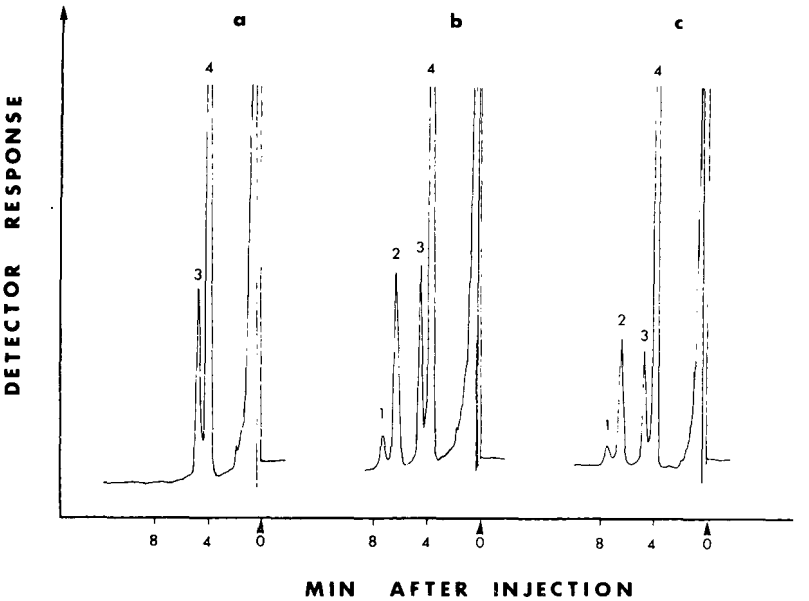

Fig. 2. Chromatograms obtained for the GLC quinidine assay of (a) blank plasma, (b) plasma standard containing 20 μmole/l quinidine and (c) plasma from a patient on chronic oral quinidine therapy. Peaks: 1 = dihydroquinidine; 2 = quinidine; 3 = dihydrocinchonidine; peak 4 = internal standard (cinchonidine). Peak areas were determined by integration.

titation. The GLC assay possessed a high degree of precision with an intra-assay coefficient of variation of 3.6% at a quinidine plasma concentration of 20 μmole/l. Recovery was 92% over the concentration range 5—20 μmole/l. Excellent correlation (r^2 = 0.96) was found between the HPLC and GLC assay (Fig. 3). The retention times of other drugs injected into the chromatograph are indicated in Table I. Since the HPLC and GLC methods rely on inde-

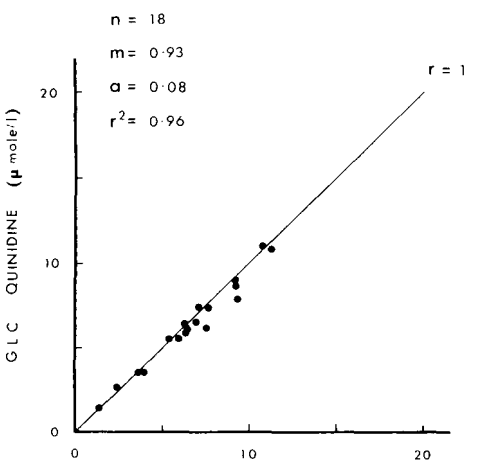

Fig. 3. Comparison of HPLC and GLC assays for plasma quinidine (m = slope, a = intercept). Samples were taken from patients on chronic oral quinidine therapy.

TABLE I

RETENTION TIMES FOR GLC ASSAY OF QUINIDINE

Compound	Retention time (sec)
Quinidine	368
Dihydroquinidine	444
Cinchonidine	222
Disopyramide	274
Procainamide	79
N-Acetylprocainamide	227

pendent means of extraction, chromatography and detection, this correlation is supportive evidence of their specificity for quinidine. The disadvantages of the GLC assay were that it did not provide an estimate of the 3-hydroxyquinidine level and required a considerably longer sample preparation time.

As expected, there was good correlation (r^2 = 0.98) between the quinidine plus metabolites level determined by the HPLC method and quinidine plus metabolites level determined by the precipitation method (Fig. 4). However, the correlations between the quinidine level measured by HPLC and quinidine plus metabolites level (HPLC) (r^2 = 0.81) (Fig. 5) and between quinidine (HPLC) and quinidine plus metabolites measured by the precipitation method (r^2 = 0.78) (Fig. 6) were relatively poor. This is consistent with published work [11, 12] and supports the observation that quinidine levels measured by the precipitation method contain a variable and significant contribution from polar metabolites which may be pharmacologically inactive.

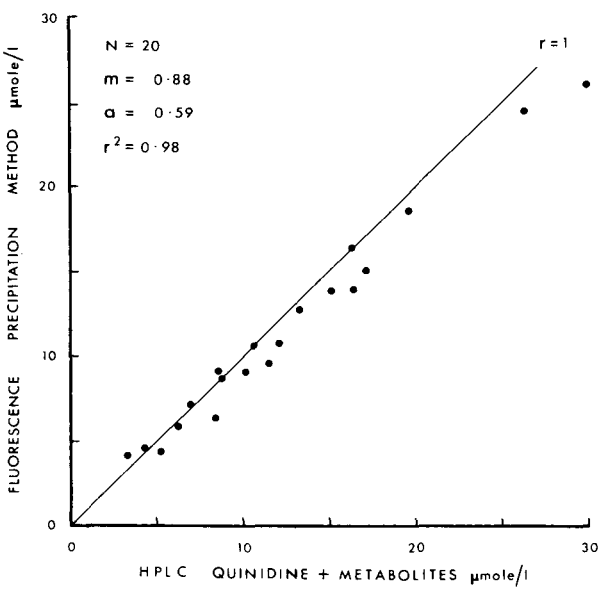

Fig. 4. Comparison of quinidine plus metabolites level determined by HPLC and levels determined by precipitation—fluorescence assay for the same plasma samples (m = slope, a = intercept).

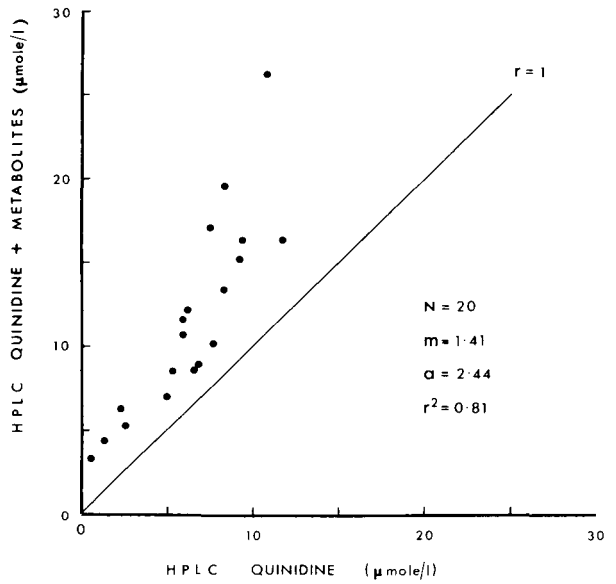

Fig. 5. Comparison of quinidine and quinidine plus metabolites plasma levels determined by HPLC (m = slope, a = intercept). Samples were taken from patients on chronic oral quinidine therapy.

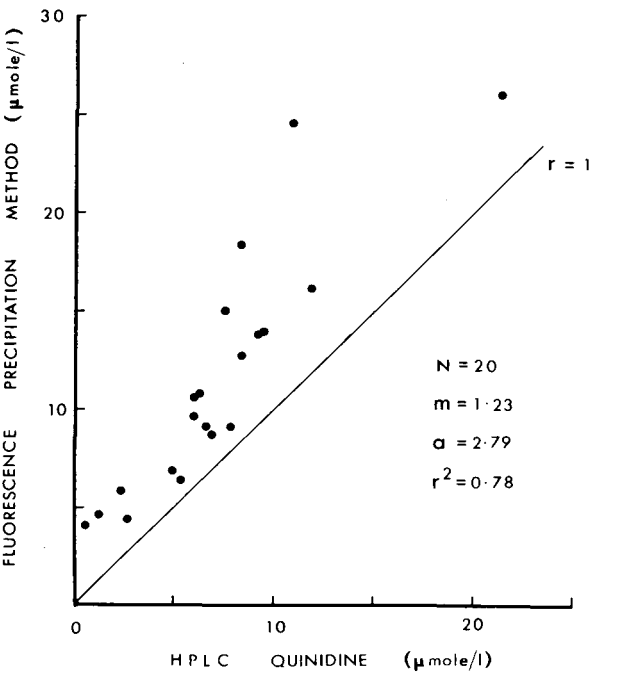

Fig. 6. Comparison of HPLC and precipitation—fluorescence assay methods for plasma quinidine (m = slope, a = intercept). Samples were taken from patients on chronic oral quinidine therapy.

TABLE II

QUINIDINE, 3-HYDROXYQUINIDINE, DIHYDROQUINIDINE AND QUINIDINE PLUS FLUORESCENT METABOLITE LEVELS (μmole/l) IN PLASMA SAMPLES FROM PATIENTS ON ORAL THERAPY

Quinidine	3-Hydroxyquinidine	Dihydroquinidine	Quinidine + metabolites*
7.4	1.5	0.3	10.8
11.4	2.4	0.4	16.1
22.2	3.3	1.0	29.4
30.0	5.2	1.2	42.0
5.8	1.4	0.4	10.7
15.2	2.5	0.7	21.5
2.2	0.5	0.1	3.3
9.7	0.6	0.2	11.9
10.0	1.7	0.2	13.3
10.2	1.7	0.3	13.6
12.6	1.9	0.5	19.2
3.3	0.6	0.1	4.6
6.8	0.5	0.2	8.3
16.6	1.9	0.6	22.5
8.0	0.3	0.3	9.4
4.6	1.5	0.2	9.9
18.0	2.6	0.7	24.3
6.0	2.7	0.3	14.8
0.6	0.5	< 0.1	3.4
6.6	0.9	0.2	8.6
Mean:			
10.4	1.71	0.40	14.9
(3.35 μg/ml)	(0.58 μg/ml)	(0.13 μg/ml)	

*Quinidine + 3-hydroxyquinidine + dihydroquinidine + other metabolites assumed to have identical fluorescence to quinidine.

The levels of quinidine, 3-hydroxyquinidine, dihydroquinidine and quinidine plus metabolites obtained for the HPLC assay of plasma samples drawn from different patients on varying oral quinidine therapy and not necessarily at steady-state are shown in Table II. The levels of 3-hydroxyquinidine and dihydroquinidine found in this mixed patient population showed similar variations to those published [3, 4, 9]. Peak 1 accounted for the majority of the additional metabolites indicated in the last column of Table II.

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CHROMBIO. 459

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND ISOLATION OF QUINIDINE AND QUININE METABOLITES IN RAT URINE

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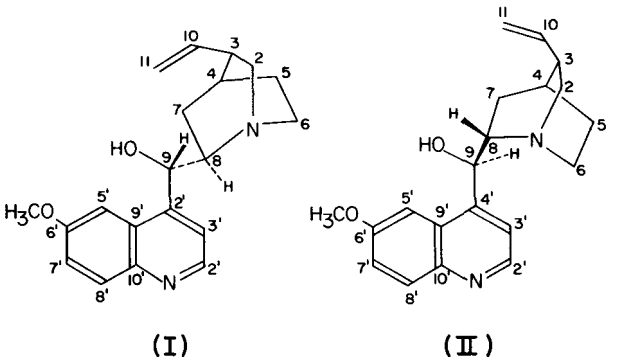
SUMMARY

A procedure for the separation and isolation of the urinary metabolites of quinidine and quinine by reversed-phase high-performance liquid chromatography is described. Nine metabolites of quinidine and eight metabolites of quinine were detected in the urine of male Sprague-Dawley rats after a single dose of quinidine or quinine (50 mg kg^{-1}). Following extraction from urine, the metabolites were separated on either an analytical or a semi-preparative reversed-phase column by gradient elution. After isolation and derivatization, the metabolites were analyzed by gas chromatography and gas chromatography—mass spectrometry.

INTRODUCTION

Quinidine (I) and quinine (II), members of the cinchona alkaloid family, have been widely used for the treatment of cardiac arrhythmias and malaria, respectively. Quinidine was introduced into medicine in 1918 [1] and is still used extensively in cardiac therapy, but quinine has been largely replaced by synthetic antimalarial drugs, including mepacrine and pamaquine. Nevertheless, quinine is still used as a bitter principle in the flavoring of carbonated table waters.

Quinidine and quinine are diastereoisomers that differ in their configuration at the C-8 and C-9 positions (Fig. 1). They are metabolized by oxidation of the quinoline and quinuclidine moieties to produce a series of phenolic and non-phenolic derivatives. The major metabolic products of quinidine found in man are (3*S*)-3-hydroxyquinidine [2—4], the carbostyril 2'-quinidinone [2, 3, 5] and O-desmethylquinidine [6, 7]. These metabolites have been detected by thin-layer chromatography (TLC) [2, 6—9] and gas chromatography—mass spectrometry (GC—MS) [2, 3] and characterized by ^{13}C NMR [3, 4], IR [3, 4]



(I) (II)

Fig. 1. Structures of quinidine (I) and quinine (II).

and UV absorption spectroscopy [2, 5] and mass spectrometry [2, 3, 6, 7]. Quinine has attracted less attention in recent years, presumably because of its declining therapeutic uses. The only significant work on quinine metabolism in man remains that of Brodie et al. [5]. These workers found that quinine is biotransformed primarily to the carbostyril and a product which was identified as 6-hydroxyquinine, but which may in fact be 3-hydroxyquinine by analogy with the metabolism of quinidine. A number of additional oxygenated metabolites of both quinidine and quinine have been suggested [2—5, 10—13] but have not been fully investigated. The identification of metabolites of quinidine and quinine by GC and GC—MS has been difficult, particularly when minor metabolites are of interest, because they are not fully resolved on packed columns. In addition, the interpretation of data is complicated by the presence of the corresponding dihydro analogs, dihydroquinidine (3—22%) or dihydroquinine (2—9%) in currently available samples [14]. These dihydro analogs are probably also metabolized by oxidative pathways. Because of these difficulties, investigators have often resorted to TLC for the separation of metabolites followed by direct inlet mass spectrometry for structural studies.

In this paper, we describe an high-performance liquid chromatographic (HPLC) method for the separation and isolation of nine metabolites of quinidine excreted in rat urine. We applied the HPLC method developed for quinidine to its isomer quinine, and eight metabolic products were isolated from rat urine.

EXPERIMENTAL

Reagents

All reagents were of analytical-reagent grade. Glass-distilled acetonitrile, tetrahydrofuran and methanol were purchased from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). HPLC-grade acetic acid was obtained from J.T. Baker (Phillipsburg, N.J., U.S.A.). Column packings of 1% SE-30 coated on Gas-Chrom Q and bistrimethylsilylacetamide (BSA) were purchased from Applied Science Labs. (State College, Pa., U.S.A.). Glusulase was obtained from Endo Labs. (Garden City, N.Y., U.S.A.). Anhydrous quinidine (found by HPLC

to contain ca. 5% of dihydroquinidine) and quinine monohydrate (containing ca. 1% of dihydroquinine) were obtained from Aldrich (Milwaukee, Wisc., U.S.A.).

Instrumentation

HPLC. μBondapak C_{18} reversed-phase analytical columns (300 × 3.9 mm I.D.) and semi-preparative columns (300 × 7.8 mm I.D.) were obtained from Waters Assoc. (Milford, Mass., U.S.A.). HPLC analyses were carried out by gradient elution using a dual solvent delivery system (Waters 6000A), a solvent programmer (Waters 660) and a UV absorbance detector (Waters 440) at 254 nm. OmniScribe recorders (Houston Instruments, Austin, Texas, U.S.A.) were employed.

GC and GC—MS. Gas chromatographic separations were carried out using silanized glass capillary columns (45 m) coated with SE-30, prepared according to a procedure developed in this laboratory [15]. Analyses were temperature programmed from 180° at 2° min^{-1}. Methylene unit (MU) values were determined with *n*-alkanes as reference compounds.

GC—MS analyses were carried out using an LKB 9000-PDP/12 bioanalytical system equipped with silanized glass coil columns (1.85 m × 2 mm I.D.) packed with 1% SE-30 on Gas-Chrom Q (100—120 mesh). All separations were programmed from 180° at 3° min^{-1}.

Animal procedure

Male Sprague-Dawley rats (ca. 200 g) were injected with a single dose of quinidine or quinine (50 mg kg^{-1}; i.p.) in dimethyl sulfoxide (0.5 ml). A control rat was injected with an equal volume of dimethyl sulfoxide. The rats were housed individually in metabolism cages and 24-h urine samples were collected. The urines were stored at −20°.

Sample preparation

After enzymatic hydrolysis of the urine samples with Glusulase at pH 4.5—4.8 for 17 h at 37°, the metabolites were extracted by an ammonium carbonate—ethyl acetate procedure [16]. For profiling metabolites by HPLC on analytical columns, an aliquot (usually one tenth) of a diluted 24-h urine sample was used. Urine extractions were carried out in centrifuge tubes fitted with Teflon-lined screw-caps. For collection of metabolites from a semi-preparative HPLC column, an ammonium carbonate—ethyl acetate extract of a 24-h urine sample was used; the extraction was carried out in separating funnels.

The extracts were evaporated to dryness in a stream of nitrogen and the residue was dissolved in methanol and transferred into Reacti-vials. The final volume of an analytical sample was 25 μl and the final volume of sample for isolation with the semi-preparative column was 750 μl. Samples were centrifuged and aliquots were injected on to the HPLC columns; 1—2 μl was required for the analytical column and 100-μl injections were used for isolation of metabolites with the semi-preparative column.

HPLC analysis

When analyses were carried out on the analytical column, a 35-min gradient

system (profile 7) was used with a column pressure of 800—1000 p.s.i. and a flow-rate of 0.9 ml min^{-1}. The solvent system consisted of a mixture of solvent A (water—acetic acid, 99:1) and solvent B (water—acetonitrile—acetic acid, 40:59:1), in which the proportion of B varied from 10 to 85%. A modified solvent system was used with the semi-preparative column and consisted a mixture of solvent A (water—acetic acid, 99:1) and solvent B (water—acetonitrile—acetic acid—tetrahydrofuran, 40:59:1:0.1), in which the proportion of B varied from 10 to 80%. A 70-min gradient (profile 8) was used with a flow-rate of 1.8 ml min^{-1} and a column pressure of 1300—1500 p.s.i.

GC and GC—MS analyses

The individual fractions from two injections on the semi-preparative column were pooled, and the organic solvent was removed (Rotovap). After freeze-drying, the residues were transferred with methanol into Reacti-vials and evaporated to dryness in a stream of nitrogen. Each residue was dissolved in pyridine (10 μl) and silylated with BSA (10 μl). After heating at 60° for 2 h, an aliquot (1—5 μl) was analysed by GC and GC—MS.

Preparation of quinidine- and quinine-10,11-dihydrodiols

Quinidine (32 mg; 0.1 m*M*) or quinine monohydrate (34 mg; 0.1 m*M*) in pyridine (0.5 ml) was added to a solution of osmium tetroxide (28 mg; 0.11 m*M*) in benzene (2 ml). The resulting yellow solution was allowed to stand at ambient temperature for 2 days. Subsequent hydrolysis with a solution of *d*-mannitol (26 mg) and sodium hydroxide (70 mg) in water (0.5 ml) gave a dark brown solution, which was extracted with ethyl acetate (5 × 10 ml). After removal of solvent (Rotovap) the quinidine or quinine products were transferred into Reacti-vials. Samples were analyzed by HPLC using the solvent system described above. Two peaks were seen on each chromatogram. The products were collected from a semi-preparative column as before and, after removal of the solvent and derivatization with BSA, the products were analyzed by GC and GC—MS.

The mass spectra indicated that the two products were stereoisomers of quinidine-10,11-dihydrodiol or quinine-10,11-dihydrodiol. Each pair of stereoisomers had essentially identical mass spectra and assigment of *R* and *S* configurations could not be made.

The methylene unit (MU) values of the quinidine-10,11-dihydrodiols with a 45-m glass capillary column were 30.8 and 31.0. The quinine dihydrodiols were not resolved on the capillary column and a single MU value of 30.9 was found.

RESULTS

Figs. 2 and 3 show the separation of the urinary metabolites of quinidine and quinine, respectively, on a semi-preparative reversed-phase HPLC column. The dotted lines show where fractions were collected. The major metabolites of quinidine, (3*S*)-3-hydroxyquinidine and O-desmethylquinidine, were found in fractions 16 and 17, respectively, and their identities were confirmed by GC—MS. 2′-Quinidinone was not detected in rat urine. Two additional major metabolites, not previously reported, were found in fractions 9 and 11. These

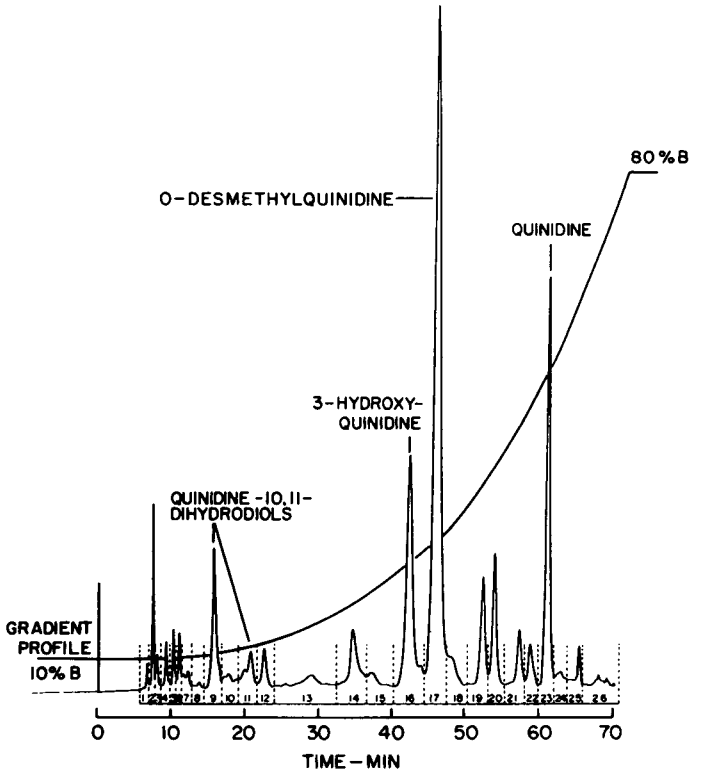


Fig. 2. Reversed-phase HPLC separation of quinidine metabolites on a semi-preparative column by gradient elution.

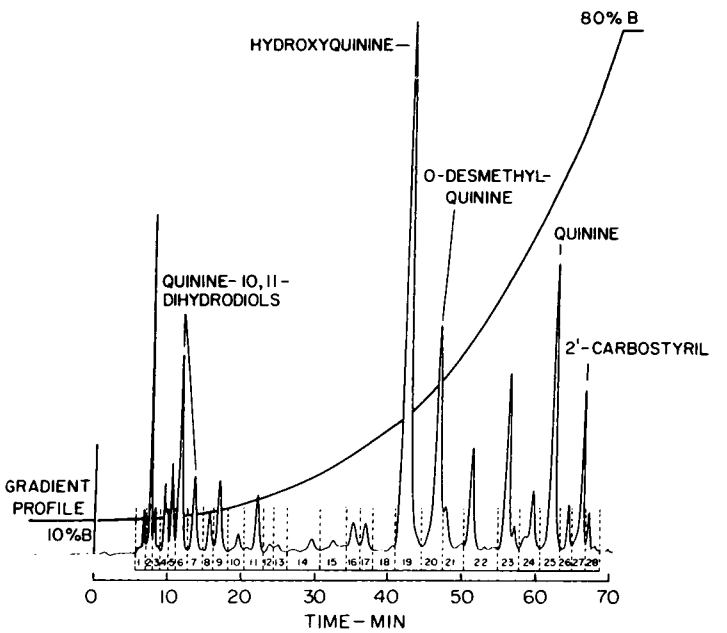


Fig. 3. Reversed-phase HPLC separation of quinine metabolites on a semi-preparative column by gradient elution.

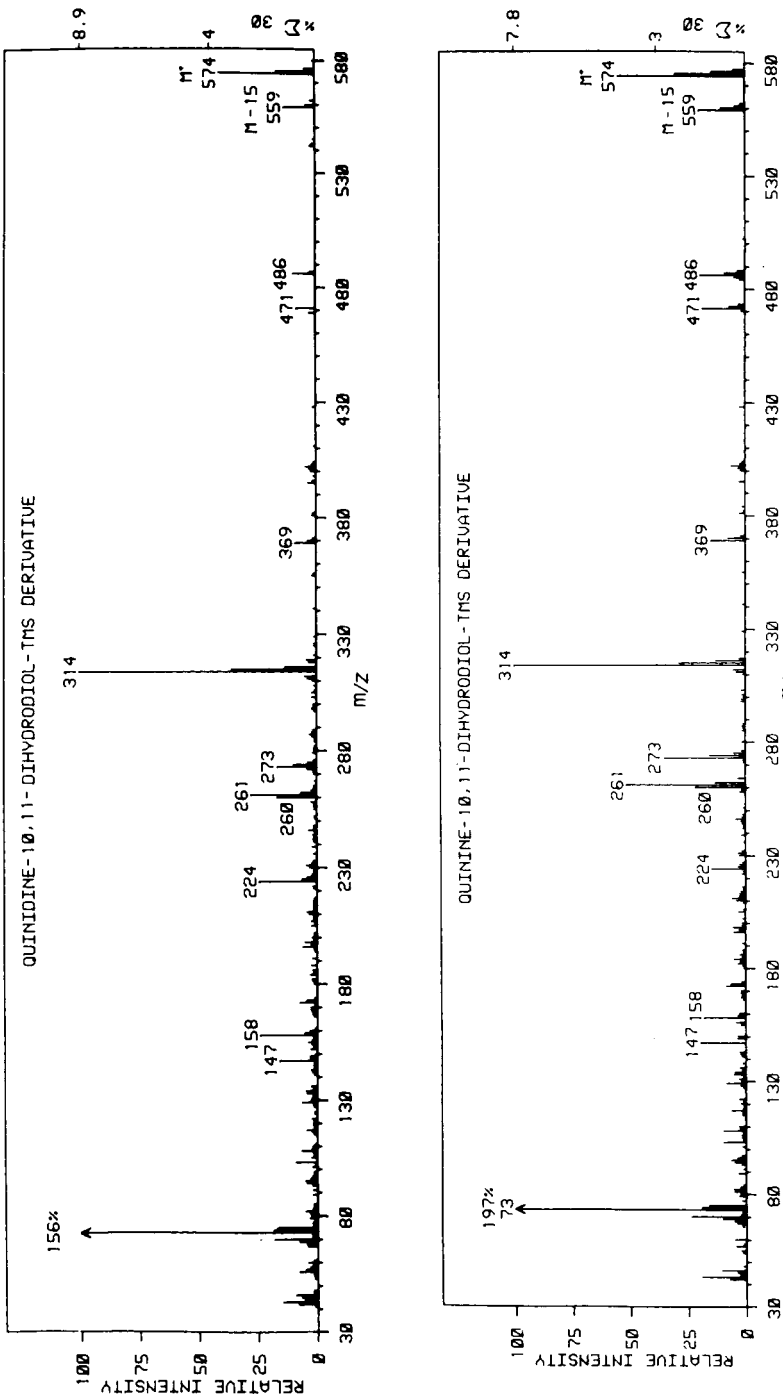


Fig. 4. Electron-impact ionization mass spectra of quinidine-10,11-dihydrodiol (TMS derivative) (upper) and of quinine-10,11-dihydrodiol (TMS derivative) (lower).

were identified as diastereoisomers of quinidine-10,11-dihydrodiol by GC—MS and by comparison with synthesized reference compounds. The mass spectrum of the quinidine-10,11-dihydrodiol collected in fraction 9 is shown in Fig. 4 (upper).

The mass spectra of quinidine, quinine and their metabolites exhibit characteristic ions formed by cleavage of the 8,9-carbon bond. In the mass spectra of the TMS derivatives of the dihydrodiols (mol. wt. = 574), these ions are observed at *m/z* 314 (base peak), corresponding to the addition of two OTMS groups to the vinyl side-chain of the quinuclidine moiety, and at *m/z* 260 and 261, corresponding to the unchanged quinoline portion of the molecule. The dihydrodiol metabolites can be distinguished from a dihydroxyquinidine substituted on the quinuclidine ring, rather than on the side-chain, by the molecular ion at *m/z* 572 (TMS derivative) and a base peak at *m/z* 312.

The two major metabolites of quinine excreted by man, hydroxyquinine and 2′-quininone [5], were found in fractions 20 and 27, respectively, and their identities were confirmed by GC—MS. 2′-Quininone was eluted from the reversed-phase column after quinidine. Drayer et al. [17] found that the corresponding quinidine metabolite, 2′-quinidinone, was eluted after quinidine from a μBondapak C_{18} column. The diastereoisomers of the 10,11-dihydrodiol of quinine and O-desmethylquinine were eluted in fractions 6, 7 and 19, respectively. The metabolites were identified by GC—MS, and in the case of the dihydrodiols, by comparison with synthesized reference compounds. The MU value of O-desmethylquinine was 27.7. The dihydrodiols were not resolved on the GC column and their MU value was 30.9. The mass spectral fragmentation of these metabolites was essentially the same as that of their quinidine analogs, but the relative peak intensities of the ions differed. The mass spectrum of the quinine-10,11-dihydrodiol collected in fraction 6 is shown in Fig. 4 (lower).

DISCUSSION

The reversed-phase HPLC procedure described here, based on gradient elution, can be used to study the metabolism of quinidine or quinine. The separation of quinidine, quinine and their metabolites on a reversed-phase (μBondapak C_{18}) HPLC column is influenced by the concentration of acetic acid in the eluting solvents. In the absence of acetic acid, quinidine and quinine were retained on the column. In the presence of 0.1% of acetic acid, both quinidine and quinine were eluted as poorly defined peaks with very long retention times. By increasing the concentration of acetic acid to 1%, both quinidine and quinine and their metabolites were eluted as well defined peaks. Comparable results were obtained by using a solvent system containing 0.1% of acetic acid and one of a series of alkylsulphonic acids (C_6—C_9), reagents which form paired ions (PIC reagents). However, it is more convenient to use acetic acid alone to generate a counter ion for the elution and separation of quinidine, quinine and their metabolites.

When the separations were carried out on an analytical column, profiles of the urinary metabolites were obtained that could be used to study the parameters affecting human metabolism and species differences in metabolism. For example, the urinary profiles of the rat and man are different. 2′-Quinidinone, a

major metabolite in man, was not detected in the rat. We found this metabolite in a urine sample obtained from a patient maintained on quinidine.

When the analyses were carried out with a semi-preparative column, nine metabolites of quinidine and eight metabolites of quinine were isolated and characterized. The time required for separation and collection of fractions for subsequent GC and GC—MS analysis was about 3 h. In addition to the dihydrodiols of quinidine and quinine and O-desmethylquinine which have not been described previously, additional oxygenated metabolites of both drugs have been characterized. The GC and GC—MS properties of these new metabolites will be reported separately.

Hydroxylation in the quinuclidine part of quinidine was shown to yield primarily (3*S*)-3-hydroxyquinidine [2—4]. Reaction at a hindered position is unusual, but in this instance the vinyl group presumably determines the site of hydroxylation. The corresponding metabolite of quinine (Fig. 3) is probably (3*S*)-3-hydroxyquinine; the isomeric 6-hydroxy structure [5] may have been incorrectly assigned. Further studies may, however, result in the identification of 6-hydroxy compounds in the group of monohydroxy metabolites derived from both quinine and quinidine.

ACKNOWLEDGEMENTS

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Biomedical Applications

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DETERMINATION OF SOME BENZODIAZEPINES AND METABOLITES IN SERUM, URINE AND SALIVA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The performance of a number of liquid—solid systems, consisting of mixtures of buffers (0.05 *M*) and methanol as mobile phase and methyl-silica as stationary phase, were investigated with respect to their use in the separation of 1,4-benzodiazepines by reversed-phase high-performance liquid chromatography with UV detection at 254 nm. Phase system selectivities and column efficiencies were determined. A nomogram is presented from which the chromatographic parameters can be calculated.

A complete separation of nine benzodiazepines within 12 min has been achieved, using methyl-silica as the stationary phase and 50% methanol as the eluent.

The results were applied to the development of a method for the determination of therapeutic levels of diazepam and its metabolites in human serum, urine and saliva. The first step in the analysis, the extraction of diazepam and its metabolites from serum and urine, was also investigated and good recoveries were achieved. A low detection limit (0.2 ng) and high precision were obtained. The concentrations of diazepam and its metabolites in human serum, urine and saliva were determined after both single and multiple oral doses of diazepam (and oxazepam).

INTRODUCTION

Since the early 1960s, benzodiazepines have been widely used as minor tranquillizers, sleep inducers and muscle relaxants. A number of methods are available for determining benzodiazepines and their metabolites in body fluids, such as UV spectrophotometry, polarography [1—4] and gas [5—7] and liquid chromatography [8—11]. UV spectrophotometry and polarography are non-specific and only give information about the overall concentration of a drug and its metabolites. Gas chromatography of the intact benzo-

diazepines is possible, but the gas chromatographic analysis of the metabolites often requires their derivatization into more volatile compounds, with possible problems.

Liquid chromatography has a greater number of possibilities for adjusting the selectivity. Moreover, because of its milder working conditions, it appears to be the most suitable technique for the analysis of thermally labile, hydrophilic and hydrophobic compounds. Until now only a few papers have described the quantitative and qualitative analysis of benzodiazepines and their metabolites using high-performance liquid chromatography (HPLC). The limit of detection of these methods is often insufficient for the needs of pharmacokinetic experiments, especially at therapeutic levels. This paper reports the determination of diazepam and its metabolites in human serum, urine and saliva, after both single and multiple oral doses of diazepam (or oxazepam).

In man, diazepam is metabolized to desmethyldiazepam, oxazepam and 3-hydroxydiazepam. Diazepam is rapidly absorbed after oral administration. A single oral dose of 10—15 mg results in peak levels in serum in the range 200—300 ng/ml within 2 h. However, there is a wide variation between individuals. Chronic oral administration of 5 mg of diazepam three times daily results in steady-state serum levels of about 200—400 ng/ml.

Desmethyldiazepam is the major metabolite of diazepam in blood. At steady-state levels the concentration of desmethyldiazepam is close to or even may exceed that of diazepam. The greater part of the metabolites in urine occur as glucuronide conjugates, while only very small amounts of diazepam are present. Determination of the metabolites of diazepam in urine requires hydrolysis of the urine samples. As a consequence of the low stability of benzodiazepines under alkaline and acidic conditions, enzymatic hydrolysis by means of β-glucuronidase is often preferred.

This paper describes the separation and quantitation of benzodiazepines in serum, saliva and urine at therapeutic levels using a highly selective and efficient reversed-phase adsorption system by HPLC with UV detection.

EXPERIMENTAL

Apparatus

The liquid chromatograph was constructed from commercially available and custom-made parts and consisted of a thermostated glass eluent reservoir, a high-pressure pump (DMP 1515, Orlita, Giessen, G.F.R.), a flow-through Bourdon-type manometer acting as damping device, a septumless sampling device Model U6K (Waters Assoc., Milford, Mass., U.S.A.), a thermostated column (stainless-steel precision-bore tubing, I.D. 2.8 mm, O.D. 6.35 mm and length 10 cm), a single-wavelength detector (Waters 440) operating at 254 nm, a flat-bed potentiometric recorder (BD8, Kipp & Zn., Delft, The Netherlands) and a computing integrator (Spectra Physics, Autolab, System I). The damping device consisted of a flow-through Bourdon tube and a flow resistance.

Columns were packed by means of a double-headed high-pressure pump (DMP 1515, Orlita) supplied with a manometer and eight flow-through Bourdon tubes.

Chemicals and materials

Organic solvents of analytical-reagent grade (Merck, Darmstadt, G.F.R.) and doubly distilled water were used. For the determination of very low concentrations, extraction solvents of high purity (Chrom AR Nanograde, Byk-Mallinckrodt, Wessel, G.F.R.) were used.

Buffer solution of pH 9.5 was prepared by adjusting the pH of saturated ammonium chloride solution to 9.5 with concentrated ammonia solution. The buffer solutions used in the mobile phase systems were prepared from 0.05 *M* solutions of phosphoric acid, sodium hydrogen phosphate and sodium dihydrogen phosphate. The benzodiazepines were kindly donated by Hoffmann-La Roche (Mijdrecht, The Netherlands) and Wyeth (Amsterdam, The Netherlands).

The methyl-silica was prepared as follows. Silica (SI 60, Merck) with a particle size range of 63—200 μm was ground in a rotating mortar. The ground materials were classified to a particle size range of 7—8 μm by means of an air classifier (Alpine MZR, Augsburg, G.F.R.). A mixture of 30 g of classified microparticulate silica, 1200 ml of toluene and 300 ml of dimethyldichlorosilane (DMCS) was refluxed with stirring for 72 h. After washing the modified silica with toluene and methanol the silica was refluxed with methanol for 6 h. The modified silica was then washed with diethyl ether and dried at 90°.

The enzyme β-D-glucuronide glucuronohydrolase (G-0251, Type B-1, Sigma, St. Louis, Mo., U.S.A.) had an activity of 10^6 Fishman units per gram of solid.

The biological samples were obtained from volunteers receiving a single dose and from patients receiving benzodiazepines therapeutically.

Column packing

Columns were packed using a slurry technique, 0.45 g of the methyl-silica was dispersed in 2 ml of carbon tetrachloride and then placed in a pre-column (I.D. 4.6 mm, O.D. 6.35 mm and length 30 cm), to which the column, closed at the bottom by a frit and a closed high-pressure valve, was attached. The pre-column was replenished with carbon tetrachloride. Then *n*-hexane was pumped into the tube, until the pressure increased to 500 bar. At this pressure, the valve at the end of the column was unscrewed. As a consequence of the change in viscosity (carbon tetrachloride 0.97 cP and *n*-hexane 0.32 cP), the pressure drop over the column decreased. After this pressure drop the pump was turned off. When the pressure had fallen completely, the column was removed and placed in the liquid chromatograph. The remaining *n*-hexane was removed by injecting three 2-ml volumes of ethanol. Then the system was ready for use.

Chromatography

The capacity ratios were calculated from the retention times of the benzodiazepines and of an unretarded compound (potassium periodate). The selectivity coefficients of a pair of compounds were calculated as the ratio of their capacity ratios. The theoretical plate height for a compound was determined from its retention time and half the peak width at 0.6 of the peak height.

Preparation of the biological samples

Urine samples. The urine was collected during 24 h. After adjusting the pH

of the urine to 5.0 with 2 *M* hydrochloric acid, a concentrated solution of 500 units of β-glucuronidase per millilitre of urine was added. After incubation at 37° for 5 h the samples were cooled to room temperature and then the benzodiazepines were extracted.

Blood samples. Blood samples of the patients receiving benzodiazepines therapeutically were taken 2—4 h after the first dose of the day. After clotting of the blood the serum was decanted and centrifuged.

Saliva samples. Saliva samples were collected from the patients immediately after taking the blood samples. The saliva was centrifuged and decanted.

Extraction

(1) Transfer 1 ml of sample, 1 ml of buffer (pH 9.5) and 1 ml of saturated potassium chloride solution [13] into a 20-ml centrifuge tube. (2) Add 10 ml of diethyl ether, mix for 60 sec (Whirlmixer), centrifuge (1000 *g* for 5 min), freeze and decant the ether phase. (3) Repeat step 2 on the aqueous phase. (4) Combine the ether phases, add 3 ml of 6 *M* hydrochloric acid, mix for 60 sec, freeze and discard the ether phase. (5) Add to the aqueous phase 1.8 ml of 10 *M* sodium hydroxide solution and 1 ml of buffer (pH 9.5), and repeat steps 2 and 3 on the aqueous phase. (6) Combine the ether phases and evaporate the solvent. (7) Dissolve the residue in 100 μl of eluent. Aliquots of 10—60 μl were analyzed by HPLC.

The determination of the higher levels does not require the complete extraction procedure. In those instances (more than 500 ng/ml), a single extraction fulfils the demands of a relatively low background. In order to achieve a rapid and efficient separation of the organic and aqueous phases after mixing, the extraction flask is placed in liquid nitrogen. The aqueous phase freezes within 50 sec and the organic phase can be decanted easily. The total extraction procedure takes less than half an hour.

RESULTS AND DISCUSSION

The choise of the phase system for a chromatographic separation should be based on the equation for the resolution, R_{ji}, for two components j and i:

$$R_{ji} = (r_{ji} - 1) \frac{\kappa_i}{1 + \kappa_i} \sqrt{N_i} \quad (1)$$

where $r_{ji} = \kappa_j/\kappa_i$ = selectivity coefficient; κ_i = capacity ratio of compound i; N_i = number of theoretical plates for compound i.

In order to determine benzodiazepines and their metabolites at very low concentrations in serum, saliva and urine, the parameters of the chromatographic process should be chosen so that both the resolution and the detection limit are adequate. The relationship between the maximum outlet concentration of the solute in the mobile phase, $c_{i,m}^{max}$, and the amount injected, Q_i, is expressed by

$$c_{i,m}^{max} = \frac{Q_i}{\sqrt{2\pi}\epsilon_m A(1 + \kappa_i)\sqrt{H_i L}} = \frac{Q_i\sqrt{N_i}}{\sqrt{2\pi}V_m(1 + \kappa_i)} \quad (2)$$

From eqn. 2 it can be concluded that highly efficient columns and small

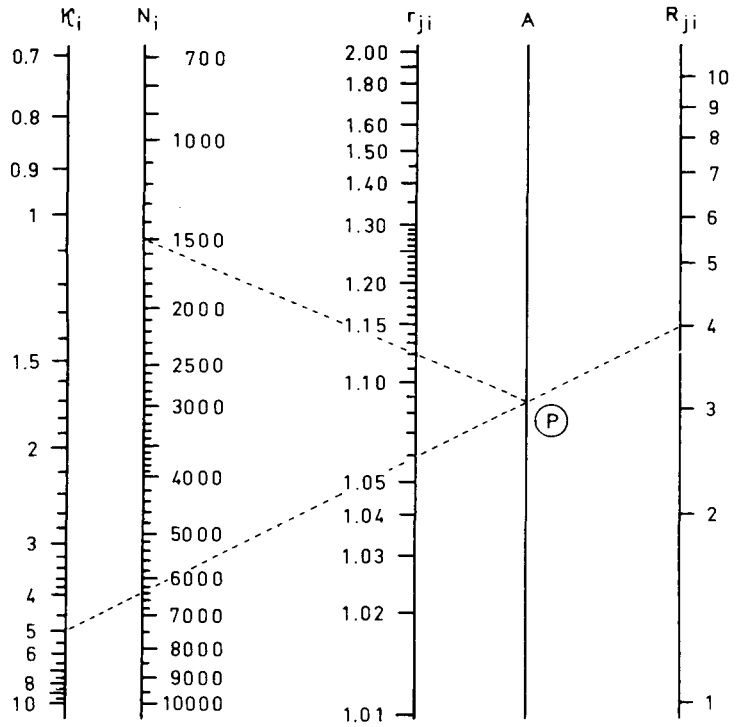


Fig. 1. Nomogram of eqn. 1.

capacity ratios are favourable for achieving low detection limits and an adequate resolution.

From eqn. 1, a nomogram can be constructed (Fig. 1). The selectivity coefficient and hence the capacity ratio of the subsequent compound can be found if the other parameters are known. In the same way, the theoretical plate number for a given phase system or the resolution can be obtained if the other parameters are known.

The nomogram is used as follows. Combining eqns. 1 and 2 it can be concluded that for minimum peak broadening and therefore a maximum outlet concentration, the resolution must be as low as practicable. In order to quantitate two components, i and j, the resolution must be equal to at least 4. The line connecting $\kappa_i = 5$ and $R_{ji} = 4$ intersects the auxiliary line A at point P. If 1500 theoretical plates are available, it can be found that the selectivity coefficient, r_{ji}, is 1.12 or $\kappa_j = 5.60$.

Similarly, if the capacity ratios and therefore the selectivity coefficient and the plate number are known, the resolution can be found easily. The line drawn through $N = 1500$ and $r_{ji} = 1.12$ intersects the line A at point P. This means that if $\kappa_i = 5$ the resolution will be 4.

Phase system selectivity

The capacity ratios and selectivity coefficients of successively eluted benzodiazepines were measured as a function of the pH of the mobile phase. The results are given in Table I.

TABLE I

CAPACITY RATIOS AND SELECTIVITY COEFFICIENTS OF A NUMBER OF BENZODIAZEPINES AS A FUNCTION OF THE pH* OF THE MOBILE PHASE (METHANOL–WATER, 40:60)

Component	pH = 2.4		pH = 4.2		pH = 5.9		pH = 7.0	
	κi	r_{ji}	κi	r_{ji}	κi	r_{ji}	κi	r_{ji}
7-Aminonitrazepam	0.00	—	1.04	—	1.10	—	1.54	—
Bromazepam	1.77	—	3.69	3.55	3.17	2.88	4.15	2.69
Nitrazepam	2.93	1.66	5.58	1.51	4.54	1.43	5.85	1.41
Oxazepam	6.75	2.30	7.22	1.29	5.63	1.24	7.32	1.25
Nor-3-hydroxyflurazepam	6.99	1.04	6.91	0.96	4.45	0.79	7.84	1.07
Clonazepam	7.33	1.05	7.58	1.10	6.39	1.44	7.97	1.02
Norchlordiazepoxide	0.86	0.12	3.41	0.45	5.95	0.93	8.09	1.02
Lorazepam	8.88	10.3	8.69	2.55	7.09	1.19	9.27	1.15
Hydroxydiazepam	9.94	1.12	10.5	1.21	8.01	1.13	10.6	1.14
Chlordiazepoxide	0.94	0.09	5.74	0.55	8.82	1.10	11.9	1.12
Desmethyldiazepam	2.90	3.09	4.84	0.84	12.0	1.36	13.8	1.16
Norflurazepam	9.00	3.10	12.7	2.62	10.1	0.84	14.1	1.02
Flurazepam	2.24	0.25	3.08	0.24	7.73	0.77	15.2	1.08
Diazepam	11.0	4.91	20.1	6.53	18.1	2.34	20.7	1.36
Prazepam	30.0	2.73	62.6	3.11	52.0	2.87	62.2	3.00
Medazepam	2.40	0.08	5.82	0.09	45.2	0.87	91.2	1.47

*pH measured in buffer solution.

If other conditions remain the same, the capacity ratios decrease with increasing methanol content and the selectivity coefficients decrease regularly with increasing methanol content. Hence there is no difference in the order of elution. However, on changing the pH of the mobile phase, it can be seen that both the selectivity coefficients and the capacity ratios are affected.

In Fig. 2 the plot of $\log \kappa$ versus pH is given for a number of benzodiazepines. Between pH 4 and 6 the traces intersect and, as a consequence, the order of elution will be inverted.

In order to detect very low amounts of, e.g., desmethyldiazepam, one has to use buffer of low pH. Because of the dependence of the maximum outlet concentration on the capacity factor, it is preferable to elute first those components which are present at the lowest concentration. In order to adjust the capacity factors to more appropriate values (e.g., $\kappa > 2$), the methanol content of the mobile phase can be decreased.

Fig. 3 shows the separation of a test mixture of benzodiazepines, showing the excellent selectivity of the phase system. It can be concluded that the phase system can be applied for qualitative determinations. In this work, the determination of diazepam and its metabolites in particular has been investigated.

Composition of the extraction solvent

The extraction of the benzodiazepines from the biological matrix is an important step in this type of trace analysis, on the one hand to remove interfering substances and on the other to enrich the benzodiazepines. Benzodiazepines are weakly basic compounds with pK_a values ranging from 1.7 to 12.0.

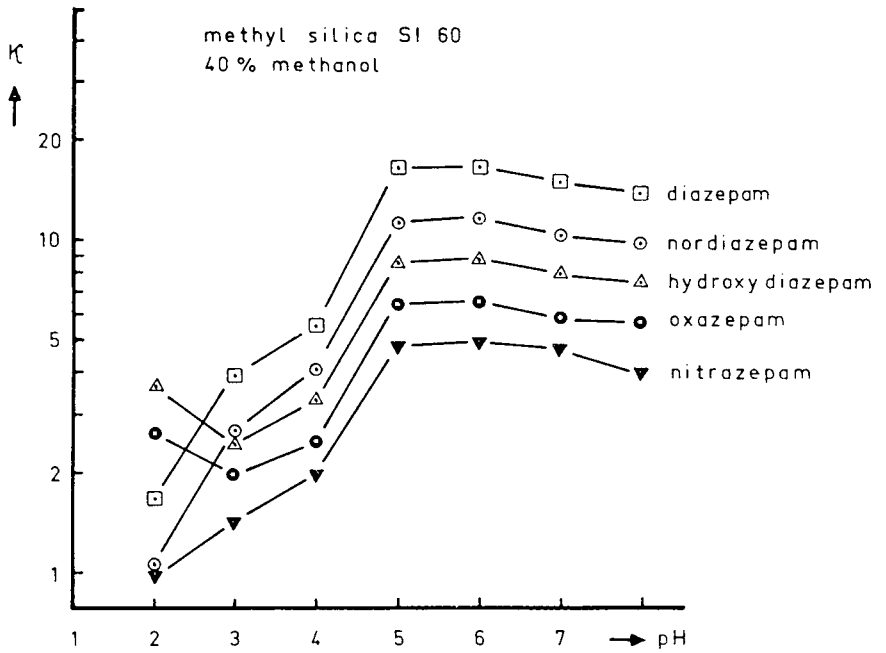


Fig. 2. Plot of the logarithm of the capacity ratio versus the pH of the mobile phase (40% methanol).

The distribution of these basic compounds between an organic solvent and an aqueous solution depends on the pH of the aqueous phase and on the pK_a value of the benzodiazepines. The total distribution coefficient of a base B, defined as the ratio of the concentrations in the organic and the aqueous phases, excluding side reactions, can be expressed by

$$K_B = \frac{[B]_{org}}{[B]_{aq} + [HB^+]_{aq}} = \frac{[B]_{org}}{[B]_{aq}} \cdot \frac{1}{1 + \frac{[H^+]}{K_a}} \quad (3)$$

where the subscripts org and aq refer to the organic and aqueous phases, respectively.

The composition of the extraction solvent determines the value of K_B. From eqn. 3 it can be seen that pH>>pK_a is favourable for obtaining high distribution coefficients, i.e., high recoveries. Many data are available in the literature on the effect of the composition of the organic phase on the distribution of the benzodiazepines. However, many of those data were obtained with extraction solvents containing highly UV-absorbing constituents (e.g., toluene or benzene). The use of this kind of solvent adversely affects determinations at the nanogram level unless the organic phase has been evaporated completely.

A number of extraction solvents were investigated. Table II gives recoveries for diazepam extracted from an aqueous solution (1 μg/ml).

The highest recoveries were obtained with diethyl ether, the addition of *n*-hexane decreased the recoveries and increased emulsification.

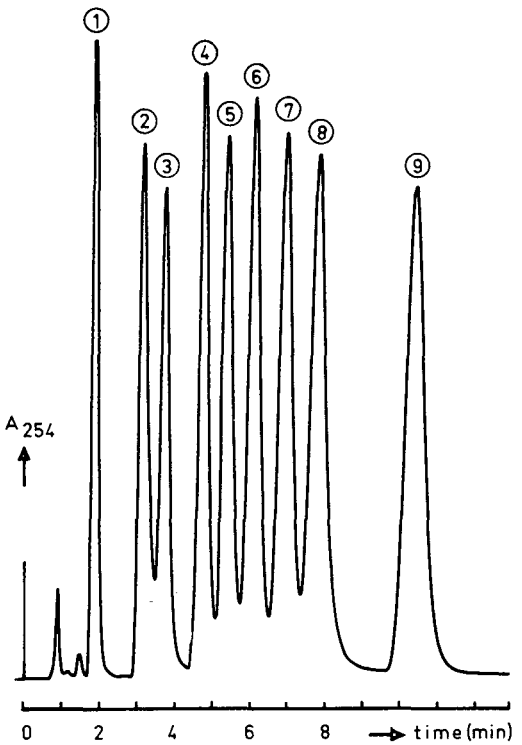


Fig. 3. HPLC separation of a test mixture of nine benzodiazepines. Column: 10 cm × 2.8 mm I.D., methyl-silica 60. Eluent: methanol—phosphate buffer (0.05 *M*, pH 6.0) (1:1). Flow-rate: 9.3 μl/sec. Peaks: 1 = 7-aminonitrazepam, 2 = bromazepam; 3 = nitrazepam; 4 = oxazepam; 5 = desmethylchlordiazepoxide; 6 = hydroxydiazepam; 7 = chlordiazepoxide; 8 = desmethyldiazepam; 9 = diazepam.

Table III gives recoveries of diazepam and its metabolites extracted from water, urine and serum, with coefficients of variation in the range from 20 to 1000 ng/ml in parentheses. The recoveries given in Table III were obtained after addition of 1 ml of saturated potassium chloride solution in the first

TABLE II

RECOVERIES OF DIAZEPAM FROM AN AQUEOUS SOLUTION (1 μg/ml) EXTRACTED WITH DIFFERENT SOLVENTS

Extraction solvent (%, v/v)			Recovery diazepam (%)
n-Hexane	Diethyl ether	*n*-Propanol	
100	—	—	52.0
50	50	—	70.1
49	49	2	68.1
48	49	3	69.9
40	60	—	79.6
—	100	—	88.3

extraction step, otherwise lower recoveries were obtained (e.g., 16% loss for desmethyldiazepam).

Preparation of urine samples

The urinary metabolites of diazepam, i.e., oxazepam, desmethyldiazepam and 3-hydroxydiazepam, are present mainly as glucuronide conjugates. As a consequence of the high polarity of these conjugates, the assay of the metabolites of diazepam in urine requires either a completely different phase system and a modified extraction procedure or an enzymatic hydrolysis. Because of difficulties in the extraction of the conjugates we chose to hydrolyse them into the free benzodiazepines. The optimal conditions for the hydrolysis were found to be pH 5.0 and 37°.

The hydrolysis is complete after 5 h; a longer period of hydrolysis results in lower recoveries, owing to the low stability of the compounds of interest.

Precision and linearity of the method

The linearity and precision of the quantitative determination of benzodiazepines by HPLC were investigated by injecting different amounts of benzodiazepines. Table III includes the coefficients of variation for test mixtures of diazepam and its metabolites. The coefficients of variation range from

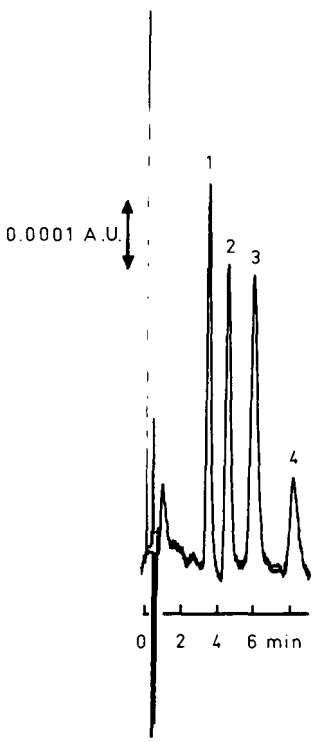
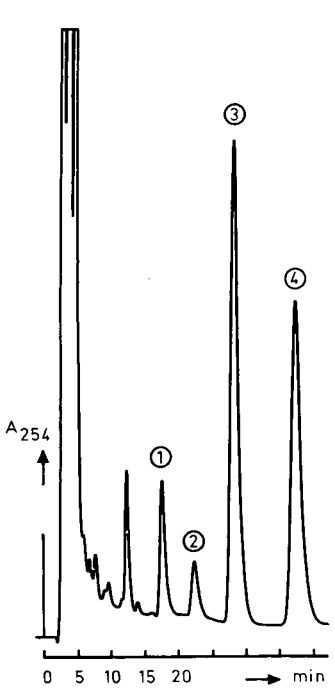


Fig. 4. HPLC separation of a test mixture of 1 ng of diazepam and 2 ng of its metabolites. Column as in Fig. 3. Eluent: methanol—phosphate buffer (0.05 *M*, pH = 7.0) (2:3). Flow-rate: 20.3 μl/sec. Peaks: 1 = oxazepam; 2 = hydroxydiazepam; 3 = desmethyldiazepam; 4 = diazepam.

TABLE III

RECOVERIES AND COEFFICIENTS OF VARIATION (C.V.) OF DIAZEPAM AND ITS METABOLITES FROM WATER, URINE AND SERUM

Component	Test mixture (20—1000 ng/ml): C.V. (%)	Mean recoveries and coefficients of variation					
		water (40—2000 ng/ml):		urine (40—2000 ng/ml):		serum (40—2000 ng/ml)	
		Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)	Recovery (%)	C.V. (%)
Oxazepam	0.5—0.2	91.4	3.5—0.7	88.8	4.0—0.7	86.3	4.2—1.7
Hydroxydiazepam	1.0—0.3	91.7	3.4—1.0	90.4	4.3—1.0	88.6	4.0—1.6
Desmethyldiazepam	1.2—0.4	92.1	3.4—1.3	91.2	3.9—1.4	89.4	4.2—1.6
Diazepam	1.3—0.5	94.8	3.3—1.2	92.0	3.5—1.4	90.3	4.3—1.9



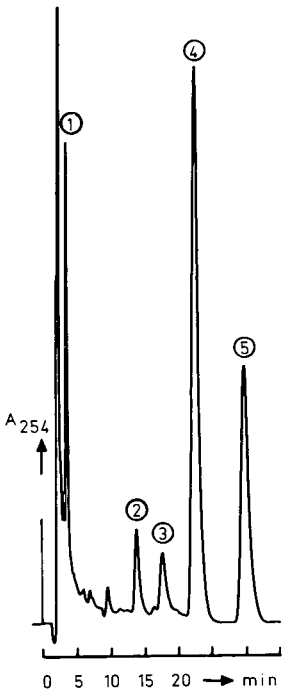


Fig. 5. Chromatogram of an extract from serum of patient B (10 mg of diazepam daily). Conditions as in Fig. 4, except flow-rate = 2.9 μl/sec. Peaks: 1 = oxazepam; 2 = hydroxydiazepam; 3 = desmethyldiazepam; 4 = diazepam.

Fig. 6. Chromatogram of an extract from saliva of patient J (40 mg of diazepam daily). Conditions as in Fig. 4, except flow-rate = 3.6 μl/sec. Peaks: 1 = caffeine; 2 = oxazepam; 3 = hydroxydiazepam; 4 = desmethyldiazepam; 5 = diazepam.

1.3% for 20 ng of diazepam injected to 0.2% for 1000 ng of oxazepam injected. The linearity of the calibration graphs, characterized by the correlation coefficients, was determined to be from 0.99990 (diazepam) to 0.99998 (oxazepam). The sensitivity of the system, defined as the slope of the graph of peak area versus amount injected expressed in integration units, ranged from 7.43 to 9.34 mV·sec/ng.

Fig. 4 shows the separation of a test mixture of diazepam and its metabolites, and it can be estimated that the limit of detection ranges from about 200 to 340 pg.

Application of the method to biological specimens

In order to test the method, serum, urine and saliva levels in volunteers and ambulant patients were determined. The results are given in Table IV.

No measurable amounts of desmethyldiazepam were seen after single doses of 5 or 10 mg, in contrast with chronic doses [14]. It appears from Table IV that after a single dose of 5 mg of diazepam, the serum levels of diazepam of the two volunteers after 2 h are about the same. However, subject R.M. showed remarkably high concentrations of desmethyldiazepam after both 2 and 9 h, which is more or less valid for the other metabolites. From a few ambulant pa-

TABLE IV

SERUM, URINE AND SALIVA LEVELS OF DIAZEPAM AND ITS METABOLITES AFTER A SINGLE DOSE AND CHRONIC ADMINISTRATION

Ox = oxazepam, Hydr. = hydroxydiazepam, Des = desmethyldiazepam, D = diazepam.

Subject	Dose	Co-medication	Serum level (ng/ml)				Urine level (μg/ml)				Saliva level (ng/ml)			
			Ox.	Hydr.	Des	D	Ox.	Hydr.	Des	D	Ox.	Hydr.	Des	D
J.P.N.*	1 × 5 mg diazepam	—	det.	~1.2	0.8	63								
J.P.N.**	(single dose)		det.	~1.2	1.6	45								
R.M.*	1 × 5 mg diazepam	—	~6	~6	15	62								
R.M.**	(single dose)		~8	~4	16	34								
R.M.*	1 × 10 mg diazepam	—	~9	~8	29	243								
R.M.**	(single dose)		~8	~6	31	98								
J***	10 mg diazepam 4 dd§	Lorazepam (2 mg) Butobarbital (100 mg) Vibramycine Rhinathiol Antalby	132	130	1025	596	14.7	8.2	4.9	—	98	82	603	486
B	10 mg diazepam 1 dd	Paracetamol Lasix	109	53	392	419					272	173	136	71
C	5 mg diazepam 1 dd	Disipal Sinemet	43	20	107	99					—	25	9	21
L§§	30 mg oxazepam 1 dd						9.8	—	—	—				

*Sample taken 2 h after intake.
**Sample taken 9 h after intake.
***Total amount of urine: 910 ml in 24 h.
§dd = daily devided dose.
§§Total amount of urine: 2460 ml in 24 h.

tients serum, saliva and urine samples were analysed for diazepam and its metabolites. All of them received diazepam orally daily and used it chronically for at least 2 weeks. All of the patients also received other drugs. The urine was collected during 24 h, but there was no special time for taking the blood or saliva samples.

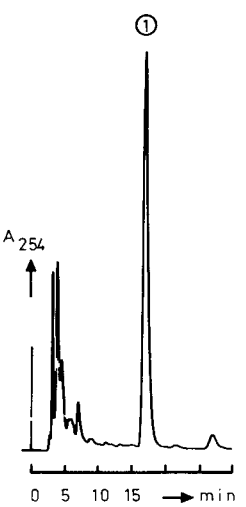
Table IV gives assays of serum levels for patients receiving various compounds as co-medication. Comparison of patient C, receiving 5 mg of diazepam daily, and the volunteers receiving the same dose (J.P.N. and R.M.) shows the great difference between the levels after single doses and chronic administration. The values in Table IV are in agreement with those given in the literature [14, 15].

Fig. 5 shows a chromatogram of an extract from serum of patient B. It can be seen that the co-medicants do not interfere with the compounds of interest.

Patient J is interesting because of the extensive co-medication involved. Many of the drugs used give acidic metabolites in blood and urine, some of which will not be extracted with the procedure described above, while others are separated chromatographically from the benzodiazepines. In none of these instances is the assay hampered by the co-medication.

The determination of saliva levels of benzodiazepines in comparison with urine and/or serum levels is interesting; however, only a few data are given in the literature [16, 17]. Fig. 6 shows a chromatogram of an extract from saliva of patient J (see Table IV).

It is notable that in the saliva from patient B the amounts of oxazepam and 3-hydroxydiazepam are higher than those of diazepam and nordiazepam,



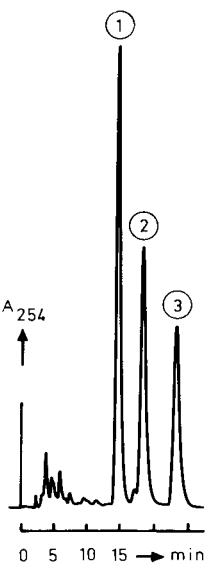


Fig. 7. Chromatogram of an extract from urine of patient L (30 mg of oxazepam daily). Conditions as in Fig. 4, except flow-rate = 2.9 μl/sec. Peaks: 1 = oxazepam.

Fig. 8. Chromatogram of an extract from urine of patient J (40 mg of diazepam daily). Conditions as in Fig. 4, except flow-rate = 3.6 μl/sec. Peaks: 1 = oxazepam; 2 = hydroxydiazepam; 3 = desmethyldiazepam.

TABLE V

AMOUNTS OF FREE METABOLITES AND CONJUGATED METABOLITES IN URINE OF PATIENT J

Component	Concentration of free metabolite in urine (ng/ml)	Total amount in urine (ng/ml)	Percentage conjugated
Diazepam	10	—	—
Oxazepam	32	14,740	99.8
Desmethyldiazepam	76	4,990	98.5
3-Hydroxydiazepam	37	8,230	99.9

while in patient J the amounts of desmethyldiazepam and diazepam are much higher than those of oxazepam and 3-hydroxydiazepam. It can be concluded from Table IV that there are great individual differences in the serum to saliva ratio for diazepam and its metabolites, which indicates that further studies should be devoted to the utility of the determination of saliva levels in order to determine serum levels. An advantage is the greater availability of saliva than blood. Caffeine and theophylline substances from coffee usually present in saliva do not interfere with the benzodiazepines. A disadvantage could be the varying amounts of saliva delivered by the patients. The same occurs with single voidings of urine. Urine was collected from patients J and L during 24 h and then analysed (Table IV). Patient L received 30 mg of Seresta® (oxazepam) daily from which oxazepam glucuronide is the only urinary metabolite (see Fig. 7).

Fig. 8 shows a chromatogram of a urine extract from patient J. It can be seen that the results agree well with the literature [17]. Although most benzodiazepines are conjugated, small amounts of free benzodiazepines can be found in urine.

Table V gives the urine results for patient J, who was receiving 40 mg of diazepam daily.

CONCLUSIONS

It can be concluded that reversed-phase HPLC is a valuable technique for the assay of benzodiazepines and their metabolites in serum and saliva. As a consequence of the hydrolysis necessary with urine samples, the method is time consuming in this instance. The phase system shows a good selectivity towards all of the benzodiazepines examined, which means that by varying the pH practical problems can be solved. Future research will be devoted to the more rapid analysis of urine samples.

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Note

Quantitation of serum tocopherols by high-performance liquid chromatography with fluorescence detection

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d-α-Tocopherol is considered to have the highest biological activity of the naturally occurring forms of vitamin E [1]. Therefore, vitamin E status in human populations is usually assessed in terms of the α-tocopherol level in blood. The other tocopherols (β-, γ- and ∂-tocopherol), however, are an important part of the daily vitamin E intake [2]. The significance of the non-α-tocopherols in human nutrition is not known. This depends to some extent on the lack of simple and rapid methods for their determination in serum samples. The chromatographic methods hitherto used to quantify the various tocopherols in serum are based on either gas chromatography (GC) [3] or thin-layer chromatography (TLC) [4]. Our previously described high-performance liquid chromatography (HPLC) method with UV detection [5] was inadequate for resolving the various tocopherols in serum from other endogenous compounds, but in a recent paper [6] it is indicated that HPLC combined with fluorescence detection can be used to separate the tocopherols in vegetable oils.

This paper describes a refined method, using *dl*-tocol as an internal standard, to quantify the various tocopherols in serum samples. The method has been applied to serum samples from healthy individuals and from mothers and their infants (cord blood).

EXPERIMENTAL

Reagents and chemicals

n-Hexane (analytical-reagent grade), redistilled once before use, was purchased from Rathburn Chemicals, Walkerburn, Great Britain. Diisopropyl

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ether and isopropanol (spectroscopic grade) were purchased from BDH, Poole, Great Britain. α-Tocopherol was obtained from Merck, Darmstadt, G.F.R. β-, γ- and ∂-tocopherol was a gift from Dr. K. Abe, Eisai Research Labs., Bunkyo-ku, Tokyo, Japan. *dl*-Tocol was obtained from Koch-Light, Colnbrook, Great Britain.

Instrumentation

Normal-phase HPLC was performed utilizing a Waters Model ALC/GPC 204 liquid chromatograph equipped with a U6K loop injector and a μPorasil column (10 μm particle size; Waters Assoc., Milford, Mass., U.S.A.). The column was eluted with *n*-hexane—diisopropyl ether (92:8) at a flow-rate of 2.5 ml/min. The fluorescence intensity of the column eluent was monitored continuously using a Schoeffel variable-wavelength spectrofluorimeter (Schoeffel Instrument Corp., Westwood, N.J., U.S.A.) equipped with a deuterium lamp. The attenuation was in the range 0.1—0.2. The excitation wavelength was set at 295 nm. The instrument was equipped with a cut-off emission filter (370 nm). For chromatographic comparison we also recorded the absorbance of the eluent at 280 nm with a Waters Model 440 UV detector.

Mass spectra were obtained on a JEOL JMS D-300 instrument equipped with a combined electron impact—chemical ionization ion source and a direct inlet probe. The mass spectrometer was coupled to a JMA 2000 mass data analysis system.

Preparation of standard solutions

Four standard mixtures with increasing concentrations of α-, β-, γ- and ∂-tocopherol and a constant concentration of the *dl*-tocol internal standard (21.5 μmol/l) were prepared and analysed by HPLC. Peak areas were determined by triangulation (peak base width × peak height). The fluorescence peak-area ratios of the standard α-, β-, γ- or ∂-tocopherol and the internal reference compound were plotted against the corresponding concentration ratios. The graphs showed good linearity in the following concentration ranges: α-tocopherol, 5—46 μmol/l; β-tocopherol, 0.5—3 μmol/l; γ-tocopherol, 1—10 μmol/l; ∂-tocopherol, 0.1—1 μmol/l. The coefficients in the equation (k = slope; l = intercept) and regression coefficients (r) are as follows: α-tocopherol, $k = 0.887$, $l = 0.024$, $r = 1.000$; β-tocopherol, $k = 1.001$, $l = 0.039$, $r = 0.999$; γ-tocopherol, $k = 0.983$, $l = 0.017$, $r = 1.000$; and ∂-tocopherol, $k = 1.142$, $l = 0.060$, $r = 0.999$.

Preparation of samples

Blood samples were obtained by venipuncture from ten healthy individuals (five males and five females), from fifteen mothers immediately after delivery and from the cord blood of their infants. The serum was removed, frozen and stored at −20° until taken for analysis. To each serum sample (aliquots of 500 μl) were added 500 μl of 99.5% ethanol containing 10.75 nmol of *dl*-tocol. After the addition of 1 ml of *n*-hexane and Vortex-mixing for 30 sec the samples were centrifuged for 5 min at 30,000 *g*. The organic layer was removed by pipette and evaporated to dryness in a stream of nitrogen. When completely dry the residue was redissolved in 50 μl of *n*-hexane. A 10—20-μl volume of

the extract was injected into the column. The same procedure was also applied to smaller serum samples (down to 100 μl).

RESULTS AND DISCUSSION

Chromatogram

Fig. 1a shows a typical chromatogram of an extract from a serum sample with *dl*-tocol as internal standard, in which α-, β- and γ-tocopherol can easily be identified. Their relative retention times are 0.37, 0.51 and 0.59, respectively. ∂-Tocopherol is not observed. It is important to note that the true retention time varied by at most 5% from day to day.

A chromatogram of an extract from the serum of an infant given a lipid emulsion intravenously (Intalipid; Vitrum, Stockholm, Sweden) is shown in Fig. 1b. Intralipid is derived from fractionated soy-bean oil, which is rich in γ- and ∂-tocopherol. In this chromatogram γ-tocopherol is the predominant vitamin E form. The presence of ∂-tocopherol in the chromatogram of this serum extract is of interest.

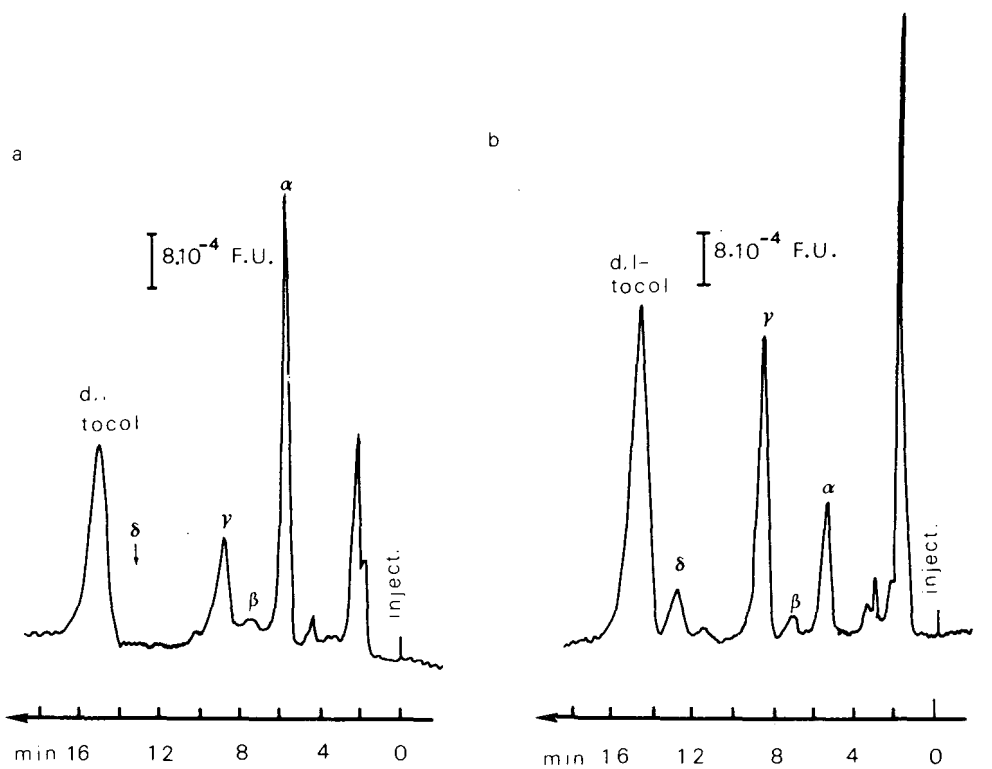

Fig. 1. HPLC separation of tocopherols in serum. (a) Normal serum; (b) serum from an infant given Intralipid. Conditions: column, μPorasil; eluent, *n*-hexane—diisopropyl ether (92:8); flow-rate, 2.5 ml/min (1000 p.s.i.); temperature, ambient; fluorescence detection; internal standard, *dl*-tocol.

Precision, sensitivity and selectivity

The recoveries of added α-, β-, γ- and ∂-tocopherol to a serum sample were found to be 92% for ∂-tocopherol and 100% for the others. The precision and reproducibility of the method were tested by analysing extracts from two different serum pools twice a day over a period of 5 days. The coefficients of variation (mean for the two serum samples) were determined to be 1.5%, 22% and 7% for α-, β- and γ-tocopherol, respectively. The high coefficient of variation for β-tocopherol is probably due to the small amount present in serum. The minimal detectable amount of injected pure α-tocopherol was 21 pmol, which corresponds to twice the noise level. The detectable amount varied only slightly for the other tocopherols, as the responses were almost identical at a given concentration.

Another chromatographic system was also tested in order to ensure that no peaks in the system described above are hidden under the tocopherol peaks. The column was exchanged for a more polar column (μ-NH_2-Bondapak, 5 μm particle size; Waters Assoc.) which was eluted with *n*-hexane—isopropanol (98:2). With this system we obtained a good separation of α- and β-tocopherol in a serum extract, but γ-tocopherol was separated less well from a new peak not observed in the other system (see Fig. 2). However, an estimation of

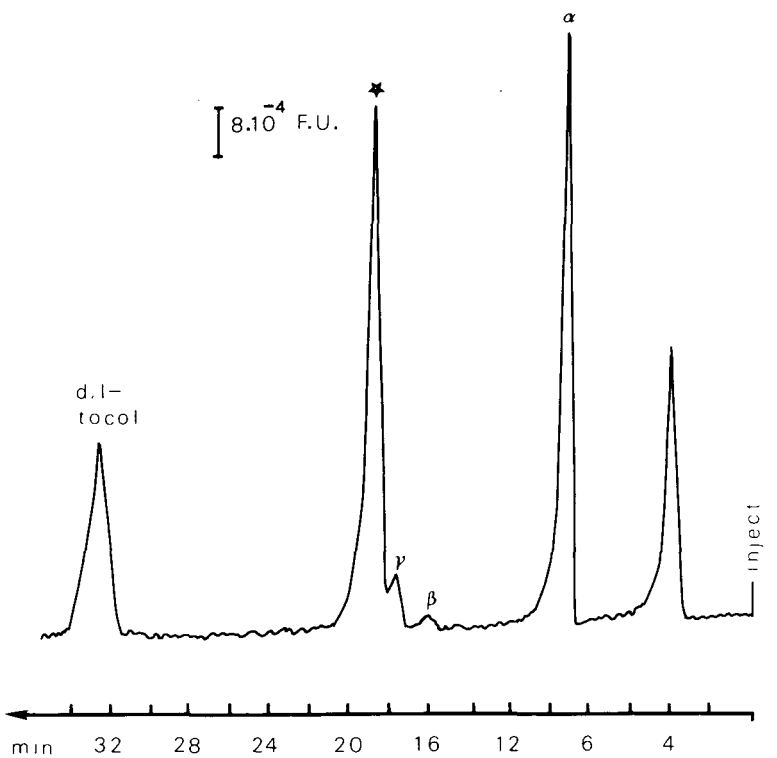

Fig. 2. HPLC separation of tocopherols in normal serum. Conditions: column, μ-NH_2-Bondapak; eluent, *n*-hexane—isopropanol (98:2); flow-rate, 1.5 ml/min (800 p.s.i.); temperature, ambient; fluorescence detection; internal standard, *dl*-tocol. The peak marked with an asterisk is not identified.

the γ-tocopherol content in a sample agreed within 10% with the result obtained on the same sample with the system described above. A fraction of the new peak in this chromatographic systems was collected and analysed by electron-impact and chemical-ionization mass spectrometry. A molar peak at m/e = 368 was found. The structure of the compound was not investigated further.

A quantitative evaluation of the α-tocopherol content in serum samples using UV detection showed agreement to within 3% with fluorescence detection.

Tocopherols in serum

Concentrations of the tocopherols in different serum extracts are presented in Table I. In normal serum α-tocopherol accounted for 87% (range 82.6—93.3%), β-tocopherol for 1.2% (range 0—2.3%) and γ-tocopherol for 11.8% (range 6.7—15.7%) of the total amount of tocopherols. ∂-Tocopherol could be detected in the chromatogram of a normal serum if the amount of the extract injected was increased approximately 20-fold (<0.024 μmol/l). The values found in this study are higher than those previously reported [2, 4] using TLC separation with spectrophotometric determination of the tocopherols. The reason for this is not necessarily related to the precision and selectivity of the earlier method [2], as these differences can also be attributed to different dietary conditions of the individuals investigated.

TABLE I

α-, β- AND γ-TOCOPHEROL LEVELS IN DIFFERENT SERA

Type of serum	No. of samples	Mean* (μmol/l)		
		α	β	γ
Normal	10	30.6 (16.3—48.4)	0.4 (0.0—0.7)	4.1 (1.9—6.0)
Maternal	15	37.4 (20.0—62.5)	0.2 (0.0—0.7)	5.8 (1.2—10.5)
Cord	15	6.0 (1.39—12.1)	—	0.2 (0.0—1.2)

*Range in parentheses.

Maternal and cord blood was also studied (Table I). The total tocopherol concentrations found in these sera are in good agreement with those obtained in earlier studies showing high maternal and low cord blood tocopherol values [7]. This may be attributed to differences in transport capacity, as the level of plasma β-lipoprotein (the principal plasma carrier of vitamin E) has been found to correlate well with the vitamin E level [8]. However, in previous studies the various tocopherols were not separated and determined individually. In the maternal serum investigated by us α-tocopherol accounted for 86% (range 73.5—96%), β-tocopherol for 0.5% (range 0—4%) and γ-tocopherol for 13.5% (range 4—24%). In cord blood serum the α-tocopherol concentration was approximately one sixth of the maternal serum level. β-Tocopherol could not be detected, but γ-tocopherol was observed in some of the serum extracts.

ACKNOWLEDGEMENTS

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Note

Gel chromatographic separation of insulin analogues in human serum

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A variety of systems for the identification of insulin molecules by chromatography have been described. Immunoreactive insulin (IRI) in human plasma is separable on Bio-Gel into a major fraction, eluted in the 6000 molecular weight region, corresponding to porcine insulin, and a smaller fraction, eluted in the 9000 molecular weight region, corresponding to porcine proinsulin [1]. Rubenstein et al. [2] fractionated plasma and urine by Sephadex gel filtration and concluded that the insulin-like component recovered in the 9000 molecular weight region was proinsulin. Permutt et al. [3] also reported on the characteristics of high-molecular-weight insulins in insulinoma patients.

Haën et al. [4] reported the existence of abnormalities in the conversion of proinsulin to insulin in normal human plasma. Kimmel and Pollock [5] and Elliot et al. [6] suggested the possibilities of an abnormal insulin in diabetic patients. Two distinct molecules of insulin in rat pancreas were reported by Smith [7, 8].

However, it is very difficult to elucidate the exact mechanism of insulin release and its molecular form in peripheral circulation of the human body, because the specific characterization of the circulating IRI components is limited by the small amount of material present in serum.

This study was undertaken in order to elucidate the heterogeneity of circulating insulin in human serum. Two groups of insulin eluted in the 6000 molecular weight region were fractionated by gel chromatography.

EXPERIMENTAL

Subjects

Four normal young adults (age 19—21 years), four adult onset diabetic patients (age 40—71 years), a borderline case of diabetes mellitus (age 47 years) and three adult onset diabetic patients (age 55—71 years) were studied. There were no significant differences in obesity.

Tests

Fifty-gram oral glucose tolerance tests (O-GTT) were given to all subjects. The serum samples taken 60 min after O-GTT were frozen at −20° until required for use.

Extraction

Extraction of the sera was carried out with minor modifications of the method reported by Oyama et al. [9] within 3 months after sampling. Serum was mixed with water (1:2) and 7.5 ml of a cold mixture of 500 ml of 99.5% ethanol and 10 ml of concentrated hydrochloric acid. The tube was allowed to stand at 4° for 20 h and, after centrifugation at 1900 *g* for 30 min at 4°, the pH of the supernatant was adjusted to 8.3 with ammonia solution. The precipitate was removed by centrifugation at 1900 *g* for 20 min at 4°. After the addition of 0.025 ml of 2 *M* ammonium acetate solution per millilitre of the supernatant, the pH of the solution was readjusted to 5.3 with hydrochloric acid. A 15-ml volume of cold 99.5% ethanol and 50 ml of diethyl ether per 10 ml of the extract were added slowly, and the solution was kept at 4° for 20 h. The precipitate was collected after centrifugation at 600 *g* for 60 min at 4°, dried with nitrogen gas and dissolved in 3 ml of 1 *M* acetic acid.

Gel chromatography

After centrifugation of the above solution, the clear supernatant was applied to a Bio-Gel P-30 column (100—200 mesh, 90 × 1.6 cm) equilibrated with 1 *M* acetic acid and eluted with the same elution buffer at 4°. The column was calibrated with porcine [^{125}I] insulin and porcine [^{125}I] proinsulin. The fraction size was 4.05 ml in eight cases, which included four normal adults and four adult onset diabetic patients, and 2.0 ml in the other four cases, which included a borderline case of diabetes mellitus and three adult onset diabetic patients.

Assay for IRI

After lyophilization, each of the fractions was dissolved in 0.6 ml of 0.1 *M* Tris—hydrochloric acid buffer of pH 7.6 (containing 0.5% of bovine serum albumin) and assayed for IRI. The radioimmunoassay of insulin was performed by the method of Horino et al. [10], utilizing anti-pork insulin guinea pig serum M 8309 and porcine monocomponent insulin (Lot. No. 834098) as standard. Single-component porcine insulin (Lot. No. 615-1082B-108-I) was used as a labelled hormone after iodination with iodine-125 [11].

Dilution tests

The dilution tests were performed with the same insulin assay system to

elucidate the immunogenicity of peaks I and II in the sera from four normal young adults.

Calculations

All values were corrected according to the reported recovery rate of extraction of insulin (0.833) [9] and the calculated recovery rate of gel filtration of labelled porcine insulin (0.647). The results were expressed as mean ± standard error of the mean. All P values were obtained by a paired Student's t-test.

RESULTS AND DISCUSSION

The sera from four normal young adults and four adult onset diabetic patients were analysed with a fraction size of 4.05 ml. The elution profiles of these samples are shown in Fig. 1, where A is from a normal adult and B from an adult onset diabetic patient. The two peaks of insulin (I and II) were well fractionated, and the position of peak II corresponded to porcine [^{125}I]insulin.

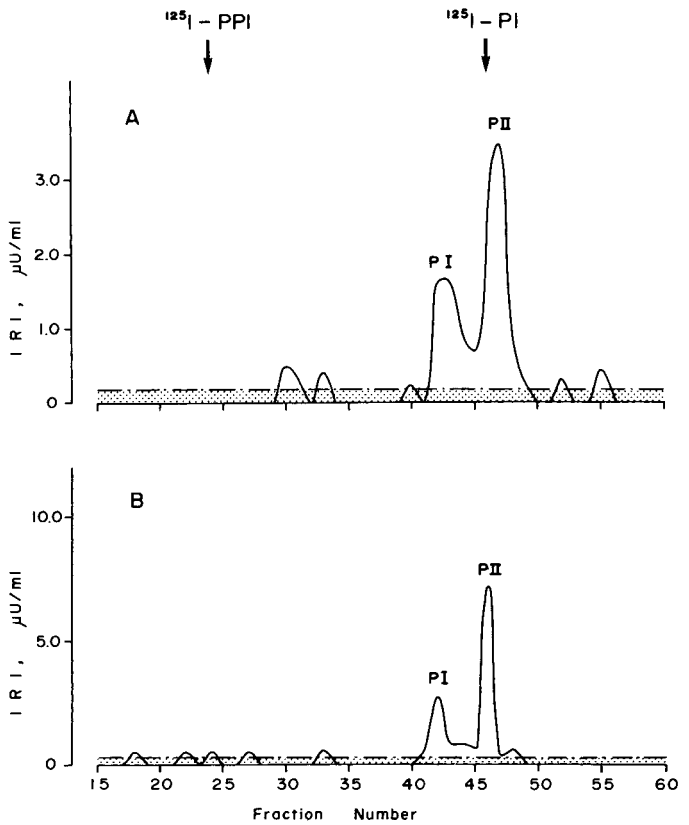

Fig. 1. Elution profiles of extracted human serum insulin on the Bio-Gel column with a 4.05-ml fraction size obtained from a normal and an adult onset diabetic patient. (A) Normal adult (K.T., 20 years old, female); (B) adult onset diabetic patient (K.A., 71 years old, female). Shaded area: detection level of IRI. PPI = porcine proinsulin; PI = porcine insulin.

TABLE I

FRACTIONATION OF INSULIN OBTAINED AT 60 MIN AFTER GLUCOSE LOAD

Results are means ± standard errors of the means. The values were corrected according to the recovery rate of extraction (0.833) and gel filtration (0.647).

Case	Maximum BS level after 50 g O-GTT (mg/dl)	Peaks (pM/ml)	
		Peak I	Peak II
Normal (n = 4)	126.5 ± 12.4*	0.04 ± 0.02**	0.08 ± 0.03***
Diabetes mellitus (n = 4)	267.8 ± 29.2*	0.15 ± 0.02**	0.15 ± 0.04***

*Significant difference ($p < 0.005$).
**Significant difference ($p < 0.01$).
***Significant difference ($p < 0.25$).

From the data in the Table I, the total insulin level (peak I plus peak II) in adult onset diabetic patients (maximum blood sugar level after 50 g of O-GTT: 267.8 ± 29.2 mg/dl) was higher than that in normal adults (maximum blood sugar level after 50 g of O-GTT: 126.5 ± 12.4 mg/dl) at 60 min after 50 g of O-GTT. This result is compatible with those reported by Yalow and Berson [12]. Peak I in adult onset diabetic patients (0.15 ± 0.02 pM/ml) was especially increased in comparison with that in normal controls (0.04 ± 0.02 pM/ml). However, the structure and function of these compounds are obscure.

In order to confirm the separabilities of these peaks, the sera of the other four adults, namely a borderline case of diabetes mellitus and three adult onset diabetic patients, were analysed by gel chromatography with a fraction size of 2.0 ml. The other conditions for extraction and assay were the same as above. The elution profiles are shown in Fig. 2, where A is from a borderline case of diabetes mellitus and B from an adult onset diabetic patient. In the borderline case, the four prominent peaks were separated. On the other hand, in the three adult onset diabetic patients, the elution profiles of serum insulin were different from that of the borderline case.

Immunoassay of serial dilutions of the insulin peaks I and II in the serum from normal young adults showed immunological identity with porcine monocomponent insulin standard, as shown in Fig. 3. Both fractions should have the same kind of antigenic determinant in their molecules. It has been reported that the anti-pork insulin serum (M 8309) reacts to both insulin and proinsulin, and its immunoreactivity to proinsulin is approximately 66% of that to insulin [13]. However, it is not known which part of the insulin molecule reacts to this antiserum at present. Therefore, the difference between insulin peaks I and II should lie in a different part to the antigenic determinant in the insulin molecule.

The immunoreactivity, the parallelism of immunogenicity among peak I, peak II and porcine monocomponent insulin and the molecular weights of the two fractions suggest that both fractions should be different from somatomedin and non-suppressible insulin-like activity, which have a kind of immunoreactivity of insulin to anti-insulin serum in their molecules. Therefore, the successful fractionation of insulins might be attributed to protein polymorphism.

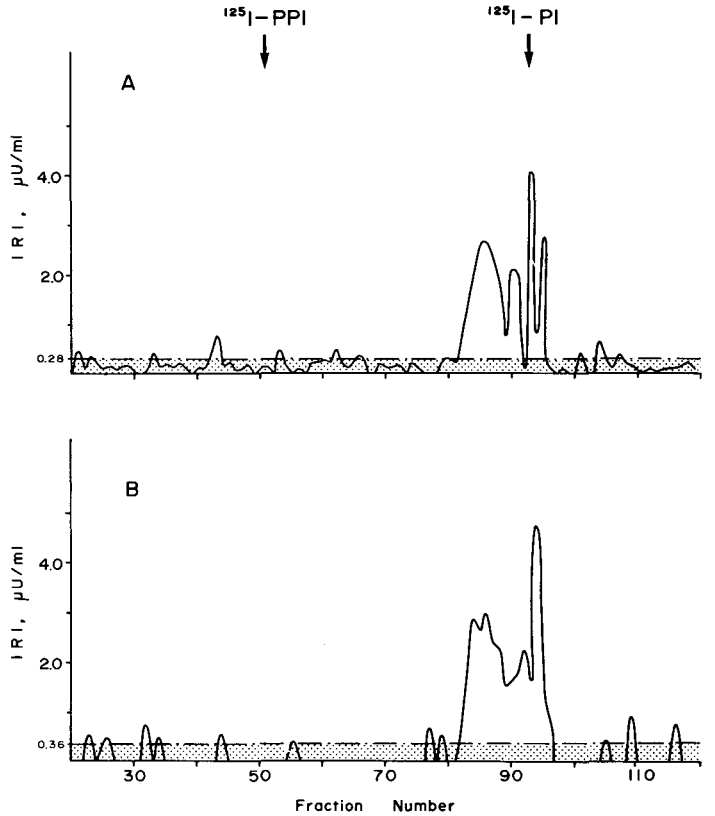

Fig. 2. Elution profiles of extracted human serum insulin on the Bio-Gel column with a 2.0-ml fraction size obtained from a borderline case of diabetes mellitus and an adult onset diabetic patient. (A) Borderline case of diabetes mellitus (F.H., 70 years old, female); (B) adult onset diabetic patient (T.Y., 55 years old, female). Shaded areas: detection level of IRI. PPI = porcine proinsulin; PI = porcine insulin.

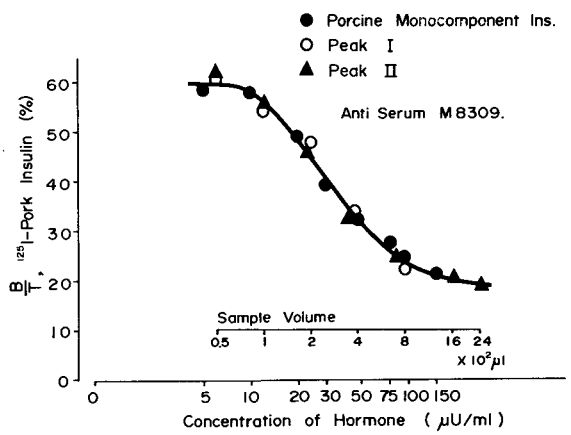

Fig. 3. Comparison of the immunoreactivity of peak I, peak II and porcine insulin in the IRI assay. Anti-pork insulin guinea pig serum (M 8309) was used. Final dilution of antiserum, $1 : 3.2 \times 10^5$. •, Mean of four determinations; ○, ▲, mean of two determinations.

The following conclusions can be drawn: (1) there are two groups of insulin in human serum, the characteristics of which might be concerned with protein polymorphism; each group could be separable into more than two subgroups; (2) a higher level of insulin peak I is observed in adult onset diabetic patients on diet therapy than that of normal adults at 60 min after 50 g of O-GTT; (3) both insulin analogues (peaks I and II) show the same immunological response with anti-pork insulin serum (M 8309).

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Note

Lipids: Thin-layer chromatographic separation in twelve fractions by three successive unidirectional developments on the same plate

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We were looking for a method capable of measuring a maximum number of lipid fractions in biological samples, in an attempt to obtain a collection of normalized lipid data of acceptable accuracy. Having selected a chromatographic technique, the problem was to try to detect all the fractions on a single chromatogram. With the methods generally used, either the neutral lipids migrate but not the phospholipids [1—9], or the phospholipids migrate but the neutral lipids remain near the solvent front with poor separation in several classes [10—13]. It seemed that no method currently used for neutral lipids can satisfactorily provide simultaneously a good separation between free cholesterol (FC) and diglycerides (DG) and between cholesterol esters (CE) and hydrocarbons such as squalene (SQ), except the procedure described by Chabard et al. [9]. However, their method still does not allow the separation of the main phospholipids. Moreover, it requires a special preparation of plates. In accordance with French and Andersen [7] we preferred to use commercial, stabilized silica gel plates, which are available anywhere, and to try to improve the separation according to the way proposed by Wildgrube et al. [14] using a succession of

solvents. With this technique, we can locate twelve well-separated fractions in three successive migrations in the same direction. At the same time, we quantify each fraction with acceptable accuracy since interference between the different lipids is avoided [15] or immediately detected.

MATERIALS AND METHODS

Solvents

Chloroform, *n*-hexane, and carbon tetrachloride were purchased from Merck (Darmstadt, G.F.R.); methanol and ethanol were from Carlo Erba (Milan, Italy). These solvents were of the best analytical grade available and were used without further purification. The following solvent systems were used: system I, chloroform—methanol—distilled water (65:25:4, v/v); system II, chloroform—*n*-hexane (3:1, v/v); and system III, carbon tetrachloride.

Lipid standards

All reference lipids were obtained from Sigma (St. Louis, Mo., U.S.A.). Standard solutions were prepared by dissolving 100 mg of each individual pure reference compound in 10 ml of chloroform—methanol (1:1, v/v), except squalene which was dissolved in chloroform—methanol (1:1) at 10 g/l. These stock solutions were diluted for use to 1 mg/ml, except for trioleylglycerol (TG) which was diluted to 2 mg/ml and for a mixed solution of cholesteryl linoleic ester (CE) at 2 mg/ml and squalene (SQ) at 1 mg/ml.

All compounds migrated as homogeneous spots on thin-layer chromatographic (TLC) plates except DG which is a mixture of 85% 1,3-dioleylglycerol (1,3DG) and 15% 1,2-dioleylglycerol (1,2DG). In our development system, the sphingomyelin (SP) standard migrated as one spot; however, in other systems [10—12] two spots were observed.

A complete migration standard solution (labeled M on the plates) was a mixture of SQ (1 mg/ml), CE (1 mg/ml), TG (1 mg/ml), 1,3DG (0.85 mg/ml), 1,2DG (0.15 mg/ml), FC (1 mg/ml), oleic acid as non-esterified fatty acid (NEFA; 1 mg/ml), monolinoleylglycerol (MG; 2 mg/ml), phosphatidyl ethanolamine (PE; 1 mg/ml), phosphatidylcholine (PC; 2 mg/ml), SP (1 mg/ml) and lysolecithin (LL; 0.5 mg/ml).

Plates

We used standard TLC plates (Merck No. 5721, 20 × 20 cm, 250 μm).

They were washed with migrating solvent system I to the top of the plate, then air dried at room temperature for 30 min and finally stored in a desiccator. Standard and sample solutions were applied with a capillary pipette (Camag, Muttenz, Switzerland) at 1.5 cm up from the lower edge of the silica gel layer. Twelve drops each of 10 μl were placed on each plate.

Development procedure

Development of the chromatograms was carried out at room temperature in a set of three covered glass tanks (Camag) internally lined with filter paper to saturate the environment. The tanks were filled with solvent to a level of 10 mm at least 4 h before use. The plates were developed to a height of 8 cm

above the origin in solvent system I (ca. 20 min) then to 13.5 cm above the origin in system II (ca. 30 min) and in system III to the upper edge of the plate (ca. 80 min).

Between each migration, the plates were allowed to dry at room temperature for 10 min. Spots were detected either by incubating the plate at room temperature in a tank saturated with iodine vapor (Fig. 1), or by spraying the plate with a 20% ethanol solution of phosphomolybdic acid, then heating it in an oven at 120° for at least 15 min [16] (Fig. 2).

RESULTS AND DISCUSSION

The advantages of two- or three-step migrations with two or three solvent systems are illustrated in Figs. 1 and 2. Fig. 1 shows the separation obtained with solvent systems I and II. Compared with migration in system I alone [10] we not only obtain the four classical phospholipid fractions (PE, PC, SP, LL), but also the separation of neutral lipids in six spots: MG, NEFA, FC, 1,2DG,

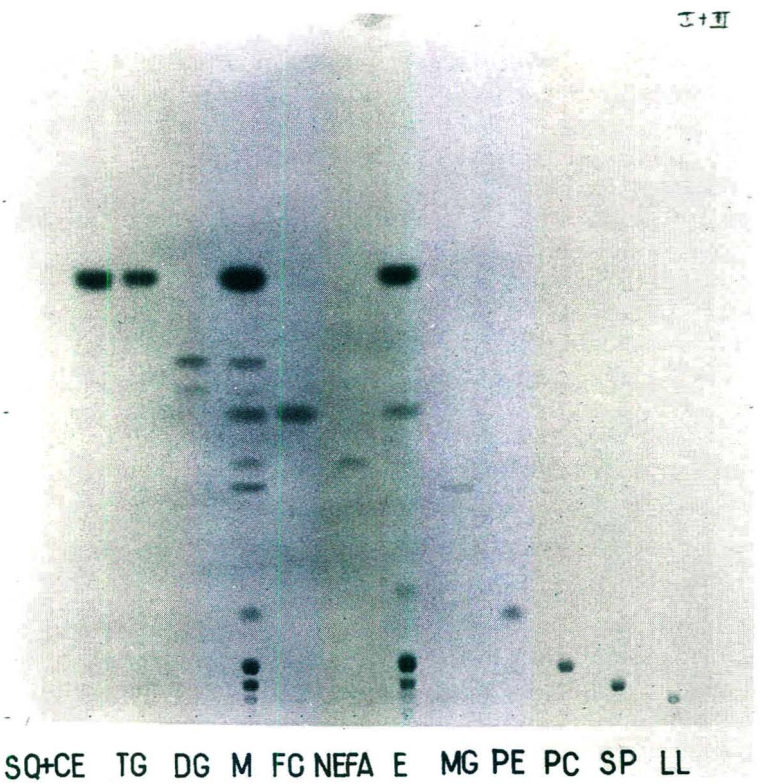

Fig. 1. Thin-layer chromatogram of lipids developed in solvent systems I + II. SQ = Squalene; CE = cholesterol ester; TG = triglycerides; DG = diglycerides (diglyceride 1,3 = 1,3DG and diglyceride 1,2 = 1,2DG); M = reference mixture of the twelve standard lipids; FC = free cholesterol; NEFA = non-esterified fatty acid; E = human serum lipid extract; MG = monoglyceride; PE = phosphatidyl ethanolamine; PC = phosphatidyl choline; SP = sphingomyelin; LL = lysolecithin.

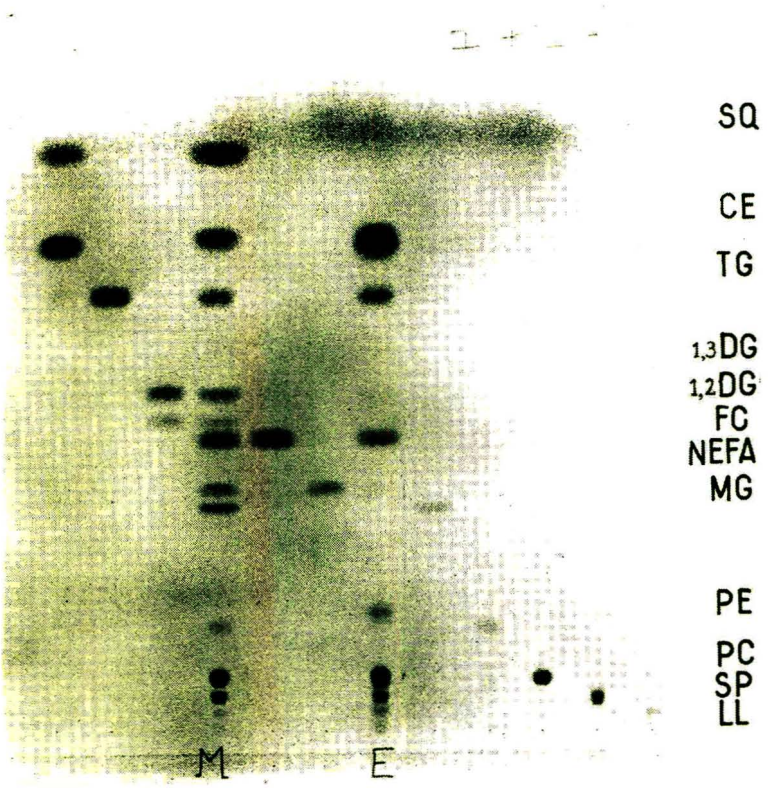

Fig. 2. Thin-layer chromatogram of lipids after subsequent development in solvent III. For abbreviations see legend to Fig. 1.

1,3DG, and a spot containing SQ, CE and TG together. The separations of MG and NEFA, which are in high concentration, and of 1,2DG and FC are particularly good.

When the third solvent system is used, TG, CE and SQ are now separated while the other compounds are unaffected (Fig. 2).

As an example of the practical use of this method we ran a sample of human serum. A blood sample was taken from a normal healthy male volunteer after 12 h of fasting. Lipids were extracted from 1 ml of serum using the method of Folch et al. [17]. The dried extract was dissolved in 1 ml of chloroform—methanol (1:1, v/v) and 10 μl of this solution were spotted on the plate at the place marked E in the figures. As in the case of the standard solution M, one observes in Fig. 2 the separation between TG and CE. One can note not only the normal absence of SQ but also the absence of DG and MG because their concentrations in serum are too low to be detected under the conditions used in this experiment. In spite of the very low NEFA concentrations in normal blood, their presence can be observed on both plates.

With the serum sample, the CE spot is larger than the one obtained with the standard mixture. This is due to the fact that blood serum contains several CE whereas in the standard mixture only cholesteryl linoleic ester was present. The

separation between CE and TG, however, is accomplished. The separation of CE can be obtained in a second step by the method of Morris [18].

The complete and accurate separation of each lipid renders this method well suited for quantitative determinations.

Of the different methods proposed up until the present study, for lipid separation in biological samples using TLC in one direction, the best results appear to be those of Wildgrube et al. [14] who obtained nine different spots on their chromatograms. We have shown that the use of three different solvent systems in a convenient order allows twelve fractions to be obtained without many experimental difficulties.

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Note

Fluorescence micro disc electrophoresis in sodium dodecyl sulfate

A simple and sensitive method applicable for routine laboratory analysis

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Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis has become a major analytical tool in protein biochemistry since its first description in 1967 [1]. However, traditional SDS polyacrylamide gel electrophoresis suffers from the disadvantage of requiring a relatively large quantity (microgram levels) of protein to give a detectable band [2]. Numerous investigators have attempted by the application of two approaches to increase the sensitivity of this method.

Diminution of column dimensions increases the sensitivity by reducing the cross-sectional area of the gel. A variety of micro-gel systems have been developed by employing capillary tube electrophoresis [3—6]. These methods involve tedious handling procedures [3—6], special apparatus [5, 6] or loss of resolution and reproducibility [5, 6]. Such methods, however, are successful in increasing sensitivity to the nanogram protein concentration range.

Conventional staining of protein bands with Coomassie brilliant blue or amido black have several inherent disadvantages. The requirements for fixation, staining, and destaining are time consuming. To be stained effectively the gels need to be removed from the glass columns. Such dye staining techniques require relatively high protein concentration and the sensitivity decreases with decreasing molecular weight.

Quantitation of these dye staining methods depends on absorption of light. Given a fluorophor with a good quantum yield, fluorescence allows an increase in sensitivity of about 10^3 in comparison to absorption [7]. Thus, fluorescence

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methods were introduced into polyacrylamide gel electrophoresis [8—13]. The most popularly used fluorescent labels are fluorescamine [8—10] and 1-dimethylaminonaphthalene 5-sulfonyl chloride (dansyl chloride) [11—13]. The fluorescent methods are sensitive down to the nanogram range and are especially useful when working with low-molecular-weight proteins. Dansylation of peptides has no effect on the linear relationship between mobility and molecular weight over a molecular weight range of 1000—12,000 [13].

The present communication describes a combination of the micro-gel approach with the dansyl chloride fluorescent labelling in SDS polyacrylamide gel electrophoresis. This technique is more sensitive than any of the reported SDS polyacrylamide gel electrophoresis methods and is suitable for routine use in ordinary laboratories.

MATERIALS AND METHODS

Acrylamide, N,N′-methylenebisacrylamide (Bis), ammonium persulfate, and N,N,N′N′-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Labs. (Richmond, Calif., U.S.A.). SDS, urea, tris(hydroxymethyl)aminomethane (Tris), tris(hydroxymethyl)aminomethane acetate (Tris acetate), potassium ferricyanide, B-mercaptoethanol and dansyl chloride were supplied by Sigma (St. Louis, Mo., U.S.A.). The column coat Dri-Film SC-87 (surfasil) was supplied by Pierce (Rockford, Ill., U.S.A.), acetone by Aldrich (Milwaukee, Wisc., U.S.A.) and Dichrol acid cleaning solution by Scientific Products (State College, Pa., U.S.A.). The protein standards ovalbumin, soybean trypsin inhibitor, lysozyme (egg white), cytochrome *c* (horse heart Type III), ribonuclease A (bovine pancreas), lima bean trypsin inhibitor, polylysine (<3000 daltons polymer), angiotensin II, oxytocin and gramicidin S (*Bacillus brevis*) were from Sigma and insulin (bovine pancreas), adrenocorticotrophic hormone (porcine) (ACTH), glucagon, bacitracin and methionine-enkephalin were from Calbiochem (Los Angeles, Calif., U.S.A.).

Procedures for dansylation of protein and preparation of gels were similar to that described by Kato and Sasaki [13] except that the dansylation process was 45 min instead of 15 min. 100-μl disposable microcapillary pipets (1 × 128 mm) (Dade Diagnostic) were acid cleaned and coated with a 0.5% solution of Dri-Film SC-87 in acetone. Micro gels were made by dipping five clean capillary pipets into a clean standard bore glass electrophoresis gel tube (6 × 130 mm) (Bio-Rad Labs.) sealed at one end with parafilm and filled with gel solution. Water was layered on top of the gel to maintain a level surface and the gel was allowed to polymerize for about 30 min. The micro gel tubes could be removed from the standard bore glass electrophoresis gel tube using a glass rod.

The electrophoresis buffer was prepared by tenfold dilution of 1 *M* tris acetate buffer, 1% SDS, pH 8.2. The gel surface was washed several times with the electrophoresis buffer before use. The gel tubes were attached to the upper reservoir of a Bio-Rad Model 155 gel electrophoresis cell by using the stoppers of ordinary vacutainer tubes. The electrophoresis cell was then filled with buffer as recommended [14]. Care was taken that no air bubbles were trapped at either end of the gel. Ten μl of the dansyl-protein solutions were layered

onto the gels using an ordinary 10-μl Hamilton microliter syringe No. 701. Electrophoresis was performed with a LKB 2103 power supply at constant current of 0.15—0.25 mA/gel. Mobility of the bands during electrophoresis can be monitored at 366 nm using a UV lamp (UV SL-25, Ultraviolet Products).

RESULTS

A typical run with 0.25 mA/gel took 6—7 h for 12.5% SDS gel and 8 h for 15.0% SDS gel. The electrophoretic patterns of authentic standard peptides run in 12.5% and 15.0% SDS gel were similar to those reported previously [12]. The brightest and fastest moving band in each gel was 1-dimethylaminonaphthalene 5-sulfonic acid (dansyl-OH) formed during the dansylation reaction [12]. One inherent shortcoming of reported micro gel methods is the relatively rapid diffusion of the protein bands after electrophoresis. However, with the present method, even with the band-spreading effect, dansylated polypeptides including cytochrome *c*, lima bean trypsin inhibitor, ACTH, bacitracin and angiotensin II gave quite discrete bands. The mobility of dansylated protein in the gel was not affected by mixing with other dansylated species as shown by the fact that the mobility of dansylated cytochrome *c* was not altered by mixing with lima bean trypsin inhibitor. The optimum amount of peptide applied was found to be 50 pmole. It was demonstrated based upon the brightness of the dansylated peptide bands that 25 pmole of certain polypeptide gave clearly distinct resolution at 366 nm.

Relative mobility of the dansylated peptide was calculated by dividing the migration distance of the dansylated peptide by that of dansyl-OH. Table I pre-

TABLE I

COMPARISON OF THE RELATIVE MOBILITIES OF PEPTIDE STANDARDS IN 12.5% AND 15.0% SDS MICRO GELS

The relative mobility was the average of five runs ± S.D.

Peptide standards	Molecular weight (reference)		Relative mobility	
			12.5% SDS	15.0% SDS
Ovalbumin	44,000	[16]	–	0.14 ± 0.01
Soybean trypsin inhibitor	21,600	[17]	0.145 ± 0.005	0.195 ± 0.005
Lysozyme	13,930	[16]	0.235 ± 0.005	0.255 ± 0.005
Ribonuclease A	13,690	[18]	0.185 ± 0.004	0.245 ± 0.006
Cytochrome *c*	12,400	[18]	0.18 ± 0.01	0.280 ± 0.003
Lima bean trypsin inhibitor	8,400	[18]	0.23 ± 0.01	0.320 ± 0.008
Insulin	5,782	[18]	0.25 ± 0.01	0.405 ± 0.005
			0.37 ± 0.01	0.505 ± 0.005
ACTH	4,600	[19]	0.250 ± 0.002	0.350 ± 0.004
Glucagon	3,483	[18]	0.223 ± 0.003	0.395 ± 0.002
Polylysine	3,000	[20]	0.22 ± 0.01	0.435 ± 0.015
Bacitracin	1,411	[19]	0.309 ± 0.005	0.47 ± 0.01
Angiotensin II	1,178	[20]	0.350 ± 0.009	0.48 ± 0.01
Oxytocin	1,000	[20]	n.d.*	n.d.*
Gramicidin S	1,000	[20]	0.340 ± 0.002	0.520 ± 0.009
Methionine-enkephalin	645	[20]	–	0.590 ± 0.008

*n.d. = not detectable.

sents the peptides and their relative mobilities in both 12.5% and 15.0% SDS gel. Relative mobilities are presented as the average of five independent experiments plus or minus standard deviations. Dansylated oxytocin was identified as a faint smear or was not detectable in either the 12.5% or 15.0% SDS gels at quantities above 100 pmole. Insulin consistently gave two bands in both gel concentrations. This was in agreement with observations reported previously [13, 15].

Fig. 1 is a plot of the logarithm of the molecular weight (MW) of the peptide standards against their relative mobilities in 12.5% SDS gel. A linear plot was obtained from soybean trypsin inhibitor (MW 21,700) to ACTH (MW 4600). The plot was close to linearity for peptides of smaller molecular weights such as bacitracin, angiotensin II and gramicidin S. There were deviations from linearity in the case of lysozyme, glucagon and polylysine. These may be due to the intrinsic charge and shape of the polypeptides as suggested by Kato and Sasaki [13]. Fig. 2 is a plot of the logarithm of molecular weight against relative mobility of the peptide standards in 15.0% SDS gel. The plot was linear from ribonuclease A (MW 12,600) to bacitracin (MW 1411). Slight deviation from linearity was observed for molecules such as soybean trypsin inhibitor, lysozyme, cytochrome *c*, angiotensin II and gramicidin S. Despite the deviation of

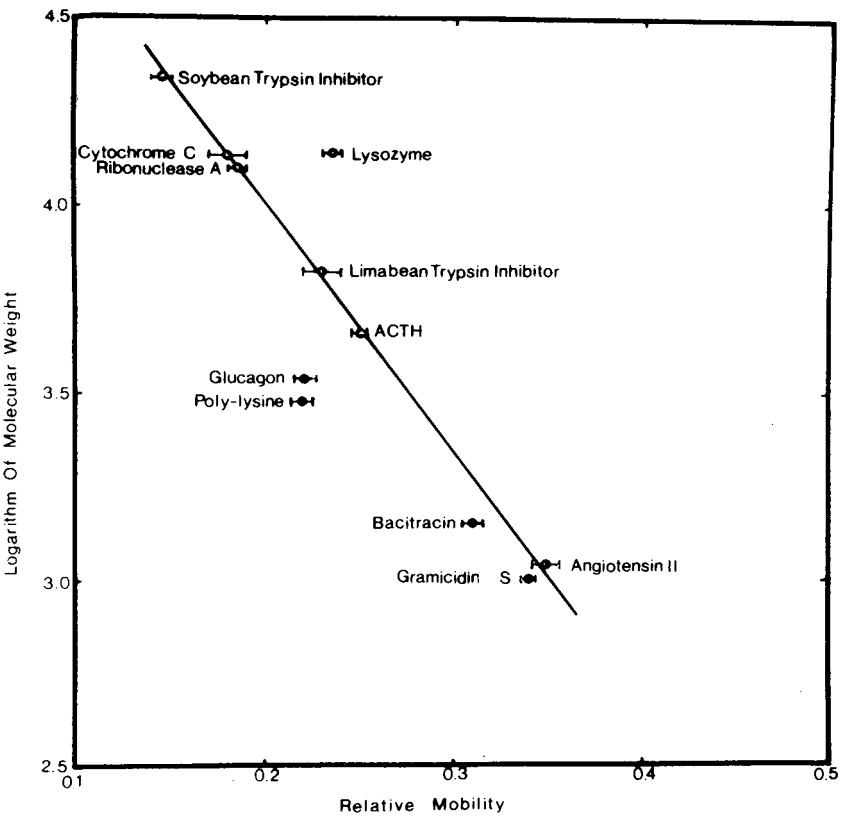

Fig. 1. Plot of the logarithm of the molecular weight of peptides versus their relative electrophoretic mobilities on 12.5% SDS micro polyacrylamide gel.

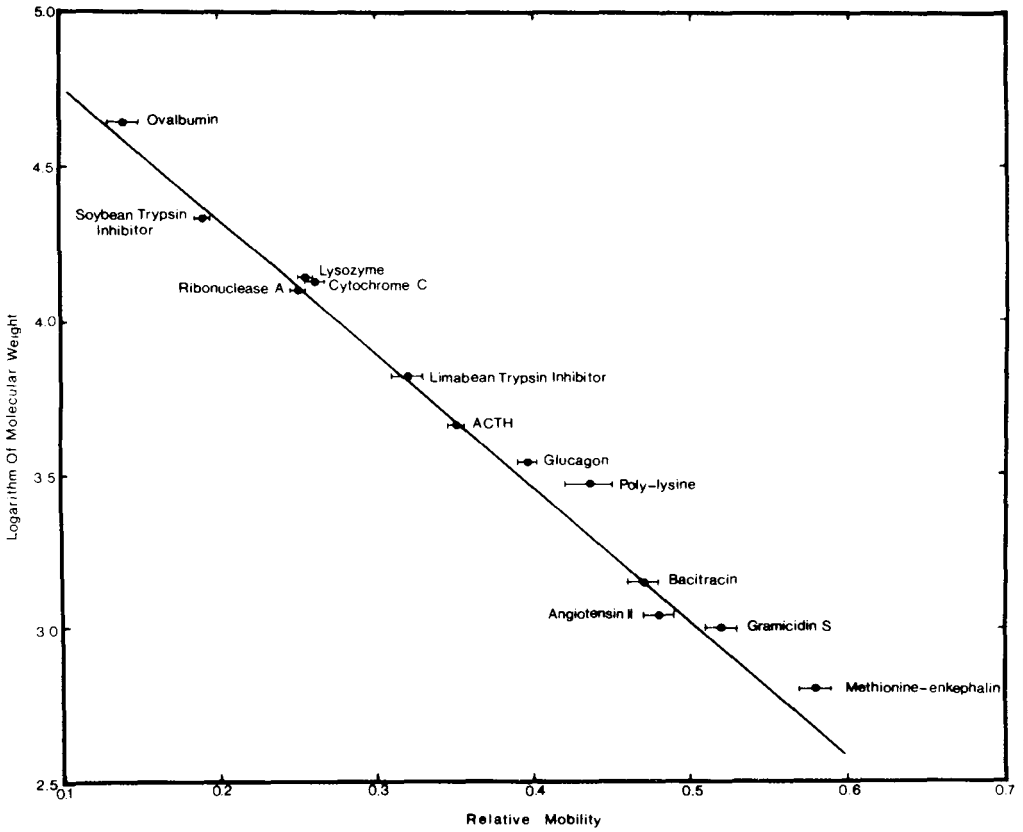

Fig. 2. Plot of the logarithm of the molecular weight of peptides versus their relative electrophoretic mobilities on 15.0% SDS micro polyacrylamide gel.

some standards, e.g. ovalbumin, glucagon, polylysine, methionine-enkephalin, from linearity, the 15.0% SDS gel gave a good linear relationship between molecular weight and relative mobility especially in the molecular weight range of 12,600—1400. Comparing the behavior of dansylated peptides in 12.5% and 15.0% gels, it is obvious that the 12.5% SDS gel is better for higher molecular weight peptides ranging from 22,00—4600 and the 15.0% SDS gel is better for lower-molecular-weight peptides.

DISCUSSION

In polyacrylamide gel electrophoresis, the gel tubes were usually siliconized so that the gel column would be removed easily following electrophoresis. This step is especially important in micro gel electrophoresis. Air bubbles easily formed along walls of untreated capillary tubes during gel polymerization. A 0.5% solution of Dri-Film SC-87 in acetone was found to be effective in preventing bubble formation.

Traditional polyacrylamide gel electrophoresis in concentrated gel (12.5%

SDS gel) containing 0.1% SDS and 8 *M* urea was reported to have the ability to fractionate peptides with molecular weights ranging from 1000 to 12,000 [13—15]. However, due to the difference in internal diameter of the conventional gel tubes (standard bore glass electrophoresis gel tube 6 × 130 mm) and the micro-capillary pipets (1 × 128 mm), the 12.5% SDS micro gel is capable of fractionating molecules with weight between 22,000—4600 with a linear relationship between relative mobility and logarithm of molecular weight. Fig. 2 shows that in 15.0% SDS micro gel, a linear plot of the logarithm of molecular weight versus relative mobility was obtained for peptides of molecular weights 12,600—1400. Thus, the fractionation capability of the 15.0% SDS micro gel is comparable with that of the conventional 12.5% SDS gel reported previously [13].

As noted in the results, most peptide standards gave distinct visible bands at quantities of 50 pmole. With some peptides, e.g. bacitracin, angiotensin II, quantities less than 25 pmole could still be visualized. On the other hand, peptides, e.g. oxytocin, which were not resolved as a distinct band, had a lower detection sensitivity than average. The reason for this difference is unclear. In general, however, the sensitivity of the present method is better than any of the fluorescence or micro gel electrophoresis procedures ever described. Besides the inherent advantages of using fluorescent labels this technique has the virtue of employing simple procedures and ordinary equipment for further improving the sensitivity. However, the major disadvantage of the present method is the difficulty of photographing the gels after electrophoresis.

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Note

Non-extractive fluorometric measurement of *p*-aminosalicylic acid in plasma by ion-pairing techniques and high-performance liquid chromatography

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The value of *p*-aminosalicyclic acid (PASA) in the treatment of tuberculosis was initially demonstrated by Lehman [1]. It has been observed that PASA in combination therapy with streptomycin and isoniazid delays the emergence of drug resistant strains of bacillus. Recently, PASA has been shown to reduce plasma cholesterol levels by 15—20% [2]. Since it is a widely available and inexpensive drug, PASA may be used as a cholesterol-lowering agent where other drugs are not effective.

A few literature methods have been reported for the analysis of PASA. These include gas chromatography [3], potentiometry [4], non-aqueous titrimetry [5], and spectrophotometry [6]. More recently, PASA has been used as an internal standard in an extractive procedure for the high-performance liquid chromatographic (HPLC) analysis of salicylazosulfapyridine metabolites in plasma [7]. Only the spectrophotometric procedure has been adapted for use in the analysis of PASA plasma levels. HPLC has been shown in these laboratories to be an effective tool for the analysis of drugs in biological fluids [8, 9]. In this paper, an HPLC separation and quantitation of PASA from plasma samples using a non-extractive approach is described. The concatenation of several recent HPLC techniques such as the use of non-extractive sample preparation, ion-pairing reversed-phase HPLC, and enhanced detector sensitivity allowed for the quantitation of therapeutic levels of PASA in as little as 100 μl of plasma.

EXPERIMENTAL

Materials

Powdered samples of *p*-aminosalicylic acid (Merck, Rahway, N.J., U.S.A.)

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and anthranilic acid (Chem-Service, Media, Pa., U.S.A.) were obtained for the preparation of standard solutions. PASA and anthranilic acid were recrystallized from ethanol and water, respectively, before use. An aqueous solution of tetrabutylammonium (TBA) hydroxide (40%) (Aldrich, Milwaukee, Wisc., U.S.A.) was obtained for use. All other chemicals and solvents used were the highest grade of commercially available materials.

HPLC conditions

The HPLC analyses were performed on a Waters Assoc. Model ALC 202 equipped with an M-6000 pump, a U6K injector, and a Perkin-Elmer Model 203 fluorometer adapted to contain an HPLC flow-through cell (Hellma Cells, Jamaica, N.Y., U.S.A.). The column was a LiChrosorb C_{18} column (250 × 3.2 mm I.D.) (Altex Scientific, Berkeley, Calif., U.S.A.). The column contained a packing material consisting of a C_{18} hydrocarbon bonded to a microparticulate silica gel (<10 μm) for reversed-phase chromatography. The mobile phase used was absolute methanol—distilled water (20:80) containing 0.005 *M* tetrabutylammonium (TBA) hydroxide and 0.01 *M* disodium acid phosphate. The pH of the mobile phase was adjusted to 5.5 with concentrated phosphoric acid and the flow-rate was set at 1.0 ml/min (1000 p.s.i.).

Fluorometric detector settings were: sensitivity = 10; selector = 10; excitation and emission wavelengths were set at 270 and 385 nm, respectively, and are uncorrected.

Standard solutions for calibration curve

A stock solution (1 mg/ml) of *p*-aminosalicylic acid was prepared by dissolving a weighed amount of the powder in distilled water. Quantities of 250, 50 and 10 μl of the stock solution were added to individual 5-ml volumetric flasks and blank human plasma was added to volume. In addition an internal standard stock solution (25 μg/ml) of anthranilic acid in mobile phase (see HPLC conditions) was prepared.

Plasma calibration procedure

Calibration curves were constructed by adding 100-μl quantities of each plasma stock solution into individual 15-ml centrifuge tubes to give the equivalent of 5, 1, and 0.2 mg *p*-aminosalicylic acid per 100 ml plasma. To each tube was added absolute methanol (100 μl) followed by mixing on a Vortex mixer (1 min) and centrifugation at 3000 r.p.m. (15 min). An aliquot (50 μl) of each supernatant was removed and transferred to a clean 15-ml centrifuge tube where 100 μl of the mobile phase containing the internal standard (stock solution) was added. After mixing (1 min), 50 μl of each solution was injected into the liquid chromatograph.

RESULTS AND DISCUSSION

It was apparent in the approach to an HPLC analysis of PASA that the amphoteric nature of the drug would limit its extractability from a biological matrix. In order to exploit the chromatographic process for the direct injection of plasma samples into the HPLC column, a non-extractive sample preparation

prior to on-column injection was necessary. Also, a reversed-phase system was chosen so that the mobile phase would be miscible with the biological fluid and the polar components in the biological matrix would be rapidly eluted. An ion-pairing mobile phase was used to increase the capacity factor (k') for PASA so that the drug would be resolved from the background components. Because of the amphoteric nature of PASA, either a cationic or anionic ion-pair reagent could be selected. The strong acidic nature of the drug (pK_a COOH = 3.25) suggested that there would be a greater probability of success with a cationic agent and this was borne out by our results. The native fluorescence of PASA was also investigated in order to achieve detection selectivity and to increase the sensitivity of the assay. Plasma volumes as small as 100 μl could be used in the resulting procedure.

Acetonitrile was initially employed in a pretreatment step as plasma protein precipitant. It was noted that the supernate obtained after centrifugation of the denatured plasma sample was slightly milky and never became clear even upon extended centrifugation. Further, there was a possibility of drug entrapment since the precipitate formed in the denaturation process was gummy. On chromatographing the acetonitrile-treated plasma, a peak associated with the plasma sample was observed in both blank and sample at a retention time of 24 min. Efforts to remove this component by pre-extraction of the plasma sample with fat soluble organic solvents such as heptane, diethyl ether or chloroform were unsuccessful. It was decided to replace the acetonitrile with absolute methanol as protein denaturant. This was advantageous since a flocculent precipitate was obtained as well as a much clearer supernate upon centrifugation. In addition, the 24-min peak that had been observed with acetonitrile did not appear.

A study of mobile phase pH versus chromatographic characteristics of the PASA—TBA ion-pair in the mobile phase was investigated. The retention time of the ion-pair decreased with increasing pH in the pH 4—8 range. A pH of 5.5 was selected since a symmetrical peak with a reasonable retention time and adequate resolution from plasma components was obtained (see Fig. 1). With the more alkaline mobile phases, there was either lack of adequate resolution for PASA and plasma components or shoulders and multi-peaks for PASA were observed. With increasing acidity of the mobile phase, there was an increase in k' which resulted in a longer analysis time.

A plot of fluorescence intensity versus mobile phase pH (Fig. 2) showed that maximum fluorescence of the ion-pair was obtainable in the pH 5—8 range, which is consistent with the pH needed for resolution from plasma components.

A comparison of the cationic ion-pairing results to those using an anionic counterion, dioctyl sodium sulfosuccinate (DOSS), was undertaken. The mobile phase consisted of absolute methanol—distilled water (20:80) containing 0.01 *M* DOSS. The chromatographic characteristics of the PASA—DOSS ion pair were investigated at pH 2.5 and 5. Fluorescence intensity was monitored at excitation and emission wavelengths of 290 and 400 nm, respectively. A double peak with a retention time of about 600 sec was observed at pH 2.5 and a single peak at or near the solvent front was observed

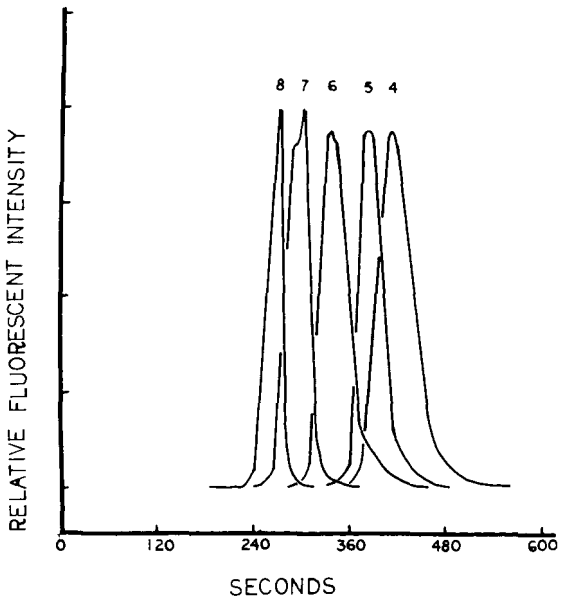

Fig. 1. Typical chromatograms of PASA in absolute methanol—distilled water (20:80) containing 0.005 *M* TBA cation and 0.01 *M* disodium acid phosphate using excitation and emission wavelengths of 270 and 385 nm, respectively, at various mobile phase pH values. The pH value is noted above each peak.

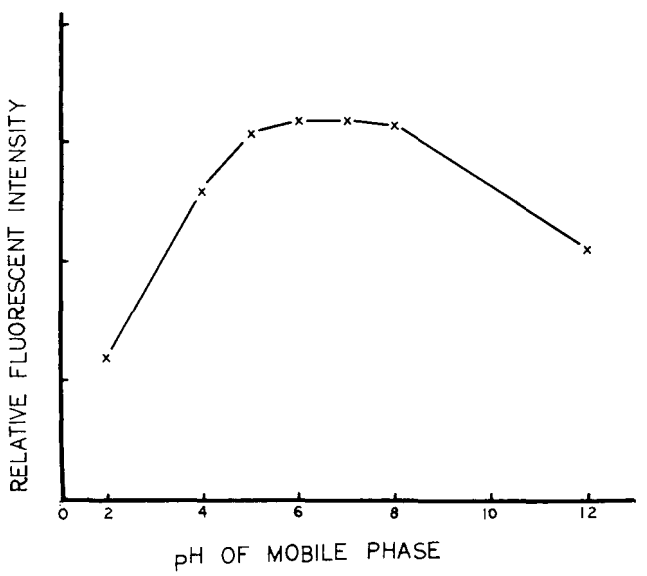

Fig. 2. pH versus PASA fluorescent intensity in absolute methanol—distilled water (20:80) containing 0.005 *M* TBA cation and 0.01 *M* disodium acid phosphate at excitation and emission wavelengths of 270 and 385 nm, respectively. Measurements were performed in duplicate on a Perkin-Elmer Model MPF-4 spectrophotofluorometer in the true emission mode.

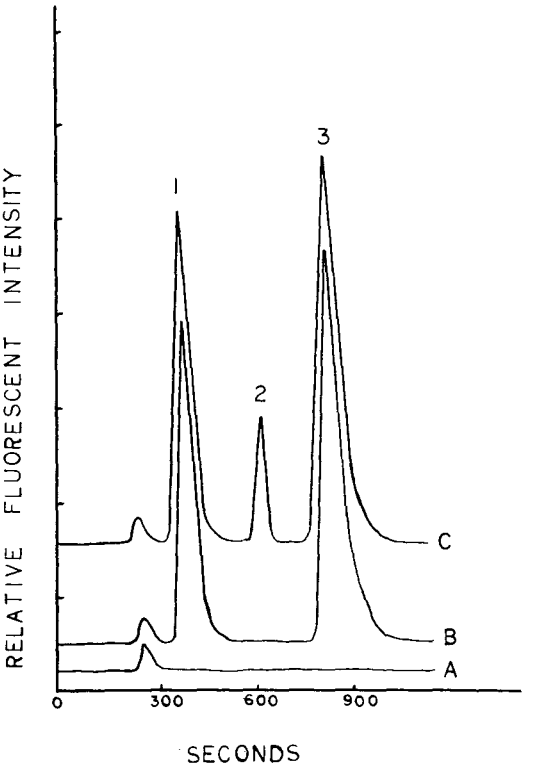

Fig. 3. Typical chromatogram of *p*-aminosalicylic acid (1) and anthranilic acid (3) (internal standard) in a spiked human plasma sample (B). For comparison, chromatograms of blank plasma (A) and of a plasma sample (C) also containing the N-acetyl metabolite of PASA (2) are shown. Conditions: Column, LiChrosorb C_{18} (250 mm × 3.2 mm I.D.), eluent, absolute methanol—distilled water (20:80) containing 0.005 *M* TBA cation and 0.01 *M* disodium acid phosphate adjusted to pH 5.5 with concentrated phosphoric acid; flow-rate, 1 ml/min; excitation and emission wavelength of 270 and 385 nm, respectively.

at pH 5. It was evident upon comparison that best results for the PASA assay could be obtained using ion-pairing with TBA counterion at pH 5.5.

Fig. 3 shows a typical chromatogram of the separation of PASA and anthranilic acid (internal standard) in a spiked human plasma sample using TBA cation at pH 5.5. Under the chromatographic conditions chosen, endogenous plasma constituents and the N-acetyl metabolite of PASA do not interfere with the assay. Furthermore, it has been shown in this laboratory that spiked plasma samples containing isoniazid (INH) and ascorbic acid, drugs commonly found in combination with PASA in pharmaceutical dosage forms also do not interfere.

A calibration curve for PASA in the therapeutic concentration range 0.2—5 mg per 100 ml of plasma [10] was performed. Anthranilic acid was found to be a suitable internal standard since it possessed good fluorescent intensity at the excitation and emission wavelengths of PASA. The area under the curve for each peak on the chromatograms was determined with an electronic integrator. The ratio of PASA peak area to the area of the internal standard (D/IS) was

calculated for each chromatogram. Regression analysis of these data at the various concentrations of PASA gave slope, 0.2043; intercept, —0.0066; and correlation coefficient, 0.9984 (n = 15). The standard error of estimate of y(D/IS) on x (PASA concentration) was ± 0.0216. The minimum detectable quantity of PASA that can be measured using this procedure is 500 pg (signal-to-noise ratio = 2).

Human plasma samples containing spiked quantities of PASA in the therapeutic concentration range were chromatographed concurrently with the calibration solutions and the ratios of drug peak areas to internal standard peak areas were calculated. The slope and intercept data from regression analysis for PASA calibration solutions were used to solve for drug concentration in the spiked samples: D/IS = (slope × concentration) + intercept. The data in Table I demonstrate the quantitative results obtained from these spiked plasma samples. The utility of HPLC in the assay of plasma levels of PASA using fluorometry and ion-pairing with tetrabutylammonium ion is clearly demonstrated with accuracy in the 1—5% range.

TABLE I

ANALYSIS OF *p*-AMINOSALICYLIC ACID IN SPIKED PLASMA SAMPLES

Initial concn. (mg/100 ml)	Concn. found* (mg/100 ml)	Relative standard deviation (%)	Accuracy (%)
0.500	0.5244 ± 0.0397	7.63	4.88
2.500	2.522 ± 0.0799	3.17	0.88

*Mean ± S.D. based on five replicate determinations of each sample.

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Note

Determination of mecillinam in urine by reversed-phase high-performance liquid chromatography

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Pivmecillinam hydrochloride (I), the pivoloyloxymethyl ester of mecillinam, and mecillinam (II) (Table I) are 6-amidinopenicillanic acid derivatives undergoing clinical evaluation as antibiotics effective against gram-negative organisms [1]. Pivmecillinam hydrochloride was specifically developed as an orally active form of mecillinam. It is a prodrug which is well absorbed from the gastrointestinal tract, and whose activity is due to its rapid biotransformation to mecillinam, the active antibiotic agent [2]. Mecillinam, however, is administered only parenterally. The synergistic activity of mecillinam with other β-lactam antibiotics is of significant clinical importance in chemotherapy [3].

The determination of mecillinam in biological fluids (plasma and urine) is performed mainly by microbiological assay methods [4], by which valuable pharmacokinetic and biopharmaceutic information in humans has been obtained [5—8]. However, these procedures require special laboratory conditions for their routine use, and a more generally applicable chemical assay was therefore sought for clinical pharmacokinetic studies.

A spectrophotometric method involving the formation of a 4-aminomethyleneimidazol-5-(4*H*)-one derivative which was measured at 330 nm, was published for the determination of mecillinam and used for the study of the degradation kinetics of the drug [9, 10]. This principle was also used for the determination of the compound in plasma or urine by high-performance liquid chromatographic analysis via post-column derivatization [10].

As mecillinam is thermally unstable and non-extractable from aqueous media owing to its amphoteric nature, analysis by high-performance liquid chromatography (HPLC) is a very practicable means for its chemical analysis. The technique has been used successfully in the analysis of several types of antibiotics, viz., kanamycin [11], tetracycline [12], gentamycin [13],

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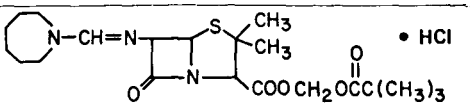
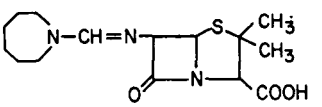
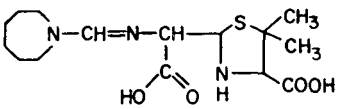
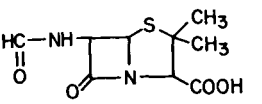
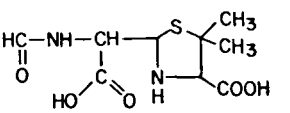
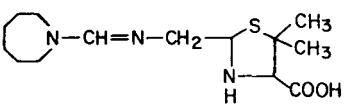
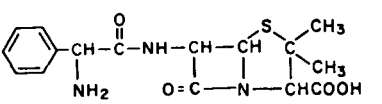
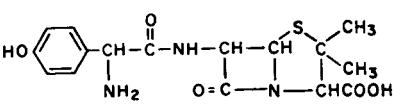
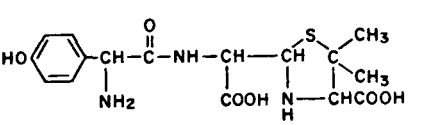
TABLE I

STRUCTURES AND CAPACITY FACTORS (k') OF PIVMECILLINAM HYDROCHLORIDE AND RELATED COMPOUNDS

Compound	Name	Structure	Retention time (min)	k'
I	Pivmecillinam		>30	—
II	Mecillinam		4.25	2.5
III			1.8	0.5
IV			1.4	<0.5
V			1.4	<0.5
VI			4.5	2.75
Related compounds	Ampicillin		2.2	0.8
	Amoxycillin		1.6	<0.5
	Amoxycillinpenicilloic acid		1.4	<0.5

cephalothin [14], chloramphenicol [15] and amoxicillin [16, 17]. These methods use either the intrinsic UV absorbance of the compound or the fluorescence of a suitable derivative (fluorescamine or *o*-phthalaldehyde), prepared post-column as the means of detection. The intrinsic UV absorbance of mecillinam at 220 nm was used for stability testing of the bulk drug and for quality assurance of its dosage forms by HPLC [18].

The HPLC procedure presented here is an adaptation of a previous method [18] and involves dilution of a urine specimen followed by reversed-phase HPLC analysis and detection of intact mecillinam by its UV absorbance at 220 nm. As the drug is extensively eliminated in urine (60% of a dose eliminated in a 12 h excretion period), determined by microbiological assays [5—8], its concentration in urine is sufficiently high to be determined by direct analysis of a dilution of urine. The HPLC assay has a sensitivity limit of 0.05 mg/ml (50 μg/ml) using a 1-ml urine sample per assay, equivalent to a minimum detectable amount of 50 ng of mecillinam injected on to the column.

EXPERIMENTAL

Column

A pre-packed, 30 cm × 4.6 mm I.D. stainless-steel column containing a 10-μm Chromegabond C_{18} reversed-phase microparticulate packing (Serial No. 289-1-29-821, E.S. Industries, Marlton, N.J., U.S.A.) was used.

Instrumental parameters

A Waters Model 6000A high-pressure liquid chromatography pump, equipped with a Model U6K injection system and a Waters pre-column filter (2 μm) (Waters Assoc., Milford, Mass., U.S.A.), was used for chromatography. A Tracor Model 970A variable-wavelength absorbance detector (Tracor Instruments, Austin, Texas, U.S.A.) was used for quantitation at 220 nm.

The isocratic mobile phase consisted of 15% acetonitrile (UV grade) in 0.01 *M* potassium buffer (pH 5.0) pumped at a constant flow-rate of 2.0 ml/min. Under these conditions the retention time of mecillinam was 4.25—4.5 min (Fig. 1). The chart speed of the Hewlett-Packard dual-channel recorder (Model 713A with option 108) was 0.5 in./min.

Reagents

All reagents were of analytical-reagent grade (A.C.S.). All aqueous buffers were prepared in distilled, carbon-filtered, deionized water filtered through a 0.2-μm filter (Type DC System, Hydro Service and Supplies, Durham, N.C., U.S.A.).

Potassium phosphate buffer, 0.01 *M* (pH 5.0), was prepared by titration of 0.01 *M* KH_2PO_4 (1.361 g/l) with a small amount of 0.01 *M* K_2HPO_4 (anhydrous) (1.742 g/l) until the desired pH was attained.

Acetonitrile (UV grade), "distilled in glass" and suitable for both spectrophotometry and liquid chromatography, was purchased from Burdick & Jackson, Muskegon, Mich., U.S.A.

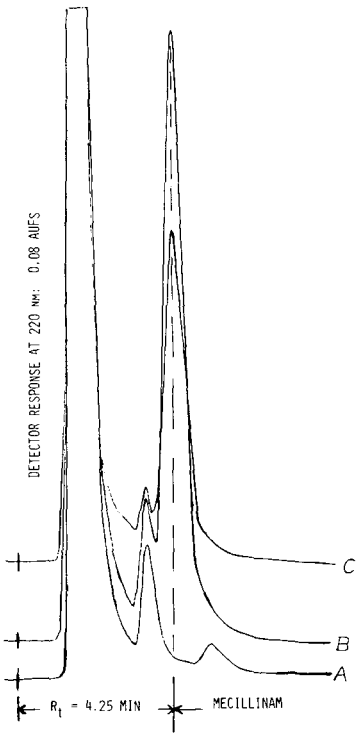


Fig. 1. Chromatograms of (A) control human urine, (B) control urine containing 2 mg/ml of authentic mecillinam added and (C) patient urine 4—6 h after a 15 mg/kg intramuscular dose.

Analytical standard solutions

Stock solutions of mecillinam, 6-β-{[(hexahydro-1*H*-azepin-1-yl)-methylene]amino}-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo(3.2.0)heptane-2-carboxylic acid, $C_{15}H_{23}N_3O_3S$, mol. wt. 325.41, m.p. 146° (with decomposition), 99% purity, were prepared on each day of analysis in fresh control human urine. Fifty milligrams of mecillinam were weighed into a 10-ml volumetric flask and dissolved in and diluted to volume with control human urine to yield a stock solution A containing 5 mg/ml. Serial dilutions of solution A were made in urine to yield standard solution B containing 1 mg/ml, solution C containing 0.2 mg/ml and solution D containing 0.05 mg/ml.

Note. All standard solutions must be made up fresh daily and discarded after use. The solutions are stable over a 24-h period at 4° (refrigerator temperature) but are unstable over a 48-h period.

Procedure

The urine specimens to be analyzed were taken from a freezer at −70° and allowed to thaw at room temperature while the working standard solutions were being prepared. If the volume of the urine sample was greater than approximately 10 ml, then the containers were partially immersed in water (20—25°) to speed up the thawing. The thawed urine specimens, together

with standard solutions A—D and control urine (E), were diluted from 1.0 ml to 10.0 ml with water in volumetric flasks and mixed well. If a diluted urine sample appeared cloudy or had a visible sediment then a 1.0-ml aliquot was filtered through a 0.45-μm pore size Millipore membrane filter for clarification.

A 10-μl aliquot of each of the clear diluted urine samples, standards A—D and control urine (E) was injected for HPLC analysis. The standard solutions A—D are used to establish a concentration—response curve for the calculation of the amount of mecillinam in unknown samples for each day of analysis.

Calculations

The concentration of mecillinam in unknown samples is determined by interpolation from the calibration graph for the working standard solutions (A—D) in the concentration range 0.05—5.0 mg/ml, processed together with the unknown samples, using the direct calibration (peak height versus concentration) technique. The same working standard solutions are also used to establish the chromatographic retention volume, and the sensitivity and linearity of the detection system for each day of analysis.

RESULTS AND DISCUSSION

Plasma levels of mecillinam determined by microbiological assay methods [4] are relatively low (C_p^{max} <10 μg/ml) following single 200-mg i.v. doses [6] owing to its rapid elimination in the urine. The concentration of the drug in urine, however, is sufficiently high [5—8] to be determined directly in diluted urine by HPLC using UV detection at 220 nm. The relatively weak UV absorbance of the compound limits the sensitivity of the method. Sample dilution is essential to reduce the introduction of endogenous particulate material onto the column, which would otherwise impair its performance. The high water solubility and amphoteric nature of mecillinam necessitates the use of reversed-phase HPLC for its analysis. The intra-assay relative standard deviation with 0.20—5.0 mg of mecillinam added per millilitre of urine is 1.5% (Table IIA), whereas the inter-assay relative standard deviation over the same concentration range is 6.3% (Table IIB).

Sample storage and handling

Studies on the kinetics and mechanism of the degradation of mecillinam in aqueous solutions [10] have demonstrated that for optimal stability, the pH should be maintained at 4.5—6.0, i.e., at about the isoelectric pH for this amphoteric drug. The acid stability of mecillinam is comparable to that of ampicillin, one of the most acid-stable penicillins. However, at neutral and weakly basic pH, mecillinam is 5—10 times more susceptible to hydrolysis of the β-lactam ring to penicilloic acid than is ampicillin [10]. Hence the stability of mecillinam at physiological pH becomes critical and can be assured only by storage at low temperature.

Mecillinam is unstable in urine even at freezer temperatures (—17 to —20°) (Table IIIA), the mean recovery declining from 88% at 16 days to 80% at

TABLE II

STATISTICAL ANALYSIS OF ASSAY PARAMETERS

Parameter	Mecillinam added (mg/ml)	Replicates (n)	Mean response (absorbance units × 10^{-4})	Mecillinam found (mg/ml)	Relative standard deviation (%)
(A) Intra-assay precision*	0.00	2	12	0.015	—
	0.05**	4	36	0.051	7.8
	0.20	4	124	0.194	1.3
	1.00	4	620	0.987	1.8
	5.00	4	3140	5.000	1.5
(B) Inter-assay reproducibility	0.05**	3	—	0.047	14.5
	0.20	6	—	0.184	8.5
	1.00	6	—	1.020	6.3
	5.00	6	—	5.090	4.2

*Calculated from least-squares curve fit. Linear least-squares regression analysis: $y = mx + b$. Slope = 1.565, intercept = +0.0065. Correlation coefficient (r) = 0.9999.
**Limit of detection.

40 days. Although storage in dry-ice for shipment is satisfactory for short-term stability (48 h), long-term stability (Table IIIB) requires storage at temperatures below —70° (e.g., Revco Ultra Low Temperature Freezer, Series/Models 800 or 1000, Rheem Refrigeration Products Div., S.C., U.S.A.).

Samples stored at —70° should be allowed to thaw gradually to room temperature, without exposure to elevated temperatures, and analyzed expeditiously, i.e., within 4 h of thawing to room temperature.

Application of the method to biological fluids

Urine specimens were collected at various time intervals over a 24-h excretion period in three subjects following a single 15 mg/kg intravenous or intramuscular administration of mecillinam (Table IV). The rapid elimination of intact mecillinam is indicated by the extensive recovery of the dose over the 0—6 h excretion interval. The data on urinary excretion obtained with the HPLC assay are in good agreement with those previously reported using microbiological assays following either the parenteral administration of mecillinam [5—8] or the oral administration of pivmecillinam [19—21].

Specificity of the assay

Pivmecillinam hydrochloride (I) when administered orally is rapidly biotransformed by de-esterification into mecillinam (II) (the active antibiotic). No measurable plasma levels of I have yet been reported [5, 21]. Under the HPLC conditions used in this assay, I is not eluted from the column even after 30 min, and it would require a more polar mobile phase for elution [22] than is described here; hence it does not interfere with the analysis of mecillinam.

TABLE III

STABILITY OF MECILLINAM IN URINE ADDED AT CONCENTRATIONS RANGING FROM 0.2 TO 5.0 mg/ml AT DIFFERENT TEMPERATURES

(A) Stability at −17°

Storage time (days)	Mean concentration found (%)	Standard deviation (%)
16	88	4.8
30	86	3.3
40	80	3.2

(B) Stability at −70°

Storage time (days)	Concentration added (mg/ml)	Concentration found (mg/ml)	Recovery (%)	Mean recovery (%)	Standard deviation (%)
10	0.2	0.21	107.0	101.5	4.7
	1.0	0.99	98.8		
	5.0	4.99	98.8		
20	0.2	0.19	98.5	94.8	11.3
	0.2	0.15	72.5		
	1.0	0.97	97.0		
	1.0	1.05	105.0		
	5.0	4.96	99.2		
	5.0	4.83	96.6		
	0.2	0.17	85.0	102.9	16.6
	0.2	0.17	86.0		
	1.0	1.08	108.0		
	1.0	1.21	121.0		
	5.0	4.77	95.4		
	5.0	6.10	122.0		
40	0.2	0.18	90.0	97.2	8.2
	0.2	0.18	92.0		
	1.0	0.98	98.3		
	1.0	0.98	98.0		
	5.0	5.29	105.8		
	5.0	4.94	98.8		
50	0.2	0.18	92.0	97.6	5.9
	1.0	0.99	99.0		
	5.0	5.01	100.2		
			Overall mean:	98.8	9.4

Several urinary metabolites of pivmecillinam and mecillinam have been reported [6, 21] (Table I). Of these, compound III, the penicilloic acid of mecillinam, and compound VI, a metabolite identified only in the rat [6, 21], have the same azomethine (C = N) chromophore as mecillinam. Compound III has a retention time of 1.8 min and is completely resolved from

TABLE IV

URINARY EXCRETION PROFILE IN HUMANS FOLLOWING A SINGLE 15 mg/kg INTRAVENOUS OR INTRAMUSCULAR DOSE OF MECILLINAM

Collection interval (h)	Patient (initials; sex; weight; dose; route)								
	L.W.; m; 77 kg; 1155 mg; i.m.			G.K.; m; 84 kg; 1260 mg; i.v.			F.K.; f; 62 kg; 930 mg; i.v.		
	Concentration (mg/ml)	Total excreted (mg)	% of dose excreted	Concentration (mg/ml)	Total excreted (mg)	% of dose excreted	Concetration (mg/ml)	Total excreted (mg)	% of dose excreted
−2 to 0	nm	—	—	nm	—	—	nm	—	—
0—1	1.79	71.6	6.2	—*	—	—	2.15	236	25.4
1—2	—*	—	—	1.07	139	11.0	—*	—	—
2—4	4.28	428.0	37.1	—*	—	—	2.32	220	23.7
4—6	0.07	12.6	1.1	2.88	262	20.8	0.54	54	5.8
6—8	nm	—	—	0.12	5.4	0.4	—*	—	—
8—12	nm	—	—	0.04	1.8	0.1	0.05	8	0.9
12—24	—	—	—	nm	—	—	nm	—	—
Total excreted		512.2	44.4		408.2	32.4		518	55.7

*Subject did not void at the end of the excretion period. Subsequent sample collected represents a "combined" pool for the two intervals.

mecillinam (retention time 4.25 min). It is a measurable urinary metabolite in man and accounted for about 6% of an oral dose of pivmecillinam [21]. The capacity factors for a number of metabolites common to pivmecillinam are also listed in Table I. Compounds IV and V are minor metabolites, accounting for 2—6% of an oral dose. They do not interfere, as they are eluted in the solvent front and are also weaker chromophores than mecillinam. Other related antibiotics which are co-adminstered with mecillinam, such as ampicillin and amoxicillin (and their respective penicilloic acids), which could be sources of interference, are all resolved from mecillinam, ensuring the specificity of the assay in urine.

The capacity factors of a number of compounds characterized as breakdown products and/or metabolites of pivmecillinam/mecillinam, using mobile phases analogous to that used on this study, have been reported by Hagel and Waysek [22] as a stability indicating reversed-phase HPLC assay for pivmecillinam hydrochloride in a capsule dosage form, and in susceptibility disks [23]. All of these breakdown products, which should not be present in frozen urine samples (—70°), would be eluted in the solvent front owing to the small k' values obtained with the mobile phase used in this study, and hence would not interfere with the specificity of the assay for mecillinam.

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Note

Estimation of serum indomethacin at therapeutic levels by means of thin-layer chromatography and spectrophotometry

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The need for an accurate, sensitive and rapid method for the estimation of indomethacin arose from studies in this department on the bioavailability of various indomethacin preparations in normal human volunteers. Previously published methods utilized radioactively labelled indomethacin [1], spectrofluorimetry [2], electron-capture gas chromatography [3—7], radioimmunoassay [8] and high-speed liquid chromatography [9]. This paper describes a procedure for such estimations, which is simple enough to permit about eighty assays to be performed in a working day by one analyst.

EXPERIMENTAL

Materials

Anaesthetic diethyl ether (Natal Cane Byproducts, Durban, Republic of South Africa) was redistilled before use. All other reagents were Merck (Darmstadt, G.F.R.) reagent grade and were used without further purification. The internal standard, piretanide (HOE-118), a new diuretic (structure shown in Fig. 1)

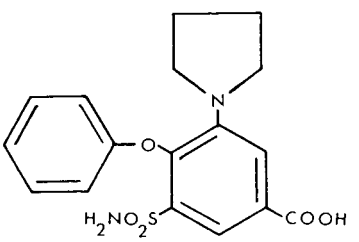

Fig. 1. Structure of piretanide (HOE-118).

was a gift from Hoechst (Frankfurt am Main, G.F.R.). High-performance thin-layer chromatographic (HPTLC) plates (silica gel 60 without fluorescence indicator) were obtained from Merck.

Apparatus

Serum was pipetted by means of an Oxford Laboratories sampler, and standards and phosphoric acid by means of a Gilford 6065 automatic pipettor-diluter. Extracts were spotted onto TLC plates by means of an EVA Chrom TLC applicator. Spectrophotometric measurements were made in the reflectance mode in a Zeiss KM 3 chromatogram spectrophotometer at 254 nm.

Method

Serum (1 ml) was pipetted into a glass-stoppered centrifuge tube (B14 Quickfit) and 20 μl of internal standard (100 mg per 100 ml acetone) was added, together with 500 μl of 1 *M* phosphoric acid. The sample was mixed for a few seconds on a Vortex mixer and 6 ml diethyl ether was then added. The contents were mixed on a horizontal shaker (100 strokes/min) for 15 min. After centrifugation (800 *g*), the diethyl ether layer was transfered to a 5-ml glass ampoule and evaporated to dryness under nitrogen. The sides of the ampoule were washed with 100 μl of acetone, and again evaporated to dryness under nitrogen. The residue was redissolved in 30 μl of acetone and 2.5 μl were spotted onto 10 × 10 cm HPTLC plates. Ten unknown and five standard extracts were spotted on each plate. The mobile phase dioxane—methanol—ammonia (7:2:1), was allowed to ascend to the 9 cm mark on the TLC plate in a Shandon N-chamber (non-saturated). The plates were then dried in a stream of cold air and scanned at 254 nm. The ratio of the peak heights of indomethacin to internal standard were calculated and plotted against indomethacin serum concentration. An equation of the calibration line was obtained by linear regression analysis and used to calculate unknowns from their peak height ratios.

RESULTS AND DISCUSSION

Fig. 2 depicts chromatograms obtained from (a) indomethacin-free serum, (b) serum spiked with indomethacin and HOE-118, and (c) serum from a

TABLE I

PRECISION AND ACCURACY OF THE TLC METHOD FOR ASSAYING INDOMETHACIN IN SPIKED SERUM

n = 10.

Spiked value (μg/ml)	Assayed value	
	Mean ± S.D. (μg/ml)	Coefficient of variation (%)
0.5	0.50 ± 0.03	6.0
1.0	1.02 ± 0.05	4.5
1.5	1.49 ± 0.03	2.2
2.0	2.00 ± 0.05	2.5
2.5	2.49 ± 0.03	1.2
5.0	4.97 ± 0.18	3.7
10.0	9.96 ± 0.08	0.8

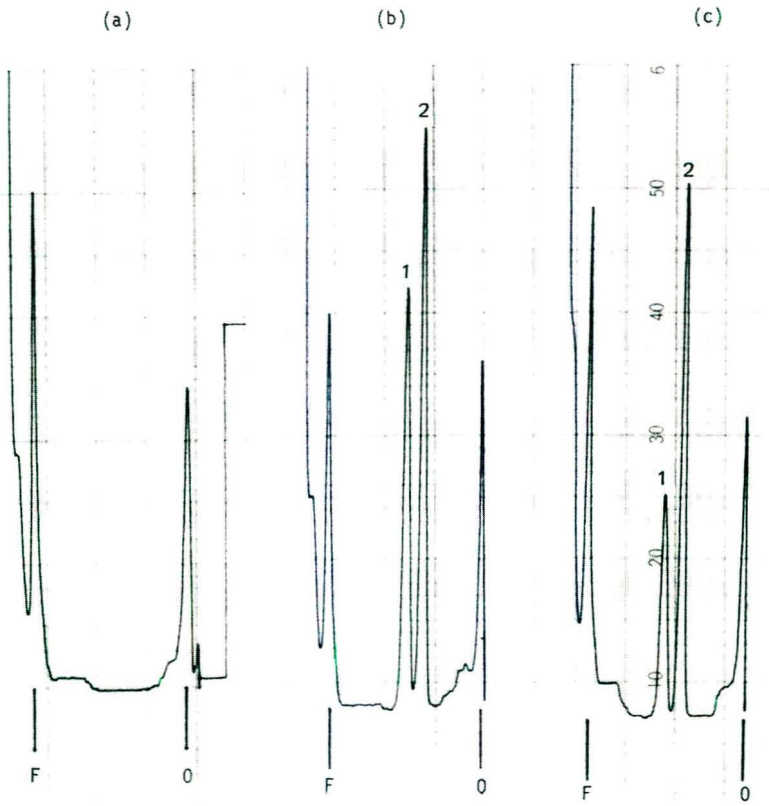

Fig. 2. Chromatograms of (a) indomethacin-free serum, (b) serum spiked with indomethacin and HOE-118, and (c) serum from volunteer after indomethacin ingestion. Peaks: 1 = indomethacin, 2 = piretanide (HOE 118).

volunteer during the trial. There are no interfering peaks from indomethacin-free serum.

The R_F value for piretanide is 0.37 and for indomethacin 0.48. Plots of the standard curves for indomethacin over the range 0.5—10 μg/ml were linear, and the mean of forty such plots is depicted in Fig. 3.

The results shown in Table I, summarize the data obtained from ten assays. The accuracy and precision of the method are excellent. The concentrations of indomethacin in the experimental samples compare favourably with the spiked values.

The method described for the quantitation of indomethacin in serum at therapeutic levels is relatively simple requiring a single extraction and separation on a TLC plate. Quantitation by means of absorption measurements is reproducible and accurate.

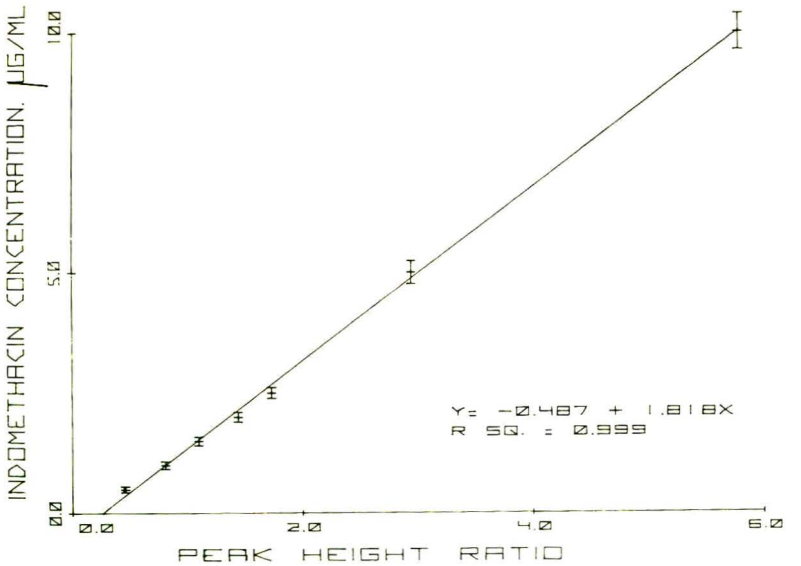

Fig. 3. Standard curve for indomethacin. Plot of indomethacin concentration versus peak height ratio of indomethacin and piretamide (n = 40).

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Letter to the Editor

Improvements to a high-performance liquid chromatographic assay for allopurinol and oxipurinol in plasma

Sir,

A recent publication from this laboratory [1] presented a simple and sensitive high-performance liquid chromatographic (HPLC) assay for allopurinol and oxipurinol in plasma. Additional experience with this assay obtained during a pharmacokinetic study [2] has led to two improvements which increase column life and sensitivity.

In the original method [1], 0.5 ml of plasma was mixed with 0.1 ml of internal standard and 0.2 ml 20% trichloroacetic acid (TCA), centrifuged, and 50 μl of the supernate injected onto a reversed-phase column eluted with a phosphate buffer (pH 6, 0.05 *M*). Due to the extreme acidity of TCA, the pH of the injected sample is very low and it was found that even though a very small volume was injected, column deterioration was occurring. To eliminate the problems, plasma samples are now precipitated using an excess of ammonium sulfate (AS) granules, with all other steps the same. This yields a supernate with a fairly neutral pH (ca. 6), well within the pH tolerance limits of the column. Sensitivity and recovery are the same as with the TCA method.

For some pharmacokinetic studies, increased sensitivity was required. Extraction of the supernate from the AS precipitation with two volumes (5 ml) of diethyl ether—propanol (6:1), combination of the organic phases, evaporation to dryness, reconstitution with 50 μl of the mobile phase and injection of 40—50 μl increased the sensitivity by a factor of 10 over that originally reported. Extraction with diethyl ether—propanol (6:1) had been suggested for fluorouracil [3]; although other ratios were tried, this combination yielded the highest and most consistent recoveries.

The two alterations to the procedure suggested above provide significant improvements to the assay. It is hoped that these will prove useful to others.

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1 A. T. James and A. J. P. Martin, *Biochem. J.*, 50 (1952) 679.
2 L. R. Snyder, *Principles of Adsorption Chromatography*, Marcel Dekker, New York, 1968, p. 201.
3 H. C. S. Wood and R. Wrigglesworth, in S. Coffey (Editor), *Rodd's Chemistry of Carbon Compounds, Vol. IV, Heterocyclic Compounds*, Part B, Elsevier, Amsterdam, Oxford, New York, 2nd ed., 1977, Ch. 11, p. 201.
4 E. C. Horning, J.-P. Thenot and M. G. Horning, in A. P. De Leenheer and R. R. Roncucci (Editors), *Proc. 1st Int. Symp. Quantitative Mass Spectrometry in Life Sciences, Ghent, June 16–18, 1976*, Elsevier, Amsterdam, Oxford, New York, 1977, p. 1.

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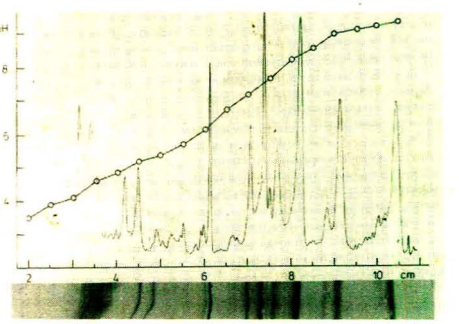

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